An Emergent Pathogen,

Phytophthora ramorum,

in Irish Woodlands

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A thesis submitted in partial fulfilment

of the requirements for the Degree of Doctor of Philosophy

Galway-Mayo Institute of Technology

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Supervisor: Dr. Elizabeth Dowling

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I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of PhD is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed: Carmel O'Connor **Candidate**

ID No.: _____

Date: 3/8/10

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Carmel O' Connor

Abstract

Phytophthora kernoviae and *Phytophthora ramorum* are both recently discovered pathogens of tree species. The disease that causes *P. ramorum* has been given the common name of Sudden Oak Death, in the US, because the expression of symptoms appears across wide swathes of forest or woodland very near the time of death of trees. Their principal host in Europe is rhododendron, primarily *Rhododendron ponticum*, which is widely distributed throughout many of Ireland's oldest oak forests. In 2003, *P. ramorum* was found, by the Department of Agriculture, Fisheries and Food (DAFF) on *Rhododendron ponticum* in the wild. Since spring 2005, sampling in this study has been carried out on susceptible foliage and in soil and watercourses from 11 susceptible forest sites in Ireland. Identification was carried out using a rapid DNA method in conjunction with morphological identification methods. Each site was sampled twice a year (2005-2008) by collecting foliage and using water and soil baits. The pathogen was identified at three sites in SW Ireland: Killarney National Park, Co. Kerry, Lauragh, Co. Kerry, and Castletownbere, Co. Cork, and was not isolated from oak trees.

Genetic variation was compared between Irish forest and nursery populations of *P. ramorum* using eight microsatellite loci. All 93 isolates had the same multilocus genotype (MG) indicating no genetic differences between wild and nursery isolates.

Up to 2008, *P. kernoviae* had only been reported in the UK, but has now been identified at two locations in New Zealand. Protocols have been developed that unequivocally discriminate the two pathogens from all other *Phytophthora* plant pathogens. Using real-time PCR, samples collected from the 11 susceptible forest sites, over the three-year period, were analysed for the presence of *P. kernoviae* and no positives in a total of 205 samples were found.

Susceptibility analyses indicated that the Irish isolate of *P. ramorum*, K2, can infect the leaves of rhododendron (*R. ponticum)*, fuschia (*Fuschia magellanica),* holly (*Ilex aquifolium),* sessile oak (*Quercus petraea)* and the strawberry tree (*Arbutus unedo)*. Additional studies on the long-term survival potential of *P. ramorum* showed that the chlamydospores of K2 are capable of surviving at temperatures of 4-20°C and in a pH range of 3-9, conditions that prevail in the soil in SW Ireland throughout the year. Extreme temperatures of -21 and 40°C and pH 2 are not suitable for the long-term survival of the pathogen.

CHAPTER 1

PHYTOPHTHORA RAMORUM

1.1. INTRODUCTION

Phytophthora ramorum is a member of the Oomycete genus *Phytophthora*, which encompasses over 67 species identified on morphological criteria. Most of these are plant pathogens, which are responsible for some of the world's most destructive diseases of crops and native vegetation (Brasier 1992). The most common example is *Phytophthora infestans*, which was the primary cause of the potato famine in Ireland in the 1840s. *P. infestans* has a devastating potential as a pathogen because it is polycyclic and can produce inoculum continuously after the initial infection, as long as conditions remain favourable (Andrianaivo 2008). *P. ramorum* was first discovered on oak trees in California between 1994 and 1995. The pathogen was given the name Sudden Oak Death (SOD) due to the way it infects and rapidly kills a large number of plant and tree species, which mainly includes oak (*Quercus*) spp. SOD was first described in Europe in 1993, in both Germany and The Netherlands (Werres et al. 2001). It was described as a twig blight disease occurring in nurseries and occasionally on large bushes. Relatively little is known about this SOD and there are concerns, particularly in Europe and North America, regarding its potential to have serious ecological, social, and economic impacts.

The symptoms that defined Sudden Oak Death (SOD) were first recognised in California between 1994 and 1995 (Rizzo and Garbelotto 2003), but again it was not immediately known what family the disease-causing organism was from. It was not until December 2000 that scientists were able to isolate the primary causal agent in Europe (Van Leeuwen et al. 2003) and give it its name, *P. ramorum*. It was in 2001 that Californian researchers, along with Clive Brasier from Alice Holt Lodge Laboratory in England, became aware of some of the morphological similarities of the North American and European isolates (Garbelotto 2003). It became clear that it was a species of Oomycete, fungus-like organisms, which are more closely related to brown algae and kelp than to 'perfect' fungi (Garbelotto 2003). The two isolates differed morphologically, genetically, and in their pathology and growth. Once the connection was made between them and further research was completed, the list of hosts grew and continues to do so, even to the present day.

1.2. LIFE CYCLE

The remarkable success of *Phytophthora* species can be accredited to their plasticity and tolerance of environmental change. *P. ramorum* has an optimum growth temperature of 20°C and a minimum and maximum temperature for growth of 2 and 30°C, respectively. It prefers areas with wet climate and constant mild temperatures for optimal disease development (Sansford et al. 2003). It spreads mostly aerially and usually infects trees and plants above the soil line.

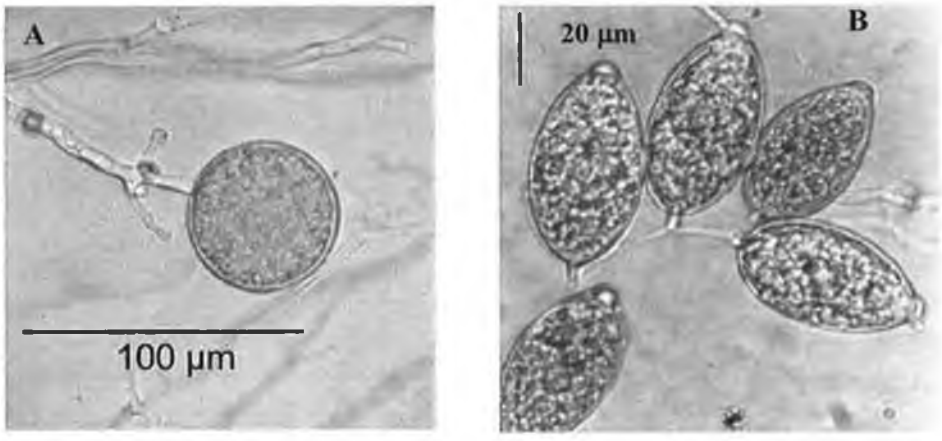

Figure 1.1. *Phytophthora ramorum* structures: A. Chlamydospores B. Semi-papillate, deciduous sporangia (http://www.plantenziektekunde.nl/pramorum).

Infection can occur through natural openings and wounds on plant tissue. Two different types of asexual spores are formed: sporangia and chlamydospores (Fig. 1.1). These can be formed on or within the plant tissue (Davidson et al. 2001). *P. ramorum* readily forms sporangia that are highly deciduous and adapted for aerial dispersal, which is thought to be the main mode of pathogen dissemination. The high number of sporangia produced by *P. ramorum* is key to driving the epidemic (Denman et al. 2007). Most sporangia production tends to occur during the rainy season and a small amount of water on the plant surface for several hours appears to aid the infection process (Garbelotto 2004). During wet weather, the sporangia on the susceptible host release a large number of zoospores, which then infect the leaf of the plant and cause leaf blight

and dieback of the host species. Since sporangia production is the stage responsible for dispersal and infection of host tissue, sporangia and zoospores are the primary propagules that drive the disease caused by *P. ramorum* (Davidson et al. 2005). *P. ramorum* does not form sporangia on bole cankers but does produce inoculum on infected foliage and stem cankers of certain host species (Brasier et al. 2004). Examples of these are tan oak (*Lithocarpus densiflorus*), Coast redwood (*Sequoia sempervirens*) and Rhododendron. The production of spores on foliar and shrub hosts underlines their importance as platforms from which disease epidemics are driven (Denman et al. 2007). In *P. ramorum* the chlamydospores are considered to play a role in the survival of the pathogen, acting as resting structures that allow the pathogen to survive either during unfavourable climates or when susceptible hosts are not present (Garbelotto 2004). When environmental conditions are conducive for vegetative growth, the chlamydospores can germinate and create new colonies vegetatively or through the production of sporangia and subsequent release of zoospores (Smith and Hansen 2007). The final and essential step in the reproductive cycle involves successful infection of new host tissue (Davidson et al. 2003a). Whether the disease successfully proceeds any further can sometimes depend on the host. *P. ramorum* is only mildly virulent on some foliar and shrub hosts, and an advantage of this, for the pathogen, is the build up of a high inoculum load along with the assurance of its long-term survival in most woodland (Garbelotto 2003). An example of such a host is Bay laurel (*Umbellularia californica*) which is a foliar host that exhibits a certain level of resistance. In California, Bay laurel may be one of the most important foliar hosts of SOD due to its observed ability to produce inoculum and its high abundance in the woodlands of coastal California (Anacker et al. 2007). Bay laurel also appears to vary in its susceptibility to *P. ramorum*. A study carried out by Anacker et al. (2007) showed that susceptibility of Bay laurel to *P. ramorum* depends on genetic, phenotypic, and environmental characteristics, as well as *P. ramorum* isolate virulence. Another example is Rhododendron, a highly susceptible shrub host (Denman et al. 2005), which could possibly be a link between the ornamental industry and the wild land components of *P. ramorum* (Garbelotto 2004). These foliar and shrub hosts are important in providing inoculum for initiating and maintaining tree disease epidemics.

