Journal of Microbiological Methods 88 (2012) 356–368

Contents lists available at SciVerse ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

A molecular method to assess *Phytophthora* diversity in environmental samples

Silvia Scibetta [a,b], Leonardo Schena [a], Antonio Chimento [b,c], Santa O. Cacciola [b], David E.L. Cooke [d,*]

[a] Dipartimento di Gestione dei Sistemi Agrari e Forestali, Università Mediterranea di Reggio Calabria, Località Feo di Vito, 89124 Reggio Calabria, Italy
[b] Dipartimento di Scienze del Farmaco, Università di Catania, Viale Andrea Doria 6, 95125 Catania, Italy
[c] Dipartimento DEMETRA, Università di Palermo, Viale delle Scienze 2, 90128 Palermo, Italy
[d] The James Hutton Institute, Dundee, DD2 5DA, UK

ARTICLE INFO

Article history:
Received 26 July 2011
Received in revised form 15 December 2011
Accepted 18 December 2011
Available online 23 December 2011

Keywords:
Phytophthora spp.
Molecular detection
Metagenomic analyses
Genus-specific primers
Natural ecosystems

ABSTRACT

Current molecular detection methods for the genus *Phytophthora* are specific to a few key species rather than the whole genus and this is a recognized weakness of protocols for ecological studies and international plant health legislation. In the present study a molecular approach was developed to detect *Phytophthora* species in soil and water samples using novel sets of genus-specific primers designed against the internal transcribed spacer (ITS) regions. Two different rDNA primer sets were tested: one assay amplified a long product including the ITS1, 5.8S and ITS2 regions (LP) and the other a shorter product including the ITS1 only (SP). Both assays specifically amplified products from *Phytophthora* species without cross-reaction with the related *Pythium s. lato*, however the SP assay proved the more sensitive and reliable. The method was validated using woodland soil and stream water from Invergowrie, Scotland. On-site use of a knapsack sprayer and in-line water filters proved more rapid and effective than centrifugation at sampling *Phytophthora* propagules. A total of 15 different *Phytophthora* phylotypes were identified which clustered within the reported ITS-clades 1, 2, 3, 6, 7 and 8. The range and type of the sequences detected varied from sample to sample and up to three and five different *Phytophthora* phylotypes were detected within a single sample of soil or water, respectively. The most frequently detected sequences were related to members of ITS-clade 6 (i.e. *P. gonapodyides*-like). The new method proved very effective at discriminating multiple species in a given sample and can also detect as yet unknown species. The reported primers and methods will prove valuable for ecological studies, biosecurity and commercial plant, soil or water (e.g. irrigation water) testing as well as the wider metagenomic sampling of this fascinating component of microbial pathogen diversity.

1. Introduction

As an important and widespread genus of plant pathogens *Phytophthora* is responsible for devastating diseases of dicotyledonous plants in crop/forest systems, horticulture and ornamental nurseries worldwide. In the past decade, surveys in natural ecosystems have recorded an increasing range of *Phytophthora* species involved in extensive decline and mortality phenomena in temperate, boreal and Mediterranean forest habitats, particularly in native vegetation where the pathogen has been inadvertently introduced. Examples include *P. ramorum*, a recently described species, responsible for a disease, referred to as "sudden oak death" (SOD) and "ramorum blight" that has severely damaged native Californian forests and caused leaf blights or dieback on under-story shrubs and ornamental nursery stock (Rizzo et al., 2005). In Europe, *P. ramorum* has been frequently reported in ornamental nurseries, from Rhododendron in natural and semi-natural ecosystems, some forest trees and is the cause of serious landscape-scale damage to Japanese larch plantations in the UK (Brasier et al., 2004a; Brasier and Webber, 2010). A second new species, *P. kernoviae*, has been described, causing bleeding lesions on beech (Brasier et al., 2005). *P. alni* (Brasier et al., 2004b) and *P. quercina* (Jung et al., 1999) are damaging to alder and oak, respectively and many other destructive *Phytophthora* species have been described (Jung et al., 2002, 2003; Rea et al., 2011). The recent decline and mortality of beech forests in Central Europe is mainly caused by *P. cactorum*, *P. cambivora* and *P. plurivora* (Jung, 2009; Jung and Burgess, 2009) while extensive dieback and mortality of *Castanea* forests and orchards (ink disease) across southern and western Europe is caused by the introduction of *P. cambivora* and *P. cinnamomi* (Vettraino et al., 2005). The ecological role of some taxa, such as *P. gonapodyides* (Brasier et al., 2003a; Reeser et al., 2011), which may be locally abundant but cause no obvious plant disease symptoms, is unclear. The recent expansion in the range and spread of species in natural ecosystems highlights limitations in our understanding of the taxonomy, diversity and dynamics of *Phytophthora* spp. in natural and managed ecosystems. There is thus an interest in investigating the relationship between taxonomic and

* Corresponding author at: The James Hutton Institute, Invergowrie, Dundee, DD2 5DA, UK. Tel.: +44 844 928 5428; fax: +44 844 928 5429.
E-mail addresses: silvia.scibetta@hotmail.it (S. Scibetta), lschena@unirc.it (L. Schena), ant_chimento@yahoo.it (A. Chimento), olgacacciola@unict.it (S.O. Cacciola), david.cooke@hutton.ac.uk (D.E.L. Cooke).

0167-7012/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.mimet.2011.12.012

functional diversity, stability and adaptability of plant hosts and their pathogens and how such knowledge could be used to understand the threats posed by pathogens such as *Phytophthora* to natural ecosystems (e.g. Mangan et al., 2010) and commercial plant production. Methodology has been a constraint on the characterization and analysis of *Phytophthora* species composition, diversity and genetic structure (Cooke et al., 2007). Technologies are required for direct detection in organic substrates, the plant host, soil or water in a relatively rapid assay and from samples of a meaningful size (Martin and English, 1997). Culture-based detection, often preceded by baiting with susceptible plant material, is the most commonly applied technique to assess the diversity of *Phytophthora* in natural communities (Jung et al., 2000; Jung, 2009; Vettraino et al., 2005; Davidson et al., 2005). The possibility of sampling bias introduced by the bait material or the isolation media may be a limitation of this method. For example, host preference among specific *Phytophthora* species and the chosen bait plant or competition for the resource offered by the bait may favor a few host-specific or dominant fast-growing *Phytophthora* species. Furthermore, the system is dependent on species producing zoospores under the baiting conditions and may leave dormant oospore populations undetected (Dick, 1966). A range of selective media have been used to isolate *Phytophthora* species from complex environmental samples (Erwin and Ribeiro, 1996). The antibacterial and antifungal chemicals which allow the growth of most *Phytophthora* species and inhibit other micro-organisms can also be toxic to a number of species of *Phytophthora* and could thus underestimate or skew measures of *Phytophthora* diversity. Baiting and isolation are also very time-consuming, which can limit the frequency and range of sampling that is practical. *Phytophthora* activity may fluctuate widely, according to environmental conditions or host availability, from dormancy to high inoculum density in a very short time (Davison and Tay, 2005); therefore, a more rapid and sensitive method for routine sampling would offer many advantages. Lastly, identification by morphological and cultural criteria is a lengthy process that can fail to discriminate some taxa (Jung et al., 2002; Brasier et al., 2003b; Jung and Burgess, 2009; Reeser et al., 2011).

In the past 15 years, DNA sequence analysis has greatly contributed to our understanding of the diversity and phylogenetic relationships in the *Phytophthora* genus. The internal transcribed spacer (ITS) regions of the rRNA gene continue to prove a valuable target for the design of many conventional and real time PCR detection assays that have complemented or replaced isolation and baiting techniques (Lee and Taylor, 1992; Cooke et al., 2000; Förster et al., 2000; Kong et al., 2003; Hughes et al., 2006; Blair et al., 2008). Such molecular assays, however, are geared to the detection of one, or very few, specific known target species (Schena et al., 2008), and, therefore unsuitable in cases where multiple or, as yet undescribed, *Phytophthora* spp. are present. This latter point is recognized as a major weakness of protocols used in international plant health legislation (Brasier, 2008).

Metagenomic DNA sequencing approaches, based on PCR amplification of single target regions such as rDNA or direct sequencing of gDNA libraries have been used to investigate “in situ” microbial communities in a range of terrestrial (e.g. Torsvik et al., 2002; Lim et al., 2010) and marine (Massana et al., 2004; Savin et al., 2004) habitats. These studies have revealed unexpectedly diverse populations and highlighted the existence of previously unknown lineages, with important evolutionary and ecological implications. Similar discoveries have also been made from the environmental sampling of fungi and oomycetes (Vandenkoornhuyse et al., 2002; Schadt et al., 2003; Jones et al., 2011; Arcate et al., 2006) demonstrating the power of such methodology and confirming that rDNA sequence diversity is a valid measure of the occurrence and distribution of phylogenetic types in natural communities. Next Generation Sequencing (NGS) is dramatically increasing the power of this approach (Lim et al., 2010; Delmont et al., 2011; Ekblom and Galindo, 2011) as a means of linking species diversity and ecosystem function.

We have sought to combine detection and identification assays into a single process to overcome some of their limitations. A time and cost-efficient molecular approach suitable for extensive application to assess the range of *Phytophthora* spp. present in environmental samples has been developed and validated. The method is based on molecular analysis by semi-nested PCR of *Phytophthora* DNA from environmental samples, cloning to construct libraries of rDNA ITS fragments and their sequence analysis (Fig. 1). The design and evaluation of a suitable PCR primer set was critical to enable detection of the genus *Phytophthora* avoiding cross reactions with the closely related *Pythium s. lato* species (Uzuhashi et al., 2010).

2. Materials and methods

2.1. Phytophthora *isolates*

Thirty-six *Phytophthora* isolates (34 species) sourced from the culture collections of the authors were used in this study. Emphasis was placed upon those known to be forest pathogens and representatives of each ITS-based *Phytophthora* clade (Cooke et al., 2000). Isolates were stored on oatmeal agar at 5 °C and grown on French bean agar for routine stock cultures. An additional 9 *Pythium s. lato* species, representative of major clades 3, 4, and 5 (Uzuhashi et al., 2010), were examined (Table 1).

