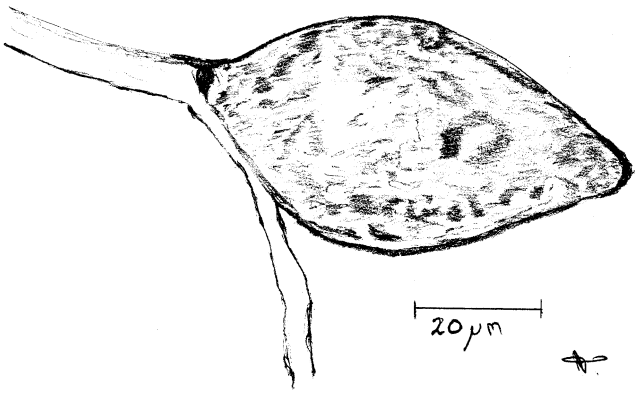
Mapping the Plant Destroyer

Phytophthora at RBGE



Malcolm A. R. Gibson

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Royal Botanic Garden Edinburgh

THE UNIVERSITY OF EDINBURGH

Abstract

The oomycetes are filamentous, eukaryotic microorganisms found within the Stramenopiles-Alveolates-Rhizaria (SAR) supergroup. One of the major taxa within the oomycetes is the genus *Phytophthora*. New *Phytophthora* species are being regularly described in the scientific literature.

Phytophthora species present a formidable pathogenic threat to forests, agriculture and ecosystems generally. Their resistant oospores and chlamydospores allow a persistent presence in soil, and are activated by the arrival of host root tissue. The dispersal of sporangia and flagellate zoospores, through rain drops, mists, or bodies of water, greatly assists the rapid infection of host plants.

There have been, and still are, instances of *Phytophthora* infecting living spermatophyte and pteridophyte hosts within RBGE. The results often involve death of the hosts, as well as local propagation of the *Phytophthora* inoculum.

This thesis explores the taxonomy, diversity and pathogenicity of the genus *Phytophthora* on a general basis, and then looks specifically at the environment within RBGE. A series of samples were taken. Selective baiting and aseptic culturing was used to isolate *Phytophthora* species. Isolates were identified using morphological and molecular techniques.

Acknowledgements

I would like to extend my personal thanks and full appreciation to my project supervisor, Katy Hayden, RBGE, who has been generous with her advice and sharing her extraordinary skills. She has also been unstinting in both her encouragement and support. I would also thank Ruth Hollands and Laura Forrest, STS, for their patience and helpful assistance answering endless questions about laboratory stuff; Rob Cubey, the Plant Records Officer, RBGE, for assistance with mapping and data provision; Kerry Walter for assistance with BG-BASE; and Michael Moeller for his Phylogeny Workshop during July 2017.

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I also openly acknowledge the long enduring enthusiasm of the late Professor Denis Garratt, Cambridge School of Botany, who introduced me to mycology in 1972. Reading an appreciation of his life and work, brought him very much back to life (Deacon 1992).

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Introduction

Phytophthora species are damaging plant pathogens with varying levels of impact upon ecosystems and human life. At their most benign, like *Phytophthora primulae* Tomlinson causing brown core root rot in *Primula* (Tomlinson 1952), the damage caused is limited, unless economic livelihood is based upon a nursery growing Polyanthus. Species such as *Phytophthora ramorum* Werres, De Cock & Man in't Veld (Werres *et al.* 2001), *Phytophthora infestans* (Mont) de Bary (Bary 1876), *Phytophthora cinnamomi* Rands (Rands 1922), *Phytophthora kernoviae* Brasier, Beales & S.A. Kirk (Brasier *et al.* 2005) and *Phytophthora sojae* Kaufmann and Gerdemann (Kaufmann & Gerdemann 1958), cause untold damage to natural ecosystems, create economic hardship to those whose livelihoods depend upon their hosts, and potentially famine for those whose lives rely upon their hosts for food and sustenance.

Even in 1996 the economic damage caused by *Phytophthora* species in the US alone was estimated to run into billions of dollars, annually (Erwin & Ribeiro 1996). These figures will have increased dramatically as the full impact of relatively new diseases, caused by species like *P. ramorum* and *P. kernoviae*, have become apparent and affecting huge areas of forests. Within England and Wales alone these two species have put an estimated £1.446 billion of public value at risk, and there is still no real sign of containment (Drake & Jones 2017).

Climate change exacerbates the seriousness of the Oomycetes such as *Phytophthora.* It is believed that global food security is threatened indirectly by triggering an increase in plant diseases caused by fungi, bacteria, viruses and oomycetes. This potentially decreases crop yields by an estimated 16% globally (Whitmee *et al.* 2015), at least partially due to heat-stressed plants being generally less able to defend against pathogen attacks.

Phytophthora presents a major threat to the world's ecosystems, the economic stability of forestry and agriculture, and even more critically to the security of global food production for an ever-increasing human population.

The living collections of The Royal Botanic Gardens Edinburgh (RBGE) are effectively a repository for biodiversity of plant life, way beyond the geographical confines of Scotland. The importance of this role is recognised globally through the wide connections RBGE has across the world. As such it is of primary importance to understand the diversity of

Phytophthora within the living collections since there is the potential for great damage to be done to the hard-won collections; and a potential knock on effect to the research capability and credibility of RBGE.

This thesis attempts to make a preliminary statement into the gathered knowledge of *Phytophthora* within the living collections in Edinburgh.

Evidence suggests that no one single type of bait, or set of culture conditions, is likely to detect all *Phytophthora* spp. present in soil or water (Parke *et al.* 2014). This means that whatever diversity of *Phytophthora* is revealed in this project, it can only ever be seen as a minimum range of the total *Phytophthora* diversity within the RBGE. To gain a total description of diversity, assuming such could ever be achieved, a wide comprehensive, and time consuming, range of sampling, baiting and culture methods would need to be deployed.

Taxonomy

Phytophthora de Bary is an important genus within the class Oomycota Arx 1967, part of the Chromalveolata, a super-kingdom proposed in 2005, (Adl *et al.* 2005), replacing Chromista Cavalier-Smith, 1997. Chromalveolata was proposed to represent the organisms descended from a single secondary endosymbiosis involving a red alga and a bikont (Keeling 2010). Based on Adl et al. the full taxonomic position of the genus *Phytophthora* can be shown thus:

Domain: Eukarya
Super-kingdom: Chromalveolata Adl et al., 2005
Kingdom: Stramenopiles Patterson, 1989, emend Adl et al., 2005
Class: Peronosporomycetes Dick, 2001 [Öomycetes Winter, 1897, emend Dick, 1976]
Order: Peronosporales E. Fisch., 1892
Family: Pythiaceae J Schröt, 1893
Genus: *Phytophthora* de Bary, 1876

The Oomycetes are heterokonts within the Stramenopiles, having two flagella on their motile reniform zoospores: a longer, forward-facing tinsel flagellum and a shorter whiplash flagellum directed backwards (Alexopoulos *et al.* 1996).

Following this major taxonomic revision of 2005 by Adl et al. the Oomycota or Oomycetes should be formally known as the Peronosporomycetes, however the original names appear to have remained in general usage, and be widely recognised.

The Oomycota are not part of Kingdom Fungi, but are ancient algal-like organisms quite closely related to the Phaeophyta and Xanthophyta, the brown and yellow-green algae respectively. Conventionally the Oomycota are often, and incorrectly, considered as fungi, with the use of the lower case “f” differentiating them from Kingdom Fungi, which uses an upper case “F”.

There are a number of key differences between the Oomycota and the true Fungi. Some of the main differences are that the Fungi are haploid or dikaryotic for most of their lifecycle whilst the Oomycetes are diploid. Fungi also have septate hyphae and chitin as a major component of their cell walls. Oomycete hyphae are non-septate and their cell walls are predominantly composed of cellulose. Additionally, both groups synthesise lysine using different pathways (Latijnhouwers *et al.* 2003).

The phylogeny (Fig. C1, p.25) shows the relationships between the major groups of eukaryotes. The oomycetes are found within the Stramenopiles, which in turn is part of a larger umbrella super-group, the SAR following the unexpected monophyly of three highly diverse divisions, Stramenopiles, Alveolates and Rhizaria (Hackett *et al.* 2007). The Fungi are found within the Opisthokonts, well removed from the Stramenopiles.

There is evidence of Oomycete-like fossils dating back 2.4 billion years (Bengtson *et al.* 2017), demonstrating the very ancient roots of this group of organisms.

The distant relationship between the Oomycetes and the True Fungi is of interest since the Oomycetes display a filamentous morphology, and have ecological roles that are very similar to those of the Fungi. Until quite recently it was believed that their morphology and lifestyles demonstrated a convergent evolution, for example (Latijnhouwers *et al.* 2003). However, it now appears more likely to be a combination of independent evolution and limited interkingdom horizontal gene transfer (HGT). Of 48 oomycete horizontally acquired genes studied, 40 show evidence of fungal origin (Savory *et al.* 2015).

Unlike the Fungi, apart from a brief period when they are haploid gametes, the Oomycetes are diploid throughout their lifecycle (Fig. 2).

The sexual phase involves the differentiation of terminal cells in the hyphae undergoing meiosis and differentiating into a female gamete, the oogonium, or a male gamete, the antheridium. Unusually, these gametes remain attached to their parent hyphae.

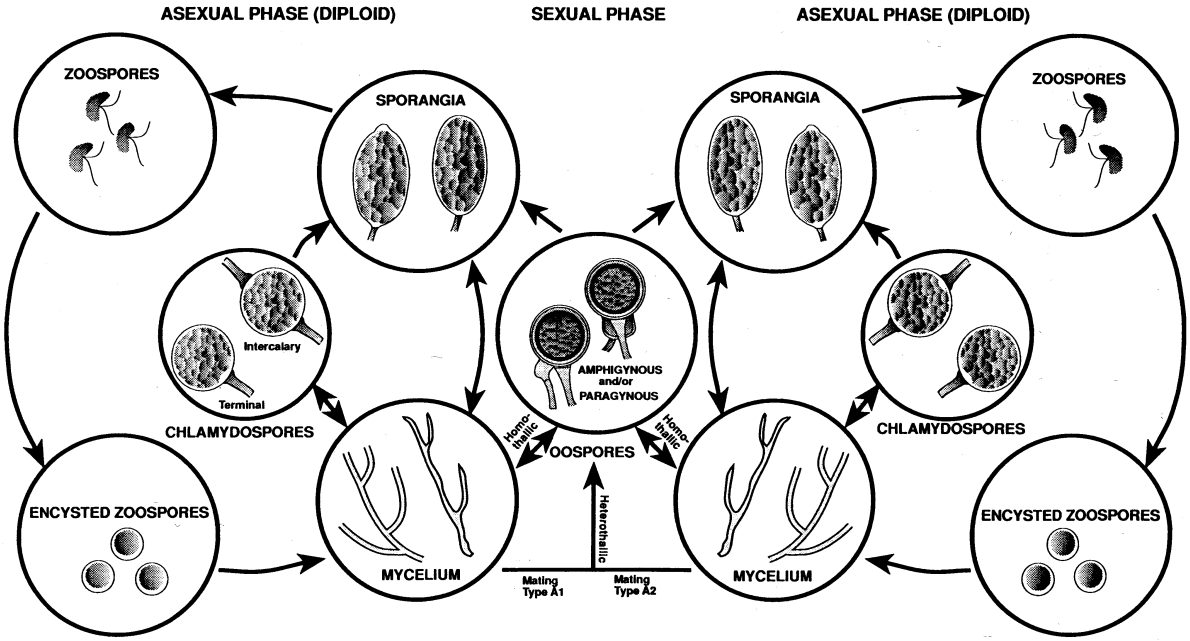

Figure 2. The life history of *Phytophthora* (Erwin & Ribeiro 1996)

Some species of Oomycete are homothallic. Thus, gametangia production and successful fertilisation can occur within a single mating type. Other species are heterothallic, meaning that two strains of opposite mating type are needed to stimulate gametangia differentiation and fertilisation. A fertilisation tube extends from the antheridium to the oogonium and a zygote is created. This usually forms into a thick-walled resistant oospore and, in due course, will germinate to yield more hyphae, but genetically differentiated from the parent hyphae due to the recombination of genes.

The Oomycetes frequently use chlamydospores as an additional survival mechanism, in the same manner as the Fungi. The chlamydospores originate as hyphal swellings, either terminal or intercalary, but develop a highly resistant cell wall. Strategically this provides a core strength, since oospores and chlamydospores, with their strong protective walls, are known to be able to survive years in soil, even in hot dry conditions, thus greatly prolonging the inoculum potential of a soil long after visible signs of infection have vanished, as evidenced through work on *Phytophthora cinnamomi* (Crone *et al.* 2013).

The asexual part of the lifecycle involves the production of sporangia, through the differentiation of hyphae. The sporangia develop motile zoospores which, on release, swim towards new host material, and then encyst. Some form of chemotaxis or electrical gradients attracting the zoospores to young root hairs has long been believed to be operational (Zentmyer 1961). With legumes, the zoospores of *Phytophthora niederhauserii* Z.G. Abad et

J.A. Abad (Abad *et al.* 2014), *P. pisi* Heyman (Heyman *et al.* 2013), *P. sojae*, and *P. vignae* Purss are positively attracted to isoflavones released by the host (Hosseini *et al.* 2014). The survival of zoospores is generally measured in terms of hours and days, but zoospore cysts have a survival expectancy measured in days and weeks. *P. cinnamomi* zoospores survive about 3 weeks in soil (Hwang 1978), and form a major stage in the pathogenic capability of *Phytophthora* to infect new hosts.

The Oomycota consist of around 500 species, grouped into five orders. The phylogenetic organisation within the class is shown in Fig 3. This shows *Phytophthora* as a highly evolved genus within the order.

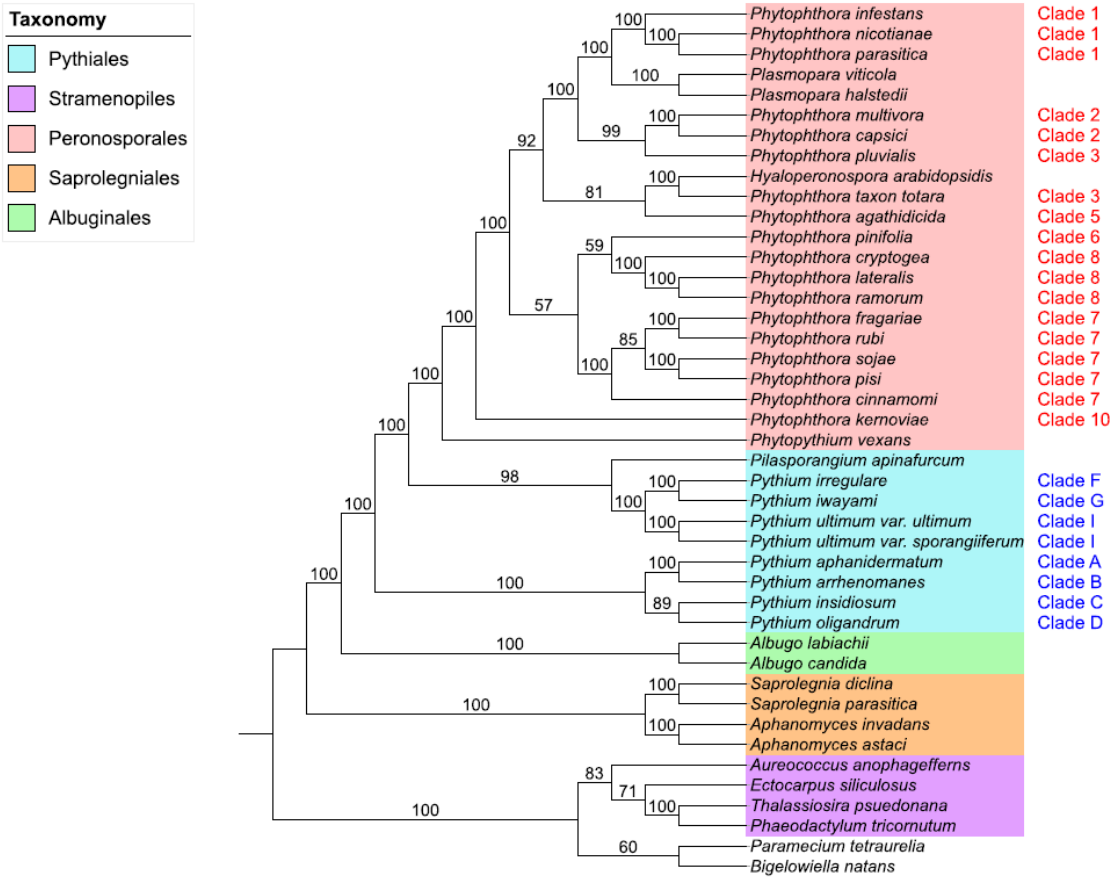

Figure 3. Matrix representation with parsimony (MRP) supertree of 37 oomycete species and 6 SAR species (2,280 source phylogenies). The supertree was generated in CLANN. The phylogeny is rooted at the SAR branch. *Phytophthora* clades as designated by Blair et al. and *Pythium* clades as designated by de Cock et al. are indicated in red and blue respectively. No colour, P. tetraurelia (Alveolata) and B, natans (Rhizaria) (Mccarthy & Fitzpatrick 2017)

The general infamy of the Oomycetes reached recorded human consciousness with the Irish Potato Famine between 1845 and 1852, caused by *Phytophthora infestans*. As a result of the Irish Potato Famine in the region of one million people died, and a further 1.5 million

emigrated. (Alexopoulos *et al.* 1996). Even allowing for the somewhat dubious Victorian political situation at the time of the Famine, given the destructive nature of *Phytophthora* infections, it is almost certainly not the first-time human life had been affected by members of the genus. *Phytophthora* species are also responsible for several recent emergences of destructive forest diseases, most likely via species introduced on nursery stock (Roy *et al.* 2014).

The genus *Phytophthora* de Bary

Studies of the *Phytophthora* genomes provide some evidence of large-scale gene duplication relatively early in their evolutionary past. Polyploids have also been identified within several species of *Phytophthora* (Sansome *et al.* 1991). A conclusion may be that *Phytophthora* is most likely an ancient polyploid (Martens & Van de Peer 2010). Such polyploidy, together with hybridisation – for example, the recent hybrid origin of *P. alni* Brasier & S.A. Kirk (Brasier *et al.* 2004) and its accumulating group of allopolyploid genotypes (Ioos *et al.* 2006) - might help to account for the high levels of plasticity, host adaptation capabilities and speciation that is observed within the genus. Allopolyploidy is widely recognised as one of the major generators of biodiversity (Abbott & Andrews 2012).

In a relatively early attempt to bring some taxonomic order to the genus *Phytophthora*, Grace Waterhouse developed a scheme of splitting it into six morphological groups (I to VI) based upon a range of criteria. She clearly stressed that "the grouping is not necessarily intended to imply that this is a 'natural' classification" (Waterhouse 1963). The groupings were later revised and adjusted (Stamps *et al.* 1990).

Developing molecular technologies allowed classification to be based on an evolutionary, as opposed to a morphological, basis. This gave rise to ground-breaking ITS-based phylogenetic analyses which suggested that *Phytophthora* is paraphyletic. All but three of the 50 taxa examined fell into a relatively recently evolved monophyletic group of eight major clades, with an additional two clades (Cooke *et al.* 2000). Cooke et al. also highlighted the recent interspecific hybridisation within the genus: one within clade 1 involving *P. nicotianae* Breda de Haan and *P. cactorum* (Leb. And Cohn) Schröeter (Man In 't Veld *et al.* 1998), and the other in clade 7 between *P. cambivora* (Petri) Buisman (Buisman 1927) and a *P. fragariae*-like taxon (Brasier *et al.* 1999).

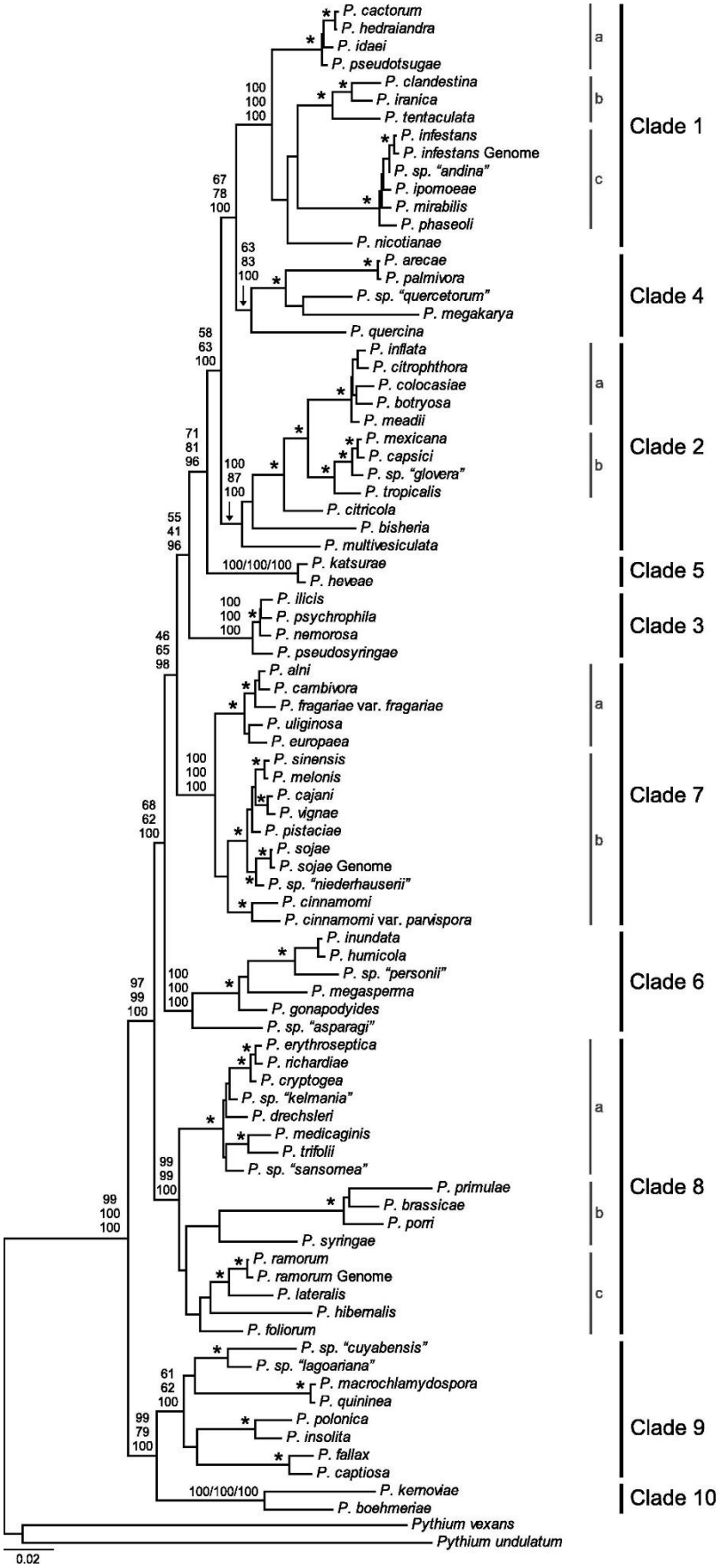

Figure 4. A genus-wide phylogeny for *Phytophthora* using seven nuclear loci. ML branch lengths shown. Numbers on nodes represent bootstrap values of maximum likelihood (top), maximum parsimony (middle) and Bayesian posterior probabilities as percentages (bottom) (Blair *et al.* 2008)

Further developments in both molecular and information technology techniques allowed substantially larger, multi-locus, phylogenetic assessments. These used the now available genome sequence data for *P. ramorum* and *P. sojae* together with large numbers of expressed sequence tags from *P. infestans, P. nicotianae* and other *Phytophthora* species, analysing some 40 million bases and identifying over 225 potential markers (Blair *et al.* 2008). A resulting phylogenetic tree (Fig. 4) broadly supported the findings of Cooke et al. from 2000, and formalised the ten clades (with clades 9 and 10 being basal to clades 1 to 8). This allowed for considerable enhancement of the understanding of the phylogenetics of *Phytophthora.*

It is extremely difficult to identify the precise number to *Phytophthora* species. This is at least partly due to the widespread use of provisional names, such as *Phytophthora* taxon Salixsoil. Since no type isolates are assigned to the provisional species it is not possible to verify whether or not newly collected isolates belong to a species with a provisional name (Kroon *et al.* 2012). In the case of the given example for a provisional name (Salixsoil), this has now been formally re-designated as a new species, *Phytophthora lacustris* Brasier, Cacciola, Nechwatal, Jung & Bakonyi. The original isolate was retrieved in June 1972, but it was forty years later, in 2012, that the species was characterised and formally described (Nechwatal *et al.* 2013).

Descriptions of new species of *Phytophthora* are regularly published with ever increasing regularity (Fig. 5). Often the species is mostly characterised by its DNA, with morphological segregation of species becoming increasingly difficult.

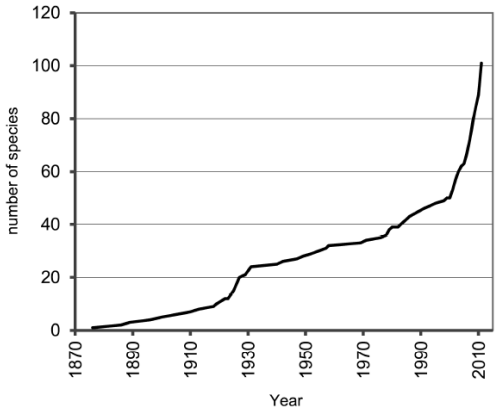

Figure 5. Increase in the number of described *Phytophthora* species over time. (Kroon *et al.* 2012)

Pathogenicity of *Phytophthora* spp.

Species of the genus *Phytophthora* are hemibiotrophs, having dual lifestyles. A suggested definition for this predominantly fungal lifestyle is:

> Fungi that have a narrow host range and initial biotrophic life-style associated with living host cells, and later switch to a necrotrophic lifestyle that kills host cells to obtain nutrients. Most taxa produce haustoria and appressoria during the initial biotrophic phase. They synthesize hydrolytic enzymes and toxins during the later necrotrophic phase (De Silva *et al.* 2016).

The high pathogenicity and invasiveness of many *Phytophthora* spp. lies within their ability to spread, persist and reproduce in new environments. This is together with a rapid lifecycle, and a propensity to reproduce asexually - producing clonal lineages, often spreading aerially, via sporangia carried on windblown rain. Furthermore, they often have resistant oospores and chlamydospores to survive through harsh climatic conditions. All these lifestyle elements are found in many of the economically damaging *Phytophthora* species, such as *P. infestans*, *P. kernoviae* and *P. ramorum*.

This combination of characteristics is further compounded by a frequent genetic capability to rapidly adapt to changing environmental diversity. This gives the capacity to create what, in the case of late potato blight (*P. infestans)*, has been described as a "nightmare disease" (Fry *et al.* 2015).

A critical component for a plant pathogen is the ability to penetrate and cross the protective layers of cutin that gives the host plant protection. Multiple copies of putative cutinases have been found in the genomes of *P. sojae* and *P. brassicae* De Cock & Man in 't Veld (Man in 't Veld *et al.* 2002). Additionally "the signature pattern for cutinases is present in the *Phytophthora* proteins, and they do encode signal peptides for secretion from the oomycete cell" (Belbahri *et al.* 2008). It is believed that the transfer probably occurred from the actinobacteria to the last common ancestor of *P. brassicae, P. sojae, P. infestans* and *P. ramorum* (Soanes & Richards 2014).

