Comparison of Methods Used to Detect the Organism Responsible for Kauri Dieback, *Phytophthora agathidicida*, From Soil Samples

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Cover image: Kauri forest in the northern Waitākere Ranges Regional Park (Upper Kauri Track), Andrew Dopheide

Comparison of methods used to detect the organism responsible for kauri dieback, *Phytophthora agathidicida,* from soil samples

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Executive summary

Kauri dieback is known to be caused by the microbial pathogen *Phytophthora agathidicida.* Trees infected by the organism show symptoms of decline including yellowing of leaves, canopy thinning, root damage, copious gum exudation, and eventually death. Since first being identified as causing disease in kauri on Great Barrier Island in the 1970s, Kauri dieback has spread throughout many stands of New Zealand's iconic kauri forest. For these reasons, kauri dieback disease has been declared an Unwanted Organism (UO) under the Biosecurity Act 1993 and is a strategic priority of the New Zealand Conservation Authority.

To study the distribution and spread of kauri dieback and the efficacy of various control strategies, it is essential to be able to accurately determine the presence and abundance of *P. agathidicida* in the environment. Methods for detecting *P. agathidicida* in soil largely centre on a culture-based approach known as baiting, but this approach is slow, taking up to 20 days to complete, and may be subject to biases in culturing and species identification that can lead to false positive or negative results. To overcome the inherent issues of baiting for *Phytophthora,* we test alternative molecular (DNA-based) methods for the detection of *P. agathidicida* in soil.

Soil samples were collected in the vicinity of trees where *P. agathidicida* is known to be present in the soil and showing various degrees of visual symptoms, in the Waitākere Ranges, west of Auckland. We sought to detect *P. agathidicida* in soil using three approaches: (i) a culture-based baiting approach, widely regarded at the standard method for the detection of *P. agathidicida* in soil whereby presumptive cells of *P. agathidicida* are isolated from soil before identification by colony and cellular morphology, (ii) a quantitative PCR (qPCR) approach whereby the genes specific to *P. agathidicida* are amplified and quantified, and (iii) a DNA sequencing approach targeting genes specific to the genus *Phytophthora,* allowing the presence of *P. agathidicida*, as well as other members of the genus to be determined. DNA sequencing approaches were used to confirm if cultures (i.e., from baiting) and DNA amplified by PCR were correctly identified as *P. agathidicida,* rather than any closely related species.

Quantitative PCR confirmed the presence of *P. agathidicida* in 93% (41/44) of samples collected in an area in which trees display symptoms of kauri dieback. Concentrations of *P. agathidicida* DNA were significantly greater in soil samples collected one metre uphill of kauri trunks than in samples collected at the same elevation as the trunk, or downhill. Only

61% (27/44) were confirmed positive for *P. agathidicida* using the standard 'baiting' approach. Assuming that *P. agathidicida* was present in every sample, the true false negative rate for detecting *P. agathidicida* using the baiting method is estimated to lie between 20-51%, with 95% confidence. This compares to an estimated false negative detection rate by quantitative PCR of between 1-19%

Our approach indicates quantitative PCR is a more sensitive method for the detection of *P. agathidicida* than baiting. We also confirm that DNA sequence analysis, using primers targeting the genus *Phytophthora* is capable of distinguishing *Phytophthora* species that are closely related to *P. agathidicida* such as *P. cinnamomi.* We recommend quantitative PCR-based analyses for the rapid screening of *P. agathidicida* in soil. To further confirm the presence of *P. agathidicida* in soil and to minimise false positive detection, we recommend the baiting of *Phytophthora*, followed by the DNA sequencing of sample material. This approach, which is faster than the traditional baiting and culturing method, still enriches *Phytophthora* biomass, allowing this material to be used for further analysis, if desired. Our combination of baiting for *Phytophthora* with DNA sequencing methods reduces the time required to screen individual samples and reduces biosecurity risks associated with culturing large quantities of unwanted organisms in the laboratory.

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1.0 Introduction

1.1 *Phytophthora agathidicida,* causal agent of kauri dieback

Phytophthora is a genus of soil- and water- borne oomycetes once thought to lie within the kingdom of Fungi. The initial classification of oomycetes within Fungi was chosen due to superficial similarities between the two groups of organisms, including the appearance of their colonies on artificial growth media, their complex life cycles, and their production of effector proteins as a mode of infection when acting as pathogens of plant species. DNA analysis, however, has shown that while the Chromista are descended from a common evolutionary ancestor, they form a paraphyletic kingdom (Rossman & Palm, 2006). There are also physiological and biochemical properties of the oomycetes that distinguish them from Fungi. These have contributed to their re-classification and include that oomycetes:

1) Form motile zoospores (i.e., spores capable of swimming by means of 'tail-like' flagella).
2) Have cell walls composed of cellulose rather than chitin.
3) Undergo distinct phases during mitosis.
4) Produce diploid hyphae (i.e., the non-sexual branching filamentous structure of the organism contains two sets of chromosomes).

The genus *Phytophthora* contains approximately 100 known species, although it is estimated that there are up to 500 additional species worldwide which have not yet been cultured and identified (Brasier, 2009). The genus includes a wide range of economically- and ecologically- significant pathogens of plants. Examples of these include *P. infestans*, causal agent of the Irish Potato Blight which was responsible for major potato crop losses in Ireland in the mid-1800s (Malcolmson, 1969), and *P. cinnamomi*, which affects hundreds of plant species in both agricultural and natural ecosystems worldwide (Zentmyer & Thorn, 1967). *P. cinnamomi* is particularly problematic in Western Australia, where it has caused major losses in native flora, including of key species such as Jarrah (*Eucalyptus marginata;* Shea *et al.,* 1983).

Approximately 30 species of *Phytophthora* are known to exist in New Zealand soils, 11 of which have been found in indigenous ecosystems (Scott & Williams, 2014). Of these, a recently discovered species, *P. agathidicida*, has received much media attention due to its impact on kauri (*Agathis australis*), an indigenous conifer species found in the upper North Island regions of New Zealand (Beever *et al.*, 2009).

P. agathidicida is a soil-borne oomycete, which is aggressively pathogenic on kauri seeds, seedlings, and trees of all ages (Horner & Hough, 2013). While it is proposed to have an

exotic origin, it is known to have been introduced to Aotea - Great Barrier Island of New Zealand during, or before, the 1970s (Gadgil, 1974). It was incorrectly identified based on cell morphology as *P. heveae* (Gadgil, 1974) after being implicated in severe kauri decline and mortality on Great Barrier Island. Initial research carried out on the pathogen concluded that the impact that it had on kauri on Great Barrier Island was anomalous, and due to unusual environmental conditions at the time (Gadgil, 1974). Since then, *P. agathidicida* has been introduced to the mainland of New Zealand, where it has been responsible for major losses of kauri throughout the Auckland Region, particularly throughout the Waitākere Ranges since the mid-2000s (Beever *et al.*, 2009, Waipara *et al.*, 2013). The range of symptoms exhibited by kauri infected by *P. agathidicida*, includes yellowing of leaves, canopy thinning, root damage and gummosis (the copious production and exudation of gum). These disease symptoms are collectively known as 'kauri dieback'. Subsequent research on areas affected by kauri dieback identified that the disease symptoms were caused by a pathogenic oomycete distinct from *P. heveae*; the pathogen was then given the interim name *Phytophthora* taxon Agathis, or PTA (Beever *et al.*, 2009). More recent research has identified that the species occurs in Clade 5 of the *Phytophthora* genus; it has since been renamed as *Phytophthora agathidicida* (Weir *et al.*, 2015).

In addition to *P. agathidicida, P. cinnamomi, P. kernoviae, P. multivora,* and *P. plurivora* have been found to be associated with kauri, although only *P. agathidicida, P. multivora* and *P. cinnamomi* have been found to cause the death of kauri (Horner & Hough, 2014, Hill *et al.*, 2017). While *P. agathidicida* is highly pathogenic on kauri, both *P. cinnamomi* and *P. multivora* known as being more of an opportunist, causing death of kauri which have had their health compromised due to environmental stress or disease (Horner & Hough, 2013).

Kauri play important ecological roles as foundation species in the indigenous forest they inhabit. They are important carbon sinks, influence nutrient cycling and plant communities in their surroundings, play host to a variety of epiphyte species, and influence the flow of water downhill after heavy rainfall through their extensive root systems (Enright & Ogden, 1987, Silvester, 2000, Wyse, 2013). They also have cultural significance to Māori and are commercially valuable, attracting large numbers of tourists to North Island forests each year due to the unique ecology of the forests they are a part of (Steward & Beveridge, 2010). Kauri dieback, caused by *P. agathidicida* therefore has the potential to cause major ecological, cultural and economic losses in New Zealand (Goldson *et al.*, 2015). The threat of kauri dieback to the kauri ecosystem was prioritised in 2008 when it was declared an Unwanted Organism under the Biosecurity Act 1993 and a national biosecurity response initiated (Waipara *et al*., 2013). In 2009, a long term disease management programme was then implemented by MAF Biosecurity New Zealand (now Ministry for Primary Industries),

Tangata Whenua, Department of Conservation, and regional councils within the natural range of kauri (Beauchamp & Waipara, 2014). The active management of kauri dieback is also listed as a strategic priority by the New Zealand Conservation Authority (NZCA, 2016).

1.2 Dispersal of *Phytophthora agathidicida* in the environment

P. agathidicida is thought be transmitted primarily through mechanical means, for instance on the soles of footwear used by hikers and recreational joggers, and through the movement of invasive feral animals which have established in natural ecosystems (Pau'Uvale *et al.*, 2010, Bassett *et al.*, 2017). To control the spread of kauri dieback, many tracks have been closed to the public. Significant funds have also been allocated towards the improvement of walking tracks (i.e., construction of wooden boardwalks to reduce the transfer of pathogen infected soil) and also the installation of footwear cleaning stations. To minimise transmission of the pathogen between kauri on footwear, regional councils and the Department of Conservation (DOC) advocate the use, and provide spray bottles of, Trigene II Advance (at 2% concentration) on public trails in indigenous kauri habitats. Trigene II Advance is a disinfectant containing a range of halogenated amines, and has been shown to kill *Phytophthora* cells in soil which have accumulated on the footwear of those travelling through *P. agathidicida*-infested areas (Bellgard *et al.*, 2013). Trigene II Advance is non-toxic to humans, and is regarded as being an environmentally-safe control measure for *P. agathidicida* (Bellgard *et al.*, 2013). While it is known to be effective in preventing the spread of the pathogen on footwear, its use is heavily dependent on public compliance. To date a number of opt-in surveys carried out by Auckland Council have identified a variable level of cooperation in terms of disinfectant use and adoption of hygiene procedures such as use of mountain bike cleaning stations (Heggie-Gracie & Robertson, 2015), A 2016 survey recorded more than 70% of Aucklanders are aware of kauri dieback disease, however, this did not translate into affirmative action on the ground as up to 83% of park visitors are not only walking past cleaning stations without scrubbing their shoes, but are also going off-track or disregarding closed tracks (Nick Waipara personal communication).

