Available online at www.sciencedirect.com
ScienceDirect
Journal of Microbiological Methods 67 (2006) 70–85
Journal of Microbiological Methods
www.elsevier.com/locate/jmicmeth

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

Leonardo Schena [a,*], David E.L. Cooke [b]

[a] *Department of Plant Protection and Applied Microbiology, Via Amendola 165/A, 70126, Bari, Italy*
[b] *Scottish Crop Research Institute, Invergowrie, Dundee, DD2 5DA, Scotland, UK*

Received 16 December 2005; received in revised form 24 February 2006; accepted 6 March 2006
Available online 17 April 2006

Abstract

Four different intergenic regions of mitochondrial DNA (mt-IGS), a fragment of the intergenic spacer (IGS) region of the rDNA (rDNA-IGS), and a fragment of the ras-related protein (*Ypt*1) gene were amplified and sequenced from a panel of 31 *Phytophthora* species representing the most significant forest pathogens and the breadth of diversity in the genus. Over 80 kbp of novel sequences were generated and alignments showed very variable (introns and non-coding regions) as well as conserved coding regions. The mitochondrial DNA regions had an AT/GC ratio ranging from 67.2 to 89.0% and were appropriate for diagnostic development and phylogeographic analysis. The IGS fragment was less variable but still appropriate to discriminate amongst some important forest pathogens. The introns of the *Ypt*1 gene were sufficiently polymorphic for the development of molecular markers for almost all *Phytophthora* species, with more conserved flanking coding regions appropriate for the design of *Phytophthora* genus-specific primers. In general, phylogenetic analysis of the sequence alignments grouped species in clades that matched those based on the ITS regions of the rDNA. In many cases the resolution was improved over ITS but in other cases sequences were too variable to align accurately and yielded phylograms inconsistent with other data. Key studies on the intraspecific variation and primer specificity remain. However the research has already yielded an enormous dataset for the identification, detection and study of the molecular evolution of *Phytophthora* species.

Keywords: *Phytophthora*; Forests; Natural ecosystems; Mitochondrial DNA; *Ypt*1 gene; Intergenic spacer (IGS) region

1. Introduction

Phytophthora is a genus in the Oomycota, responsible for some of the most serious and economically important plant diseases (Judelson and Blanco, 2005). Some species also damage important natural ecosystems, altering the composition of the flora. In W. Australia, *P. cinnamomi*, an introduced pathogen, has destroyed large areas of World Heritage 'Jarrah' eucalypt forest and its unique understorey flora (Shearer et al., 2004). Similarly, a newly discovered species, *P. ramorum*, has destroyed large areas of native Californian oak forest, killing native oaks and other trees from a range of genera (Werres et al., 2001; Rizzo and Garbelotto, 2003; Hansen et al., 2005). In Europe *P. ramorum* has been frequently reported on *Rhododendron* and other shrubs in nurseries and recently it has been isolated from a number of trees (Brasier et al., 2004a). In

* Corresponding author. Tel.: +39 080 5443055; fax: +39 080 5442911.
E-mail address: leonardo.schena@agr.uniba.it (L. Schena).

0167-7012/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.mimet.2006.03.003

November 2003, a second new species called *P. kernoviae* was discovered causing bleeding cankers on beech (Brasier et al., 2005). Like *P. ramorum*, *P. kernoviae* is spreading on rhododendrons and is also aerial or splash-dispersed via caducous sporangia. Hundreds of thousands of alders have been killed across a broad swathe of Central and Northern Europe by another newly described species, *P. alni* (Brasier et al., 2004b). In this case the threat appears to have emerged from a hybridization event (Brasier et al., 1999). One of the most widespread and most frequently isolated *Phytophthora* across Europe is *P. quercina*, another newly described species very aggressive against fine roots of oaks and implicated in a rapid decline of oaks growing on acid, well-drained soils (Cooke et al., 1999, 2005; Jung et al., 1999). Six other 'new' species have been isolated recently from important trees: *P. uliginosa* and *P. europaea* (oaks), *P. pseudosyringae* (oak and beech), *P. psychrophila* (oak), *P. inundata* (different hosts), and *P. nemorosa* (different hosts) (Brasier et al., 2003; Jung et al., 2002, 2003; Hansen et al., 2003). Together with *P. citricola* (many hosts) and *P. ilicis* (oak and holly), the above species are frequently found in 'clusters', on the same sites or sometimes even same tree, usually where mature trees are declining rapidly (Jung et al., 2002; Vettraino et al., 2002, 2005).

The discovery of so many pathogens in such a short time (~ 5 years), is in part attributable to improved detection methods but other factors are undoubtedly involved, such as climate change 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 the wholesale trade. In recent years, conventional and real-time PCR has emerged as an important 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., 2004). Molecular detection methods are available for a number of *Phytophthora* species which are known to cause diseases in forest trees including *P. ramorum* (Martin et al., 2004; Hayden et al., 2004; Tomlinson et al., 2005), *P. quercina* (Schubert et al., 1999; Nechwatal et al., 2001), *P. citricola* (Schubert et al., 1999; Nechwatal et al., 2001), *P. cambivora* (Schubert et al., 1999), *P. lateralis* (Winton and Hansen, 2001), and *P. cinnamomi* (Kong et al., 2003a). The above diagnostic assays are geared to the detection of particular species and therefore are not suitable to assess what *Phytophthora* species might be present in mixed forest and natural ecosystem samples. Furthermore most of the above detection methods are based on the internal transcribed spacer (ITS) regions. The nuclear-encoded ribosomal RNA genes (rDNA) provide attractive targets to design specific primers since they are highly stable, can be amplified and sequenced with universal primers, occur in multiple copies, and possess conserved as well as variable sequences (White et al., 1990). 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. Important *Phytophthora* pathogens such as *P. nemorosa*, *P. ilicis*, *P. psychrophila*, and *P. pseudosyringae* have very similar ITS regions sequences and the design of effective and robust specific primer sets is very challenging (Martin and Tooley, 2003a,b). Similarly *P. alni*, *P. cambivora*, *P. fragariae*, and *P. europaea* are phylogenetically closely related and challenging to distinguish via ITS sequences (Brasier et al., 2004b). *P. ramorum* is closely related to *P. lateralis* differing by only 11 single nucleotide in the ITS regions (Werres et al., 2001). To discriminate the two taxa lengthy procedures such as single strand polymorphism (SSCP) analysis (Kong et al., 2004) or a double amplification with two different primer pairs (Hayden et al., 2004) were required.

Recent molecular analyses have substantially increased our understanding of the phylogenetic relationships between *Phytophthora* species and provide an enormous source of data to develop molecular detection methods. These analyses were based on the ITS1 and ITS2 region (Cooke et al., 2000), the mitochondrial encoded cytochrome oxidase II (*Cox*II) and I (*Cox*I) genes (Martin and Tooley, 2003a) and on a combination of different coding genes of nuclear (transaction elongation factor 1α and β-Tubulin) and mitochondrial (*Cox*I; NADH dehydrogenase) genome (Kroon et al., 2004). As previously mentioned, ITS sequences in some circumstances fail in discriminate among closely related taxa. Similarly, phylogenetic analyses by Martin and Tooley (2003a) and Kroon et al. (2004) were based on coding sequence with a relatively low mutation rate and therefore real limited target sites for diagnostic development. 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 (Kong et al., 2003a,b) but neither genes contain introns and are unlikely to be variable enough to distinguish a broad range of species.

Introns and intergenic portions of the nuclear and mitochondrial genome may prove more variable and therefore more promising targets for diagnostic development. The analysis of sequence variation in five different intergenic mitochondrial DNA spacer (mtDNA-IGS) regions showed the presence of intra- and inter-taxon variation for *P. infestans* and four related taxa (Wattier et al., 2003). Similarly, intergenic regions of mt-DNA were suited to the development of specific detection methods for *P. ramorum*, *P. nemorosa* and *P. pseudosyringae* (Martin et al., 2004).

The aim of the present research was to assess the suitability of other highly variable genomic regions for the development of specific detection methods for a broad range of *Phytophthora* species with an emphasis on species known to cause diseases on forest trees. Inter- and intraspecific variation was examined and any phylogenetic inference considered.

2. Materials and methods

2.1. Isolates and DNA extraction

Forty-five isolates (31 *Phytophthora* species) sourced from the culture collections of the authors and from CABI Biosciences (Egham, UK) were used in this study (Table 1).

