Development of a DNA-based method for detection and identification of *Phytophthora* species

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Abstract. *Phytophthora* diseases cause major losses to agricultural and horticultural production in Australia and worldwide. Most *Phytophthora* diseases are soilborne and difficult to control, making disease prevention an important component of many disease management strategies. Detection and identification of the causal agent, therefore, is an essential part of effective disease management. This paper describes the development and validation of a DNA-based diagnostic assay that can detect and identify 27 different *Phytophthora* species. We have designed PCR primers that are specific to the genus *Phytophthora*. The resulting amplicon after PCR is subjected to digestion by restriction enzymes to yield a specific restriction pattern or fingerprint unique to each species. The restriction patterns are compared with a key comprising restriction patterns of type specimens or representative isolates of 27 different *Phytophthora* species. A number of fundamental issues, such as genetic diversity within and among species which underpin the development and validation of DNA-based diagnostic assays, are addressed in this paper.

Introduction

Since Anton de Bary created the genus *Phytophthora* and described the first species, *P. infestans*, in 1876 (de Bary 1876), over 70 different species have been described. All species of *Phytophthora* are plant pathogens and cause severe devastation to a large variety of trees, ornamental and crop plant species. The economic losses caused by *Phytophthora* diseases are considerable and it has been estimated that *Phytophthora* diseases currently cost Australian plant-based industries in excess of AU$250 million/year and impose severe limitations to the expansion of several industries, especially in the fruit and ornamental sectors (Irwin *et al*. 1995). In addition, some diseases, such as Jarrah Dieback caused by *P. cinnamomi* in West Australia (Newell 1998), Oak Decline in Europe caused by *Phytophthora alni* (Brasier *et al*. 2004) and Sudden Oak Death caused by *Phytophthora ramorum* in the USA (Rizzo *et al*. 2002), also have a serious effect on native ecosystems in some parts of the world.

Disease management of *Phytophthora* is heavily geared towards prevention of infection through the production of clean planting material, disease-free soil, high levels of nursery hygiene, resistant rootstock, and improving drainage and soil health in the field. Since hygiene is a relevant part of integrated disease management of *Phytophthora*, it is important to accurately determine the absence of *Phytophthora* in plant material, potting mixes and soil samples. The use of disease-free planting material is important in halting the spread of *Phytophthora* pathogens from nurseries to orchards and fields but it is also highly relevant to limiting the spread of *Phytophthora* pathogens across the globe. Some *Phytophthora* species such as *P. megakarya*, *P. ramorum*, *P. alni* and *P. fragariae* var. *fragariae* have a limited global distribution and quarantine measures are an important strategy for excluding these pathogens from agricultural production systems and native ecosystems. The species of *Phytophthora* causing disease on a particular host are, in a limited number of cases, easy to identify based on distinct symptoms on above-ground plant parts. For example, *P. colocasiae* causing Leaf Blight on taro and *P. infestans* causing Late Blight on potatoes. Other *Phytophthora* species, especially root-infecting *Phytophthora* species, show rather ill-defined symptoms, including yellowing and wilting of

the aboveground foliage, which can be caused by different species of *Phytophthora*, as well as by a large array of other microorganisms, including *Pythium* species. Many diseases caused by *Phytophthora* have in the past not been rigorously identified, or have been incorrectly attributed to secondary invaders as outlined by Tsao (1990). Hence, there is a clear rationale for accurate detection and identification of *Phytophthora* species.

Conventional diagnostic tests are based on isolating *Phytophthora* from diseased plant tissue using culture media containing a cocktail of antibiotics (Ribeiro 1978; Erwin and Ribeiro 1996; Drenth and Sendall 2004), induction of spore formation to reveal characters of taxonomic value and subsequent microscopic examination of the morphology of the spores. Other characters such as the presence of chlamydospores, hyphal swellings and structures associated with the formation of oospores are also taken into account when identifying *Phytophthora* to the species level. This is a labour-intensive process, which takes considerable skill and time. Soil samples expected to contain *Phytophthora* may need to be baited, using lupin seedlings (Pratt and Heather 1972), pear fruit (McIntosh 1964), citrus leaf discs (Grimm and Hutchison 1973), cocoa pods (Newhook and Jackson 1977) or a range of other baits (Drenth and Sendall 2004; Erwin and Ribeiro 1996). From lesions appearing on the plant baits, *Phytophthora* may subsequently be isolated using the same method as for diseased plant tissue. For many *Phytophthora* species, these conventional tests are a very sensitive and accurate method to determine the causal agent of disease. However, they are somewhat time-consuming and, therefore, are not well suited for routine screening of large numbers of samples. In addition, the limited number of evolutionarily relevant morphological characters available and the difficulties with inducing the production of informative structures in axenic culture, may give rise to mis-identification of many *Phytophthora* species.

Alternative methods, such as the generation of protein profiles (Gill and Zentmyer 1978; Erselius and de Vallaviele 1984; Erwin and Ribeiro 1996, Table 4.4) have been developed for identification of *Phytophthora* species. Isozyme patterns have also been established for many species (Tooley *et al*. 1985; Oudemans and Coffey 1991; Erwin and Ribeiro 1996, Table 4.4) and isozymes have been extensively used to determine variation within, rather than between, species. In order to improve the efficiency and accuracy of the detection of *Phytophthora* species, alternative methods such as enzyme immunoassays have been developed (Hardham *et al*. 1986; Gabor *et al*. 1993; Devergne *et al*. 1994) as well as a dipstick immunoassay for specific detection of *P. cinnamomi* (Cahill and Hardham 1994). None of these methods have found their way into routine large-scale detection of *Phytophthora* from plant material and soil. Four important reasons why these tests are not

widely used include: (i) lack of specificity leading to cross reactivity with other pathogens, (ii) failure to detect low levels of infestation due to lack of sensitivity, (iii) the cost and time needed to conduct some of these tests, and (iv) the lack of validation of these tests under a range of different conditions. Hence, the search for a more accurate method to detect and identify *Phytophthora* species has continued.

Molecular biological experimentation was accelerated through the development of the polymerase chain reaction (PCR) (Mullis *et al*. 1986, 1994). PCR has allowed significant advances to be made in the detection and identification of microorganisms, as it enabled rapid amplification of small amounts of DNA. High levels of specificity can be achieved through the design of oligonucleotide primers, and high sensitivity can be obtained through the choice of reaction conditions and boosted by the cyclic amplification process. Other significant developments have concerned the ease with which DNA sequence information could be obtained. Coupled with the development of algorithms and bioinformatics software specifically designed for analysing and manipulating large amounts of DNA sequence data, a solid framework was erected which allowed researchers to analyse or compare many different DNA sequences to reveal evolutionary relationships among different species and allow the design of specific primers for PCR.