Feral animals such as goats and pigs are hypothesised to be vectors of the disease, by transferring soil-borne *P. agathidicida* cells between kauri through their foraging activity. In a study based in the Waitākere Ranges by Krull *et al*., (2013), the pathogen was not recovered from trotters and snouts of feral pigs shot by hunters contracted by the Auckland Council. The negative results were reported as likely being false-negative outcomes, however, and were thought to be the result of limitations of the method used to detect the pathogen. In particular, it was hypothesised that inhibitors present in the snouts and trotters of the feral pigs prevented growth of the *Phytophthora* cells on artificial

medium. Based on observing the foraging patterns of the pigs in the environment, and their proximity to kauri root tissue when foraging, it was concluded that feral pigs were likely to be vectors of the disease in the Waitākere Ranges (Krull *et al.*, 2013).

A subsequent study detected viable *P. agathidicida* from a kauri root retrieved from captive-fed pig faeces, providing proof of concept that pigs can vector *P. agathidicida* following ingestion of infected root material (Bassett *et al.*, 2017). However, it was only detected in one contaminated sample, despite processing a total of 11.2 m of passaged kauri root and 800 passaged millet seeds, from 12 pigs. The study did isolate five other *Phytophthora* species including *P. multivora* and *P. cinnamomi*. Ingestion of contaminated roots and soil by feral pigs is probably a minor pathway for spread of *P. agathidicida*.

Feral pigs are currently declared a pest in the Auckland Regional Pest Management Strategy and an extensive culling programme is underway in the Waitākere Ranges Regional Park to mitigate their impacts to native biodiversity and vectoring potential of kauri dieback (Nick Waipara, personal communication).

Based on current research, it is hypothesised that *P. agathidicida* is unlikely to be spread by water, unlike other *Phytophthora* species which are routinely isolated from streams and ponds within natural ecosystems. This is largely based on the work of Randall (2014), who, in a year-long study of freshwater catchments within the Waitākere Ranges, found no *P. agathidicida* cells when using a method known as baiting to detect the pathogen. Despite this, other *Phytophthora* species such as *P. cinnamomi*, *P. multivora* and an unknown species of *Phytophthora* were isolated in the study.

The current uncertainty concerning both the present day distribution of *P. agathidicida* and mechanisms for dispersal mean that more sensitive and accurate methods to detect the presence of the pathogen in a wide range of media are highly desirable.

1.3 Control of *Phytophthora agathidicida* in the environment

Currently there is no effective control for trees and environments infected with *Phytophthora agathidicida*. Glasshouse trials have shown that phosphite (PO_3^-) application either to the soil, in foliar sprays, or by direct injection into trunk tissue have some success at abating symptoms in seedlings infected with kauri dieback (Horner & Hough, 2013). Subsequent trials on mature kauri in forest ecosystems showed that trees with dieback symptoms caused by *P. agathidicida* displayed less severe symptoms of infection (such as developing fewer, and less active lesions on leaves) over a period of 3 years (Horner *et al.*, 2015). Phosphite appears to reduce plant susceptibility to *Phytophthora* pathogens by improving the ability of the host to resist infection, apparently by enhancing the response

of stress-related signalling pathways, increasing expression of defence-related genes, and inducing production of anti-microbial compounds such as hydrogen peroxide (Eshraghi *et al.*, 2011). However, trials in Western Australia for controlling *P. cinnamomi*-mediated Jarrah Dieback have shown that this is only a temporary solution, with multiple applications of phosphite required to prevent the pathogen from re-infecting the tree (Colquhoun & Hardy, 2000). With Jarrah forest covering a vast amount of land in Western Australia, the costs of materials and labour required for carrying out these applications in the long-term are high, despite the low cost of phosphite application per tree, and management of the pathogen is focussed on controlling its spread rather than eradicating it completely (Hardy *et al.*, 2001). These issues mean that for the management of kauri dieback in New Zealand, phosphite may only be effective for preventing damage on particular trees or kauri stands which have particular value due to their age or cultural significance (Horner *et al.*, 2015). There are known to be potential issues with phytotoxicity when phosphite is applied at high rates and in high dosages, particularly in trees under physiological stress due to pathogens or environmental factors (Graham, 2011). To study the efficacy of this and other control measures, it is important to be able to accurately determine the presence and abundance of *P. agathidicida* in the environment, allowing the fate of the organism to be tracked following intervention measures.

1.4 Methods for detecting *Phytophthora agathidicida* in soil

Given that there is no viable method for treating kauri infected with *Phytophthora agathidicida* in the environment, current management plans centre on restricting the spread of the pathogen in the environment, which requires its accurate detection in soil samples (Waipara *et al.*, 2013). The methods for detecting *P. agathidicida* in soil largely centre on a culture-based approach known as baiting (Erwin & Ribeiro, 1996), although more recently a quantitative PCR (or qPCR) protocol was developed by Than *et al*., (2013). To date no qPCR technique has been successfully validated for the detection of *P. agathidicida* in any environmental samples.

1.4.1 Culture-based isolation of *Phytophthora agathidicida* from soil

Direct isolation of *Phytophthora* propagules from soil is extremely difficult due to the complex nature of the soil matrix. Culture-based isolation is also challenging due to the large number of organisms present, many of which are able to take up nutrients in artificial growth media at a faster rate than *Phytophthora,* which may then be outcompeted. Organisms including bacteria, other chromists and fungi such as yeasts and ascomycetes may further inhibit the growth of *Phytophthora* in culture via the production of antimicrobial compounds. To overcome these issues, ‘baiting’ is often carried out to increase the numbers of *Phytophthora,* where present in the sample media, before attempting to selectively culture *Phytophthora* in the laboratory. To achieve this, soil samples are placed

in a container and left to air-dry, before the soil is flooded with sterile water. The addition of water triggers the *Phytophthora* cells to form motile zoospores (i.e., asexual reproductive cells that can move throughout the environment using 'tail-like' structures called flagella). Tissue baits, often consisting of the ends of lupin (*Lupin angustifolis*) radicle roots, are then suspended on the surface of the water. The lupin root cells produce signalling compounds such as lectins which encourage migration of *Phytophthora* sp. from the surrounding soil towards the root tips, before colonising the tissue via the vascular system. The root tips of the lupins, which may now be enriched with *Phytophthora* zoospores can then be surface-sterilised with ethanol and plated on selective growth media.

Phytophthora species are commonly isolated on artificial growth media such as V8 Juice Agar (V8A) (Miller, 1955), Corn Meal Agar (CMA) and Potato Dextrose Agar (PDA), amended with selective antimicrobial compounds to prevent the growth of unwanted organisms. Plated cultures are incubated and sub-cultured until pure cultures are obtained. The organisms growing on the agar plates may then be identified based on cell and colony morphology to confirm the presence of *P. agathidicida.*

A disadvantage of this approach is that it takes up to 20 days to complete, representing a major bottleneck for the effective management of areas vulnerable to kauri dieback. A high level of taxonomic expertise is also required to identify the isolated cells based on morphology, due to the resemblance of *P. agathidicida* to other *Phytophthora* species at all stages of its life cycle.

1.4.2 Quantitative PCR of *Phytophthora agathidicida*

To overcome the inherent issues of baiting for *Phytophthora*, a quantitative PCR (or qPCR) protocol was developed by Than *et al*., (2013). This approach is based on Taqman chemistry, and uses DNA primers which amplify a 60 bp region of a non-coding DNA fragment between the genes encoding 18S and 5.8S rRNA genes in eukaryotic organisms. The sequence of the 60 bp region amplified by the DNA primers is unique to *P. agathidicida*, allowing for a highly specific diagnosis (i.e., DNA should only be amplified during PCR if the organism is present).

The Taqman approach involves the use of a fluorescent dye, or fluorophore, which emits a fluorescent signal during DNA amplification. This increase in fluorescence is recorded by the qPCR machine. Since this signal is only released upon the amplification of the chosen DNA region, fluorescence intensity is directly related to the concentration of the target DNA within the sample. Than *et al.,* (2013) report this protocol is sensitive enough to detect *P. agathidicida* DNA in concentrations as low as 2 fg from DNA extracted from pure culture, and 20 fg for DNA extracted from soil. Similar protocols have successfully been

developed for the detection of other *Phytophthora* species from soil, including *P. fragariae* var. *rubi* (Schlenzig *et al.*, 2005), *P. ramorum* (Chandelier *et al.*, 2006) and *P. cinnamomi* (Williams *et al.*, 2009).

1.4.3 Sequencing DNA from *Phytophthora*

Advances in DNA sequencing technology have allowed for population-level profiling of microbial assemblages from various ecosystems. In the context of soil microbiology, this involves extracting DNA from soil and amplifying a selected region (amplicon) using PCR. The PCR products are then purified and loaded onto a DNA sequencing machine, such as the Illumina Miseq (http://www.illumina.com/systems/miseq.html). The DNA sequences generated are then analysed to assess the similarity of the amplified DNA sequences to sequences in microbial DNA databases. In contrast to highly-specific methods of detecting organisms of interest from soil samples, such as qPCR, DNA sequencing is most commonly used to study microorganisms at the community-level. For example, in their study of *Phytophthora* communities in European forest soils, Scibetta *et al.,* (2012) proposed that by using genus-specific primers for the initial PCR steps, Illumina sequencing is an effective way of capturing the diversity of all *Phytophthora* species from a particular sample. By identifying all *Phytophthora* species associated with diseased and healthy trees, researchers would then be able to gain insights into how closely-related pathogens of *P. agathidicida* might act synergistically to establish infection and cause the symptoms observed.