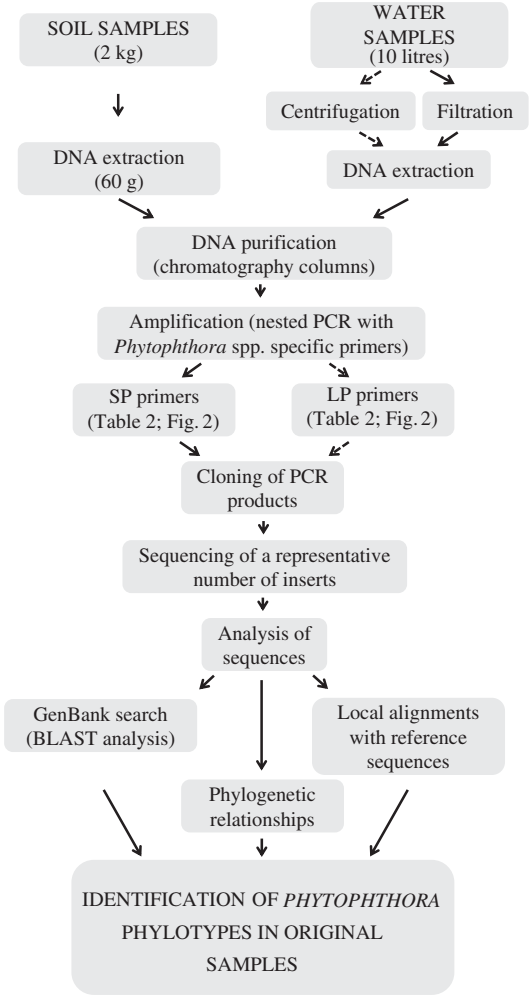

Fig. 1. Schematic representation of the molecular method developed in the present study to monitor the abundance and diversity of *Phytophthora* species in water and soil samples. Broken lines indicate strategies that proved less effective and were not further pursued.

2.2. DNA extractions

DNA extractions from pure cultures of *Phytophthora* spp. and *Pythium s. lato* spp. were made according to the protocol described by Schena and Cooke (2006). Total DNA was suspended in HPLC water and stored at −20 °C. For routine analysis DNA was diluted to 10 ng/μl and maintained at 4 °C.

To extract DNA from soil, published methods (Cullen et al., 2002; Brierley et al., 2009) were slightly modified. After thorough mixing of the 2 kg soil sample, 60 g was taken and shaken at 300 rpm for 5 min in a 250 ml jar containing 120 ml of lysis buffer (0.12 M Na_2HPO_4, 1.5 M NaCl, 2% CTAB hexadecyltrimethylammonium bromide; pH 8) and 12 stainless steel ball bearings (∅ 20 mm) using a ball mill (PM400, Retsch, Germany). The extraction mixture was kept on ice. Three 1.5 ml aliquots of each sample were transferred into 2 ml Eppendorf tubes and the upper phase was recovered by centrifugation at 13,000 rpm for 5 min. DNA was extracted with an equal volume of 100% chloroform, precipitated with isopropanol and washed with 70% ethanol according to standard procedures (Sambrook et al., 1989). DNA was air dried, resuspended in 100 μl of HPLC water and stored at −20 °C.

Two different strategies, centrifugation and filtration, were investigated to extract DNA from propagules in water samples. In the first case, 10 l of water was centrifuged in 200 ml tubes for 10 min at 10,000 rpm with a Sorvall RC-5C PLUS Centrifuge (Du Pont, Newtown, USA). The pellet was then progressively collected in a 2 ml tube by further centrifugations for 15 min at 5000 rpm (GS-15R Centrifuge, Beckman) and 5 min at 10,000 rpm with a bench centrifuge (5415D Eppendorf). The pellet was blended in a Mini-BeadBeater-8 (Bio-Spec Products, Bartlesville, USA), at 5000 rpm for 1 min, with 700 μl of CTAB buffer, 0.6 g of a mix of silica beads (∅ 0.1 and 1 mm) and 2 metal beads (∅ 3 mm) (BioSpec Products Inc.). The upper phase was extracted with 100% chloroform following the same procedure described for soil. DNA was resuspended in 50 μl of HPLC water and stored at −20 °C. To extract DNA from water using the filtration strategy, wet filters were cut into ca. 5 mm squares, subdivided between three 2 ml Eppendorf tubes and freeze-dried for 2 h. Each sub-sample was blended in a Mini-BeadBeater-8 for three 1 min periods at 3000 rpm with 1.5 ml of SDS lysis buffer (200 mM Tris–HCl [pH 8], 250 mM NaCl, 25 mM EDTA, 0.5% SDS) and 0.6 g of a mix of silica beads (∅ 0.1 and 1 mm). Tubes were centrifuged at 13,000 rpm for 15 min and the upper phase was extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and 100% chloroform (−20 °C), respectively. DNA was precipitated and washed as reported for soil and resuspended in 50 μl of HPLC water.

Total DNA from soil was purified through a single chromatography column (Micro Bio-Spin Columns, Bio-Rad, Hercules, CA) filled with 15 mm of polyvinylpolypyrrolidone (PVPP) (Sigma-Aldrich, UK). Before use, the PVPP columns were conditioned by two sequential additions of 150 μl of HPLC water, each followed by a 3 min centrifugation at 5000 rpm. The DNA extracted from water filters was purified using the same columns, but filled with 500 μl of Sepharose 2B (Sigma-Aldrich, UK) (Miller, 2001). All DNA samples (50 μl) were added to the top of the columns and centrifuged for 3 min at 3000 rpm. Purified eluates were collected in a sterile 1.5 ml tube and stored at −20 °C (long-term) or 4 °C (routine use).

Purified environmental DNA samples were analyzed by electrophoresis in 1.5% agarose gel with SYBR Safe™ DNA gel stain (Invitrogen, UK). Nanodrop (Thermo Fisher Scientific Inc.) was used to measure absorbance at 260, 280 and 230 nm and estimate concentration and contamination with protein and humic acid. Furthermore to confirm that all DNA samples were of sufficient quality to be amplified by PCR, 1 μl of each DNA sample (undiluted and ten times diluted) was amplified using the universal primers ITS3–ITS4 (White et al., 1990).

Table 1
Phytophthora and *Pythium s. lato* isolates included in this study.

Species	Isolate codes[a]	Origin		
		Host	Country	Year
Phytophthora alni subsp. *alni*[2]	P669; SCRP2	*Alnus* sp.	UK	1995
	P818; SCRP4	*Alnus* sp.	Germany	1995
	IMI 392317; SCRP8	*Alnus* sp.	France	1996
P. alni subsp. *multiformis*[2]	P770; SCRP3	*Alnus* sp.	Netherlands	1995
P. cactorum	IMI 296524; SCRP27	*Rubus idaeus*	Wales	1985
P. cambivora	IMI 296831; SCRP67	*Rubus idaeus*	Scotland	1985
P. capsici	IMI 352321; SCRP103	*Piper nigrum*	India	1989
P. cinnamomi	CBS270.55; SCRP115	*Chamaecyparis lawsoniana*	Netherlands	1993
P. citricola-like	SCRP130	*Rubus idaeus*	Scotland	1986
P. citrophthora	IMI 332632; SCRP179	*Actinidia chinensis*	Chile	1989
P. cryptogea	IMI 045168; SCRP207	*Lycopersicon esculentum*	New Zealand	1951
P. drechsleri	ATCC46724; SCRP232	*Beta vulgaris*	USA	1935
P. erythroseptica	SCRP240	*Solanum tuberosum*	Netherlands	
P. europaea[1]	CBS109053; SCRP622	*Quercus robur*	Germany	1995
P. fragariae	SCRP245	*Fragaria* × *ananassa*	England	1945
P. rubi	IMI 355974; SCRP333	*Rubus idaeus*	Scotland	1985
P. idaei	CBS968.95; SCRP370	*Rubus idaeus*	Scotland	1985
P. ilicis	SCRP377	*Ilex aquifolium*	UK	1995
P. infestans	SCRP03.26.3.3	*Solanum tuberosum*	Scotland	2003
P. insolita	IMI 288805; SCRP385	Soil	Taiwan	1979
P. inundata	IMI 389751; SCRP644	*Salix* sp.	UK	1972
P. katsurae	SCRP388		France	1996
P. kernoviae[2]	IMI 393170; SCRP722	*Fagus sylvatica*	England	2003
P. lateralis	IMI 040503; SCRP390	*Chamaecyparis* sp.	USA	1942
P. medicaginis	SCRP407	*Medicago* sp.	Iran	1989
P. megasperma	IMI 133317; SCRP435	*Malus sylvestris*	Australia	1968
P. nemorosa[2]	SCRP910			2004
P. nicotianae	IMI 268688; SCRP468	*Citrus* sp.	Trinidad	
P. palmivora	SCRP526	*Hevea brasiliensis*	Thailand	1995
P. pistaciae	IMI 386658; SCRP533	*Pistacia vera*	Iran	1986
P. pseudosyringae	IMI 390500; SCRP674	*Malus pumila*	Italy	2001
P. psychrophila[1]	SCRP630	*Quercus ilex*	France	1996
P. quercina[1]	CBS 784.95; SCRP541	*Quercus robur*	Germany	1995
P. ramorum[2]	SCRP911	*Rhododendron* sp.	Scotland	2004
P. syringae[3]	Ph36	Soil bait	Italy	1999
P. sojae	SCRP555	*Glycine max*	USA	1995
Pythium pyrilobum	IMI 308312			
Py. catenulatum	IMI 323121			
Py. torulosum	IMI 308268	Soil	UK	1981
Py. dissotocum	UQ2623	Root rot	Australia	1988
Py. aphanidermatum	UQ2071	Sugarcane soil	Australia	1992
Globisporangium ultimum	UQ1496	*Euphorbia pulcherrima*	USA	
G. intermedium	SCRP723	Myristica fragrans	Grenada	
G. splendens	IMI 391319			
Elongisporangium undulatum	IMI 337230; SCRP667	*Larix* sp.	Scotland	1989

Isolates kindly provided by [1]Thomas Jung (Brannenburg, Germany), [2]Clive Brasier (Forest Research, Farnham, UK) and [3]Antonio Ippolito (University of Bari, Italy).

[a] Culture collections: SCRP = Scottish Crop Research Institute (http://www.scri.ac.uk/), IMI = CABI (http://www.cabi.org/), CBS = Centraalbureau voor Schimmelcultures (http://www.cbs.knaw.nl/), ATCC = American Type Culture Collection (http://www.lgcstandards-atcc.org/); UQ = University of Queensland.

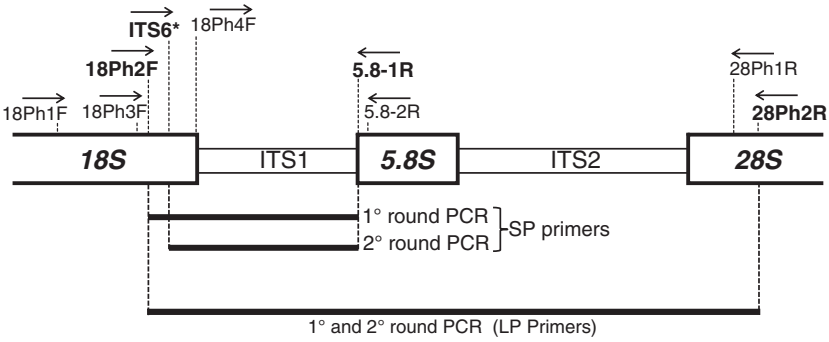

Fig. 2. Schematic representation of the internal transcribed spacer (ITS) regions of the ribosomal DNA (rDNA) with location of primers utilized in this study. Bold highlighted primers are those selected for field surveys. *Primer reported by Cooke et al. (2000).