One of the first questions one encounters concerns the selection of DNA sequences useful for specific detection of the genus *Phytophthora* and identification of species within this genus. There must be sufficient variation in the targeted DNA sequence between different species to allow species-specific detection, and sufficiently conserved areas within species to allow detection of all isolates within any given species or within the genus as a whole. Genes encoding rRNA in eukaryotic organisms possess these qualities as they are essential for cellular viability and exhibit regions that differ in nucleotide sequence variability between species (White *et al*. 1990). In addition to a suitable level of sequence variation between different species, the use of rDNA can boost the sensitivity of a diagnostic test since the gene exists in a high copy number (100–200 copies) in the genome. In most eukaryotic organisms, the rRNA (rDNA) genes comprise a multigene family in which the copies are arranged in tandem repeats (White *et al*. 1990). As in most eukaryotes, each repeat in Oomycete organisms consists of a single transcription unit that includes the small subunit, the 5.8S, and the large subunit genes separated by two internal transcribed spacers, ITS1 and ITS2, and the intergenic spacer (IGS) (Lee and Taylor 1992). The availability of these new tools gave rise to numerous studies that aimed to identify and delineate different *Phytophthora* species (Crawford *et al*. 1996; Cooke *et al*. 2000; Förster *et al*. 2000; Kroon *et al*. 2004*a*). These studies have given rise to a significant increase in our understanding of the

evolutionary relationships among most species within the genus *Phytophthora*.

The ever-growing amount of available DNA sequence data and a growing understanding of the evolutionary relationships among the different *Phytophthora* species, combined with the available DNA amplification technology, allowed for the development of a range of molecular diagnostic assays. Numerous diagnostic assays have been developed based on ITS, the internal transcribed spacers, of the rRNA repeat unit through the design of oligonucleotide primers that are highly specific to individual species of *Phytophthora* including *P. palmivora*, *P. nicotianae*, *P. cryptogea*, *P. cinnamomi* (Drenth *et al*. 1999*a*), *P. ramorum* (Kroon *et al*. 2004*b*) and *P. fragariae* (Bonants *et al*. 1997), whereas others use restriction digest patterns of the mitochondrial DNA to distinguish between species (Martin and Tooley 2004). Although most of these techniques answered one or more relevant biological questions at the time, none of them was suitable for routine detection and identification of a wide range of different *Phytophthora* species. What these tests have in common is that they are specific to one or a few different species but they are not well suited to identify a range of *Phytophthora* species when no prior information is available concerning the species likely to be involved. Thus, in order to develop an assay, which is suitable for routine identification of a large range of species within the genus *Phytophthora*, a different approach was needed.

The overall objective of the research described in this paper was to develop a DNA-based diagnostic assay for detection and identification of a wide range of *Phytophthora* species of economic importance in Australia and elsewhere. More specific aims were (i) obtain rDNA sequence information from a number of representative strains of economically important species within the genus *Phytophthora*, (ii) design PCR primers that are specific to the genus *Phytophthora*, (iii) develop and validate a rapid and easy to use assay for the identification of a wide range of different *Phytophthora* species, and (iv) validate the diagnostic assays for routine detection and identification of *Phytophthora* species. A rapid way to detect and identify species within the genus *Phytophthora* provides an important tool in pathogen detection and determining the causal agent of many diseases. It may also be used directly on plant tissue, without the need for obtaining pure cultures of the *Phytophthora* pathogen; this may speed up the diagnosis considerably and underpin decisions in disease management. In addition, it allows for identification of emerging and exotic *Phytophthora* species in a standardised and routine manner.

Methods

The development of our PCR-based identification for *Phytophthora* was undertaken in a systematic manner that involved a detailed study of the evolutionary relationships between different *Phytophthora* species to ensure species could be accurately identified. We have identified and adhered to the following successive steps in the development of a DNA-based detection and identification assay for *Phytophthora*:

(i) assess levels of genetic diversity present in targeted *Phytophthora* species and select authentic and representative isolates from each species;

(ii) obtain rDNA sequence information from representative strains of each of the species;

(iii) reveal evolutionary relationships among the different *Phytophthora* species based on rDNA sequence information and relate this to relevant biological species;

(iv) use rDNA sequence information to design and synthesise PCR primers which are highly specific for each of the targeted species;

(v) test primers under predetermined testing conditions to reveal the optimal primer combinations for effective amplification of the rDNA target area;

(vi) test primers for specificity on pre-determined tester series consisting of *Phytophthora* and *Pythium* species;

(vii) test sensitivity of highly specific primer combinations;

(viii) validation of the PCR-based diagnostic test through comparison of the assay with conventional diagnostic assays on pure cultures and infected plant material; and

(ix) optimisation of the DNA-based diagnostic test for routine use.

Significant parts of the first three steps of this process with regard to genetic diversity within *Phytophthora* species have already been published (Drenth *et al*. 1996; Irwin *et al*. 1996; Drenth and Goodwin 1999; Linde *et al*. 1999; Purwantara *et al*. 2001) and the evolutionary makeup of the genus *Phytophthora* has been elucidated based on ITS sequence data (Crawford *et al*. 1996; Cooke *et al*. 2000; Förster *et al*. 2000). More recent studies (Kroon *et al*. 2004*a*; Martin and Tooley 2004) have provided additional sequence data from other genomic and mitochondrial sequences. Taken together, these provide an in-depth overview of the genetic diversity and evolutionary relationships among *Phytophthora* species such that no further details are provided in this paper.

Phytophthora *culture collection*

In order to design species- and genus-specific DNA-based diagnostic tests, sequence information from the rRNA (rDNA) repeat was obtained from representative isolates from many different *Phytophthora* species, isolates within each species, as well as from species of the closely related genus *Pythium* (Table 1). Before and during the conduct of this project, the CRC for Tropical Plant Protection and its predecessor, CRC for Tropical Plant Pathology, had developed a relatively large *Phytophthora* collection currently consisting of 3511 entries. From the collection, a number of representative isolates from each species was chosen for DNA sequence analysis (Table 1). Collection details are provided for the strains, as listed in Table 3 and shown in Fig. 2 but, for most species, numerous isolates were sequenced as summarised in the final column of Table 1. Isolates were maintained on 20% clarified juice agar (Ribeiro 1978) at 25°C. After 5 to 7 days, mycelium was scraped from the plates and inoculated into 200 mL of 20% clarified juice in a 500 mL flask. Cultures were grown for 5 to 7 days at 25°C on an orbital shaker (150 rpm). Fungal mycelium was harvested by vacuum filtration through Miracloth (Calbiochem Inc., Australia), freeze-dried and then stored at −70°C until used for DNA extractions. Deoxyribonucleic acid was extracted from fungal mycelium, ground with a mortar and pestle, by a method modified from Raeder and Broda (1985) by the addition of 10 mg/mL RNase A (Sigma, Australia) and incubation at 37°C for 1 h after the isopropanol precipitation. This was followed by precipitation

Table 1.   Details of representative *Phytophthora* species and their ITS1–5.8S-ITS2 DNA sequence used in this study