Scibetta *et al*., (2012) used a nested PCR approach in their study of *Phytophthora* community DNA. Nested PCR protocols are typically more sensitive than standard PCR and so using this approach, Scibetta *et al.,* (2012) were able to increase the probability of successfully amplifying DNA from *Phytophthora* species in soil. This is significant, as *Phytophthora* cells typically occur in low abundances in soil relative to other microorganisms such as fungi (Borneman & Hartin, 2000). The first round of the PCR protocol used by Scibetta *et al.,* (2012) involves the primers 18Ph2F (forward) and 5.8S1-R (reverse) to amplify the internal transcribed spacer region between the 18S and 5.8S regions of ribosomal RNA present in all eukaryotes. During a second round of PCR, the primer ITS6, developed to amplify DNA only from *Phytophthora* species (Cooke *et al.*, 2000), is used as the forward primer, and 5.8S1-R was once again used as the reverse primer. Thus in the second round of PCR, only the DNA of *Phytophthora* and closely related organisms is amplified. Using this approach, Scibetta *et al.,* (2012) report a 1 fg detection limit of *Phytophthora* DNA from soil.

1.5 Aims

We first aimed to confirm the specificity and reliability of established baiting protocols for detecting *P. agathidicida* from soil samples in the vicinity of trees displaying symptoms of kauri dieback disease. Then we sought to investigate the specificity and reliability of molecular, or DNA-based methods for the detection of *P. agathidicida* DNA from the same soil, to compare the ability of both methods to accurately detect *P. agathidicida* presence in natural samples. If molecular methods can be reliably used for the detection of kauri dieback disease they present a number of advantages, particularly since these methods do not require the pathogen to be first cultured to confirm its presence in a sample.

2.0 Methods

2.1 Sampling

2.1.1 Study site

Soil sampling was carried out near Huia (13/08/2015), a settlement which lies within the Waitākere Ranges in west Auckland, New Zealand (Figure 1). The study site chosen is situated on a ridge approximately 120 m above sea level, within an area of forest consisting of kauri stands, and contains a combination of mostly indigenous, but also some introduced trees. Some kauri within this area exhibit advanced dieback symptoms as a result of infection by *P. agathidicida*. The presence of *P. agathidicida* in soils was previously confirmed by field surveys carried out by Auckland Council and the University of Auckland. In 2016 a kauri health survey of the Waitākere Ranges Regional Park confirmed that *P. agathidicida* is widespread in all kauri stands within in the Lower Huia catchment area, in which this study site is located (Hill *et al*., 2017) Other *Phytophthora* species previously identified at the site include *P. cinnamomi* and *P. multivora* (van der Westhuizen, 2014). We identified eleven of the trees previously-studied by van der Westhuizen (2014), their coordinates and elevations are summarised in Table 1. The trees formed distinct clusters on the upper and lower portions of the ridge where they occurred and exhibit varying severities of dieback symptoms.

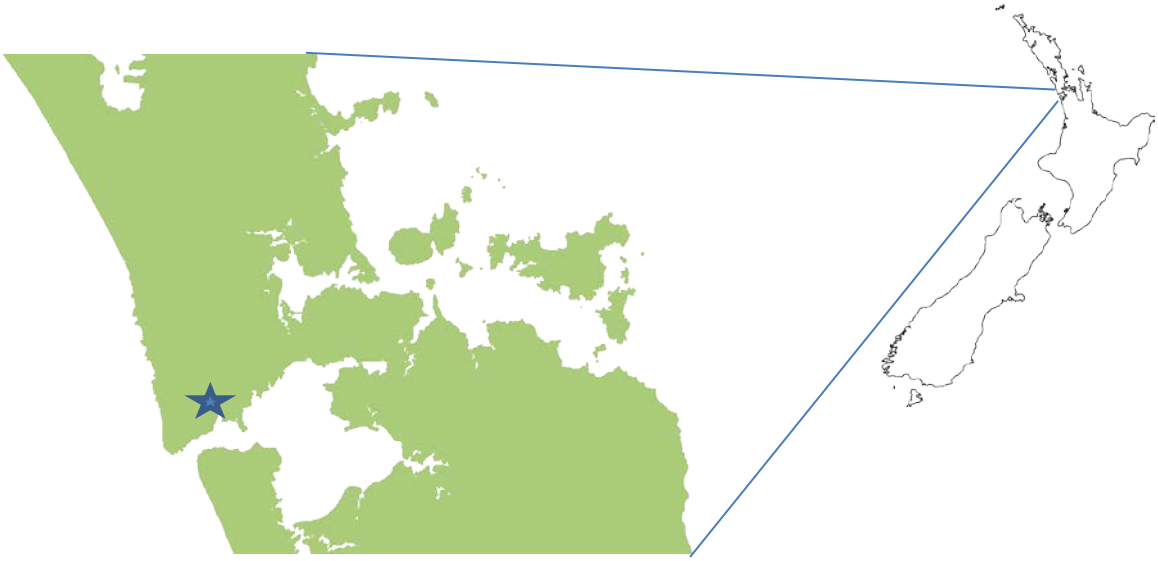

Figure 1. Location of sampling site in the locality of Huia, within the Waitākere Ranges, New Zealand.

Table 1. Coordinates and elevation of kauri in an area known to be impacted by kauri dieback, and a summary of other *Phytophthora* species found in addition to *P. agathidicida* at each of the trees (modified from van der Westhuizen *et al*., (2014)).

Tree I.D.	Coordinates (NZTMP)	Elevation (m.a.s.l.)	*Phytophthora* species detected in addition to *P. agathidicida*
2	E1738749 N5903162	132	*P. cinnamomi, P. multivora*
3	E1738745 N5903158	127	*P. cinnamomi*
4	E1738740 N5903156	124	–
5	E1738743 N5903143	124	*P. cinnamomi*
6	E1738746 N5903144	137	*P. cinnamomi*
8	E1738781 N5903147	134	–
9	E1738812 N5903153	145	*P. cinnamomi*
10	E1738800 N5903100	140	*P. cinnamomi*
11	E1738801 N5903111	127	–
12	E1738808 N5903110	134	*P. cinnamomi*
13	E1738788 N5903163	132	–

2.1.2 Collection protocol

To maximise our chances of detecting *P. agathidicida* we chose to sample in an area with trees showing visual symptoms of kauri dieback. Using a metal soil corer (1.5 cm diameter, 10 cm depth), four soil samples were collected 1 m from each of the selected Kauri. The cores were taken at the cardinal points around each tree (

Figure 2) to ensure that the results gathered were not biased by the topography of the landscape, the trees exposure to sunlight, or build-up of organic matter such as leaf litter. Care was taken to avoid or remove coarse roots, leaves, soil-dwelling macroorganisms (e.g., earthworms) and other organic matter while collecting the samples. The soil sampling method used at the study site is modified from the standardised sampling method used in the national kauri dieback soil surveillance programme (Beauchamp, 2016).

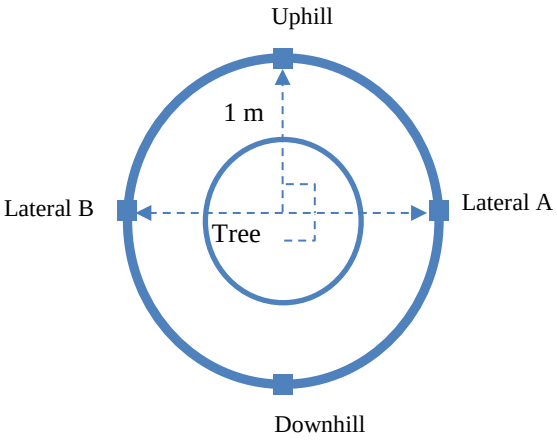

Figure 2. Representation of sampling scheme around each tree. The blue squares represent where soil samples were collected; uphill, downhill and at lateral points 1 m from the base of the trunk.

Samples were placed into individual snap-lock bags and put in a chilly-bin, on ice, for transport to the laboratory. In the laboratory, soil samples were mixed within their bags to homogenise the sample material, then placed into sterile beakers to a depth of 10 cm for the baiting procedure. Soil not used for baiting was stored at -20°C for subsequent molecular analysis.

2.1.3 Baiting

The baiting procedure followed Beever *et al*., (2010) in their report on the detection of *P. agathidicida*, prepared for the Ministry of Agriculture and Forestry and Biosecurity New Zealand.

Blue lupin seeds (*Lupinus angustifolius*), sourced from New Zealand, were surface-sterilised in 70% ethanol and rinsed in distilled water. They were then soaked in distilled water for three hours, and mixed with vermiculite in a plastic tray to a depth of 3 cm. The seeds were then watered with distilled water and left to germinate at 20°C under light until the emerging radicals were ~3 cm in length (3 days).

Soil samples were left to air-dry for 3 days, before they were moistened with distilled water to stimulate zoospore production. The moistened soil was incubated under ambient light for 4 days at room temperature, with a lid placed loosely on each tube to prevent evaporation and subsequent dehydration of soil.

At the end of the 4-day incubation, the soils were flooded with distilled water so that the water level in each 250 ml beaker was ~5 mm below the opening. Each beaker was then covered with aluminium foil punched with three holes 1.0 cm in diameter. Three of the prepared radical baits were then threaded through each of the holes in the aluminium sheet so that their roots were submerged in the water below. The baits were then left at room temperature on a laboratory bench top for 2 days.

The lupin baits were removed from the foil and rinsed under distilled running water. The distal 2 cm portion of each lupin radical was then removed using a sterile scalpel, soaked in 70% ethanol for 30 s and rinsed under distilled running water. The root tips were then dried on paper towels, and plated on solid P_5ARPH-CMA media (Jeffers & Martin, 1986). Each set of root tips was placed on individual P_5ARPH-CMA agar plates (see Appendix A). The inoculated P_5ARPH-CMA plates were covered with aluminium foil to prevent degradation of the light sensitive antibiotics (ampicillin, rifampicin, hymexazol) included in the media and placed in an incubator at 20°C for 2 days.