An additional biochemical feature found in, but not restricted to, *P. sojae* and *P. capsici* Leonian, is the use of 'necrosis and ethylene-inducing peptide 1' -like proteins (NLPs) to disrupt host plant membranes. Phylogenetic analysis suggests that oomycete NLPs arose by horizontal gene transfer from a fungal genome (Richards *et al.* 2011).

As well as the ability to penetrate host cells, further sophisticated mechanisms are needed to provide the ability to evade, manipulate or overcome host immune systems. Work with *P. sojae* suggests that the majority of effector proteins encoded in the genome have the potential to suppress plant defence, but that a small minority of strongly expressed, rapidly evolving principal effectors may make large indispensable contributions to successful infection (Wang *et al.* 2011). It is likely that timely expression of these effector proteins provides the delicate control of biotrophic and necrotrophic phases that is so critical for hemibiotrophs (Dou & Zhou 2012).

The impact of these multiple origin HGTs has been to provide *Phytophthora* with the capability for a diverse and particularly effective means of penetrating, and then manipulating, host plant cells. Without this core capability, all the other characteristics which greatly aid the virulence of *Phytophthora* infections would have been severely modulated.

A fine assessment of the pathogenic capability of *Phytophthora* is given by Jung *et al.*

> The specific life strategy of *Phytophthora* spp., based on highly resistant oospores facilitating long-term survival, chlamydospores for medium- and short-term survival, the multicyclic production of sporangia releasing motile zoospores able to infect healthy unwounded tissues of vigorous hosts, their often high aggressiveness, and the either wide host ranges or very specific adaptations to certain host plants, arguably make *Phytophthora* species the most notorious and successful invasive plant pathogens in existence (Jung *et al.* 2016).

Diversity of *Phytophthora* species.

The literature tends to deal with the diversity of *Phytophthora* being explored either on an ecological niche basis, such as riparian alder ecosystems of western Oregon (Sims *et al.* 2017), or *Phytophthora* diversity in nursery-grown ornamental and fruit plants (Cooke *et al.* 2015), as an individual species, or as a host-species relationship, than in terms of a more global diversity. This is perhaps a feature of the devastating ecological and economic impact that many *Phytophthora* species can inflict.

Some broad biodiversity generalisations are possible. *Phytophthora* species are plant pathogens. They are hemibiotrophic. No species are known to persist solely as a saprophyte

(Judelson 2012), though there is some evidence that *P. cinnamomi* chlamydospores can persist substantially longer in the presence of dead plant material (Weste & Vithanage 1979).

Phytophthora species are soil centric, and generally build a highly effective inoculum within the soil using resistant and persistent oospores and chlamydospores. Critically the Oomycetes need free water to complete their life cycle (so the motile zoospores swim to new host tissues). This connection with water is an invidious one in relation to *Phytophthora* pathogenicity since it has a major impact upon their ability to spread zoospores. Sporangia and zoospores can be distributed by rain drop splash, washed downstream in rivers, or even carried aerially for significant distances on mists and in wind-driven rain (Fig. 6).

This dispersal capability, coupled with rapid sporangial development and highly resistant, persistent, oospores and chlamydospores allows a *Phytophthora* infection to endure unfavourable conditions, but to very rapidly escalate into an epidemic when suitable hosts and favourable climatic conditions come together.

Some *Phytophthora* are very host specific, such as *Phytophthora eriugena* Clancy and Kavanagh, with just *Chamaecyparis lawsoniana* (A.Murray bis) Parl. being recorded as its host. Other species, such as *Phytophthora fragariae* Hickman (Hickman 1940), have a relationship with a range of hosts, but with *P. fragariae* they all tend to be members of the Angiosperm family Rosaceae. This would suggest some common genetic pattern within the Rosaceae is open to exploitation by *P. fragariae*. Yet a further group, with species such as *P. capsici, P. ramorum and P. cinnamomi* tend to be complete generalists with hosts ranging across almost the entire plant kingdom.

Figure 6. Spore trapping on a hill top in RBGE Benmore Gardens (image courtesy K. Hayden)

Another variable concerns the type of plant disease caused by the *Phytophthora*. Some species, such as *Phytophthora erythroseptica* Pethybridge, cause predominantly soil borne infection with root and collar rots; others, for instance *Phytophthora megakarya* Brasier and Griffin, tend to cause disease in the more aerial parts of their hosts, creating blights, fruit and crown rots. As with host specificity there is a third group of extreme generalists with different types of disease in different hosts. *Phytophthora citricola* Sawada causes, amongst many diseases, root rot in *Phaseolus vulgaris* L., Brown fruit rot in *Citrus* spp., crown rot in *Pseudotsuga menziesii* (Mirb.) Franco., and collar rot in *Acer saccharum* Marsh. (Erwin & Ribeiro 1996)

In terms of global distribution *Phytophthora* is almost cosmopolitan, though records from the Arctic and Antarctica appear to be absent. This is possibly a consequence of the unavailability of free water, due to permafrost and ice conditions. There is already evidence of a widespread biological response to recent rapid warming on the Antarctic peninsula with abrupt shifts in microbial population change, growth and mass accumulation rates (Amesbury *et al.* 2017). Antarctica, originally part of Gondwana, was largely temperate rainforest up until around 32 mya. In consequence, it is highly probable that *Phytophthora* was present in Antarctica historically.

An important consideration in terms of the biogeographical diversity of Phytophthora is the degree of global redistribution of species away from their native areas into new geographical regions. Many bryophytes, lichens and bacteria have spores that are minute and highly resistant. They get carried on high altitude air currents, and often have an almost global distribution, leading to the inspired Baas Becking tenet, "*alles is overall*: maar *het milieu selecteert*" (Baas Becking 1934). Oomycetes have no such global distribution mechanisms. Their resistant oospores and chlamydospores are relatively large, and usually soil based; their motile zoospores, though much smaller, are not resistant. The biology of the Oomycetes allows for localised dominance, but not for global distribution.

The main reason for the geographical redistributions identified in *Phytophthora* would seem to be found in the frequent introductions of "exotic" plants, for economic, agricultural and horticultural reasons. These appear to have often led to *Phytophthora* species being introduced to new environments through unintentional translocation of spores, or even mycelium, in either the plants or in the soil they are transplanted in.

Sometimes the *Phytophthora* fail to get a foothold, but equally they can become invasive, and are frequently able to make large jumps in their host relationships (Prospero & Cleary 2017). These can be dramatic since in many cases the new host has no natural resistance to the *Phytophthora* as there has been no shared co-evolution.

An early example of *Phytophthora* as an invasive pathogen can be seen in *P. cinnamomi*, which has the ability to infect a very wide and diverse range of plant species (Hardham 2005). *P. cinnamomi* appears to have been spreading around the world for more than 150 years. It is presumed to originate within south east Asia, but now poses a substantial threat to forests and ecosystems generally across the globe. It is known to have caused serious mortality among the native *Castanea* in forests in southeastern USA and southern Europe. It is also responsible for 'Jarrah dieback' affecting many forest ecosystems in southwestern Australia (Fig. C7, p.25), one of the 25 globally recognised biodiversity hotspots (Myers *et al.* 2000).

There was an epidemic of *P. cinnamomi* in UK and European plant nurseries during the 1960s-70s. This mainly affected *Chamaecyparis, Rhododendron* and *Erica* species (Brasier 2008).

An example of a more recent, and at least as devastating, invasive *Phytophthora* is seen with *P. ramorum*. This species causes shoot dieback and stem bleeding lesions on a variety of trees and ornamentals, such as species of the genera *Rhododendron, Viburnum, Quercus* and *Fagus*. It is thought to have originated in Eastern Asia but then spread globally via imported European nursery stock in the 1990s (Brasier 2008).

By the mid-1990s Sudden Oak Death was first observed in the San Francisco Bay area of California, but it was not until the summer of 2000 that the causal agent, *P. ramorum*, was isolated (Frankel 2008). A recent, highly damaging, development in the epidemiology has been a spread to native *Vaccinium* swards and in late 2009 a "jump" to Japanese Larch, *Larix kaempferi*, was discovered (Webber *et al.* 2010). This jump has had severe impacts within the UK forestry industry, particularly larch plantations in the west of Scotland. It also poses a substantial threat to the RBGE accessions, as evidenced at Benmore.

Potential impact of anthropogenic climate change

There is significant scientific evidence for anthropogenic climate change. The main area for debate revolves around what the local effects of the global climate change may be. Much of this debate focuses on natural variability, but a certain negativity is created by what are sometimes perceived as speculative climate models, future emissions, and uncertainties in the UKCP09 probabilistic projections and future prospects (Murphy *et al.* 2010).

As far back as 2000 the predictions for changes in the impact of *Phytophthora cinnamomi* were dramatic, with very regional changes in impact across the globe, due to changing temperatures and precipitation (Fig. C8, p.26). Mostly these changes are a decrease in impact across equatorial and tropical areas coupled with an increase in impact in more northerly and southerly regions. There are, however, some equatorial and tropical hotspots where the impact is predicted to rise.

Assessing the impact of climate change on the activities of *Phytophthora* species is not straightforward. Three factors need to be considered: the reaction of the *Phytophthora* to the changed climate; the impact of the changes upon the potential host plants (they may be weakened by climate stresses, particularly drought and heat stress, may thrive, or may be severely threatened by the changes); and finally, the effect of the climate change in all its aspects upon the relationship between the *Phytophthora* and the host (Allen *et al.* 2010).

There is evidence for climate change having a strong impact on the dynamics and distribution of plant-infecting fungi and more directly so than with animal-infecting fungi. This is supported by the number of disease alerts in the ProMED database for pathogenic fungi. The rate has risen much more rapidly between 1999 and 2010 for plant-infecting than for animal-infecting fungi (Fisher *et al.* 2012).

An additional factor is the tendency towards monoculture, both within agriculture and forestry. Whilst economically useful, such practices make the crops ever more susceptible to damage by *Phytophthora* epidemics (Li *et al.* 2016), which also tend to potentially strengthen the *Phytophthora* genome even further.

An illustration of these impacts can be seen in the major change observed in potato late-blight (*P. infestans*) in Finland. The epidemics began 15 days earlier on average from 1996 to 2002 than from 1991 to 1995. Additionally, the mean defoliation rate rose from 70% to 99% in the years 1996 to 2002. Analysis of the climate data gathered at eight observation sites between

1983 and 2002 supports the hypothesis that the increased frequency of rain and higher early-season temperature has affected the onset time of the epidemics (Hannukkala *et al.* 2007).

The UK Climate Projections from 2009 provide key findings relating to UK regions.

Table 1. Changes in daily mean (summer and winter averages), and summer-mean daily maximum and minimum temperatures, averaged over administrative regions, by the 2050s under the Medium emissions scenario. Wider range is defined as the range from the lowest to highest value of change for all emissions scenarios and all three (10, 50 and 90%) probability levels by the 2050s (Murphy *et al.* 2010)

Variable	Mean temperature, winter °C					Mean temperature, summer °C					Mean daily maximum temperature, summer °C					Mean daily minimum temperature, summer °C				
Probability level	10%	50%	90%	Wider range		10%	50%	90%	Wider range		10%	50%	90%	Wider range		10%	50%	90%	Wider range	
North Scotland	0.6	1.7	2.8	0.6	3.0	0.9	2.0	3.4	0.9	3.9	0.8	2.5	4.5	0.9	5.3	0.9	2.3	3.9	0.9	4.4
East Scotland	0.7	1.7	2.9	0.6	3.1	1.1	2.3	3.9	1.0	4.5	1.0	3.0	5.4	1.0	6.3	1.1	2.5	4.3	1.0	4.9
West Scotland	1.0	1.9	3.0	0.8	3.3	1.1	2.4	3.8	1.0	4.4	0.9	3.0	5.2	0.9	5.9	0.9	2.4	4.2	0.9	4.7

There is likely to be a clear rise in temperature across the East of Scotland. The summers are likely to become substantially hotter, up to a 5°C increase, than at present. There is also a projected warming of the winters, potentially substantial, in the worst-case scenario (Table 1). Both the mean maximum and minimum temperatures in summer increase considerably.

Table 2. Changes in annual-, winter- and summer-mean precipitation, averaged over administrative regions, by the 2050s under the Medium emissions scenario. Wider range is defined as the range from the lowest to highest value of change for all emissions scenarios and all three (10, 50 and 90%) probability levels by the 2050s (Murphy *et al.* 2010)

Variable	Annual mean precipitation %					Winter mean precipitation %					Summer mean precipitation %				
Probability level	10%	50%	90%	Wider range		10%	50%	90%	Wider range		10%	50%	90%	Wider range	
North Scotland	−6	0	+5	−7	+6	+3	+13	+24	0	+26	−23	−10	+2	−23	+6
East Scotland	−4	0	+5	−5	+6	+2	+10	+20	−1	+21	−26	−12	+1	−27	+6
West Scotland	−6	0	+5	−7	+6	+5	+15	+28	0	+30	−26	−12	+1	−27	+6

The range of change in precipitation is very much wider than for temperature (Table 2). There is more confidence that drier summers will be experienced, but that the winters may be drier, or wetter. In the west, unsurprisingly, the increase in winter precipitation is likely to be more significant than in the east, which could impact upon the health of the Benmore and Logan gardens.

It seems probable that the ability of the various *Phytophthora* species to rapidly evolve new genetic forms, either through mutation or hybridisation, all aided by their very rapid life cycle, does not bode well for RBGE given the likelihood of warmer and wetter winters in East Scotland. The potentially drier summers may contain some of the spread of *Phytophthora* through the possible limitation of zoospore dispersal in the absence of rain.

Past RBGE *Phytophthora* diversity

A critical paper in the consideration of *Phytophthora* in Scotland is Cooke's recent review (Cooke 2015). This examines the 42 species of Phytophthora reported in Scotland, and nine species that are present in the UK but not yet confirmed in Scotland (as of 2015). However, given the extreme biodiversity of the flora within the RBGE Gardens, the listing, whilst being a useful start, is almost certainly insufficiently definitive.

An important aspect of particular relevance to RBGE is that, by its very nature, the plant communities found within the gardens are usually unnatural. Plants from all around the world are planted together in communities that are unlikely to be reproduced in the natural environment. Because of this it is important to record the plant communities that are actually found within RBGE when trying to identify possible hosts.

It is known that there is a diversity of *Phytophthora* within the RBGE gardens. In 2015 Forest Research reported *Phytophthora austrocedri* Gresl. & E.M. Hansen (Greslebin *et al.* 2007), and this resulted in the removal of a number of trees (Fig. 9) and shrubs (Hayden & Connell-Skinner 2016).

Specific findings for *Phytophthora* include the recent discovery of *Phytophthora ilicis* Buddenh. & Roy A. Young (Buddenhagen & Young 1957) from an *Ilex* sp. in the Southeast corner of the gardens in 2017. In 2016 *Phytophthora pseudosyringae* T. Jung & Delatour (Jung *et al.* 2003) was isolated from *Betula papyrifera* Marshall (1963.3188), and also from soil beneath both *Juniperus squamata* Buch.-Ham. ex D.Don (1969.4936A), and *Nothofagus obliqua* (Mirb.) Heenan & Smissen, the plant having since died. Prior to this, at some point between 2013 and 2015, *Phytophthora cambivora* had been isolated from *Fagus sylvatica* L. (all K. Hayden, RBGE, pers. comm.).

Additionally, there have been persistent occurrences of *Phytophthora* within the RBGE nursery. A number of infections were ultimately traced back to the propagation bed E19. As a result of this, a query was run by RBGE Horticulture to identify all the plants which had been planted on from bed E19, and where they were planted within the main garden. This list can be found in Appendix Four.

Figure 9. *Phytophthora austrocedri* at RBGE, on *Juniperus rigida* (Stephan Helfer)

Searching BG-BASE, the RBGE accessions database, revealed that 50 records from RBGE Edinburgh mentioned *Phytophthora* (there were many more for Benmore). Identification to species level is rarely recorded, so some care must be made with interpretation of this data, but the observations were made by skilled and experienced horticulturists and mycologists, even if not backed up by culturing or DNA analysis, so some confidence can be applied to the results.

Of these 50 records some 31 apply to accessions of *Fitzroya cupressoides* (Molina) I.M.Johnst. in 1996 and 1998 and grown in the experimental beds E44 and E48. All records are tagged as suspected *Phytophthora*. Intriguingly specimens from the multiple accessions that have been grown on outside the experimental beds are the only survivors. 19961389*F, which was moved from the experimental bed in 2000 to Fota Arboretum, is the only survivor from the seven original plants in that accession. 19981643*AI/AO, which were moved from bed E48 in 2002 to Vogrie Country Park, are the only two survivors of 26 original plants in the accession. This strongly suggests that the specific infections were not with the individual trees, but were environmental and localised to RBGE.

Of the remaining 19 records one is from 2002, three from 2014, two from 2015, ten from 2016 and already there are four records for 2017, though potentially more since there is likely to be a time lag in recording. 11 of the records are for gymnosperms, with eight of them in the genus *Juniperus*. Of the remainder, four are in the Ericales whilst there are also records for *Ilex, Tilia* and *Fagus.*

There would appear to be evidence of a growing problem with *Phytophthora* within the RBGE estates, as well as most likely Scotland generally, and this suggests an increase in

Phytophthora activity and success, possibly led, or at least aided by anthropogenic climate change.

Ongoing sampling has highlighted concerns, both within the gardens and the nursery. The aim of this thesis is to develop an enhanced understanding of the diversity of *Phytophthora* within RBGE Edinburgh.

Colour illustrations I

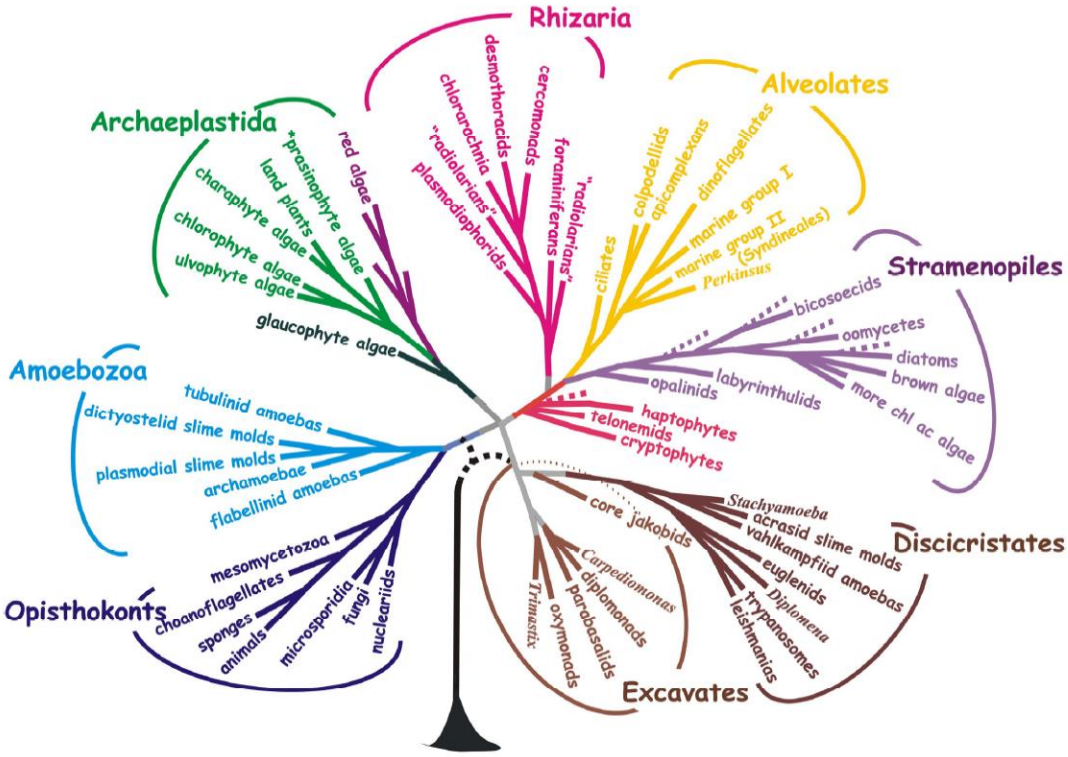

Figure C1. A consensus phylogeny of the major groups of eukaryotes based on published molecular phylogenetic and ultrasound data. (Baldauf 2008).

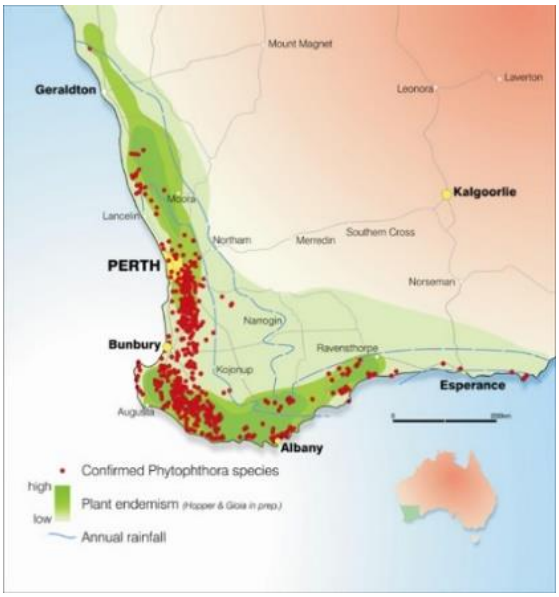

Figure C7. Map showing the extent of *Phytophthora* species invasion within SW Australia (red dots). The dark green shading shows areas of high plant endemism (Western Australia Department of Biodiversity 2017).

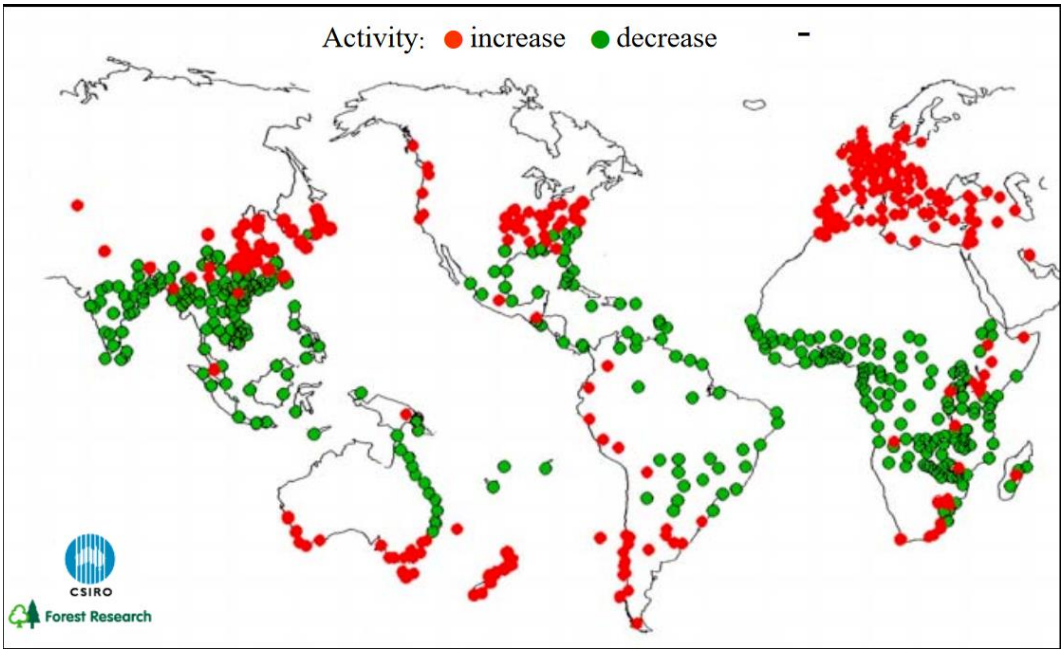

Figure C8. Predicted worldwide activity of *P. cinnamomi*: contrast between current climates and a +3°C mean increase (Brasier 2000)

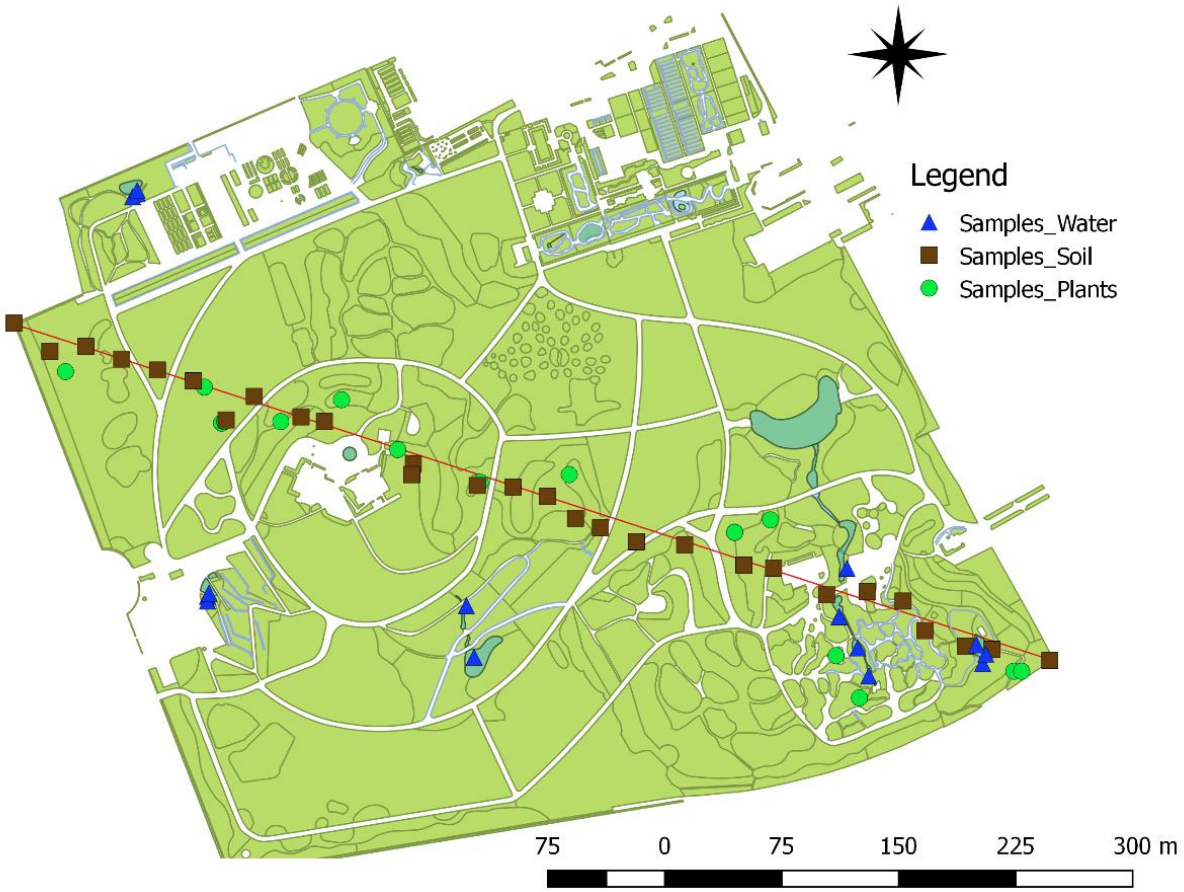

Figure C12. GIS mapping of RBGE showing soil, water and symptomatic plant sample points (Quantum GIS).