From the shrub hosts, the spores can be splashed from the leaf surface onto the bark, stem or leaves of nearby foliar hosts and begin a new infection (Davidson et al. 2003a). Spores landing in streams or irrigation runoff have the potential to travel long distances (Davidson et al. 2003a). Once the outer bark of a tree is breeched, the inner bark becomes infected (Cave et al. 2007). Death of the tree occurs when the pathogen kills the cambium around the entire stem circumference, which in turn destroys the tree's vascular system (Garbelotto 2003). According to Garbelotto (2004), it has been estimated that a few years (minimum two) may be required for the disease to cause mortality at any infested site. Sporangia are not produced on the cankers of trees, once the pathogen has infected a tree it cannot reproduce asexually. This would suggest that infection of trees was an accident of nature for *P. ramorum* (Sandra Denman, pers. comm.). Figure 1.2 shows the probable life cycle of *P. ramorum* and has been adapted from the disease cycle of the Late blight caused by *P. infestans* (http://rapra.csl.gov.uk/background/lifecycle.cfm).

P. ramorum reproduces asexually via the sporangia and the chlamydospores (Fig. 1.2). The sporangia release motile zoospores that can swim and are moved passively in moving water to new infection sites; these zoospores must encyst before infection can take place. *P. ramorum* is heterothallic, which means it requires two separate mating types, A1 and A2, for sexual reproduction to occur. Both mating types of the pathogen are only distinguishable by a small range of phenotypic traits. To date, oospores have not been observed in nature, which indicates that the two mating types may not be currently sexually reproducing in the environments to which they have been introduced, but it is expected that *P. ramorum* participates in sexual reproduction in its native habitat. Sexual reproduction has been successfully carried out in the laboratory between the two *P. ramorum* mating types (Werres and Zielke 2003).

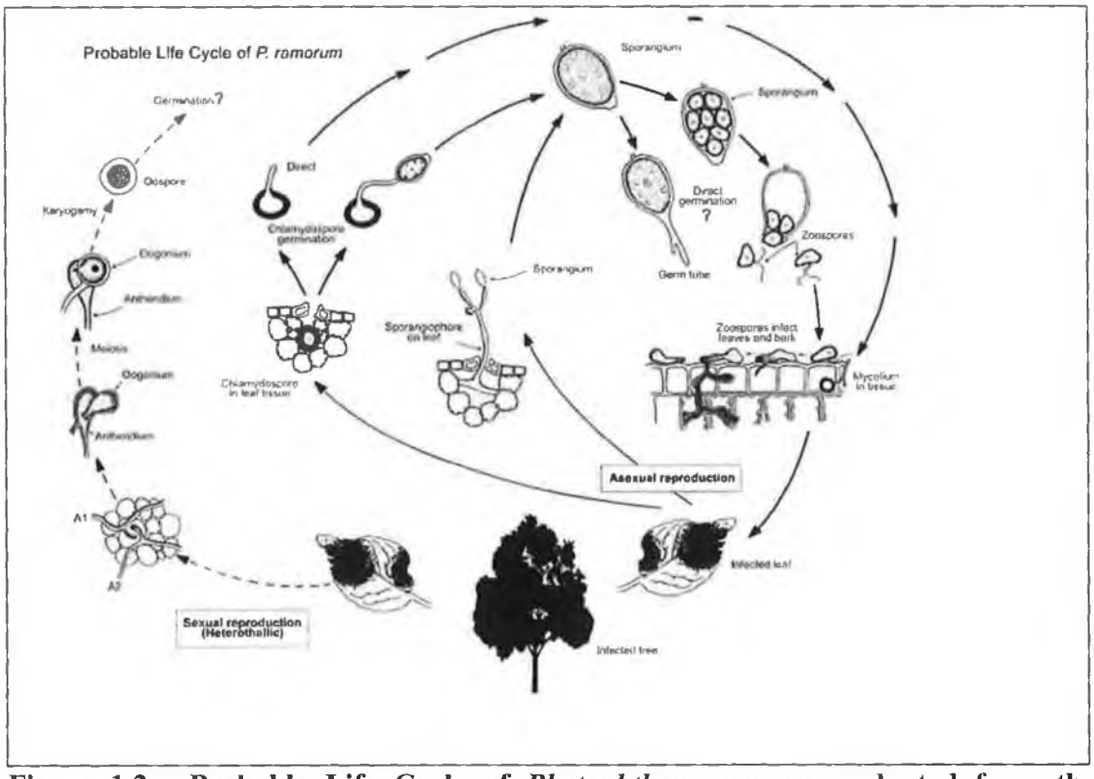

Figure 1.2. Probable Life Cycle of *Phytophthora ramorum* adapted from the disease cycle of the Late blight caused by *Phytophthora infestans* (http://rapra.csl.gov.uk/background/lifecycle.cfm).

Initially, most European isolates were thought to be the A1 mating type. However, an isolate collected in 2002 in Belgium was found to be of the A2 mating type (Werres and Zielke 2003). All North American woodland isolates have previously been of the A2 mating type, but in 2003 the first European A1 mating type was identified in a nursery in northern Oregon (Ivors et al. 2004). According to Garbelotto (2004), the A1 and A2 isolates are not distinguished solely by mating type; they also differ with respect to their morphology and pathogenicity on a range of hosts. Another of the distinguishing characters between the two isolates is that European isolates tend to grow faster than the North American isolates under most of the environmental conditions tested (Garbelotto 2004). Microsatellite markers have distinguished three lineages of *P. ramorum* (Ivors et al. 2006). The predominant European clade of *P. ramorum* was

designated the EU1 lineage, the predominant North American clade was designated the NA1 lineage, while the rare third clade, found in a Washington nursery during the Ivors et al. (2006) study, was designated as the NA2 lineage (Brasier et al. 2006). The small amount of molecular variation shown between each of the three lineages is consistent with their having originated from an original, more variable gene pool (Ivors et al. 2006). Brasier et al. (2006) studied the adaptive variation of each lineage and found that the two lineages share important behavioural responses, such as the shape of their growth, temperature curves, and their host range profiles. The tests carried out also revealed important differences in the behaviour of EU1 and NA1 lineages. This included their mean growth rates, colony stability and their pathogenic aggressiveness. It was suggested that the two main molecular lineages of *P. ramorum* found in North American and Europe, NA1 and EU1, probably represent different founder genotypes from a more variable ancestral *P. ramorum* gene pool in the pathogen's geographic centre of origin.

1.3. DISEASE SYMPTOMS

Disease symptoms caused by *P. ramorum* depend on the host plant species, but at present three different disease symptoms exist: stem canker, twig blight, and leaf necrosis (Davidson et al. 2003b). These symptoms indicate different infecting processes and the development of the disease varies from host to host (Pogoda and Werres 2004). Since the symptoms are not always obvious, it is extremely difficult to determine whether *P. ramorum* is present or not. It is not unusual to have an asymptomatic plant harbouring the pathogen (Garbelotto 2003). The best way to distinguish SOD from other causes of oak mortality, when out in the field, is to look for symptoms on adjacent known hosts, such as *Rhododendron* spp., which exhibit leaf blight and die back symptoms (Fig. 1.3A). If the symptoms are found on several hosts next to the dying oak then it is probable that the infection is due to *P. ramorum*. The most useful diagnostic symptom for *P. ramorum* on trees is the development of cankers on the trunk. Cankers have red-brown to black discoloration and seep dark red to black sap (Fig. 1.3B).

Not all host species are affected to the same extent. It is believed that the pathogen will first colonise the leaves of shrub hosts, which could be a strategy to increase the chances of survival since these are not as affected by the disease as most oak trees. In oak species, only adult plants are significantly affected. Infection of seedlings is unreported in nature, and infection of saplings appears to be extremely rare (Davidson et al. 2003b). Another problem in identifying *P. ramorum* in the field is that some of the foliar disease symptoms caused by it are indistinguishable from other *Phytophthora* species such as *P. nemorosa* and *P. pseudosyringae* (Martin et al. 2004).

