INVITED REVIEW

TOOLS TO DETECT, IDENTIFY AND MONITOR *PHYTOPHTHORA* SPECIES IN NATURAL ECOSYSTEMS

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SUMMARY

Phytophthora is a genus of plant pathogens responsible for severe damage to crops, natural vegetation and forestry worldwide. Accurate detection, diagnosis and species identification is fundamental to disease management, clear scientific communication and the statutory measures to prevent pathogen spread and yet this often proves challenging. Many serious disease problems have emerged in recent years, often associated with previously undescribed *Phytophthora* species and damaging plants in both commercial production systems and natural ecosystems. Many advances in DNA-based molecular diagnostics and DNA sequencing have been made recently that increased our ability to accurately detect and characterise Phytophthoras. However, there is a continual need for improvement and an increasing interest in a broader monitoring of *Phytophthora* species in natural ecosystems to get a better feel for their distribution and impact through understanding their ecology. This review examines the technical advances in the field and the rationale for such studies on *Phytophthora*.

Key words: *Phytophthora*, natural ecosystems, ecology, molecular detection, target genes.

INTRODUCTION

There are numerous threats to forests and other natural ecosystems worldwide; of which the direct impacts of logging and forest clearance for agriculture are the most apparent. However, the contribution of pathogens to rapid mortality (termed 'dieback') and a more general 'decline' of particular vegetation systems is also significant. Many cases of 'dieback' and 'decline', particularly of tree species, have been reported but in many cases the cause(s) are not fully understood due to the complexity of the interacting biotic and abiotic factors (Jönsson, 2006). Understanding the driving forces in such processes is obviously critical for effective forest and ecosystem management. Pests and pathogens are often implicated and, in this context, two key factors are worthy of greater attention. Firstly, the impacts of climate change on diseases of wild, forest and agricultural plants. With temperature increases and changes in humidity and rainfall patterns predicted, changes in the range and activity of organisms responsible for forest declines seem inevitable (e.g. Brasier and Scott, 1994). It is thus important that scientists, as well as those responsible for managing such natural resources, are prepared for the consequences of climate change on the distribution, biology and ecology of pathogens. Secondly, the introduction and invasive spread of pests and pathogens into new ecological niches *via* natural processes or increased international trade of plant material is an ongoing and significant threat (e.g. Levine and D'Antonio, 2003). Predicting either factor separately is difficult so the challenges are significantly increased when both are considered together. Biodiversity is considered a key factor in providing ecosystems with sufficient plasticity, or resilience, to buffer such climate change or pest and pathogen load. However the impact of pathogens combined with unsustainable management of natural, forest or agricultural ecosystems may reduce biodiversity further. An additional consequence of natural ecosystem decline or forest mortality induced by plant disease is the reduced fixation of carbon into plant material, in direct conflict with one of the most important goals of the Kyoto protocol on climate change.

Among the plant pathogens threatening forests and other natural ecosystems *Phytophthora* stands out, with a significant number of the 70 or so described species proven to be aggressive agents that threaten ecosystem stability and plant productivity on a global scale (Erwin and Ribeiro 1996; Ristaino and Gumpertz, 2000; Jung *et al*., 2005; Rizzo *et al*., 2005). Several well known examples of severe mortality and progressive dieback have been reported. In some cases, such as sudden oak death

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caused by *Phytophthora ramorum* and jarrah dieback caused by *P. cinnamomi*, *Phytophthora* has been demonstrated as a primary factor. In other circumstances, the role of *Phytophthora*, while not apparent from the outset, now has very strong support. Such cases include the rapid mortality and decline of Iberian and cork oak by *P. cinnamomi* (Robin *et al*., 2001; Luque, *et al*., 2002) and the clear involvement of *P. quercina* in the, often more gradual, decline of *Quercus robur* and *Q. petraea* (Jung *et al*., 2000; Jönsson *et al*., 2005; Cooke *et al*., 2005) in Europe and parts of Asia minor (Balci and Halmschlager, 2003). The discovery of a *Phytophthora* species (Greslebin and Hansen, 2006) strongly implicated in a previously unexplained dieback of *Austrocedrus chilensis* ('mal de cipres') in Patagonia (Filip and Rosso, 1999) is a recent example in this vein. Although the evidence for the involvement of *Phytophthora* is clear, much remains to be learnt about the specific interactions between the pathogen, host and environment that result in plant disease and ecosystem decline. For example, the role of factors such as environmental stresses, including the effects of direct and indirect human activities in predisposing forest trees and cultivated plants to *Phytophthora* infection is poorly understood.

P. ramorum, a pathogen isolated in the early 1990s (Werres *et al*., 2001) but unknown as the cause of Sudden Oak Death until 2000, exemplifies the threat posed by *Phytophthora*. In a suitable environment such as that in the Californian coastal evergreen and tanoak/redwood forests within the fog belt its impact is devastating, having, to date, killed over a million native oaks and tanoaks (http://nature.berkeley.edu/comtf). Evidence that *P. ramorum* was introduced and distributed by man (Ivors *et al*., 2006) has focussed attention on forest health and monitoring of *Phytophthora* species. A different scenario is presented by a periodic decline of oaks (principally *Q. robur* and *Q. petraea*) in Europe. In this case, many causes have been proposed but recent data suggests that under some circumstances it is initiated by fine root mortality due to infection by *P. quercina* (Jung *et al*., 1999; Jung *et al*., 2000; Jönsson *et al*., 2005). Many other *Phytophthora* species cause serious damage; some examples follow. In November 2003, the newly discovered species *P. kernoviae* was reported causing bleeding lesions on mature beech trees associated with infected Rhododendrons (Brasier *et al*., 2005) and has since been the subject of a Plant Health Order to assist in local containment and eradication schemes. Hundreds of thousands of alders have been killed across large areas of Central and Northern Europe by another newly described species, *P. alni* (Brasier *et al*., 2004). This species appears to have emerged from a hybridization event (Brasier *et al*., 1999) and represents a threat to natural and managed alder stands and riparian ecosystems in Europe. *P. cambivora* together with *P. cinnamomi* is responsible for Ink Disease of sweet chestnut (Vettraino *et al*., 2005) which is endemic in many chestnut-growing areas in Italy and plays a significant role in *Castanea sativa* mortality (Fleischmann *et al*., 2004). In the USA, *P. inflata* was found to be the most important species on *Fagus sylvatica* (Jung *et al*., 2005), whereas *P. lateralis* is a major cause of death of the valuable timber species, Port Orford cedar (Lawson cypress) in its natural habitat, in Oregon (Hansen *et al*., 2000). More recently *P. lateralis* has also been discovered in oak forests in Europe (Hansen and Delatour, 1999). The impact of other *Phytophthora* species found recently in natural forest, such as *P. pseudosyringae* associated with bleeding canker on beech trees in Europe (Cacciola *et al*., 2005; Motta *et al*., 2003; Jung *et al*., 2003), deserves further investigation.