Materials and methods

Three sampling strategies have been deployed within this study.

1. A transect band across the RBGE garden (soil samples and baiting)
2. Tissue collection from symptomatic plants close to or in the transect band.
3. Open water courses and bodies of water (water samples and baiting)

Analysis was conducted in the Government licenced Pathology Lab where full bio-security protocols are deployed (Fig. 10). No materials may be removed from the lab without being autoclaved.

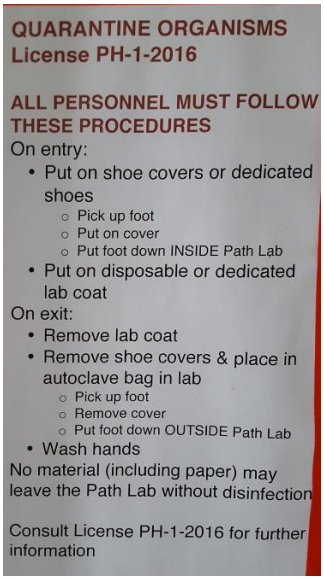

Figure 10. Pathology Lab Quarantine Notice

Best practice and recognised laboratory methods were employed across the collection, culturing and molecular phases of the project to minimise the risks of cross-infection between samples.

Baits

In order to isolate species of *Phytophthora* living within the collected samples baiting was used. Results from the baiting were cultured and then isolated. Unripe pears were chosen since their thick cuticle prevents many of the other organisms in the soil or water from penetrating the fruit. *Phytophthora* zoospores are, however, able to infect the pears (K. Hayden, RBGE, pers. com.).

Both the soil and water samples had a firm pear added as bait. The pears were gently washed with detergent and allowed to dry, or were lightly wiped with 70% ethanol, before use, to reduce any surface organisms. The baited samples were then incubated within closed plastic boxes to reduce the risk of mites getting out of the samples. Prior to use the pears have any pre-exiting blemishes circled with an indelible pen (Fig.11). This allows new blemishes caused through contact with the soil or water sample to be quickly identified.

Figure 11. Pear bait showing marking of pre-existing blemishes.

Both Packham and Forelle pears, due to availability, were used during the sampling. The Packham pears were generally more suited to the immersion in water since they tended to sit square in the bag, having a recessed calyx attachment, giving a flattened base to the fruit. The Forelle pears had a very rounded base, so it was more difficult to keep the pear sitting upright with the stalk clear of the water surface

With each baiting exercise several pears from each batch, all identically treated, were incubated in sterile distilled water under the same conditions as the soil and water samples, as a control measure. The use of pears as bait for *Phytophthora* spp. is well documented (Yamak *et al.* 2002; Gevens *et al.* 2007; Wang *et al.* 2009).

Sampling - soil

A transect band was run between the southeast and northwest corners of the Edinburgh gardens where historic *Phytophthora* infections were known.

The transect length is just under 700 metres, and soil samples were taken at approximately 25 metre intervals to give 28 samples (Fig. C12, p.26). Where it was impossible to sample at the pre-determined point, e.g. bare rock, tarmac, or potential injury to plants, then the sample was

taken from the nearest free soil. GPS coordinates for all samples were taken with a Garmin GPSMAP64 set to British Grid using the Ordnance Survey GB datum which ties in with the coordinates system for the GIS mapping used by RBGE.

Best practices aiming to minimise any risk of damage to plants, transfer of pathogens between locations, or cross contamination were followed. The details for each sample point are shown (Table 3). Full details of the plant communities, including the taxonomic authority, around each sampling point are presented in Appendix One.

Approximately 250g of each of the soil samples was placed within its own strong plastic bag, coded with the sample number, with 300ml of distilled water added to the soil. A cleaned pear was then carefully placed within the bag. The bags were incubated within the laboratory.

The pears were examined after two days. If there were any new lesions the pear was removed from the sample and lightly wiped down with 70% ethanol to remove any surface organisms. A nursery tag with the sample code was attached to the stalk of the pear and photographs taken to show the developments whilst it was kept within the sample. The pear was then further incubated in a separate box. Paper tissue was used to keep the pears apart from one another. Each day the isolated pears were checked and photographs of lesion progress taken.

Table 3. Soil sampling points on transect line between SE and NW corners of RBGE Edinburgh gardens. Soil samples were collected on Monday 22nd May 2017. The conditions were damp, still, light rain and 13°C in the morning. By lunchtime it had dried and become warmer.

Sample	**Coordinates**	**Nearest plants**	**Accession**
001	NT24990 75218	Small dead tree	
002	NT24953 75225	*Heracleum sphondylium*, *Iris*, 3m from *Juniperus*	
003	NT24936 75227	*Betula*, *Prunus* (possible fungal infection)	
004	NT 24910 75237	*Pinus*	
005	NT24896 75256	*Vaccinium uliginosum*	2007.1246 A
006	NT24873 75262	*Helianthemum* 'Rhodanthe Carneum'	1969.4691 C
007	NT24847 75260	Ericaceae	
008	NT24813 75277	*Thuja plicata*	1969.9317 A
009	NT24794 75279	*Pinus peuce*	1925.1014 A
010	NT24756 75292	*Rhododendron*	
011	NT24725 75294	*Betula papyrifera*	1963.3188 A

012	NT24702 75303	*Pieris formosa*	2003.1602 A
013	NT2468675309	*Rubus foliaceistipulatus*	2005.1835 A
014	NT4668 75309	*Fraxinus sogdiana*	1947.0116 A
015	NT24646 75329	*Ilex* (dying)	
016	NT24623 75330	*Rhododendron wallichii*	1981.3602 E
017	NT24582 75344	*Ilex*	
018	NT24581 75337	*Buxus sempervirens*	1975.4038 A
019	NT24525 75371	*Paeonia anomala* var. *intermedia*	1957.0170 A
020	NT24510 75374	*Rhododendron thomsonii* ssp. *thomsonii*	1937.0196 C
021	NT24480 75387	*Quercus* x *rosacea*	1969.8322 A
022	NT24462 75372	*Magnolia stellata*	2006.1599 C
023	NT24441 75397	*Quercus frainetto*	1969.9293 A
024	NT24418 75404	*Nothofagus pumilo*	1967.1402 A
025	NT24395 75411	*Berberis soulieana*	
026	NT24372 75419	*Euonymus sieboldianus*	2006.2309 A
027	NT24349 75426	*Castanea dentata*	1940.1006 A
028	NT24326 75434	*Taxus* and *Ilex* hedges	

When lesions had properly developed the pear was removed to a freshly cleaned laminar flow cabinet. Small sections of the pear skin at the edge of the lesions are removed using a flame sterilised scalpel, and then transposed onto a plate of V8 PARP agar. Six sections of skin were plated in one dish, with adequate space between sections. The plate was coded with the sample number and then sealed with Parafilm. The plates were incubated upside down in the laboratory, within double sealed plastic bags, for the first 24 hours. They were then permanently removed to the Pathology Lab.

Sampling – symptomatic plants

Plants were identified that showed symptoms of infection, potentially with *Phytophthora*. These plants were photographed, GPS coordinates logged (Table 4 & Fig. 12), and tissue samples from diseased areas were taken. Latex gloves were worn, and liberal use of an ethanol spray was used on both gloves and feet. Samples were collected into new medium sized Ziploc bags.

Due to the very high temperatures on the day that the collections were made, samples were not made from members of the Cupressaceae. Isolation of *P. austrocedri* from plant tissue has been observed to fail at temperatures above 16°C (S. Green, pers. comm.). All the sealed bags containing plant samples that had been collected were immediately placed in a cool box containing a large, double-bagged, quantity of crushed ice to keep them cool.

With one bush, *Chrysolepis chrysophylla* 'Obovata' (Fagaceae), clearly almost completely dead, trunk scrapings were taken from the area showing a demarcation between dead and still live wood. This was the only specimen where any physical damage was made to the plant. In all other cases only leaves or twigs were removed. One tree, *Betula papyrifera* (Betulaceae), was on the transect line and clearly diseased – almost certainly with *Phytophthora* judging by the extensive black bleeding at the base of the trunk – but was left un-sampled since it was not appropriate to risk damaging the tree. A soil sample from the root area had, however, been taken earlier.

Details of the taxonomic authorities for all the symptomatic plants sampled are given in Appendix Two.

Table 4. Symptomatic plant sampling points close to transect line between SE and NW corners of RBGE Edinburgh gardens. Samples were collected on Thursday 25th May 2017. The conditions were extremely hot and sunny. An ice box was used to keep the samples cool.

Code	Accession	GPS	Species	Description
201	1975.4074E	NT24788 75300	*Rhododendron meddianum* var. *atrokermesinum*	Dead twigs mottled. Buds aborted. Leaf dying from tip backwards towards reddened petiole
202	1975.4074B	NT24788 75300	*Rhododendron meddianum* var. *atrokermesinum*	Dead buds; cigar tube brown dry leaves. Mottled trigs
203	1969.3777A	NT24868 75194	*Chrysolepis chrysophylla* 'Obovata'	Shrub; completely dead. Leaves all dried and brown. Bark scrapings taken from base of shrub where dead wood and live wood meet.
204		NT24967 75211	*Ilex* spp.	Tree trunk bleeding. Leaves seem to go darkened, almost necrotic. Splits and lesions on twigs
205		NT24972 75221	*Ilex aquifolium* sapling	Possible mycelial growth around twigs where split. Leaves blackening from margins inwards
206		NT24853 75221	Ericaceae	Dried leaves brown/reddish; aborted fruits; dead twigs. Some mottling on twigs
207	1974.4227A	NT24811 75308	*Rhododendron* aff. *arboreum* hybrid	Leaves dying, curling inwards. Mottling on twigs.
208	2005.1828A	NT24682 75337	*Rubus* spp.	Many leaves yellow with red/black mottling; sometimes only on part of leaf,

				with remainder green. Some pronounced black scab-like bits near petiole
209	1981.3602D	NT24625 75332	*Rhododendron wallichii*	Dead buds and dead twigs. Mottling and some black spots on twigs. Leaves with some blotchy, particularly on underside
210	1932.0188E	NT24609 75301	*Rhododendron argyrophyllum* ssp. *nankingense*	Quite large dead part with twigs, leaves & buds all dead. Green leaves showing some peripheral mottling around leaf tips and margins. Many leaves brown, dry and in-curled like a cigar.
211	1980.1760B	NT24572 75353	*Trochodendron aralioides*	Dying leaves very red. Black spots on twigs
212	1968.7718A	NT24536 75385	*Kamia latifolia*	Leaves appear to be dying from tips, brown to reddish margin, Twigs mottled with whitish colour.
213	1987.0444B	NT24497 75371	*Rhododendron thomsonii* hybrid	Small black spots on twigs on greyish background. Some leaves quite mottled – tending to yellow with brown areas/ dark perimeter, reddish petioles. Some buds dead
214	1968.7759A	NT24459 75370	*Lithocarpus densiflorus*	Some leaf tips browned with distinct reddish margin line and further discolouration within brown area. Other leaves mottled brown but still with narrow green margin around midrib.
215	2011.1933A	NT24461 75371	*Quercus coccifera*	Leaves seem very small; some brown and dead. Dead twigs appear to have lesions. Soil sample needed.
216	2005.2392C	NT24448 75393	*Quercus pyrenaica*	Many leaves with numerous brown blotches often over whole leaf surface. Blotches showing clear delineation between dark perimeter and lighter interior; small dark spots on many leaves. In some leaves, there is pronounced discolouration at point where petiole meets the leaf lamina.
217	2006.1572F	NT24359 75403	*Trochodendron aralioides*	Many leaves showing distinct red discolouration, almost pixelated in places. Some insect damage nibbled around margins. Leaves ultimately dying to yellow/brown.
218	2009.0006C	NT24357 75434	*Polystichum acrostichoides*	Pinnae browning from margin inwards and dying. Rachis dark brown underside but greenish above. Base of rachis seems to have distinct lesions.

Sampling - water

The third strand involved water samples from various water courses and bodies of water within the gardens. *Phytophthora* needs free water for the dispersal of its motile zoospores As a result sampling the open water courses was essential. In order to ensure safe access to the various water courses a simple home-made sampling system was fabricated using a walking pole, a wide tin can, wire and cable ties (Fig. 13).

Figure 13. Water sampling device allowing easy and safe access to water courses.

This allowed good access to the water even when access would have been otherwise difficult. The can and pole were dried with paper tissue after each "dipping" and then sprayed with 70% ethanol. Latex gloves were also used to protect against contamination, and possible leptospirosis. Approximately 300ml samples were taken, and the coordinates for the point of sampling logged (Fig. 12 & Table 5).

Table 5. Water course sampling points within RBGE Edinburgh gardens. Samples were collected on Thursday 1st June 2017. The conditions were dry, breezy and 17°C.

Sample	GPS	Location	Notes
101	NT24405 75517	Pond near Botanics Cottage	Mostly *Alnus*
102	NT24402 75515	Pond near Botanics Cottage	Mostly *Alnus*
103	NT24405 75519	Pond near Botanics Cottage	Mostly *Alnus*
104	NT24439 75239	John Hope Gateway, lower pool	*Gunnera*
105	NT24450 75256	John Hope Gateway, lower pool	*Gunnera*
106	NT24451 75261	John Hope Gateway, upper pool	
107	NT24616 75253	Chinese Garden, upstream of top bridge	
108	NT24621 75220	Chinese Garden, bottom pond	
109	NT24947 75216	Memorial Pond	*Alnus, Betula, Pinus*
110	NT24949 75222	Memorial Pond	*Alnus, Betula, Pinus*
111	NT24943 75228	Memorial Pond	*Alnus, Betula, Pinus*
112	NT24874 75208	Rockery, just downstream of top bridge	Mostly Cupressaceae
113	NT24867 75226	Rockery, just upstream of middle bridge	Mostly Cupressaceae
114	NT24855 75246	Rockery, pond just upstream of Caledonian Hall bridge	Mostly Cupressaceae
115	NT24860 75277	Downstream of Caledonian Hall bridge	

Growth media

Buffered and clarified V8 juice was prepared separately by adding 1g calcium carbonate per 100mls of V8 juice, mixing thoroughly, and then running the mixture in a centrifuge for 10 minutes at 7,200rpm. The resulting supernatant was decanted in 25ml portions in aliquots and stored at -20°C for usage as required.

To prepare V8 PARP agar plates two one litre media bottles were each filled with 25ml V8 juice, buffered and clarified, 7.5g agar and 475ml distilled water. A magnetic stir bar was added, very loosely attaching a cap, and then the bottles were autoclaved using the Media 121:15 cycle in an LTE Touchclave-R Autoclave. After sterilisation, the caps of the media bottles were tightened to seal, and then placed in a water bath set at 60°C to cool. When the media has cooled the still hot bottles were carefully transferred to a laminar flow cabinet using protective gloves, and placed on a heated (55°C) stirring machine. After mixing, the following antibiotics were added to the agar:

- 0.4ml Pimaricin 2.5% aqueous solution
- 0.25g Ampicillin
- 5ml Pentachloronitrobenzene stock 1g PCNB in 200ml 100% ethanol)
- 1.5ml Rifamycin 2%

Mixing, using the magnetic stir bar, was continued for a few minutes to ensure even dispersal of the antibiotics throughout the medium; and then plates were poured. Once fully set the plates were bagged, and stored in the dark (wrapped in tin foil) at 4°C. All protocols are as described (Jeffers & Martin 1986; Ferguson & Jeffers 1999).

The selective PARP medium is used to try to prevent other organisms, particularly bacteria, other fungi, and *Pythium* from overwhelming any *Phytophthora*. Subsequently, selected hyphae are transferred to a growth medium of 10% V8 agar (V8A).

The procedure for V8A is almost identical to that used for the V8-PARP media, except in each litre media bottle the V8 juice volume is doubled, the distilled water reduced by 25ml, and that no antibiotics are added. The concentration of V8 is thus increased to 10% to allow nutrients for growth.

Culturing process

All agar plates were seated with Parafilm, once they had been inoculated, in order to prevent the plates drying out, and also to seal the plates against possible cross contamination or fungus mites. A consequential benefit of this was that if a plate was accidentally knocked over there was no bio-hazard. The plates were generally stored upside down so that any evaporation gathered on the lid rather than dropping onto the culture. Once finished with all plates were autoclaved.

The V8 PARP agar plates, whether from tissue, soil or water sampling, were examined daily using a dissecting microscope within the quarantine area. When hyphae were observed within the agar, the areas were marked on the underside of the plate with an indelible pen. Ideally young hyphal tips were selected since these were less likely to be cross contaminated.

The selected areas were aseptically transferred to V8A plates, coded as a sub-set of the original PARP codes. After sub-culturing, the plates were sealed with Parafilm and stored upside down in the 18°C incubator. After ten to twelve day's growth the V8A plates were ready for harvesting.

Time constraints meant that no attempt was made to culture the isolates on alternative media, such as pea broth, carrot, cornmeal, potato dextrose or malt extract agar.

Incubation issues

An electro-mechanical fault with the climate control system in the laboratory resulted in the temperature dropping to 11°C. The climate control system was shut down, and ambient temperatures levelled to between 20° and 25°C (Fig. 14).

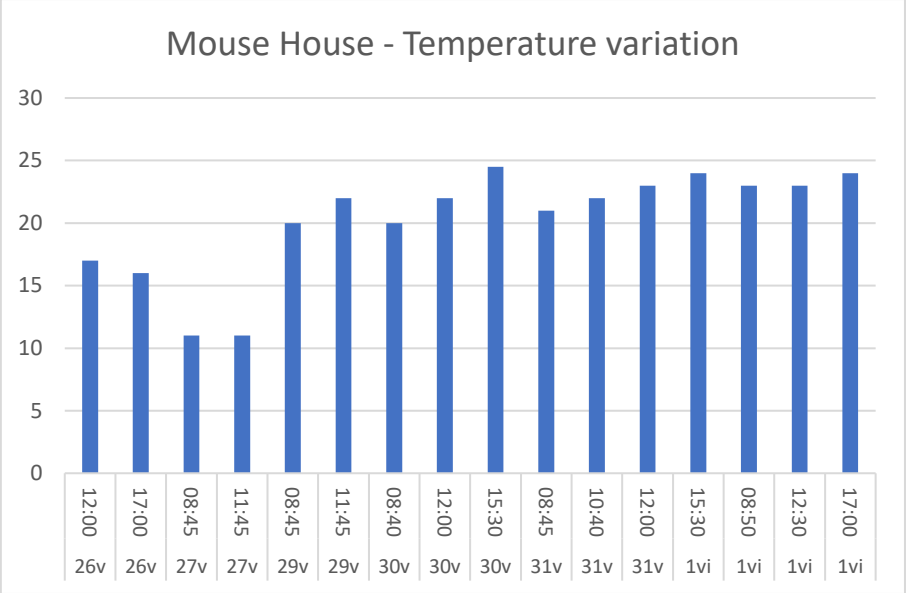

Figure 14. Graph showing temperature at various points during period 26th May 2017 to 1st June 2017. There are no readings for night-time temperatures, but it is expected that these will fall a little below the first morning temperature recorded.

The lack of climate control within the quarantine zone meant that ambient temperatures were variable, and generally high. It seems very likely that the resulting incubation temperatures were too hot for many of the plates, and prevented growth of possible *Phytophthora* inoculum.

Harvesting hyphae

The recommended procedure for harvesting hyphae from the V8A isolate plates involved the collection of aerial hyphae on sterile cocktail sticks, transfer to 100µl sterile water in a 1.5ml format tube using a scrubbing action, capped, and then denaturing by heat. Two samples were taken from each isolate plate. A chart was maintained so that each coded tube in the 96-well block was immediately related to a specific V8A plate. The tubes were stored at -20°C.

PCR

PCR tubes were set up with full duplicate sets for the hyphal extractions. One set would use *Phytophthora* specific primers, and the other universal ITS primers (Fig. 15). Assuming all hyphal extraction samples contain DNA, then all should give a positive result for the ITS primers, but only those hyphae from *Phytophthora* should give a result with the *Phytophthora*

specific primers. This allows for a positive identification of any samples where the hyphae were of *Phytophthora* origin.

18Ph2F^{-1}	GGATAGACTGTTGCAATTTTCAGT
5.8s-1R^{-1}	GCARRGACTTTCGTCCCYR
ITS3^{-2}	GCATCGATGAAGAACGCAGC
ITS4^{-2}	TCCTCCGCTTATTGATATGC

Figure 15. Primers used for PCR. (1) (Scibetta *et al.* 2012) (2) (White *et al.* 1990)

The PCR tubes are built in a laminar flow cabinet in the low DNA laboratory, so that the risk of cross contamination from post PCR products, which would compromise the diagnostics, would be minimised. No PCR products are allowed in the low DNA room, and all equipment is wiped down with a 5 to 10% solution of bleach to degrade any remnant DNA in the environment.

When each row of tubes is completed a strip of caps is put onto the row to seal them. A procedure was established where the end of the caps strip with a perforation was always positioned at tube 1 in the strip of tubes. This would help to minimise the cross infection risk of putting the caps back onto the tubes the wrong way round.

PCR was performed in 20µl reactions using 1x reaction buffers, 2mM dNTPs, primers and 2 µl of template. Controls were set up for each PCR reaction. One set had the water used for the hyphal extraction, another set for the water used to make up the stock PCR solutions; and a set for the positive control, *Phytophthora austrocedri* isolate GA3 (B. Henricot, Forest Research), diluted to 1ng/µL.

The PCR tubes were processed in BioRad 100 PCR machines using the following cycling conditions.

Table 6. Protocol for *Phytophthora* specific primers with DNA from Oomycete hyphal extractions. ** For PHYT61 the temperature at stage 3 is 61°C, but for PHYT58 the temperature is 58°C

Lid 105°C Volume: 20µl	
1	94°C for three minutes
2	95°C for 30 seconds
3**	61°C for 30 seconds
4	72°C for one minute
5	GOTO step 2, 39x
6	75°C for five minutes
7	10°C, ∞

Gel-electrophoresis was run for the PCR products using 1% agarose gels, SYBR safe[1] DNA gel stain, and 1kB plus ladder. A Syngene G: BOX F3 Fluorescence Imaging System was used to view the gels.

Purification

Prior to purification and sequencing, morphologically distinctive isolates were selected from the plates which had given a positive result with the *Phytophthora* specific primers. The selection was made generously, given the aim was to allow the full diversity of *Phytophthora* in the study to be sequenced.

PCR product from the selected isolates was treated with ExoSAP-IT (Bell 2008). This works using the enzyme Exonuclease I to degrade residual single-stranded primers and any extraneous single stranded DNA products produced by the PCR, then Shrimp Alkaline Phosphatase to hydrolyse any remaining dNTPs from the PCR mixture.

2µl of ExoSAP IT was aliquoted into numbered tubes, then 5µL of defrosted PCR product was added to a mapped tube. The tubes were then processed in a BioRad 100 PCR machine. The ExoSAP protocol was run which involves incubation at 37°C for 15 minutes followed by heating to 80°C for 15 minutes to inactivate the enzyme.

The BigDye reaction was then set up, running forward (18Pf2F) and reverse (5.8s-1R) primers on replicate tubes for each selected, and purified, product. The composition for a single reaction was 4.5µl deionised water, 0.5 µl BigDye, 2 µl of 5x sequencing buffer and 0.32 µl 10µM of the relevant primer. 7.64µl of the reaction was aliquoted into the relevant set of numbered tubes and then 1µl of purified PCR product added to the relevant tubes in both sets.

The PCR tubes are then put into a BioRad 100 PCR machine and are run through the BIGDYE protocol which runs 25 cycles of 95°C for 30 seconds, 50°C for 20 seconds and 60°C for 4 minutes, before reverting to 4°C forever at the end of the process. After the protocol had finished the tubes were moved to the sequencing freezer for collection. The relevant EDNA paperwork was completed and submitted.

[1] SYBER safe DNA gel stain is harmful. Blue nitrile gloves must be used when handling the chemical. Toxic waste bins must be used for disposing of the pipette tips used.

A similar process was subsequently run using the ITS primer PCR products for selected probable *Phytophthora* extractions. This allowed sequencing the ITS2 region.

Species characterisation

Nine isolates were chosen as typical of the groupings found within the "*Phytophthora*" collection of positive samples.

As part of the species characterisation, additional to the morphological detailing, a series of new isolates of the nine selected isolates were sub-cultured onto 10% V8A, but with a piece of autoclaved Watermans No. 1 filter paper laid on top of the agar. The hyphae grow through the filter paper which can then be removed and dried in a container with silica gel giving a source of pure hyphae for high quality DNA extraction.

A temperature-growth rate study was established. A series of 2mm plugs were plated onto fresh 10% V8A plates. The diameter of the plates was 45mm, so a radial growth of 21.5mm would fill the plate, assuming the original inoculum was central. A set of twelve replicates of each sample were made. These were then separated into four batches of three plates for each sample for incubation at 12°C, 16°C, 18°C and 30°C. The plates were examined after three days (14vii17) and a line drawn around the extent of growth. Similarly, after a further two days (16vii17), and four days (18vii17), or until the plate was filled.

At the end of the study an acetate with concentric measuring circles was placed over the growth with the centre over the original inoculum (Fig. C20), and an image of the plate was recorded using a Logitech HD web-cam. This allows the growth for each period to be measured, and the daily growth rate can be averaged.

A zoospore release study was undertaken with the nine selected samples. Agar plugs containing the isolates were incubated for three days whilst submerged in sterile soil extract to the point where the surface of the plug was just covered. The chamber holding the plugs was then placed in a refrigerator at 4°C for 30 minutes and then incubated at 18°C for two hours to stimulate zoospore production (Chapman & Vujicic 1965; Pfender *et al.* 1977).

No pathogenicity tests were conducted with the cultures since Koch's postulates were not being explored as part of the study.

Statistical analysis

It had been noticed that the isolates from soil sample 026 looked remarkably similar but were sparse in morphological detail. They had also grouped closely in the phylogenetic tree. As a means of ascertaining the degree of similarity between the MG026A samples sporangia measurements were recorded. A one-way analysis of variance with Tukey's post hoc testing was used to determine significant differences ($p<0.05$) in length and width between isolates from sample 026. Statistical analysis was undertaken in R (R Development Core Team 2008). The data was checked for normal distribution.

Phylogenetics

Data on sequences from the selected PCR outcomes that had been purified and sent for sequencing were assembled in Sequencher 5.1 Build 1067 ("Sequencher 5.4.6 DNA sequence analysis software"). Once the sequences were assembled they were run against three web resources to get indications of identity. NCBI Blast[2], Phytophthora-ID[3] and PhytophthoraDB[4] were used to see what identification consensus was achieved.

ITS sequences for a number of *Phytophthora* species were downloaded from the PhytophthoraDB website (Ivors *et al.* 2007). The intention was to have at least one species from each of the ten recognised clades. An ITS sequence for *Pythium zingiberum*, for use as part of an outgroup, was downloaded from Genbank (Benson *et al.* 2005). All downloaded sequences were taken as described on the web site (Table 7).

Table 7. Details of all downloaded sequences for *Phytophthora* and *Pythium* species for building a supporting phylogenetic tree to assist with sample identification and positioning.