The P_5ARPH-CMA plates were inspected for growth. Mycelia from individual colonies from each plate were macerated, then sub-cultured in duplicate on clarified V8 juice agar (V8A) and potato dextrose agar (PDA) and incubated for 6 days at 20°C in the dark. Details of the growth media used for culturing the recovered *Phytophthora* can be found in Appendix A. *Phytophthora* colonies obtained through sub-cultures were initially identified by microscopy and further identified through DNA sequence analysis of the ITS1 region of the *Phytophthora* genome, as described in Appendix B.

2.1.4 Detection and quantitation of *Phytophthora* with quantitative PCR

We used the approach of Than *et al*., (2013) to selectively amplify DNA from *P. agathidicida.* To confirm the sensitivity of this molecular approach to detect *P. agathidicida* DNA*,* but not the DNA of closely related species, we amplified DNA extracted from cultures of *P. kernoviae, P. hibernalis, P. plurivora,* and *P. cinnamomi,* as well as *Pythium vexans* and *P. irregulare.* We used the same approach to amplify *P. agathidicida* DNA from DNA extracts of soil and also lupin roots. Quantitative PCR allows the user to quantify the amount of target DNA that is amplified and from this to infer the abundance of target DNA (e.g., *P. agathidicida* DNA) in the original sample media, for example as ng DNA μl^{-1} of the extraction solution. The qPCR protocol is outlined in Appendix C.

2.1.5 Identification of *Phytophthora agathidicida* by DNA sequence analysis

We used DNA sequence analysis, following the protocol of Scibetta *et al*., (2012), in an attempt to identify *P. agathidicida* present in soil DNA extracts, but also to confirm if cultures (i.e. from baiting) and DNA amplified by qPCR were correctly identified as *P. agathidicida*, rather than any closely related species. For the lupin root baits, DNA was extracted from four pieces of cut root tissue per sample using Extract-N-Amp™ Plant Tissue PCR Kits (Sigma-Aldrich).

To determine whether the PCR protocol used by Scibetta *et al*., (2012) was suitable for amplifying a range of *Phytophthora* species found in New Zealand, including *P. agathidicida*, a series of positive controls were prepared. These consisted of individual DNA extracts from each of the species listed in section 2.1.4 to ensure that the primers used in the Scibetta *et al*., (2012) study did not amplify DNA from non-target oomycetes.

PCR was carried out on a dilution series of each soil DNA extract or DNA extracted from the lupin bait (undiluted; 1:10, 1:100 and 1:1000 dilutions), as well as on *Phytophthora* genomic DNA positive controls (undiluted; 1:10, 1:100 and 1:1000 dilutions), as further detailed in Appendix D.

2.1.6 Statistical analyses

Sensitivity analysis was carried out to obtain the estimated false negative detection rate for each technique, using the method of Gardner *et al*., (2006). All other analyses were performed using the statistical analysis software R (R Core Team, 2005). A generalised linear model (GLM) was carried out to determine whether the observed differences between methods were statistically significant. Analysis of variance (ANOVA) was used to determine whether differences among the means among multiple sample groups were significant. Tukey-Kramer honesty significant difference tests were used to assess whether any particular group of samples were significantly different from another.

2.2 Results

2.2.1 Baiting

Baiting resulted in successful isolation of *P. agathidicida* from 27 of the 44 sampling points (i.e., 4 soil cores x 11 trees). No pathogen was detected at two trees (Table 2). There was only one tree from which *P. agathidicida* was recovered from all baits. *P. cinnamomi* was also commonly identified as growing on the P_5ARPH-CMA plates, it was isolated from 32 of the 44 samples (Figure 3). The two *Phytophthora* species were distinguished in culture through examination of their chlamydospore and colony morphologies (Figure 3). *P. cinnamomi* colonies also had a faster mean growth rate on PDA than *P. agathidicida* (3.5 mm/day and 2.8 mm/day respectively, at 20°C).

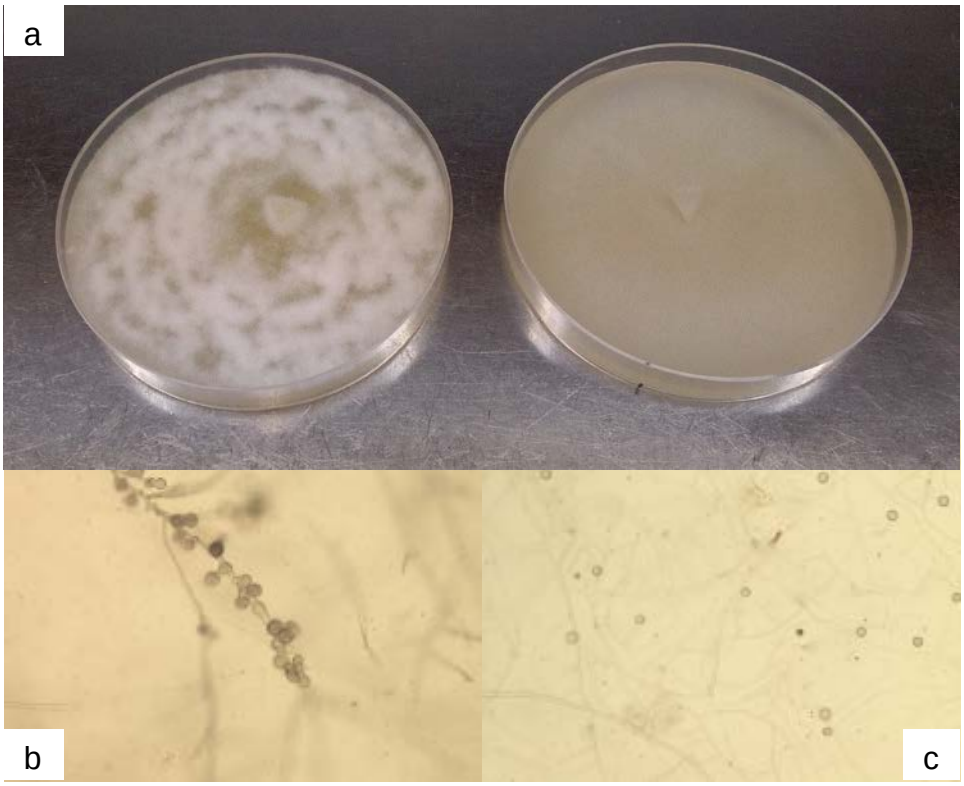

Figure 3. *Phytophthora* species isolated from lupin baits. a) *P. cinnamomi* (left) and *P. agathidicida* (right) colonies on PDA; b) hyphal swellings characteristic of *P. cinnamomi* chlamydospores; c) *P. agathidicida* chlamydospores.

Table 2. Summary of baiting results. Cells shaded blue indicate successful *Phytophthora agathidicida* isolations from baited soil samples, while unfilled cells indicate that the pathogen was not isolated from the sample. Cells marked with an asterisk (*) indicate that *P. cinnamomi* was isolated from the sample.

Sample	Uphill	Lateral A	Downhill	Lateral B
Tree 2		*		
Tree 3	*		*	*
Tree 4	*	*		*
Tree 5	*		*	*
Tree 6	*		*	*
Tree 8	*			*
Tree 9	*	*	*	*
Tree 10		*	*	
Tree 11		*	*	*
Tree 12	*	*	*	
Tree 13	*	*	*	*

The results of a Generalised Linear Model indicate that there was no statistically significant relationship between baiting success and the location of each sample around the trees, elevation or position of the tree on the ridge ($p = 0.7$, $n = 41$). Assuming that *P. agathidicida* was present in every sample, for the observed detection rate of 61%, the true false negative rate for detecting *P. agathidicida* using the baiting method was estimated to lie between 20-51%, with 95% confidence.

2.2.2 Quantitative Polymerase Chain Reaction (qPCR) assays

P. agathidicida standards with DNA concentrations below 5.2 fg/µL had 'undetermined' cycle threshold (CT) readings signifying that the *P. agathidicida* DNA in these samples were too low in concentration for any quantifiable amplification to occur and identifying the detection limits of our qPCR assay. The standards which had DNA concentrations between 5.2 fg/µL and 5.2 ng/µL produced positive CT readings. The concentration of *P. agathidicida* DNA was plotted against their CT value outputs from the qPCR machine to create a standard curve to confirm the strength of relationship between the concentration of target DNA in a sample and the ease that *P. agathidicida* DNA could be amplified from it

by qPCR. The R^2 value of this curve was 0.9978, which indicates our qPCR assay was sensitive and linear over six orders of magnitude.

Both pure solutions of *P. agathidicida* DNA and solutions combined with the DNA of *P. kernoviae, P. hibernalis, P. plurivora* and *P. cinnamomi* and *Pythium vexans* produced CT values which corresponded with concentrations of DNA within the detection limits of 5.2 fg/µL to 5.2 ng/µL. This indicates that the qPCR protocol is able to selectively amplify, and accurately quantify, ITS rRNA genes from the pathogen in pure genomic DNA extracts and from mixed Pythiaceae community DNA.

Of the 44 soil samples taken, 41 generated positive qPCR results that fell within the detection range of our approach. The inferred concentrations of *P. agathidicida* DNA for these samples ranged between 91 and 1010 fg/µL. The three samples for which no *P. agathidicida* DNA was detected were collected 1 m downhill from the tree. For the observed detection rate of 97%, we estimated the false negative rate for any one sample (with 95% confidence) as between 1-19%. Concentrations of *P. agathidicida* DNA varied among individual kauri (Figure 4a), but *P. agathidicida* was detected in the vicinity of every tree. Samples collected uphill of the subject tree had slightly higher concentrations of the target DNA than those from lateral points; those taken from downhill had markedly lower concentrations than the other samples (Figure 4b). Concentrations of *P. agathidicida* DNA appeared to be greater in samples taken on the upper part of the ridge but there was no discernible relationship between the elevation of the trees and inferred DNA concentrations (Figure 4c). There was a significant difference among concentrations of *P. agathidicida* DNA amplified from soil surrounding different trees $F(9,3) = 4.3$, $p = 0.007$). Post-hoc Tukey's HSD tests showed that concentrations of *P. agathidicida* DNA amplified from soil 1 m uphill of the trunk were significantly greater than those 1 m downhill, at the 0.05 level of significance. The average DNA concentration determined for uphill and downhill samples was 68.5 and 11.5 fg/µL, respectively. All other comparisons were non-significant.