The high-profile disease outbreaks discussed above have focussed attention on the damage *Phytophthora* can cause in natural ecosystems and three international meetings organised under the auspices of the IUFRO working party "*Phytophthora* diseases on forest trees (7.02.09)" (http://www.iufro.org) have certainly helped to stimulate the research community in this field. Many previously described *Phytophthora* species have been isolated from forests and other natural ecosystems including *P. cryptogea, P. megasperma*, *P. syringae, P. ilicis, P. drechsleri, P. gonapodyides* and *P. citricola* (Vettraino *et al*., 2002, 2005, Jung *et al*., 2000). However, since the year 2000 at least eight new species have been recovered: *P. uliginosa* and *P. europaea* (on oaks), *P. pseudosyringae* (on oak and beech) *P. psychrophila* (on oak), *P. inundata* (on different hosts), *P. nemorosa* (on different hosts) and *P. fallax* and *P. captiosa* (on eucalyptus) (Brasier *et al*., 2003; Jung *et al*., 2002, 2003; Hansen *et al*., 2003; Dick *et al*., 2006). This raises the obvious questions as to whether these new cases are a result of recent introductions of invasive pathogens, improved methods of isolation and characterisation, more intensive sampling, a change in environmental conditions or a combination of these and other factors. Although all *Phytophthora* species are considered specialist invaders of living plant tissue and therefore of potential concern it is also important to have a realistic measure of the relative risks posed by the presence of a particular species (or combination of species) in a given habitat. Some species such as *P. gonapodyides*, *P. inundata* and a series of related taxa awaiting formal description in ITS clade 6 (Cooke *et al*., 2000; Brasier *et al*., 2003) are frequently isolated from natural ecosystems but, in general, considered only weakly pathogenic and thus of minor importance. Their ecological role in, for example, fine root turnover or leaf decomposition has not been studied. Further evaluation of such cases and consideration of the role of *Phytophthora* species in the functioning of 'natural' and 'healthy' ecosystems is also important.

It is very likely that the presence and activity of *Phytophthora* species in natural ecosystems are underesti-

mated. Clearly it is important to better understand the significance of these Phytophthoras in the decline of forests and other natural ecosystems and for this more information on their distribution, biology, pathogenicity and ecology is urgently needed. Much of our current knowledge is based on traditional methods of detection (selective media and baiting techniques) and morphological identification which have several shortcomings when used for ecological surveys to assess species occurrence and distribution. However several, mainly molecular-based, approaches have recently been developed and their application in the study of Phytophthoras in natural ecosystems is opening an incredible number of research opportunities. Technological advances, increased automation and reduced costs of sequencing have contributed to the success of new approaches to the study of microbial diversity *via* molecular methods. There is a trend towards 'Microbial Observatories' (Kane, 2004) that catalogue microbial diversity in a given habitat at a fine scale. The aim of this review is to examine the currently available methods to detect and identify *Phytophthora* species in forest and other natural ecosystems as well as the rationale and future research opportunities in the field.

TRADITIONAL DETECTION METHODS

An extensive description of conventional detection methods applicable to *Phytophthora* species is reported by Erwin and Ribeiro (1996). Briefly, conventional methods include: i) direct microscopic examination of diseased material, ii) baiting with plant materials, and iii) isolation of the pathogens from infected plant tissues, water and soil using general or selective agar media. Visual detection for signs of *Phytophthora* on infected plant parts is very difficult and limited to a restricted number of host-pathogen combinations in which airborne spores are visible as a downy mass of sporulation. Much more important is the isolation of *Phytophthora* from soil and host tissues by baiting with selective hosts. This method is based on the use of highly susceptible hosts that are rapidly infected by a *Phytophthora* species and was widely utilised as the only effective method to isolate *Phytophthora* before the advent of selective media. Baiting exploits the selective pathogenicity of a *Phytophthora* species to living host tissue and, for example in the case of *Phytophthora fragariae* var *fragariae* on strawberry plants (Duncan, 1980), yields highly specific symptoms indicating the presence of the pathogen. More commonly, however, a visible lesion is formed and subsequently plated out on selective media to enable a more accurate identification of the species. A representative list of reference baits including leaf disks, cotyledons, and various fruits and seedlings was reported by Erwin and Riberio (1996). Among them rhododendron and oak leaves are widely reported for the isolation of forest Phytophthoras (Jung *et al*., 2002; Vettraino *et al*., 2005). Bating is less commonly used to detect *Phytophthora* species in infected host tissues but is more appropriate for isolation from soil and water samples. In the first case the baiting substrate is put into flooded soils to attract pathogen zoospores whereas for the detection of *Phytophthora* in rivers, streams and irrigation canals suitable baits can be directly incubated in the body of water. Baiting is cost effective, simple and familiar to many workers and thus remains a common and appropriate approach. An additional advantage in the case of river sampling is that the bait can be left in the substrate for relatively long periods and may thus recover low levels of *Phytophthora*. However, baiting has a number of drawbacks when used to assess the occurrence and distribution of a specific species since the selective colonization and development on different types of baits can strongly reduce the number of species that can be isolated. Even if a particular species can initially colonize baits, some species may competitively exclude others during the incubation process, leaving relatively few species to dominate baits. For example, *P. quercina* was isolated *via* a carefully refined oak-leaf baiting system but not using apple or pear baits that worked well for other *Phytophthora* species (Jung *et al*., 1999). Furthermore, in the case of laboratory baiting the system selects species that produce zoospores under the specific conditions provided and may for example leave oospore populations undetected. Because oospores serve as survival structures and likely constitute a large proportion of the *Phytophthora* inoculum in soils, assessments based solely on baiting are likely to underestimate the diversity. The isolation of *Phytophthora* directly from soil and water samples requires the use of selective media to prevent the masking or inhibition of the relatively slow growing *Phytophthora* by the tremendous range of other micro-organisms. Generic media could only be utilised to isolate *Phytophthora* from host and/or baiting tissues however the use of selective media has markedly increased the succes of isolation. At the present a number of different selective media are available; among these the P10 ARP medium prepared amending corn meal agar (CMA) with a number of chemicals is one of the most effective (Erwin and Riberio, 1996). This medium contains a number of antibacterial and antifungal chemicals which enable the growth of most *Phytophthora* species but prevent the growth of other micro-organisms with the exception of *Pythium* and *Mortierella*, two genera found abundantly in the soil. Hymexazol can be added to the media to prevent the development of many *Pythium* and *Mortierella* species however it is also toxic to a number Phytophthoras and therefore could yield an unrepresentative measure of *Phytophthora* diversity.