2.3. PCR reactions

All PCR reactions were carried out in the same conditions using a Primus 96^{plus} Thermalcycler (MWG-Biotech). In nested PCR, the first and second rounds of PCR amplifications were performed in a volume of 15 µl and 25 µl respectively. PCR reaction contained 10 mM Tris–HCl [pH 9], 50 mM KCl, 0.1% Triton X-100, 50 µg BSA, 1.5 mM $MgCl_2$, 40 µM dNTPs, 1 unit of *Taq* polymerase (*Taq* DNA polymerase, Promega Corporation, WI, USA), 0.4 µM of primers and 0.5 µl of DNA (1 µl of the 1st round-product was added in the 2nd round mix). Amplification conditions for *Phytophthora* spp. specific primers consisted of 1 cycle of 95 °C for 2 min, 40 cycles (1st round) or 35 cycles (2nd round) of 95 °C for 20 s, 61 °C for 25 s, 72 °C for 1 min (LP primers) or 30 s (SP primers) and a final cycle of 72 °C for 5 min. Similar amplification conditions were utilized for the universal primers ITS3–ITS4, but a single round PCR was utilized and annealing temperature was reduced to 55 °C.

Amplicons were separated by electrophoresis in 2% agarose gels containing SYBR Safe™ DNA gel stain (Invitrogen), in TBE buffer and visualized on UV light. The size of each band was compared with a 100 bp DNA ladder.

Many precautions were taken to avoid DNA contamination of the PCR reactions. In nested PCR, first and second round PCR reactions were set up in separate laminar flow hoods localized in separate areas. Both laminar flow hoods were cleaned with 0.2 M NaOH and 70% EtOH. A special set of pipettes was maintained only for these amplifications and filter tips (Axygen Scientific, UC, USA) were used at all times. Prior to use, the HPLC water was UV-treated to denature any contaminating nucleic acids (Spectrolinker XL-1500, Spectronics Corporation).

2.4. Design and selection of genus-specific primers

Considerable effort was made to design primers that could be used under standardized conditions to amplify target regions from all *Phytophthora* species but not from other soilborne related genera and, in particular, from ubiquitous species of the genus *Pythium s. lato*. A comprehensive range of ribosomal DNA (rDNA) sequences from *Phytophthora* species and from related Oomycetes in local and GenBank databases were aligned and examined for *Phytophthora*-specific target regions. We did not specifically attempt to exclude the closely related aerial downy mildew taxa such as *Peronospora*.

A number of candidate genus-specific primers were designed against the conserved 18S, 5.8S, and 28S genes and across the ITS1 and ITS2 junctions (Fig. 2; Table 2). Primers were designed to amplify a long product (LP) flanking the ITS1, 5.8S and ITS2 regions or a short product (SP) flanking the ITS1 region. Initial tests were conducted with LP primers. The SP assay was subsequently developed to increase the assay's sensitivity. To this aim, two new reverse primers were designed at the start of the 5.8S region (5.8S-1R and 5.8S-2R) and tested in combination with the previously selected forward primer 18Ph2F or the universal primer ITS6 (Fig. 2).

All primers were designed using the Primer 3 software (Rozen and Skaletsky, 2000) to have the same amplification requirements, with a melting temperature between 58 and 62 °C and an AT/CG ratio between 31.2 and 67.8% (Table 2).

Specificity of all primers was preliminarily evaluated against the whole GenBank database by in silico analyses using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1997). Furthermore, primers were experimentally evaluated by PCR using all possible primer combinations and target DNA from 36 different *Phytophthora* isolates and 9 *Pythium s. lato* species (Table 3).

The sensitivity of selected LP and SP assays was evaluated using one and two rounds of PCR to amplify total DNA extracted from a pure culture of *P. erythroseptica*, serially diluted to concentrations from 10 ng μl^{-1} to 100 ag μl^{-1}. In the case of two rounds of PCR, 1 µl of the amplified product with selected LP primers (18Ph2F/28Ph2R) was re-amplified in a second round PCR with the same primers. In the SP assay, primers 18Ph2F/5.8S-1R and ITS6/5.8S-1R were used in the first and second rounds, respectively.

Table 2
Sequences and features of primers investigated in the present study. Bold highlighted primers are those selected for the analysis of environmental samples.

Primer code	Location	Sense	Length (bp)	Primer sequence (5′–3′)	AT/CG (%)
18Ph1F	18S	Forward	21	CCATTTTTGGTAGGTTTGTGG	57.1
18Ph2F	**18S**	**Forward**	**24**	**GGATAGACTGTTGCAATTTTCAGT**	**62.2**
18Ph3F	18S	Forward	25	TAGACTGTTGCAATTTTCAGTCTTG	64.0
18Ph4F	18S	Forward	22	TGGATTGATGGGAACTTTTTTA	68.8
ITS6[a]	**18S**	**Forward**	**21**	**GAAGGTGAAGTCGTAACAAGG**	**52.4**
28Ph1R	28S	Reverse	16	CAAGCGCCCACGCTGA	31.2
28Ph2R	**28S**	**Reverse**	**18**	**AAGGAACTTGCCCCAAGC**	**44.4**
5.8S-1R	**5.8S**	**Reverse**	**20**	**GCARRGACTTTCGTCCCYRC**	**35.0–50.0**
5.8S-2R	5.8S	Reverse	28	GAAAGTTGCTATCTAGTTAAAAGCARRG	60.7–67.8

[a] Primer reported by Cooke et al. (2000).

Table 3
Results of specificity tests conducted with *Phytophthora*-genus-specific primers using DNA from 34 species of *Phytophthora* (36 isolates) and 9 species of *Pythium s. lato*. Primers in bold are those selected for the analysis of environmental samples.

Target species[a]	Clades[b]	LP primers								SP primers					
		18Ph1F/ 28Ph1R	18Ph1F/ 28Ph2R	18Ph2F/ 28Ph1R	**18Ph2F/ 28Ph2R**	18Ph3F/ 28Ph1R	18Ph3F/ 28Ph2R	18Ph4F/ 28Ph1R	18Ph4F/ 28Ph2R	**18Ph2F/ 5.8S-1R**	18Ph2F/ 5.8S-2R	18Ph3F/ 5.8S-1R	18Ph3F/ 5.8S-2R	**ITS6/5.8S-1R**	ITS6/5.8S-2R
P. nicotianae	1	nd	nd	nd	+	nd	nd	nd	nd	+	+	+	+	+	+
P. cactorum	1a	−	−	+	+	+	+	+	+	+	+	+	+	+	+
P. idaei	1a	−	−	+	+	+	+	+	+	+	+	+	+	+	+
P. infestans	1c	+	+	+	+	+	+	+	+	+	+	+	+	+	+
P. citricola-like	2	+	+	+	+	+	+	+	+	+	+	+	+	+	+
P. citrophthora	2a	−	−	+	+	+	+	+	+	+	+	+	+	+	+
P. capsici	2b	−	−	+	+	+	+	+	+	+	+	+	+	+	+
P. ilicis	3	+	+	+	+	+	+	+	+	+	+	+	+	+	+
P. nemorosa	3	−	−	+	+	+	+	+	+	+	+	+	+	+	+
P. pseudosyringae	3	−	−	+	+	+	+	+	+	+	+	+	+	+	+
P. psychrophila	3	−	−	+	+	+	+	+	+	+	+	+	+	+	+
P. palmivora	4	−	−	+	+	+	+	nd	nd	+	+	+	+	+	+
P. quercina	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+
P. katsurae	5	−	−	+	+	+	nd	+	+	+	+	+	+	+	+
P. inundata	6	−	−	+	+	+	+	+	+	+	+	+	+	+	+
P. megasperma	6	−	−	+	+	+	+	+	+	+	+	+	+	+	+
P. alni subsp. *alni* (SCRP2)	7a	+	+	+	+	+	+	+	+	+	+	+	+	+	+
P. alni subsp. *alni* (SCRP4)	7a	+	+	+	+	+	+	+	+	+	+	+	+	+	+
P. alni subsp. *alni* SCRP8)	7a	+	+	+	+	+	+	+	+	+	+	+	+	+	+
P. alni subsp. *multiformis*	7a	−	−	+	+	+	+	+	+	+	+	+	+	+	+
P. cambivora	7a	−	−	+	+	+	+	nd	nd	+	+	+	+	+	+
P. europaea	7a	+	+	+	+	+	+	+	+	+	+	+	+	+	+
P. fragariae var. *fragariae*	7a	+	+	+	+	+	+	nd	nd	+	+	+	+	+	+
P. rubi	7a	+	+	+	+	+	+	nd	+	+	+	+	+	+	+
P. cinnamomi	7b	+	+	+	+	+	+	nd	nd	+	+	+	+	+	+
P. pistaciae	7b	−	−	+	+	+	+	+	+	+	+	+	+	+	+
P. sojae	7b	−	−	+	+	+	+	nd	+	+	+	+	+	+	+
P. cryptogea	8a	−	−	+	+	+	+	nd	nd	+	+	+	+	+	+
P. drechsleri	8a	−	−	+	+	+	+	nd	nd	+	+	+	+	+	+
P. erythroseptica	8a	−	−	+	+	nd	+	nd	nd	+	+	+	+	+	+
P. medicaginis	8a	−	−	+	+	nd	+	nd	nd	+	+	+	+	+	+
P. syringae	8b	−	−	+	+	+	+	nd	nd	+	+	+	+	+	+
P. lateralis	8c	−	−	+	+	+	+	nd	nd	+	+	+	+	+	+
P. ramorum	8c	−	−	nd	+	+	+	nd	nd	+	+	+	+	+	+
P. insolita	9	−	−	+	+	+	+	nd	nd	+	+	+	+	+	+
P. kernoviae	10	nd	nd	+	+	+	+	nd	+	+	+	+	+	+	+
Py. aphanidermatum	3	−	−	−	−	−	−	−	−	−	−	−	−	−	+
Py. pyrilobum	3	−	−	−	−	−	−	−	−	−	+	−	+	−	−
Py. catenulatum	3	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Py. torulosum	3	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Py. dissotocum	3	−	−	−	−	−	−	−	−	−	−	−	−	−	−
G. intermedium	4	−	−	−	−	−	−	−	−	−	−	−	+	−	−
G. splendens	4	−	−	−	+	+	+	−	−	−	−	+	+	+	+
G. ultimum	4	−	−	−	−	−	−	−	−	−	−	−	+	−	−
E. undulatum	5	−	−	−	−	−	−	−	−	−	+	+	+	+	+

[a] Genus abbreviations: P = *Phytophthora*, Py = *Pythium*, G = *Globisporangium*, E = *Elongisporangium*.
[b] *Phytophthora* and *Pythium s. lato* clades according to Blair et al. (2008) and Uzuhashi et al. (2010), respectively; + = positive amplification; − = negative amplification; nd = not determined.