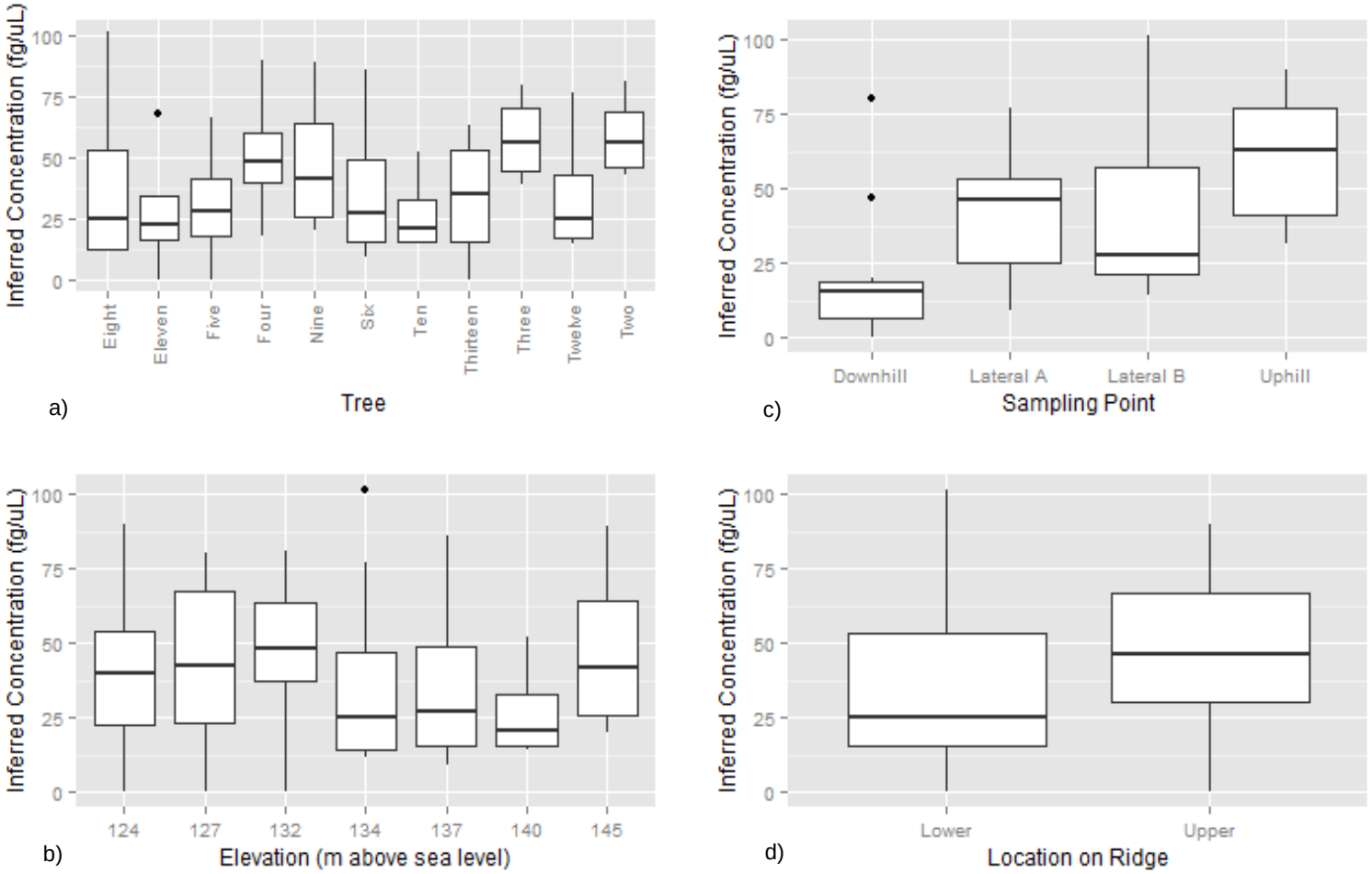

Figure 4. Boxplots displaying how the inferred *P. agathidicida* DNA concentrations varied according to, a) the tree they were sampled from, b) the elevation of the sampled tree above sea level, c) sampling point around each tree, and d) location of sample on the ridge. Boxplots show median and 1.5 x interquartile range. Error bars are 1 x standard error.

2.2.3 Comparison of baiting and qPCR results

Although examination of the data suggests that *P. agathidicida* is more likely to be detected by baiting sample material with higher concentrations of *P. agathidicida* as inferred by qPCR (Figure 5), there was little to no correlation found between baiting outcome and *P. agathidicida* DNA concentration inferred by qPCR (Spearman's correlation = 0.15).

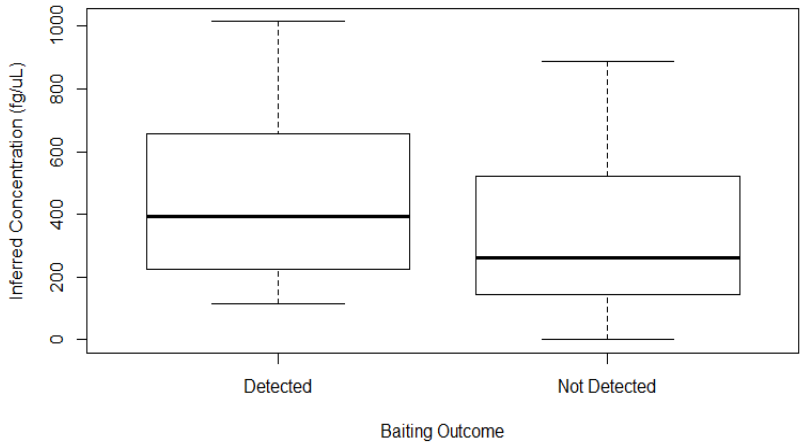

Figure 5. Boxplots displaying concentration of *P. agathidicida* DNA inferred by qPCR against baiting outcome (Spearman's correlation = 0.15). Boxplots show median and 1.5 x interquartile range. Error bars are 1 x standard error.

2.2.4 DNA sequencing of *Phytophthora* DNA

DNA extracted from each of the six species of *Phytophthora* were successfully amplified using the primers of Scibetta *et al*., (2012) to target the DNA of members of the genus *Phytophthora.* The DNA of a non-*Phytopthora* species (*Pythium vexans*) was not amplified. A dilution series of *P. agathidicida* DNA confirmed amplification could be achieved from samples containing more than 5.2 ng/µL *Phytophthora* genomic DNA. *Phytophthora* DNA could not be detected from soil DNA extracts using the protocol from Scibetta *et al.,* (2012), at any of the dilutions trialled. However, *Phytophthora* DNA could be amplified from lupin tissue used to bait these same soil samples for *Phytophthora.* DNA sequencing of 15 DNA fragments, isolated from lupin root baits confirmed that six of these sequences were most closely related to *P. agathidicida* and nine to *P. cinnamomi* (Table 3).

Table 3. Counts of *Phytophthora* species detected from lupin radicles by DNA sequencing, following the approach of Scibetta *et al.,* (2012).

Sample	*Phytophthora agathidicida*	*Phytophthora cinnamomi*
Uphill	1	2
Lateral A	3	1
Downhill	1	3
Lateral B	1	3
Total	6	9

3.0 Discussion

3.1 Performance of baiting protocol

3.1.1 Observed detection rate

The overall detection rate when isolating *P. agathidicida* using baiting in this study was 61%, with the pathogen being recovered from around nine of the eleven trees sampled from. These detection rates compare reasonably with the results of baiting from indigenous forest soils by Beauchamp (2013) and are consistent with unpublished results of surveillance carried out by staff at Landcare Research (personal communication, Stanley Bellgard).

3.1.2 Species isolated using baiting protocol

The baiting approach used in this study to isolate *P. agathidicida* from the forest soil samples is used to culture *Phytophthora* at genus-, rather than species-level (i.e., the baiting approach is not specific for *P agathidicida*). As a result, the isolation of non-targeted *Phytophthora* was expected in this study. As well as *P. agathidicida*, *P. cinnamomi* was commonly isolated from the soil samples; in fact, this species was detected more frequently than *P. agathidicida*. The frequency at which *P. cinnamomi* was isolated is not surprising for two reasons. Firstly, *P. cinnamomi* has been long known to occur extensively throughout indigenous forest in the Waitākere Ranges (Newhook, 1970), and in particular the trees sampled from in this study (van der Westhuizen, 2014). While it is not as aggressive a pathogen of kauri as *P. agathidicida,* it is known to colonise and cause dieback symptoms on kauri which have had their health compromised through abiotic stresses such as drought and nutrient deficiency, and biotic stresses such as infection by pathogens (Horner & Hough, 2014). A number of the kauri (i.e., trees 2, 3, 4 and 6) proximal to our soil sampling locations were in advanced stages of decline due to dieback symptoms, and could therefore have provided an opportunity for colonisation by *P. cinnamomi*. This in turn could have led to its increased abundance in soil, and its high frequency of isolation. Secondly, *P. cinnamomi* exhibit a faster growth rate than *P. agathidicida* in culture. In cases where lupin bait tissue was colonised by both *Phytophthora* species before being plated on CMA-P_5ARPH, *P. cinnamomi* may have been able to out-compete *P. agathidicida* by taking up nutrients in the agar at a faster rate, thereby inhibiting *P. agathidicida* colony growth.

P. multivora was another *Phytophthora* species previously found at the study site (at Tree 2; van der Westhuizen (2014)), however this species was not detected using baiting in this study. This may have simply been due to its absence from the site at the time sample material was collected, low concentrations or patchy distribution of the cells or ill health of any cells which may have been picked up while sampling, or due to it being out-competed by other microorganisms on selective media in the laboratory.

3.2 Performance of the quantitative Polymerase Chain Reaction (qPCR) protocol

The length of time required to culture baited organisms as well as the uncertainty associated with the species-level identification of organisms by colony and cellular morphology are seen as major method limitations. For this reason, molecular methods for pathogen detection are increasingly desirable. *P. agathidicida* was detected from 41/44 of the samples collected using qPCR, with the pathogen being detected at all 11 kauri. The detection limit of 5.21 fg/μL from this study was an order of magnitude lower than limit for detecting *P. agathidicida* from soil reported by Than *et al*., (2013) study (20 fg/μL), but was in the same order of magnitude for detecting DNA isolated from *P. agathidicida* isolates in pure culture. This suggests a good level of sensitivity for the method but also highlights the need to determine pathogen detection limits of an ecosystem or site specific basis. The qPCR protocol was able to be used to accurately amplify and infer the concentration of *P. agathidicida* ITS rDNA from mixed community DNA.