Table 1
Isolates of *Phytophthora* included in the study, their designations, and origins

Species	Isolate numbers	Origin		
		Host	Country	Year
P. alni	SCRP2	*Alnus* sp.	UK	1995
P. cactorum	SCRP27 (IMI296524)	*Rubus idaeus*	Wales	1985
P. cambivora	SCRP67 (IMI296831)	*Rubus idaeus*	Scotland	1985
	SCRP75	*Fagus* sp.	UK	1995
	SCRP80	*Castanea sativa*	Italy	
	SCRP82	*Eucalyptus* sp.	Australia	
P. capsici	SCRP103 (IMI352321)	*Piper nigrum*	India	1989
P. cinnamomi	SCRP115 (CBS270.55)	*Chamaecyparis lawsoniana*	Netherlands	1993
P. citricola	SCRP130	*Rubus idaeus*	Scotland	1986
	SCRP136	Soil	UK	1995
	SCRP140	*Taxus* sp.	UK	1995
	SCRP143	*Quercus robur*	Germany	
P. citrophthora	SCRP179 (IMI332632)	*Actinidia chinensis*	Chile	1989
P. cryptogea	SCRP207 (IMI045168)	*Lycopersicon esculentum*	New Zealand	1951
P. drechsleri	SCRP232 (ATCC46724)	*Beta vulgaris*	U.S.A.	1935
P. erythroseptica	SCRP240	*Solanum tuberosum*	Netherlands	
P. europaea	SCRP622	*Quercus robur*	Switzerland	
P. fragariae var. *fragariae*	SCRP245	*Fragaria* × *ananassa*	England	1945
P. fragariae var. *rubi*	SCRP333 (IMI355974)	*Rubus idaeus*	Scotland	1985
P. ilicis	SCRP377	*Ilex aquilifolium*	UK	1995
	SCRP379	*Ilex aquilifolium*	UK	
P. infestans	SC03.26.3.3	*Solanum tuberosum*	Scotland	2003
P. insolita	SCRP385 (IMI288805)	Soil	Taiwan	1979
P. inundata	SCRP644 (IMI389751)	*Salix* sp.	UK	1972
	SCRP643 (IMI389750)	*Aesculus hippocastanum*	UK	1970
	SCRP647	*Vitis* sp.	S. America	1997
	SCRP649	*Alnus glutinosus*	Denmark	1995
P. katsurae	SCRP388		France	1996
P. kernoviae	SCRP722	*Fagus sylvatica*	England	2003
P. lateralis	SCRP390 (IMI040503)	*Chamaecyparis* sp.	U.S.A.	1942
P. medicaginis	SCRP407	*Medicago* sp.	Iran	1989
P. megasperma	SCRP435 (IMI133317)	*Malus sylvestris*	Australia	1968
P. nemorosa	SCRP910		USA	2004
P. nicotianae	SCRP468 (IMI268688)	*Citrus* sp.	Trinidad	
P. palmivora	SCRP526	*Hevea brasiliensis*	Thailand	1995
P. pistaciae	SCRP533 (IMI386658)	*Pistacia vera*	Iran	1986
P. pseudosyringae	SCRP674 (IMI390500)	*Malus pumila*	Italy	2001
	SCRP734	*Fagus sylvatica*	Italy	2003
P. psychrophila	SCRP630	*Quercus ilex*	Germany	
P. quercina	SCRP541	*Quercus robur*	Germany	1995
	SCRP547	*Quercus cerris*	Germany	1995
	SCRP549	*Quercus ilex*	Italy	1995
	SCRP550	*Quercus robur*	Germany	1995
P. ramorum	SCRP911	*Rhododendron* sp.	Scotland	2004
P. sojae	SCRP555	*Glycine max*	USA	1995

Isolates were stored on oatmeal agar at 5 °C and grown on French bean agar for routine stock cultures.

For DNA extraction phytophthoras were grown in 20 ml still culture of a sucrose/asparagine/mineral salts broth containing 30 μg ml^{-1} β-sitosterol (Elliott et al., 1966). After vacuum filtration, the mycelium was freeze-dried for extended storage at −20 °C. To extract total DNA 10–20 mg of dry mycelia were suspended in 800 μl of breaking buffer (200 mM Tri–HCl [pH 8], 250 mM NaCl, 25 mM EDTA, 0.5% SDS) and 800 μl of phenol/chloroform/isoamyl alcohol (25:24:1) in the presence of 0.2 g each of 0.1 mm diameter zirconia/silica beads and 1.0 mm diameter glass beads. The extraction mixture was blended in a Mini Bead Beater (Bio-Spec Products, Bartlesville, OK., USA) at 5000 rpm for 60 s and centrifuged at 13,000 ×*g* for 5 min. The upper phase was extracted twice with 800 μl of phenol/chloroform/isoamyl alcohol (25:24:1) and 700 μl of chloroform/isoamyl alcohol (24:1), respectively. DNA was precipitated with an equal volume of isopropanol for 1 h at 5 °C, washed with 70% cold ethanol (−20 °C), dried, resuspended in sterile distilled water and stored at

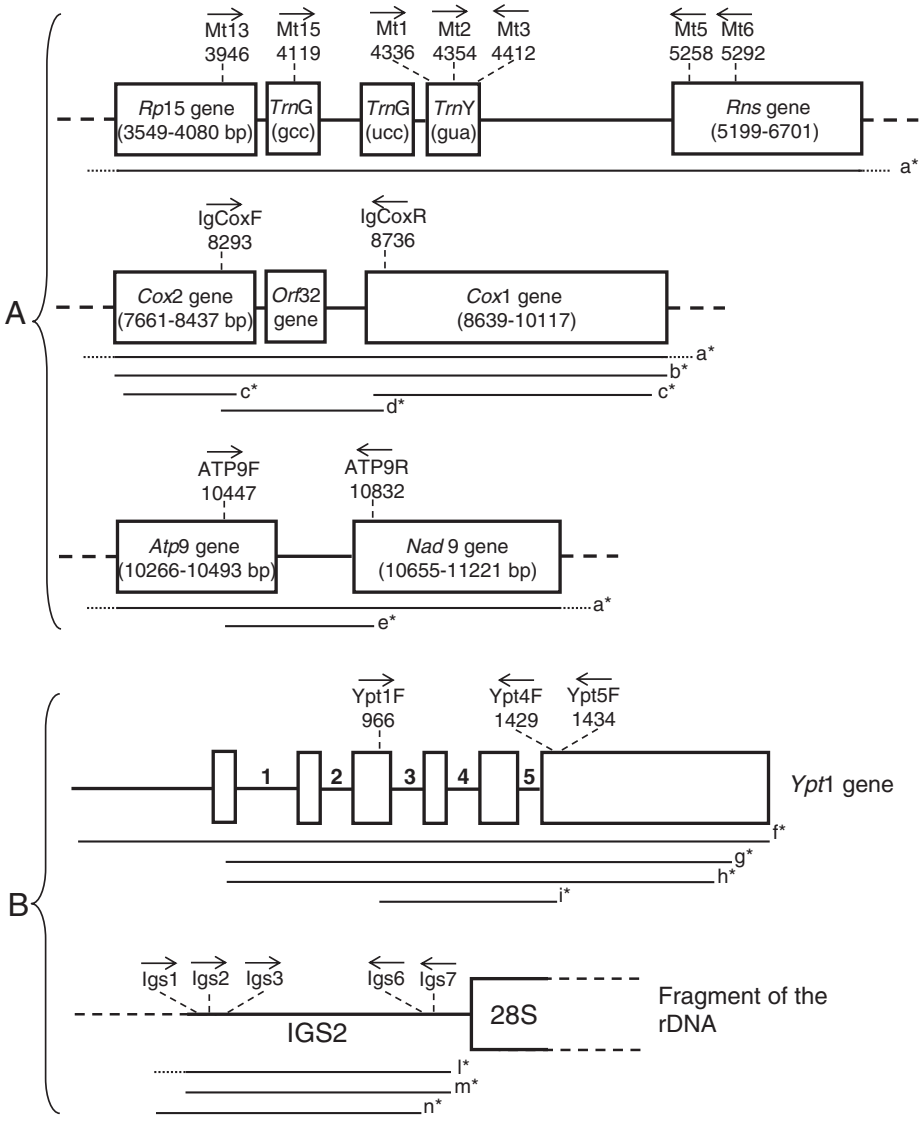

Fig. 1. Schematic representation of mitochondrial (A) and nuclear (B) DNA regions examined in this study with location of selected primers above and reference to the available DNA databases below each statement (*). Arrows on primers indicate orientation. *a) Paquin et al. (1997), b) Sachay et al. (1993), c) Martin and Tooley (2003a), d) Martin et al. (2004), e) Wattier et al. (2003), f) Chen and Roxby (1996), g) *P. ramorum* scaffold_16:280386–282150, h) *P. sojae* scaffold_30:367461–369077, i) Moorman et al. (2002), l) Liew et al. (1998), m) *P. sojae* scaffold_6:111816–112266, n) *P. ramorum* scaffold_1053:6938–7389.

−20 °C. For routine amplifications, DNA was diluted to 10 ng/μl and maintained at 5 °C.

2.2. Primer selection

To amplify different regions of nuclear and mitochondrial DNA 82 different primers were selected either from the literature or designed using the Primer3 Software (Rozen and Skaletsky, 2000) on the bases of published DNA sequences. The *P. megasperma Cox*2 and *Cox*1 gene sequence (Sachay et al., 1993) and/or the complete *P. infestans* mitochondrial DNA sequence (accession number U17009) reported by Paquin et al. (1997) were used as template for the design of mitochondrial primers (Fig. 1; Table 2). Among these, primers ATP9F–ATP9R were already reported by Wattier et al. (2003) and primers IgCoxF–IgCoxR were designed by adding two degenerations to the forward primer reported by Martin et al. (2004) (FMPh-8b and FMPh-10b) (Table 2). Primers utilised to amplify a region of the rDNA-IGS were designed from alignments of sequences reported by Liew et al. (1998) for *P. medicaginis*, *P. megasperma* and *P. trifolii* with a fragment of *P. sojae* [scaffold_6:111816–112266 (http://www.jgi.doe.gov/)] and a fragment of *P. ramorum* [scaffold_1053:6938–7389 (http://www.jgi.doe.gov/)] (Fig. 1; Table 2). To design primers for the *Ypt*1 gene the complete sequence of this gene from *P. infestans* reported by Chen and Roxby (1996) was aligned with a portion of the same gene available for *P. cinnamomi*, *P. cryptogea*, and *P. citricola* (Moorman et al., 2002), *P. sojae* [scaffold_30:36746–369077 (http://www.jgi.doe.gov/)] and *P. ramorum* [scaffold_16:280386–282150 (http://www.jgi.doe.gov/)] (Fig. 1; Table 2).