The list of natural host species for *P. ramorum* is constantly growing. The two main groups of oak that are susceptible to *P. ramorum* are from the red oak and white oak groups. To date, only oaks in the red oak group have been found to be susceptible to infection by *P. ramorum* in California (Davidson et al. 2003b). Hosts within this group include coast live oak (*Quercus agrifolia*), California black oak (*Quercus kelloggii*), Shreves's oak (*Quercus parvula* var. *shrevei*), Northern red oak (*Quercus rubra*), and Southern red oak (*Quercus falcata*) (Davidson et al. 2003b and Rizzo et al. 2002). The white oak group is only mildly susceptible to *P. ramorum*; some of the hosts include Sessile oak and English oak (*Quercus robur*). In relation to *P. ramorum* in Ireland, this is good news as our native oaks are from the white oak group. However, *P. ramorum* is not restricted to just oak species. Depending on the continent, it is very difficult to find out the number of true, natural hosts for *P. ramorum* since numbers vary from different countries (personal perusal of online information). According to UK findings, the number is currently at about 120 species, from 36 families (http://www.defra.gov.uk/planth/newsitems/suscept.pdf). Some of the hosts affected in the UK include *Camellia*, *Viburnum* and *Rhododendron*, with the latter being the most affected by the pathogen (http://www.defra.gov.uk/planth/newsitems/suscept.pdf). In addition, some tree species have been affected. These include a non-native Southern red oak, Turkey oak (*Q. cerris*), and foliar infection of European Holm oak (*Quercus ilex*).

Figure 1.3. Classic symptoms of the disease caused by *Phytophthora ramorum* on ornamental and tree hosts: A, aerial and petiole dieback on *Rhododendron* spp. and leaf base necrosis (inset); B, bleeding canker on *Quercus falcata* (American southern red oak) (http://www.defra.gov.uk/planth/pestnote/newram.pdf).

1.4. GEOGRAPHIC DISTRIBUTION

As explained above, there are three lineages of *P. ramorum*; EU1, NA1 and NA2. The A1 and A2 mating types have been identified in the EU1 lineage, whereas isolates of the NA1 lineage are of the A2 mating type. Both the American and European outbreaks are believed to have resulted from the introduction of the pathogen via the plant trade. The geographic centre of the origin of *P. ramorum* remains unknown, although suggestions to its source include Yunnan in western China, the Himalayas and Taiwan (Brasier et al. 2006).

In California, oak mortality has reached epidemic levels along the whole Californian and Southern Oregon coasts (Fig. 1.4). The pathogen has spread to 14 coastal Californian counties and killed well over a million trees (http://nature.berkeley.edu/comtf/pdf/Press/4.16.08_Release_study_Identifies_SOD_Intr

oduction_Points_Into_CA_Forests_and_Finds_Pathogen_isEvolving.pdf). The map of the distribution of SOD along the western coast of the US has not changed much since the first versions were put online in 2001. A small number of newly infested sites have since been reported, some of which were in Humboldt County in Northern California. Between autumn 2005 and summer 2006, tan oak mortality increased considerably in almost all directions from the original centre of infestation in the Redway/Garberville area within Humboldt County. Most of the mortality was described as being due to the county's two wet winters, which would have favoured prolific pathogen reproduction and spread (Valachovic and Lee 2006). At present, the main concern in California is that the level of tan oak mortality exceeds 80% and local extinction of the species seems increasingly likely. While tan oak is considered to have little economic value in the timber industry in the US, being viewed as an aggressive weed; it is, nevertheless, still important as an early coloniser in many forests and as a stabiliser of disturbed areas (Hansen 2007). From 2001 to 2005, studies were carried out on 499 circular plots (500 m^2) at 38 sites along the entire Californian coastline in order to determine the distribution of *P. ramorum* and two other *Phytophthora* spp.; *P. ramorum* was recovered from 40% of the plots at 22 sites (Murphy et al. 2007). In California, it is believed that the pathogen is well-established in many areas and that it is still spreading in others (Hansen 2007). In 2000, within a few months of identifying *P. ramorum* as the cause of SOD, Oregon began early detection surveys and this state began eradication efforts within weeks of finding the first *P. ramorum* infestation (Hansen 2007). Despite five years of eradication efforts in Oregon, 36 new infested sites were found in 2006, with new infections detected beyond the quarantine boundary (Kanaskie et al. 2007). This increase in infected trees was attributed to two consecutive years of unusually wet weather in spring and early summer (Kanaskie et al. 2007).

Figure 1.4. Distribution of Sudden Oak Death in Oregon and California counties, as of 5 June 2008 (http://nature.berkeley.edu/comtf/pdf/2007Year-endSummary.pdf).

In the EU the pathogen has been detected in Belgium, Denmark, France, Germany, the Netherlands, Poland, Slovenia, Spain, Sweden and the UK, and more recently in Italy, Norway (interceptions), Switzerland and the Czech Republic (Fig. 1.5;

Table 1.1). In both Poland and Slovenia the pathogen was detected on intercepted imported hosts (http://nature.berkeley.edu/comtf/pdf/2007Year-endSummary.pdf). *P. ramorum* has been found on trees in just two countries within the EU, namely the UK and the Netherlands (Webber 2007). In the UK and Ireland, *P. ramorum* has been found in both nurseries and in the wild. After the discovery of the pathogen in the south of the UK in 2002 (Lane et al. 2003), emergency EC legislation was implemented to prevent the spread of the pathogen within the European Community. These measures were amended in April 2004 and again in March 2007 (Slawson et al. 2007). The current measures require member states of the EU to conduct official surveys, to apply import controls and internal movement controls on *Rhododendron* spp., *Viburnum* spp., and *Camellia* spp., and require that eradication measures be taken when *P. ramorum* is discovered. Nurseries are subject to at least two official inspection visits annually, to confirm that *P. ramorum* is not present, and when the pathogen is detected, further eradication measures are required (Slawson et al. 2007). In the UK from April 2002 to date, *P. ramorum* has been found at 718 sites, in nurseries, garden centres and in the wild (http://www.defra.gov.uk/planth/pramorum3.htm).

On the 10th of October 2005, the presence of *P. ramorum* in Killarney National Park was first reported, via the *Irish Times* (Fig. 1.6). It has since been confirmed by the Department of Agriculture, Fisheries and Food (DAFF) that the pathogen was found at a total of 44 sites in relation to the commercial industry; 27 in garden centres, 14 in nurseries, and 3 in public and private greens, from November 2007 to September 2008 (Matthew Clarke, pers. comm.). Recent DAFF surveys carried out in the wild showed that six sites were recorded as positive in 2008, compared to three in 2007 (Gerard Cahalane, pers. comm.). In the wild, the pathogen has only been found on *R. ponticum*. To date there are no known trees in Ireland that have tested positive for *P. ramorum*; the positive results from both the commercial industry and the wild have been from ornamental hosts, such as *Viburnum* spp. and *Rhododendron* spp (Matthew Clarke and Gerard Cahalane, pers. comm.). It is still unknown how the pathogen was introduced into Ireland and the UK.

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Table 1.1. The European distribution of naturally infected hosts of *Phytophthora ramorum* (http://rapra.csl.gov.uk/objectives/wp1/naturalhostresults.cfm).

Country	Hosts	Situation of findings	Number of records
Ireland*	*Rhododendron* spp., *Viburnum* spp., *Camellia* spp., *Photinia* spp. and *Magnolia* spp. *Rhododendron* spp. only *R. ponticum* only	10 Nurseries and 11 Garden Centres 35 Public Green sites 23 Forestry sites	33 (nurseries) 34 (garden centres) 2 sites 8 +ve samples 3 sites 35 +ve samples
Belgium[5]	*Rhododendron* spp. *Viburnum* spp.	Nursery Nursery	1 1
Denmark[5]	*Rhododendron* spp.	Nursery, unknown Unknown	2, 1 1
Finland[5]	*Rhododendron* spp.	Nursery, unknown	2, 1
France[5]	*Camellia* spp. *Pieris japonica* *Rhododendron* spp. *Viburnum* spp.	Nursery Nursery Nursery Nursery	1 2 139 42
Germany[5]	*Pieris japonica* *Rhododendron* spp. *Viburnum* spp.	Outdoor Nursery, unknown, outdoor Nursery, unknown	1 26, 3, 5 19, 4
Italy[5]	*Rhododendron* spp.	Nursery	1
Norway[5]	*Rhododendron* spp. *Viburnum* spp.	Nursery, Outdoor Outdoor	1, 22 3
Poland[5]	*Calluna vulgaris* *Photinia fraseri* *Pieris japonica* *Rhododendron hybrid*	Nursery Nursery Nursery Nursery	1 1 1 1
Slovenia[5]	*Kalmia* spp. *Rhododendron* spp. *Viburnum* spp.	Nursery, unknown Nursery, unknown, outdoor Nursery, outdoor	4, 1 23, 1, 1 5, 2