On the whole, traditional methods are based on mor-

phological and cultural criteria and therefore require skilled and specialised microbiological expertise, which often takes many years to be acquired. These methods are very time consuming requiring days or weeks to complete and results are not always conclusive, e.g. when closely related organisms need to be discriminated. Furthermore, traditional methods may not be sensitive enough to detect the pathogen in presymptomatic infections and it is generally accepted that failure to detect *Phytophthora* species with baiting techniques does not necessarily indicate their absence (Erwin and Ribeiro, 1996). Although Phytophthoras are aggressive pathogens, they are also seasonally active and ephemeral organisms that may be quickly replaced in host tissues and soils by other micro-organisms. Therefore, the absence of *Phytophthora* in a natural ecosystem must be interpreted with caution since their population can fluctuate from non-detectable to a high inoculum density in a very short period of time. Isolation of *P. cinnamomi* or the alder Phytophthoras by traditional baiting methods is usually only 10-20% successful even from fresh, active bark lesions.

One rather obvious advantage of these methods is that successful isolation yields objective proof of the presence of the pathogen and a *Phytophthora* culture that is available for further characterisation. Such records and resultant key culture collections provide the essential raw data for subsequent analysis. However, the limitations of such approaches, as described above, have lead to the development of novel methods for detecting and identifying *Phytophthora* species as well as other plant pathogens over the last decade. Among them greatest effort has been focussed on the development of diagnostics based on antibodies and nucleic acid technologies (McCartney *et al*., 2003).

SEROLOGICAL METHODS

Serological methods are based on the use of antibodies i.e. molecules produced by the mammalian immune systems to identify invading organisms or substances. Methods for producing antibodies for plant pathogen diagnostics and their use have been reviewed by several authors (Torrance, 1998). Initially attempts were made to develop specific polyclonal antibodies (PAbs) which recognize multiple epitopes of the pathogen. However these antibodies, widely utilised for the detection of plant viruses, do not usually have the desired degree of specificity for more complex micro-organisms such as fungi and Oomycetes and, importantly, the specificity may vary with each newly produced batch (Hahn and Werres, 1997).

The development of the monoclonal antibody approach in the mid-1980s opened new opportunities. Being produced by individual cell lines all antibodies in a specific serum are identical, identify a single target epitope and, therefore, are potentially much more specific. Monoclonal antibodies have been utilised for the production of *Phytophthora* species and genus specific commercial detection kits using both membrane-based and ELISA formats (Ali-Shtayeh *et al*., 1991). Some of these kits proved appropriate for the identification of important forest Phytophthoras such as *P. cactorum* and *P. cinnamomi* (Benson, 1991). These antibodies subsequently formed the basis of a series of diagnostic tests for use in *P. cinnamomi* identification, including immunofluorescence assays, ELISAs and a dipstick assay (Cahill and Hardham, 1994). The dipstick assay, in particular, combines synthetic baits (chemoattractants) with a species-specific immunoassay to detect Phytophthoras in the field. However, monoclonal antibodies are generally slow to produce, expensive to both produce and maintain, and occasionally cell lines may die or stop producing the required antibody. A new method of producing antibodies named "phage display" has been used to develop immuno-diagnostic assays for plant pathogens (Ziegler and Torrance, 2002). Immuno-diagnostic assays have been developed using this technique for a number of plant viruses and proved to be effective also for the identification of more complex organism such as *P. infestans* (Gough *et al*., 1999).

Although such antibody-based tests have been compared experimentally (Pettitt *et al*., 2002) it was not until the test format was improved that their practical use has increased. Lateral flow devices that display a simple colour change in the presence of *Phytophthora* antigens (e.g. http://pdiag.csl.gov.uk/) have, for example, proved useful in the field as a pre-screen to focus further sampling efforts (Jung *et al.*, 2005).

MOLECULAR METHODS

Contemporary approaches to identify, diagnose and study *Phytophthora* species as well as other plant pathogens have moved overwhelmingly towards those that exploit nucleic acid sequence differences between species. Prior to the introduction of the polymerase chain reaction (PCR), nucleic acid based diagnostics generally involved the use of hybridisation probes, and they continued to be used as an alternative to PCR for identification of plant pathogens including *Phytophthora* species until the mid 1990s. Randomly selected probes from DNA libraries were utilised to detect *P. nicotianae* (Goodwin *et al*., 1989), *P. citrophthora* (Goodwin *et al*., 1990) and *P. cinnamomi* (Judelson and Messenger-Routh, 1996). However, in recent years hybridisation has been displaced by PCR-based approaches because of their greater sensitivity, simplicity and speed, and because it is possible to use much simpler protocols to extract the nucleic acid samples for detection.

Conventional PCR. The polymerase chain reaction (PCR) is a method for synthesising (amplifying) millions of copies of specific DNA sequences identified by two short oligonucleotides (primers) using a thermostable enzyme (Taq DNA polymerase) and repeated cycles of denaturation, polymerisation and elongation at different temperatures (Mullis and Faloona 1987; Ward *et al.*, 2004). The whole process is repeated many times, so that after 30-35 cycles (approximately 2 hours) millions of copies of the sequence have been produced. Being primer specific the amplification of an appropriate sized fragment can be used as an indication of the presence of a specific organism. The procedures are, in principal, very simple; requiring only the mixing of a few ingredients (DNA, buffer, deoxyribonucleotide triphosphates (dNTPs), oligonucleotide primers and DNA polymerase) to be incubated in a PCR machine programmed to switch between the different temperatures required. Depending on the design of the primers, selectivity at either narrow or broad taxonomic levels is possible, thus enabling the detection of a single pathogen at the genus, species or strain level. The presence of the amplified DNA is usually checked by agarose gel electrophoresis, but alternative detection formats include using colorimetric (Mutasa *et al.*, 1996) or fluorometric assays (Fraaije *et al.*, 1999).