With the exception of primers for the rDNA-IGS, all primers were designed in coding regions to amplify flanking introns or non-coding intergenic regions (Fig. 1). When more GenBank DNA sequences from different species were available, sequences were aligned using the MultAlin software (Corpet, 1988) and primers designed in the more conserved regions. Degenerate primers were designed when required.

2.3. DNA amplification and sequencing

Considerable effort was made to obtain successful amplification of as many species as possible. This involved identification of the best primer pairs for each genomic region and for each *Phytophthora* species and adjustment of $MgCl_2$ concentration and annealing

Table 2
Selected primers used in this study

Target DNA	Primers	Sequence (5′–3′)	Reference [a]
Mitochondrial genome region between gene *trn*G (gcc) and gene *trn*Y (gua)	Mt13F	ACAGTTTTTCGAATTAAAAACAGAA	Paquin et al. (1997)
	Mt15F	TTGCCAAGGTTAATGTTGAGG	
	Mt3R	GGAGAAAGTAGGATTCGAACCT	
Mitochondrial genome region between gene *trn*Y (gua) and gene *Rns*	Mt1F	TGGCTGAGTGGTTAAAGGTG	Paquin et al. (1997)
	Mt2F	TGGCAGACTGTAAATTTGTTGAA	
	Mt5R	TTGCATGTGTTAAGCATACCG	
	Mt6R	CTCACCCGTTCGCTATGTTT	
Mitochondrial genome region between gene *Cox*2 and gene *Cox*1	IgCoxF	AAAAGAGARGGTGTTTTTTAYGGA	Paquin et al. (1997)
	IgCoxR	GCAAAAGCACTAAAAATTAAATATAA	Sachay et al. (1993), Martin and Tooley (2003a), and Martin et al. (2004)
Mitochondrial genome region between gene *Atp*9 and gene *Nad*9	ATPF	TTTATTCTGTTTAATGATGGC	Paquin et al. (1997)
	ATPR	CAGCACAAATTTCAGATAATAC	Wattier et al. (2003)
Ras-related protein (*Ypt*1) gene	Ypt1F	CGACCATYGGYGTKGACTTT	Chen and Roxby (1996)
	Ypt4R	TTSACGTTCTCRCAGGCGTA	Moorman et al. (2002)
	Ypt5R	GCAGCTTGTTSACGTTCTCR	*P. ramorum* [b]
			P. sojae [c]
Intergenic spacer (IGS) region of the rDNA	Igs1F	AAAGTGRKGMGGWGWWGCKGA	Liew et al. (1998)
	Igs2F	AAGTRYMTKAACAACGCTCT	*P. ramorum* [d]
	Igs3F	GYGCGAAGGWKTGCTG	*P. sojae* [e]
	Igs6R	CCCAGCRYAAACAACAACAC	
	Igs7R	ATATCCTCCATACGWAAGAAGACG	

[a] Reference to available DNA sequences on which primers were based.
[b] Scaffold_16:280386–282150 (http://www.jgi.doe.gov/).
[c] Scaffold_30:36746–|369077 (http://www.jgi.doe.gov/).
[d] Scaffold_1053:6938–7389 (http://www.jgi.doe.gov/).
[e] Scaffold_6:111816–112266 (http://www.jgi.doe.gov/).

temperatures for PCR reactions. Genomic regions that were difficult to amplify from many species were excluded. To amplify the mtDNA-IGS, PCR reactions were performed in a total volume of 50 μl containing 30 ng of genomic DNA, 10 mM Tris–HCl (pH 9), 50 mM KCl, 0.1% Triton X-100, 100 μM dNTPs, 7 mM $MgCl_2$, 50 μg BSA, 2 unit of *Taq* polymerase (Taq DNA polymerase, Promega Corporation, WI, USA) and 1 μM of primers. PCR amplification conditions consisted of: 1 cycle of 95 °C for 2 min; 35 cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 60 s; and a final cycle of 72 °C for 10 min. To amplify the *Ypt*1 gene and the rDNA-IGS the concentration of $MgCl_2$ was reduced to 1.5 mM and annealing temperature increased to 55 °C. In both cases, successful amplification was confirmed by gel electrophoresis on 2% agarose gels and ethidium bromide staining.

Single PCR bands were purified with the MinElute PCR Purification Kit (Quiagen Ltd. West Sussex, UK) to remove excess primers and nucleotides. Sequencing was carried out with the same primers utilised for the amplification in a dye-terminator cycle-sequencing reaction (FS sequencing kit, Applied Biosystems, Warrington, UK) and run on an ABI373 automated sequencer (Applied Biosystems).

2.4. Sequence analysis

Prior to analysis all sequences were trimmed to a common start and end point: the first amplified nucleotide of the most internal primers utilised for each region. In a few cases sequences were trimmed to the first amplified nucleotide of the external primers and in these cases the few obtained with internal primer sequences included the primer sequence itself to maximise the sequence length available.

In each case, all sequences were aligned using ClustalX (Thompson et al., 1997) and introduced to TOPALi for phylogenetic analysis with the Neighbor-Joining method based on Jukes–Cantor distances as implemented in TOPALi (Milne et al., 2004). Parametric bootstrapping using the DSS statistic was used to compare tree topologies (Goldman et al., 2000).

3. Results and discussion

3.1. Amplification and analysis of intergenic regions of mitochondrial DNA (mt-IGS)

The results of the analysis of four different mt-IGS regions are summarised in Table 4. The portion between genes *Trn*G (gcc) and *Trn*Y (gua) (*Trn*G–*Trn*Y) was amplified and sequenced from 25 different *Phytophthora* species using two different primer combinations (Mt13F–Mt3R or Mt15F–Mt3R) (Fig. 1, Tables 2 and 3). The sequences comprised three coding and two non-coding regions (Fig. 1), had an average AT/GC ratio of 67.2%, a length ranging from 250 to 290 bp (Table 3) and produced an alignment totalling 318 bp. Approximately one third of sites across the alignment (103) were phylogenetically informative and the average pairwise distance was 0.12.

The mtDNA fragment between genes *Trn*Y (gua) and *Rns* (*Trn*Y–*Rns*) was amplified with primers Mt2F–Mt5R from *P. fragariae* var. *fragariae* and var. *rubi* or primers Mt1F–Mt6R (all other species). Sequences were obtained from 35 different isolates (24 different species) (Fig. 1, Tables 2 and 3), comprised two short coding regions flanking a large intergenic region and had an average AT/GC ratio of 77.6%. Sequence length was very variable ranging from 285 bp for *P. capsici* to 660 bp for *P. nemorosa*. An alignment totalling 674 bp in length was generated and 482 phylogenetic informative sites identified with an average pairwise distance of 0.68. Intraspecific polymorphisms were identified in all the species where multiple isolates were examined with higher levels in *P. citricola*, *P. quercina* and *P. inundata* (Fig. 2B). *P. quercina* and *P. inundata* in particular showed deletions of 55 and 42 bp respectively compared to other isolates of the same species (Table 3).

The genomic region between genes *Cox*2 and *Cox*1 (*Cox*2–*Cox*1) was amplified with primers IgCoxF–IgCoxR from 27 different species (Fig. 1, Tables 2 and 3). DNA fragments comprised two small coding regions of *Cox*2 and *Cox*1 genes flanking two small intergenic regions and the *Orf*32 gene (Fig. 1). The sequences had an average AT/GC ratio of 81.3%, a length ranging from 387 to 428 bp (Table 3) and resulted in an alignment of 445 bp. Approximately one third (151) of the sites were phylogenetically informative and the average pairwise distance was 0.12.

The genomic region between genes *Atp*9 and *Nad*9 (*Atp*9–*Nad*9) was amplified with primers ATP9F–ATP9R from 17 different species (Fig. 1, Tables 2 and 3). DNA fragments comprised two small coding regions of *Atp*9 and *Nad*9 genes flanking an intergenic region (Fig. 1). Analysed sequences had an average AT/GC ratio of 89.0%, a length ranging from 322 to 355 bp (Table 3) and produced an alignment of 370 bp. Approximately forty percent (151) of the sites were phylogenetically informative and the average pairwise distance was 0.31.

In the present work we analysed 4 different mitochondrial regions from many *Phytophthora* species. Some of

Table 3
Data coverage for accessions included in the analysis, list of primers optimized for amplification of specific *Phytophthora* species and length of amplified fragments as trimmed to exclude primer sequences