2.5. Detection of Phytophthora species from environmental samples

The detection efficiency, resolving power, and the specificity of selected primers and DNA extraction procedures were evaluated by analyzing 15 soil samples, collected from an area of mixed woodland (oak, alder, cherry, hazel, birch) planted in agricultural soil in November 1997, and 10 water samples collected from a stream nearby. In all field experiments an additional *Phytophthora*-free soil sample and several sterile water samples were processed exactly as collected samples and served as negative controls.

Soil samples (approximately 2 kg) consisted of a well mixed series of 3–4 sub-samples collected with a trowel around single selected trees at a distance of 50 cm from the collar. The sub-samples were collected from a 25 by 25 cm hole dug at least 30 cm deep to avoid the upper soil horizons (location 56°27′50″N; 03°04′13″W).

Water samples (10 l) were collected from a fast flowing section of a small stream (Invergowrie Burn), close to the woodland area (location 56°27′50″N; 03°03′52″W). Samples analyzed using the centrifugation strategy (Fig. 1) were immediately returned to the laboratory in plastic containers and stored in a cold room at 4 °C for no longer than 24 h prior to DNA extraction.

For filtration, the same quantity of water was filtered directly in the field using a clean knap-sack sprayer to pressurize an in-line polypropylene filter holder (XX4304700, Millipore, UK) into which a mixed cellulose esters filter (RW1904700, Millipore, UK) was fitted (diameter 47 mm and porosity 1.2 μm). The cellulose filters were stored in sterile tubes maintained on ice (in the field) or at 4 °C (in the laboratory) and processed to extract DNA within 24 h. The support tools were carefully washed with water and 70% ethanol before being used for the next sample.

Two surveys were carried out in May and November 2005 when the environmental conditions were considered favorable for *Phytophthora* activity (Table 4). In the first survey (May) 15 samples (5 water and 10 soil) were analyzed with the LP assay (Figs. 1 and 2), 5 of which (2 water and 3 soil) were also later assayed with the SP primers (Figs. 1 and 2) to compare the sensitivity of the two tests. The centrifugation strategy was utilized to extract DNA from all water samples.

In the second sampling (November) five water and five soil samples were analyzed using the SP primers and a filtration strategy was utilized to extract DNA from water samples (Table 4).

2.5.1. Cloning and sequencing of PCR fragments

All PCR products obtained with the LP and/or SP assays from water and soil samples processed as described above, were purified through chromatography columns (Wizard SV Gel and PCR Clean-up System, Promega) and cloned into competent cells of *Escherichia coli* (JM109 Competent Cells HE) using the pGEM-T Easy Vector System (Promega) according to the manufacturer's protocol apart from a reduced ligation reaction volume (5 instead of 10 μl). For each cloned sample, 96 white clones were selected and cultured in 130 μl of Luria Bertani-Ampicillin medium (LB-Amp) in a 96×200 μl plate. After 24 h randomly selected clones were amplified in 25 μl PCR reactions using the generic primers T7 and SP6 (0.4 μM) and 0.3 μl of bacterial suspension as DNA template. Amplification mixtures were prepared as described before and cycled as follows: 1 cycle of 95 °C for 2 min, 30 cycles of 95 °C for 20 s, 55 °C for 25 s, 72 °C for 50 s and a final cycle of 72 °C for 5 min. Successful amplification was confirmed by electrophoresis on 2% agarose gels. After amplification 70 μl of 50% sterile glycerol was added to all LB-Amp cultures and they were stored at −80 °C.

Amplification products from clones were purified with ExoSAP-IT (USB, Staufen, Germany), sequenced following the manufacturer's protocol for BigDye Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems, Warrington, UK) and run on ABI3730 automated sequencer (Applied Biosystems). Sequencing was initiated using forward primers ITS6 (LP) or T7 (SP) and reverse primer ITS4 (LP) or SP6 (SP). For each cloned sample around 15 cloned fragments were sequenced. The number of clones sequenced from each sample depended on the number of phylotypes detected in an initial screen with more sampled (up to 25) in cases of higher variability.

Sequence Navigator software (Applied Biosystems) was used to carefully edit the forward and reverse sequences to generate a consensus sequence. Sequences in which either forward or reverse reads were unclear were discarded or re-sequenced. Representative consensus sequences were deposited in GenBank [JF300182–JF300282].

Sequences were compared by alignment with locally held ITS database and by Basic Local Alignment Search Tool (BLAST) analysis with GenBank (NCBI) by (http://blast.ncbi.nlm.nih.gov/). Sequences obtained in the present study and reference sequences from local and GenBank databases were aligned using ClustalX (Thompson et al., 1997) and introduced to TOPALi for phylogenetic analysis with the PhyML and MrBayes methods based on Maximum Likelihood and Bayesian Tree Estimation respectively (Milne et al., 2008).

Table 4
Results of field surveys conducted on soil and water samples collected in Invergowrie (Scotland, UK) in May and November 2005. Water samples W1–W5 and W6–W10 were processed by centrifugation and filtration, respectively. DNA samples were amplified by nested PCR using LP, SP or both primer pairs (samples in bold) in order to compare the sensitivity of the two assays.

	Sample	Origin	DNA extraction results			Amplification results[a]	
			Concentration (ng/μl)	A_{260}/A_{280}	A_{260}/A_{230}	LP primers	SP primers
A. May							
Water	W1	Stream	68.1	2.2	0.8	+	nd
	W2	Stream	69.6	2.2	0.8	−	nd
	W3	Stream	28.5	2.5	0.3	−	nd
	W4	**Stream**	34.1	2.1	0.5	−	+
	W5	**Stream**	34.2	2.6	0.6	−	+
Soil	S1	*Prunus avium*	106.2	1.9	0.9	+	nd
	S2	*Quercus* sp.	304.3	2.2	0.8	+	nd
	S3	*Quercus* sp.	88.2	1.9	0.8	+	nd
	S4	*Quercus* sp.	65.2	2.2	0.5	+	nd
	S5	*Acer* sp.	119.2	1.8	0.7	+	nd
	S6	***Acer* sp.**	284.7	1.8	1.2	−	+
	S7	***Prunus avium***	160.1	1.7	0.7	−	+
	S8	*Quercus* sp.	128.8	1.8	0.6	−	nd
	S9	*Quercus* sp.	156.1	1.6	0.6	−	nd
	S10	***Quercus* sp.**	84.6	1.9	0.6	−	−
B. November							
Water	W6	Stream	56.8	2.1	1.3	nd	+
	W7	Stream	36.6	1.9	1.6	nd	+
	W8	Stream	30.8	2.0	0.6	nd	+
	W9	Stream	26.0	1.9	1.8	nd	+
	W10	Stream	18.4	1.9	1.7	nd	+
Soil	S11	*Quercus* sp.	335.6	1.7	0.7	nd	−
	S12	*Quercus* sp.	312.7	1.7	0.6	nd	−
	S13	*Alnus glutinosa*	159.1	1.8	0.8	nd	+
	S14	*Alnus glutinosa*	88.7	2.0	0.7	nd	+
	S15	*Fraxinus excelsior*	77.4	1.8	0.8	nd	+

[a] nd = not determined.

3. Results

3.1. DNA extraction from soil and water

A number of DNA extraction protocols from soil and water samples were preliminarily tested to increase the amount and purity of

DNA, to speed up and simplify extractions and to reduce nucleic acid loss during processing. A purification step with chromatography columns was always required to attain DNA of a quality suitable for PCR amplification from both soil and water samples (data not shown).

The protocol reported by Brierley et al. (2009) with minor modifications was selected to extract DNA from soil and enabled the extraction of total nucleic acid suitable for PCR amplification in about 2 h. The concentration of extracted DNA ranged from 65.2 to 335.6 ng/μl with zero or low protein (260A/280A ratios of 1.6–2.2) or humic acid (260A/230A ratios of 0.5–1.2) contamination (Table 4).

Centrifugation and filtration methods were investigated to extract DNA from water samples. In the filtration strategy, DNA recovery ranged from 18.4 to 56.8 ng/μl with no protein contamination (260A/280A ratios of 1.9–2.1) and very low levels of humic acids (260A/230A ratios of 0.6–1.8) (Table 4). Similar results were obtained with centrifugation (Table 4) but filtration was a more rapid and simple approach. Each ten liter water sample took approximately 15 min to be filtered compared to more than 1 h for centrifugation. More importantly, filtration is a field-based assay.

Most water and soil samples produced a positive amplification with the universal primers ITS3–ITS4 (data not shown). The few samples that did not produce any amplification were preliminarily excluded from the analyses. In some cases a better amplification (brighter bands) was achieved with the ten times diluted samples as compared to undiluted ones. In these cases the diluted samples were analyzed with the *Phytophthora* spp. specific primers.

3.2. Design and selection of the genus-specific primers

In silico searches confirmed that none of the LP or SP primers matched any non-*Phytophthora* sequences available in GenBank. Furthermore, most LP primer pairs showed a high level of specificity in PCR reactions using all possible primer combinations and target DNA from 36 different *Phytophthora* isolates and 9 *Pythium s. lato* species (Table 3). Positive amplification was obtained with target DNA from the complete set of *Phytophthora* species but not from *Pythium s. lato* (Table 3). The primer pair 18Ph2F–28Ph2R amplifying a 1200 bp PCR fragment (LP) was selected as providing the best compromise between specificity, efficient amplification and absence of primer–dimers.

For the SP assay, both 5.8S primers combined with the forward primer 18Ph2F gave a positive signal for all *Phytophthora* species included in this study. The 5.8S-1R primer was more specific for *Phytophthora* detection than 5.8S-2R with no cross reaction to *Pythium s. lato* (Table 3). The same primer provided a lower level of specificity when combined with ITS6, a universal primer modified to match oomycete sequences (Cooke et al., 2000), with faint amplification of the approximately 260 bp products from *Globisporangium undulatum* and *G. splendens* (Table 3). Full specificity to *Phytophthora* was however achieved in a semi-nested approach in which an ITS6-5.8S-1R second round PCR with 1 μl of first round amplified product from primers 18Ph2F-5.8S-1R was utilized as target DNA (data not shown).

Sensitivity tests showed that LP primers 18Ph2F/28Ph2R detected down to 10 pg of total DNA in a single round PCR. This detection limit was reduced to 100 fg by re-amplifying 1 μl of the amplified product from the first round in a second round PCR with the same primers. The SP assay using primers 18Ph2F/5.8S-1R for the first round and ITS6/5.8S-1R in the second round was ten- and one hundred fold more sensitive than the LP assay amplifying 1 pg and 1 fg in the first and second round PCR, respectively.

3.3. Detection of Phytophthora species from environmental samples

3.3.1. Amplification results

A nested approach was always required to yield suitable PCR products for both LP and SP assays since no amplification was obtained with a single round PCR for either soil or water samples (data not shown). In the first survey (May), LP primers produced a positive amplification from a single water sample and from 5 of the 10 soil samples analyzed (Table 4). SP primers yielded amplification products from 2 soil and 2 water samples which tested negative with the LP primers suggesting they increase the assay sensitivity. In the second sampling (November), SP primers produced a positive amplification from all water and three soil samples (Table 4). Considering data from both field surveys (Table 4) six of 15 samples (40%) were positive with the LP primers (50% from soil and 20% from water) and 12 out of 15 samples (80%) with the SP primers (62.5% from soil and 100% of water samples).