Spain[$]	*Aesculus hippocastanum* *Arbutus unedo* *Camellia* spp. *Rhododendron* spp. *Syringa vulgaris* *Taxus* spp. *Viburnum* spp.	Unknown Nursery, unknown Nursery, unknown Nursery, unknown Unknown Unknown Nursery, unknown, outdoor	1 1, 1 3, 1 5, 2 1 1 4, 7, 1
Sweden[$]	*Rhododendron* spp.	Nursery, unknown	2, 12
Switzerland[$]	*Rhododendron* spp. *Viburnum* spp.	Nursery Nursery, outdoor	3 7, 2
The Czech Republic[$]	*Viburnum* spp.	Nursery	1
The Netherlands[$]	*Fagus sylvatica* *Quercus rubra* *Rhododendron* spp. *Taxus media* *Viburnum* spp.	Outdoor Outdoor Nursery, outdoor Nursery Nursery, unknown	6 8 12, 24 1 9, 1
UK, Northern Ireland[$]	*Rhododendron* spp. *Viburnum* spp. *Acer* species *Aesculus hippocastanum* *Arbutus unedo* *Camellia* spp. *Castanea* spp. *Castanopsis orthacantha* *Cinnamomium camphora* *Cornus* spp. *Cydonia oblonga* *Drimys winterii*	Nursery, unknown Nursery, unknown Outdoor Outdoor Unknown Nursery, unknown, outdoor Outdoor Outdoor Outdoor Outdoor Outdoor Outdoor	11, 9 4, 3 2 1 1 7, 34, 4 4 1 2 4 1 1

UK, England and Wales[$]	*Eucalyptus haemastoma*	Outdoor	1
	Fagus sylvatica	Outdoor	6
	Fraxinus excelsior	Outdoor	2
	Garrya elliptica	Nursery	1
	Grevillia spp.	Outdoor	1
	Griselinia littoralis	Nursery	1
	Hamamelis spp.	Nursery, unknown, outdoor	1, 1, 1
	Kalmia spp.	Nursery, unknown, outdoor	3, 10, 1
	Laurus nobilis	Nursery, outdoor	1, 1
	Leucothoe spp.	Nursery, unknown	1, 2
	Lonicera hispidula	Unknown	1
	Magnolia spp.	Nursery, unknown, outdoor	2, 2, 31
	Michelia spp.	Outdoor	9
	Nothofagus obliqua	Outdoor	2
	Osmanthus spp.	Nursery, outdoor	1, 1
	Parrotia persica	Nursery	1
	Pieris spp.	Nursery, unknown, outdoor	6, 14, 16
	Quercus spp.	Outdoor	29
	Rhododendron spp.	Nursery, unknown, outdoor	227, 265, 442
	Salix caprea	Unknown	1
	Schima spp.	Outdoor	2
	Soil/gravel	Outdoor	1
	Syringa spp.	Nursery, unknown, outdoor	1, 1, 1
	Taxus baccata	Nursery, unknown	1,1
	Umbellularia californica	Outdoor	1
	Viburnum spp.	Nursery, unknown, outdoor	207, 179, 11
UK, Scotland[$]	*Rhododendron* spp.	Nursery, unknown	11, 4
	Syringa vulgaris	Nursery, outdoor	1, 1
	Viburnum spp.	Nursery, unknown, outdoor	25, 8, 2

* Data supplied by the Department of Agriculture, Fisheries and Food (DAFF), Irish survey results for 2007 (Gerard Cahalane, pers. comm.).

$ Data compiled from the Risk Analysis for *Phytophthora ramorum* (RAPRA) website.

Figure 1.5. Distribution of *Phytophthora ramorum* in Europe based on records from Risk Analysis *Phytophthora ramorum* website (RAPRA) and European Union Member States (EUMS) up to the end of 2006: Nursery outbreaks include those found in nurseries, garden centres, shops and retail centres and outdoor outbreaks include those found in gardens, parks, public greens and woodlands (http://rapra.csl.gov.uk/objectives/wp1/HistDistribution.cfm).

Concern as deadly oak disease strikes national park in Kerry

By Anne Lucey

An aggressive plant disease which has the potential to devastate Ireland's oldest remaining oak woods has struck a number of rhododendron bushes in Killarney National Park, Co Kerry.

The disease, known as sudden oak death, is already dispersed throughout the 26,000-acre park and has been reported in major tourist areas such as Torc and Ross Island where it has affected rhododendrons. Leaf spots, needle-tip blight and canker are among the symptoms.

Surveys so far have identified 25 infected rhododendron ponticum bushes and park staff are cutting out affected areas.

Amid concern the disease could spread, warning signs were erected at park entrances in Killarney this weekend. The public is being asked not to remove any vegetation from the park to prevent a further spread of the disease, scientifically

"There's a serious threat to the native woodlands and everything possible is being done to protect them," said NPWS divisional manager Paddy O'Sullivan.

So far no traces of the disease, believed to have come into the country on imported plants, have been found on oak trees in the Killarney area.

Arbutus and yew trees were also at risk, Mr O'Sullivan said.

First reported in the US in the mid-1990s, the disease attacks azaleas, big-leaf maple, huckleberry, laurel, camellia, along with honeysuckle and Douglas fir. It has destroyed thousands of oak in California and Oregon.

There have also been a number of findings in Britain and the Netherlands.

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Figure 1.6. *Irish Times* article (10 October, 2005) announcing, for the first time, the presence of *Phytophthora ramorum* in Ireland (http://www.irishtimes.com/newspaper/ireland/2005/1010/1127148489340.html).

1.5. MODES OF DISPERSAL

From the biology and temperature-growth requirements of *P. ramorum*, the pathogen appears to have adapted to aerial dispersal in temperate or Mediterranean-type climates (Brasier et al. 2004). Identifying the pathways of entry of *P. ramorum*, especially into uninfected areas, has become a priority. In both North America and Europe, the introduction of the pathogen seems to have occurred relatively recently and separately, through the trade and sales of ornamental plants (Brasier et al. 2004). The pathogen can

also be transported long distances via contaminated soil, leaf litter, and infected bark. The more natural forms of dispersal are thought to be by rain splash, wind-driven rain, irrigation or ground water. As mentioned before, shrub hosts play an important role in the spread of *P. ramorum*. It has been reported that maximum levels of mortality in tan oaks occurs where Bay laurels are present, and Coast live oak mortality is positively correlated with local abundance of Bay laurel trees (Anacker et al. 2007). Upon infection by *P. ramorum*, foliar hosts such as Bay laurel express non-fatal lesions from which large amounts of inoculum can be produced and spread to neighbouring hosts, such as oak species (Anacker et al. 2007). A study was carried out by Swiecki and Bernhardt (2007) between 2005 and 2006, to determine if there was a safe distance between Bay laurel and coast live oak beyond which the risk of the disease is acceptably low. They found that the risk of disease, severe symptom development and mortality were highest when the distance between Bay laurel and coast live oak trees was less than 1.5 m. In the UK, *P. ramorum* has been mainly found in planted woodland gardens, which contain a wide range of non-native and exotic plants, but particularly where species of rhododendron dominate. Where the pathogen has been found on infected trees in woodlands in the UK, the key under-storey component is *R. ponticum* (Webber 2007). Laboratory tests carried out by Denman et al. (2006) showed that rhododendron is highly susceptible to infection by *P. ramorum* and can also support abundant sporulation. Rhododendron has played a key role in disease escape into the natural and semi-natural environments and the subsequent spread to trees. It is the most frequently infected host in non-nursery outbreaks in some European countries (Webber 2007).

Other common pathways of movement are vectors such as humans and animals. Humans walking through an infected site, such as a wildlife park, and then visiting an uninfected site could become a major factor in the rapid spread of *P. ramorum*. Simple pieces of equipment such as secatures and gloves, even footwear, can easily contribute to the spread of *P. ramorum* through the reuse of the equipment on different hosts without prior sterilising (Cushman et al. 2007).

1.6. GENETIC VARIATION

The *P. ramorum* population structure has all the indications of a recently introduced organism. Low population diversity of a pathogen in a specific geographic region is frequently used to support the hypothesis of its non-native character (Prospero et al. 2007). Studies carried out by Ivors et al. (2004), using amplified fragment length polymorphism (AFLP) analyses, demonstrated differences between the EU and US isolates of *P. ramorum*. Their results showed that EU and US isolates clustered into separate clades, but each displayed low levels of genetic variation. Ivors et al. (2006) reported variation between European and North American nursery isolates using 12 DNA microsatellite loci. Prospero et al. (2007) also confirmed the distinction between the EU and US isolates, using 10 microsatellite loci, but once again failed to detect variation within each grouping.

1.7. AIMS

The recent discovery and spread of *P. ramorum* in Ireland provided a unique opportunity to study the ecology and genetics of this quarantine organism.

The aims and hypotheses were to:

- Sample for *P. ramorum* using soil and water baits and foliage collection at susceptible forest sites in Ireland, using both morphological and molecular methods to determine whether or not the pathogen can be detected in the soil or water, or on the foliage of susceptible hosts.
  H_0: *P. ramorum* cannot be detected from soil and water baits, and from susceptible foliage using morphological and molecular methods.

- Sample for *P. ramorum* in locations other than those previously found positive for *P. ramorum* by DAFF. Eleven sites were sampled which comprised oak forests with an under-storey of *R. ponticum*.
  H_0: *P. ramorum* is not present at other Irish locations besides those already identified by DAFF.

- Determine if *P. ramorum* is present on trees in Ireland close to infected rhododendron. The best way to distinguish *P. ramorum* from other causes of oak mortality, when out in the field, is to look for symptoms on adjacent known hosts, such as *Rhododendron* spp., which exhibit leaf blight and die back symptoms (Garbelotto 2003).
  H_0: *P. ramorum* is not present on trees in Ireland.