Source	Identification code	Taxon	Downloaded
PhytophthoraDB	PD_00050_ITS	*P. alni* subsp. *alni*	05vii17
PhytophthoraDB	PD_00908_ITS	*P. alni* subsp. *alni*	05vii17
PhytophthoraDB	PD_02801_ITS	*P. alni* subsp. *multiformis*	05vii17
PhytophthoraDB	PD_02131_ITS	*P. alni* subsp. *uniformis*	05vii17
PhytophthoraDB	PD_01849_ITS	*P. asparagi*	13vii17
PhytophthoraDB	PD_01281_ITS	*P. asparagi*	13vii17
PhytophthoraDB	PD_01648_ITS	*P. austrocedri*	05vii17

[2] https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch
[3] http://phytophthora-id.org/seq-id.html
[4] http://www.phytophthoradb.org/blast.php

PhytophthoraDB	PD_01657_ITS	*P. austrocedri*	05vii17
PhytophthoraDB	PD_01665_ITS	*P. boehmeriae*	13vii17
PhytophthoraDB	PD_01666_ITS	*P. boehmeriae*	13vii17
PhytophthoraDB	PD_00347_ITS	*P. cactorum*	05vii17
PhytophthoraDB	PD_00410_ITS	*P. cactorum*	05vii17
PhytophthoraDB	PD_00425_ITS	*P. cactorum*	05vii17
PhytophthoraDB	PD_00565_ITS	*P. cactorum*	05vii17
PhytophthoraDB	PD_00950_ITS	*P. cactorum*	05vii17
PhytophthoraDB	PD_00959_ITS	*P. cactorum*	05vii17
PhytophthoraDB	PD_00994_ITS	*P. cactorum*	05vii17
PhytophthoraDB	PD_00994_ITS_2	*P. cactorum*	05vii17
PhytophthoraDB	PD_02150_ITS	*P. cactorum*	05vii17
PhytophthoraDB	PD_02154_ITS	*P. cactorum*	05vii17
PhytophthoraDB	PD_00691_ITS	*P. cambivora*	05vii17
PhytophthoraDB	PD_00708_ITS	*P. cambivora*	05vii17
PhytophthoraDB	PD_00767_ITS	*P. cambivora*	05vii17
PhytophthoraDB	PD_02161_ITS	*P. cambivora*	05vii17
PhytophthoraDB	PD_02163_ITS	*P. cambivora*	05vii17
PhytophthoraDB	PD_00442_ITS	*P. capsici*	13vii17
PhytophthoraDB	PD_00443_ITS	*P. capsici*	13vii17
PhytophthoraDB	PD_00674_ITS	*P. capsici*	13vii17
PhytophthoraDB	PD_01975_ITS	*P. capsici*	13vii17
PhytophthoraDB	PD_00713_ITS	*P. cinnamomi*	13vii17
PhytophthoraDB	PD_00718_ITS	*P. cinnamomi*	13vii17
PhytophthoraDB	PD_00982_ITS	*P. cinnamomi*	13vii17
PhytophthoraDB	PD_00233_ITS	*P. citricola*	05vii17
PhytophthoraDB	PD_00615_ITS	*P. citricola*	05vii17
PhytophthoraDB	PD_01136_ITS	*P. citricola*	05vii17
PhytophthoraDB	PD_01862_ITS	*P. citricola*	05vii17
PhytophthoraDB	PD_02185_ITS	*P. citricola*	05vii17
PhytophthoraDB	PD_00524_ITS	*P. citrophthora*	13vii17
PhytophthoraDB	PD_01704_ITS	*P. citrophthora*	13vii17
PhytophthoraDB	PD_01705_ITS	*P. citrophthora*	13vii17
PhytophthoraDB	PD_00300_ITS	*P. colocasiae*	13vii17
PhytophthoraDB	PD_00314_ITS	*P. colocasiae*	13vii17
PhytophthoraDB	PD_00338-ITS	*P. colocasiae*	13vii17
PhytophthoraDB	PD_00511_ITS	*P. cryptogea*	14vii17
PhytophthoraDB	PD_01764_ITS	*P. cryptogea*	14vii17

PhytophthoraDB	PD_01725_ITS	*P. cryptogea*	14vii17
PhytophthoraDB	PD_00498_ITS	*P. drechsleri*	14vii17
PhytophthoraDB	PD_00469_ITS	*P. drechsleri*	14vii17
PhytophthoraDB	PD_00441_ITS	*P. drechsleri*	14vii17
PhytophthoraDB	PD_00030_ITS	*P. erythroseptica*	14vii17
PhytophthoraDB	PD_00014_ITS	*P. erythroseptica*	14vii17
PhytophthoraDB	PD_00036_ITS	*P. erythroseptica*	14vii17
PhytophthoraDB	PD_00082_ITS	*P. europaea*	05vii17
PhytophthoraDB	PD_00084_ITS	*P. europaea*	05vii17
PhytophthoraDB	PD_00914_ITS	*P. fragariae*	05vii17
PhytophthoraDB	PD_00922_ITS	*P. fragariae*	05vii17
PhytophthoraDB	PD_02042_ITS	*P. fragariae*	05vii17
PhytophthoraDB	PD_02776_ITS	*P. gallica*	05vii17
PhytophthoraDB	PD_00481_ITS	*P. gonapodyides*	05vii17
PhytophthoraDB	PD_00783_ITS	*P. gonapodyides*	05vii17
PhytophthoraDB	PD_01776_ITS	*P. gonapodyides*	05vii17
PhytophthoraDB	PD_02276_ITS	*P. gonapodyides*	05vii17
PhytophthoraDB	PD_00969_ITS	*P. heveae*	13vii17
PhytophthoraDB	PD_00971_ITS	*P. heveae*	13vii17
PhytophthoraDB	PD_02777_ITS	*P. hydropathica*	13vii17
PhytophthoraDB	PD_00133_ITS	*P. ilicis*	05vii17
PhytophthoraDB	PD_02053_ITS	*P. ilicis*	05vii17
PhytophthoraDB	PD_00799_ITS	*P. infestans*	13vii17
PhytophthoraDB	PD_00870_ITS	*P. infestans*	13vii17
PhytophthoraDB	PD_02365_ITS	*P. infestans*	13vii17
PhytophthoraDB	PD_00145_ITS	*P. inundata*	13vii17
PhytophthoraDB	PD_02778_ITS	*P. irrigata*	13vii17
PhytophthoraDB	PD_00128_ITS	*P. katsurae*	13vii17
PhytophthoraDB	PD_02400_ITS	*P. katsurae*	13vii17
PhytophthoraDB	PD_00105_ITS	*P. kernoviae*	05vii17
PhytophthoraDB	PD_00164_ITS	*P. kernoviae*	05vii17
Genbank	JQ582465.1	*P. lateralis*	15vii17
PhytophthoraDB	PD_01829_ITS	*P. lateralis*	13vii17
PhytophthoraDB	PD_00147_ITS	*P. megakarya*	13vii17
PhytophthoraDB	PD_01832_ITS	*P. megakarya*	13vii17
PhytophthoraDB	PD_000467_ITS	*P. megasperma*	05vii17
PhytophthoraDB	PD_00704_ITS	*P. megasperma*	05vii17
PhytophthoraDB	PD_02428_ITS	*P. megasperma*	05vii17

PhytophthoraDB	PD_02431_ITS	*P. megasperma*	05vii17
PhytophthoraDB	PD_02432_ITS	*P. megasperma*	05vii17
PhytophthoraDB	PD_00449_ITS	*P. nicotianae*	13vii17
PhytophthoraDB	PD_00581_ITS	*P. nicotianae*	13vii17
PhytophthoraDB	PD_00589_ITS	*P. nicotianae*	13vii17
PhytophthoraDB	PD_00652_ITS	*P. nicotianae*	13vii17
PhytophthoraDB	PD_02100_ITS	*P. pinifolia*	13vii17
PhytophthoraDB	PD_00231_ITS	*P. plurivora*	05vii17
PhytophthoraDB	PD_00232_ITS	*P. plurivora*	05vii17
PhytophthoraDB	PD_00272_ITS	*P. plurivora*	05vii17
PhytophthoraDB	PD_00373_ITS	*P. plurivora*	05vii17
PhytophthoraDB	PD_00395_ITS	*P. plurivora*	05vii17
PhytophthoraDB	PD_00446_ITS	*P. plurivora*	05vii17
PhytophthoraDB	PD_00531_ITS	*P. plurivora*	05vii17
PhytophthoraDB	PD_00533_ITS	*P. plurivora*	05vii17
PhytophthoraDB	PD_01132_ITS	*P. plurivora*	05vii17
PhytophthoraDB	PD_01107_ITS	*P. polonica*	13vii17
PhytophthoraDB	PD_02506_ITS	*P. porri*	13vii17
PhytophthoraDB	PD_02507_ITS	*P. porri*	13vii17
Genbank	KU321521.1	*P. pseudosyringae*	15vii17
Genbank	KP757325.1	*P. pseudosyringae*	15vii17
PhytophthoraDB	PD_0039_ITA	*P. psychrophila*	13vii17
PhytophthoraDB	PD_01143_ITS	*P. quercetorum*	05vii17
PhytophthoraDB	PD_0064_ITS	*P. ramorum*	14vii17
PhytophthoraDB	PD_0065_ITS	*P. ramorum*	14vii17
PhytophthoraDB	PD_01115_ITS	*P. sansomeana*	14vii17
PhytophthoraDB	PD_00764_ITS	*P. syringae*	05vii17
PhytophthoraDB	PD_00788_ITS	*P. syringae*	05vii17
PhytophthoraDB	PD_00044_ITS	*P. syringae*	05vii17
PhytophthoraDB	PD_01865_ITS	*P. syringae*	05vii17
PhytophthoraDB	PD_00317-ITS	*P. tropicalis*	13vii17
PhytophthoraDB	PD_00471_ITS	*P. tropicalis*	13vii17
PhytophthoraDB	PD_00539_ITS	*P. tropicalis*	13vii17
PhytophthoraDB	PD_00540_ITS	*P. tropicalis*	13vii17
Genebank	AJ233465.1	*Pythium zingiberum*	13vii17

The combined sequences (isolates, 43 *Phytophthora* spp., 1 *Pythium* spp.) were then imported into Mesquite Version 3.10 Build 765 (Maddison & Maddison 2017). Alignment was

undertaken using BioEdit Version 7.2.5 (12/11/2013) (Hall 1999), Mesquite and MAFFT (Katoh *et al.* 2005). The alignments were processed using PAUP4* (Swofford 2002) to produce Maximum Parsimony trees with bootstrap values. FigTree (Rambaut 2016) was used for presentation of trees.

Species hypotheses

Preliminary identification hypotheses are postulated from the results of the phylogenetic work. The support for these identification hypotheses will then be judged by comparison to published data for *Phytophthora* spp. using a combination of:

- Morphological measurements and observations;
- Growth rates at set temperatures;
- DNA evidence, including phylogenetic analysis.

Morphological characterisation

The V8A isolates of samples which had shown positive for *Phytophthora* in the gel electrophoresis of PCR products were separated from the other V8A plates and their morphology noted. Any other plates within the general batch of V8A plates that displayed a similar colony morphology, typically roseate or chrysanthemum shaped patterning, were also separated. Examples of such colony morphologies from a published study are shown in Fig. 16.

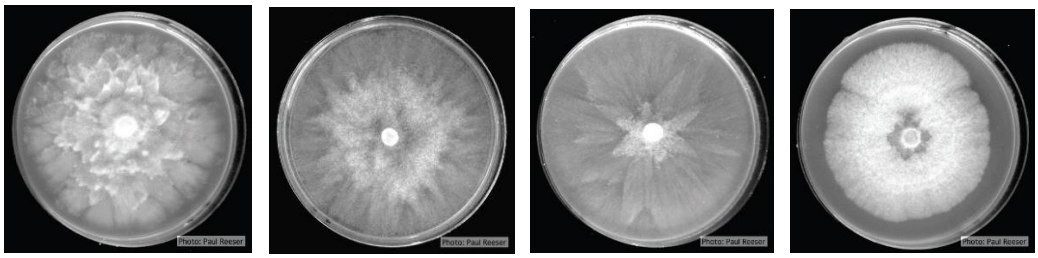

Figure 16. Variety of colony morphologies for *Phytophthora* growing on V8A. From left to right – *P. pseudosyringae, P. cactorum, P. siskiyouensis, P. ramorum* (Parke & Eberhart)

Morphological details of the following characters were then recorded. Generally the terminology used in "Phytophthora Diseases Worldwide" (Erwin & Ribeiro 1996) was used.

- Colony morphology
- Hyphae

- Hyphal swellings
- Chlamydospores
- Oogonia and/or oospores
- Antheridia
- Sporangia

Assuming the isolates were pure, then oogonia, antheridia and oospores would only be seen in homothallic species; that is, species where the mycelium can self-fertilise. In heterothallic species, no sexual organs will be produced unless both mating types for the species make contact, making morphological identification much harder. There are several *Phytophthora* species where sexual organs are either not produced, or they have never been seen.

Data recording

The study always had the potential for a vast proliferation of disparate data. Almost 50 samples were collected and numerous agar plates were cultured. It is critical that at any point in time the progress of a specific culture plate can be confidently reviewed and traced back to an original sample. A Microsoft Access database was devised and built to store the varied range of data that could originate from the project. All tables were normalised and the relationships between tables (Fig. 17) designed to optimise operational requirements.

A copy of the Access database is included on the DVD of supplementary data attached to this thesis.

Figure 17. Table relationships in the bespoke MS Access database

Colour illustrations II

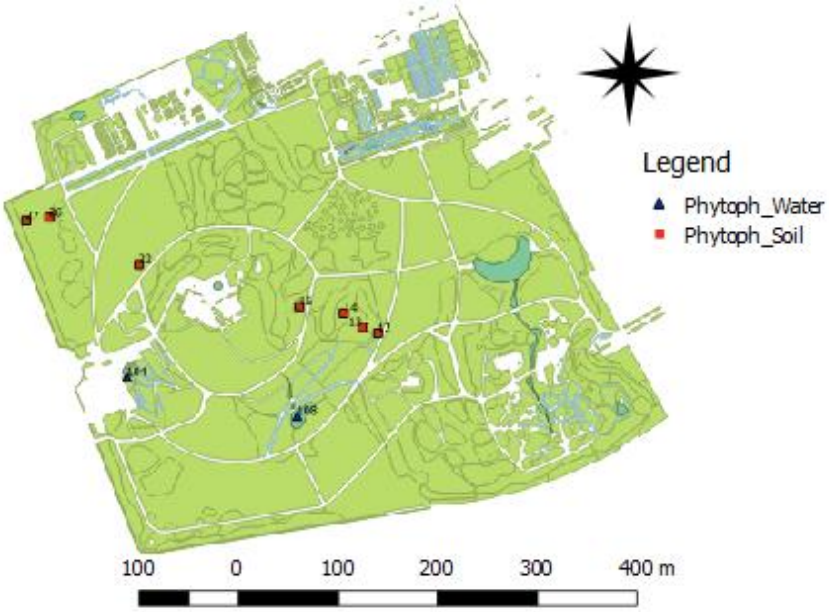

Figure C18. Location within RBGE of all samples that gave a positive result for *Phytophthora*. (Quantum GIS)

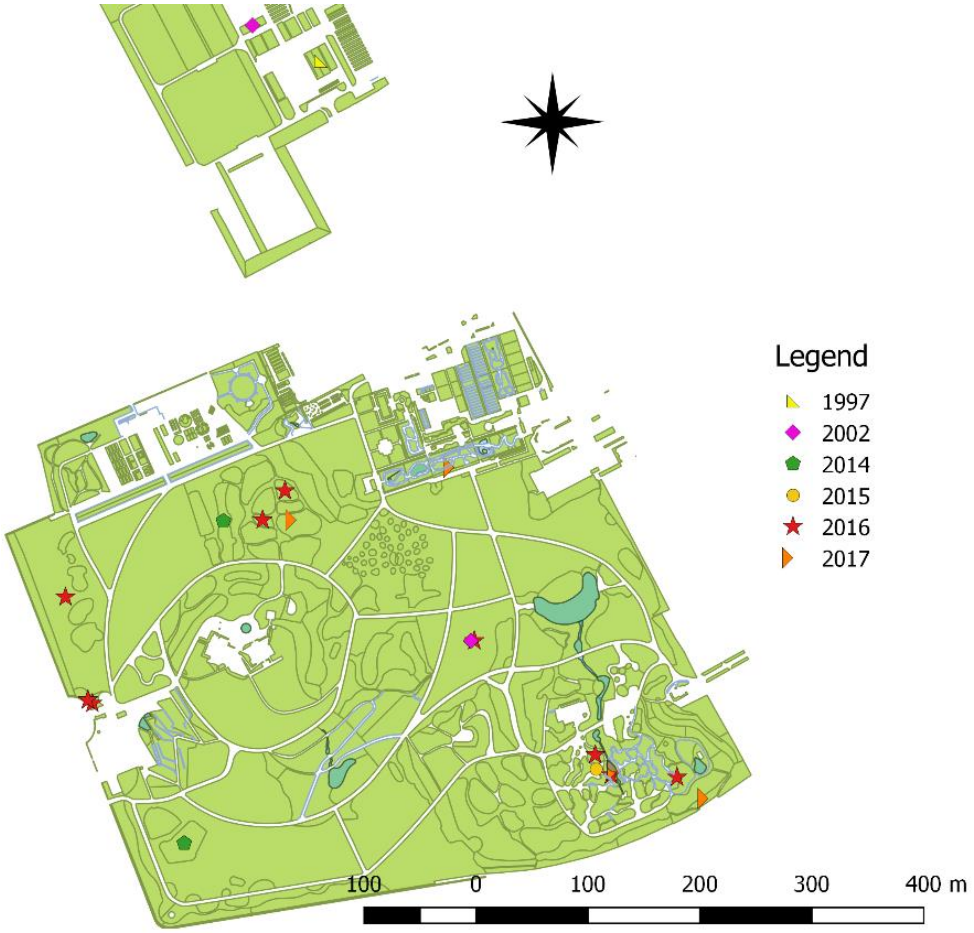

Figure C19. GIS map of RBGE showing the location of historic suspected or confirmed *Phytophthora* infections by year. Positions are estimated on basis of bed locations given in BG-BASE. (Quantum GIS)

Figure C20. One plate from each of the temperature incubations for isolate MG108-2. A was at 12°C, B at 16°C, C at 18°C and D at 30°C. The black line marks the growth at 3 days, the blue line at 5 days and the red line at 7 days.

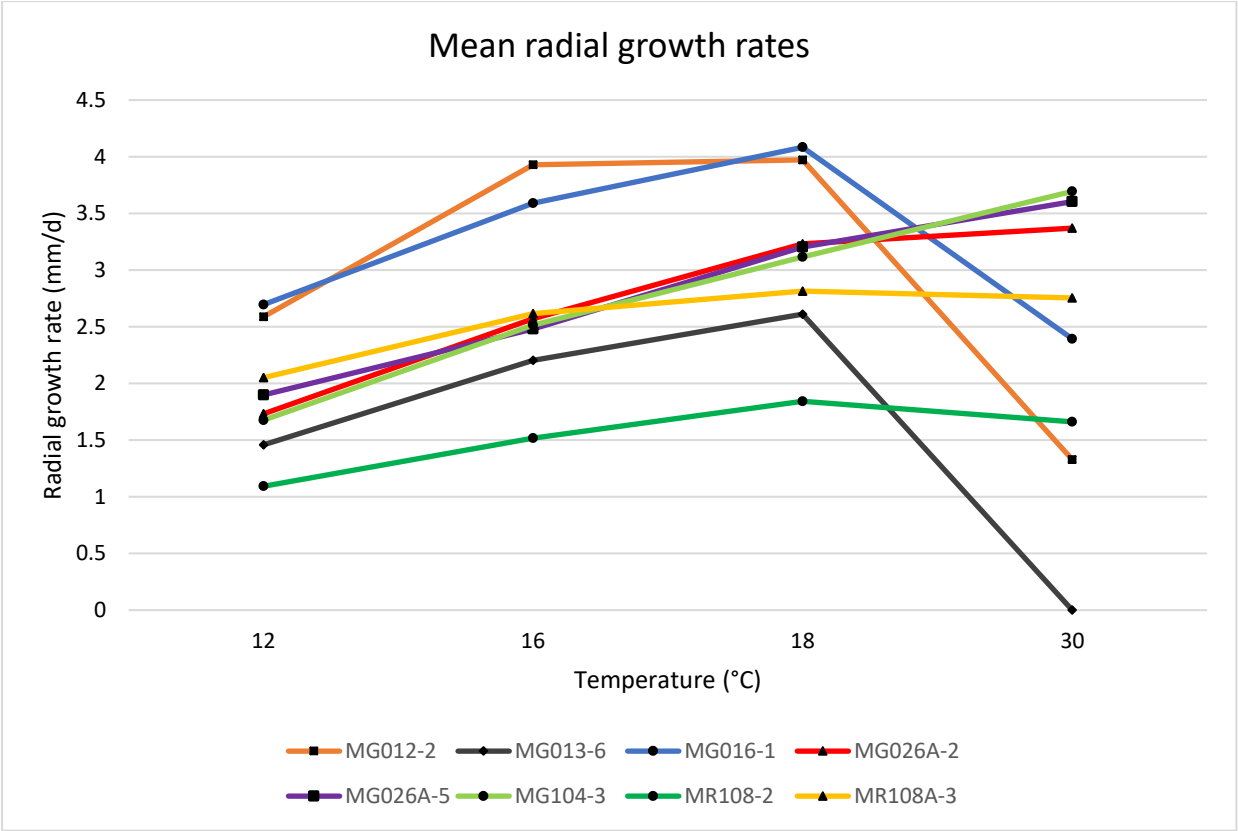

Figure C21. Growth rates for the selected isolates on 10% V8A plates at set incubation temperatures of 12°, 16°, 18 and 30°C. Measurements taken on days three, five and seven. The mean and standard deviation for each set of three isolate replicates per temperature was calculated. A "grand" mean was then calculated to give an averaged growth rate for the isolate at the temperature. The "grand" standard deviation was calculated *in quadrature* but is not shown on the chart for the sake of clarity.

Results

Baits

All three randomly selected control pears from the batch of Packham pears for the soil samples were devoid of any lesions. Control-3 had shown some signs of water splitting. These were cultured on PARP plates, but no signs of fungal growth were observed. This demonstrates that the pears, and the distilled water used to hydrate the soil samples, were generally free from any pre-exiting *Phytophthora* infection.

Two randomly selected control pears for the batch of Forelle pears for the water samples were devoid of any lesions. Both showed some signs of water splitting and were cultured on PARP plates but no signs of fungal growth were observed. This demonstrates that the pears themselves were generally free from any pre-exiting *Phytophthora* infection.

Images of all the baits are included in the DVD of supplementary data which is attached to this thesis.

Sampling

Phytophthora was isolated from seven of the 28 soil samples. Full details of the culturing can be found in Appendix One. None of the tissue samples gave a positive result for *Phytophthora*; very few of the agar plates grew much at all. Culture details are provided in Appendix Two. *Phytophthora* was isolated from two of the 15 water samples. Full details of the culturing can be found in Appendix Three. The distribution of these nine samples across RBGE is shown (Fig. C18, p.47).

The results of the BG-BASE historical *Phytophthora* query show how scattered infections have been (Fig. C19, p.47). There are no species-level identifications available. In the Southeast corner, there is a profound cluster of *Phytophthora* instances. These are all associated with *Juniperus* and occurred since 2015. This probably represents *P. austrocedri* running through susceptible members of the genus. These were not newly established plants. The date of accession ranges from 1932 to 1979, so they were well-established, yet all the plants died between 2015 and 2017.

PCR results

Comparison of the results for each sample with the *Phytophthora* specific and the ITS primers revealed a total of 27 V8A plates gave a positive result for the *Phytophthora* specific primers. In many cases these were replicate plates from the same original lesion on pears and morphological examination suggested they were common species.

All the negative controls showed blank on gel electrophoresis results, other than one in the very first PCR which gave a feint positive for the *Phytophthora* specific primers but a firm negative with the ITS primers. This would appear to have been a slight cross-contamination.

Species characterisation

The measurements from the various incubator growth rate plates were averaged for each isolate across all three plates for each temperature. An example of a set of plates replicated across the four temperatures (Fig. C20, p.48) is shown

At all the tested temperatures, the plates for isolate MG026A-1 were completely covered when they were first reviewed three days after inoculation. As a result, the growth rate can only be considered as greater than 7mm per day since it is impossible to tell precisely when the edge of the plates was reached. The results of the study, excluding isolate MG026A-1 are charted (Fig. C21, p.48).

The only complete failure to grow amongst the samples was with isolate MG013-6 at 30°C, where none of the three plates showed any growth. All other plates showed some result at all temperatures. As might be expected with all isolates the growth rate at 18°C was faster than at 16°C; and the growth rate at 16°C was faster than at 12°C.

However, the behaviour at 30°C was quite different. Isolate MG013-6 failed to grow at all. The growth rate for isolates MG012-2 and MG016-1 dropped dramatically; the growth rates for isolates MG108-2 and MG108A-3 fell back, the former more so than the latter; the growth rate for isolates MG026A-2, MG026A-5 and MG104-3 increased.

The optimum growth temperature, from the incubation temperatures used, was 18°C for all isolates tested except MG026A-2, MG026A-5 and MG104-3. For these isolates the optimal growth was 30°C.

After eight days, there was no sign of any gametangia on the plates used for the MG026A crossing experiment. There was a pronounced difference between the plates which contained the isolate MG026A-3 as one of the crosses. All the crosses between isolates MG026A-2, MG026A-4 and MG026A-5 resulted in one homogenous colony with no visible demarcation between the two original inocula (Fig. 22-R). However, when isolate MG026A-3 was involved in the crosses there was a narrow distinctive demarcation zone between the two colonies (Fig. 22-L). The illustration does not show it well since a low wattage halogen light had to be used to back-light the plate, however the zone can be seen by the light not being obstructed by dense mycelial growth.

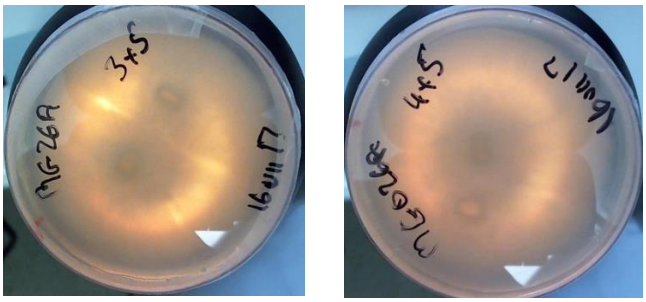

Figure 22. Colony growth on experimental crosses of MG026A isolates. Left, cross involving isolate MG026A-3; Right, cross not involving isolate MG026A-3

Statistical assessment of MG026A sporangia

Sporangia were observed (n=35) on isolates MG026A-2, -4 and -5. There is a strong correlation (r=0.81) between length and width. Data is presented as mean ± standard deviation. The mean dimensions (length x width) for the isolates were 66.34 ± 11.05 x 37.9 ± 3.72µm (MG026A-2), 58.15 ± 11.41 x 34.5 ± 4.47µm (MG026A-4) and 52.44 ± 9.37 x 32.62 ± 5.48µm (MG026A-5). One-way analyses of variance revealed there is a significant difference (p<0.05) between the three isolates for both length and width.