PCR is the most important and sensitive technique presently available for the detection of plant pathogens (Ward *et al.*, 2004). This technique has the potential to detect single copies of the target gene contained in single propagules (Lee and Taylor, 1990) and is widely reported as an effective detection method for a number of *Phytophthora* species including those prevalent in forest ecosystems (Table 1). However, in some circumstances, a nested PCR approach is used to improve the sensitivity and/or specificity of the assay. This involves two consecutive PCR reactions, in which the use of the first primer pair is followed with a second pair recognising a DNA region within the PCR product amplified by the first set (Ippolito *et al.*, 2002). The use of nested PCR increases the risks of false positives due to cross contamination and involves more time and effort (Kwok, 1990). However, the availability of *Phytophthora* genus-specific primers for a common first amplification could be useful to reduce the number of required amplifications with beneficial effects on the costs of the analysis and, to some extent, on the risks of false positives. A primer amplifying the ITS1 and ITS2 regions (see below) from all members of the Peronosporales in combination with the universal primer ITS4 was reported by Cooke *et al.* (2000). Ippolito *et al.* (2002) reported a primer combination (Ph2-ITS4) amplifying DNA from all *Phytophthora* species but did not, however, test their specificity against the abundant soil-borne genus *Pythium*. A pair of *Phytophthora*-specific primers amplifying a fragment of the ras-related protein gene have also been published (Schena *et al.* 2006a). Finally a set of primers amplifying the ITS1 and ITS2 regions from all *Phytophthora* species and not cross reacting with *Pythium* species has been utilised to develop a new method for the monitoring of *Phytophthora* diversity in soil and water environments (see below).

Multiplex PCR, based on the use of several PCR primers in the same reaction, can be used to detect several pathogens simultaneously and reduce time and costs. In conventional PCR, multiplex assays are difficult to develop because different targets need to be differentiated by product size on agarose gels and yet the efficiency of amplification is strongly influenced by amplicon size. Consequently it is difficult to identify good amplification conditions for all amplicons and shorter amplicons may be amplified preferentially over longer ones (Henegariu *et al.*, 1997).

Several attempts have been made to use conventional PCR as a quantitative detection method. However, it remains very difficult to relate the quantity of PCR products generated during PCR to the concentration of the target DNA originally present in the sample. This is due to a decrease in the efficiency of the reaction towards the final cycles of the amplification that means the final amount of PCR product is no longer proportional to the amount of original template present. A method, called competitive PCR, has been developed to quantify DNA of a number of target pathogens including *P. infestans* (Judelson and Tooley, 2000); however the method is very laborious and not sufficiently accurate (Mahuku and Platt, 2002).

There are several significant advantages of PCR-based detection methods over the traditional methods of diagnosis; for example, micro-organisms do not need to be cultured, the potential to detect a single target molecule in a complex mixture and their speed and versatility. Despite this, the adoption of PCR for routine detection of plant pathogens has been relatively slow, often due to technical limitations related to the post-amplification amplicon detection procedures.

Real-time PCR. With emerging diseases such as sudden oak death caused by *P. ramorum* (Hayden *et al.*, 2004), which have brought with them a realisation of the threat to entire ecosystems and industries, real-time PCR approaches have been adopted to provide a more rapid means of screening water, plant, and soil samples (Schaad *et al.*, 2003). Several reviews covering the most common real-time PCR chemistries and their application to the study of plant pathogens have been recently published (Schaad and Frederick, 2002; McCartney *et al.*, 2003; Schena *et al.*, 2004a; Ward *et al.*, 2004; Mumford *et al.*, 2006). In particular, real-time PCR chemistries applied to the study of *Phytophthora* species include amplicon sequence non-specific (SYBR Green) and specific (TaqMan, Molecular beacons and Scorpion

Table 1. Updated list of primers reported for the identification and detection of *Phytophthora* species known to threaten forests and other natural ecosystems.

Phytophthora species	Primer F	Primer R	Target	Chemistry	Reference
Alder Phytophthoras	D16F	D16R	SCAR	Conventional PCR	De Merlier *et al*., 2005
P. alni subsp. *alni*	PA-F	PA-R	SCAR	Conventional PCR	Ioos *et al*., 2005
P. alni subsp. *multiformis*	PAM-F	PAM-R	SCAR	Conventional PCR	Ioos *et al*., 2005
P. alni subsp. *uniformis*	PAU-F	PAU-R	SCAR	Conventional PCR	Ioos *et al*., 2005
P. cactorum	PC1	PC2	SCAR	Conventional PCR	Causin *et al*., 2005
P. cambivora	CAMB3	CAMB4	RAPD	Conventional PCR	Schubert *et al*., 1999
P. cinnamomi	95.422	96.007	Cina-6a gene	Other*	Coelho *et al*., 1997
	LPV2 F	LPV2 R	Lpv gene	Conventional PCR	Kong *et al*., 2003
	LPV3 F	LPV3 R	Lpv gene	Conventional PCR	Kong *et al*., 2003
P. citricola	CITR1	CITR2	ITS region	Conventional PCR	Schubert *et al*., 1999
	P5	P6	ITS region	TaqMan	Böhm *et al*., 1999
	Ycit3F	Ycit4R	Ypt1	TaqMan	Schena *et al*., 2006a
P. kernoviae	Yptc3F	Yptc4R	Ypt1	Taq Man	Schena *et al*., 2006a
P. lateralis	Platf	Platr	ITS region	Conventional PCR	Winton and Hansen, 2001
P. nemorosa	FMnem-1	FMnem-3	Cox2-Cox1	Conventional PCR	Martin *et al*., 2004
P. pseudosyringae	FMPps1c	FMPps2c	mtDNA	TaqMan	Tooley *et al*., 2006
	FMPps1c	FMPps2c	Cox2-Cox1	Conventional PCR	Martin *et al*., 2004
P. quercina	QUERC1	QUERC2	SCAR	Conventional PCR	Schubert *et al*., 1999
	QUERC3	QUERC4	SCAR	Conventional PCR	Nechwatal and Oßwald, 2001
	Yque3F	Yque4R	Ypt1	TaqMan	Schena *et al*., 2006a
P. ramorum	Phyto1	Phyto4	ITS region	SYBR Green	Hayden *et al*., 2004
	FMPr-1a	FMPr-7	Cox2-Cox1	Conventional PCR	Martin *et al*., 2004
	Pram-114Fc	*Pram*-190R	ITS region	TaqMan	Hughes *et al*., 2006
	FMPr-1a	FMPr-7	mtDNA	TaqMan	Tooley *et al*., 2006
	Yram4F	Yram3R	Ypt1	TaqMan	Schena *et al*., 2006a

*colorimetric assay which involves an oligonucleotide capture probe covalently immobilised on microtitration wells, a multi-biotinylated oligonucleotide detection probe and the PCR-amplified target DNA.