- Analyse samples collected from susceptible forest sites, over a three-year period, for the presence of *Phytophthora kernoviae*, using real-time PCR. In 2004, a series of surveys carried out in Cornwall, in the southwest of England, detected a new *Phytophthora* in woodland from a mature European beech

(*Fagus sylvatica*), and at a nearby woodland site from *R. ponticum*; it was subsequently named *Phytophthora kernoviae* (Brasier et al. 2005). At present, *P. kernoviae* has only been found in the UK and New Zealand (Webber 2007), but is of concern due to Ireland's close proximity to the UK.

H_0: *P. kernoviae* is not present in Ireland.

- Determine and compare the genetic variation present in Irish wild and nursery populations of *P. ramorum*, using a selection of DNA microsatellite loci.

  H_0: There is no genetic variation either within or between wild and nursery populations of the pathogen.

- Investigate the susceptibility of (i) four under-storey species [rhododendron (*Rhododendron ponticum)*, fuschia (*Fuschia magellanica),* hydrangea (*Hydrangea macrophylla)* and holly (*Ilex aquifolium)*] and (ii) of two tree species [sessile oak (*Quercus petraea),* and the strawberry tree (*Arbutus unedo)*] to an Irish isolate of *P. ramorum* (isolated from *R. ponticum*, from Killarney National Park, 30.05.2005). Within this study, determine if wounding the leaves on the upper leaf surface, prior to applying a mycelial plug of *P. ramorum* on top of the wound, increases the level of infection.

  H_0: The leaves of the four under-storey and two tree species are not susceptible to *P. ramorum*.

  H_0: Wounding the leaves on the upper surface does not increase the level of infection caused by *P. ramorum*.

- Ascertain the survival potential of chlamydospores, of *P. ramorum*, by examining the effects of temperature (-21, 4, 20 and 40°C) and pH (2, 3, 4, 5, 7, 9) on chlamydospores, which are associated with the long-term survival of *P. ramorum*.

  H_0: Neither temperature nor pH effect the *in vitro* survival of chlamydospores of *P. ramorum*.

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Sampling and identification of *Phytophthora ramorum* in Ireland from 2005-2008

Abstract

Over a three year period, sampling for the presence of *Phytophthora ramorum* was carried out at 11 susceptible forest sites in Ireland. The site selection criterion was oak forests with an understorey of *Rhododendron ponticum*. Sampling was also carried out at locations other than those previously found positive for *P. ramorum* to determine if the pathogen was spreading in Ireland. Sampling methods included foliage collection of *R. ponticum* and the use of both soil and water baits. A total of 314 samples were tested using both morphological and molecular methods, to determine if the pathogen was present in the sample material. Real-time PCR was also used to test for the presence of *Phytophthora kernoviae*, a new *Phytophthora* pathogen of trees and shrubs discovered in 2003 during surveys of woodlands in Cornwall, south-west of the UK. Of the 11 sites investigated *P. ramorum* was found on *R. ponticum* at three locations in the southwest of Ireland, Killarney National Park, Co. Kerry, Lauragh, Co. Kerry and Dunboy Wood in Castletownbere, Co. Cork. The latter site was a new finding for Ireland. There were no findings of trees infected with the pathogen, nor of the new pathogen, *P. kernoviae*.

CHAPTER 2

SAMPLING AND IDENTIFICATION OF *PHYTOPHTHORA RAMORUM* IN IRELAND FROM 2005-2008

2.1. INTRODUCTION

Since the discovery of *Phytophthora ramorum* in the mid 1990s, it has had devastating effects in Europe and along the west coast of the United States (Ivors et al. 2004). In Europe, *P. ramorum* has been detected in 15 countries, including Belgium, the Czech Republic, Denmark, France, Germany, Italy, Ireland, the Netherlands, Norway, Poland, Slovenia, Spain, Sweden, Switzerland, and the UK (Sansford et al. 2008). In the UK and Ireland, *P. ramorum* has been found in both nurseries and the wild. The pathogen was first found in the south of the UK in 2002 and since then around 718 outbreaks have been reported in nurseries, garden centres, and in the wild (Sansford et al. 2008). In Ireland, *P. ramorum* has been found at six locations in the wild, on *Rhododendron ponticum* (Gerard Cahalane, pers. comm.) and there were 44 positives reported in nurseries and garden centres from November, 2007 to September 2008 (Matthew Clarke, pers. comm.). In California, oak mortality has reached epidemic levels along the whole Californian and Southern Oregon coasts. The pathogen has spread to 14 coastal Californian counties and it has killed well over a million trees (Palmieri 2008).

In view of the damage that has occurred in the US and Europe, it is important to develop a clear picture of the status of *P. ramorum* in Ireland. Therefore information on where the pathogen is found and levels of infection at infected sites is needed to assess the potential damage that *P. ramorum* may cause in Irish woodlands. Detection and discrimination of disease caused by this pathogen can be challenging. Symptoms of *P. ramorum* are not unique; the leaf spots, tip dieback and bleeding associated with the pathogen differ only subtly from symptoms caused by other leaf-inhabiting or canker-causing pathogens (Goheen 2003). On oak (*Quercus*) spp. and Tanoak (*Lithocarpus densiflorus*), *P. ramorum* kills subsections of the bark, which results in cankers or lesions and eventually leads to complete crown mortality and death of the tree (Davidson et al. 2003). On foliar hosts, such as rhododendron, leaves are readily infected by the pathogen and brown-black lesions often develop on the leaf portion where water accumulates. *P. ramorum* can also infect and kill branches of several rhododendron species, and may progress to kill entire plants (Davidson et al. 2003). Some seasonal restrictions also apply to sampling; *P. ramorum* is most readily isolated from its woody

hosts during cool and moist periods, and samples that are allowed to dry to any degree rarely yield the pathogen in culture (Goheen 2003).

P. ramorum can be identified using either morphological or molecular methods. Successful culturing of the pathogen seems to depend on the environmental conditions that prevail at sampling locations, the host response and the presence of competing organisms in the plant tissue, all of which may inhibit culturing of the pathogen (Martin et al. 2004). Morphologically, the most distinguishing feature of *P. ramorum* is the presence of large chlamydospores (Fig. 2.1A) that are mainly formed on the terminus of hyphae (Davidson et al. 2003). The pathogen is generally characterised by semi-papillate, deciduous sporangia (Fig. 2.1B), by its slow growth rate and its ability to survive at low temperatures (Kong et al. 2004). The papilla refers to the plug or thickening at the apical end of the sporangium through which the zoospores exit (Fig. 2.1C). Very often the sporangia can be mistaken as being papillate instead of semi-papillate since a swollen papilla may be due to the fact that the sporangium is about to germinate (Fig. 2.1C). Identifying the pathogen solely on morphological criteria requires considerable experience in species identification and can be time consuming because it is very easy to confuse *P. ramorum* with other *Phytophthora* species that have a similar morphology (Martin et al. 2004). It is also common to recover *Phytophthora nemorosa* or *Phytophthora pseudosyringae* from forest samples that show similar symptoms and a similar geographic distribution to *P. ramorum* (Martin et al. 2004). *P. ramorum* has a unique molecular sequence at the Internal Transcribed Spacer (ITS) region of the nrRNA gene, which distinguishes it from all other known *Phytophthora* species (Davidson et al. 2003).

In November 2003, the first case of *P. ramorum* infecting a UK tree was confirmed on a mature specimen of southern red oak (*Quercus falcata*) in Sussex (Brasier et al. 2004). The fact that the source of the tree infection appeared to come from diseased rhododendrons initiated detailed sampling surveys for *P. ramorum* on trees growing in close proximity to *R. ponticum* (Brasier et al. 2005). In 2003, a series of these surveys carried out in Cornwall, in the southwest of England, detected a new

Phytophthora isolated from both a large aerial bleeding lesion on a mature European beech (*Fagus sylvatica*) and from foliage of *R. ponticum* growing in a nearby woodland; it was subsequently named *Phytophthora kernoviae* (Brasier et al. 2005). In the UK, *P. kernoviae* has been associated with wide-spread foliar necrosis and shoot die-back of rhododendron (Brasier 2005); bleeding cankers have been reported mainly on beech, along with two oak trees (*Quercus robur*) and one tulip tree (*Liriodendron tulipifera*) (Webber 2007). It behaves in a similar fashion to *P. ramorum*, sporulating on rhododendrons and causing lethal stem cankers on trees, but it appears to be more aggressive than *P. ramorum* (Brasier et al. 2005). *P. kernoviae* exhibits a unique combination of behavioral and morphological properties including its breeding systems, gametangial morphology, sporangial morphology, growth-temperature relationships and colony patterns (Brasier et al. 2005). Its nrDNA sequence is also unique, and unrelated to that of *P. ramorum* (David E.L. Cooke and Kelvin J.D. Hughes, pers. comm., cited in Brasier et al. 2005). To date, *P. kernoviae* has been found at 69 sites in England and Wales, with most findings on rhododendron in small areas of woodland in Cornwall (EPPO 2008). The origin of *P. kernoviae* is still unknown (Webber 2007). It was initially thought that *P. kernovaie* was only present in the UK, but in March 2006 the pathogen was found at two locations on the North Island of New Zealand. It was isolated from soil in Trounson Kauri Park as a result of a research project being carried out to determine the species of *Phytophthora* present in the park, and at a second site (location not mentioned) on the edible Custard apple (*Annona cherimola*) (Webber 2007).