The results of the specificity analysis are similar to those from other studies trialling qPCR protocols based on Taqman chemistry for the detection of *Phytophthora* species. The Taqman protocol for detecting *P. ramorum* as validated by Chandelier *et al.,* (2006) reported a high degree of specificity for the method, after using it to amplify and quantify DNA from the pathogen from a mixture of DNA extracted from 16 species of *Phytophthora.* Tomlinson *et al.,* (2007), reported a detection limit of 10 fg/μL when detecting DNA from *P. ramorum* from forest soil samples. Other studies have demonstrated that the Taqman qPCR approach has achieved comparable results for detecting *P. nicotiane* (Li *et al.*, 2013), *P. cactorum* (Li *et al.*, 2013) and *P. cinnamomi* (Williams *et al.*, 2009).

The only physical variable which seemed to affect inferred *P. agathidicida* DNA concentration was the location of the sample around each kauri, with those taken uphill of

the trunk tending to show higher inferred concentrations. This suggests that *P. agathidicida* is more abundant uphill of each of the kauri in the study. The reason for this is unclear, but might possibly be related to the increased movement of soil water downhill of the tree as a result of the trunk directing a larger volume of water downhill, which might 'dilute' the numbers of *P. agathidicida* present. While Randall *et al.,* (2010) were unable to find *P. agathidicida* in freshwater catchments in the Waitākere Ranges, they did not rule out the possibility of the pathogen being spread via water due to constraints of the baiting method. Further research is needed using additional baiting and qPCR trials, to confirm whether *P. agathidicida* is able to be spread via the movement of water, like other oomycetes such as *P. cinnamomi* (Tippett *et al.*, 1987).

3.3 Detection of *Phytophthora agathidicida* by sequence analysis of DNA amplified from soil

The PCR trials carried out on DNA isolated from individual *Phytophthora* species confirmed the Scibetta *et al.,* (2012) protocol was able to amplify and discriminate DNA from a wide range of *Phytophthora* species. Importantly, this included *P. agathidicida*, confirming that in principal, the method could be used to detect the pathogen in soil, provided that its DNA is of sufficient proportion in the total soil DNA extracts for amplification to occur. The estimated detection limit of 52 fg/µL for DNA extracted from *P. agathidicida* isolates in pure culture was higher than the 1 fg/µL limit reported by Scibetta *et al.,* (2012). The successful amplification of *P. cinnamomi* was encouraging, as the species is prevalent in soil throughout west Auckland forest (Podger & Newhook, 1971), and is hypothesised to be an opportunistic pathogen of kauri weakened by other biotic and abiotic stresses (Horner & Hough, 2014). We confirm that the method of Scibetta et al (2012) is sufficient to discriminate between these closely related species.

The negative PCR result from the *Pythium vexans* DNA sample shows that there was no cross-reactivity between the primers and this taxon. However, more work is needed on a wider range of oomycetes to confirm the specificity of the primer set for *Phytophthora* oomycetes.

We were unable to replicate the results of Scibetta *et al.,* (2012), in that there was no detectable amplification of *Phytophthora* directly from the soil, suggesting lower sensitivity of the protocol than originally suggested by these authors. The estimated detection limit of 52 fg/µL was based on *P. agathidicida* DNA extracted from pure cultures of the pathogen.

The minimum amount of DNA needed to detect *Phytophthora* in environmental soil samples using the protocol may have been higher than 52 fg/µL, due to the presence of PCR inhibitory substances within the soil such as clay and humic material. For example, DNA binds strongly to clay particles (Frostegard *et al.*, 1999, Cai *et al.*, 2006) preventing the isolation of DNA into the extraction supernatant whereas the presence of humic material in DNA extracts inhibits the activity of some enzymes including DNA polymerases (Dong *et al.*, 2006). Where the presence of only *P. agathidicida* is of interest it may be appropriate to design primers that are more specific, amplifying only the DNA of this pathogen. However, in many cases information on the presence of a broader range of *Phytophthora*, is considered desirable.

3.4 Detection and identification of *Phytophthora* sp. by sequence analysis of DNA amplified from lupin baits

In contrast to the PCRs using DNA extracted directly from soil, DNA was amplified from lupin seedling roots used for baiting soil samples from the same sites. This suggests that baiting acted as an enrichment step prior to the PCR, allowing the detection of Phytopthora species. This 'hybrid' method constitutes an intermediate step between the purely molecular approach of using PCR directly on soil DNA extracts, and the culture-based approach of inoculating selective agar plates with baited lupin root tissue and making a diagnosis based on the cells which are able to grow on them. Despite the opportunities provided by the use of molecular methods, baiting for *P. agathidicida* can nevertheless be considered desirable as, by relying on the baiting step, this 'hybrid' method requires the cells within the soil sample to be physiologically capable of colonising the bait tissue. Hence the detection of Phytophthora in baiting experiments confirms not only that the organism is present, but also that it is capable of infection. The ability of this approach to detect infective cells is a major advantage over the use of purely molecular methods in which positive results do not necessarily reflect the physiological state or viability of the organisms they target. Since this method does not rely on the cells to be able to grow on artificial growth media, it reduces issues with sample contamination during culturing and speeds the process of sample analysis as cells do not need to be incubated on culture media before identification. Using a DNA sequencing approach, we were further able to discriminate sequence data originating from closely related strains such as *P. agathidicida, P. cinnamomi* and *P. multivora*.

3.5 Conclusions

Overall, the results of the study show that the baiting approach was not as specific or sensitive as the qPCR method for detecting the presence of *P. agathidicida* in soil samples. The analysis also suggests a high degree of reproducibility and specificity for the qPCR protocol developed by Than *et al.,* (2013). These factors are crucial for any diagnostic technique, and given the presence of other *Phytophthora* species in forest soils, the results of this study are in support of implementing the qPCR protocol in routine ecological surveys in areas vulnerable to kauri dieback.

Our results highlight that the PCR protocol from Scibetta *et al.,* (2012) is not suitable for detecting *P. agathidicida* directly from forest soil, as it currently stands. Using the protocol in conjunction with baiting improved the rate of successful amplification of *Phytophthora* DNA from soil, including DNA from *P. agathidicida*. However, this additional step introduces biases such as requiring the *Phytophthora* cells to be physiologically capable of forming zoospores to colonise bait tissue, and to do so in abundances that are high enough for the PCR protocol to successfully amplify the DNA. These are biases that purely molecular-based approaches of detecting *Phytophthora* tend to avoid, thereby negating some of the advantages in using them over culture-based methods. A further optimised PCR protocol with higher amplification rates would be more appropriate for coupling with next-generation DNA sequencing methods (such as Illumina sequencing) for profiling *Phytophthora* species in New Zealand forest soils, and studying broad-scale trends in their population dynamics across different landscapes.

The baiting method still has an inherent advantage over the molecular approaches, however, in that by targeting *Phytophthora* at the genus level, it allows for the isolation and characterisation of potentially novel species, which may end up having previously-unknown ecological roles. The ability to culture cells in the laboratory from various locations within, or between landscapes, also allows for studies on biogeographical patterns in pathogenicity, which would in turn allow insights into how kauri dieback may be controlled, e.g. greater resources allocated to areas with more aggressive strains of *P. agathidicida*. DNA sequencing also offers the potential for discovering new species (for instance in Scibetta *et al*., (2012)), and allowing the pathogenicity of *Phytophthora* to be inferred via genome sequencing (e.g. Studholme *et al*., (2016)). However, studies using DNA sequencing to understand microbial populations are largely considered hypothesis-

generating exercises, and their results may need to be confirmed experimentally using live cultures before they are accepted into scientific theory (Hennekam & Biesecker, 2012).

The choice of detection method used, therefore, should reflect whether land managers are interested in simply detecting broad-scale distributions of the pathogen or require highly accurate knowledge as to the presence or absence of the pathogen in specific location. Both baiting and molecular-based techniques can be employed to allow for informed decisions to be made on how kauri dieback should be managed. Another consideration is the per sample cost of analysis. This remains very hard to estimate as costs will vary depending on the facilities used and the personnel costs for sample processing. For this reason we provide an estimate of the costs associated with the analysis of ~100 soil samples by qPCR and also using DNA sequence analysis (Tables 4 and 5) but we caution that the real costs may vary substantially compared this present day estimate (22/02/2017).

Table 4. Presumptive costs associated with the analysis of 100 samples by qPCR

Step	Per sample cost (NZ $)	Sample processing hours
DNA extraction	9.00	10
DNA amplification	2.00	3
Consumables –pipette tips, PCR plates, gloves, etc.	3.00	-
Total	$14.00	13 h

Note: *Not included in the price estimate are costs associated with the creation and use of positive PCR controls, or costs associated with data analysis and interpretation. Human resources (i.e., sample processing hours) are not included in the per sample costs.*

Table 5. Costs associated with the DNA sequence analysis of 100 samples for the detection and identification of *Phytophthora* species.

Step	Per sample cost (NZ $)	Sample processing hours
DNA extraction	9.00	10
DNA amplification	1.20	3
DNA primers	0.20	
DNA purification	2.70	4
DNA Quantification	1.50	3
DNA Sequence Analysis (using an Illumina MiSeq machine)	56.00	-
Consumables –pipette tips, PCR plates, gloves, etc.	10.00	-
Total	$80.60	20 h

***Note**: This cost estimate is for the analysis of sample DNA using an Illumina MiSeq DNA sequencing machine, rather than the Sanger Sequencing approach described in this document as Illumina sequencing is more cost competitive for the analysis of large sample numbers (e.g., 100+ samples). Not included in this price estimate are costs associated with the creation and use of positive PCR controls, or costs associated with data analysis and interpretation, which can be substantial for large DNA sequence datasets. Human resources (i.e., sample processing hours) are not included in the per sample costs.*

3.6 Recommendations

Molecular methods such as qPCR are a more sensitive approach for the detection of *P. agathidicida* than assessments reliant on baiting the organism from soil followed by morphological identification of isolated cells and colonies. However, molecular methods such as qPCR are prone to false positive and negative detection of target DNA, at rates which remain difficult to quantify. For this reason, the qPCR-based method of Than *et al.,* (2013) is recommended for the rapid and relatively cost-effective screening of large sample numbers to indicate the likely presence of *P. agathidicida*. However, where the most accurate detection of *P. agathidicida* in soil is desirable, for example to confirm the presence of the organism in sites not previously known to harbour it, we recommend the baiting of *Phytophthora*-like organisms from soil to increase their abundance in the sample material. This approach, when followed by DNA sequence analysis of *Phytophthora* DNA (Scibetta *et al.*, 2012) to confirm the genetic identify of organism within the pool of extracted DNA is faster than traditional methods for baiting and isolating *Phytophthora* from soil. This combination of baiting and molecular methods also removes the likelihood of misidentifying closely related *Phytophthora* species by assessment of their morphological characteristics alone. Our combination of baiting for *Phytophthora* followed by species identification using modern DNA sequencing methods reduces the time required to screen individual samples compared to traditional baiting and culturing techniques, by removing the 10 day period otherwise needed to culture sample material on agar plates. It also reduces biosecurity risks associated with culturing large quantities of unwanted organism in the laboratory.