PCR) methods (Table 1). Compared to conventional PCR, real-time PCR eliminates the requirement for post-amplification processing steps thus saving time and labour. Without ethidium bromide, health risks for operators and environmental contamination are reduced and the throughput of PCR testing as an automated diagnostic system suitable for large-scale applications increases. This is particularly true when, as reported for *P. ramorum*, specific on-site methods are developed to extract and amplify DNA in the field using a portable real-time PCR platform (Cepheid SmartCycler) (Tomlinson *et al*., 2005). In this system, the analysis may be completed in two hours and unlike other methods does not require centrifugation steps, organic solvents, or the use of liquid nitrogen for sample homogenization. The ability to test plant samples for specific infections rapidly and at the point of sampling is likely to have a number of useful applications. Epidemiological studies in the field or at remote locations, for example, could greatly benefit from the ability to perform molecular testing without the need to return samples to a laboratory. Also, decisions regarding control strategies including eradication measures, which may need to be taken rapidly, could be better informed by the availability of reliable real-time PCR data on-site and within few hours of inspection.

The potential of real-time PCR as a large-scale detection method can be further improved by the use of multiple primers to amplify and detect multiple templates within a single reaction (multiplex real-time PCR). This is particularly important for *Phytophthora* species in forest and natural ecosystems where they are frequently

found in 'clusters' on the same site or sometimes even on the same tree (Vettraino *et al*., 2002, 2005; Jung *et al*., 2002). Unlike conventional PCR, with real-time detection technology, differentiation can be achieved using a different fluorescent dye for each species, and thus amplicons of the same length can be used. The number of target micro-organisms that can be simultaneously detected by real-time PCR is usually limited by the number of fluorophores available. The recent discovery of non-fluorescent quencher (dark quencher) has made available additional wavelength emissions that were previously occupied by the emission of the quenchers themselves (Mackay *et al*., 2002). A practical challenge in the application of multiplex real-time PCR is the optimisation of complex PCR reactions containing several primers and probes to avoid reductions in sensitivity and/or specificity (Ippolito *et al*., 2004). In an effort to simultaneously detect two pathogens, a three-way multiplex amplification was evaluated using markers for *P. ramorum, P. pseudosyringae*, and the host plant to serve as a positive control (Tooley *et al*., 2006). While multiplexing had no effect on the sensitivity of the *P. ramorum* and plant markers, there was a reduction in the detection sensitivity for the *P. pseudosyringae* markers. Recently, however, Schena *et al*. (2006a) demonstrated that the optimization of a series of factors including primer and probe design, amplification conditions and length of the amplified fragments enabled the simultaneous detection of four target pathogens (*P. ramorum, P. kernoviae, P. citricola* and *P. quercina*) without any reduction of sensitivity compared to single amplifications. In particular, the amplification of very short amplicons and the use of dark quenchers to avoid overlapping of fluorescence emission peaks from fluorophores and quenchers seemed to be the key step to the success. Multiplex PCR can be used to minimise the risk of false negatives due to a variety of naturally occurring compounds, such as humic acids, tannins, and lignin associated compounds that can interfere with PCR and inhibit DNA amplification (Bridge and Spooner, 2001). Specific primer-probe systems for *P. ramorum* and/or *P. pseudosyringae* were combined in a multiplex assay with a plant primer-probe system to allow plant DNA present in extracted samples to serve as a positive control in each reaction (Tooley *et al*., 2006; Hughes *et al*., 2006).

Another significant advantage of real-time PCR is the increased sensitivity in comparison to other detection methods including conventional PCR. For a number of forest Phytophthoras detection sensitivity ranging from 1 to 100 fg of target DNA has been reported for methods based on different target genes including the ITS regions (Hughes *et al*., 2006), the ras-related protein gene (Schena *et al*., 2006a) and an intergenic region of the mitochondrial genome (Tooley *et al*., 2006). Schena *et al*. (2006a) found clear evidence that these levels of sensitivity are sufficient to detect single copies of target DNA as testified by the total DNA content per nucleus of related Oomycetes such as *Achlya* spp. (40 fg), *Saprolegnia* spp. (200 fg), *Albugo* spp. (47-80 fg), *Bremia* spp. (72-147 fg), *Paraperonospora* spp. (152-144 fg), *Peronospora* spp. (47-138 fg), *Plasmopara* spp. (92-163 fg), and *Pseudoperonospora* spp. (82-90 fg) (Voglmayr and Greilhuber, 1998). A nested approach with conventional (first amplification) and labelled primers (second amplification) can further increase sensitivity. Such an approach enabled the detection of *P. nicotianae* and *P. citrophthora* in naturally infected substrates with very low pathogen concentrations but significantly increased the risks of false positives due to cross contamination of reaction mixtures (Ippolito *et al*., 2004). In practice, the reduced risk of cross-contaminations is a major advantage of real-time PCR. Although false positives can result from sample-to-sample contamination, a more serious source of false positive is the carry-over of DNA from a previous amplification of the same target (Kwok, 1990). Since reaction tubes do not need to be opened at the end of the reaction, the amplified fragments do not represent a source of contamination for future amplifications. This aspect is particularly important for quarantine pathogens such as *P. ramorum* and *P. kernoviae* in which results of a molecular analysis are likely to impact large-scale eradication schemes or plant trade.

Another possible source of false positives in all molecular based techniques is the detection of nucleic acids originating from dead cells; this risk is higher in real-time PCR due to the very high sensitivity of the technique. Nucleases are widely diffused in environmental samples and can potentially degrade DNA after the death of micro-organisms. However, the degradation rate strongly depends on environmental conditions. Several studies based on real-time PCR for quantitative assessment of degradation kinetics of DNA in different micro-organisms showed that degradation of DNA could be rapid in some cases, such as after heat treatment, or slower, according to DNA binding potential of the substrate (Wolffs *et al*. 2005). Schena and Ippolito (2003) found that DNA of *R. necatrix* is degraded rapidly in soil and minimizes the risks of false positives due to the presence of dead cells. However, other studies have shown that DNA can persist in soil for long period of time by forming complexes with soil components (England *et al*., 1997). These findings imply that caution should be taken in studies where DNA degradation data are used to assess risks of false-positives. In order to deal with risks of false-positive PCR results, many researchers have investigated the use of mRNA as a viability marker, on the basis of its rapid degradation compared to DNA (Alifano *et al*., 1994). However, the detection of mRNA requires a reverse transcriptase (RT) PCR approach which is more complex and expensive; furthermore, the choice of mRNA extraction method and mRNA target influence the mRNA degradation rate

(Sheridan *et al*., 1998).