Phytophthora species	Isolates	Mitochondrial DNA				Nuclear DNA	
		*Trn*G–*Trn*Y	*Trn*Y–*Rns*	*Cox*2–*Cox*1	*Atp*9–*Nad*9	rDNA-IGS	*Ypt*1
P. alni	SCRP2	DQ162893 Mt13F-3R 278 bp	DQ162924 Mt1F-6R 384 bp	DQ162846 IgCoxF-R 415 bp	DQ162884 ATPF-R 336 bp	DQ162993 Igs2F-7R 434 bp	DQ162953 Ypt1F-5R 459 bp
P. cactorum	SCRP27	DQ162892 Mt13F-3R 261 bp	DQ162935 Mt1F-6R 406 bp	DQ162854 IgCoxF-R 393 bp		DQ162994 Igs2F-7R 429 bp	DQ162960 Ypt1F-5R 429 bp
P. cambivora	SCRP67	DQ162894 Mt13F-3R 278 bp	DQ162927 Mt1F-6R 413 bp	DQ162847 IgCoxF-R 415 bp	DQ162885 ATPF-R 336 bp	DQ162995 Igs2F-7R 435 bp	DQ162954 Ypt1F-5R 461 bp
P. cambivora	SCRP75		DQ162925 Mt1F-6R 413 bp				
P. cambivora	SCRP80		DQ162928 Mt1F-6R 413 bp				DQ162955 Ypt1F-5R 461 bp
P. cambivora	SCRP82		DQ162926 Mt1F-6R 413 bp				DQ162956 Ypt1F-5R 461 bp
P. capsici	SCRP103	DQ162899 Mt15F-3R 263 bp	DQ162915 Mt1F-6R 285 bp	DQ162863 IgCoxF-R 417 bp	DQ162880 ATPF-R 352 bp	DQ162996 Igs2F-7R 418 bp	DQ162972 Ypt1F-5R 449 bp
P. cinnamomi	SCRP115	DQ162897 Mt13F-3R 271 bp		DQ162849 IgCoxF-R 419 bp	DQ162889 ATPF-R 355 bp	DQ162997 Igs2F-7R 435 bp	DQ162959 Ypt1F-4R 441 bp
P. citricola	SCRP130	DQ162900 Mt15F-3R 262 b	DQ162917 Mt1F-6R 297 bp	DQ162865 IgCoxF-R 417 bp		DQ162998 Igs2F-7R 422 bp	DQ162968 Ypt1F-5R 463 b
P. citricola	SCRP136						DQ162969 Ypt1F-5R 463 bp
P. citricola	SCRP140		DQ162918 Mt 1F-6R 296 bp				DQ162970 Ypt1F-5R 463 bp
P. citricola	SCRP143		DQ162919 Mt1F-6R 296 bp				DQ162971 Ypt1F-5R 463 bp
P. citrophthora	SCRP179	DQ162901 Mt15F-3R 263 bp	DQ162916 Mt1F-6R 295 bp	DQ162864 IgCoxF-R 417 bp		DQ162999 Igs2F-7R 423 bp	DQ162973 Ypt1F-4R 441 bp
P. cryptogea	SCRP207	DQ162908 Mt13F-3R 255 bp	DQ162921 Mt1F-6R 310 bp	DQ162859 IgCoxF-R 416 bp		DQ163000 Igs2F-7R 423 bp	DQ162987 Ypt1F-5R 457 bp
P. drechsleri	SCRP232	DQ162910 Mt13F-3R 257 bp		DQ162862 IgCoxF-R 415 bp		DQ163001 Igs2F-7R 421 bp	DQ162989 Ypt1F-5R 445 bp
P. erythroseptica	SCRP240	DQ162909 Mt13F-3R 255 bp	DQ162922 Mt1F-6R 310 bp	DQ162860 IgCoxF-R 416 bp		DQ163002 Igs2F-7R 423 bp	DQ162988 Ypt1F-5R 457 bp
P. europaea	SCRP622	DQ162895 Mt13F-3R 278 bp	DQ162932 Mt1F-6R 473 bp	DQ162848 IgCoxF-R 416 bp	DQ162886 ATPF-R 329 bp	DQ163003 Igs2F-7R 435 bp	DQ162952 Ypt1F-5R 449 bp
P. fragariae var. *fragariae*	SCRP245	DQ162896 Mt13F-3R 278 bp	DQ162929 Mt2F-5R 472 bp			DQ163004 Igs2F-6R 411 bp	DQ162950 Ypt1F-5R 459 bp

Table 3 (*continued*)

Phytophthora species	Isolates	Mitochondrial DNA				Nuclear DNA	
		*Trn*G–*Trn*Y	*Trn*Y–*Rns*	*Cox*2–*Cox*1	*Atp*9–*Nad*9	rDNA-IGS	*Ypt*1
P. fragariae var. *rubi*	SCRP333		DQ162930 Mt2F-5R 467 bp				DQ162951 Ypt1F-5R 459 bp
P. ilicis	SCRP377		DQ162936 Mt1F-6R 567 bp			DQ163005 Igs2F-6R 382 bp	DQ162962 Ypt1F-5R 463 bp
P. ilicis	SCRP379		DQ162937 Mt1F-6R 568 bp				DQ162963 Ypt1F-5R 463 bp
P. infestans	SC03.26.3.3	DQ162890 Mt13F-3R 250 bp		DQ162855 IgCoxF-R 392 bp	DQ162873 ATPF-R 342 bp	DQ163006 Igs2F-7R 434 bp	DQ162961 Ypt1F-5R 435 bp
P. insolita	SCRP385		DQ162931 Mt1F-6R 340 bp				DQ162974 Ypt1F-5R 430 bp
P. inundata	SCRP644	DQ162902 Mt13F-3R 259 bp	DQ162941 Mt1F-6R 642 bp	DQ162870 IgCoxF-R 424 bp	DQ162882 ATPF-R 335 bp	DQ163007 Igs2F-7R 442 bp	DQ162982 Ypt1F-5R 459 bp
P. inundata	SCRP643		DQ162940 Mt1F-6R 642 bp				DQ162983 Ypt1F-5R 459 bp
P. inundata	SCRP647		DQ162943 Mt1F-6R 600 bp				DQ162984 Ypt1F-5R 459 bp
P. inundata	SCRP649		DQ162942 Mt1F-6R 642 bp				DQ162985 Ypt1F-5R 459 bp
P. katsurae	SCRP388	DQ162904 Mt13F-3R 290 bp	DQ162920 Mt1F-6R 349 bp	DQ162857 IgCoxF-R 411 bp	DQ162877 ATPF-R 344 bp	DQ163008 Igs3F-6R 395 bp	DQ162980 Ypt1F-5R 426 bp
P. kernoviae	SCRP722	DQ162914 Mt13F-3R 279 bp		DQ162872 IgCoxF-R 411 bp			DQ162975 Ypt1F-5R 457 bp
P. lateralis	SCRP390	DQ162912 Mt13F-3R 267 bp	DQ162949 Mt1F-6R 511 bp	DQ162850 IgCoxF-R 408 bp	DQ162878 ATPF-R 324 bp	DQ163009 Igs2F-7R 421 bp	DQ162991 Ypt1F-4R 461 bp
P. medicaginis	SCRP407	DQ162911 Mt13F-3R 253 bp	DQ162923 Mt1F-6R 337 bp	DQ162861 IgCoxF-R 418 bp		DQ163010 Igs2F-7R 421 bp	DQ162990 Ypt1F-5R 458 bp
P. megasperma	SCRP435	DQ162903 Mt13F-3R 270 bp		DQ162871 IgCoxF-R 428 bp	DQ162883 ATPF-R 335 bp	DQ163011 Igs2F-7R 434 bp	DQ162986 Ypt1F-5R 454 bp
P. nemorosa	SCRP910		DQ162938 Mt1F-6R 660 bp	DQ162866 IgCoxF-R 410 bp		DQ163012 Igs2F-7R 382 bp	DQ162965 Ypt1F-5R 463 bp
P. nicotianae	SCRP468	DQ162891 Mt13F-3R 268 bp	DQ162933 Mt1F-6R 440 bp	DQ162856 IgCoxF-R 387 bp	DQ162874 ATPF-R 341 bp	DQ163013 Igs2F-7R 429 bp	DQ162981 Ypt1F-5R 419 bp
P. palmivora	SCRP526	DQ162906 Mt13F-3R 273 bp		DQ162858 IgCoxF-R 390 bp	DQ162875 ATPF-R 345 bp	DQ163014 Igs2F-7R 526 bp	
P. pistaciae	SCRP533		DQ162948 Mt1F-6R 585 bp	DQ162852 IgCoxF-R 414 bp	DQ162887 ATPF-R 352 bp	DQ163015 Igs2F-7R 434 bp	DQ162957 Ypt1F-5R 473 bp
P. pseudosyringae	SCRP674	DQ162907 Mt13F-3R 259 bp		DQ162868 IgCoxF-R 408 bp		DQ163016 Igs1F-6R 382 bp	DQ162966 Ypt1F-5R 472 bp

(continued on next page)

Table 3 (*continued*)

Phytophthora species	Isolates	Mitochondrial DNA				Nuclear DNA	
		*Trn*G–*Trn*Y	*Trn*Y–*Rns*	*Cox*2–*Cox*1	*Atp*9–*Nad*9	rDNA-IGS	*Ypt*1
P. pseudosyringae	SCRP734						DQ162967 Ypt1F-5R 472 bp
P. psychrophila	SCRP630		DQ162939 Mt1F-6R 635 bp	DQ162867 IgCoxF-R 410 bp	DQ162881 ATPF-R 330 bp	DQ163017 Igs2F-7R 405 bp	DQ162964 Ypt1F-5R 477 bp
P. quercina	SCRP541	DQ162905 Mt13F-3R 266 bp	DQ162944 Mt1F-6R 559 bp	DQ162869 IgCoxF-R 428 bp	DQ162876 ATPF-R 337 bp	DQ163018 Igs2F-7R 416 bp	DQ162976 Ypt1F-5R 450 bp
P. quercina	SCRP547		DQ162945 Mt1F-6R 559 bp				DQ162977 Ypt1F-5R 450 bp
P. quercina	SCRP549		DQ162946 Mt1F-6R 559 bp				DQ162978 Ypt1F-5R 450 bp
P. quercina	SCRP550		DQ162947 Mt1F-6R 504 bp				DQ162979 Ypt1F-5R 450 bp
P. ramorum	SCRP911	DQ162913 Mt13F-3R 268 bp	DQ162934 Mt1F-6R 362 bp	DQ162851 IgCoxF-R 408 bp	DQ162879 ATPF-R 322 bp	DQ163019 Igs2F-7R 421 bp	DQ162992 Ypt1F-4R 459 bp
P. sojae	SCRP555	DQ162898 Mt13F-3R 276 bp		DQ162853 IgCoxF-R 413 bp	DQ162888 ATPF-R 344 bp	DQ163020 Igs2F-7R 434 bp	DQ162958 Ypt1F-5R 478 bp

these regions were easily amplified and sequenced however others were more challenging. In some cases even testing multiple primer combinations using different annealing temperatures and different $MgCl_2$ concentrations amplification was not possible probably due to high mutation rates around the primer sites or due to rearrangements in gene order/orientation. Additional mitochondrial regions of potential interest reported by Wattier et al. (2003) or identified during this project in the *Rns*–*Orf* 79 and *Orf* 79–*Cox*2 gene regions were only amplified from a limited number of *Phytophthora* species and therefore not investigated further (data not shown).