3.7 Study limitations

While our study provides interesting insights into the advantages and disadvantages of each of the detection methods used, the study site did not span an area large enough to address geographical differences in soil type and composition, which may have an influence on the performance of each method. There was no temporal element to the study; since this study was only conducted during the winter season in New Zealand, variation in climatic variables such as rainfall and temperature over various seasons could not be considered. This is of importance since the dispersal of *Phytophthora* is expected to be strongly impacted by climatic conditions, particularly precipitation, as high soil moisture is required for the dispersal of zoospores (Newhook, 1959).

3.8 Future research

The limitations of the study can be addressed by trialling the detection methods over a larger geographical area and over multiple seasons, so that the effects of soil type and chemistry, geography and climatic factors on the success of each detection method can be better understood. The Taqman qPCR approach used in this study could be combined with protocols developed for *P. cinnamomi* and other key *Phytophthora* species in a multiplex qPCR protocol (e.g. Li *et al*., 2013). This would allow for rapid and quantifiable detection of multiple *Phytophthora* species within a single sample and qPCR reaction. Despite the opportunities provided by these molecular methods, baiting for *P. agathidicida* is often considered desirable, as in comparison to molecular approaches which can amplify DNA from dead organisms, only live organisms are detected by culturing. However, the length of time required to culture baited organisms as well as the uncertainty associated with the species-level identification of organisms by colony and cellular morphology are seen as major method limitations. The use of high-throughput molecular assays to analyse the DNA of baited material can increase the rate at which samples can be screened, and provide a useful and alternative approach for the correct taxonomic identification of baited organisms. Using such methods, the isolation and culturing of potential pathogens is no longer required as DNA is extracted directly from the bait material. This reduces the time required to screen individual samples but also reduces the biosecurity risks otherwise associated with culturing large quantities of unwanted organisms in the laboratory environment.

4.0 References

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Appendix A Recipes for growth media

Cornmeal Agar amended with antibiotics, P_5ARPH-CMA

For isolation of *Phytophthora* species from soil (Jeffers & Martin, 1986)

Item	Quantity
Solution A:	
Distilled Water	900 ml
Corn meal agar (Difco)	17 g
Solution B:	
Distilled Water	100 ml
Primaricin; antifungal	5 mg
Ampicillin; antibiotic	250 mg
Rifampicin; antibiotic	10 mg
Pentachloronitrobenzene (PCNB); antifungal	100 mg
Hymexazol; antifungal	50 mg

Directions:

- Autoclave solution A for 15 min at 121^{o}C and 15 p.s.i.
- Wait until cooled to 50^{o}C, then add solution B through a sterile 0.2 µm filter before pouring plates.

Clarified V8 Juice Agar, cV8A

For inducing sporulation of *Phytophthora* species (Tuite, 1969)

Item	Quantity
Distilled water	800 ml
Clarified V8 juice	200 ml
Bacto agar (Difco)	15 g

Directions:

- Autoclave solution for 15 min at 121^{o}C and 15 p.s.i.

Clarified V8 Juice

Item	Quantity
Well shaken original-flavour V8 Juice (Campbell Soup Company)	200 mL
Calcium carbonate ($CaCO_3$)	2 g

Directions:

- Centrifuge at 7,000 rpm for 10 min.
- Retain supernatant, freeze in 50 ml aliquots for later use

Potato Dextrose Agar

To support growth of *Phytophthora* cultures (Jeffers & Martin, 1986)

Item	Quantity
Distilled water	1000 mL
Potato dextrose agar (Difco)	39 g

Directions:

- Autoclave solution for 15 min at 121^{o}C and 15 p.s.i., wait until cooled to 50^{o}C before pouring plates

Appendix B Identification of sub-cultured microorganisms

Microscopic inspection of colonies

Colonies sub-cultured on each of the V8A and PDA plates were inspected at the end of the incubation period using a compound microscope, under 100X and 200X magnification. To observe the oospores from the plates more closely, slides of cellular material were also prepared for inspection under 400X magnification. Agar plugs were taken from these plates and soaked in an 85%-strength lactic acid solution overnight to dissolve the agar, leaving behind the mycelial mats. The retained mycelia were then fixed on slides using clear lactoglycerol, before hyphal structures were observed using a stereomicroscope. At least 25 oospores were inspected for each colony isolated at the various magnifications. The appearance of the colonies on PDA and cV8A, hyphal characteristics, size of spores and colony growth rate (mm/day) were recorded. With the assistance of a trained *Phytophthora* specialist at Landcare Research, the isolates were then classified to species or genus level where possible, thus completing the traditional method of isolating and identifying *Phytophthora* species from environmental samples.

Verification of species identities using DNA sequencing

To verify the accuracy of the visual identifications, a subset of 10 isolates grown on PDA and identified as being in the *Phytophthora* genus, were selected for DNA sequencing. Agar plugs from areas on the PDA plates supporting hyphal growth were taken, and DNA extracted from these using a Qiagen X-tractor Tissue kit and an X-tractor Gene robot (Corbett Life Sciences).

PCR was then performed on the DNA extracts, using the universal ITS4 and ITS6 primers designed for the study of oomycetes, fungi and fungal-like organisms (see Table 6 for sequences). This primer set in particular has been identified by Weir *et al.,* (2015) as being suitable for amplifying a region of oomycete ITS1 rDNA that is variable enough to differentiate between *Phytophthora* species present in New Zealand soils. The PCR cocktail for each sample had a total volume of 25 µL, and consisted of 1 µL of the DNA extract solution, 0.5 µL each of forward and reverse primers at a concentration of 10 µM, 10 µL of Promega GoTaq® Green Master Mix (a proprietary master-mix for PCR containing *Taq* Polymerase, Mg^{2+} ions, dinucleotide triphosphates and reaction buffers) and 13 µL of nuclease-free UltraPure water (Invitrogen). A negative control consisted of 1 µL of sterile water instead of DNA template. The PCR was carried out on a GeneAmp®

PCR System 9700 (Applied Biosystems®) thermal cycler. Cycling conditions consisted of an initial denaturing phase of 4 min at 94°C, followed by 38 cycles of denaturing for 30 s at 94°C, annealing for 45 s at 52°C, extension for 45 s at 72°C, and a final extension phase for 7 min at 72°C.

Table 6. Primers used in the PCR-amplification of Phytophthora isolates for Sanger sequencing.

Primer	Sequence (5'-3')	Reference
ITS4 (forward)	TCCTCCGCTTATTGATATGC	White et al. (1990)
ITS6 (reverse)	GAAGGTGAAGTCGTAACAAGG	Cooke et al. (2000)

PCR products were visualised using agarose gel electrophoresis to verify that: 1) successful DNA amplification had occurred, 2) the DNA products obtained were of the expected size (812 bp), and 3) that there was no contamination introduced while the PCR was being set up. The electrophoresis was carried out using 3 µL of PCR product on a 1% (w/v) agarose gel which was stained with SYBR® Safe (Thermofisher), immersed in 0.5 x TBE buffer and run for 40 min at 100 V. Bands on the agarose gel were visualised using the Gel Doc XR+ System (Bio-Rad).

PCR products were purified using a spin-column-based purification method (Zymo Research DNA Clean & Concentrator^{TM} kit). The DNA concentrations of the purified products were then measured using a Nanodrop® ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, USA). The DNA concentrations of the samples were normalised to 5 ng/µL, and volumes of 5 µL submitted to New Zealand Genomics Limited (NZGL) for Sanger DNA Sequencing (Sanger *et al.*, 1977).

Once the Sanger DNA Sequencing had been carried out, sequences for each of the isolates were viewed using the DNA sequence analysis software, Chromas (version 2.4.4; Technelysium). This software allows the bases in a sequenced DNA fragment to be viewed alongside a chromatogram generated during sequencing, which displays the probability that each base in a given sequence has been identified correctly (Figure 6). The trailing ends of each sequence are typically considered to be of lower quality than the central region, due to errors invariably introduced during the initial and terminal phases of the sequencing process. These low-quality ends (~80 bp) were then trimmed, before each sequence was interrogated against the National Center for Biological Information (NCBI; http://www.ncbi.nlm.nih.gov/) database using a nucleotide BLAST® (Basic Local

Assignment Search Tool; Boratyn *et al.*, (2013)) query. The sequences were then assigned to taxa based on their similarity to reference ITS1 sequences from known *Phytophthora* species within the database.