Species	Accession number	Isolate details					
		Isolation number			Origins		
		International[A]	GenBank[B]	Host	Country	Collection	No. of isolates sequenced[C]
Phytophthora							
P. arecae	UQ2820	IMI348342	AF266781	*Cocos nucifera*	Indonesia	1991	1
P. boehmeria	UQ4505	–	–	–	Australia	1999	1
P. cactorum	CAC2	IMI296524	AF266772	*Rubus idaeus*	Wales	1985	3
P. cambivora	UQ2633	IMI296831	AF266763	–	–	–	1
P. cambivora	UQ2634	–	–	–	–	–	1
P. capsici	UQ2819	IMI352321	AF266787	*Piper nigrum*	India	1989	2
P. capsici	UQ1529	–	–	–	Brazil	1987	1
P. cinnamomi	UQ881	–	AF266764	*Syzygium aromaticum*	Malaysia	–	17
P. cinnamomi	UQ733	–	–	–	Australia	1981	2
P. citricola	UQ2621	IMI031372	AF266788	–	Australia	1989	2
P. citrophthora	UQ1320	IMI332632	AF266785	–	–	1994	3
P. clandestina	UQ726	IMI287317	–	*Trifolium subterranea*	Australia	1990	2
P. cryptogea	UQ843	IMI045168	AF266769	–	Australia	–	27
P. drechsleri	UQ3041	IMI040500	AF266798	*Solanum tuberosum*	Argentina	1935	7
P. erythroseptica	UQ1569	–	AF266797	*Solanum tuberosum*	USA	1994	4
P. fragariae v. *fragariae*	UQ3668	IMI330736	AF266762	*Fragaria ananassa* x	Scotland	1972	3
P. gonapodyides	UQ2823	IMI06875	AF266793	–	UK	1989	3
P. infestans	UQ2118	–	AF266779	*Solanum tuberosum*	Netherlands	1988	4
P. iranica	UQ2132	IMI158964	–	*Solanum melongena*	Iran	1969	1
P. katsurae	UQ4058	IMI 360596	AF266771	*Rambutan*	Australia	1998	2
P. medicaginis	UQ125		AF266277	*Medicargo sativa*	Australia	1987	3
P. megakarya	UQ2822	IMI337104	AF266782	*Theobroma cacao*	Ghana	1990	2
P. megasperma	UQ3043	IMI133317	AF266794	*Malus sylvestris*	Australia	1968	18
P. mirabilis	UQ1691	ATCC64130	AF266777	*Mirabilis jalapa*	Mexico	1995	2
P. nicotianae	UQ848	–	AF266776	–	Australia	–	3
P. palmivora	UQ1294	–	AF266780	*Theobroma cacao*	Papua New Guinea	1994	5
P. parasitica[D]	UQ630	–	–	–	Australia	–	2
P. sojae	UQ336	–	AF266769	*Glycine max*	Australia	1994	6
P. syringae	UQ2635	–	AF266803	–	–	–	3
P. trifolii	UQ2143	–	AF266800	*Trifolium*	–	–	2
P. vignae	UQ136	–	AF266766	*Vigna sinesis*	Australia	1988	2
Pythium							
Py. aphanidermatum	UQ2071	–	AF271227	Sugarcane soil	Australia	1992	1
Py. dissotoctum	UQ2623	–	AF271228	Root rot	Australia	1988	1
Py. graminicola	UQ604	–	AF271229	Root rot	Australia	1993	3
Py. irregulare	UQ2622	–	AF271226	Root rot	Australia	–	5
Py. undulatum	–	IMI337230	AF271230	*Larix* sp.	Scotland	1989	1
Py. arrhenomanes	UQ608	–	–	–	–	–	1
Py. dissotocum	UQ2623	–	–	–	–	–	1
Pythium ultimum	UQ1406	–	AF271225	*Euphorbia pulcherrima*	USA	–	3
Pythium debaryanum	UQ2624	–	–	–	–	–	1

[A]International designations: ATCC, American Type Culture Collection, USA; IMI, CABI Bioscience (International Mycological Institute), United Kingdom.
[B]Accession numbers for GenBank database (no sequence was submitted if a near identical sequence was already in the database).
[C]When the number is >1, additional isolates of the same species were sequenced to determine if sequence variation occurred within the rDNA repeat of that species.
[D]*P. parasitica* is conspecific to *P. nicotianae* and the name *P. nicotianae* has priority (See Erwin and Ribeiro 1996).

with 2 volumes of 100% ethanol and centrifugation at 10 000*g* for 10 min. The pellet was rinsed with 70% ethanol, dried under vacuum and resuspended in TE buffer (pH 8.0). Deoxyribonucleic acid was stored at −20°C.

PCR amplification

The region of the ribosomal repeat from the 3′ end of 18S gene (through ITS1, the 5.8S gene and ITS2) to the 5′ end of the 28S gene defined by the oligonucleotide primers TW81 and AB28 (Howlett *et al*. 1992) was amplified by PCR. PCR was performed in a 50 µL reaction containing 50–100 ng genomic DNA, 100 ng of each oligonucleotide primer, 67 mM Tris-HCL (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 0.45% v/v Triton X-100, 200 µg/mL gelatin, 5 mM $MgCl_2$, 200 µM each of dATP, dCTP, dGTP and dTTP (Biotech Ltd, Australia) and 3 Units *Tth* plus DNA Polymerase (Biotech Ltd, Australia). Mineral oil (30 µL, Sigma, Australia) was overlaid on the reaction mix and the tube was briefly centrifuged. The reaction was performed in a Perkin Elmer DNA Thermocycler 4800 (Perkin Elmer, USA) for 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 45 s and extension at 72°C for 2 min, with an initial denaturation of 3 min at 94°C before cycling and a final extension of 10 min at 72°C after cycling. A portion (3 µL) of the amplified products was run in a 0.8% agarose gel in Tris-Acetate-EDTA (TAE) buffer (Sambrook 1989). The presence of a single bright band (850–950 bp) for each sample was a check for successful amplification. The PCR product was purified directly from the remainder of the PCR amplification reaction (47 µL) using the Wizard PCR Preps Purification system (Promega Corporation, Australia). All purified DNA samples were stored at –20°C.

DNA sequencing

Sequencing was conducted on an Applied Biosystems Model 373A DNA Sequencer (Applied Biosystems Inc., Australia) as recommended by the manufacturer. In order to sequence both strands, internal oligonucleotide primers S1 to S6 (Crawford *et al*. 1996) were synthesised on an Oligo 1000 DNA Synthesiser (Beckman, Australia) to obtain a series of overlapping amplicons. The sequence data were checked between complementary strands and the resulting sequences were aligned using the computer software package, CLUSTAL V (Higgins *et al*. 1992). The alignment of all sequences was also checked visually.

Using this methodology, we obtained rDNA sequence information from the ITS1 and ITS2 regions of 132 different isolates representing 27 different *Phytophthora* species (Table 1). For the design of primers specific to the genus *Phytophthora*, we also obtained rDNA sequence data from an array of different *Pythium* species (Table 1). Due to great variability among *Pythium* species, we designed a series of additional primers, 3A-D and 5B to obtain the ITS1–5.8S-ITS2 sequence of these species (Drenth *et al*. 1999*b*).