3.3.2. Analysis of PCR sequences from water and soil samples

A total of 260 cloned ITS fragments were sequenced from the 18 soil and water samples that produced a positive amplification with LP or SP primers. Some challenges were raised during the analysis of the cloned sequences. When using the LP assay, chimeric sequences as a result of PCR-driven recombination were found in two soil samples at a frequency of 30% and 54%. One or two recombination

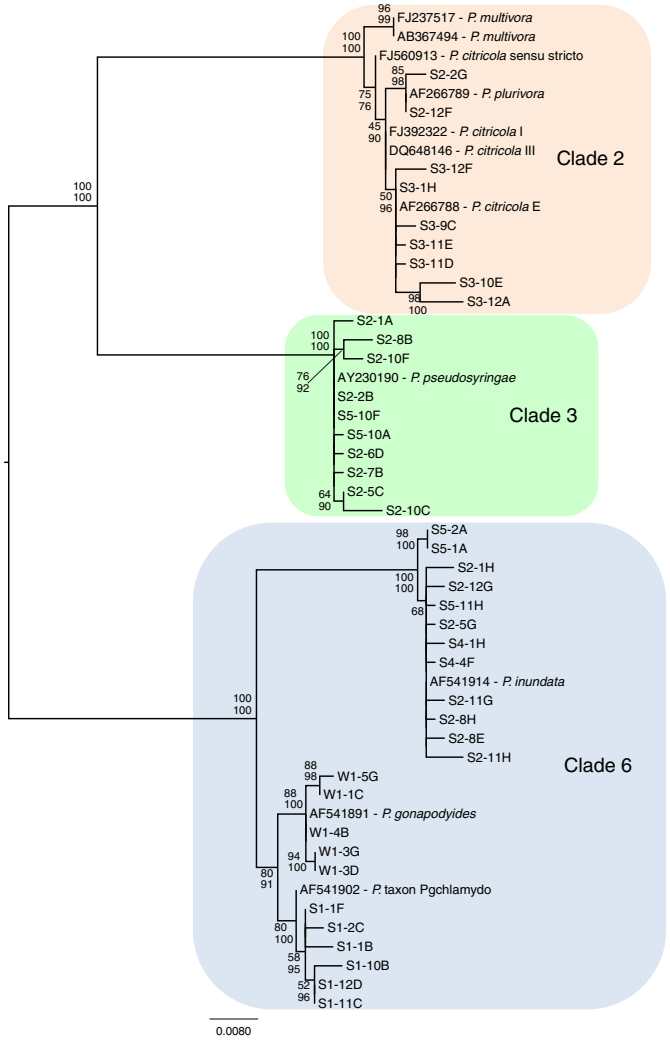

Fig. 3. Phylogenetic tree constructed with sequences generated using LP primers from water (W) and soil (S) samples and relative closest BLAST sequences retrieved from GenBank. Maximum likelihood branch lengths (PhyML) are shown. Numbers on nodes represent bootstrap support values for maximum likelihood (upper) and Bayesian probabilities presented as percentages (lower). Scale bar indicates number of substitutions per site.

breakpoints were evident and frequently associated with the highly conserved 5.8S region. Amplifying a shorter fragment with the SP assay reduced this markedly to only 1.7% of the clones (n = 174).

Phylogenetic analysis of LP and SP fragments revealed sequences that grouped within six of the ten main clades of the genus (Cooke et al., 2000; Blair et al., 2008) ascribed to 15 different *Phytophthora* species (phylotypes). Species detected in the samples collected in May with the LP assay grouped in clades 2, 3 and 6 (Fig. 3), while those collected in November and tested with the SP assay clustered in clades 1, 6, 7, and 8 (Fig. 4). None of the cloned sequences showed any matches to sequences of *Pythium s. lato* spp. Some of the detected sequences were 100% identical to GenBank deposited accessions but many others grouped within the above-mentioned clades, but did not perfectly match any known sequence and constituted specific subgroups which were frequently supported by high bootstrap values (Figs. 3 and 4). *P. syringae* (clade 8) was the most frequently sampled single taxon (18.8% of the clones) while the heterogeneous group of sequences attributable to clade 6 *Phytophthora* phylotypes were the most frequently detected group. A diverse mixture of over 40 different clade 6 types were amplified of which only 5 were identical to previously documented taxa such as *Phytophthora* taxon Salixsoil, *P.* taxon Pgchlamydo, *P. gonapodyides*, *P. inundata* and *P. megasperma* (Figs. 3 and 4). *P. citricola* E and *P. plurivora* (Jung and Burgess, 2009) (clade 2), *P. drechsleri* (clade 8), *P. nicotianae* (clade 1) and *P. pseudosyringae* (clade 3) were also prevalent whereas only few clones contained sequences ascribable to *P. cactorum* (clade 1), *P. cambivora*, *P. alni* and *P. pistaciae* (clade 7). Although some *Phytophthora* species were detected at both sampling dates with both SP and LP assays, the majority of the species were only detected with one of the two tests (Fig. 5). Six and 11 different *Phytophthora* phylotypes were detected

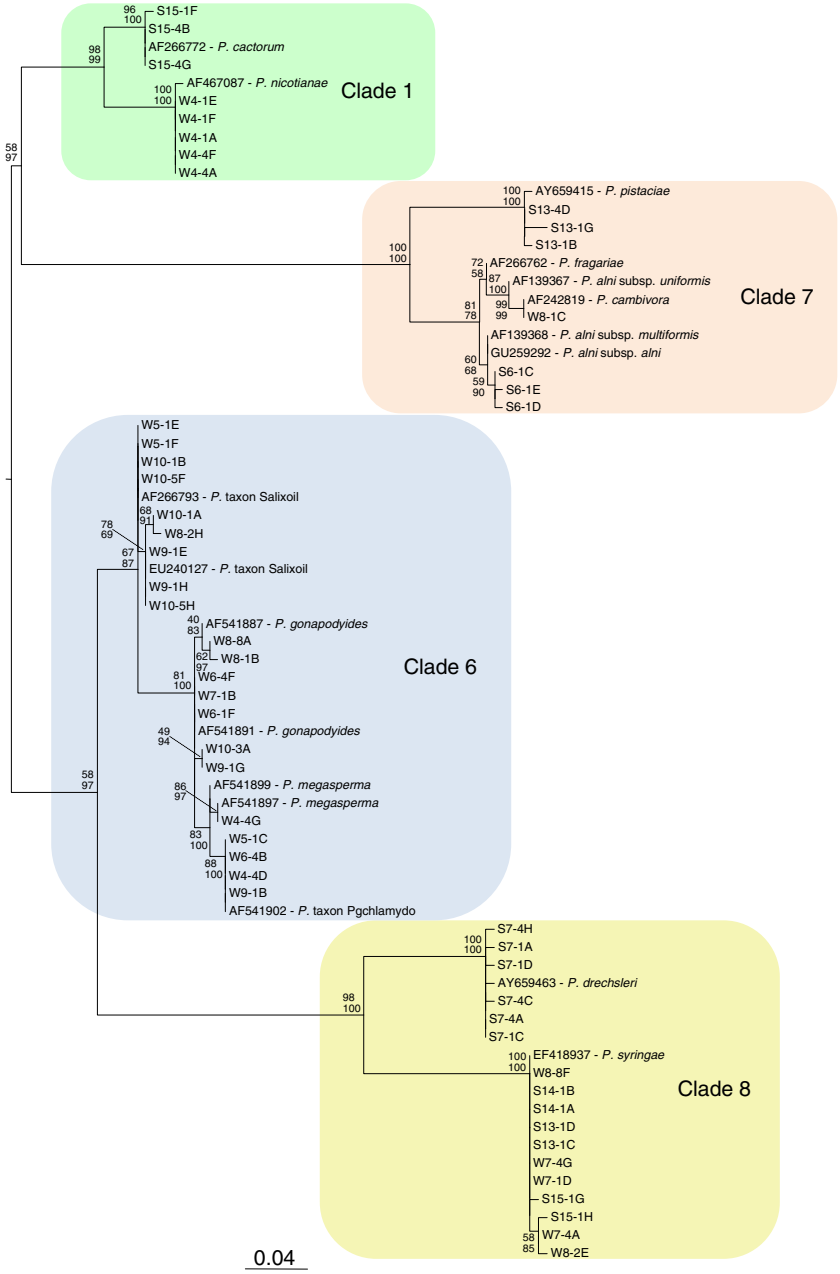

Fig. 4. Phylogenetic tree constructed with sequences generated using SP primers from water (W) and soil (S) samples and relative closest BLAST sequences retrieved from GenBank. Maximum likelihood branch lengths (PhyML) are shown. Numbers on nodes represent bootstrap support values for maximum likelihood (upper) and Bayesian probabilities presented as percentages (lower). Scale bar indicates number of substitutions per site.

with the LP and SP assays by sequencing 72 and 141 clones respectively. Ten *Phytophthora* phylotypes were detected in soil and 9 in water samples (Table 5). All positive water samples contained at least two different *Phytophthora* phylotypes, and 5 different phylotypes were detected in the W8 sample (Table 5). In contrast, only 1 or 2 *Phytophthora* phylotypes were found within the positive soil samples (Table 5). Interestingly, most phylotypes detected in the water samples belonged to clade 6 (Table 5; Figs. 3 and 4).

Two additional phylotypes, matching *Peronospora ervi* and *Pe. aparines* and *Plasmopara densa*, were observed at a low frequency indicating that these primers can cross-react with the predominantly aerial downy mildew species (data not shown).

4. Discussion

The aim of the present study was to develop a metagenomic approach to monitor the abundance and diversity of *Phytophthora* in water and soil samples. The fundamental challenge of the project was the choice of a suitable PCR primer set that would detect all *Phytophthora* species but not cross-react with members of the closely related genus *Pythium s. lato*. Two different primer sets were designed to specifically amplify a long product (LP) flanking the ITS1, 5.8S and ITS2 regions or a short product (SP) flanking the ITS1 region only. In laboratory tests LP and SP primer sets proved successful, amplifying all of the 36 *Phytophthora* species tested and either no product or very weak amplification from the tested *Pythium s. lato* species. The *Phytophthora* species tested were carefully selected to represent species from all clades defined by current molecular phylogenetic analyses (Cooke et al., 2000; Blair et al., 2008) and reduce the likelihood of false negatives due to *Phytophthora* spp. not amplifying. Selected *Pythium s. lato* species were less representative of the breadth of diversity within this genus since only 3 out of 5 phylogenetic clades were included (Uzuhashi et al., 2010). However, in silico comparisons conducted by BLAST analyses confirmed the match of selected primers with corresponding sequences from all *Phytophthora* taxa known to date and the absence of any close matches to any *Pythium s. lato* species known to date.