Among the analysed regions the *Trn*G–*Trn*Y was the least variable and therefore unsuitable as a target region for the design of species specific diagnostics. Higher levels of sequence diversity were found in the *Atp*9–*Nad*9 region although this region was only amplified and sequenced from a limited number of species (17) (Fig. 2A, D). The occurrence of intraspecific variability in the *Atp*9–*Nad*9 region is reported for *P. infestans* and closely related species (Wattier et al., 2003). More appropriate for identification, taxonomic and phylogenetic studies seems to be the *Cox*2–*Cox*1 region (Fig. 2C). This region can be easily amplified and aligned as the total length is quite similar in all phytophthoras and it has a combination of conserved and more variable portions. This region was utilised to develop a specific molecular method for the detection of *P. ramorum*, *P. nemorosa* and *P. pseudosyringae* in planta (Martin et al., 2004). Of 24 species where multiple isolates were examined, intraspecific polymorphism was not observed for 16 species while 5 species (*P. cactorum*, *P. citricola*, *P. megakarya*, *P. megasperma*, and *P. syringae*) exhibited limited intraspecific polymorphism (http://pwa.ars.usda.gov/salinas/cipru/frank/phyto.htm). Our data demonstrate that the same region has potential for the detection of numerous other species, although limited interspecific diversity was noted among isolates of *P. cambivora*, *P. europaea* and *P. alni* subsp. *alni* and between *P. nemorosa* and *P. psychrophila*. *P. psychrophila* was not included in the panel of species utilised by Martin et al. (2004) to assess specificity of *P. nemorosa* primers.

Of all the mitochondrial regions investigated in this study the one flanked by genes *trn*Y (gua) and *Rns* has the greatest potential to be used as target in the design of molecular detection methods for almost all the *Phytophthora* species examined and likely many more. In particular, this region seems to be ideal to develop assays to discriminate closely related species or even sequence variants for studies on intraspecific variation that cannot be detected using more conserved genomic regions (Fig. 2B). As an example, sufficient diversity was found

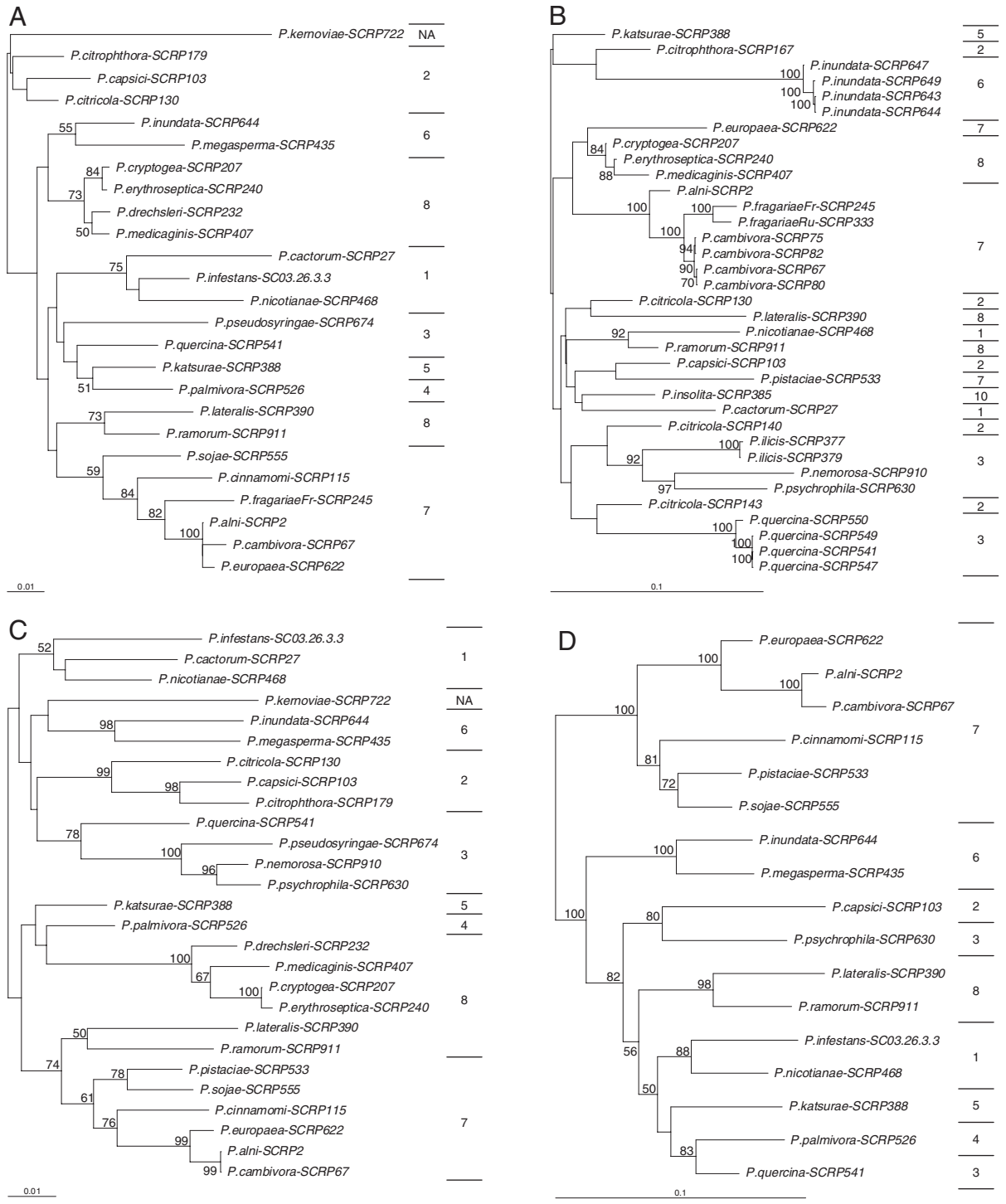

Fig. 2. Detailed phylograms of *Phytophthora* species using DNA sequence data from four spacers between mitochondrial genes: *Trn*G–*Trn*Y (A), *Trn*Y–*Rns* (B), *Cox*2–*Cox*1 (C), and *Atp*9–*Nad*9 (D). Phylograms were constructed after DNA distance-based analysis of each genomic region. The numbers at the branch points indicate the percentages of bootstrap values (based on 500 bootstraps). The numbers in the columns on the right of each phylogram refer to ITS clades as defined by Cooke et al. (2000).

to design specific primers that can distinguish between *P. fragariae* var. *fragariae* and *P. fragariae* var. *rubi* (Schena and Cooke, unpublished data). Obviously intraspecific variation could seriously compromise the suitability of species-specific primer sets. However, careful consideration of target regions and appropriate design and placement of primers followed by assays specific to the study can be carried out to develop detection methods with desired levels of specificity.

The mitochondrial genome is present in multiple copies per cell, thereby improving the sensitivity of the detection system. Furthermore, all mitochondrial regions were amplified using *Phytophthora* universal primers designed on conserved coding genes to amplify the flanked intergenic portion. This organization can enable the development of very sensitive detection methods since genus-specific primers designed in the conserved coding regions can be nested with species-specific primers designed in the intergenic regions. A similar approach shown to be effective in a large number of phytopathogenic fungi (Schena and Ippolito, 2003; Ippolito et al., 2004) and enabled the detection of 2 fg of target DNA with mitochondrial based primers (Martin et al., 2004). A general disadvantage of mitochondrial DNA is the very high AT/GC ratio. In some intergenic regions the AT/GC ratio can easily reach the 80–90% making the design of effective primers quite difficult; however mitochondrial primers with a very high AT/GC ratio were designed for *P. ramorum*, *P. nemorosa* and *P. pseudosyringae* (Martin et al., 2004). Furthermore, mitochondrial DNA is generally more difficult to amplify and requires higher concentration of $MgCl_2$ compared to genomic DNA. Another potential complication of using mitochondrial based marker system for identification of pathogens at the species level is the presence of species hybrids (Brasier et al., 1999; Delcan and Brasier, 2001). The mitochondrial genome is uniparentally inherited; therefore, the hybrids would have a single mitochondrial genome of one of the parents. Depending on which species functioned as the maternal parent and contributed the mitochondria, the use of species-specific primers pair may amplify a diagnostic band indicating the presence of a particular species when, in fact, it is a hybrid. This was observed in natural hybrids of *P. nicotianae* and *P. cactorum*, all of which had the mitochondrial DNA restriction fragment length polymorphism of *P. nicotianae* (Man in 't Veld et al., 1998).