Design and testing of genus and species-specific primers

In order to design oligonucleotide primers specific to the genus *Phytophthora* as a whole, the sequences of all 27 different *Phytophthora* species were aligned using the computer software package Sequencer 2.1 (Gene Codes Corporation, Ann Harbor, MI, USA). The aligned sequences were printed in a parallel format. Specific sequences, showing no variation within the *Phytophthora* genus, were visually targeted for potential oligonucleotide primer binding sites. Each potential oligonucleotide primer sequence was analysed for specificity, melting temperature, self-homology, cross-homology, internal stability, PCR product size and compatibility with potential complementary oligonucleotide primers to ascertain its ability to yield a suitable oligonucleotide primer using the computer package Oligo 4.1 (Rychlik, W. National Biosciences, Plymouth,

MN, USA). The following parameters were used in the design of the primers:

(i) Melting temperature: melting temperature (T_m) depends on the length of the primer and the GC/AT ratio. The GC/AT ratio of the diagnostic *Phytophthora* oligonucleotide primers was sought within the 40–60% range and the difference in T_m between different oligonucleotide primers was ideally 5°C or less. Although larger oligonucleotide primers have increased T_m values (higher annealing temperatures) they do not necessarily make better or more specific oligonucleotide primers.

(ii) Self and cross homology: oligonucleotide primers were designed free of 3′ complementary ends to reduce the chance of primer-dimer artefact formation which inevitably leads to a reduction in yield. Designed oligonucleotide primers were also checked for self-complementarities using the computer package Oligo 4.1 (Rychlik, W. National Biosciences, Plymouth, MN, USA). Self-complementary oligonucleotide primers form hairpin loops, which are troublesome, particularly at the 3′end, because this can cause internal primer extension, thus preventing amplification of the target DNA. Potential hybridisation of the *Phytophthora* species-specific oligonucleotide primer with the rDNA repeat of *Pythium* species was checked using computer programs.

(iii) Internal stability: oligonucleotide primers were designed in such a way that their stability was high at the 5′ end but somewhat less stable at the 3′ end. This effectively eliminates false priming. An oligonucleotide primer with low stability at its 3′ end (low GC:AT ratio) will function well in PCR because false priming with non-target sites will not be stable enough to initiate synthesis. We used a ΔG (at 25°C) at the 3′end no lower than −9 kcal/mol. We sought to avoid oligonucleotide primers with GC-rich 3′ termini as they have high stability and can efficiently anneal with non-target sequences, resulting in non-specific product synthesis.

Synthesis and purification of oligonucleotide primers

Oligonucleotide primers were synthesised on a Beckman Oligo 1000 DNA synthesiser (Beckman, Australia) according to the manufacturer's instructions. Purification was by the ultra-fast DNA cleavage and deprotection kit (Beckman, Australia). Oligonucleotide primers were incubated for 5 min in 0.6 mL of ammonium hydroxide at room temperature, 10 min at 65°C and then 5 min at 4°C. After the solution had been dried down under vacuum for about 2 h, the pellet was resuspended in 200 µL MilliQ water before adding 30 µL 2 M NaOAc and 400 µL EtOH and placed for 30 min at 4°C to precipitate the synthesised oligonucleotides. The DNA was collected by centrifugation for 5 min, the supernatant discarded and the DNA pellet washed once in 0.5 mL 70% EtOH. The DNA was centrifuged and dried before dissolving in 100 µL MilliQ water. The oligonucleotide concentration was measured on a GeneQuant RNA/DNA calculator (Pharmacia, USA) and diluted to 2.1 pmol/µL. Never more than one primer was handled per day to reduce the risk of contamination when purifying or diluting out stock primer.

Polymerase chain reaction

The standard PCR protocol used for testing oligonucleotide primers was: 10× PCR Buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl; 0.15 mM $MgCl_2$; 0.001% gelatin), 0.2 µM dNTPs (50 µM of each dNTP), 0.25 µM for each primer, 0.5 Unit *Tth* Plus DNA polymerase (Fisher-Biotech, Perth Australia), and 50 ng *Phytophthora* DNA in a total reaction volume of 25 µL. The PCR reactions were overlaid with a drop of liquid paraffin. The following cycling regime was standard: initial denaturation 94°C for 5 min, denaturation 94°C for 30 s, annealing temp (60–70°C) for 30 s and extension for 1 min at 72°C. All PCR reactions were conducted in duplicate.

Primers were subjected to the following three tests to ascertain their suitability as *Phytophthora* genus-specific primers.

(i) Annealing temperature: the maximum and optimum annealing temperature per primer pair was empirically investigated starting with a range of annealing temperatures with steps of 2°C starting at 60°C.

(ii) PCR cycles: the specificity and efficiency of selected primer pairs in the PCR was tested from 15 to 40 cycles in steps of 5. Only those primer pairs yielding a single amplification product identical in size from 15 cycles to 40 cycles were tested further.

(iii) Specificity: after successful amplification of the target DNA, the specificity of the primer pairs was checked on the range of different *Pythium* species. When no cross-reactivity was shown, the primer pairs were checked against the full range of *Phytophthora* species. In order to develop a robust protocol for amplification of the rDNA amplicon from a wide range of *Phytophthora* species, the PCR protocol was also tested by halving and doubling the concentration of each component, while keeping all other components constant.

Restriction digests of the amplicon

In order to obtain diagnostic DNA fingerprints, the genus-specific PCR amplicon obtained using forward primer A2 in combination with reverse primer I2 (Table 2), was subjected to three different restriction enzymes, *Msp*I, *Rsa*I and *Taq*I. Digestion was conducted in a total volume of 10 μL comprising of 5 μL of the amplified PCR product, 3 μL MilliQ water, 1 μL restriction enzyme (*Msp*I U/20 μL, *Rsa*I U/10 μL and *Taq*I U/20 μL) and 1 μL 10× CA buffer (200 mM Tris-HCL pH 7.2; 70 mM $MgCl_2$; 1 M KCl; 20 mM β-Mercaptoethanol; 1 mg/mL BSA. Samples were spun for 5 s before incubation at 37°C for 30 min for *Msp*I and *Rsa*I and at 65°C for *Taq*I digests.

Gel electrophoresis

In order to clearly separate DNA fragments within a range of 50–500 bp, 3% high-resolution agarose gels (Progen, Australia or Amresco, Ohio, USA) were used. These gels were run in modified TBE buffer comprising of 90 mM Tris-borate and 2 mM EDTA. We also reduced the thickness of the gel to 3.8 mm. This significantly increased the sharpness of the bands and reduced the time needed to run the gels while at the same time making the running of high resolution agarose gels far more cost effective.