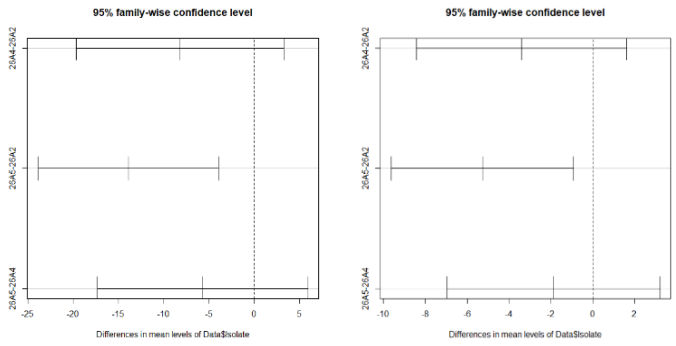

Figure 23. Differences in mean values for sporangium length (left) and sporangium width (right) from Tukey's post hoc analysis of isolates MG026A-2, MG026A-4 and MG026A-5.

Tukey's post hoc analysis (Fig. 23) further determined this difference was between isolates MG026A-2 and MG026A-5 for length (p=0.00645) and for width (p=0.0186).

Phylogenetics

The sequences from the DNA analysis were all tentatively identified using the combination of BLAST, Phytophthora-ID and PhytophthoraDB. The ITS1 sequences from the *Phytophthora* specific primers were very ambiguous, but the results for ITS2, from the universal ITS primers, were more useful, with the first ten results being either one or two species, which narrowed identification down.

The ITS2 sample sequences and the downloaded sequences for known species were aligned and then run together in PAUP*, random stepwise, 1000 replicates, and saving no more than 100 trees with a score >1 for each replicate. 57,300 trees were saved. These trees were processed to give a strict consensus tree (Fig. 24). This allowed more confidence in assessing the relationships between the unknown isolates and the known species. As a result, a more definitive assessment of the species was gained than BLAST allowed.

The bootstrap support values for the strict consensus tree are generally good, but some branch support values are relatively sparse. Additionally, whilst all the clades are represented on the tree, they do not fall in line neatly. This is almost certainly because the downloaded sequences were much larger than the ITS2 sequences for the isolates, and much trimming at the beginning and end of the downloaded sequences was needed so that all the sequences aligned well. There is, however, a reasonable degree of similarity to the *Phytophthora* trees built from multiple gene sequences.

On the basis of the positioning of each of the isolates within the strict consensus tree (Fig. 24) the following groupings were observed:

- Isolates MG012-1, MG014-1 and MG016-1 group with *P. plurivora* T. Jung & T.I. Burgess (Jung & Burgess 2009)
- Isolate MG013-6 groups with *P. ilicis.*
- Isolates MG022B-6 and MG022B-11 group with *P. alni* subsp. *uniformis* Brasier & S.A. Kirk (Brasier *et al.* 2004)

- Isolates MG027A-3, MG027A-6, MG027A-11 and MG027A-4 group with *P. cambivora.*
- Isolate MG104-7 groups with *P. gonapodyides* (Petersen) Buisman (Buisman 1927)
- Isolates MG026A-3, MG026A-4 and MG026A-5 group with *P. gonapodyides*.
    - However, they may be a distinct variant of *P. gonapodyides,* or even a new species.
- Isolates MG108-1 and MG108A-4 group with *P. gallica* T. Jung & J. Nechwatal (Jung & Nechwatal 2008)

The phylogenetic tree below is spread over two pages to allow for better legibility of taxon names. The upper-case letters ‘A’, ‘B’, ‘C’ and ‘D’ show where the upper and lower halves of the tree join. The numerals are the bootstrap values from 10,000 replicates bootstrap in PAUP.

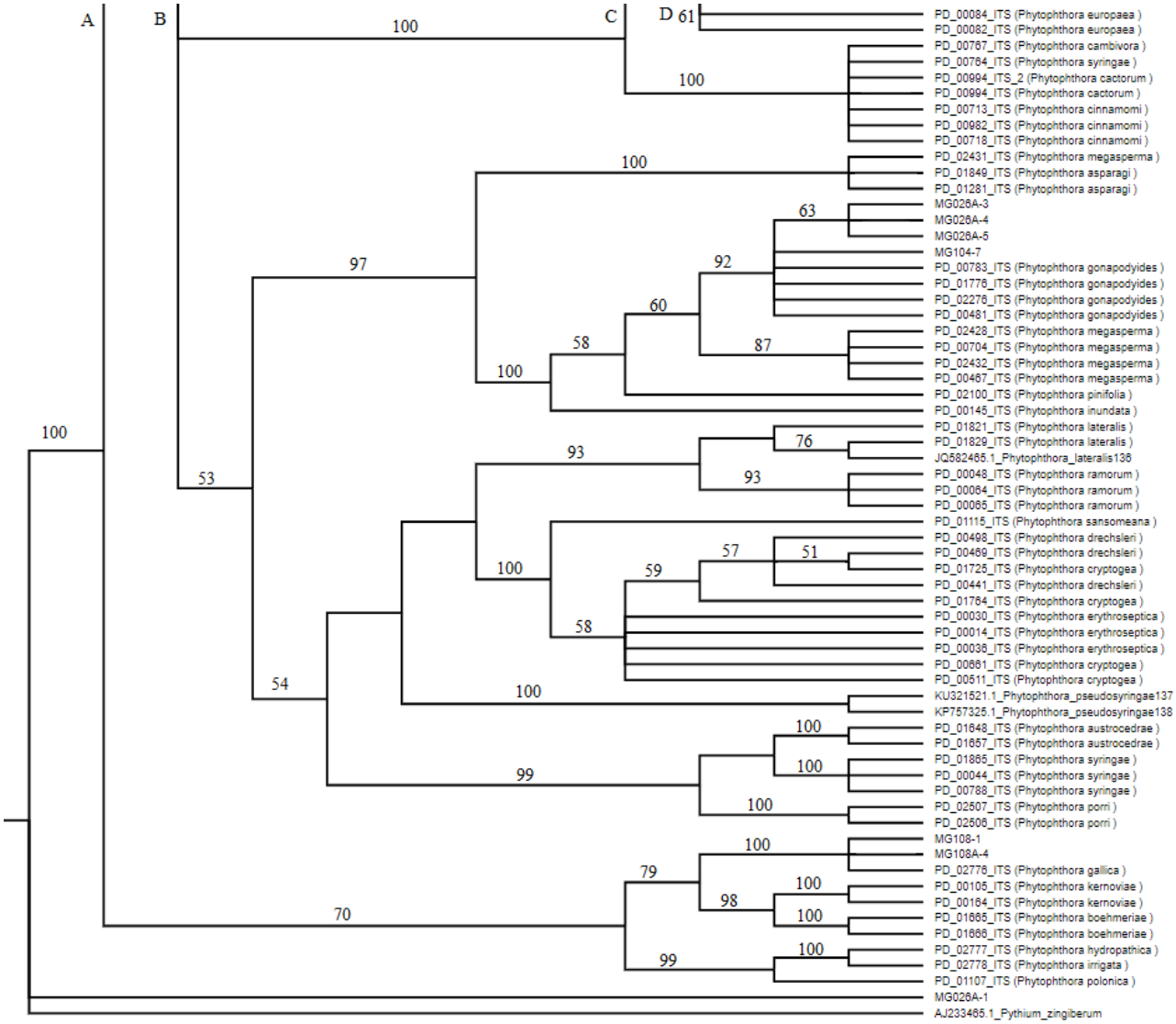

Figure 24. Phylogeny for *Phytophthora* based on the study isolates with a range of illustrative sequences (PhytophthoraDB and GenBank downloads) using ITS2 locus. Maximum parsimony strict consensus tree with bootstrap values added. *Pythium zingiberum* as outgroup.

Morphological characterisation

Oogonia were not found on many of the isolates, and even when found were not always numerous. This may have been down to the species found, the culture conditions, or simply the age of the culture plates. Where they were found images were taken. A representative selection is shown (Fig. 25). It is not always possible to fully see, and interpret, the position of the antheridium. (Table 9).

All microscopy images are included on the DVD of supplementary data which is attached to this thesis.

Table 9. Dimensions and antheridial attachment for observed oogonia. Note, the number of observations per isolate is variable, and at best too small for reliable recording.

Isolate	Dimensions	Antheridial attachment
MG012-2 (nine oogonia)	28.72 ± 2.19 µm	Paragynous
MG014-2 (two oogonia)	28.05 ± 1.23 µm	Paragynous
MG016-1 (nine oogonia)	29.13 ± 1.66 µm	Paragynous
MG-16-2 (three oogonia)	26.57 ± 0.97 µm	Paragynous
MG026A-6 (one oogonium)	16.86 µm	Paragynous

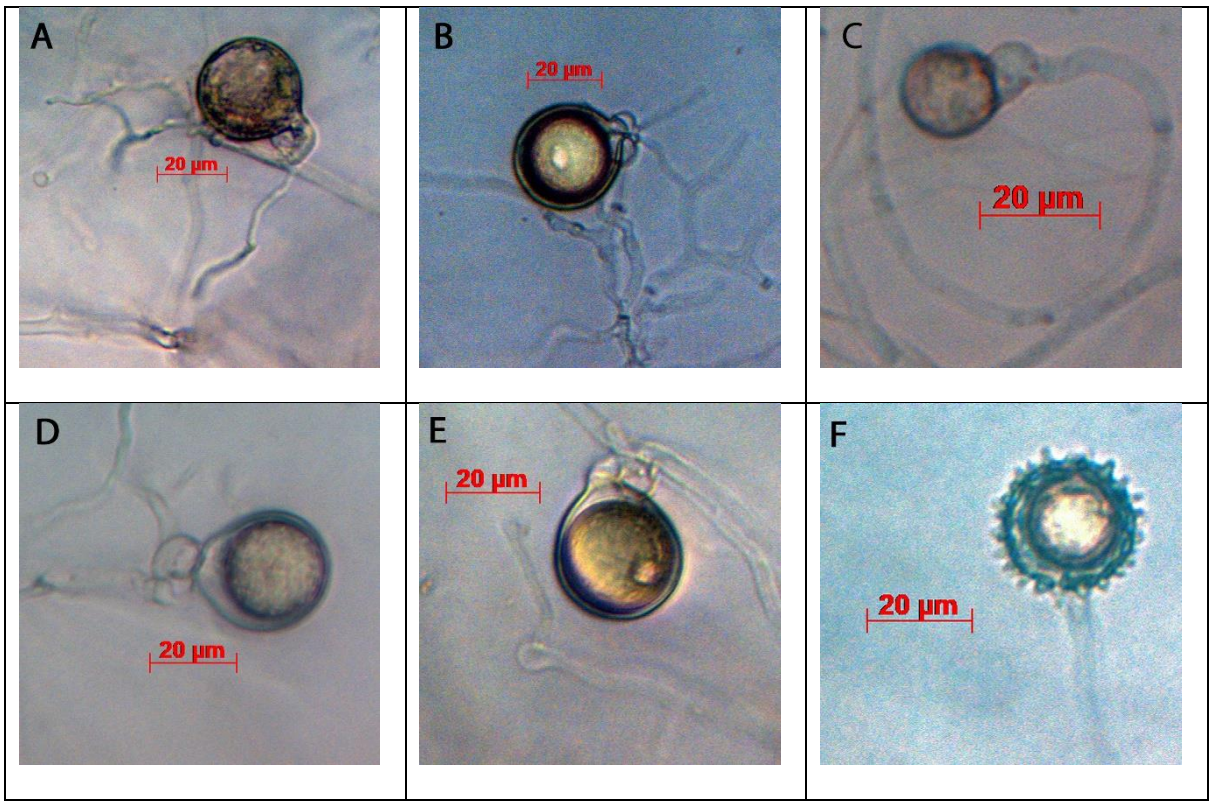

Figure 25. Oogonia and oospores: A. MG012-2; B. MG014-2; C. MG026A-6; D: MG016-1; E. MG016-2; F. MG026A-1;

Sporangia were seen more often than oogonia. The size and shape showed rather more variation than seen with the oogonia (Fig.26).

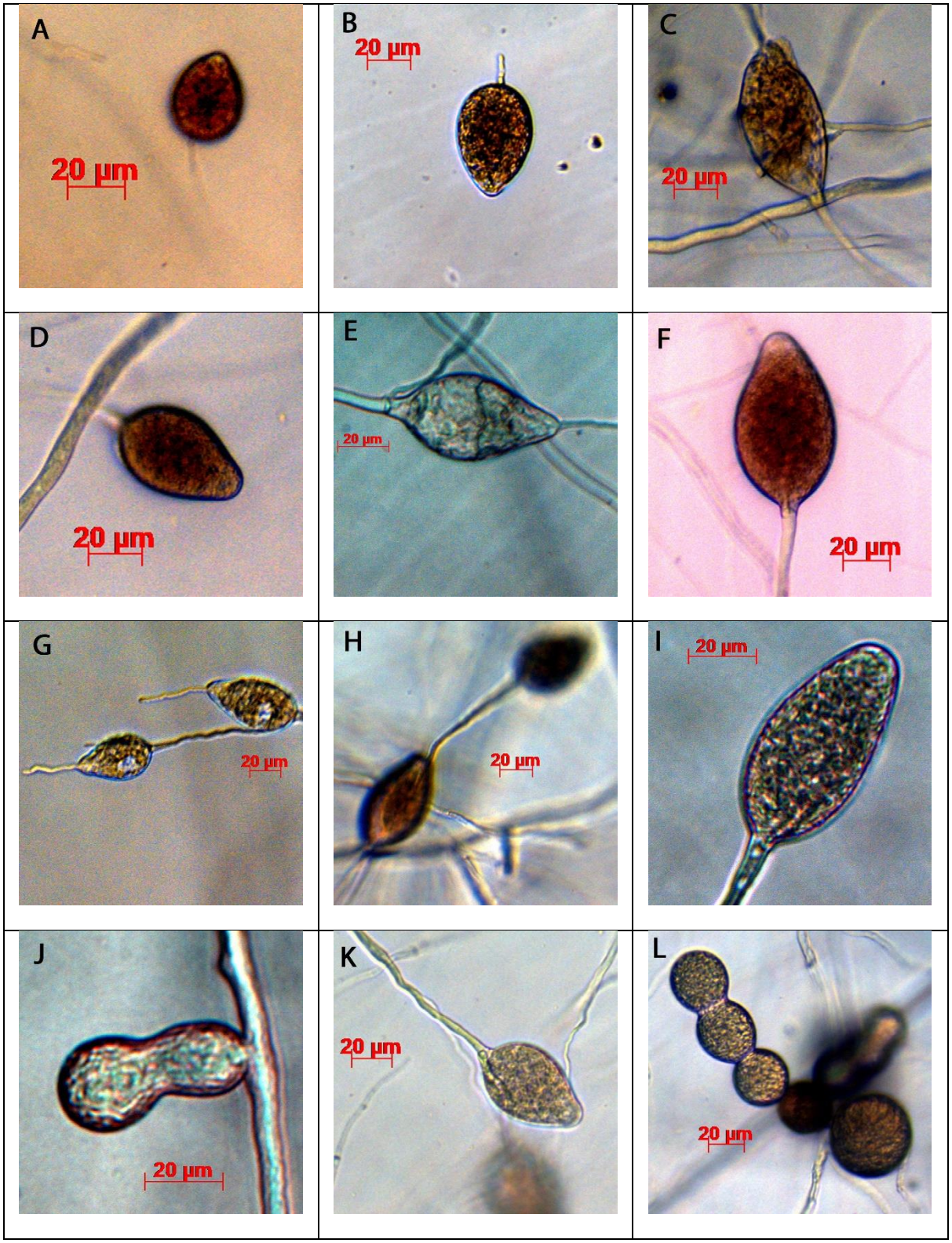

Figure 26. Sporangia: A. MG012-2; B. MG013-6; C. MG026A-2; D. MG026A-2; E. MG026A-4; F. MG026A-5; G. MG-26A-2 sporangiophore; H. MG026A-5 proliferation; I. MG104-7; J. MG108-5 peanut; K. MG108A-3 sporangium; L. MG108A-3 peanut sporangium and/or chlamydospores

A number of isolates had internally proliferating sporangia (Fig. 26H), and there some isolates exhibiting sporangiophores (Fig. 26G), often sympodial.

Generally, the shape varied between almost globose (Fig. 26A) to almost ellipsoid (Fig. 26I), but some isolates have a distinctly limoniform shape (Fig 26D and 26F). The most extravagant shape was the "peanut-shape" sporangium which was found in isolate MG108-2 (Fig. 26J). It is possible that the peanut shaped structure from isolate MG108A-3 (Fig. 26L) may be a sporangium or could be a grouping of chlamydospores,

Measurements for the sporangia observed across an isolate are presented in Table 10.

Table 10. Shape and dimensions for observed sporangia. Note, the number of observations per isolate is variable, and at best too small for reliable recording.

Isolate	**Shape**	**Dimensions (L x B)**	**Size range**	**Observations**
MG012-2 (two sporangia)	Ovoid	30.5 x 24.9 µm	29.4-31.6 x 22.4-27.3 µm	
MG013-6 (one sporangium)	Ovoid	50.9 x 36.4 µm		Caducous; pedicel 12.0 µm
MG016-5 (one sporangium)	Ovoid	22.5 x 17.8 µm		
MG026A-2 (14 sporangia)	Varied	66.3 x 37.9 µm	48.4-73.8 x 32.4-43.9 µm	Some proliferation
MG026A-4 (eight sporangia)	Limoniform	58.1 x 34.5 µm	41.2-73.6 x 27.2-39.2 µm	
MG026A-5 (13 sporangia)	Limoniform	52.4 x 32.6 µm	40.4-76.2 x 25.7-46.3 µm	Some proliferation
MG027A-11 (one sporangium)	Ellipsoid	42 x 17.5 µm		
MG104-7 (two sporangia	Ovoid/ellipsoid	55.2 x 31.6 µm	44.2-66.1 x 30.4-32.9 µm	
MG108-5 (three sporangia)		36.7 x 27.4 µm	29.8-50.4 x 23.9-30 µm	
MG108A-3 (six sporangia)	Limoniform	61.7 x 35.4 µm	52-80.6 x 29.2-47.1 µm	

In addition to gametangia and sporangia there are a number of other morphological characteristics which may have some diagnostic value. Images of these assorted characters are shown (Fig. 27). Some isolates had chlamydospores (Fig. 27E, G), and in others there was a coiling of hyphae (Fig. 27A, 27B), or odd hyphal swellings (Fig. 27D), which were quite distinctive. An aborted chlamydospore, or possibly an aborted oogonium, was noticed in isolate MG108-2 (Fig. 27H).

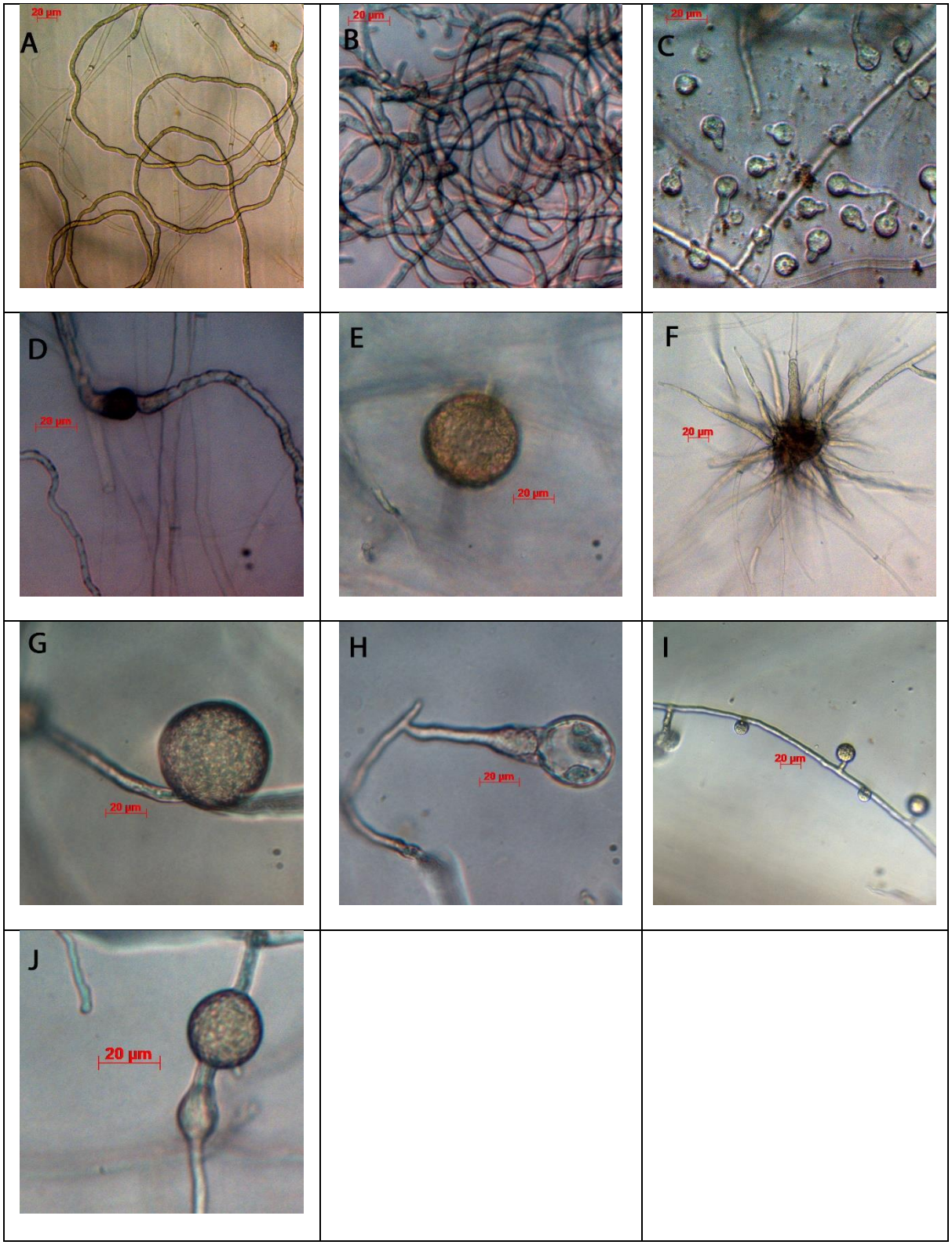

Figure 27. Miscellaneous morphology: A. Coiling hyphae, MG026A-4; B. Dense coiling hyphae, MG026A-5; C. Zoocysts, MG026A-5; D. Unidentified structure MG027A-3, possibly a zygomycete contaminant; E. Chlamydospore, MG108-2; F. Sporangium germinating like a starburst, MG104-1; G. Intercalary chlamydospore, MG108A-3; H. Aborted chlamydospore, MG108-2; I. Row of chlamydospores, MG108A-3; J. Possible chlamydospore, MG013-6.

The image (Fig. 27J) is potentially difficult. It could be an oogonium viewed from above, so no sight of the antheridium, or it could be a chlamydospore.

Based on the observed characteristics of the various isolates the "Lucid Key to common species of *Phytophthora*" (Ristaino 2012) was used to key out the results. The key does not cover *P. gallica* or *P. plurivora* so it was not possible to use it for these species.

Usable characters for the isolates that were keyed are given (Table 11).

Table 11. Morphological characteristics for use with the Lucid Key. There were insufficient characters available to get any meaningful results from running the key for MG022B isolates.

Character	**MG013-6**	**MG026A**	**MG027A**
Sporangium	Caducous	Non-caducous	Non-caducous
Pedicel	Up to 20µm		
Sporangium	Ovoid	Ovoid	Ellipsoid, nonpapillate
Length to breadth ratio	<1.6	<1.6	
Sporangium length	45-75 µm	45-75 µm	
Sporangiophore		Simple sympodial	
Homo/Hetero-thallic		Heterothallic	Heterothallic
Hyphal swellings	Absent	Absent	Present
Chlamydospores	Not seen	Not seen	Not seen
Temperature optimum	< 22°C		
Growth rate	Slow		
Colony			No rosette on V8A

The results from the Lucid Key suggests isolate MG013-6 was *Phytophthora ilicis*, that isolate MG026A was *P. gonapodyides*, and that isolate MG027A was either *P. cambivora* or *P. cryptogea*.

These results were obtained whilst it was believed that Fig. 26J was an oogonium. If the search criteria are entered but with chlamydospores present, then the Lucid Key is unable to classify the isolate.

Data recording

The MS Access database was used to appraise summaries of the data found. An example of a data form is shown (Fig.28).

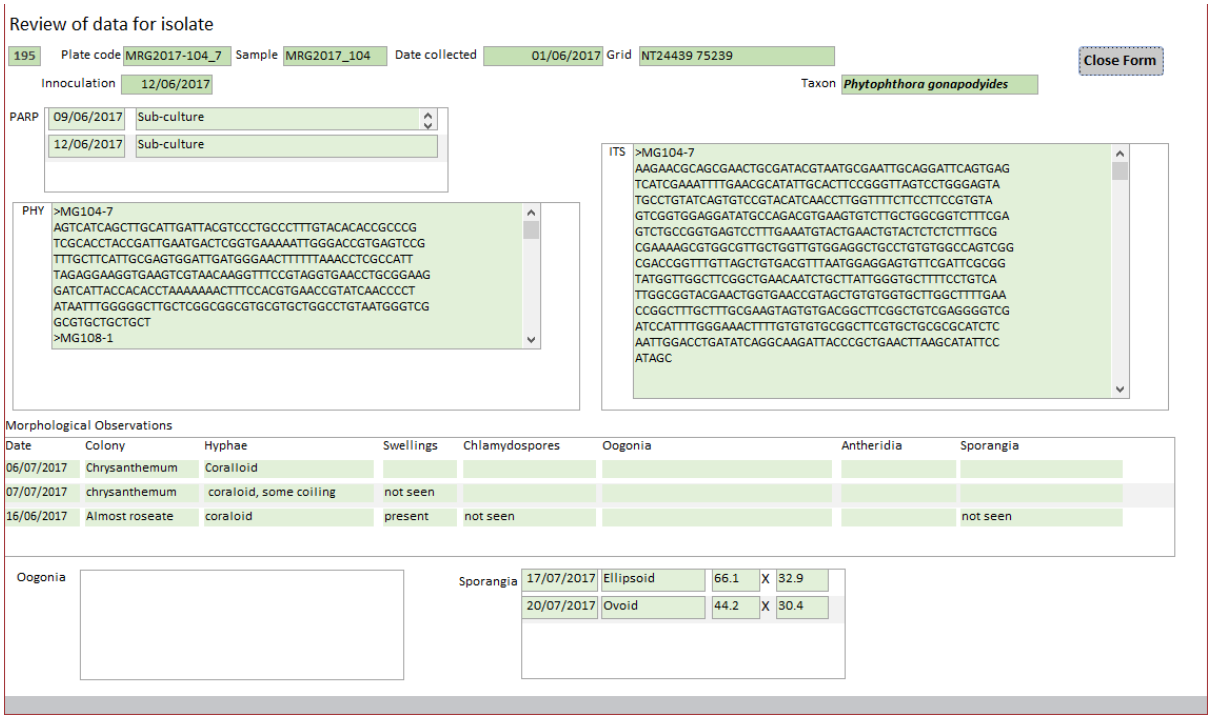

Figure 28. Bespoke data form in MS Access.