The phylogenetic trees generated from the alignment of the mtDNA-IGS region sequences are presented in Fig. 2. Trees generated from the *Trn*G–*Trn*Y, *Coc*2–*Cox*1 and *Atp*9–*Nad*9 regions showed clustering of taxa that, in general, is concordant with that determined by the analysis of the ITS regions (Cooke et al., 2000) and a combination of different coding genes of nuclear and mitochondrial DNA (Martin and Tooley, 2003a; Kroon et al., 2004) (Fig. 2A, C, D). However, compared to the ITS regions, the higher mutation rate in these regions yielded longer branch lengths between taxa and, in some cases modified the clustering of some major clades. These data indicated that the short variable *Trn*G–*Trn*Y, *Cox*2–*Cox*1 and *Atp*9–*Nad*9 regions are poorly suited for broad scale phylogenetic analysis but can be utilised to improve the resolution of ITS and other reported genomic region for studies focussing on subgroups of more closely related *Phytophthora* species. The *Trn*Y–*Rns* region was too variable to align accurately and the phylogenetic tree is thus inconsistent with those reported from other genomic regions (Fig. 2B). This region cannot be utilised for a broad scale phylogenetic analysis, but is more appropriate for the examination of intraspecific variation and for the analysis of very closely related species. In particular, considering the increasing interest in the origin of newly introduced phytophthoras the *Trn*Y–*Rns* region will likely serve as a very powerful target region for the reconstruction of phylogenetic history of isolates of a species in relation to their geographic origin. Such phylogeographic analyses will aid in the reconstruction of pathways of global pathogen spread. In *P. quercina*, for example, sequencing this region would add valuable details to the recent analysis based on AFLP's (Cooke et al., 2005).

Table 4
Summary table showing results of sequence analyses

Amplified fragments	No. of sequences	Alignment length	Accession numbers [a]	No. of phylogenetic informative sites	Nucleotides (%)				Average pairwise distance
					A	T	G	C	
*Trn*G–*Trn*Y	25	318	73917338	103	31.3	35.9	19.8	13.1	0.12
*Trn*Y–*Rns*	35	674	73917364	482	38.6	39.0	11.6	10.8	0.68
*Cox*2–*Cox*1	27	445	73917175	151	42.2	39.1	10.7	8.0	0.12
*Atp*9–*Nad*9	17	370	73917285	151	41.8	47.2	3.8	7.2	0.31
rDNA-IGS	28	472	73917497	259	12.9	30.2	37.5	19.4	0.27
*Ypt*1	43	512	73917404	318	24.5	21.6	28.8	25.1	0.81

[a] GenBank accession numbers for the Popsets associated with each amplified fragment.

3.2. Amplification and analysis of a fragment of the intergenic spacer region of the rDNA (rDNA-IGS)

The results of the analysis of the rDNA-IGS fragment are summarised in Table 4. This fragment was amplified using a combination of 5 different primers from 28 different species (Fig. 1, Tables 2 and 3). The analysed sequences had an average AT/GC ratio of 43.1%, a length ranging from 382 to 526 bp (Table 3) and produced an alignment of 472 bp. Approximately half of the sites (259) were phylogenetically informative and the average pairwise distance was 0.27. Alignment and comparison of the sequences of this region of the IGS showed a level of polymorphism comparable to that observed in the ITS1 and ITS2 regions (Fig. 3A).

The IGS1 and IGS2 regions have great potential since, like the ITS regions, they are multicopy (up to 200 copies per haploid genome) (Bruns et al., 1991) and their length (4000–5000 bp) provides considerable scope for primer development. However, their utilisation as targets to develop specific molecular markers has been limited mainly because of the difficulties related to the amplification of a long fragment (4000–5000 bp) and the lack of effective universal primers. The present study provides such a set of universal primers for the amplification of a short fragment that can be easily sequenced and characterised from a large number of *Phytophthora* species. The sequences from 28 different *Phytophthora* species reported in this study are an important starting point to facilitate the amplification of the same region from other *Phytophthora* species and the amplification and characterization of the potentially more variable flanking regions. Furthermore these 28 sequences represent an important advance as they can be used to develop molecular diagnostics for important species such as *P. quercina*. The sequence variation is not, however, sufficient to allow specific assays for all the *Phytophthora* species included in the present study. Specific primers to detect *P. medicaginis* were developed on the IGS2 region because the ITS regions were not sufficiently polymorphic to allow the discrimination of closely related species (Liew et al., 1998).

The phylogenetic tree generated from the alignment of the rDNA-IGS region sequences (Fig. 3A) matched closely that based on ITS analysis (Cooke et al., 2000) with clades 1–5 grouping together and the non-papillate taxa in clades 7 and 8 at a basal position in the tree.

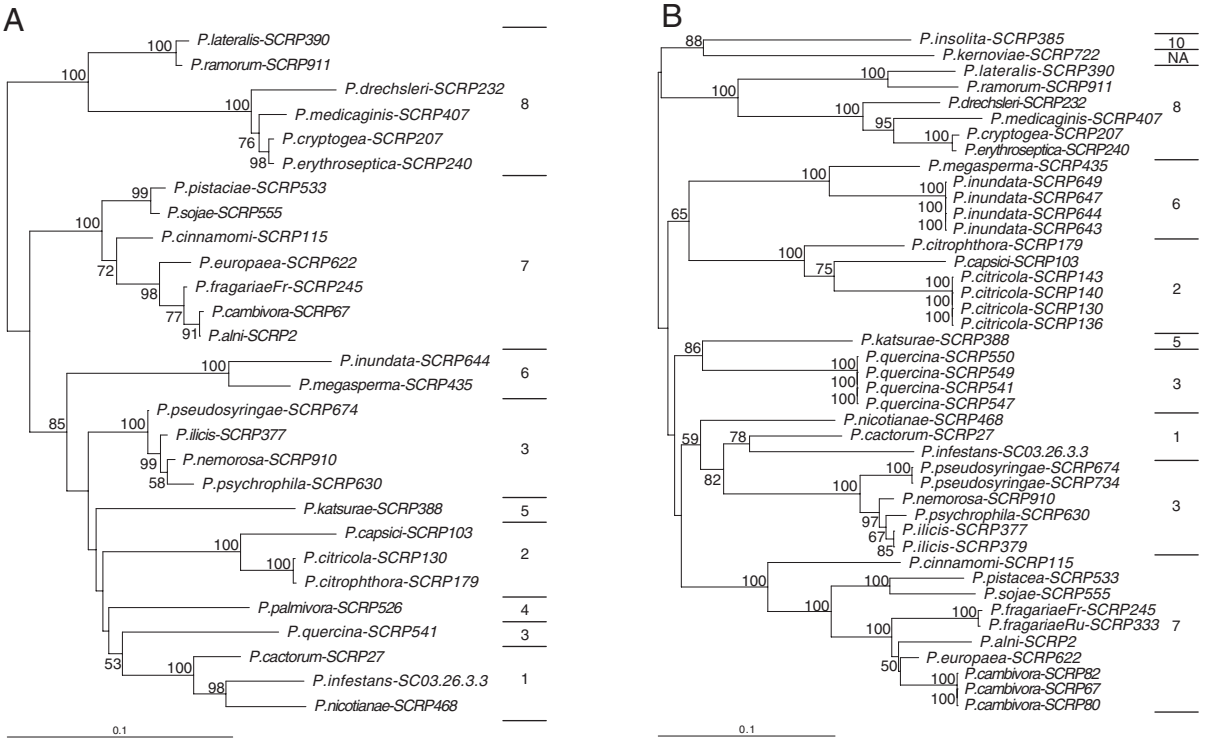

Fig. 3. Detailed phylograms of *Phytophthora* species using DNA sequence data from a fragment of the intergenic spacers (IGS) region (A) and a fragment of the ras-related protein (*Ypt*1) gene (B). Phylograms were constructed after DNA distance-based analysis of each genomic region. The numbers at the branch points indicate the percentages of bootstrap values (based on 500 bootstraps). The numbers in the columns on the right of each phylogram refer to ITS clades as defined by Cooke et al. (2000).

3.3. Amplification and analysis of a fragment of the ras-related protein (Ypt1) gene

Results of the analysis of the *Ypt*1 gene are summarised in Table 4. This gene was amplified using a combination of 3 different primers from 43 different isolates (29 species) (Fig. 1, Tables 2 and 3). Amplified fragments comprised 2 small portions of exons flanking 2 exons and 3 introns (introns 3, 4, 5) (Fig. 1). Sequences had an average AT/GC ratio of 46.1% a length ranging from 419 to 478 bp (Table 3) and produced an alignment of 512 bp (Fig. 3). Two thirds of the sites (318) were phylogenetically informative and the average pairwise distance was 0.81.

Alignment of the *Ypt*1 gene sequences obtained from different species reveals the presence of conserved coding regions flanking very variable introns (Fig. 4). This organization, was expected as the *Ypt*1 gene is similar to eukaryotic genes, but dissimilar to other *P. infestans* genes in containing introns (Chen and Roxby, 1996). Introns have high potential as targets for specific molecular detection methods. The highly polymorphic nature of these regions enables the differentiation of closely related species such as *P. pseudosyringae*, *P. nemorosa*, *P. psychrophila*, and *P. ilicis* that have almost identical ITS regions (Fig. 3B). Similarly, sufficient polymorphism is available among *P. cambivora*, *P. alni* subsp. *alni*, *P. europaea* and *P. fragariae* and between *P. ramorum* and *P. lateralis*. Furthermore, the levels of sequence diversity appear sufficient to design species-specific primers for other pathogens known to cause diseases on forest trees such as *P. inundata*, *P. megasperma*, *P. cinnamomi*, *P. kernoviae*, *P. citricola*, *P. cactorum* and likely many more. It should also be considered that in the present work only a portion of the *Ypt*1 gene was investigated and that the entire gene could provide additional potential for species discrimination (Fig. 1). In the 6 species where multiple isolates were examined (*P. inundata*, *P. citricola*, *P. quercina*, *P. pseudosyringae*, *P. ilicis*, and *P. cambivora*) intraspecific polymorphism was not observed. A single polymorphic nucleotide was identified comparing the sequence of the European isolate of *P. ramorum* utilised in the present research and the sequence available from the genome sequencing project (http://genome.jgi-psf.org/-scaffold_16|280386|282150). Although other isolates need to be sequenced and analysed to confirm the lack of intraspecific polymorphism, this data suggests that the *Ypt*1 gene is not subject to intraspecific variation that could cause problems for diagnostic assays.