Sample preparation

Once we had identified primer pair A/2-I/2 that consistently generated a *Phytophthora* genus-specific amplicon, we wanted to ascertain if this amplicon could be generated from DNA extracted from infected plant tissue. An important part of DNA-based diagnostics is the ability to effectively obtain DNA from an infected plant sample with minimal contamination by components which may inhibit the PCR amplification process. Because *Phytophthora* species can attack a wide range of different plants, we tried a range of different published DNA isolation techniques from plant material (Edwards *et al.* 1991; Guillemaut and Maréchal-Drouard 1992; Cheung *et al.* 1993; Klimyuk *et al.* 1993; Wang *et al.* 1993). The method below adapted from McDonald *et al.* (1994) worked best with mycelium from axenic cultures and

Table 2.   Sequence of *Phytophthora* genus-specific oligonucleotide primers

Primer name	Oligonucleotide sequence (5′–3′)
A2—forward	ACTTTCCACGTGAACCGTTTCAA
I2—reverse	GATATCAGGTCCAATTGAGATGC

Phytophthora from infected tissue types such as infected root tips and leaf lesions. Approximately 20 mg of mycelium scraped with a scalpel from a culture plate, or infected tissue cut from the margin of a lesion on plant material, or a freshly infected root tip, was ground in an Eppendorf tube initially in 200 μL of extraction buffer (200 mM Tris-HCl (pH 7.5), 200 mM NaCl, 25 mm EDTA, 0.5% w/v SDS, made up in MilliQ water), then further macerated with the addition of another 800 μL of extraction buffer. The sample was then vortexed for 30 s, followed by centrifugation (10 000*g*) for 10 min at room temperature. Supernatant (750 μL) was transferred to a new tube and DNA precipitated with the addition of 600 μL of isopropanol. After 5 min incubation at room temperature, the DNA was recovered by centrifugation (10 000*g*) for 10 min at room temperature. The DNA pellet was washed with 750 μL 70% ethanol and centrifuged (10 000*g*) for 5 min. The ethanol was poured off and the DNA pellet was vacuum dried. The pellet was finally resuspended in 50 μL MilliQ water.

Results

The generic primers TW81 and AB28 were able to amplify all 27 different *Phytophthora* species and nine different species of *Pythium*. The use of the internal ITS primers allowed us to obtain complete sequence information from all *Phytophthora* and *Pythium* species listed in Table 1. For the *Pythium* species, some alternative internal primers, 3A-D and 5B, were designed (Drenth *et al.* 1999*b*) to amplify internal sectors of the rDNA to obtain full sequence information.

One primer pair, A2 (forward) and I2 (reverse) (Table 2) produced an amplicon from all 27 different species of *Phytophthora* with equal efficiency, as exemplified in Fig. 1 for 2 isolates each of 3 different *Phytophthora* species. None of the *Pythium* species tested produced an amplicon with these primers. This so called 'genus-specific' primer pair was also able to produce a visible amplicon on an agarose gel after 15 cycles when starting with 50 ng of genomic DNA of *Phytophthora*. The sensitivity of these primers was also tested when varying the amount of template used in a dilution series and varying the number of cycles in the PCR. The sensitivity of the PCR-based diagnostic assay was tested under our standard conditions on 10-fold dilutions of purified DNA of *P. cinnamomi* (strain UQ734) and we were able to detect as little as 2 pg of target DNA in a single sample. This amount of DNA corresponds to approximately the material in two nuclei in the *Phytophthora* genome. Since developing these primers, we have further tested them on 1660 *Phytophthora* isolates comprising a wide range of different species and in all cases a single amplicon was amplified of a size between 752 and 832 base pairs, which encompasses the ITS1–5.8S-ITS2 region of the rDNA of *Phytophthora* species.

Species identification

To be able to distinguish between the different *Phytophthora* species under investigation, we used the restriction enzymes *Msp*I, *Rsa*I and *Taq*I on the resulting amplicon obtained using the genus-specific primers. Details of the sizes of the restriction fragment patterns for each species based on

Table 3.   Amplicon size and band sizes (bp) derived from DNA sequence information of rDNA produced when using the genus-specific PCR primers of different *Phytophthora* species after restriction digests of the amplicon

Phytophthora species	Accession number	PCR amplicon	*Msp*I band sizes	*Rsa*I band sizes	*Taq*I band sizes
P. palmivora	UQ1294	788	467, 321	359, 314, 105, 10	283, 235, 147, 59, 57, 7
P. arecae	UQ2820	788	467, 321	359, 314, 105, 10	283, 235, 147, 59, 57, 7
P. megakarya	UQ2822	791	464, 327	359, 278, 105, 31, 10	235, 193, 146, 88, 59, 57, 7
P. nicotianae	UQ848	804	349, 335, 120	373, 293, 105, 33	249, 193, 148, 90, 59, 58, 7
P. parasitica	UQ630	804	349, 335, 120	373, 293, 105, 33	249, 193, 148, 90, 58, 44, 15, 7
P. boehmeria	UQ4505	828	515, 313	351, 303, 174	256, 227, 197, 89, 59
P. katsurae	UQ4058	776	302, 254, 220	340, 181, 140, 105, 10	216, 150, 142, 89, 62, 59, 51, 7
P. citrophthora	UQ1320	781	301, 254, 226	339, 321, 111, 10	215, 199, 149, 90, 59, 32, 30, 7
P. capsici	UQ1529	752	281, 250, 221	318, 274, 105, 45, 10	194, 194, 149, 90, 60, 59, 7
P. capsici	UQ2819	752	250, 221, 204, 77	318, 274, 105, 45, 10	194, 149, 148, 90, 60, 59, 46, 7
P. iranica	UQ2132	793	323, 249, 221	361, 284, 105, 33, 10	439, 237, 59, 58
P. mirabilis	UQ1691	797	329, 247, 221	367, 282, 105, 33, 10	283, 243, 155, 59, 57
P. infestans	UQ2118	797	329, 247, 221	367, 282, 105, 33, 10	283, 243, 155, 59, 57
P. vignae	UQ136	827	368, 340, 119	378, 332, 107, 10	195, 195, 150, 90, 72, 59, 59, 7
P. cinnamomi	UQ733	828	339, 221, 146, 122	377, 170, 165, 106, 10	194, 181, 150, 90, 74, 60, 59, 12, 7
P. cinnamomi	UQ881	829	368[A], 339, 221, 147, 122	377, 171, 165, 106, 10	194, 181, 151, 90, 74, 60, 59, 12, 7
P. fragariae	UQ3668	832	340, 221, 146, 125	378, 170, 168, 106, 10	194, 194, 151, 90, 81, 60, 59, 3
P. sojae	UQ336	828	341, 222, 146, 119	379, 332, 107, 10	195, 194, 150, 90, 72, 60, 59, 7
P. cactorum	UQ2620	794	326, 220, 140, 108	364, 282, 105, 33, 10	240, 193, 148, 89, 59, 58, 7
P. syringae	UQ2635	814	314, 222, 180, 98	352, 176 149, 107, 20, 10	228, 195, 166, 90, 69, 59, 7
P. citricola	UQ2621	761	291, 220, 178, 72	329, 317, 105, 10	205, 193, 149, 90, 59, 58, 7
P. clandestina	UQ726	792	249, 221, 202, 120	360, 284, 105, 33, 10	432, 236, 59, 58, 7
P. erythroseptica	UQ1569	797	257, 145, 122, 100, 85, 52, 32, 4	351, 329, 107, 10	227, 195, 167, 90, 59, 59
P. cryptogea	UQ843	801	261, 145, 122, 100, 85, 52, 32, 4	354, 329, 107, 10	230, 195, 167, 90, 59, 59
P. drechsleri	UQ3041	799	262, 146,122, 117, 100, 52	352, 330, 107, 10	228, 195, 167, 91, 59, 59
P. medicaginis	UQ125	795	257, 177, 122, 100, 84, 51, 4	350, 192, 136, 107, 10	226, 195, 167, 90, 59, 58
P. gonapodyides	UQ2823	819	334, 147, 125, 115, 98	372, 172, 158, 107, 10	150, 143, 143, 130, 118, 69, 59, 8
P. megasperma	UQ3043	814	334, 143, 125, 114, 98	372, 325, 107, 10	149, 143, 140, 130, 118, 67, 59, 8
P. cambivora	UQ2633	832	340[A], 294, 221, 146, 125, 46	378, 170, 168, 106, 10	194, 194, 151, 90, 84, 60, 59
P. cambivora	UQ2634	832	340[A], 294, 221, 146, 125, 46	378, 170, 168, 100, 10	194, 194, 151, 90, 76, 60, 59, 8
P. trifolii	UQ2143	795	277, 257, 122, 84, 51, 4	350, 328, 107, 10	226, 200, 167, 90, 58, 54