One of the main advantages of real-time PCR is its suitability for estimating plant pathogen biomass in host tissues or environmental samples (Schaad and Frederick 2002; McCartney *et al*. 2003; Ward *et al*. 2004; Schena *et al*., 2004a, 2004b). Despite the very limited number of reports to date on the use of real-time quantitative PCR for *Phytophthora* detection in forests and natural ecosystems (Hayden *et al*., 2004; Schena *et al*., 2006a) a number of important applications are immediately apparent. The quantification of pathogen biomass in the soil is important in the study of pathogen population dynamics and ecology. Furthermore, quantitative analyses could be utilised to determine inoculum thresholds necessary for disease development in a number of host-pathogen combinations. Unlike baiting and cultural methods, real-time PCR is not affected by external factors such as other fungal species that could conceal the presence of the pathogen on, for example, selective agar media. A strong and significant correlation was found between inoculum density of *P. nicotianae* assessed with a selective medium (propagules per gram of soil), and the corresponding Ct values in real time PCR (Ippolito *et al*., 2004). Quantitative detection methods have been developed for a number of other soil-borne pathogens including *Helminthosporium solani* (Cullen *et al*., 2001), *Colletotrichum coccodes* (Cullen *et al*., 2002), *Spongospora subterranea* (van de Graaf *et al*., 2003), *Fusarium solani* f. sp. *phaseoli* and the arbuscolar mycorrhizal fungus *Glomus intraradices* (Filion *et al*., 2003). Quantitative detection methods can also be developed for the quantification of pathogen propagules in other potentially contaminated environments such as water and air which play an important role in the dispersal of *Phytophthora* species (Ristaino and Gumpertz, 2000).

The quantification of pathogen DNA in the host could be utilised to evaluate the extent of spread in the tissues or to determine the presence of *Phytophthora* in symptomless plant parts or whole plants. In comparison to fruiting body abundance, ergosterol content and dot blot analysis, TaqMan real-time PCR provided the most accurate calculation of the relative growth of *Phaeocryptopus gaeumannii* within Douglas-fir needles and was the only method able to quantify the pathogen early in the disease cycle (Winton *et al*., 2003). In alfalfa plants infected with *P. medicaginis* significant correlations were found between the amount of pathogen DNA and disease severity, suggesting that real-time PCR can be utilised for the scoring of plant resistance when samples are indistinguishable on the basis of visual disease severity assessment (Vandemark and Barker, 2003).

DNA micro- and macro-arrays. DNA arrays and chips are powerful new tools for gene expression profiling but can also be used for identification and differentiation of micro-organisms (Wu *et al*., 2001). Recently DNA micro- and macro-arrays have been proposed as an alternative new method for the simultaneous detection of many plant pathogens (Anderson *et al*., 2006; Lievens and Thomma, 2005) and may prove a powerful tool to reveal clusters of Phytophthoras reported from natural ecosystems. Detection methods based on conventional and real-time PCR are the most sensitive means of detecting pathogens but only one or few target pathogens can be detected simultaneously in a single reaction mixture (Schena *et al*., 2004a). In DNA arrays, species-specific oligonucleotides are immobilized on a solid support that can be a flexible membrane, such as nylon, or a rigid support such as silicon or glass (Anderson *et al*., 2006; Lievens *et al*., 2005a). The target DNA's are labeled PCR fragments amplified using consensus primers spanning a genomic region that includes species-specific sequences. Subsequently, labeled amplicons are exposed to the array and those that hybridise represent the species present in the sample. In theory, the range of organisms detectable in a single PCR is limited only by the number of spots on the array and the extent of the diversity within the selected genomic target region. Initially this technology was proposed for the diagnosis of human diseases and genetic disorders (Saiki *et al*., 1989) however several other applications have been reported recently including the detection of oomycete plant pathogens using specific oligonucleotides designed on the ITS regions (Anderson *et al*., 2006; Lievens *et al*., 2005b). Macroarrays have proved appropriate for the multiplex detection and identification of plant pathogens from complex environmental samples including those derived from soils and plants and, to some extent, also provide quantitative information about the abundance of the target pathogens (Lievens *et al*., 2005a). Recently, genus-specific primers have been developed that amplified ITS and flanking regions from all *Phytophthora* species but none of the *Pythium* species tested (Chimento *et al*., 2005; S. Scibetta, personal communication). These primers, in combination with array technology should prove a powerful tool that is suited to large-scale surveys of *Phytophthora* species in natural ecosystems.

Direct sequencing from environmental samples. Despite the large number of studies conducted in the past 10 years using traditional detection methods (selective media and baiting techniques), and to a lesser extent serological and molecular approaches, the presence and activity of *Phytophthora* in forest and other natural ecosystems is probably still underestimated. As discussed, traditional selective media and baiting techniques have a number of shortcomings when used to assess the occurrence and distribution of even single species. When the objective is to study the diversity of *Phytophthora* species present, the problems are even greater as no single method will be optimal for all

species. Although molecular detection methods have overcome many of the problems of isolation and baiting techniques, the majority of diagnostic assays are also specifically designed to detect only a single species. As such, they too are inappropriate for broader surveys of *Phytophthora* diversity and distribution in natural ecosystems in which a system capable of detecting multiple species or even undescribed species is required. Recent molecular analyses of bacterial communities in terrestrial or marine habitats have revealed an incredible diversity of previously undescribed and potentially non-culturable organisms (Torsvik *et al*., 2002). Similar discoveries are being made from analysis of fungal (Vandenkoornhuyse *et al*., 2002; Lynch and Thorn, 2006) and stramenopile communities (Massana *et al*., 2004) including Oomycetes associated with the rhizosphere of different hosts (Arcate *et al*., 2006). Recently an innovative molecular approach for the study of *Phytophthora* species in forests and other natural ecosystems has been developed in the Scottish Crop Research Institute (SCRI), UK (S. Scibetta, personal communication). This method is based on a new set of *Phytophthora* genus-specific primers enabling the amplification of the internal transcribed spacer (ITS) regions of the ribosomal DNA (rDNA) repeat regions. The method is based on DNA extraction and purification from soil and water samples, amplification of the ITS regions from all *Phytophthora* species by nested PCR and the cloning of the second round PCR product (Chimento *et al*., 2005). Database comparisons of the DNA sequence derived from the cloned fragments are used for species identification. Primer specificity was widely tested using a large number of different *Phytophthora* species representatives of the breath of diversity in the genus and a representative number of isolates from the related genus *Pythium*. A key advance from previously published methods was the lack of cross-reaction with the ubiquitous *Pythium* species. In preliminary tests with samples collected in Scottish woodlands the method has proved very effective, enabling the detection of many *Phytophthora* species. The range and type of species present varied from sample to sample and in one single 500 g soil sample four different *Phytophthora* species were detected. Furthermore novel unreported ITS sequences were also identified, suggesting the presence of new species (S. Scibetta, personal communication).