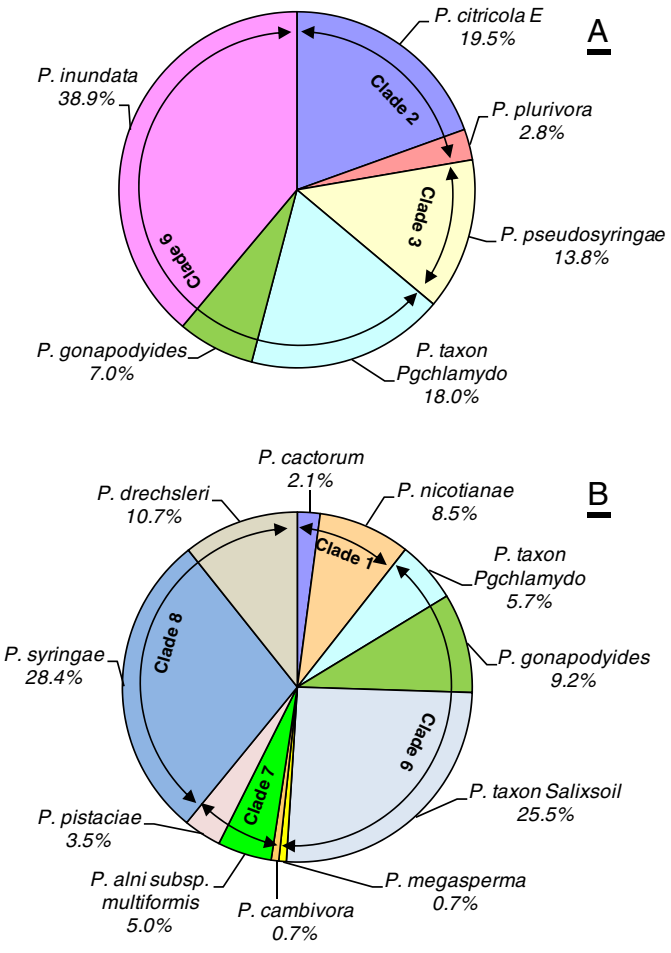

Fig. 5. Frequency of cloned ITS *Phytophthora* phylotypes detected with LP (A) and SP (B) primers and their matches to known species in GenBank. The number of clones represented in A and B was 72 and 141 respectively.

Table 5
Phytophthora phylotypes detected in soil and water samples producing a positive amplification with LP and/or SP primers.

Number of phylotypes detected per sample	Soil samples		Water samples	
	Samples	Detected phylotypes	Samples	Detected phylotypes
1	S1	*P.* taxon Pgchlamydo		
	S3	*P. citricola* E		
	S4	*P. inundata*		
	S6	*P. alni*		
	S7	*P. drechsleri*		
	S14	*P. syringae*		
2	S5	*P. inundata*	W5	*P.* taxon Salixsoil
		P. pseudosyringae		*P.* taxon Pgchlamydo
	S13	*P. syringae*	W7	*P. syringae*
		P. pistaciae		*P. gonapodyides*
	S15	*P. syringae*	W10	*P.* taxon Salixsoil
		P. cactorum		*P. gonapodyides*
3	S2	*P. pseudosyringae*	W1	*P. citricola* E
		P. inundata		*P. gonapodyides*
		P. plurivora		*P. inundata*
			W4	*P. nicotianae*
				P. taxon Pgchlamydo
				P. megasperma
			W9	*P.* taxon Salixsoil
				P. gonapodyides
				P. taxon Pgchlamydo
4			W6	*P. gonapodyides*
				P. taxon Pgchlamydo
				P. taxon Salixsoil
				P. syringae
5			W8	*P. syringae*
				P. gonapodyides
				P. taxon Salixsoil
				P. taxon Pgchlamydo
				P. cambivora

The complete absence of sequences matching *Pythium s. lato* species among the 260 clones from field testing was strong support for this *Phytophthora* specificity. *Pythium s. lato* is generally considered more abundant in environmental samples than *Phytophthora*, which was demonstrated in a preliminary phase of this project. A pair of primers available for nested PCR detection of the Peronosporales and Pythiales (DC6 and ITS4 followed by ITS6 and ITS4) (White et al., 1990; Cooke et al., 2000) were tested against water samples from the same stream used for all subsequent sampling. Multiple clones of the PCR product were sequenced, but all matched two species of *Pythium s. lato* (data not shown). Similarly, an investigation of the molecular diversity of the oomycete community inhabiting the rhizosphere of three plant species showed a dominance of *Pythium s. lato* species with very few *Phytophthora* phylotypes recovered (Arcate et al., 2006). The primers have not been tested against downy mildew genera but the frequency of propagules of such taxa in soil and water is likely to be lower than that of *Phytophthora.* The very low frequency among the samples tested in this study appears to bear this out.

The first test performed with the LP primers (May) was successful in demonstrating the potential of the method, but highlighted areas in need of improvement. The absence of amplification from more than 70% of the samples tested in this phase was unexpected and,

while it may have reflected an absence of *Phytophthora* in these samples, or an issue with DNA quality, it was considered more likely to have been caused by the limited sensitivity of the assay. This was confirmed by preliminary tests of sensitivity with pure culture DNA and the positive amplification achieved with SP primers from 4 out of 5 samples which tested negative with LP primers (Table 4). This result is in agreement with the reduced PCR product size from the 1200 bp of the LP assay to 490 bp (first round PCR) and 260 bp (second round PCR) of the SP assay. Shorter amplicons favor higher levels of sensitivity in conventional as well as in real-time PCR reactions and are preferentially amplified from environmental samples containing DNA from a mixture of different species (Schena et al., 2006).

An additional problem with the longer PCR product was PCR artifacts or chimeric sequences in which the ITS1 of one species becomes combined in a single clone with the ITS2 region of a second species. This phenomenon is a recognized problem of PCR (e.g. Bradley and Hillis, 1997) and must be considered in PCR-based environmental monitoring studies where multiple species are amplified in the same reaction. The highly conserved 5.8S region dramatically increased the risk by providing a block of identical sequence in the central region of all PCR products allowing the annealing and extension of partially extended PCR fragments. The SP assay all but removed this problem of chimeric sequences.

Although the SP primers provided significant higher levels of sensitivity than the LP primers nested PCR was still required to detect *Phytophthora* DNA from environmental samples. This matches the experience of other studies detecting very low levels of pathogen DNA from soil, due to the limited hyphal growth and relatively low numbers of resting spores within fine roots and root fragments from which releasing DNA can be challenging (Borneman and Hartin, 2000; Lees et al., in press; Brierley et al., 2009). A drawback of nested PCR is the increased risk of false positives due to cross contamination. In the present study, extreme care was taken to avoid cross-contamination between samples and carry-over of amplified sequences as recommended by Kwok (1990). In particular, pre- and post-PCR amplification steps were performed in separate rooms. Nonetheless, cross-contamination due to the presence of ITS PCR product or DNA or mycelial fragments from liquid nitrogen based grinding in the laboratory is hard to exclude completely and may explain rare cases of *Phytophthora infestans* (data not shown) and *P. pistaciae* (Fig. 4; Table 5) detection in this study. These species may not be considered present at any significant level in the Scottish natural ecosystems sampled. However, considering that the ecosystem investigated (Invergowrie, UK) was an agricultural field prior to 1997 and is adjacent to cultivated farmland, the presence of *P. infestans* propagules in the water samples is plausible. In the case of *P. pistaciae* the detected clones clearly clustered with reference sequences of this species but none were identical and the existence of variants of the species as yet unknown to the scientific community cannot be excluded. In any case, contamination was considered as sporadic and very infrequent, as confirmed by the lack of amplification from the many water-only nested negative control reactions.

Previous culture-independent approaches to study eukaryotic microbial communities have been based on conserved genes such as the small subunit (SSU) (Vandenkoornhuyse et al., 2002; Massana et al., 2004) and the large subunit (LSU) (Arcate et al., 2006) of the ribosomal DNA. Such conserved genes are not appropriate to discriminate among most *Phytophthora* species. The variation found in the ITS regions, however, proved suitable to discriminate most *Phytophthora* species (Robideau et al., 2011) and even minor sequence 'variants' in complex groupings of taxa such as those found in ITS clade 6 (Brasier et al., 2003a). Furthermore, the multi-copy nature of the ITS makes the region an appealing target for sequencing environmental substrates where the quantity of DNA present is low.

The assay developed in this study complements proposals of the ITS region as one of the preferred DNA barcoding markers for species identification of single taxa in 'environmental DNA barcoding' (Seifert, 2009; Bellemain et al., 2010; Robideau et al., 2011). A large number of *Phytophthora* ITS sequences are currently deposited in the international nucleotide sequence databases (Nilsson et al., 2009) providing a wide range of reference material for the identification of taxa. Issues with unreliable annotations of sequences in public DNA repositories remain an obstacle to all sequence-based species identification (Nilsson et al., 2006); this drawback, however, can be attenuated by primarily using sequences from ex-type isolates (Jung et al., 2011) and will be progressively solved with the standardization process needed for the use of ITS sequences as fungal and oomycete barcoding markers. Furthermore, effective and reliable species-level data sets are available for *Phytophthora* (e.g. www.phytophthoradb.org) and a sequence-based identification tool (www.Phytophthora-ID.org) has been recently developed and is freely available on the web (Grünwald et al., 2011). The ITS1 region possesses the most important characteristics of a desirable locus for DNA barcoding since it is present in all the taxa of interest, can be easily amplified without species-specific PCR primers and is short enough to be easily sequenced with current technology. Furthermore, a shorter amplicon is simple to clone and appropriate for the next-generation sequencing strategies in which thousands of sequences can be analyzed from a single environmental sample, enabling in-depth analysis of the microbial diversity. Other high-throughput studies have targeted either the ITS1 or the ITS2 region (Buée et al., 2009; Jumpponen and Jones, 2009), as the entire ITS region is too long (average length 654 bp in oomycetes (Seifert, 2009; Robideau et al., 2011)) for next generation sequencing methods.

The range and frequency of phylotypes detected by the LP and SP assays differed; six and 11 taxa belonging to 3 and 4 different clades were detected with LP and SP primers respectively, with only species of clade 6 being detected with both primer pairs (Fig. 5). The higher sensitivity of SP primers likely explains the greater number of taxa detected, including those with a very low incidence in water and soil samples. Species such as *P. pseudosyringae* and those related to *P. citricola* (*P. plurivora* and others) (Jung and Burgess, 2009) were only detected with LP primers. It should also be noted that relatively few samples were positive with the LP primers and these were sampled at a different time of the year to the SP samples (Tables 4 and 5) which may have introduced a bias. Subsequent larger-scale sampling in Scotland and Southern Italy (unpublished data) in which *P. pseudosyringae* and *P. citricola*-like phylotypes were detected with SP primers suggests that primer specificity is not an issue.