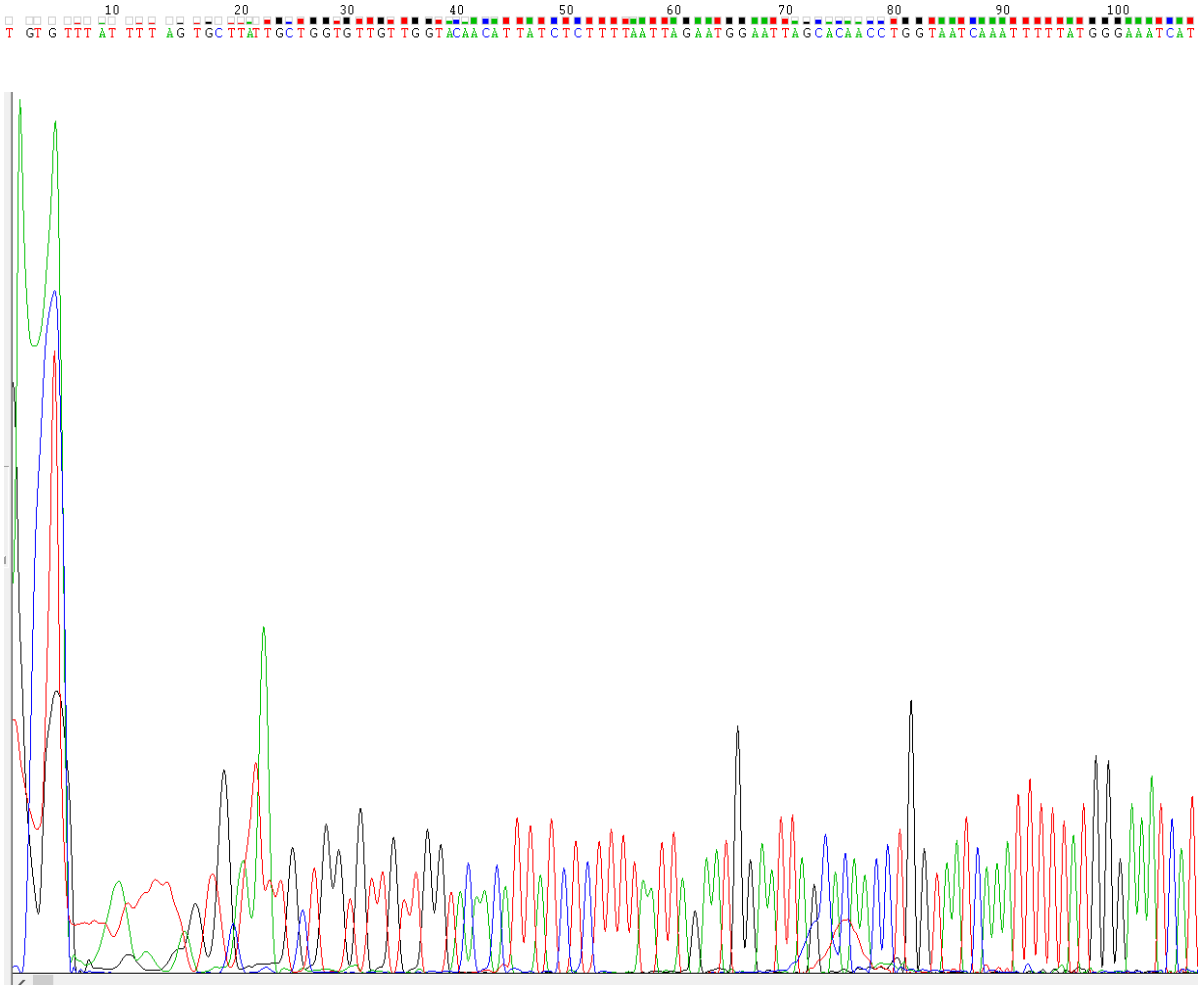

Figure 6. Chromatogram highlighting Sanger sequencing output for the first 106 bases of a *Phytophthora cinnamomi* PCR amplicon. The heights of the peaks of the graph represent the degree of uncertainty with which each of the bases were identified.

Appendix C Detection and quantification of *Phytophthora agathidicida* by quantitative polymerase chain reaction (qPCR)

Extraction of DNA from soil

We used PowerSoil®DNA Isolation Kits (MO BIO Laboratories Inc., Solana Beach, CA, USA) to extract DNA directly from soil. It is the protocol recommended by the Earth Microbiome Project (www.earthmicrobiome.org), and has been used successfully in soil environments despite the presence of humic acids that can interfere with PCR (Gilbert *et al.*, 2014). DNA was extracted from 0.25 g of soil homogenised from each sample using this kit. DNA extracts were then stored at -20°C until required for qPCR.

Quantitative Polymerase Chain Reaction (qPCR) protocol

Each of the environmental DNA extracts were amplified in triplicate using the protocol of Than *et al.,* (2013). Each reaction mixture had a total volume of 15 µL and contained 1 µL of the DNA extract to be amplified, 0.525 µL each of forward and reverse primers at 10 µM, 7.5 µL of TaqMan® Environmental Master Mix 2.0 (ThermoFisher), 0.24 µL of probe at 80 nM and 5.21 µL of sterile, nuclease-free UltraPure water (Invitrogen). Primer and probe sequences used in the protocol are summarised in Table 7. The probe is included in the PCR mixture to increase the specificity of the qPCR, as first reported by Holland et al (1991).

Table 7. Sequences of primers and probe used in Taqman-based qPCR targeting *Phytophthora agathidicida* ITS rRNA gene developed by Than *et al.,* (2013).

Reaction component	Sequence (5'-3')
PTA_ITS_F2 (forward primer)	AACCAATAGTTGGGGGCGA
PTA_ITS_R3 (reverse primer)	CTCGCCATGATAGAGCTCGTC
PTA_ITS_Probe	GGCGGCTGCTGGCTTTGGCT

PCR cycling conditions consisted of a denaturing phase at 95°C for 10 minutes, followed by 40 cycles of annealing at 95°C for 15 s and extension at 61°C for 60 s. The qPCR was carried out on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). The output, containing the amplification profiles recorded in real time during the qPCR process, was analysed using the qPCR analysis software, SDSv2.4 (Applied Biosystems).

qPCR controls

Positive controls were included in all qPCR runs and consisted of two additional genomic DNA extracts from *P. agathidicida* obtained from the SCION, New Zealand, at concentrations of 4.21 ng/µL and 4.36 ng/µL. Negative controls consisted of sterile, nuclease-free water. In addition, *DNA* extracts of known concentrations from five *Phytophthora*, and two *Pythium* species, were obtained from the International Collection of Microorganism from Plants (ICMP) at Landcare Research. *Pythium* is a genus of oomycetes, along with *Phytophthora*, in the Family Pythiaceae. Sample material (5 µL) from each extract were combined to create a mock DNA community that was amplified at the same time as control and environmental DNA. This was done to assess the ability of the protocol to selectively amplify and quantify *P. agathidicida* ITS1 rRNA genes amongst that of closely-related species. The DNA used and their respective concentrations are summarised in Table 8.

Table 8. DNA extracts from the species of Pythium and Phytophthora used to create mock Pythiaceae community DNA, and their respective concentrations.

Species	Concentration of DNA in the initial extract (ng/µL)	Percentage of DNA in final solution (%)
Pythium vexans	2.48	14.74
Pythium irregulare	0.74	4.40
Phytophthora kernoviae	0.82	4.88
Phytophthora agathidicida	4.63	27.53
Phytophthora hibernalis	4.96	29.49
Phytophthora plurivora	2.29	13.61
Phytophthora cinnamomi	0.90	5.35

qPCR standards

A sample of DNA extracted from *P. agathidicida* in pure culture was obtained from the International Collection of Microorganisms from Plants (ICMP), with a concentration of 5.21 ng/µL. Sterile water was used to carry out ten-fold serial dilutions, so that a dilution series with DNA concentrations between 5.21 ng/µL to 5.21×10^{-10} ng/µL were obtained. The absolute quantification method was used to infer the concentration of *P. agathidicida* DNA in each of the samples. Firstly, a standard curve was generated by plotting the log DNA concentration (to base 10) of each of the standards, against their mean CT value as

determined by inspecting the amplification profiles during the exponential phase of the qPCR process. The linear relationship between these values was then modelled, before the strength this relationship was determined by calculating its R^2 value, which is a measurement of goodness of fit for a linear model (McCullagh & Nelder, 1989).

To infer the concentration of *P. agathidicida* in each of the samples, their mean CT value was calculated. This was then used to interpolate their log DNA concentration from the standard curve; their antilogs were then calculated to find the inferred concentration of *P. agathidicida* DNA in each sample, in ng/µL.

Appendix D Identification of *Phytophthora agathidicida* by DNA sequence analysis

The sequences of the primers used to amplify DNA from the genus *Phytophthora* are summarised in Table 9. PCR conditions for the first round consisted of an initial denaturing phase at 95°C for 2 min, followed by 30 cycles of denaturing at 95°C for 20 s, annealing at 60°C for 30s, and extension at 72°C for 30 s, with a final extension phase at 72°C for 7 min. For the second PCR, 1 µL of PCR product from the first round was used. PCR conditions for the second round were the same as for the first, except this time there were 25 cycles carried out instead of 30. Agarose gel electrophoresis and gel imaging were used to verify that successful PCR amplification had occurred.

Table 9. Primers used in the nested PCR used by Scibetta *et al.,* (2012) in their study of Phytophthora species in forest soil and stream water, and their sequences.

Primer	**Round**	**Sequence (5'-3')**
18Ph2F (forward)	1	GGATAGACTGTTGCAATTTTCAGT
ITS6 (forward)	2	GAAGGTGAAGTCGTAACAAGG
5.8S-1R	1 and 2	GCARRGACTTTCGTCCCYRC

DNA sequence analysis

Amplified PCR products were purified using a commercial spin-column-based purification kit (Zymo Research DNA Clean & Concentrator^{TM}kit) before being used to create a DNA clone library. This involved the use of the pGEM®-T Easy Vector System (Promega), and transformation of competent *E. coli* cells (JM109 High Efficiency Competent Cells - Promega). Following the plating and incubation steps in the pGEM®-T Easy Vector System (Promega) protocol, white colonies of *E. coli* (indicative of successful transformation and recombination) were selected and individually sub-cultured in 5 mL of LB broth overnight at 37°C. To extract DNA from the sub-cultured cells, 200 µL of LB broth was then incubated at 95°C for 5 min to heat-lyse the cells within. PCR was then carried out using the primers and cycling conditions for the second round of the nested PCR developed by Scibetta *et al.,* (2012), with 1 µL of the LB broth containing the heat-lysed

cells acting as the template. Agarose gel electrophoresis was then carried out to confirm that the cloned DNA fragments were of the expected size (250-300 bp), and that successful amplification had occurred, PCR products were purified using the Zymo Research DNA Clean & Concentrator^{TM} kit, and were stored at -20°C until required for Sanger Sequencing by New Zealand Genomics Limited (NZGL).

Find out more: phone 09 301 0101, email rimu@aucklandcouncil.govt.nz or visit aucklandcouncil.govt.nz **and knowledgeauckland.org.nz**

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