The UK Central Science Laboratory developed two real-time PCR molecular methods, based on ITS sequences that were highly specific for *P. ramorum* and *P. kernoviae*. The assay for *P. ramorum* had a limit of detection of 10 pg of DNA (Hughes et al. 2006) and for *P. kernoviae* they were able to detect the pathogen at concentrations as low as 1 pg per reaction volume (DEFRA and CSL 2005). The use of real-time PCR methods allowed Hayden et al. (2006) to confirm *P. ramorum* infection in over 204 symptomatic plant samples collected from 33 sites in California. The method also

allowed them to expand the confirmed host range of *P. ramorum* to include 10 additional plant species.

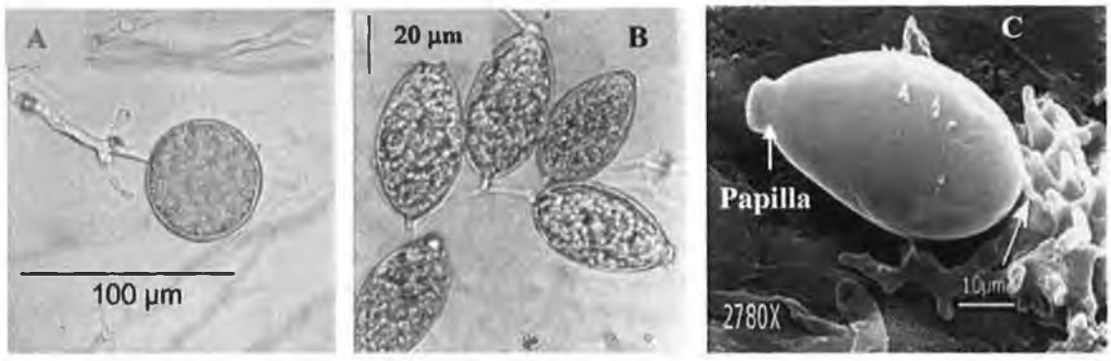

Figure 2.1. *Phytophthora ramorum* structures: A Chlamydospore; B Semi-papillate, deciduous sporangia (http://www.plantenziektekunde.nl/pramorum); C Shape of papilla on sporangium prior to germination (Florance 2004).

The aims of this study were to sample for *P. ramorum* at 11 susceptible forest sites in Ireland using the site selection criterion of oak forests with an under-storey of *R. ponticum*. At present, the Department of Agriculture, Fisheries and Food (DAFF) sample throughout Ireland for *P. ramorum* (Gerard Cahalane, pers. comm.). We also wanted to determine if *P. ramorum* was present at other locations other than those previously found positive by DAFF. Another aim was to determine if *P. ramorum* was present on trees in Ireland close to infected rhododendron. Sampling included foliage collection of *R. ponticum*, and the use of both soil and water baits. Both morphological and molecular methods were used to detect the pathogen in sampled material. In addition, samples that were negative for *P. ramorum* were also tested for the presence of *P. kernoviae*, using real-time PCR.

2.2. MATERIALS AND METHODS

2.2.1. Sampling Sites

Sampling began in 2005, where the possible locations of three *P. ramorum* infected areas were made known to me by Patrick Walsh, Forestry Department, Galway-Mayo Institute of Technology (GMIT). These sites were Killarney National Park and Lauragh, Co. Kerry, and the Vee, Co. Waterford. It was noted that all three sites were heavily infested with rhododendron and also that sessile oak (*Quercus petraea*) was present. Therefore, from June 2005 to July 2008, field sampling was carried out using the site selection criterion of oak forests with an under-storey of *R. ponticum*. Sampling was carried out primarily by collecting foliage, but also by using water and soil baits, and lateral flow devices, which were supplied by the Central Science Laboratory (CSL) in York, UK. Culturing, isolation and DNA extraction of the pathogen was carried out using the modified methods of Van Leeuwen et al. 2003.

Sampling for the presence of *P. ramorum* was carried out from 2005 to 2008. In 2005, seven sites were sampled; in both 2006 and 2007 ten sites were sampled; in 2008 any positive sites found during the previous three years were revisited (Table 2.1). Each site was sampled a minimum of once a year, and any sites found to contain *P. ramorum* were sampled twice a year (when budget allowed). Overall, eleven sites were sampled throughout the course of this study, with 314 samples collected over the four-year period (Fig. 2.2; Table 2.1). Site description and Ordnance Survey maps of sites sampled are in Appendix I.

Figure 2.2. Location of 11 sites sampled for *Phytophthora ramorum* throughout Ireland from 2005-2008. See Appendix II, Tables A1 to A11 for a description of the characteristics of each site, and Table 2.1 for the sampling strategies used at each site.

Table 2.1. Location of sites, sampling dates (2005-2008) and sampling strategies used.

Site No.	Site name	Date	N[S]	Type of sampling[F]
1	Glenveagh National Park, Co. Donegal	07.12.05	17	SB, WB, F
		12.07.06	6	F
		10.08.07	10	SB, WB, F
2	Brackloon Woods, Co. Mayo	14.08.06	4	S, WB, F
		09.08.07	8	F
3	Connemara National Park, Co. Galway	24.11.05	6	SB, WB, F
		16.04.06	3	F
		30.03.07	7	SB, WB, F
4	Killarney National Park, Co. Kerry	30.05.05	6	F
		28.06.05	4	F
		02.08.05	7	Soil and Bark
		23.02.06	9	SB, WB, F
		24.06.06	30	SB, WB, F
		01.07.07	16	SB, WB, F
		09.05.07	9	F
		17.05.08	9	F
		24.06.08	8	F
5	Lauragh, Co. Kerry	28.07.05	11	F
		26.02.06	2	F
		26.06.06	6	F
		02.07.06	10	F
		08.05.07	9	F
		19.05.08	12	F
6	Dunboy Wood, Castletownbere, Co. Cork	05.06.08	9	F
		15.06.08	7	F
7	Glengarriff, Co. Cork	31.05.05	2	F
		25.02.06	5	SB, WB, F
		02.07.06	6	F
		08.05.07	9	F
		17.05.08	10	F
8	Lismore, the Vee, Co. Waterford	31.05.05	2	F
		27.06.06	3	F
		03.07.07	4	F
9	Killballyboy Wood, the Vee, Co. Waterford	31.05.05	2	F
		27.06.06	9	F
		03.07.07	5	F
10	John F. Kennedy Arboretum, Co. Wexford	27.09.06	5	F
		04.07.07	7	F
11	Tomnafinnoge Wood, Co. Wicklow	26.09.06	6	F
		04.07.07	9	F
	Samples supplied by DAFF from the Vee, (county unknown)	30.11.07	5	Leaf material isolated on P_5ARP^* agar plates

[S]N = the number of samples collected. [F]SB = soil bait containing *Rhododendron ponticum* leaves; WB = water baits containing *Rhododendron ponticum* leaves; F = foliage. * PARP = Pimaricin-ampicillin-rifampicin-pentachloronitrobenzene (P_5ARP) (Van Leewen et al. 2003).

2.2.2. Sampling methods

At each sampling site the nearest hosts were recorded and a brief description of the surrounding area was noted e.g. buildings, roads, watercourses. Random sampling was carried out for the presence of *P. ramorum*. Foliage showing symptoms of infection were photographed and collected (Figure 2.3). During the first year of sampling, plants that were sampled were marked with blue electrical tape in order re-sample on return trips. Unfortunately, most of the markings were not present on return trips, presumably due to either plant or foliate removal. Each foliage sample comprised enough material to fill one large ziplock bag (35 x 37 cm). The foliage was wrapped in moist tissue to prevent the sample from drying out, and stored in the labelled ziplock bag, along with the gloves used to collect the sample. All ziplock bags from one sampling location were then placed into a clear, large, labelled, biohazard bag and stored in the trunk of the car in order to separate sampling sites, and keep the samples out of direct sunlight.

Figure 2.3. Foliage of *Rhododendron ponticum* showing symptoms of leaf necrosis, caused by *Phytophthora ramorum*, from Castletownbere, Co. Cork.

Baits were made using 25 cm^2 sections of sterile muslin cloth, into which was placed five pieces of sterile gravel (for weight), approximately eight pieces of cut up *R. ponticum* leaves (surface sterilised using 70% ethanol), and two pieces of polystyrene

packaging to aid floatation near the water surface (for water baits only, not necessary for soil baits). The cloth was tied off, not too tight, with approximately 2.5 m of string (only necessary for water baits) (Paul Beales, pers. comm.). These bags were frozen for several months before use and were brought to the sampling sites in polythene bags so as the leaves did not dry out (as recommended by Paul Beales). Water baits were tied to the bank of the river to allow the bait to move with the current, while soil baits were placed a couple of cm in the soil, directly under foliage of *R. ponticum*. Baits were left in the water and soil for three days and were stored in labelled ziplock bags upon collection. Baits were then stored in a cooler box containing ice packs to keep the samples cool and transferred to the laboratory by car.