Colour illustrations III

Figure C31. Left - *Ilex* sapling at NT 24648 75331, 44.4m away from sample site 013. Right – leaves removed from an *Ilex* tree known to be infected with *Phytophthora ilicis*.

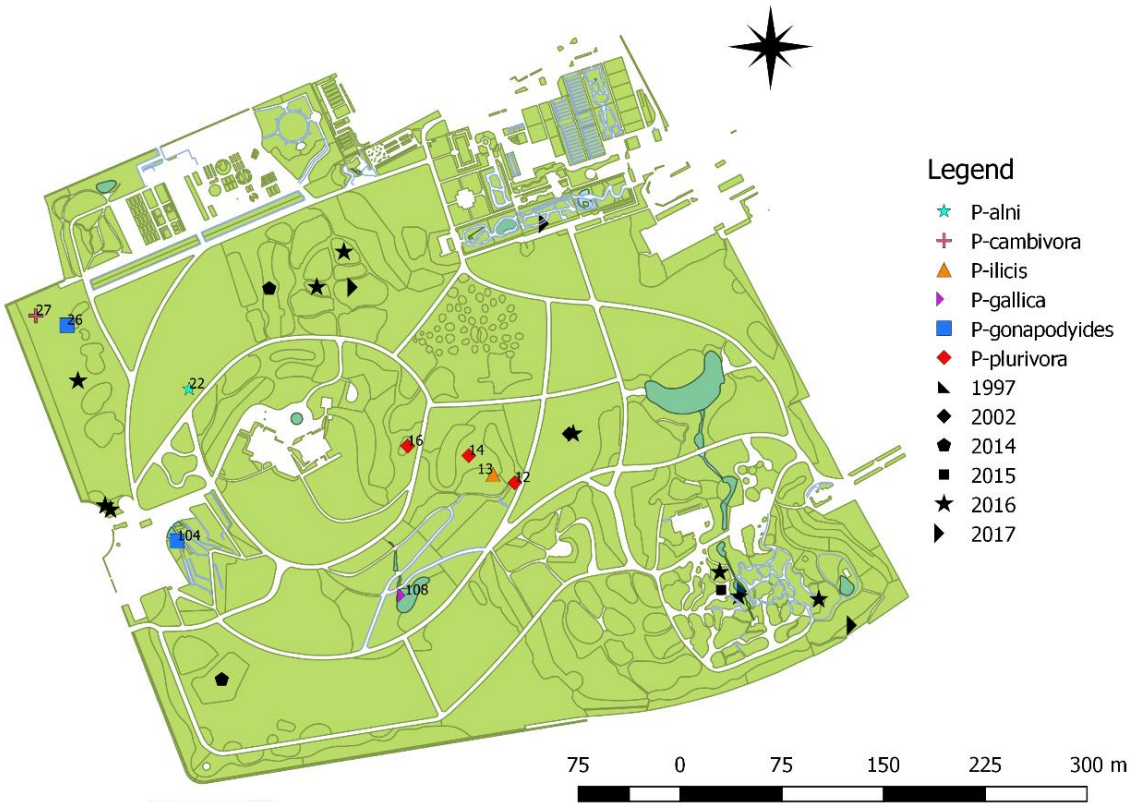

Figure C32. GIS map of RBGE showing historical records for *Phytophthora* (as shown in Fig. C19) together with records of *Phytophthora* spp. identified in current sampling. (Quantum GIS)

Discussion

Species identification

Based upon the results of the morphological assessment, the growth-rate analysis, the Lucid Key results and the phylogenetic table it is possible to attribute a species-level identification for the *Phytophthora* that were isolated. The confidence of such identification depends upon the degree of support from the various criteria used.

Isolates MG012-2, MG014-1 and MG016-1

Phylogenetically these three isolates all group with *P. plurivora* in Clade 2.

P. plurivora is known to have multiple hosts, be homothallic, have paragynous antheridial attachment and semi-papillate sporangia (Kroon *et al.* 2012)

The almost pleurotic oogonium with a paragynous antheridium of MG012-2 (Fig. 25A) appears typical of those found in all three isolates, although it is possible that the antheridial attachment in Fig. 25B is potentially amphigynous. Dimensionally, the oogonia fit comfortably within the range of mean diameters for *P. plurivora*, (Jung & Burgess 2009) being slightly too small to be *P. citricola* or *P. inflata* Caroselli and Tucker (Caroselli & Tucker 1949), but slightly too large to be *P. multivora* P.M. Scott & T. Jung (Scott *et al.* 2009).

Sporangia were not found on isolate MG014-1 but the sporangia on the other two isolates appear close in shape and size (e.g. Fig. 26A). The sporangia are substantially smaller than the range of isolate means quoted when the species was formally published: 39.6-52.3 x 28.9-38.8 µm (Jung & Burgess 2009), but the number of sporangia observed is low, and those seen may have been immature sporangia.. The report also states that gametangia were readily produced in single culture by all the isolates examined, and this is confirmed within the RBGE isolates.

The maximum temperature for *P. plurivora* is 32°C with an optimum of 25°C. The growth rate at optimum temperature is 8.1±0.18 mm/d. The growth rate on V8A at 20°C is 6.3±0.1 mm/d (Jung & Burgess 2009). These precise temperatures were not used in this study but it is clear the measured rates for the RBGE isolates (Fig. 21) are lower than this. MG012-2 gave

4±0.7 mm/d and MG016-1 gave 4.1±0.3 mm/d at 18°C. This was their peak rate at the temperatures tested.

The hypothesis that these isolates, all from different soil samples, are *P. plurivora* would appear to be partially, though not categorically, supported.

Isolate MG013-6

Morphologically it is difficult to separate *P. ilicis* and *P. pseudosyringae*. The phylogenetic analysis shows this isolate grouping categorically alongside *P. ilicis* in Clade 3, and well separated from *P. pseudosyringae*.

P. ilicis is known to have *Ilex aquifolium* as its host, be homothallic, have amphigynous antheridial attachment and semi-papillate sporangia (Kroon *et al.* 2012)

Fig. 26B shows a caducous obpyriform, semipapillate sporangium in which zoospores have formed immediately prior to emergence, following a chill shock treatment. The pedicel of the sporangium is 12.05μm. The sporangium is large for *P. ilicis*, but the length-breadth ratio (at 1.39:1) fits comfortably into the range given (Erwin & Ribeiro 1996). The small objects, just visible in the image, appear to be motile zoospores, approximately 3.5μm in diameter, from other sporangia in the soil extract, but it was impossible to get them into focus for a camera shot due to the speed of their movement.

The colony formation on agar is very similar to published images (Scanu *et al.* 2014). The growth for MG013-6 (Fig. 21) peaks at 18°C with a rate of 2.6±0.5 mm/d. There was no growth at 30°C. There is some correspondence with the growth rate figures of 20°C as optimum and a maximum of 25°C (Erwin & Ribeiro 1996).

The Lucid Key gave only one outcome for the morphological characters of this isolate, on the assumption the structure seen (Fig. 27J) was an oogonium, *P. ilicis*. However, if the structure is entered as a chlamydospore the Lucid Key is unable to give an outcome. Within the Lucid Key chlamydospores are regarded as not found in *P. ilicis* - though they are reported as rare (Erwin & Ribeiro 1996).

The hypothesis that isolate MG013-6 is *P. ilicis* is well, but not absolutely, supported.

Isolates MG022B-6 and MG022B-11

Phylogenetically these two isolates both group with *Phytophthora alni* subsp. *uniformis* in Clade 7.

P. alni is known to have *Alnus* spp. as its host, be homothallic, have amphigynous antheridial attachment and nonpapillate sporangia (Kroon *et al.* 2012)

There was very little morphological differentiation on either of the two culture plates. No gametangia, chlamydospores or sporangia appear to have formed, but there was a strongly pronounced form of coralloid hyphae (Fig. 29), and abundant aerial growth in both isolates.

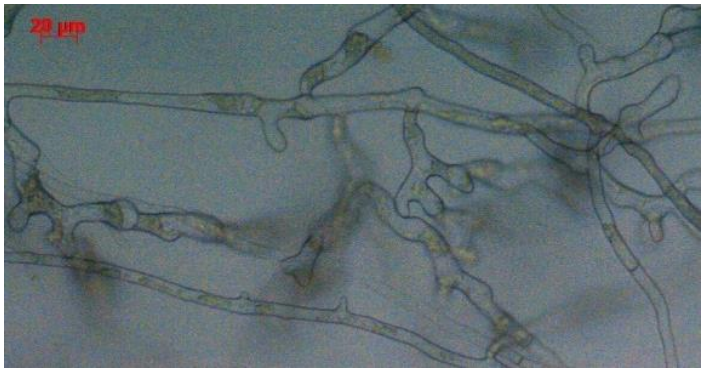

Figure 29. Coralloid hyphae from isolate MG022B-7

There seems some correspondence with the description given when the species was published (Brasier *et al.* 2004) where *P. alni* subsp. *uniformis* is described as "an irregular appressed colony, often with a little woolly aerial mycelium in the colony centre but submerged growth at the edge. Gametangia generally frequent". The colony formation fits, but there were no gametangia to be seen. These isolates were not included in the growth rate study, so no comparative data is available.

On the evidence that is available, there is nothing to either support or reject the hypothesis that isolates MG022B-6 and MG022B-11 are *Phytophthora alni* subsp. *uniformis*. For a positive identification, further investigation, particularly culturing to stimulate the formation gametangia, would be needed.

Isolate MG026A-1

This isolate showed up as a positive with the *Phytophthora* specific primers, however it differed from the other isolates in that it grew very fast – in the growth rate study isolate MG026A-1 had filled the V8A plate before the day three examination for all temperatures. It also had very numerous and very distinctive spiky oospores (Fig. 25F). When the DNA sequence was run through BLAST it shows as *Pythium anandrum* Drechsler, and comparison with images of *Pythium anandrum* oospores confirmed the identification as highly likely. The fact that it shows up with the *Phytophthora* specific primers suggests that it potentially has

more ITS1 similarity with *Phytophthora* than do most of the other members of the sister genus *Pythium*.

Isolates MG026A-2, MG026A-3, MG026A-4 and MG026A-5

Phylogenetically this grouping of isolates sits close to *Phytophthora gonapodyides* in Clade 6, but relatively distinct from it. This raises the possibility of the isolates forming a new, cryptic, species.

P. gonapodyides is thought to be a rather minor pathogen and is suspected to be largely saprophytic on roots. Oogonia are unknown or rarely produced, antheridial attachment is unknown, and nonpapillate sporangia (Kroon *et al.* 2012).

The images of sporangia (Figs. 26D to 26H) bear a similarity to published images (Erwin & Ribeiro 1996) of sporangia for *P. gonapodyides*, and the dimensions also appear to fit, although the width is towards, and slightly beyond, the high end of published accounts when the sporangia were from cultures growing in sterile soil extract. Internal proliferation (Fig. 26H) is typical for the species.

It is suggested (Erwin & Ribeiro 1996) that the sporangiophores of *P. gonapodyides* are sympodial only in water (Pittis & Colhoun 1984), but Fig. 26G was from the isolate growing on 10% V8A. However, careful reading of the original Pittis and Colhoun paper shows references to the aquatic associations of *P. gonapodyides*, but no mention of sporangiophores only being sympodial in water was found.

In the MG026A crosses there is a pronounced difference between isolate MG026A-3 and the other similar isolates, with some form of defence mechanism either being produced by isolate MG026A-3, or else being produced by the other isolates against MG026A-3. It is reasonable to believe the other isolates were all essentially the same type.

The growth for MG026A-2 (Fig. 21) peaks at 30°C with a rate of 3.4±0.3 mm/d. MG026A-5 peaks at 30°C with a rate of 3.6±0.4 mm/d. This pattern seems to broadly tally with information given (Erwin & Ribeiro 1996) but there is a potentially troublesome mention by Brasier *et al*. (1993) reporting “slow growth at 20, 25 and 30°C”.

Referring to the original 1993 paper (Brasier *et al.* 1993) the words “grew comparatively slowly” is used, as mentioned in Erwin & Ribeiro, but the data given in the actual paper suggests an average colony diameter for British isolates, after 4 days, of 26.5±5.5mm at 20°, 30mm at 25°, and 31.0±5.9mm at 30°. The temperatures and observation intervals do not

directly match the growth rate study undertaken, but working from the observed data[5] colony diameter for the two isolates in the growth rate study would be 24.8mm at 18°C, and 30.97mm at 30°C. Thus, the growth rate study data for isolates MG026A-2 and MG026A-5 corresponds very closely with the Brasier *et al*. data.

The evidence from the experimental crosses within the MG026A isolates showed no sign of gametangia, but does suggest that isolate MG026A-3 is different in some way from the other isolates in the group, though it is not possible at this stage to say how or what the difference is. Further, the apparent proximity of isolates MG026A-2, -4 and -5 is not fully supported by the statistical analysis of sporangia dimensions. This would suggest that all the relevant MG026A isolates are different strains, but that -2, -4 and -5 are closer to each other than they are to -3.

The Lucid Key gave only one outcome for the morphological characters of this isolate, *P. gonapodyides*. Weighing matters, the new species hypothesis is only supported by the phylogenetic evidence. All the other lines of evidence support the hypothesis that these isolates are *P. gonapodyides*, which is not strongly disputed by the phylogenetic evidence.

Thus, it would seem likely that the isolates are *P. gonapodyides*, but that the uncertainty would warrant further investigation. Such investigation would involve culturing on different media, incubation at differing temperatures, robust morphological assessment supported by statistical analysis of the observed results, and more extensive DNA analysis.

<u>Isolates MG027A-3, MG027A-6, MG027A-8 and MG027A-11</u>

Phylogenetically these isolates sit alongside a grouping of *Phytophthora cambivora, P. fragariae*, and *P. alni* subsp. *alni*. All these species are in Clade 7.

P. cambivora has multiple hosts and infects roots, is heterothallic and when oogonia are produced (i.e. with two different mating strains present) the antheridial attachment is amphigynous. The sporangia are nonpapillate (Kroon *et al.* 2012).

No oogonia were found in any of the isolates, which would tally with the *P. cambivora* being heterothallic. Very few sporangia were found but, the one that was found proved difficult to photograph (Fig. 30-L). It would appear to be nonpapillate. The mycelium has coralloid

[5] Adjusting to 4 days by taking an average of the 3- and 5-day readings, and then converting the measured radii to colony diameters, and averaging between the two isolates.

hyphae which are often distorted into irregular shapes (Erwin & Ribeiro 1996) which fits with the observations (Fig. 30-R)

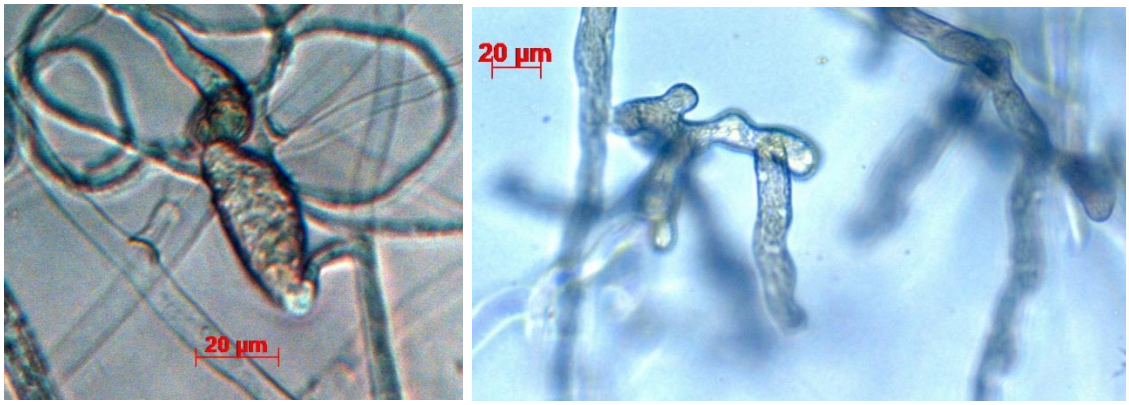

Figure 30. Left – MG027A-11 sporangium; Right – MG027A-8 coralloid hyphae.

These isolates were not included in the growth rate study, but since the optimum temperature is 22 to 24°C and cessation of growth occurs at 32°C the temperatures used in the growth rate study were unlikely to have yielded any definitive information.

The Lucid Key gave two possible outcomes for the morphological characters of this isolate, *P. cambivora* or *Phytophthora cryptogea* Pethybridge and Lafferty (Pethybridge & Lafferty 1919). Looking at the phylogenetics of these two species, *P. cambivora* is in Clade 7 whilst *P. cryptogea* is in Clade 8. Within the maximum parsimony strict consensus tree that was run for the isolates and various downloaded sequences to give reference markers (Fig. 24) the MG027A isolates sit within a clade that contains *P. alni*, *P. cambivora* and *P. fragariae*. *P cryptogea*, however, sits on a completely different branch, demonstrating a phylogenetic distance between the two species. It is thus more likely that the isolates are *P. cambivora* than *P. cryptogea*.

The hypothesis that these isolates are *P. cambivora* would appear to be quite well, though not categorically, supported.

Isolates MG104-3 and MG104-7

Phylogenetically this sits between the grouping of isolates MG026-3, -4 and -5 and *P. gonapodyides*, though phylogenetically closer to *P. gonapodyides* than to the MG026 isolates. The general description of *P. gonapodyides* is given for the MG026A isolates.

The shape and size of the sporangia found on isolate MG104-7 (Fig. 26I) fits within the brackets that are given for sporangia of *P. gonapodyides* in Erwin & Ribeiro. One feature noted in the cultures was relatively frequent germinating sporangia (Fig. 27F) resembling

starbursts. If these were germinating spores from contamination then hyphae would be expected to be septate, but these seem to be oomycete hyphae. This characteristic does not appear to have any diagnostic value.

The growth for isolate MG104-3 (Fig. 21) peaks at 30°C with a rate of 3.7±1 mm/d. An optimum temperature for *P. gonapodyides* is given as 25°C with a maximum of 30 to 35°C (Erwin & Ribeiro 1996). Applying the same criteria as given above, the comparison to the Brasier et al. (1993) growth rates shows the 4-day colony diameter for the isolate to be 27.9mm at 18°C, and 28.6mm at 30°C. These values are within one standard deviation of the Brasier *et al*. results.

The hypothesis that isolates MG104-3 and MG104-7 are *P. gonapodyides* would appear to be well supported.

Isolates MG108-1 and MG108A-3

Phylogenetically these two isolates sit in a grouping with *Phytophthora gallica* in Clade 10. *P. gallica* is known to have *Quercus robur* as its host, and is often found in wet places. Oogonia are unknown or rarely produced, antheridial attachment is unknown, and sporangia are nonpapillate (Kroon *et al.* 2012).

A characteristic feature of the cultures was the range of form the sporangia took, and in particular the occurrence of peanut-shaped sporangia (Figs. 26J and 26L). The cultures also showed growth of thin-walled spherical chlamydospores (Figs. 27G, 27H and 27I). In all, 12 chlamydospores were photographed. The diameter range was from 26.16 to 47.66µm, with a mean of 34.7 ±6.3µm. The growth for MG108-2 (Fig. 21) peaks at 18°C with a rate of 1.8±0.3 mm/d. For isolate MG108A-3 the peak is similarly 18°C but the rate is 2.8±0.7 mm/d.

Neither of the cultures showed any sign of oogonia so *P. kernoviae*, being homothallic and having an absence of chlamydospores, can be morphologically discounted. Similarly, *P. boehmeriae* can be ruled out as it also is homothallic and produces papillate caducous sporangia (Jung & Nechwatal 2008), which were not found within the cultures.

The temperature-growth relationship for MG108A-3 fits well with the published range for *P. gallica* being grown on V8A, but MG108-2 falls below the range given (Jung & Nechwatal 2008).

Thus, the hypothesis that the isolates from sample 108 are *P. gallica* is well supported. However, there are quite substantial differences between the two isolates which almost

certainly merits further morphological and phylogenetic investigation. This is particularly so, given that *P. gallica* has only once previously been isolated in Scotland (Cooke 2015).

Identification of *Phytophthora* to species level with any certainty is clearly a very much more complex and complicated process than might be thought. Compared with e.g. the Angiosperms, distinctive characters are often sparse, and their development is frequently dependent upon specific culturing conditions. The cryptic nature of the Oomycetes, and the substantial similarity of form, demands that multiple lines of investigation converge in order to confirm the taxon. Even then DNA analysis is an almost *de facto* requirement.

Plant community analysis

An analysis of the surrounding plant communities, to species level, for those soil samples where *Phytophthora* has been isolated (Table 12) was undertaken. With the water samples, such an analysis is not practical.

Seven soil samples (012, 013, 014, 016, 022, 026 and 027) and five putative *Phytophthora* species (*P. alni*, *P. cambivora, P. gonapodyides, P. ilicis* and *P. plurivora)* are involved.

Table 12. Plant communities around *Phytophthora* positive soil sample points. Where the specific plant has appeared in the nursery propagation bed E19 trace forward analysis it is shown in bold.

Sample	Community
012	*Pieris formosa, Fraxinus angustifolia, Syringa vulgaris, Fraxinus excelsior*.
013	*Rubus foliaceistipulatus, Potentilla fruticosa, Syringa komarowii, Ostrya carpinifolia, Sorbus caloneura*
014	*Fraxinus sogdiana, Abies forrestii,* ***Betula utilis****, Berberis, Lonicera tangutica, Spiraea mongolica, Ligustrum delavayanum, Sorbus eburnean, Acer franchetii, Ligustrum compactum, Cotoneaster, Liquidambar styraciflua,* ***Syringa tomentella****.*
016	*Rhododendron wallichii,* ***Magnolia kobus****, Rhododendron thayerianum, Prunus serotina, Liriodendron tulipifera*
022	***Magnolia stellata****,* ***Quercus pyrenaica****, Pinus ponderosa, Lithocarpus densiflorus, Quercus robur*
026	*Euonymus sieboldianus,* ***Sorbus commixta****, Juniperus communis, Torreya taxifolia, Abies homolepis*
027	*Castanea dentata, Staphylea bumalda,* ***Cercidiphyllum japonicum****, Myrica californica, Sambucus tigranii, Fitzroya cupressoides, Cornus, Helwingia japonica (dead), Abies spectabilis, Polystichum acrostichoides, Sorbus commixta, Malus, Pinus balfouriana, Cephalotaxus harringtonii, Betula alleghaniensis*

Samples 012, 014 and 016

P. plurivora is pathogenic towards numerous and frequently occurring woody hosts. This list includes species such as *Abies alba*, *Alnus* spp., *Acer* spp., *Aesculus hippocastanum*, *Carpinus betulus*, *Fagus sylvatica*, *Fraxinus excelsior*, *Quercus* spp., Rhododendron *spp.*, *Syringa vulgaris*, *Tilia* spp., and *Tsuga canadensis* (Jung & Burgess 2009).

Amongst the plant communities around these sample points such species are widely found. Additionally, several of the plants around these three sample points have originated from propagation bed E19, so these too may be considered a potential source of the *Phytophtora* infection.

Sample 013

The only known hosts for *P. ilicis* are *Ilex aquifolium* and *I. crenata* (Ristaino 2017a). However, these plants are not found in the immediate community at sample point 013. The only *Ilex* spp. within a 100m radius of the sample site was a small, rather sickly-looking sapling (Fig. C31-L, p.62) tucked into the undergrowth. It is noteworthy that some of the foliage on the sapling is a reddish-brown. Where *Ilex* is infected with *P. ilicis* the foliage usually looks a washed up grey-green (Fig. C31-R, p.62), so it is possible that, although sickly, the cause is not *Phytophthora*.

Sample 022

Four members of the genus *Alnus* (*A. alnobetula*, *A, cordata*, *A. glutinosa*, and *A. incana*) are the recognised hosts for *P. alni* ("Phytophthora alni (phytophthora disease of alders)" 2017). None of these hosts was in the immediate vicinity of sample point 022. Despite careful checking no *Alnus* spp. was found within a 100m radius of the sample site.

Sample 026

A range of hosts is given for *P. gonapodyides* (Ristaino 2017b) but the only link between the plant community at sample point 026 and the listing is *Abies* spp., with *Abies homolepis* growing in the community. However, it must also be noted that the specimen of *Sorbus commixta* in this community originated in the nursery propagation bed E19.

Sample 027

The classic host for *P. cambivora* is *Castanea dentata* (Erwin & Ribeiro 1996) and this is the dominant tree in the local community at sample point 027. The sample was taken at the edge of the drip zone from the tree. However, also within that community is a *Cercidiphyllum* that

originated in the nursery propagation bed E19. Further testing of this location would be valuable to try and identify if the source of the *Phytophthora* that was found is associated with the *Castanea* or the *Cercidiphyllum*.

No clear pattern of association between host and pathogen emerges from this analysis. The sample size is admittedly small. The fact that five of the seven communities included at least one plant transplanted from propagation bed E19, where there are known to have been *Phytophthora* infections, at the very least merits further investigation to test its significance.

The composite diversity of *Phytophthora* within RBGE

Now that both the historic distributions of *Phytophthora* within RBGE, albeit partial and without any species-level identification, and the taxa identified within the current survey are available it is possible to combine these data into a single GIS map of the gardens (Fig. C32, p.62). It must be stressed that the survey results show the minimum *Phytophthora* presence within RBGE. Additional culturing methodologies, use of different baits, and replicate baiting of the samples might all reveal the presence of additional *Phytophthora* species.

The striking thing about the distribution is its serendipitous nature. There is very little overlap between the current and historic distributions. Had conditions been cooler it is possible that there may well have been *P. austrocedri* showing up in samples in the Southeast corner of the gardens, and this would have given a degree of overlap.

Looking at the plants which, according to BG-BASE records, died from suspected *Phytophthora* infections in 2016 (symbol = black star), they are scattered across almost the whole gardens, a tree here, a tree there. Given there are no *Phytophthora* species-level identification given in the BG-BASE records it is impossible to take this further. However, with the considerable time delay between planting and death, it does seem clear that the infection was not introduced with the trees at the time of planting. The deaths would appear to be either brought about through some infected soil element being introduced to the locality; or by spores that were carried from either inside, or outside RBGE by wind-driven rain, mists, animals or human feet.

Given that *Phytophthora* spp. are plant pathogens, and often highly destructive ones, it might be hoped that they would be absent from RBGE. However, given the nature of RBGE, with its near global collection of plants, and the fact that *Phytophthora* spp. form an integral part of the global plant biodiversity, it is almost inevitable that they will be found in RBGE.

Six species of *Phytophthora* were found during the sampling, but this is a minimum and it is highly likely more species would be found with wider sampling and more extensive culturing and investigation. For some, the identification was robust, but for others the identification was more tentative, due mostly to the available time limiting the range of culture possibilities.

The species found shows a broad cross section of the genus *Phytophthora*, with five of the ten clades being represented.