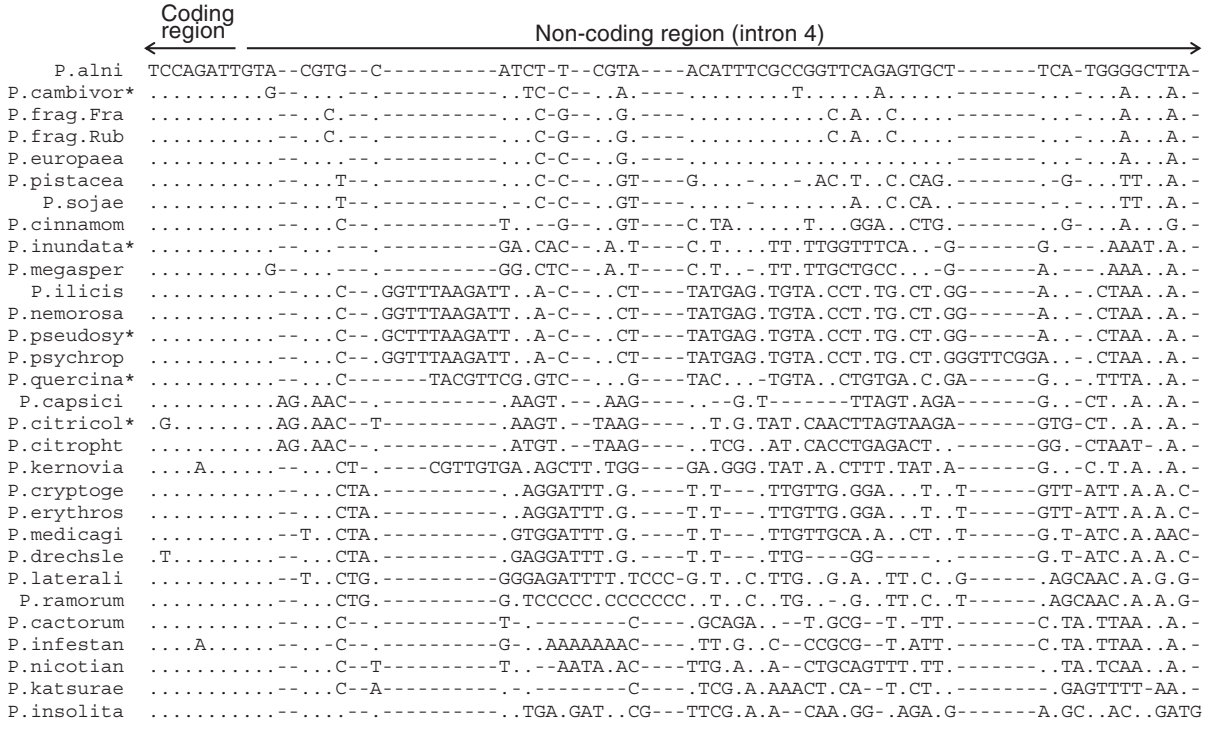

Fig. 4. Example of a DNA sequence alignment of a section of the ras-related protein (*Ypt*1) gene comprising part of intron 4 and part of the preceding coding region. In all species where multiple isolates were sequenced (*), no intraspecific polymorphism was observed (. sequence identical to and – deletion from that of *P. alni*).

A DNA fragment corresponding to the ones sequenced in the present research for *Phytophthora* has been recently amplified and sequenced from 12 different *Pythium* species (Moorman et al., 2002). Alignment of all *Phytophthora* and *Pythium* fragments revealed the possibility of using coding regions to design *Phytophthora*-specific primers (data not shown). Such an assay will be of great benefit in the study of Phytophthoras in forests and natural ecosystems. Recent surveys have shown that *Phytophthora* species are frequently in 'clusters', on the same sites or sometimes even same tree (Vettraino et al., 2002, 2005). *Phytophthora*-specific primers can give important information about the abundance of *Phytophthora* species in a particular habitat and combined with sequencing of cloned amplified fragments will likely facilitate the identification of new species. Furthermore, the genetic structure of the *Ypt*1 gene (alternate conserved and variable regions) enables the development of nested approaches in which a first round with genus-specific primers is combined with a second round with species-specific primers. Compared to other available target sequences the *Ypt*1 gene has the enormous advantage to enable the design of all specific primers in a limited DNA region. This aspect enables the use of a common amplified product from the first amplification as template for all nested specific primers with a significant reduction of times and costs for analyses. A concern in PCR detection of a pathogen in planta and natural ecosystems is the low concentration of the pathogen DNA that may result in the masking of the pathogen presence. PCR, however, is known to be extremely sensitive, and capable of detecting a single molecule of template DNA. Compared to the rDNA genes the *Ypt*1 gene has the disadvantage of being a single copy gene (Chen and Roxby, 1996). However, the detection limit of a single amplification with primers designed on the *Ypt*1 gene was 10 pg of target DNA and was increased by 100-fold (to 100 fg) combining this primers with a first round amplification with genus-specific primer (Schena and Cooke, personal communication). Similar detection limits have been reported for a number of *Phytophthora* species and shown to be sufficient to detect low pathogen concentrations. Detection limits ranging from 2 to 100 pg enabled the detection of *P. cambivora*, *P. quercina* and *P. citricola* in seedlings of pedunculate oaks and European beech (Schubert et al., 1999).

The *Ypt*1 gene has been studied extensively because of its important roles in a large number of very diverse organisms (Segev and Botstein, 1987; McCormick, 1995), however its use to date as molecular marker to identify species has been limited to some *Pythium* species (Moorman et al., 2002). In the study of Moorman et al. (2002) the *Ypt*1 gene sequences were found less informative than those of the ITS regions and therefore not useful in *Pythium* identification. Why the *Ypt*1 gene is very variable in *Phytophthora* and quite conserved in *Pythium* is a matter worthy of further investigations. What we already know is that the intron 4 found in *P. infestans* (Chen and Roxby, 1996) and during this research in all *Phytophthora* species was not found in several species of *Pythium* (Moorman et al., 2002).

In conclusion, in the present study six genomic regions were amplified and sequenced from a large number of *Phytophthora* species and their potential use for a range of applications such as diagnostics was assessed. Key studies on the inter- and intraspecific variation remain, however the detailed groundwork needed to amplify these regions from such a diverse collection of species has been completed and a foundation laid for future research. The comprehensive dataset generated offers great potential for the identification, detection and study of the molecular evolution of *Phytophthora* species. This comprehensive dataset integrates, strengthens and improves information provided by other recently studied genomic and mitochondrial regions and provides an important foundation for future research in this highly damaging group of plant pathogens.

Acknowledgments

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 C.M. Brasier for providing isolates, N. Williams for invaluable help with the culture collection and J.M. Duncan for his support in establishing the co-operation between the Department of Plant Protection and Applied Microbiology and the Scottish Crop Research Institute. Cultures were held under SASA License No. PH/37/2005.

References

Brasier, C.M., Cooke, D.E.L., Duncan, J.M., 1999. Origin of a new *Phytophthora* pathogen through interspecific hybridization. Proc. Natl. Acad. Sci. U. S. A. 96, 5878–5883.

Brasier, C.M., Sanchez-Hernandez, E., Kirk, S.A., 2003. *Phytophthora inundata* sp. nov., a part heterothallic of trees and shrubs in wet or flooded soils. Mycol. Res. 107, 477–484.

Brasier, C., Denman, S., Brown, A., Webber, J., 2004a. Sudden oak death (*Phytophthora ramorum*) discovered on trees in Europe. Mycol. Res. 108, 1107–1110.

Brasier, C.M., Kirk, S.A., Delcan, J., Cooke, D.E.L., Jung, T., Man In't Veld, W.A., 2004b. *Phytophthora alni* sp. nov. and its variants: designation of emerging heteroploid hybrid pathogens spreading on *Alnus* trees. Mycol. Res. 108, 1172–1184.