All tools used to collect samples and to place baits, and the soles of shoes were disinfected with 70% ethanol (Beales et al. 2003; Scianna et al. 2003) prior to moving to the next sampling site and after completing sampling for the day.

2.2.3. Culturing of *Phytophthora ramorum*

Pimaricin-ampicillin-rifampicin-pentachloronitrobenzene (P_5ARP) medium (Van Leewen et al. 2003) was used to isolate *Phytophthora* from leaf material, as described in Van Leewen et al., 2003. Between three and five leaves per foliage/bait sample were incubated on P_5ARP (See Appendix II Tables A.1 to A.11). For each leaf, four pieces of symptomatic foliage material (4 x 2 cm^2), including the leading edge, were excised using sterile secatures (sterilised using 70% ethanol). The sample pieces were then surface cleaned by washing them with distilled water in a plastic bag. The wash water was changed between samples and kept for autoclaving, to ensure that any spores that might have been washed off leaf surface were destroyed. Gloves were changed between samples to prevent cross-contamination between samples.

The four washed pieces of symptomatic foliage were plated onto one plate of P_5ARP. Sample plates were incubated for 5-10 days at 20°C, away from direct sunlight. After incubation, isolates were examined for the following features: colony growth rate, mycelium type, chlamydospores and sporangia. A positive *P. ramorum* was recorded if

there was 1 cm^2 per week colony growth rate, with a feathery/granular appearance and weakly coralloid mycelium with repeated branching in addition to large chlamydospores (not always present) 22-72 µm diameter, and deciduous semi-papillate sporangia (40-80 x 20-32 µm) with one narrow papilla (5-8 µm) (Van leewen et al. 2003). Isolates showing any signs of growth were then sub-cultured onto a fresh plate of P_5ARP. DNA extracted from emerging cultures was also subjected to conventional and real time PCR (RT-PCR) analysis.

2.2.4. DNA extraction

DNA was extracted from all cultures obtained from foliar, and water and soil bait samples collected within this study. Samples aseptically excised from P_5ARP cultures (approximately 0.5 cm x 1 cm) were placed in 1.5 ml Eppendorf tubes. A small amount of dry ice was added to each tube and samples were homogenised using a glass pestle tissue grinder.

DNA was extracted from homogenized samples using the NucleoSpin plant DNA extraction kit (Macherey-Nagel, Düren, Germany). Three hundred µl of C1 buffer (commercial kit components) was added to the Eppendorf tube and the sample was homogenized for 30 s, and then vortexed for 30 s. This procedure was repeated five times. The sample was then centrifuged at 11 000 rpm for 5 min, after which the aqueous phase was pipetted off into a fresh 1.5 ml Eppendorf tube. The removed aqueous phase was incubated at 65°C for 30 min. Three hundred µl of C4 buffer (commercial kit components) and 200 µl of 100% ethanol were added to the Eppendorf tube, and the sample was vortexed for 30 s. A labelled Nucleospin plant column, containing a silica membrane onto which the DNA binds, was placed into an Eppendorf tube and the sample mixture was pipetted onto the column. The sample was centrifuged at 10 000 rpm for 1 min, and the flow-through was discarded. This was repeated if necessary, if more sample mixture remained in the silica membrane. Four hundred µl of CW buffer (commercial kit components) was pipetted onto the column, the sample was centrifuged at 10 000 rpm for 1 min and the flow-through was discarded. Seven hundred µl of C5 buffer (commercial kit components) was pipetted onto the column, the

sample was centrifuged at 10 000 rpm for 1 min, and the flow-through was discarded. Another 200 µl of C5 buffer was pipetted onto the column and the sample was centrifuged at full speed (14 000 rpm) for 2 min to remove the C5 buffer completely. The flow-through was discarded. The column was placed into a fresh 1.5 ml Eppendorf tube. Fifty µl elution buffer, preheated to 70°C, was pipetted onto the column. The sample was incubated at room temperature for 5 min and then centrifuged at full speed for 1 min in order to collect DNA at the bottom of the column. The DNA was pipetted into a labelled 0.5 ml PCR tube and stored at 4°C until it was analysed (within 2-3 weeks).

2.2.5. PCR amplification and electrophoresis of *P. ramorum*-specific product

Conventional PCR was used to determine if DNA from cultures isolated from P_5ARP were *P. ramorum.* PCR reactions were performed in duplicate for each DNA extract.

2.2.5.1. PCR reagents

PCR amplification of a partial sequence of the ITS1 5.8s (conserved region situated between the ITS1 and ITS2 regions) and a partial sequence of the ITS 2 region was carried out using primers PramF1 and Pram R1, designed by Kelvin Hughes (CSL) (Beales et al. 2003). Amplification took place in a 23 µl reaction mixture containing 5 µM of each primer (Promega, UK), 10 x reaction buffer containing $MgCl_2$ (15 mM) (MWG Biotech AG, Germany), 2.5 mM of each dNTP (MWG Biotech AG, Germany), 5 U Taq polymerase (MWG Biotech AG, Germany) and deionised water (see Appendix III Table A1). For each reaction 2 µl of template DNA was added. The reaction master mix was made up x20 to reduce sampling error when measuring small volumes. Each PCR run also included a positive sample comprising master mix plus *P. ramorum* DNA [provided by Central Science Laboratory (CSL), UK] and a negative sample comprising master mix plus water.

2.2.5.2. PCR conditions

PCR amplification was carried out in thin-walled PCR tubes (Sarstedt, Ireland) in a Biometra^{TM} T1 Thermal Cycler. Amplifications were achieved by running 30 cycles of 94°C for 2 min, followed by 30 cycles, consisting of 94°C for 30 s, 57°C for 30 s and 72°C for 30 s, and a final extension of 10 min at 72°C (Beales et al. 2003) (see Appendix III, Table A2). Following PCR amplification, the samples were either run immediately on agarose gels or stored at –20°C.

2.2.5.3. Agarose gel electrophoresis

Agarose gels were run using a wide mini-sub cell GT electrophoresis cell (Bio-Rad, UK). Gels comprised 3 µl ethidium bromide (Sigma, Ireland), 1.5 % agarose (Sigma, Ireland) dissolved in 100 ml of 1 X TBE (Tris-Borate EDTA) buffer (Sigma, Ireland); gels were prepared as previously described in (Van Leewen at al. 2003). The agarose gel was submerged in 800 ml of 1 X TBE running buffer.

The PCR samples were prepared for electrophoresis by adding 2 µl of Blue/Orange 6 X loading dye (Promega, UK) to 10 µl of PCR product and 10 µl of this mix was loaded into a gel well. A 100 bp DNA ladder (Promega, UK), a negative control and one positive control sample (CSL) were run on each gel. One hundred volts was applied to the gel for ~ 1 h or until the loading solution had run ¾ of the way down the gel. The gel was then removed from the gel rig and visualised on a UV light box (Hoefer, Mighty Bright) at 240 nm (Fig. 2.3). Gel images, captured using a digital camera (Canon Powershot A300), were downloaded onto a desktop computer.

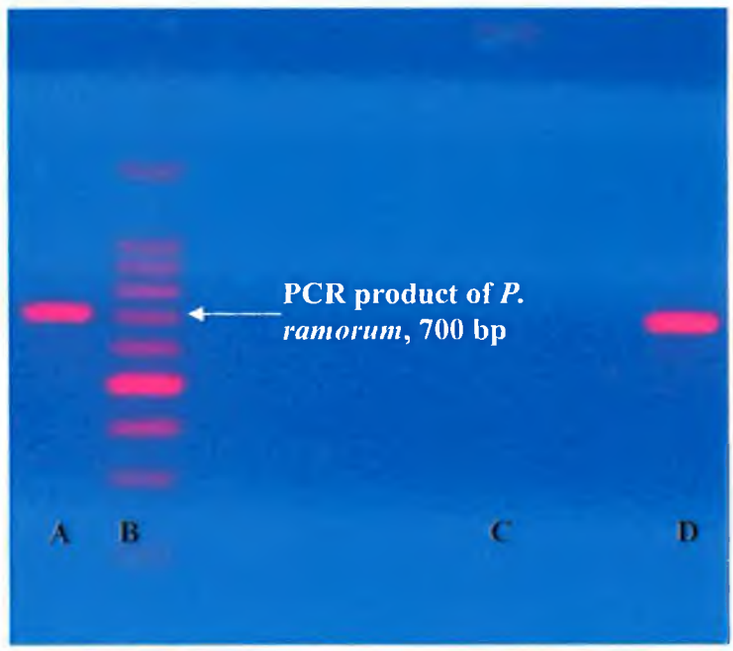

Figure 2.3. Visualisation of *Phytophthora ramorum*-specific polymerase chain reaction products by agarose gel electrophoresis. Lanes: A: positive control (*P. ramorum* isolate), B: 100bp DNA Ladder, C: negative control, D: *P. ramorum* strain K002.