- Clade 2 – *P. plurivora*
- Clade 3 – *P. ilicis*
- Clade 6 – *P. gonapodyides*
- Clade 7 – *P. alni; P. cambivora*
- Clade 10 – *P. gallica*

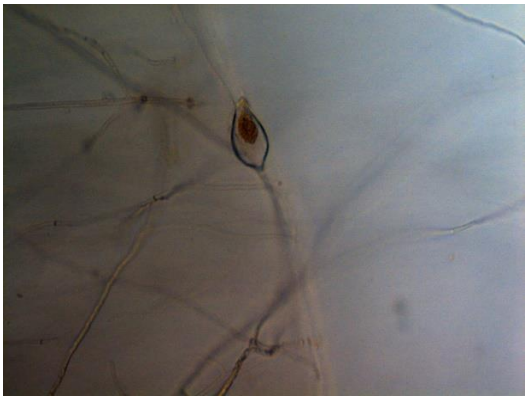

Figure 33. Sporangium of *Phytophthora gallica*. The width of the sporangium, at its widest point, is 30.81µm.

Fortunately, none of the notorious species, such as *P. ramorum*, *P. kernoviae* or *P. austrocedri* were isolated, although the evidence for presence of *P. austrocedri*, particularly in the rock garden area, was substantial. Whilst not isolating the highly aggressive, and very damaging, species within RBGE, the collective profiles of the *Phytophthora* spp. that were isolated, give little room for complacency:

- *P. alni* is a damaging pathogen but is highly restricted to trees in the genus *Alnus*. The symptoms are often observed as sparse, excessively small, yellow leaves and often a

high production of cones, which is symptomatic of stress in *Alnus* ("Phytophthora alni" 2017).

- *P. cambivora* is an invasive and persistent pathogen causing disease, such as ink disease on chestnut and more generally stem canker, to a wide range of hosts. Its spread is supported by its capacity to survive as a saprophyte and the persistence of highly resistant oospores in the soil ("Phytophthora cambivora (root rot of forest trees)" 2017).
- *P. gallica* appears to be a weak pathogen (Jung & Nechwatal 2008), unlike the other members of Clade 10. Reported hosts include *Quercus robur*, *Fagus sylvatica*, *Salix alba* and *Alnus glutinosa* (Sullivan & Bulluck 2010). It is thus, potentially an issue within forested areas within northern temperate regions. *P. gallica* appears to have only been identified once before in Scotland (Cooke 2015). A sporangium is shown (Fig. 33)
- *P. gonapodyides* is considered a minor pathogen causing root rots on a number of ornamentals. It is also believed to be able to live as a saprophyte on the twigs of a number of species (Ristaino 2017b).
- *P. ilicis* is restricted to trees in the genus *Ilex* but is potentially very damaging, as evidenced by recent mature tree removals at RBGE. It was recently (June 2016) found in Germany (Schrader & Werre 2016), so is clearly expanding its geographical range.
- *P. plurivora* acts in forests as a fine root pathogen and appears involved in widespread declines of *Fagus sylvatica* and *Quercus* spp. (Jung & Burgess 2009). In Serbia, *P. plurivora* has been found to be a more aggressive pathogen of *F. sylvatica* than *P. cactorum*, which was previously considered to be the most harmful to beech trees (Milenković *et al.* 2012).

An additional consideration is that there is evidence of some propensity towards hybridisation within the genus *Phytophthora* e.g. (Brasier *et al.* 1999; Cooke *et al.* 2000). Given multiple species have been isolated within a reasonably small area of ground that is extremely rich in angiosperm diversity, the possibility of hybridisation occurring cannot be ignored. A solitary hybridisation may not be successful, but if it was, and succeeded in generating a hybrid swarm with potentially novel pathogenic capacity, then the implications could be extremely serious.

Two major questions arise from these observations. Firstly, how did the *Phytophthora* get to RBGE; and secondly, what can be done to limit any impact of the occurrences?

There would appear to be two generic routes of entry. A known possibility, though very carefully monitored at RBGE, is the introduction of disease through new plantings. A second possibility is through the unintended introduction of *Phytophthora* spores. Such spores could be carried into RBGE on wind-driven rain; by accidental translocation of soil, either through tools, tractor wheels or human boots; or by animal movements, such as squirrels or birds. In each of these cases the transfer could be from a *Phytophthora* infection either outside RBGE or internally, moving spores from an already established infection.

In the case of the nursery propagation bed E19, it seems likely that an initial *Phytophthora* infection escaped the quarantine examination (early stages of *Phytophthora* infections are often not at all obvious), and then proliferated in the bed. Subsequently, as plants were moved into the garden, any infections picked up in the nursery bed through either direct contact or aggressive *Phytophthora* spread, were transferred into the main RBGE garden.

In terms of limiting the impact of identified infections, there are not many viable options. There appear to be no real chemical or biotic control measures. Removal, albeit a blunt instrument, seems to be the only option. Within agricultural monocultures removal is often problematic since *Phytophthora* have the capability to move an infection to an epidemic very rapidly. In agriculture, once *Phytophthora* is identified then it is often too late, since the infection has already spread rapidly through the crop. In forestry situations, there is more likelihood that removal, and surrounding clearance, may be successful so long as it is spotted soon enough, and effective and robust sanitation precautions are taken (Forestry Commission 2010).

All new accessions to RBGE are put in quarantine and, if developing symptoms warrant, they are tested for *Phytophthora*. There is an ongoing sampling regime operational within the nursery.

RBGE has a robust policy of removing plants that are potentially infected with *Phytophthora*, especially where it may be one of the invasive species, so local spread can be effectively curtailed. The critical element for this policy to work is that symptomatic plants need to be correctly identified at an early a stage of the infection.

Over recent years the technology and techniques that allow for detection and identification of *Phytophthora* spp. have been rapidly improving. Quantitative real-time PCR, using TaqMan,

now allows reliable detection and identification of *P. ramorum, P. kernoviae* and *P. austrocedri* from soil samples (Elliot *et al.* 2015). Molecular diagnostics also allow for faster and much more precise identification than was previously possible. These tools potentially allow for the tracking of the spread of *Phytophthora* infections and for implementing swifter intervention.

The risks that *Phytophthora* poses to a world-leading botanic garden, such as RBGE, are potentially enormous. As knowledge of the threat has become clearer so better horticultural practices, assisted by scientific support and understanding, have developed. Inevitably such practices are more time consuming and labour intensive. They usually require investment in new equipment and resources. In current economic conditions, these constraints are extremely challenging. However, with the continued global movement of *Phytophthora*, which seems very likely to be aided by the effects of anthropogenic climate change, the conflict between oomycete (Fig. 33) and angiosperm (Fig. 34) appears to be deepening.

Figure 34. *Phytophthora* damage to *Betula papyrifera* within RBGE

Further investigation

There have undoubtedly been limitations in this investigation into the diversity of *Phytophthora* within RBGE. Some have been due to the relatively short period of investigation, given all samples had to be found and cultured before any further work progressed; others were due to weather conditions, inexperience or equipment shortcomings.

It seems highly likely that the inability to accurately and consistently regulate the incubation temperatures for the baited soil samples, water samples, and the PARP plates has, at best, been unhelpful, and more likely damaging in terms of the baiting and culturing.

That none of the tissue samples from symptomatic plants revealed the presence of *Phytophthora* does not necessarily mean that *Phytophthora* was absent; merely that the culturing process, and temperature range during collection and subsequent incubation did not permit growth. Ambient temperatures on the day of collection were high. Using a cool box with bagged ice was an attempt to limit the effect on the samples but may not have been adequate.

More time would have allowed a greater level of sub-culturing of the V8A plates. This would have allowed greater certainty that all the isolated cultures were a single strain and completely uncontaminated, particularly as experience in aseptic technique improved.

Notwithstanding these limitations the results have provided information on *Phytophthora* within RBGE that was not previously available. These results have also highlighted areas where further investigation would be helpful. Collecting more samples, and then culturing them for *Phytophthora* would allow new avenues to be explored. These could include:

- More precise determination of taxon identity where there is some ambiguity. This may, quite realistically, lead to the characterisation of new *Phytophthora* species.
- Utilising real time quantitative PCR methods to assess not only the presence, but importantly the intensity, of *Phytophthora* species within RBGE.
- Area mapping of *Phytophthora* species coverage may help inform future planting plans – i.e. selection of less susceptible host species, or at least avoidance on host species with known susceptibility.
- Investigating the population genetics of the *Phytophthora* spp. found would provide valuable insights into the way the Oomycete is behaving in the highly, but unnaturally, diverse environment of RBGE. It might also shed light on the origins of individual pathogens within RBGE, and help understand hybridisation within the genus.

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Appendix One – Soil samples

Plant communities are given as the immediate plant closest to the point of sampling, and major plants growing within an approximate 5m radius of the crown of the immediate plant, or where crown of plant encroaches on the 5m radius of the immediate plant. Accession number details are given where they were found. In cases where the accession has been propagated in bed E19 in the nursery and then planted out, the plant is indicated after the accession number. Taxonomic authorities from the RBGE Catalogue of the Living Collection.

A range of soil samples (012, 013, 014, 016, 022, 026, and 027) gave a strong positive PCR result for *Phytophthora*. The control pears did not give a *Phytophthora* result.

These results do not necessarily mean that *Phytophthora* was absent in the other samples, nor indeed that all species of *Phytophthora* in the original soil samples were successfully cultured; merely that the culturing process, and temperature range during incubation, only permitted growth in a limited number of instances. It is also possible that the ambient temperature on the day of collecting the soil samples had some impact.

Soil sample 001

- **Plant community**: *Sorbus aucuparia* L. (1998.0200H, dead), *Rhododendron ponticum* L. (1999.0746B), *Prunus padus* L. (1998.0199D), *Ilex* x *alterclerensis* (Hort. Ex Loudon) Dallim. 'Camellifolia' (1999.1051 A)
- Collected on 22v17; baited on 23v17.
- Bait removed to incubation box on 29v17 due to signs of water damage.
- No lesions developed on pear during incubation. Disposed of on 5vi17.
- No *Phytophthora* cultured from sample.

Soil sample 002

- **Plant community**: *Sorbus arranensis* Hedl. (1999.0040A), *Alnus glutinosa* (L.) Gaertn. (1998.0786B), *Juniperus communis* L. (1995.2725B) *J. communis* (1995.2717K, dead), *Prunus padus* (1998.0199A3), *Iris pseudocorus* L., *Heracleum spondylium* L..
- Collected on 22v17; baited on 23v17.
- Bait removed to incubation box on 29v17 due to signs of lesions.
- Lesions developed; tissue samples from pear plated onto PARP agar (MG2017 002) on 30v17.
- No *Phytophthora* cultured from sample.

Soil sample 003

- **Plant community**: *Prunus padus* (1998.0199 C1, C2 and C3), *Sorbus aucuparia* L. (1998.0200E), *Betula pendula* Roth, *Ilex aquifolium* L. seedlings, *Juniperus communis* (1938.0203C), *Pinus sylvestris* L. (1975.1732A), *Taxus baccata* L. seedlings.
- Collected on 22v17; baited on 23v17.
- Bait removed to incubation box on 31v17.
- Lesions well developed; tissue samples from pear plated onto PARP agar (MG2017 003) on 31v17.
- Plate MG2017 003 sub-cultured onto V8A (four plates) on 01vi17.
- Hyphae from V8A plates MG2017 003-1 and MG2017 003-3 isolated for DNA on 13vi17
- No *Phytophthora* cultured from sample.

Soil sample 004

- **Plant community**: *Betula pendula*, *Ulex europaeus* L., *Sorbus aucuparia*, *Olearia odorata* Petrie (1938.0034A), *Rhododendron kiusianum* Makino var. *kiusianum* (1921.0003B), *Ophiopogon* (1984.5019A). *Spiraea nervosa* Franch. & Sav. (2006.1421B), *Cotoneaster linearifolius* (G.Klotz) G.Klotz (1979,0416A), *Pinus sylvestris* L. 'Aurea' (1963.3820A)
- Collected on 22v17; baited on 23v17.
- Bait removed to incubation box on 31v17.
- No lesions developed on pear. Disposed of on 5vi17.
- No *Phytophthora* cultured from sample.

Soil sample 005

- **Plant community**: *Vaccinium uliginosum* L. (2007.1246A), *Arctostaphylos uva-ursi* (L.) Spreng. (2008.1480A), *Kalmia procumbens* (L.) Gift & Kron. (2008.0985A), *Salix reticulata* L. (2008.1193B), *Salix lanata* L. (2008.2037A and 2008.2027A), *Betula pendula*, *Salix arbuscula* L. (2007.0081B), *Salix lapponum* L. (2008.2059A, dead)
- Collected on 22v17; baited on 23v17.
- Bait removed to incubation box on 29v17 due to signs of lesions.
- Lesions developed; tissue samples from pear plated onto PARP agar (MG2017 005) on 01vi17.
- Plate MG2017 005 sub-cultured onto V8A (three plates) on 02vi17.
- PARP plate MG2017 005 to autoclave on 05vi17.
- Hyphae from V8A plates MG2017 005-1 and MG2017 005-3 isolated for DNA on 13vi17
- No *Phytophthora* cultured from sample.

Soil sample 006

- **Plant community**: *Helianthemum* 'Rhodanthe Carneum' (1969.4691C), *Ilex crenata* Thunb. Ex A.Murray 'Mariesii' (1936.1024A), *Chamaecyparis obtusa* (Siebold & Zucc) Endl. 'Pygmeae' (1965.3087D), *Aurinia saxatilis* (L.) Desv. (1990.1748A), *Betula pendula* Roth 'Tristis' (1968.7193B)

- Collected on 22v17; baited on 23v17.
- Bait removed to incubation box on 29v17 due to signs of lesions.
- Lesions developed; tissue samples from pear plated onto PARP agar (MG2017 006) on 01vi17.
- Plate MG2017 006 sub-cultured onto V8A (six plates) on 02vi17.
- PARP plate MG2017 006 to autoclave on 05vi17.
- Hyphae from V8A plates MG2017 006-7 isolated for DNA on 13vi17
- No *Phytophthora* cultured from sample.

Soil sample 007

- **Plant community:** *Betula utilis* D.Don var. *jacquemontii* (Spach) Winkl. (2012.0055A), *Berberis lijiangensis* C.Y.Wu ex S.Y.Bao(1994.4018E)
- Collected on 22v17; baited on 23v17.
- Bait removed to incubation box on 30v17 due to signs of lesions.
- Lesions well developed; tissue samples from pear plated onto PARP agar (MG2017 007) on 31v17.
- Plate MG2017 007 sub-cultured onto V8A (six plates) on 01vi17.
- PARP plate MG2017 007 to autoclave on 05vi17.
- Hyphae from V8A plates MG2017 007-3 and MG2017 007-5 isolated for DNA on 13vi17
- No *Phytophthora* cultured from sample.

Soil sample 008

- **Plant community:** *Thujopsis dolabrata* (Thunb. Ex L.f.) Siebold & Zucc. (1978.3563E), *Thuja plicata* D.Don (1969.9317A, 1986.1200A3 and 1986.1200A2), *Rhododendron yunnanense* Franch. (1977.4117A), *Osmanthus heterophyllus* (G.Don) P.S.Green (1966.5077B)
- Collected on 22v17; baited on 23v17.
- Bait removed to incubation box on 31v17.
- Lesions well developed; tissue samples from pear plated onto PARP agar (MG2017 008) on 05vi17.
- Plate MG2017 008 sub-cultured onto V8A (six plates) on 06vi17.
- PARP plate MG2017 008 to autoclave on 06vi17.
- Hyphae from V8 Aplate MG2017 008-2 isolated for DNA on 13vi17
- No *Phytophthora* cultured from sample.

Soil sample 009

- **Plant community:** *Pinus peuce* Griseb. (1925.1014A), *Thuja plicata* (1969.9316E and 1969.9317O), *Chamaecyparis lawsoniana* (A.Murray bis) Parl. (1968.7285I), *Cryptomeria japonica* (Thunb. Ex L.f.) D.Don (1975.1712A), *Rhododendron hanceanum* Hemsl. 'Nanum' (1944.0188A), *Podocarpus salignus* D.Don (1957.1001A)
- Collected on 22v17; baited on 23v17.
- Bait removed to incubation box on 31v17.
- No lesions developed on pear. Disposed of on 5vi17.

- No *Phytophthora* cultured from sample.

Soil sample 010

- **Plant community:** *Rhododendron*, *Picea sitchensis* (Bong.) Carière (1982.5052A), *Acer spicatum* Lam. (2010.1133A)
- Collected on 22v17; baited on 23v17.
- Bait removed to incubation box on 29v17 due to signs of lesions.
- Lesions well developed; tissue samples from pear plated onto PARP agar on 31v17 (MG2017 010A and MG2017 010B).
- Plate MG2017 010A sub-cultured onto V8A (5 plates) on 01vi17.
- Plate MG2017 010B sub-cultured onto V8A (five plates) on 01vi17.
- PARP plates MG2017 010A and MG2017 010B to autoclave on 05vi17.
- Hyphae from V8A plates MG2017 010A-1 and MG2017 010A-4 isolated for DNA on 13vi17
- Hyphae from V8A plates MG2017 010B-1, MG2017 010B-3 and MG2017 010B-5 isolated for DNA on 13vi17
- No *Phytophthora* cultured from sample.

Soil sample 011

- **Plant community:** *Betula papyrifera* Marshall (1963.3188A), *Carpinus betulus* L. 'Incisa' (1904.0011A), *Platanus* x *acerifolia* (Aiton) Willd. 'Hispanica' (1969.8196A), *Meliosma dillenifolia* (Wall. ex Wight & Arn.) Walp. ssp. *tenuis* (Maxim.) Beusekom (2006.1565B), *Fraxinus latifolia* Benth. (1986.1516A)
- Collected on 22v17; baited on 23v17.
- Bait removed to incubation box on 31v17.
- Lesions well developed; tissue samples from pear plated onto PARP agar (MG2017 011) on 05vi17.
- Plate MG2017 011 sub-cultured onto V8A (four plates) on 06vi17.
- PARP plate MG2017 011 to autoclave on 06vi17.
- Hyphae from V8A plates MG2017 011-1 and MG2017 011-2 isolated for DNA on 13vi17
- No *Phytophthora* cultured from sample.

Soil sample 012

- **Plant community:** *Pieris formosa* (Wall.) D.Don (2003.1602A), *Fraxinus angustifolia* Vahl var. *australis* (Gay) C.K.Schneid. (1968.7564A), *Syringa vulgaris* L. 'Nigricans' (1969.9292A), *Fraxinus excelsior* L. (1971.6101A).
- Collected on 22v17; baited on 23v17.
- Bait removed to incubation box on 31v17.
- Lesions developed; tissue samples from pear plated onto PARP agar (MG2017 012) on 01vi17.
- Plate MG2017 012 sub-cultured onto V8A (five plates) on 02vi17.
- PARP plate MG2017 012 to autoclave on 05vi17.
- Hyphae from V8A plates MG2017 012-3 and MG2017 012-3 isolated for DNA on 13vi17

- PCR from 19vi17 shows a positive result for *Phytophthora* from plate 012-2.
- Taxon identified as *Phytophthora plurivora*

Soil sample 013

- **Plant community:** *Rubus foliaceistipulatus* T.T.Yu & L.T.Lu (2005.1835A), *Potentilla fruticosa* L. (2003.1477A), *Syringa komarowii* C.K.Schneid. (1995.1056B), *Ostrya carpinifolia* Scop. (1996.5037A), *Sorbus caloneura* (Stapf) Rehder (1981.0837A)
- Collected on 22v17; baited on 23v17.
- Bait removed to incubation box on 29v17 due to signs of lesions.
- Lesions developed; tissue samples from pear plated onto PARP agar (MG2017 013) on 01vi17.
- Plate MG2017 013 sub-cultured onto V8A (five plates) on 02vi17, and one additional V8A plate on 05vi17.
- PARP plate MG2017 013 to autoclave on 05vi17.
- PCR from 19vi17 shows a positive result for *Phytophthora* from plate 013-6.
- Taxon identified as *Phytophthora ilicis*

Soil sample 014

- **Plant community:** *Fraxinus sogdiana* Bunge (1947.0116A), *Abies forrestii* Coltm.-Rog. var. *georgei* (Orr) Farjon (1994.4016D, 1994.4014G and 1994.3735Z), *Betula* aff. *utilis* (1994.4133R, T and V), *Berberis*, *Lonicera tangutica* Maxim. (1994.0722A), *Spiraea mongolica* Maxim. (1998.0131C) *Ligustrum delavayanum* Har. (2003.1545A), *Sorbus eburnean* McAll. (1994.0251M), *Acer franchelli* Pax (1997.0295B), *Ligustrum compactum* Hook.f. & Thomson (1915.1003A) *Cotoneaster* (2003.1547A), *Liquidambar styraciflua* L. (1923.1028E), *Syringa tormentella* Bureau & Franch. (1923.1028E -E19), *Betula utilis* D.Don (1996.0521K – E19)
- Collected on 22v17; baited on 23v17.
- Bait removed to incubation box on 31v17.
- Lesions developed; tissue samples from pear plated onto PARP agar (MG2017 014) on 01vi17.
- Plate MG2017 014 sub-cultured onto V8A (five plates) on 02vi17.
- PARP plate MG2017 014 to autoclave on 05vi17.
- PCR from 19vi17 shows a positive result for *Phytophthora* from plate 014-1.
- Taxon identified as *Phytophthora plurivora*

Soil sample 015

- **Plant community:** *Ilex aquifolium*, *Rubus fruticosus* L., *Solanum nigrum* L., *Rhododendron makinoi* Tagg (1938.0170A), *Acer davidii* Franch. ssp. *grossseri* (Pax) deJong (1997.0249A), *Platanus occidentalis* (1946.0096A), *Osmanthus delavayi* Franch. (1966.5073B)
- Collected on 22v17; baited on 23v17.
- Bait removed to incubation box on 25v17 due to water spoilage
- Lesions well developed; tissue samples from pear plated onto PARP agar (MG2017 015) on 31v17.

- Plate MG2017 015 sub-cultured onto V8A (two plates) on 05vi17.
- Hyphae from V8A plate MG2017 015-2 isolated for DNA
- No *Phytophthora* cultured from sample.

Soil sample 016

- **Plant community:** *Rhododendron wallichii* Hook.f. (1981.3602E), *Magnolia kobus* A.D.C. (2005.1975G – E19, and 1966.5007B), *Rhododendron thayerianum* Rehder & E.H.Wilson (1934.1012D), *Prunus serotina* Ehrh. (1985.1311A), *Liriodendron tulipifera* L. (1968.7757A)
- Collected on 22v17; baited on 23v17.
- Bait removed to incubation box on 31v17.
- Lesions developed; tissue samples from pear plated onto PARP agar (MG2017 016) on 01vi17.
- Plate MG2017 016 sub-cultured onto V8A (five plates) on 02vi17.
- PARP plate MG2017 016 to autoclave on 05vi17.
- Hyphae from V8A plates MG2017 016-1 and MG2017 016-5 isolated for DNA
- PCR from 15vi17 and 19vi17 show a positive result for *Phytophthora* from plates 016-1 and 016-5.
- Taxon identified as *Phytophthora plurivora*

Soil sample 017

- **Plant community:** *Ilex aquifolium* (1968.7676B), *Chamaecyparis pisifera* (Siebold & Zucc.) Endl. 'Filifera' (1973.4692A), B*uddleja globosa* Hope (1980.3533B), *Corylopsis sinensis* Hemsl. var. *calvescens* Rehder & E.H.Wilson (1997.3542A – E19), *Thuja plicata* (1969.9317A), *Rubus* –dead (2005.1826B, *Rhododendron* aff. *ririei* Hemsl. & E.H.Wilson (1973.4056E)
- Collected on 22v17; baited on 23v17.
- Bait removed to incubation box on 29v17 due to signs of lesions.
- No lesions developed on pear. Disposed of on 5vi17.
- No *Phytophthora* cultured from sample.

Soil sample 018

- **Plant community:** *Buxus sempervirens* L. (1975.4038A and H), *Chamaecyparis lawsoniana* (1987.5211D), *Rhododendron hunnewellainum* Rehder & E.H.Wilson ssp. *hunnewellianum* (1921.004A), *Ilex aquifolium, Diospyros kaki* L.f. (1983.1526A), *Trochodendron araloides* Siebold & Zucc. (1980.1760B)
- Collected on 22v17; baited on 23v17.
- Bait removed to incubation box on 29v17 due to signs of lesions.
- Lesions developed; tissue samples from pear plated onto PARP agar (MG2017 018) on 30v17.
- Plate MG2017 018 sub-cultured onto V8A (four plates) on 31v17.
- PARP plate MG2017 018 to autoclave on 01vi17.
- Hyphae from V8A plate MG2017 018-4 isolated for DNA on 13vi17
- No *Phytophthora* cultured from sample.

Soil sample 019

- **Plant community:** *Paeonia anomala* L. var. *intermedia* (C.A.Mey.) O.Fedtsch. & B.Fedtsch. (1957.0170A). *Acer pseudoplanatus* L., *Paeonia delavayi* Franch. x *lutea* Franch. (1951.0062A), *Tilia* x *europaea* L. (1969.9323J)
- Collected on 22v17; baited on 23v17.
- Bait removed to incubation box on 29v17 due to signs of lesions.
- Lesions developed; tissue samples from pear plated onto PARP agar on 30v17 (MG2017 019A and MG2017 019B).
- Plate MG2017 019A sub-cultured onto V8A (5 plates) on 31v17.
- Plate MG2017 019B sub-cultured onto V8A (five plates) on 31v17.
- PARP plates MG2017 019A and MG2017 019B to autoclave on 01vi17.
- Hyphae from V8A plates MG2017 019-3 and MG2017 019-4 isolated for DNA on 13vi17
- Hyphae from V8A plate MG2017 019B-5 isolated for DNA on 13vi17
- No *Phytophthora* cultured from sample.

Soil sample 020

- **Plant community:** *Rhododendron thomsonii* Hook.f. ssp. *thomsonii* (1937.0196C), *Fargesia murieliae* (Gamble) T.P.Yi (1992.5039A), *R. fulvum* Balf.f. & W.W.Sm. spp. *fulvoides* (Balf.f. & Forrest) D.F.Chamb. (1969.8595E), *Syringa emodi* (1985.1859F), *Vibirnum lantana* L., *Ilex*
- Collected on 22v17; baited on 23v17.
- Bait removed to incubation box on 31v17.
- Lesions developed; tissue samples from pear plated onto PARP agar (MG2017 020) on 05vi17.
- Plate MG2017 020 sub-cultured onto V8A (three plates) on 06vi17.
- PARP plate MG2017 020 to autoclave on 08vi17.
- Hyphae from V8A plates MG2017 020-1 and MG2017 020-3 isolated for DNA on 13vi17
- No *Phytophthora* cultured from sample.

Soil sample 021

- **Plant community:** *Quercus* x *rosacea* Bechst (1969.8322A), *Pinus tabuliformis* Hort. Ex Carière (1969.8187B), *Quercus velutina* Lam. (1936.0399A), *Pinus nigra* J.F.Arnold ssp. *pallasiana* (Lamb.) Holmboe (1969.8179C), *Ilex aquifolium* L. 'Handsworth New Silver' (1968.7660), *Quercus pontica* K.Koch (1969.8314A), *Quercus robur* L. ssp. *pedunculiflora* (C.Koch) Menitsky (1919.0013A), *Quercus ilhaburensis* Decne. ssp. *microlepsis* (Kotschy) Hedge & Yalt. (1969.8284A), *Quercus pubescens* Willd. (1969.8315A)
- Collected on 22v17; baited on 23v17.
- Bait removed to incubation box on 31v17. Showing lesions.
- Lesions well developed; tissue samples from pear plated onto PARP agar on 31v17 (MG2017 021A and MG2017 021B).
- Plate MG2017 021B sub-cultured onto V8A (five plates) on 01vi17.
- No sub-culturing was required from plate MG2017 021A since no hyphae developed from the plated pear samples.