TARGET DNA REGIONS FOR THE DEVELOPMENT OF DIAGNOSTIC ASSAYS

Whichever of the above molecular detection methods is selected, the crucial step in assay development is the identification of an appropriate target DNA region. A good target gene should be sufficiently variable to enable the differentiation of closely related species but, at the same time, should not contain intraspecific variation that would jeopardise the detection of all strains. A single optimal target gene for all *Phytophthora* species and assay requirements is unlikely to exist so compromises are required. For some assays, a highly conserved target region is critical to enable the design of generic primers that amplify target DNA from a group of species (e.g. all *Phytophthora* species) whereas for another assay the requirement may be to differentiate lineages of a single species. General features of a good target gene are as follows: the gene should be easily amplified and sequenced and ideally multi-copy to enable the development of sensitive detection methods.

Recent molecular analyses have substantially increased our understanding of the phylogenetic relationships between *Phytophthora* species and provide an enormous source of data on target genes to develop molecular detection methods (Cooke *et al*., 2000; Kroon *et al*., 2004; Ioos *et al*., 2006; Schena and Cooke, 2006). Furthermore, the exponential increase of sequences in databases together with the availability of data from three genome sequencing projects for *P. sojae* (http://genome.jgi-psf.org/), *P. ramorum* (http://genome.jgi-psf.org/) and *P. infestans* (www.broad.mit.edu) will give new insights for developing appropriate molecular markers for the identification, detection and study of the genus *Phytophthora*.

Internal Transcribed Spacer (ITS) regions. The ITS regions of the nuclear ribosomal DNA (rDNA) array are the most commonly sequenced regions for *Phytophthora* and have been widely utilised for phylogenetic studies (Cooke *et al*., 2000; Kroon *et al*., 2004) and diagnostic assay development (e.g. Silvar *et al*., 2005). The ITS regions provide attractive targets because they are highly stable, can be easily amplified and sequenced with universal primers, occur in multiple copies, and possess conserved as well as variable sequences (White *et al*., 1990). ITS-based PCR primers have been reported and are available for the detection of a number of *Phytophthora* species including those important for their impact on natural ecosystems (Table 1). Furthermore, the same regions have been utilised to design specific oligonucleotide arrays for a number of plant pathogens (Anderson *et al*., 2006; Lievens and Thomma, 2005). However, in recent years, the discovery and ITS sequencing of many new *Phytophthora* species have raised concerns about the specificity of some ITS-based molecular detection methods. This is due to cases where the ITS sequences are not sufficiently variable, making the design of primers to identify and detect closely related taxa very difficult or impossible. Important forest Phytophthoras 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 Too-

ley, 2003; Schena and Cooke, 2006). Similarly *P. alni*, *P. cambivora, P. fragariae*, and *P. europaea* are phylogenetically closely related and challenging to distinguish via ITS sequences (Brasier *et al*., 2004). The PCR assay used widely for *P. ramorum* detection was recently found to cross-react with *P. foliorum* a newly discovered and closely related species of unknown importance (Donahoo *et al*., 2006). The discrimination of *P. lateralis* from *P. ramorum* requires two 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). For the same reason in the real-time PCR detection method developed by Hughes *et al.* (2006) it was necessary to introduce a base substitution in one primer to increase specificity but at a cost of decreased sensitivity.

Intergenic spacer (IGS) region. The intergenic (IGS1 and IGS2) regions of rDNA seem to have great potential as alternative to the ITS regions. Like the ITS regions, they are multicopy (up to 200 copies per haploid genome) but their length (4000-5000 bp) also provides considerable scope for primer development. Specific primers to detect *P. medicaginis* were developed on the IGS2 region because of difficulties in discriminating the related species on the basis of ITS (Liew *et al*., 1998). However, the utilisation of the IGS regions as targets to develop specific molecular markers has been limited, primarily due to the difficulties in amplifying a long fragment (4000-5000 bp) and the lack of effective universal primers. The complete sequence of the IGS region of *P. megasperma* has been recently determined and utilised to develop a primer set to identify *P. megasperma* isolates (Nigro *et al*., 2005). Recently, a set of universal primers has been developed and utilised for the amplification of a PCR fragment of approximately 450 bp in a region of the IGS very close to the 28S rDNA gene (Schena and Cooke, 2006). The alignment and comparison of this region from 28 different *Phytophthora* species showed a level of genetic variability comparable to those of the ITS regions. However the new sequences do allow the discrimination of some additional *Phytophthora* species with very similar ITS regions and should facilitate the amplification and characterization of the potentially more variable flanking regions.

Random DNA fragments. Another approach to identify specific target sequences is the random amplification of regions of the genome with PCR-based techniques such as random amplified polymorphic DNA (RAPD) or arbitrarily primed PCR (AP-PCR) (Welsh and McClelland, 1990). Randomly amplified PCR fragments have been utilized as targets for the design of sequence characterized amplified region (SCAR) primers specific for a number of *Phytophthora* species (Table 1). This approach is particularly useful when closely related species or specific strains need to be identified. SCAR primers have been recently utilized to identify the three subspecies of *P. alni* (*P. alni* subsp. *alni*, *P. alni* subsp. *uniformis* and *P. alni* subsp. *multiformis*) (Ioos *et al*., 2005). However, the identification of potentially specific target regions is very laborious and time consuming. Furthermore, in most cases, no background information regarding the origin, localisation, function or stability of the selected fragments is available. There is thus a need for time consuming screening to confirm the specificity, reliability and stability of the selected primer sets against a large and representative selection of isolates of both the target species and those of related species. Even after such screening, monitoring of the pathogen population may be needed to check for any false negatives resulting from deletions or mutations in the target region.