[A]Additional band within species due to the presence of different amplicons within one species.

sequence information of the rDNA are given in Table 3. To rapidly identify a species by its restriction pattern, the patterns were arranged according to the number of fragments visibly obtained with the restriction enzyme *Msp*I to form a molecular identification key (Fig. 2). The combination of high-resolution agarose and thinner gels allowed for clear resolution in the shortest possible time available.

A few species that showed high levels of sequence homology (*P. infestans* and *P. mirabilis*, and *P. cryptogea* and *P. erythroseptica*) could not be distinguished using this three-enzyme combination. Although sequence variability

exists between *P. erythroseptica* and *P. cryptogea*, the three restriction enzymes in combination with the size fractionation on agarose gels used in our diagnostic test are unable to differentiate between these closely related species. However, DNA sequence differences exist between these species (Cooke *et al.* 2000; Kroon *et al.* 2004*a*) and they are considered to be different species. *P. palmivora* and *P. arecae* also showed an identical restriction pattern but it has also been suggested that these species are conspecific (Ho 1992; Mchau and Coffey 1994). Nevertheless, different diseases are caused by *P. arecae* and *P. palmivora*. Clearly
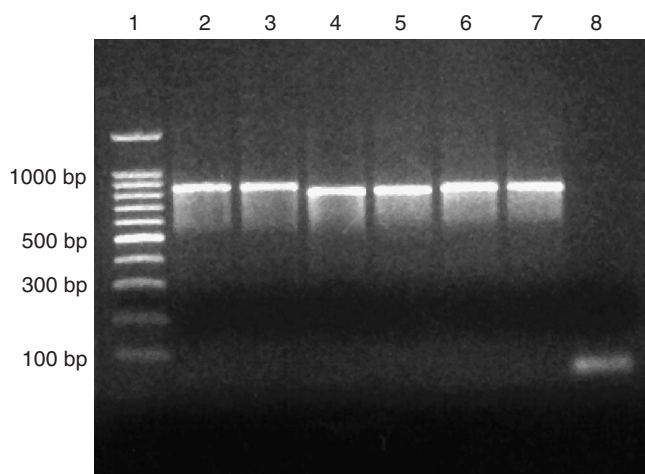
Fig. 1. Genus PCR product of six *Phytophthora* isolates of three different species. Lane 1: 100 bp ladder; lane 2: *P. cinnamomi* UQ733; lane 3: *P. cinnamomi* UQ734; *P. palmivora* UQ230; lane 5: *P. palmivora* UQ639; lane 6: *P. nicotianae* UQ747; lane 7: *P. nicotianae* UQ852; lane 8: negative control.

more work is needed to clarify the status of *P. arecae* as a separate species.

Deoxyribonucleic acid sequence data of the rDNA region under investigation had already alerted us to variability in the rDNA sequence within a single species. Obtaining a restriction pattern of one rDNA amplicon will, of course, ignore most small mutations unless they happen to fall within the recognition sequence of the restriction enzyme. For example, an extra *Msp*I restriction site occurs in *P. capsici* isolate UQ2819, producing four fragments instead of the three produced by digesting *P. capsici* isolate UQ1529 (Fig. 2: Table 3). In this case, the mutation is fixed in all rDNA tandem repeats of the template DNA, or at a level such that any alternate mutations are masked and not visualised through the PCR and RFLP process. In this instance, the sum of the RFLP fragments is equal to the size of the PCR amplicon. Variability within species was also observed in *P. nicotianae* where an extra restriction site was observed in UQ630.

A more complex issue that may confound species identification arises from polymorphisms in rDNA repeat units within a single isolate of *Phytophthora* that may give rise to apparently alternative or aberrant RFLP patterns. For example, isolates of *P. cinnamomi* are known to produce different *Msp*I RFLP profiles from genus PCR amplicons of slightly different size; UQ733: 339, 221, 146, 122 bp; UQ881: 368, 339, 221, 147, 122 bp. The isolate UQ881 produces an additional RFLP fragment, 368 bp, which is produced from amplicons that have lost the *Msp*I site between the 146 bp and 222 bp fragments. The relative intensity of the 368 bp fragment in electrophoresis gels of the restriction fragment mix depends on the relative proportion of rDNA tandem gene copies that carry the mutation that results in the loss of a diagnostic *Msp*I site. The diploid nature of

Phytophthora species may also give rise to heterozygosity for the rDNA repeat, in which case we would expect bands to be of equal intensity. Aberrant RFLP profiles of this type, which result in the partial loss or gain of restriction sites in some amplicons but not others, can be recognised because the sum of the RFLP fragments is greater than the size of the genus PCR amplicon. Variability within species and within rDNA repeat units within a single isolate is expected to be present in all species but, in our experience, is typically only detected when large numbers of samples are screened in a routine manner. Since 1996, we have applied our assay to over 1660 different isolates of *Phytophthora* from Australia and overseas which has allowed us to identify such variants. This extensive screening has also lead to many refinements of the procedures and truly validates the assay as a rapid tool for identification of a wide range of *Phytophthora* species.

To enhance the usefulness of the identification assay, we also tried to match the amplification technique with a reliable and robust DNA extraction technique. We adapted a quick and very effective DNA extraction technique from mycelium growing *in vitro*, infected leaves and root tips. In order for PCR-based diagnostic tests to work successfully, a consistent and reliable DNA isolation method from different plant parts, as outlined in Methods, is a vital part of this assay. Since in Phytophthora diagnostics we are dealing with such a large range of different diseases and affected tissues, it is unlikely that one technique could meet all requirements.

Discussion

Identification of representative isolates of each species and determining the evolutionary relationships among the different *Phytophthora* species based on the isolates used in this study have been published previously (Crawford *et al.* 1996; Cooke *et al.* 2000). Since that time, additional studies have been conducted using ITS sequence information (Förster *et al.* 2000) and a combination of mitochondrial and nuclear gene sequences (Kroon *et al.* 2004*a*; Martin and Tooley 2004) that all support the evolutionary relationships among an extensive set of *Phytophthora* species, as first put forward by Cooke *et al.* (2000).