- PARP plate MG2017 021B to autoclave on 05vi17.
- Hyphae from V8A plates MG2017 021B-3, MG2017 021B-4 and MG2017 021B-5 isolated for DNA on 13vi17
- No *Phytophthora* cultured from sample.

Soil sample 022

- **Plant community:** *Magnolia stellata* Maxim. (2006.1599B and C – E19), *Quercus pyrenaica* Willd. (2011.1933A – E19), *Pinus ponderosa* Dawson ex C.Lawson var *scopulorum* Engelm. (1969.8183A), *Lithocarpus densiflorus* (Hook. & Arn.) Rehder (1968.7759A), *Quercus robur* L. (1969.8318A), *Quercus robur* L. 'Pulverulenta' (1964.4085A)
- Collected on 22v17; baited on 23v17.
- Bait removed to incubation box on 29v17 due to signs of lesions.
- Lesions developed; tissue samples from pear plated onto PARP agar on 30v17 (MG2017 022A and MG2017 022B).
- Plate MG2017 022A sub-cultured onto V8A (six plates) on 01vi17, and further (three plates) on 02vi17.
- Plate MG2017 022B sub-cultured onto V8A (six plates) on 01vi17 and further (five plates) on 02vi17.
- PARP plates MG2017 022A and MG2017 022B to autoclave on 02vi17.
- Hyphae from V8A plates MG2017 022A-1 and MG2017 022A-4 isolated for DNA on 13vi17
- Hyphae from V8A plates MG2017 022B-3, MG2017 022B-6 and MG2017 022B-11 isolated for DNA on 13vi17
- PCR from 20vi17 shows positive results for *Phytophthora* from plates 022A-1, 022A-4, 022B-6 and 022B-11.
- Taxon identified as most likely *Phytophthora alni* subsp. *uniformis*

Soil sample 023

- **Plant community:** *Quercus frainetto* Ten. (1969.8293A), *Picea purpurea* Mast. (1969.8142A), *Nothofagus pumelo* (Poepp. & Endl.) Krasser (1967.1402A), *Quercus kelloggii* Newb. (1915.0033A), *Fagus sylvatica* L. 'Zlatia' (1973.4007A), *Quercus rubra* L. (1935.0109A), *Quercus variablis* Blume (1969.8283B)
- Collected on 22v17; baited on 23v17.
- Bait removed to incubation box on 29v17 due to signs of lesions.
- No lesions developed on pear. Disposed of on 5vi17.
- No *Phytophthora* cultured from sample.

Soil sample 024

- **Plant community:** *Nothofagus pumelo* (1967.1402A), *Picea purpurea* (1969.8142A), *Fagus sylvatica* L. 'Aspenifolia' (1968.7544A), *Quercus glandulifera* Blume (1970.2531A), *Q. pubescens* (1969.8297A)
- Collected on 22v17; baited on 23v17.
- Bait removed to incubation box on 31v17.

- Lesions developed; tissue samples from pear plated onto PARP agar (MG2017 024) on 01vi17.
- Plate MG2017 024 sub-cultured onto V8A (four plates) on 02vi17.
- PARP plate MG2017 024 to autoclave on 05vi17.
- Hyphae from V8A plates MG2017 024-1 and MG2017 024-3 isolated for DNA on 13vi17
- Hyphae from V8A plates MG2017 024-1 and MG2017 024-3 isolated for DNA on 13vi17
- No *Phytophthora* cultured from sample.

Soil sample 025

- **Plant community:** *Fagus grandifolia* Ehrh. (1957.0262A), *Berberis souliena* C.K.Schneid. (1982.5022A and 1917.1008A), *Larix gmelinii* var *japonica* (1915.1010A), *B. hamiltoniana* Ahrendt (1953 1007A), *B. francisci-ferdinandi* C.K.Schneid. (1918.0046A), *Pinus koraiensis* (1937.0258A), *Quercus palustris* (1906.1009A), *B. darwinii* (1976.1087A), *P. brachytyla* var *brachytyla* (1980.2525A), *Berberis lempergiana* Ahrendt (1944.1005A), *Quercus acutissima* Carruth. (1978.4154A), *F. sylvatica* 'Aspenifolia' (1968.7544A)
- Collected on 22v17; baited on 23v17.
- Bait removed to incubation box on 31v17.
- Lesions developed; tissue samples from pear plated onto PARP agar (MG2017 025) on 01vi17.
- Plate MG2017 025 sub-cultured onto V8A (five plates) on 02vi17.
- PARP plate MG2017 025 to autoclave on 05vi17.
- Hyphae from V8A plates MG2017 025-2 and MG2017 025-3 isolated for DNA on 13vi17
- No *Phytophthora* cultured from sample.

Soil sample 026

- **Plant community:** *Euonymus sieboldianus* Blume (2006.1309A), *Sorbus commixta* Hedl. (2005.1902B – E19), *Juniperus communis* (2007.1541B), *Torreya taxifolia* Arn (2002.2356F), *Abies homolepsis* Siebold & Zucc. var *umbrellata* (Mayr) E.H.Wilson (1915.1007A)
- Collected on 22v17; baited on 23v17.
- Bait removed to incubation box on 29v17 due to signs of lesions.
- Lesions developed; tissue samples from pear plated onto PARP agar on 30v17 (MG2017 026A and MG2017 026B).
- Plate MG2017 026A sub-cultured onto V8A (four plates) on 31v17, and further (two plates) on 01vi17.
- Plate MG2017 026B sub-cultured onto V8A (three plates) on 31v17.
- PARP plates MG2017 026A and MG2017 026B to autoclave on 01vi17.
- Hyphae from V8A plates MG2017 026A-1, MG2017 026A-2, MG2017 026A-3 and MG2017 026A-4 isolated for DNA on 13vi17
- Hyphae from V8A plates MG2017 026A-5, MG2017 026B-2 and MG2017 026B-3 isolated for DNA on 20vi17

- PCR from 21vi17 shows positive results for *Phytophthora* from plates 026A-1, 026A-2, 026A-3, 026A-4, 026A-5, 026B-2 and 026B-3.
- Taxon identified as almost certainly *Phytophthora gonapodyides*

Soil sample 027

- **Plant community:** *Castanea dentata* (Marshall) Borkh. (1940.1006A), *Staphylea bumalda* D.C. (2007.1301D), *Cercidiphyllum japonicum* Siebold & Zucc. (2006.1584I – E19), *Myrica californica* Cham. (2009.1875A), *Sambucus tigranii* Troitsky (2009.1805D), *Fitzroya cupressoides* (Molina) I.M.Johnst. (1958.8381K), *Cornus* (2010.1844B and C), *Helwingia japonica* (Thumnb.) F.Dietr. (dead) (2007.1361B), *Abies spectabilis* (D.Don) Spach. (1983.2576A), *Polystichum acrostichoides* (Michx.) Schott (2009.006C), *Sorbus commixta* (2005.1278A), *Malus* (2013.2167), *Pinus balfouriana* Balf. ssp. *austrina* R.Mastrogiuseppe & J.Mastrogiuseppe (1969.8161B), *Cephalotaxus harringtonii* (Forbes) K.Koch 'Fastigata' (1968.7278A), *Betula alleghianiensis* Britton (2008.1960P)
- Collected on 22v17; baited on 23v17.
- Bait removed to incubation box on 29v17 due to signs of lesions.
- Lesions developed; tissue samples from pear plated onto PARP agar on 30v17 (MG2017 027A and MG2017 027B).
- Plate MG2017 027A sub-cultured onto V8A (seven plates) on 01vi17, and further (five plates) on 02vi17.
- Plate MG2017 027B sub-cultured (five plates) on 31v17.
- PARP plate MG2017 027A to autoclave on 02vi17, and plate MG2017 027B on 31v17.
- Hyphae from V8A plates MG2017 027A-3, MG2017 027A-6, MG2017 027A-8 and MG2017 027A-11 isolated for DNA on 20vi17
- Hyphae from V8A plate MG2017 027B-2 isolated for DNA on 20vi17
- PCR from 21vi17 shows positive results for *Phytophthora* from plates 027A-3, 027A-6, 027A-8, 027A-11 and 027B-2.
- Taxon identified as *Phytophthora cambivora*

Soil sample 028

- **Plant community:** *Taxus baccata* L. 'Fastigata' (2007.1849O – E19), *Ilex aquifolium*, *Tilia* (outside RBGE), *Ulmus* (possibly *glabra*), *Fraxinus*, *Pinus tabuliformis* (1969.8187C), *Tsuga canadensis* (L.) Carière (2008.1942H), *Abies*, *Spiraea japonica* L.f. var. *acuta* T.T.Yu (1994.1196G), an Araliaceae
- Collected on 22v17; baited on 23v17.
- Bait removed to incubation box on 29v17 due to signs of lesions.
- Lesions developed; tissue samples from pear plated onto PARP agar (MG2017 028) on 30v17.
- No *Phytophthora* cultured from sample.

Control 1

- Bait added to 200ml deionised and filtered water on 23v17.
- Removed to incubation box on 30v17 due to signs of water damage.

- No lesions developed on pear. Disposed of on 5vi17.
- No *Phytophthora* cultured from the control.

Control 2

- Bait added to 200ml deionised and filtered water on 23v17.
- Removed to incubation box on 31v17.
- No lesions developed on pear. Disposed of on 5vi17.
- No *Phytophthora* cultured from the control.

Control 3

- Bait added to 200ml deionised and filtered water on 23v17.
- Water splitting visible on 29v17 so pear removed to incubation box.
- Tissue samples from the pear were plated onto PARP on 31v17. By 08vi17 there was no sign of anything growing on the culture plate.
- No *Phytophthora* cultured from the control.

Appendix Two - Tissue samples

No tissue samples gave a positive result for *Phytophthora*. This does not necessarily mean that *Phytophthora* was absent; merely that the culturing process, and temperature range during incubation, did not permit growth.

Tissue sample 201

Rhododendron meddianum Forrest var. *atrokermesinum* Tagg

- Collected on 25v17.
- Plated onto PARP agar (MG2017 201) on 26v17.
- Sufficient signs of hyphal activity to sub-culture onto V8A (four plates) on 06vi17.
- PARP plate MG2017 201 autoclaved on 13vi17

Tissue sample 202

Rhododendron meddianum var. *atrokermesinum*

- Collected on 25v17.
- Plated onto PARP agar (MG2017 202) on 26v17.
- PARP plate MG2017 202 autoclaved on 13vi17

Tissue sample 203

Chrysolepis chrysophylla (Douglas ex Hook.) Hjelmq. 'Obovata'

- Collected on 25v17.
- Plated onto PARP agar (MG2017 203) on 26v17.
- PARP plate MG2017 203 autoclaved on 13vi17

Tissue sample 204

Ilex spp.

- Collected on 25v17.
- Plated onto PARP agar on 26v17 (MG2017 204A and MG2017 204B).
- Sufficient signs of hyphal activity on plate MG2017 204A to sub-culture onto V8A (one plate) on 06vi17.
- PARP plates MG2017 204A and MG2017 204B autoclaved on 13vi17

Tissue sample 205

Ilex aquifolium sapling

- Collected on 25v17.
- Plated onto PARP agar (MG2017 205) on 26v17.
- Sufficient signs of hyphal activity to sub-culture onto V8A (one plate) on 08vi17.
- PARP plate MG2017-205 autoclaved on 13vi17

Tissue sample 206

- Collected on 25v17.
- Plated onto PARP agar (MG2017 206) on 26v17.
- PARP plate MG2017-206 autoclaved on 13vi17
- Hyphae from V8A plate MG2017 206-4 isolated for DNA on 20vi17

- No *Phytophthora* cultured from sample.

Tissue sample 207

Rhododendron aff. *arboreum* Sm. hybrid

- Collected on 25v17.
- Plated onto PARP agar (MG2017 207) on 26v17.
- PARP plate MG2017-207 autoclaved on 13vi17

Tissue sample 208

Rubus spp.

- Collected on 25v17.
- Plated onto PARP agar (MG2017 208) on 26v17.
- Sufficient signs of hyphal activity to sub-culture onto V8A (six plates) on 06vi17.
- PARP plate MG2017-208 autoclaved on 13vi17

Tissue sample 209

Rhododendron wallichii Hook.f.

- Collected on 25v17.
- Plated onto PARP agar (MG2017 209) on 26v17.
- PARP plate MG2017-209 autoclaved on 13vi17

Tissue sample 210

Rhododendron argyrophyllum Franch. ssp. *nankingense* (Cowan) D.F.Chamb.

- Collected on 25v17.
- Plated onto PARP agar (MG2017 210) on 26v17.
- Sufficient signs of hyphal activity to sub-culture onto V8A (one plate) on 06vi17.
- PARP plate MG2017-210 autoclaved on 13vi17

Tissue sample 211

Trochodendron aralioides Siebold & Zucc.

- Collected on 25v17.
- Plated onto PARP agar (MG2017 211) on 26v17.
- PARP plate MG2017-211 autoclaved on 13vi17

Tissue sample 212

Kamia latifolia L.

- Collected on 25v17.
- Plated onto PARP agar on 26v17 (MG2017 212A and MG2017 212B)
- PARP plate MG2017-212 autoclaved on 13vi17

Tissue sample 213

Rhododendron thomsonii Hook.f. hybrid

- Collected on 25v17.
- Plated onto PARP agar (MG2017 213) on 26v17.
- PARP plate MG2017-213 autoclaved on 13vi17

Tissue sample 214

Lithocarpus densiflorus

- Collected on 25v17.
- Plated onto PARP agar (MG2017 214A and MG2017 214B) on 26v17.
- PARP plates MG2017 214A and MG2017 214B autoclaved on 13vi17

Tissue sample 215

Quercus coccifera L.

- Collected on 25v17.
- Plated onto PARP agar (MG2017 215) on 26v17.
- PARP plate MG2017-215 autoclaved on 13vi17

Tissue sample 216

Quercus pyrenaica Willd.

- Collected on 25v17.
- Plated onto PARP agar (MG2017 216) on 26v17.
- PARP plate MG2017-216 autoclaved on 13vi17

Tissue sample 217

Trochodendron aralioides

- Collected on 25v17.
- Plated onto PARP agar (MG2017 217) on 26v17.
- PARP plate MG2017-217 autoclaved on 13vi17

Tissue sample 218

Polystichum acrostichoides (Michx.) Schott

- Collected on 25v17.
- Plated onto PARP agar (MG2017 218) on 26v17.
- PARP plate MG2017-218 autoclaved on 13vi17

Appendix Three - Water samples

Two water samples (104 and 108) gave a strong positive PCR result for *Phytophthora*; there were also two samples (102 and 106) which gave inconclusive PCR results. The control pears did not give a *Phytophthora* result.

These results do not necessarily mean that *Phytophthora* was absent in the other samples, nor indeed that all species of *Phytophthora* in the samples were successfully cultured; merely that the culturing process, and temperature range during incubation, only permitted growth in a limited number of instances.

Water sample 101

- Collected on 01vi17. Baited on 02vi17.
- Bait removed from water sample with water splits on 12vi17
- Plated onto PARP agar (MG2017 101A and MG2017 101B) on 12vi17.
- No *Phytophthora* cultured from sample.

Water sample 102

- Collected on 01vi17. Baited on 02vi17.
- Bait removed to incubation box on 09vi17 due to water spoil and signs of lesions.
- Plated onto PARP agar (MG2017 102) on 12vi17.
- Hyphae from V8A plate MG2017 102-3 isolated for DNA on 20vi17
- Tentative *Phytophthora* response from plate 102-3.

Water sample 103

- Collected on 01vi17. Baited on 02vi17.
- Bait removed from water sample with water damage on 12vi17
- Plated onto PARP agar (MG2017 103) on 12vi17.
- No *Phytophthora* cultured from sample.

Water sample 104

- Collected on 01vi17. Baited on 02vi17.
- Bait removed to incubation box on 06vi17 due to water spoil and signs of lesions.
- Plated onto PARP agar (MG2017 104) on 07vi17.
- Plate MG2017 104 sub-cultured onto V8A (five plates) on 09vi17.
- Plate MG2017 104 sub-cultured onto V8A (four plates) on 12vi17.
- PARP plate MG2017 104 to autoclave on 12vi17.
- Hyphae from V8A plates MG2017 104-3, MG2017 104-5 and MG2017 104-7 isolated for DNA on 20vi17
- PCR from 21vi17 shows a positive result for *Phytophthora* from plates 104-3 and 104-7.
- Taxon identified as *Phytophthora gonapodyides*

Water sample 105

- Collected on 01vi17. Baited on 02vi17.
- Bait removed to incubation box on 09vi17 due to signs of lesions.
- Plated onto PARP agar (MG2017 105) on 12vi17.
- Plate MG2017 105 sub-cultured onto V8A (two plates) on 13vi17.
- Hyphae from V8A plate MG2017 105-1 isolated for DNA on 20vi17
- No *Phytophthora* cultured from sample.

Water sample 106

- Collected on 01vi17. Baited on 02vi17.
- Bait removed to incubation box on 07vi17 due to water spoil and signs of lesions.
- Plated onto PARP agar (MG2017 106) on 08vi17.
- Plate MG2017 106 sub-cultured onto V8A (two plates) on 12vi17.
- PARP plate MG2017 106 to autoclave on 12vi17.
- Hyphae from V8A plate MG2017 106-1 isolated for DNA on 20vi17
- Tentative *Phytophthora* response from plate 106-1.

Water sample 107

- Collected on 01vi17. Baited on 02vi17.
- Bait removed to incubation box on 09vi17 due to water splits at base.
- Plated onto PARP agar (MG2017 107) on 12vi17.
- No *Phytophthora* cultured from sample.

Water sample 108

- Collected on 01vi17. Baited on 02vi17.
- Bait removed to incubation box on 07vi17 due to water spoil and signs of lesions.
- Plated onto PARP agar (MG2017 108 and MG2017 108A) on 08vi17.
- Plate MG2017 108 sub-cultured onto V8A (one plate) on 09vi17.
- Plate MG2017 108A sub-cultured onto V8A (four plates) on 12vi17.
- Plate MG2017 108 sub-cultured onto V8A (further seven plates) on 12vi17.
- Plate MG2017 108A sub-cultured onto V8A (further five plates) on 13vi17.
- Hyphae from V8A plates MG2017 108-1, MG2017 108A-1 and MG2017 108A-4 isolated for DNA on 20vi17
- PCR from 21vi17 shows a positive result for *Phytophthora* from plates 108-1, 108A-1 and 108A-4.
- Taxon identified as *Phytophthora gallica*

Water sample 109

- Collected on 01vi17. Baited on 02vi17.
- Bait removed to incubation box on 09vi17 due to large soft lesion on pre-existing blemish.
- Plated onto PARP agar (MG2017 109) on 12vi17.
- Plate MG2017 109 sub-cultured onto V8A (four plates) on 13vi17.
- Hyphae from V8A plate MG2017 109-2 isolated for DNA on 20vi17
- No *Phytophthora* cultured from sample.

Water sample 110

- Collected on 01vi17. Baited on 02vi17.
- Bait removed to incubation box on 09vi17 due to water splits at base.
- Plated onto PARP agar (MG2017 120) on 12vi17.
- No *Phytophthora* cultured from sample.

Water sample 111

- Collected on 01vi17. Baited on 02vi17.
- Bait removed to incubation box on 06vi17 due to water spoil and signs of lesions.
- Plated onto PARP agar (MG2017 111) on 07vi17.
- Plate MG2017 111 sub-cultured onto V8A (three plates) on 09vi17.
- Hyphae from V8A plates MG2017 11-1 and MG2017 11-3 isolated for DNA on 20vi17
- No *Phytophthora* cultured from sample.

Water sample 112

- Collected on 01vi17. Baited on 02vi17.
- Plated onto PARP agar (MG2017 112) on 12vi17.
- No *Phytophthora* cultured from sample.

Water sample 113

- Collected on 01vi17. Baited on 02vi17.
- Bait removed from water sample with water spoil at base on 12vi17
- Plated onto PARP agar (MG2017 113) on 12vi17.
- No *Phytophthora* cultured from sample.

Water sample 114

- Collected on 01vi17. Baited on 02vi17.
- Bait removed to incubation box on 08vi17 due to water splits at base.
- Plated onto PARP agar (MG2017 114) on 08vi17.
- No *Phytophthora* cultured from sample.

Water sample 115

- Collected on 01vi17. Baited on 02vi17.
- Bait removed from water sample on 12vi17
- Plated onto PARP agar (MG2017 115) on 12vi17.
- No *Phytophthora* cultured from sample.

Control 4

- Bait added to 200ml deionised and filtered water on 02vi17.
- Bait removed to incubation box on 09vi17 due to water splits at base.
- Plated onto PARP agar (MG2017 Ctrl 4) on 12vi17.
- No *Phytophthora* cultured from sample.

Control 5

- Bait added to 200ml deionised and filtered water on 02vi17.
- Bait removed to incubation box on 06vi17 due to water splits at base.
- Plated onto PARP agar (MG2017 Ctrl5) on 08vi17.
- No *Phytophthora* cultured from sample.

Appendix Four - E19 trace-forward plants in beds crossed by transect line.

Bed	Taxon	Accession	Identifier
B01	*Alnus fauriei*	2007	1561
B01	*Alnus firma*	2007	1317
B01	*Alnus firma*	2005	1905
B01	*Betula grossa*	2006	1408
B01	*Chamaecyparis obtusa*	1968	7303
B01	*Cryptomeria japonica* var. *sinensis*	1949	0126
B01	*Cupressus macrocarpa*	2009	0071
B01	*Cupressus sargentii*	2009	0070
B01	*Lithocarpus densiflorus*	2008	0001
B01	*Magnolia kobus*	2007	1276
B01	*Mallotus japonicus*	2006	1435
B01	*Neoshirakia japonica*	2005	2173
B01	*Sorbus commixta*	2005	1902
B01	*Sorbus commixta*	2007	1394
B01	*Zanthoxylum ailanthoides*	2005	0056
B03	*Carpinus kawakamii*	2007	0817
B03	*Carya ovata*	2008	1873
B03	*Fitzroya cupressoides*	1958	8381
B04	*Carpinus caroliniana*	2008	1879
B04	*Fitzroya cupressoides*	1958	8381
C01	*Acer australe*	2005	2165
C01	*Acer sieboldianum*	2006	1369
C01	*Magnolia stellata*	2007	1286
C01	*Magnolia stellata*	2006	1599
C01	*Quercus cerris*	2011	2240
C01	*Quercus coccifera*	2004	1959
C01	*Quercus coccifera*	2005	2392
C01	*Quercus macranthera* ssp. *syspirensis*	2004	1955
C01	*Quercus macranthera* ssp. *syspirensis*	2004	1958
C01	*Quercus macrolepis*	2009	1410
C01	*Quercus mongolica*	2006	1376
C01	*Quercus muehlenbergii*	2007	0962
C01	*Quercus petraea*	2005	2413
C01	*Quercus pyrenaica*	2011	1933
C01	*Quercus serrata*	2005	2012
C01	*Quercus serrata*	2005	2012
C01	*Quercus trojana*	2005	2403
C01	*Quercus variabilis*	2007	1588
C01	*Quercus variabilis*	2006	1462

C01	*Styrax japonicus*	2007	1363
F05/N	*Acer crataegifolium*	2005	2132
F05/N	*Acer rufinerve*	2006	1460
F05/N	*Betula utilis*	2004	0965
F05/N	*Juglans californica*	2008	0094
F05/N	*Magnolia kobus*	2007	1276
F05/N	*Magnolia stellata*	2007	1286
F05/N	*Magnolia stellata*	2007	1286
F05/N	*Pterostyrax hispida*	2007	1284
F05/S	*Quercus prinus*	2007	0964
F11	*Magnolia stellata*	2005	2138
F11	*Thuja plicata*	1969	9317
F11	*Thuja plicata x standishii*	1974	1830
F11	*Thuja plicata x standishii*	1969	9317
F11	*Thuja plicata x standishii*	1969	9317
F12	*Piptanthus nepalensis*	2014	1177
F12	*Rhus javanica* var. *roxburghiana*	2006	1442
F15/S	*Magnolia salicifolia*	2005	2023
H22	*Acer*	2001	1100
H22	*Acer*	2008	1536
H22	*Castanea sativa*	2008	1546
H22	*Platanus orientalis*	2012	0696
H22	*Syringa tomentella*	1923	1028
H25	*Spiraea*	1991	0520
H27	*Corylopsis sinensis* var. *calvescens*	1997	3542
K02	*Alnus firma*	2006	1391
K02	*Alnus firma*	2006	1536
K02	*Alnus japonica*	2006	1441
K02	*Cornus* cf. *controversa*	2007	1496
K02	*Cornus macrophylla*	2006	1385
K02	*Cornus racemosa*	2008	1877
K02	*Corylus avellana*	2008	1523
K02	*Corylus avellana*	2008	1523
K02	*Corylus avellana*	2008	1523
K02	*Corylus avellana*	2008	1523
K02	*Corylus heterophylla*	2007	1281
K02	*Corylus sieboldiana*	2007	1444
K02	*Corylus sieboldiana*	2005	1954
K02	*Euptelea polyandra*	2007	1565
K02	*Frangula alnus*	2004	0005
K02	*Fraxinus americana*	2006	0366
K02	*Fraxinus americana*	2008	1866

K02	*Fraxinus angustifolia*	2010	0570
K02	*Fraxinus apertisquamifera*	2007	1420
K02	*Fraxinus dipetala*	2008	0822
K02	*Fraxinus lanuginosa* forma *serrata*	2005	2156
K02	*Fraxinus pennsylvanica*	2011	1935
K02	*Lindera benzoin*	2009	0744
K02	*Lindera benzoin*	2010	1061
K02	*Lindera obtusiloba*	2005	2098
K02	*Lindera sericea*	2006	1354
K02	*Lindera triloba*	2005	2155
K02	*Mallotus japonicus*	2006	1435
K02	*Meliosma dilleniifolia* ssp. *tenuis*	2006	1565
K02	*Populus maximowiczii*	1924	1015
K02	*Populus nigra* 'Plantierensis'	1969	8212
K02	*Populus nigra* var. *betulifolia (female)*	1969	8209
K02	*Populus x canadensis* 'Eugenei'	1969	8202
K02	*Rhamnus grandifolia*	2005	1737
K02	*Salix*	2008	2011
K02	*Salix fragilis*	2008	2009
M21	*Acer laxiflorum* var. *longilobum*	2007	1770
M21	*Euptelea*	2002	2291
M21	*Sorbus commixta*	2003	1211
M22	*Chamaecyparis lawsoniana*	1968	7285
M22	*Chamaecyparis lawsoniana*	1968	7285
M22	*Chamaecyparis lawsoniana*	1968	7285
M22	*Chamaecyparis obtua*	1968	7303
M22	*Magnolia kobus*	2005	1969
M22	*Magnolia salicifolia*	2005	2023
M22	*Magnolia sieboldii*	2005	2109
R02	*Betula calcicola*	2005	0411
R23	*Carpinus laxiflora*	1994	2830
U09	*Juniperus communis* 'Barradalensis'	1965	3282
W02/E	*Acer pictum* ssp. *trichobasis*	2012	0694