Brasier, C.M., Beales, P.A., Kirk, S.A., Denman, S., Rose, J., 2005. *Phytophthora kernoviae* sp. nov., an invasive pathogen causing

bleeding stem lesions on forest trees and foliar necrosis of ornamentals in Britain. Mycol. Res. 109, 853–859.
Bruns, T.D., White, T.J., Taylor, J.W., 1991. Fungal molecular systematics. Ann. Rev. Ecolog. Syst. 22, 525–564.
Chen, Y., Roxby, R., 1996. Characterization of a *Phytophthora infestans* gene involved in the vesicle transport. Gene 181, 89–94.
Cooke, D.E.L., Drenth, A., Duncan, J.M., Wagels, G., Brasier, C.M., 2000. A molecular phylogeny of *Phytophthora* and related *Oomycetes*. Fungal Genet. Biol. 30, 17–32.
Cooke, D.E.L., Jung, T., Williams, N.A., Schubert, R., Bahnweg, G., Oßwald, W., Duncan, J.M., 1999. Molecular evidence supports *Phytophthora quercina* as distinct species. Mycol. Res. 103, 799–804.
Cooke, D.E.L., Jung, T., Williams, N.A., Schubert, R., Oßwald, W., Duncan, J.M., 2005. Genetic diversity of European populations of the oak fine-root pathogen *Phytophthora quercina*. For. Pathol. 35, 57–70.
Corpet, F., 1988. Multiple sequence alignment with hierarchical clustering. Nucleic Acids Res. 16, 10881–10890.
Delcan, J., Brasier, C.M., 2001. Oospore viability and variation in zoospore and hyphal tip derivatives of the hybrid alder phytophthoras. For. Pathol. 31, 65–83.
Elliott, C.G., Hendrie, M.R., Knights, B.A., 1966. The sterol requirement of *Phytophthora cactorum*. J. Gen. Microbiol. 42, 425–435.
Goldman, N., Anderson, J.P, Rodrigo, A.G., 2000. Likelihood-based tests of topologies in phylogenetics. Syst. Biol. 49, 652–670.
Hansen, E.M., Parke, J.L., Sutton, W., 2005. Susceptibility of Oregon forest trees and shrubs to *Phytophthora ramorum*: a comparison of artificial inoculation and natural infection. Plant Dis. 89, 63–70.
Hansen, E.M., Reeser, P.W., Davidson, J.M., Garbelotto, M., Ivors, K., Douhan, L., Rizzo, D.M., 2003. *Phytophthora nemorosa*, a new species causing cankers and leaf blight of forest trees in California and Oregon, U.S.A. Mycotaxon 88, 129–138.
Hayden, K.J., 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, 1075–1083.
Ippolito, A., Schena, L., Soleti Ligorio, V., Yaseen, T., Nigro, F., 2004. Real-time detection of *Phytophthora nicotianae* and *P. citrophthora* in citrus roots and soils. Eur. J. Plant Pathol. 110, 833–843.
Judelson, H.S., Blanco, F.A., 2005. The spores of *Phytophthora*: weapons of the plant destroyer. Nat. Rev., Microbiol. 3, 47–58.
Jung, T., Cooke, D.E.L., Blaschke, H., Duncan, J.M., Oßwald, W., 1999. *Phytophthora quercina* sp. nov., causing root rot of European oaks. Mycol. Res. 103, 785–798.
Jung, T., Hansen, E.M., Winton, L., Oßwald, W., Delatour, C., 2002. Three new species of *Phytophthora* from European oak forests. Mycol. Res. 106, 397–411.
Jung, T., Nechwatal, J., Cooke, D.E.L., Hartmann, G., Blaschke, M., Oßwald, W.F., Duncan, J.M., Delatour, C., 2003. *Phytophthora pseudosyringae* sp. nov., a new species causing root and collar rot of deciduous tree species in Europe. Mycol. Res. 107, 772–789.
Kong, P., Hong, C.X., Richardson, P.A., 2003a. Rapid detection of *Phytophthora cinnamomi* using PCR with primers derived from the Lpv putative storage protein genes. Plant Pathol. 52, 681–693.
Kong, P., Hong, C.X., Jeffers, S.N., Richardson, P.A., 2003b. A species-specific polymerase chain reaction assay for rapid detection of *Phytophthora nicotianae* in irrigation water. Phytopathology 93, 822–831.
Kong, P., Hong, C.X., Tooley, P.W., Ivors, K., Garbelotto, M., Richardson, P.A., 2004. Rapid identification of *Phytophthora ramorum* using PCR-SSCP analysis of ribosomal DNA ITS-1. Lett. Appl. Microbiol. 38, 433–439.
Kroon, L.P.N.M., Bakker, F.T., van den Bosch, G.B.M., Bonants, P.J.M., Flier, W.G., 2004. Phylogenetic analysis of *Phytophthora* species based on mitochondrial and nuclear DNA sequences. Fungal Genet. Biol. 41, 766–782.
Liew, E.C.Y., Maclean, D.J., Irwin, J.A.G., 1998. Specific PCR based detection of *Phytophthora medicaginis* using the intergenic spacer region of the ribosomal DNA. Mycol. Res. 102, 73–80.
Man in 't Veld, W.A., Veenbaas-Rijks, W.J., Ilieva, E., de Cock, A.W.A.M., Bonants, P.J.M., Pieters, R., 1998. Natural hybrids of *Phytophthora nicotianae* and *Phytophthora cactorum* demonstrated by isozyme analysis and random amplified polymorphic DNA. Phytopathology 88, 922–929.
Martin, F.N., Tooley, P.W., 2003a. Phylogenetic relationships among *Phytophthora* species inferred from sequence analysis of mitochondrially encoded cytochrome oxidase I and II genes. Mycologia 95, 269–284.
Martin, F.N., Tooley, P.W., 2003b. Phylogenetic relationships of *Phytophthora ramorum*, *P. nemorosa*, and *P. pseudosyringae*, three species recovered from areas in California with sudden oak death. Mycol. Res. 107, 1379–1391.
Martin, F.N., Tooley, P.W., Blomquist, C., 2004. Molecular detection of *Phytophthora ramorum*, the causal agent of sudden oak death in California, and two additional species commonly recovered from diseased plant material. Phytopathology 94, 621–631.
McCormick, F., 1995. Ras-related proteins in signal transduction and growth control. Mol. Reprod. Dev. 42, 500–506.
Milne, I., Wright, F., Rowe, G., Marshal, D.F., Husmeier, D., McGuire, G., 2004. TOPALi: software for automatic identification of recombinant sequences within DNA multiple alignments. Bioinformatics 20, 1806–1807.
Moorman, G.W., Kang, S., Geiser, D.M., Kim, S.H., 2002. Identification and characterization of *Pythium* species associated with greenhouse floral crops in Pennsylvania. Plant Dis. 86, 1227–1231.
Nechwatal, J., Schlenzing, A., Jung, T., Cooke, D.E.L., Duncan, J.M., Oßwald, W.F., 2001. A combination of baiting and PCR techniques for the detection of *Phytophthora quercina* and *P. citricola* in soil samples from oak stands. For. Pathol. 31, 85–97.
Paquin, B., Laforest, M.J., Forget, L., Roewer, I., Wang, Z., Longcore, J., Lang, B.F., 1997. The fungal mitochondrial genome project: evolution of fungal mitochondrial genomes and their gene expression. Curr. Genet. 31, 380–395.
Rizzo, D.M., Garbelotto, M., 2003. Sudden oak death: endangering California and Oregon forest ecosystems. Front. Ecol. Environ. 1, 197–204.
Rozen, S., Skaletsky, H.J., 2000. Primer3 on the WWW for general users and for biologist programmers. In: Krawetz, S., Misener, S. (Eds.), Bioinformatics Methods and Protocols: Methods in Molecular Biology. Humana Press, Totowa, NJ, pp. 365–386.
Sachay, D.J., Hudspeth, D.S.S., Nadler, S.A., Hudspeth, M.E.S., 1993. Oomycete mtDNA: *Phytophthora* genes for cytochrome c oxidase use an unmodified genetic code and encode proteins most similar to plants. Exp. Mycol. 17, 7–23.
Schena, L., Ippolito, A., 2003. Rapid and sensitive detection of *Rosellinia necatrix* in roots and soils by real time Scorpion-PCR. J. Plant Pathol. 85, 15–25.
Schena, L., Nigro, F., Ippolito, A., Gallitelli, D., 2004. Real-time quantitative PCR: a new technology to detect and study phytopathogenic and antagonistic fungi. Eur. J. Plant Pathol. 110, 893–908.
Schubert, R., Bahnweg, G., Nechwatal, J., Jung, T., Cooke, D.E.L., Duncan, J.M., Müller-Starck, G., Langebartels, C., Sandermann

Jr., H., Oßwald, W.F., 1999. Detection and quantification of *Phytophthora* species which are associated with root-rot diseases in European deciduous forests by species-specific polymerase chain reaction. For. Pathol. 29, 169–188.

Segev, N., Botstein, D., 1987. The ras-like yeast *Ypt*1 gene is itself essential for growth, sporulation, and starvation response. Mol. Cell. Biol. 7, 2367–2377.

Shearer, B.L., Crane, C.E., Cochrane, A., 2004. Quantification of the susceptibility of the native flora of the south-west Botanical Province, western Australia, to *Phytophthora cinnamomi*. Aust. J. Bot. 52, 435–443.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 24, 4876–4882.

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

Vettraino, A.M., Barzanti, G.P., Bianco, M.C., Ragazzi, A., Capretti, P., Paoletti, E., Luisi, N., Anselmi, N., Tannini, A., 2002. Occurrence of *Phytophthora* species in oak stands in Italy and their association with declining oak trees. For. Pathol. 32, 19–28.

Vettraino, A.M., 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. Eur. J. Plant Pathol. 111, 169–180.

Wattier, R.A.M., Gathercole, L.L., Assinder, S.J., Gliddon, C.J., Deahl, K.L., Shaw, D.S., Mills, D.I., 2003. Sequence variation of intergenic mitochondrial DNA spacers (mtDNA-IGS) of *Phytophthora infestans* (Oomycetes) and related species. Mol. Ecol. Notes 3, 136–138.

Werres, S., Marwitz, R., Man in 't Veld, W.A., De Cock, A.W.A.M., Bonants, P.J.M., De Weerdt, M., Themann, K., Ilieva, E., Baayen, R.P., 2001. *Phytophthora ramorum* sp. nov., a new pathogen on *Rhododendron* and *Viburnum*. Mycol. Res. 105, 1155–1165.

White, T.J., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.), PCR Protocols: a Guide to Methods and Applications. Academic Press, Inc., New York, USA, pp. 315–322.

Winton, L.M., Hansen, E.M., 2001. Molecular diagnosis of *Phytophthora lateralis* in trees, water, and foliage baits using multiplex polymerase chain reaction. For. Pathol. 31, 275–283.