The aim of the present study was method development rather than its widespread application. However, these results provide an interesting insight into *Phytophthora* populations in a natural ecosystem. Overall, 15 different taxa were detected with most also being widely reported in natural ecosystems (Goheen and Frankel, 2009) and recovered during recent extensive surveys in forest streams in Oregon and Alaska (Reeser et al., 2011). It is not known whether taxa detected in the present study are endemic to the region but it is probable that some were introduced in 1997 with the commercially supplied tree seedlings. Species of clade 6 were detected frequently in water samples which is consistent with other reports of taxa such as *P. gonapodyides*, *P. inundata* and *P.* taxon Salixsoil flourishing in aquatic habitats or riparian ecosystems where they may also play a role in the breakdown of plant debris such as fallen leaves (Brasier et al., 2003b; Jung et al., 2011; Reeser et al., 2011). Other clade 6 taxa such as *P. megasperma* are economically important pathogens of commercial horticulture causing severe collar and root rots of ornamentals, fruit trees and woody perennials (Brasier et al., 2003a).

In agreement with similar metagenomic investigations conducted with marine Stramenopiles (Massana et al., 2004), Peronosporomycete rhizosphere communities (Arcate et al., 2006) and fungi in forest soils (Buée et al., 2009) these results demonstrate the feasibility of using the molecular approach to reveal *Phytophthora* biodiversity.

Unlike traditional detection method (see Introduction), the molecular approach can detect target DNA from a range of pathogen life stages and, due to its high sensitivity, even species with a very low incidence can be detected in complex environmental samples in which other species may predominate. In the present study, several novel phylotypes were identified, and although they were clustered within well known clades of *Phytophthora* species (Cooke et al., 2000), they represent ITS types not yet reported in GenBank. Furthermore, subsequent large scale investigation conducted using the SP assay in other ecosystems have amplified *Phytophthora*-like sequences that were distinct from any known *Phytophthora* species for which ITS1 sequences are available (unpublished data). A challenge for all such metagenomic studies is delimitation of taxa based on molecular phylotypes. At present there are no established criteria for determining whether two sequences represent genetic variants of the same species, distinct taxa, or SNPs caused by PCR errors. Indeed, no single level of variability should be used for estimates of conspecificity in different species (Nilsson et al., 2009). It is highly likely that many new species of *Phytophthora* will be found to be widespread in agricultural and natural ecosystems (Brasier, 2007).

One rather obvious disadvantage of the molecular method compared to conventional methods is the absence of an isolate of the pathogen. However, such molecular analysis provides a means of flagging up potentially damaging pathogens to be followed up with more detailed analysis via baiting and pathogen isolation. Another potential limit of such molecular detection methods is the risk of detecting DNA from dead cells. Nucleases can degrade DNA after the death of microorganisms, but the degradation rate is strongly dependent on the environmental conditions. Schena and Ippolito (2003) found that DNA of *Rosellinia necatrix* is degraded rapidly in soil and minimizes the risks of false positives due to the presence of dead cells. Hussain et al. (2005) reported a progressive reduction in the efficiency of detection of *P. infestans* mycelial inoculum which was not detectable beyond 18 months burial in the soil. However, further research is necessary to assess the persistence of DNA under different environmental conditions and in relation to the pathogen propagules. The DNA from thick walled oospores, for example, is likely to be more difficult to detect than that from actively growing mycelium in host tissue (Lees et al., in press). Although several reports indicate that nucleic acids are quickly digested by DNases in the soil (England et al., 1998), other studies show DNA persisting in soil for long periods in complexes with soil components (England et al., 1997). The detection of RNA is another option (Chimento et al., 2011) as it degrades quickly after tissue death. However, the reverse transcription step and degradation during sample processing may lead to false negatives.

An important step in the optimization of the present method was the system for sampling and processing water samples. Three different filters from Millipore (PVDF-polyvinylidene fluoride, glass fiber and mixed cellulose esters) were preliminarily selected according to their porosity in relation to *Phytophthora* zoospore diameter, flow rate, practicality for in-field use and suitability of material for direct DNA extraction (i.e. absence or minimization of substances that may inhibit PCR). The mixed cellulose esters filter was used in the present study since it provided a higher efficiency compared to other investigated membranes (data not shown). The direct in-field filtration using a simple knapsack sprayer fitted with an in-line filter support was very efficient and portable means of field sampling in remote locations and took only 15 min. Furthermore, the time saved using the DNA extraction procedure directly from the filter was considerable compared to the lengthy centrifugation method. The 1.2 μm pore size of the selected membrane proved suitable for the capture of the approximately 10 μm *Phytophthora* zoospores. The Sepharose purification used in the DNA extraction proved an efficient means of obtaining amplifiable DNA. The filter DNA extraction protocol was markedly improved using SDS extraction lysis buffer rather than CTAB buffer. Data from analytic DNA evaluation supported this and the method's efficiency was confirmed by the almost 100% success rate in amplifications from water sample-DNA.

Despite these improvements and considerable optimization of the protocols, there remain great challenges in consistent PCR amplification from soil. Dilution of DNA samples in this study illustrated the problem as, in some cases, a 1 in 10 dilution was needed and in other cases better amplification was achieved in undiluted samples. Caution should thus be applied in interpreting the results of soil detection analyses with appropriate control reactions to ensure that negative results really represent the absence of detectable *Phytophthora* DNA rather than simply a DNA sample in which inhibitors prevent efficient PCR amplification. Despite this, the positive PCR amplification with universal rDNA primers demonstrated the DNA extraction and purification protocols used in this study resulted in DNA suitable for molecular analysis.

5. Conclusions

A practical method based on PCR amplification of DNA directly extracted from environmental samples, cloning and sequencing has been developed and proved to be effective in detecting the abundance and diversity of *Phytophthora* species in natural ecosystems. Results can be achieved in approximately 3–4 working days as compared to the 2–3 weeks of conventional approaches. The method was particularly efficient for detecting *Phytophthora* in aquatic habitats since water sampling offered the advantages of speed, effectiveness and high efficiency enabling the detection of a greater range of species compared to soil sampling. The likely objective of projects using such technology are to sample as widely as possible in order to either estimate overall *Phytophthora* diversity or maximize the likelihood of detecting specific quarantine species such as *P. ramorum*.

The science of metagenomics is currently in its pioneering stages and the present method has great scope for further implementation with new approaches such as pyrosequencing and denaturing high performance liquid chromatography (DHPLC) (Barlaan et al., 2005; Petrosino et al., 2009; Lim et al., 2010). However, the genus-specific primers, zoospore filtration and DNA extraction protocols from soil and filters already represent an important step forward in this field and we foresee their value in many applications. They include biosecurity as a monitoring tool for tracking known or unknown pathogenic *Phytophthora* species in commercial plant, soil or associated water samples. Although naturally infected host tissues have not been analyzed in the present study, this implementation of the method will be straightforward considering that a number of commercial and lab-based DNA extraction methods are available. Monitoring *Phytophthora* diversity in natural ecosystems has been demonstrated and one may now examine the natural ecology of *Phytophthora* under circumstances with and without observable disease symptoms, test for the presence of potentially non-culturable or as yet uncultured taxa to aid the search for the center of diversity and investigate novel host–pathogen associations and threats posed by 'wild' species moving from one habitat to another.

Acknowledgments

This research was partially funded by grants from the University of Palermo and the Italian Ministry of University and Research with support from the Scottish Government Rural and Environment Research and Analysis Directorate. S. Scibetta and A. Chimento were supported by PhD fellowships from the Mediterranean University of Reggio Calabria and the University of Palermo, respectively. The authors thank Naomi Williams at the Scottish Crop Research Institute, Dundee, Scotland, for invaluable help with the culture collection and Clive Brasier, Forest Research, UK and others who contributed key *Phytophthora* cultures. Cultures were held under SASA License No. PH/11/2008.

References

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped-BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.

Arcate, J.M., Karp, M.A., Nelson, E.B., 2006. Diversity of *Peronosporomycete* (oomycete) communities associated with the rhizosphere of different plant species. Microb. Ecol. 51, 36–50.

Barlaan, E.A., Sugimori, M., Furukawa, S., Takeuchi, K., 2005. Profiling and monitoring of microbial populations by denaturing high performance liquid chromatography. J. Microbiol. Methods 61, 399–412.

Bellemain, E., Carlsen, T., Brochmann, C., Coissac, E., Taberlet, P., Kauserud, H., 2010. ITS as an environmental DNA barcode for fungi: an in silico approach reveals potential PCR biases. BMC Microbiol. 10, 189.

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

Bradley, R.D., Hillis, D.M., 1997. Recombinant DNA sequences generated by PCR amplification. Mol. Biol. Evol. 14, 592–593.

Borneman, J., Hartin, R.J., 2000. PCR primers that amplify fungal rRNA genes from environmental samples. Appl. Environ. Microbiol. 66, 4356–4360.

Brasier, C.M., Cooke, D.E.L., Duncan, J.M., Hansen, E.M., 2003a. Multiple new phenotypic taxa from trees and riparian ecosystems in *Phytophthora gonapodyides–P. megasperma* ITS clade 6, which tend to be high-temperature tolerant and either inbreeding or sterile. Mycol. Res. 107, 277–290.

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

Brasier, C.M., 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 the UK. Mycol. Res. 109, 853–859.

Brasier, C.M., 2007. *Phytophthora* biodiversity: how many *Phytophthora* species are there? In: Goheen, E.M., Frankel, S.J. (Eds.), Proceedings of the Fourth Meeting of the International Union of Forest Research Organizations (IUFRO) Working Party S07.02.09 Albany, CA. Phytophthoras in Forests and Natural Ecosystems, GTR-221. U.S.D.A. Forest Service Pacific Southwest Research Station, pp. 101–115.

Brasier, C.M., 2008. The biosecurity threat to the UK and global environment from international trade in plants. Plant Pathol. 57, 792–808.

Brasier, C.M., Webber, J., 2010. Plant pathology: sudden larch death. Nature 466, 824–825.

Brierley, J.L., Stewart, J.A., Lees, A.K., 2009. Quantifying potato pathogen DNA in soil. Appl. Soil Ecol. 41, 234–238.

Buée, M., Reich, M., Murat, C., Morin, E., Nilsson, R., Uroz, S., Martin, F., 2009. 454 pyrosequencing analyses of forest soils reveal an unexpectedly high fungal diversity. New Phytol. 2, 449–456.

Chimento, A., Cacciola, S.O., Garbelotto, M., 2011. Detection of mRNA by reverse-transcription PCR as an indicator of viability in *Phytophthora ramorum*. Forest Pathol. doi:10.1111/j.1439-0329.2011.00717.x.

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., Schena, L., Cacciola, S.O., 2007. Tools to detect, identify and monitor *Phytophthora* species in natural ecosystems. J. Plant Pathol. 89, 13–28.

Cullen, D.W., Lees, A.K., Toth, I.K., Duncan, J.M., 2002. Detection of *Colletotrichum coccodes* from soil and potato tubers by conventional and quantitative real-time PCR. Plant Pathol. 51, 281–292.