Alternative genomic regions. Among alternative genomic regions the Cina-6a gene (Coelho *et al*., 1997) and the putative storage protein gene (*Lpv*) (Kong *et al*., 2003) where utilised as targets for specific identification of *P. cinnamomi*, whereas the elicitin *parA*1 gene (Lacourt and Duncan, 1997) proved to be an effective target for *P. nicotianae*. However none of these genes appeared to be sufficiently variable to be utilised as a target to distinguish a broad range of *Phytophthora* species. A very promising target gene for phylogenetic studies and for the development of specific molecular detection methods is the ras-related protein (*Ypt*1) gene (Chen and Roxby, 1996; Ioos *et al*., 2006). The non-coding regions of the *Ypt*1 gene showed sufficient variation to differentiate *Phytophthora* species that are almost identical in ITS sequence (Schena and Cooke, 2006) and were utilised to develop a molecular tool box for the identification and detection of 15 different forest Phytophthoras (Schena *et al*., 2006b) and a multiplex real time PCR detection method to simultaneously detect and quantify *P. ramorum*, *P. kernoviae*, *P. citricola* and *P. quercina* (Schena *et al*., 2006a). Since the Ypt1 gene is single copy the detection limits of molecular methods based on this gene (100 fg of target DNA) were lower than those based on multi-copy genes (Ippolito *et al*., 2004; Hayden *et al*., 2004). However, Schena *et al.* (2006a) demonstrated that, from a practical standpoint, the lower level of sensitivity achieved with the *Ypt*1 gene is a minor problem since even single copies of the gene, and therefore, even single propagules of target *Phytophthora* species, could be detected by the highly efficient single round multiplex real-time PCR assay. Furthermore, methods based on single copy genes are not affected by the number of tandem repeats as in multi-copy genes and there is the potential to accurately correlate Ct values with the pathogen biomass and/or with the number of propagules.

Mitochondrial DNA (mtDNA). The sequencing of the entire mtDNA of *P. infestans* (Paquin *et al*. 1997) has prompted the use of this cytoplasmic genome as a useful target for *Phytophthora* phylogenetic analysis and species identification. The sequencing of coding regions of mtDNA from a large number of *Phytophthora* species has shown that genes such as the *Cox*2 and *Cox*1 are appropriate for broad phylogenetic analyses in the genus *Phytophthora* (Martin and Tooley, 2003; Kroon *et al*., 2004). Some variable intergenic regions have also proved suitable for assays to discriminate closely related species and for phylogenetic studies targeting a single species (interspecific variability) or few phylogenetically related sister species (Schena and Cooke, 2006). The intergenic region between the *Cox*2 and *Cox*1 genes of mt-DNA was exploited to develop specific primers to detect *P. ramorum, P. nemorosa* and *P. pseudosyringae* by conventional PCR (Martin *et al*., 2004) and *P. ramorum* and *P. pseudosyringae* by multiplex real-time PCR (Tooley *et al*., 2006). The mitochondrial DNA is present in multiple copies per cell thereby molecular methods based on this target are potentially very sensitive and enabled the detection of 2 fg of target DNA (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 very challenging. Furthermore, mitochondrial DNA is generally more difficult to amplify and requires a higher concentration of $MgCl_2$ compared to genomic DNA. Another potential complication of using only mitochondrial-based marker systems for pathogen identification is the presence of species hybrids (Brasier *et al*., 1999). The mitochondrial genome is uniparentally inherited along with the maternal (oogonial) line. Thus species hybrids may inherit the mitochondrial genome of either parent and confound the results of diagnostic assays. A potentially damaging hybrid species may be mis-identified as one of the parental species or, if the assay is not designed to detect both parental mtDNA haplotypes, remain undetected.

CONCLUDING REMARKS AND FUTURE CHALLENGES

Predicting the threats posed by native pathogens while minimising the risks of further invasive *Phytophthora* diseases is a challenge. This is especially true in an era in which climate change is 'moving the goal posts' and increasing global trade in plant material is increasing the risk of invasive pathogens (continuing the football theme, that might be considered an 'own goal'). Molecular methods for detection, identification and monitoring of *Phytophthora* species have proved important tools to help meet this challenge. They have allowed the rapid characterisation of new species and design and exploitation of specific DNA-based assays to accurately diagnose the cause of diseases and detect the presence of the pathogen in soil, water and plant material. Of course, molecular methods alone cannot provide all the answers but have proved valuable tools that teams of experienced plant pathologists, mycologists and ecologists have at their disposal. Despite the technological advances reported in this article, there is considerable scope for improvement. There is an ongoing need for field kits for rapid and accurate on-site detection of specific pathogens and advances in automation and miniaturisation via 'lab-on-a-chip' (e.g. Neuzil *et al*., 2006) and array-based technology to increase sample throughput are on the horizon. There is also a great need for generic *Phytophthora* detection systems that will help circumvent some of the weaknesses inherent in the current plant health systems as described by Brasier (2004).

Molecular techniques also have great potential in providing a different perspective on the way we view *Phytophthora* in natural ecosytems. Molecular approaches are increasingly used to survey microbial diversity in a range of terrestrial and marine environments (Torvisk *et al*., 2002; Kane, 2004; Massana *et al*., 2004). The observed trend is clear; greater than expected levels of microbial diversity are found. A study of basidiomycete diversity in agricultural soil, for example, revealed that only 24 of 241 'species' matched named reference species in GenBank (Lynch and Thorn, 2006). Defining and naming species in the absence of a culture is a current challenge and commonly such molecular 'species' are referred to as 'Operational Taxonomic Units' or 'Phylotypes' and defined according to sequence similarity (Schloss and Handelsman, 2005). Bioinformatics tools are being generated to automate such species determination from raw sequence data and studies are moving from describing microbial diversity to making relevant comparisons of community structure at a range of scales (Schloss and Handelsman, 2006). Defining *Phytophthora*, or perhaps wider Oomycetes, diversity at such scales has not yet been attempted but clearly provides an opportunity to increase our understanding of this group of pathogens and their impact on natural and managed vegetation systems. In the case of *Phytophthora*, standard approaches and improved primer sets are needed to examine *Phytophthora* prevalence at the community level with structured habitat-specific sampling regimes. Where novel 'molecular-species' are discovered it will serve to focus isolation attempts and standard approaches to recover and define the key attributes of the taxonomy, ecology and pathology of the species. It is an intriguing possibility that we may be only scratching at the surface of *Phytophthora* diversity and that species exist that have not to date been cultured using current methods.

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