Analysis of genetic diversity within and between *Phytophthora* populations through the use of numerous isolates for each species under investigation is important as it not only allows for accurate assessment of evolutionary relationships between species but also ensures that any diagnostic assay developed does detect and identify the widest possible range of individuals within a species. A population approach, as adhered to in this paper in which we accessed large numbers of strains from each of the species, allowed us to select areas of the rDNA repeat that were conserved among all *Phytophthora* species. This in turn enabled identification of sites for a forward primer
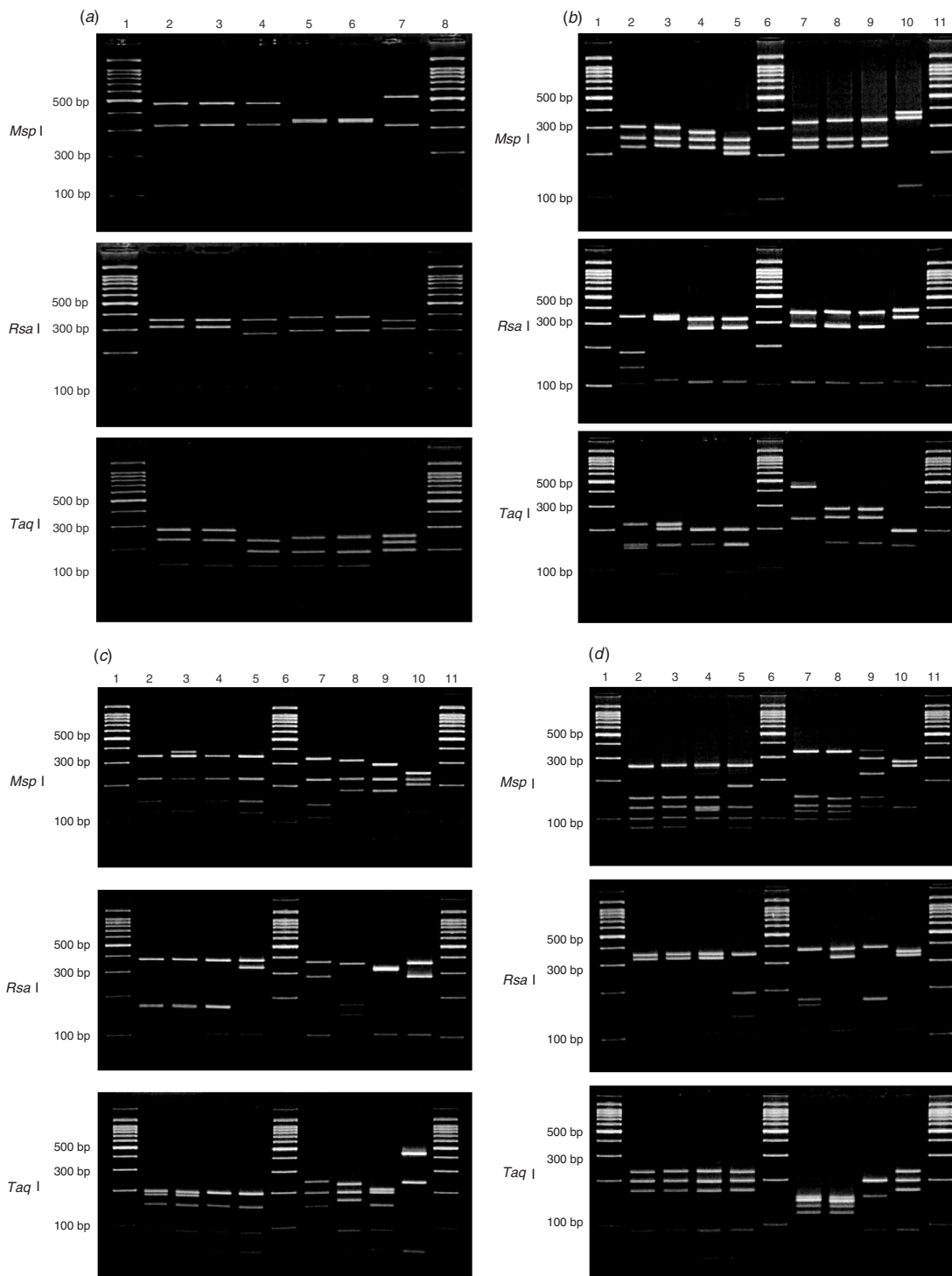
Fig. 2. Restriction digest patterns of the amplicon after PCR using *Phytophthora* genus-specific primer pair A2-I2. Details of the sizes of the restriction fragments are listed in Table 3. Patterns are organised based on the number of visible bands obtained with *Msp*I. (*a*) Two band species. Lane 1: 100 bp ladder; lane 2: *P. palmivora*; lane 3: *P. arecae*; lane 4: *P. megakarya*; lane 5: *P. nicotianae*; lane 6: *P. parasitica*; lane 7: *P. boehmeria*; lane 8: 100 bp ladder. (*b*) Three band species. Lane 1: 100 bp ladder; lane 2: *P. katsurae*; lane 3: *P. citrophthora*; lane 4: *P. capsici* UQ1529; lane 5: *P. capsici* UQ2819; lane 6: 100 bp ladder; lane 7: *P. iranica*; lane 8: *P. mirabilis*; lane 9: *P. infestans*; lane 10: *P vignae*; lane 11: 100 bp ladder. (*c*) Four band species. Lane 1: 100 bp ladder; lane 2: *P. cinnamomi* UQ733; lane 3: *P. cinnamomi* UQ881; lane 4: *P. fragariae*; lane 5: *P. sojae*; lane 6: 100 bp ladder; lane 7: *P. cactorum*; lane 8: *P. syringae*; lane 9: *P. citricola*; lane 10: *P. clandestina*; lane 11: 100 bp ladder. (*d*) Five band species. Lane 1: 100 bp ladder; lane 2: *P. erythroseptica*; lane 3: *P. cryptogea*; lane 4: *P. drechsleri*; lane 5: *P. medicaginis*; lane 6: 100 bp ladder; lane 7: *P. gonapodyides*; lane 8: *P. megasperma*; lane 9: *P. cambivora*; lane 10: *P.trifolii*; lane 11: 100 bp ladder.

and a reverse primer with the ability to specifically amplify DNA from a wide range of different *Phytophthora* species. Sequencing of multiple isolates of the same species revealed that intra-specific variation in the rDNA ITS sequence is low and stable, but not absent. For example, *P. capsici* shows variation for the number of *Msp*I restriction sites, while other species, such as *P. cinnamomi*, showed variation among the rDNA repeat units within a single isolate. Sufficient sequence variation is present between species, allowing identification of all *Phytophthora* species targeted in this study based on ITS1 and ITS2 sequence alone (Cooke *et al*. 2000).