Davidson, J.M., Wickland, A.C., Patterson, H.A., Falk, K.R., Rizzo, D.M., 2005. Transmission of *Phytophthora ramorum* in mixed-evergreen forest in California. Phytopathol. 95, 587–596.

Davison, E.M., Tay, F.C.S., 2005. How many soil samples are needed to show that *Phytophthora* is absent from sites in the south-west of Western Australia? Australas. Plant Path. 34, 293–297.

Delmont, T.O., Robe, P., Cecillon, S., Clark, I.M., Constancias, F., Simonet, P., Hirsch, P.R., Vogel, T.M., 2011. Accessing the soil metagenome for studies of microbial diversity. Appl. Environ. Microbiol. 77, 1315–1324.

Dick, M.W., 1966. The Saprolegniaceae of the environs of Blelham Tarn: sampling techniques and the estimation of propagules numbers. J. Gen. Microbiol. 42, 257–282.

Ekblom, R., Galindo, J., 2011. Applications of next generation sequencing in molecular ecology of non-model organisms. Heredity 107, 1–15.

England, L.S., Lee, H., Trevors, J.T., 1997. Persistence of *Pseudomonas aureofaciens* strains and DNA in soil. Soil Biol. Biochem. 29, 1521–1527.

England, L.S., Holmes, S.B., Trevors, J.T., 1998. Persistence of viruses and DNA in soil. World J. Microb. Biot. 14, 163–169.

Erwin, D.C., Ribeiro, O.K., 1996. *Phytophthora* Diseases Worldwide. APS Press, St Paul.

Förster, H., Cummings, M.P., Coffey, M.D., 2000. Phylogenetic relationships of *Phytophthora* species based on ribosomal ITS1 DNA sequence analysis with an emphasis on Waterhouse groups V and VI. Mycol. Res. 104, 1055–1061.

Goheen, E.M., Frankel, S.J., 2009. Phytophthoras in forests and natural ecosystems. Proceedings of the Fourth Meeting of the International Union of Forest Research Organizations (IUFRO) Working Party S07.02.09. U.S.D.A. Forest Service, Pacific Southwest Research Station, Albany, CA (Tech. Coords).

Grünwald, N.J., Martin, F.N., Larsen, M.M., Sullivan, Press, C.M., Coffey, M.D., Hansen, E.M., Parke, J.L., 2011. Phytophthora-ID.org: A sequence-based Phytophthora identification tool. Plant Disease 95, 337–342.

Hughes, K.J.D., Tomlinson, J.A., Griffin, R.L., Boonham, N., Inman, A.J., Lane, C.R., 2006. Development of a one-step real-time PCR assay for diagnosis of *Phytophthora ramorum*. Phytopathol. 96, 975–981.

Hussain, S., Lees, A.K., Duncan, J.M., Cooke, D.E.L., 2005. Development of a species-specific and sensitive detection assay for *Phytophthora infestans* and its application for monitoring of inoculum in tubers and soil. Plant Pathol. 54, 373–382.

Jones, M.D.M., Forn, I., Gadelha, C., Egan, M.J., Bass, D., Massana, R., Richards, T.A., 2011. Discovery of novel intermediate forms redefines the fungal tree of life. Nature 474, 200–203.

Jumpponen, A., Jones, K., 2009. Massively parallel 454 sequencing indicates hyperdiverse fungal communities in temperate *Quercus macrocarpa* phyllosphere. New Phytol. 184, 438–448.

Jung, T., Cooke, D.E.L., Blaschke, H., Duncan, J.M., Oßwald, W.F., 1999. *Phytophthora quercina* sp. nov., causing root rot of European oaks. Mycol. Res. 103, 785–798.

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 Pathol. 49, 706–718.

Jung, T., Hansen, E.M., Winton, L., Oßwald, W.F., 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.

Jung, T., 2009. Beech decline in Central Europe driven by the interaction between *Phytophthora* infections and climatic extremes. Forest Pathol. 39, 73–94.

Jung, T., Stukely, M.J.C., Hardy, G.E., St, J., White, D., Paap, T., Dunstan, W.A., Burgess, T.I., 2011. Multiple new *Phytophthora* species from ITS Clade 6 associated with natural ecosystems in Australia: evolutionary and ecological implications. Persoonia 26, 13–39.

Jung, T., Burgess, T.I., 2009. Re-evaluation of *Phytophthora citricola* isolates from multiple woody hosts in Europe and North America reveals a new species, *Phytophthora plurivora* sp. nov. Persoonia 22, 95–110.

Kong, P., Hong, C.X., Jeffers, S.N., Richardson, P.A., 2003. A species-specific polymerase chain reaction assay for rapid detection of *Phytophthora nicotianae* in irrigation water. Phytopathol. 93, 822–831.

Kwok, S., 1990. Procedures to minimize PCR-product carry-over. 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, pp. 142–145.

Lee, S.B., Taylor, J.W., 1992. Phylogeny of five fungus-like protoctistan *Phytophthora* species, inferred from the internal transcribed spacers of ribosomal DNA. Mol. Biol. Evol. 9, 636–653.

Lees, A.K., Sullivan, L., Lynott, J.S., Cullen D.W., in press. Development of a quantitative real-time PCR assay for *Phytophthora infestans* and its applicability to leaf, tuber and soil samples. Plant Pathol. doi:10.1111/j.1365-3059.2011.02574.x.

Lim, Y.W., Kim, B.K., Kim, C., Jung, H.S., Kim, B.S., Lee, J.H., Chun, J., 2010. Assessment of soil fungal communities using pyrosequencing. J. Microbiol. 48, 284–289.

Mangan, S.A., Schnitzer, S.A., Herre, E.A., Mack, K.M.L., Valencia, M.C., Sanchez, E.I., Bever, J.D., 2010. Negative plant–soil feedback predicts tree-species relative abundance in a tropical forest. Nature 466, 752–755.

Martin, F.N., English, J.T., 1997. Population genetics of soilborne fungal plant pathogens. Phytopathol. 87, 446–447.

Massana, R., Castresana, J., Balague, V., Guillou, L., Romari, K., Groisillier, A., Valentin, K., Pedros-Alio, C., 2004. Phylogenetic and ecological analysis of novel marine Stramenopiles. Appl. Environ. Microbiol. 70, 3528–3534.

Miller, D.N., 2001. Evaluation of gel filtration resins for the removal of PCR-inhibitory substances from soils and sediments. J. Microbiol. Meth. 44, 49–58.

Milne, I., Lindner, D., Bayer, M., Husmeier, D., McGuire, G., Marshall, D.F., Wright, F., 2008. TOPALi v2: a rich graphical interface for evolutionary analyses of multiple alignments on HPC clusters and multi-core desktops. Bioinformatics 25, 126–127.

Nilsson, R., Ryberg, M., Kristiansson, E., Abarenkov, K., Larsson, K., Koljalg, U., 2006. Taxonomic reliability of DNA sequences in public sequence databases: a fungal perspective. PLoS One 1, e59.

Nilsson, R., Ryberg, M., Abarenkov, K., Sjökvist, E., Kristiansson, E., 2009. The ITS region as a target for characterization of fungal communities using emerging sequencing technologies. FEMS Microbiol. Lett. 296, 97–101.

Petrosino, J.F., Highlander, S., Luna, R.A., Gibbs, R.A., Versalovic, J., 2009. Metagenomic pyrosequencing and microbial identification. Clin. Chem. 55, 856–866.

Rea, J., Burgess, T.I., Hardy, G.E., St, J., Stukely, M.J.C., Jung, T., 2011. Two novel and potentially endemic species of *Phytophthora* associated with episodic dieback of Kwongan vegetation in the south-west of Western Australia. Plant Pathol. doi:10.1111/j.1365-3059.2011.02463.x.

Reeser, P.W., Sutton, W., Hansen, E.M., Remigi, P., Adams, G.C., 2011. *Phytophthora* species in forest streams in Oregon and Alaska. Mycologia 103, 22–35.

Rizzo, D.M., Garbelotto, M., Hansen, E.M., 2005. Integrative research and management of an emerging pathogen in California and Oregon forests. Annu. Rev. Phytopathol. 43, 309–336.

Robideau, G.P., De Cock, A.W.A.M., Coffey, M.D., Voglmayr, H., Brouwer, H., Bala, K., Chitty, D.W., Désaulniers, N., Eggertson, Q.A., Gachon, C.M.M., Hu, C.-H., Küpper, F.C., Rintoul, T.L., Sarhan, E., Verstappen, E.C.P., Zhang, Y., Bonants, P.J.M., Ristaino, J.B., Lévesque, C.A., 2011. DNA barcoding of oomycetes with cytochrome c oxidase subunit I and internal transcribed spacer. Mol. Ecol. Resour.. doi:10.1111/j.1755-0998.2011.03041.x

Rozen, S., Skaletsky, H., 2000. Primer3 on the WWW for general users and for biologist programmers. Methods Mol. Biol. 132, 365–386.

Sambrook, J., Fitsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Press, Cold Spring Harbor.

Savin, M.C., Martin, J.L., LeGresley, M., Giewat, M., Rooney-Varga, J., 2004. Plankton diversity in the Bay of Fundy as measured by morphological and molecular methods. Microbial Ecol. 48, 51–65.
Schadt, C.W., Martin, A.P., Lipson, D.A., Schmidt, S.K., 2003. Seasonal dynamics of previously unknown fungal lineages in tundra soils. Science 301, 1359–1361.
Schena, L., Ippolito, A., 2003. Rapid and sensitive detection of *Rosellinia necatrix* in roots and soils by real time Scorpion-PCR. J. Plant Pathol. 8, 15–25.
Schena, L., Hughes, K.J.D., Cooke, D.E.L., 2006. Detection and quantification of *P. ramorum*, *P. kernoviae*, *P. citricola* and *P. quercina* in symptomatic leaves by multiplex real-time PCR. Mol. Plant Pathol 7, 365–379.
Schena, L., Cooke, D.E.L., 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. J. Microbiol. Meth. 67, 70–85.
Schena, L., Duncan, J.M., Cooke, D.E.L., 2008. Development and application of a PCR based "molecular tool box" for the detection of *Phytophthora* species damaging forests and natural ecosystems. Plant Pathol. 57, 64–75.
Seifert, K.A., 2009. Progress towards DNA barcoding of fungi. Mol. Ecol. Resour. 9, 83–89.
Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 24, 4876–4882.
Torsvik, V., Ovreas, L., Thingstad, T.F., 2002. Prokaryotic diversity — magnitude, dynamics, and controlling factors. Science 296, 1064–1066.
Uzuhashi, S., Tojo, M., Kakishima, M., 2010. Phylogeny of the genus *Pythium* and description of new genera. Mycoscience 51, 337–365.
Vandenkoornhuyse, P., Baldauf, S.L., Leyval, C., Straczek, J., Young, J.P.W., 2002. Evolution-extensive fungal diversity in plant roots. Science 295, 2051.
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.
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, pp. 315–322.