PCR-RFLP patterns may mimic partial or incomplete digestion products due to the failure of the restriction enzyme to cut some or all genus PCR amplicons at a specific site. It must be remembered that the PCR amplicons generated by the PCR process are *in vitro* amplification products from an rRNA multigene family, in which copies are arranged in tandem repeats in the genomic template DNA. Concerted evolution acts to homogenise gene sequence composition in these tandem repeats, which are generally quite uniform, but mutation may also act to change nucleotide bases. There may be a propensity for mutations to accumulate with greater frequency at some sites in the gene sequence than at others. Mutations in the sequence motifs recognised by a restriction enzyme are not be precluded from this process. This can result in the loss or gain of new sites, affecting the resultant RFLP patterns derived from different tandem repeats in an isolate, or a mutation may be fixed in all tandem repeats of an isolate. Examples of variation among isolates within a single species have been highlighted for *P. capsici* while variation within a single isolate is detailed for *P. cinnamomi*.

The genus-specific primer pairs A2/I2 were tested on 27 different *Phytophthora* species so as to ensure that they consistently amplified a fragment of the correct size in a consistent manner. The sensitivity of the test was assessed in a dilution series of template DNA and by varying the number of PCR cycles to test the robustness of the assay. After optimisation of the reaction conditions, the assay was validated through applying it to identification of over 1660 isolates of a range of different *Phytophthora* species which now form part of our *Phytophthora* collection, and reproducible detection at the genus level was obtained.

Instead of developing primers for a number of individual *Phytophthora* species, we considered it more effective to develop primers for the genus as a whole, and then use the DNA sequence information to find a selection of restriction enzymes able to provide fingerprints capable of differentiating between species. Faced with large numbers of different species to identify, analysis of a shared amplicon among species is a more effective strategy compared with deploying large numbers of species-specific primers used separately, or combinations of primers in nested PCR strategies. The restriction patterns collated in our

molecular identification key are easy to generate once the *Phytophthora* amplicon has been obtained by digesting with restriction enzymes, *Msp*I, *Rsa*I and *Taq*I. This DNA restriction pattern can be compared with restriction patterns from type specimens or representative isolates of a large number of species in our molecular diagnostic key (Fig. 2). The combination of rDNA sequence data, genus-specific primers and a molecular key, based on restriction digests of the amplicon, provides an effective tool to identify a suspect *Phytophthora* diagnostic sample down to species level.

Effective implementation of DNA-based diagnostic assays in plant pathology is to a large degree dependent on the effectiveness of obtaining DNA from the sample. With Phytophthora diagnostics, four different sample types were typically encountered; pure cultures, infested plant material, infested soil and water. Mycelium simply scraped from the surface of pure cultures is easy to standardise with regard to amount, purity, level of inhibitors and efficiency of the DNA extraction. The rapid procedure outlined in the methods works effectively and consistently with cultures from all *Phytophthora* species tested. If no amplicon was present after a PCR with genus primers, dilutions of 1/10 and 1/50 of the template ensured that a negative result was not due to the presence of high levels of inhibitors. Although, the amount of template DNA can be quantified, this is not routinely done due to the time and cost involved as well as the limited biological significance of quantifying the amount of *Phytophthora* present in a sample.

Due to the large number of *Phytophthora* species recorded, over 70, and the vast number of Phytophthora diseases they cause on different plant parts on a myriad of different plant species, it is beyond the scope of this paper to validate an effective DNA extraction procedure from plant tissue. It is difficult to standardise the amount of infected tissue to use as it may include plant organs as diverse as roots, bark, leaves, fruit and tubers, and the quantity of *Phytophthora* mycelium in these different tissues will also vary greatly. Mycelium of *Phytophthora* also disintegrates quickly once the cells it feeds upon die. Different plant tissue and different plant species will also contain a wide range of different inhibitors of PCR at varying levels. Basic plant pathology skills are paramount as careful excising of infected plant tissue at the advancing margin of a *Phytophthora* lesion is a very important aspect of correct detection and identification of *Phytophthora* pathogens using this and other methods.

The enormous range and variability of soil types such as sand, loam, clay, peat and soil-free potting mixes that may harbour *Phytophthora* also present a challenge to develop a single effective strategy to obtain DNA for molecular diagnostic assays. The variability encountered in physical soil structure, chemical composition and the presence of inhibitors is enormous, which will make validation of DNA-

based assays directly from a heterogeneous substance such as soil rather difficult. However, our *Phytophthora* detection and identification method is generic and its usefulness would increase if combined with an effective DNA extraction technique from soil. In order to overcome the variability among soil samples, we routinely use the lupin baiting method as developed and validated by Chee and Newhook (1965). This method takes advantage of the motile nature of zoospores of *Phytophthora* and has been validated over time (Pratt and Heather 1972). Root tips of infected lupin radicals can be directly submitted for DNA isolation, followed by the genus-specific PCR for detection and identification of the *Phytophthora* species involved. At the same time, the infested root sample can be used to obtain a pure culture if needed for further work or as a reference. Depending on the species under investigation, a wide range of different types of baits can be used (Erwin and Ribeiro 1996).

Rapid and correct identification of *Phytophthora* species will help resolve problems in crop species from which *Phytophthora* is hard to isolate and in root diseases on crops from which *Phytophthora* has not been reported before. New and exotic species of *Phytophthora* can be more easily detected and accurately identified than before. The availability of rapid diagnostic tests for *Phytophthora*, in concert with effective DNA extraction techniques, may change the way in which diagnostics are used in the nursery and horticultural industries. The availability of affordable tests, which can be routinely applied, will lead to improvements in nursery hygiene, since the routine testing of planting material and soil forms an integral part of accreditation schemes. Implementation of our diagnostic test offers increased sample handling capabilities. This will lead to better disease management strategies as diseased plants can be identified more quickly before the spread of *Phytophthora* can take place. Rapid identification and detection of species is also important in the importation and export of plant material. Australia is currently free from a range of exotic *Phytophthora* species with the potential to seriously affect our plant based industries and native flora. The ability to rapidly detect and identify such *Phytophthora* species in infected plant tissue is important for Australia's diagnostic capability.

Our test is different in a number of ways from the rDNA restriction patterns put forward by Cooke and Smith (2000) to differentiate species of *Phytophthora*. Firstly, our assay uses primers that are specific to the genus *Phytophthora* instead of using generic ITS sequencing primers that can amplify a large range of different organisms including Oomycetes, such as most species within the genus *Pythium*. Secondly, our PCR-RFLPs are generated through the use of thin high-resolution agarose gels, which produce sharp clear bands in a short running time of 1 h, compared with 4 h. Thirdly, we use different restriction enzymes, *Msp*I, *Rsa*I and *Taq*I instead of *Msp*I, *Taq*I and *Alu*I. Fourthly, our range of target

species is somewhat different and more relevant to Australian conditions. Fifthly, our PCR-RFLP patterns are printed on sheets for easy use in the laboratory without the need for expensive computer software. Our focus has been to develop and especially validate a system that would be able to identify commonly encountered Phytophthora diseases in Australia and Southeast Asia together with a range of *Phytophthora* species, such as *P. fragariae*, *P. megakarya* and *P. colocasiae*, which are of quarantine significance to Australia. We have created an open-ended system so that other species and new exotic species, such as *P. ramorum*, can be added, if needed. This is important, as in the last decade more then 10 new species of *Phytophthora*, including *P. ramorum*, have been described.

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