2.2.6. Real-time PCR analysis of *P. ramorum* and *P. kernoviae*

In 2008, a real-time PCR machine became available for use. Culture samples that tested negative for *P. ramorum* by conventional PCR were thereafter analysed for the presence of either *P. kernoviae* or *P. ramorum* using RT- PCR (Table 2.2). Primers and probes used were those specific for *P. kernoviae* (*Pkern* 60F, *Pkern* 121R and probe *Pkern* 84T; DEFRA and CSL 2005) or *P. ramorum* (*Pram*114-F, *Pram*1527-190-R and probe *Pram*1527-134-T; Hughes et al 2006).

Individual real-time PCR reactions were performed for each pathogen and reactions were run in an ABI Prism 7500 System (Applied Biosystems, UK). Real time PCR reaction mixes (45 µl) contained 100 ng of DNA, 0.3 µM of each forward and

reverse primer and 0.1 μM of *Pkern*84T Probe (MWG Biotech AG, Germany), 1x Internal Positive Control (IPC) mix and 1x IPC DNA (Applied Biosystems), Taqman Universal PCR Master Mix (Applied Biosystems) consisting of 1x buffer A (50 mM KCL, 10 mM Tris-HCL, pH 8.3, ROX passive reference dye) and AmpliTaq Gold at 0.025 U/μl, plus 0.2 mM each dNTP and 5.5 mM $MgCl_2$, and deionised water (see Appendix III, Table A3). During each PCR run, *P. kernoviae* and *P. ramorum* DNA, provided by CSL, was used as a positive control, deionised water was used as a negative control, and an Internal Positive Control (IPC) was used to ensure that negative results were not due to a failed PCR.

Sealed microtitre plates containing the PCR reaction components were vortexed for 5 s and centrifuged at 1000 rpm on a Universal 320R bench-top centrifuge (Mason Technology). A pre-read run, conducted prior to RT-PCR, recorded the background fluorescence of each well. The pre-read fluorescence is subtracted from the post-read fluorescence to take into account the pre-amplification background fluorescence. PCR reaction conditions for *P. kernoviae* consisted of: 50°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min (DEFRA and CSL 2005). Those for *P. ramorum* consisted of 94°C for 10 min, followed by 40 cycles of 94°C for 15 s and 60°C for 60 s (Hughes et al. 2006). The cycle threshold (CT: the cycle at which a significant increase in fluorescence occurs due to real-time PCR amplification) values were automatically recorded using the standard factory settings. According to the DEFRA and CSL 2005 protocol, samples with a CT values less than 30 were considered to be *P. kernoviae* positive, a CT value greater than 30 indicated a negative result. Once the amplification run was complete the post-read run was preformed. Samples with a CT value less then 36 are considered to be *P. ramorum* positive; a CT value greater than 36 indicated a negative result (Hughes et al. 2005).

Table 2.2. Number of culture samples per site that were tested for the presence of *Phytophthora kernoviae* and *Phytophthora ramorum* using real-time PCR.

Site	Number of samples tested*
Glenveagh National Park, Co. Donegal	29
Brackloon Woods, Co. Mayo	11
Connemara National Park, Co. Galway	15
Killarney National Park, Co. Kerry	60
Lauragh, Co. Kerry	29
Dunboy Wood, Castletownbere, Co. Cork	6
Glengarriff, Co. Kerry	26
Lismore, the Vee, Co. Waterford	6
Killballyboy Wood, the Vee, Co. Waterford	10
John F. Kennedy Arboretum, Co. Wexford	10
Tomnafinnoge Wood, Co. Wicklow	12

*Each culture was derived from a different sample.

2.2.7. Statistical analysis

Tests of association between morphological and molecular data (see Appendix II, Tables A4-A6) were performed using the χ^2-square analysis function in Minitab v. 15.

2.3. RESULTS

2.3.1. Morphological and Conventional PCR

Official surveys carried out in Ireland by DAFF since 2003 have confirmed the presence of *P. ramorum* at five locations in the wild (Gerard Cahalane, pers. comm.; Table 2.3). Of the 11 sites investigated during this study, the pathogen was found at three locations in the southwest of Ireland over the course of four years sampling (Fig. 2.2). These were Killarney National Park and Lauragh (in Co. Kerry) and Dunboy Wood (Castletownbere Co. Cork). The latter site was a new finding for Ireland and it was identified during this study in June 2008.

The first positive found, during the course of this study, was in May 2005 on *R. ponticum* foliage in Killarney National Park. In total, 98 samples were collected from Killarney National Park. Seventy-two were *R. ponticum* foliage, 14 were water baits, five were soil baits and seven were from Turkey oak (*Quercus laevis*) supplied by Patrick Walsh (Forestry Department, Galway-Mayo Institute of Technology) (see Appendix II, Table A4). Shortly after the first positive finding, DAFF intensively surveyed the entire boundary of the park and signs were erected throughout the park, forbidding the removal of susceptible foliage. In February 2006, we detected the first water-positive recording of *P. ramorum* in Ireland, in Killarney National Park. The site was located just off a main road and consisted of a body of still water, which had a stream flowing from it into a large lake. Since there are three interconnected lakes in Killarney National Park, a further 10 water baits were placed along a five mile stretch of the watercourse four months later, with no further positives found. From the 98 foliage and water and soil bait samples collected over the four-year sampling period, 27 were positive for *P. ramorum*, both morphologically and molecularly. Of these 27 positives two were from soil baits, one from a water bait, and 24 from *R. ponticum* foliage (Table 2.3), 60 exhibited growth of unknown species and 11 showed no growth at all (see Appendix II, Table A4).

The second positive site found was in the village of Lauragh, Co. Kerry in 2006, and the pathogen was isolated from samples of *R. ponticum* foliage only. In total, 50

samples of *R. ponticum* foliage were collected, of which 14 were found positive, both morphologically and molecularly, for *P. ramorum* (Table 2.3), 29 exhibited unknown growth, and seven showed no signs of growth (see Appendix II, Table A5). This site is a privately owned site and is currently under containment in accordance with the EC legal requirements of findings in the wild (Gerard Cahalane, pers. comm.).

The third positive finding was in Dunboy Woods, Castletownbere, Co. Cork in 2008, and the pathogen was isolated from samples of *R. ponticum* foliage. Upon examining the samples back in the laboratory and identifying *P. ramorum* both morphologically and molecularly the finding was reported to DAFF and a detailed survey was carried out on the site, over a three- week period (Gerard Cahalane, pers. comm.). This was a new finding in the wild for Ireland. In total, 16 samples of *R. ponticum* foliage were collected from this site, of which 10 were found positive for *P. ramorum*, both morphologically and molecularly (Table 2.3) and six exhibited unknown growth (see Appendix II, Table A6). Following the surveys carried out in Castletownbere, an additional two forest sites, location unknown, were found in the wild by DAFF (Gerard Cahalane, pers. comm.).

In October 2003, DAFF located its first positive finding in the Vee in the southeast of the country. Eradication efforts were carried out under quarantine control measures but in 2006 the pathogen was still found, but at extremely low levels. During the course of this study, 25 samples of *R. ponticum* foliage were collected from this site; 16 exhibited unknown fungal growth and nine showed no signs of growth (see Appendix II, Tables A8 and A9). Within this study, no positive was found in the Vee.

In total 314 samples were tested over the course of the four-year study. The number of leaves sampled per sample, and number of extractions per sample can be seen in Appendix II, Tables A1-A11.

Table 2.3. Summary of *Phytophthora ramorum* positive sites in Ireland (2005-2008).

Sites	Host	Year	Number of samples tested	Positive samples[£]
Killarney National Park, Co. Kerry[$]	*Rhododendron ponticum*	2005	98	27; 2 SB, 1 WB and 24 F
Lauragh, Co. Kerry[$]	*R. ponticum*	2006	50	14 F
Dunboy Wood, Castletownbere, Co. Cork[*]	*R. ponticum*	2008	16	10 F
The Vee, Co. Waterford[$]	*R. ponticum*	2003	25	0
Two privately owned areas of land, location unknown[$]	*R. ponticum*	2008	N/A	N/A

N/A = Information not available.
Positive = Samples positive for *Phytophthora ramorum*.
[$] = Discovered by the Department of Agriculture Fisheries and Food (DAFF).
[*] = Discovered during this study.
[£]SB = soil bait containing *Rhododendron ponticum* leaves; WB = water baits containing *Rhododendron ponticum* leaves; F = foliage.

2.3.2. Real-time PCR

The 214 samples that tested negative for *P. ramorum* using conventional PCR were subsequently tested for *P. kernoviae* and *P. ramorum* using RT-PCR. There were no positive findings of *P. kernoviae* or *P. ramorum* in the 214 samples taken from the 11 sites throughout Ireland, during 2005-2008, as determined by RT-PCR. One hundred and ninety six of the samples gave CT values > 36. The remaining 18 samples gave CT values of 40, which, according to the DEFRA and CSL (2005) protocol, could be due to non-amplifiable DNA. The positive control of *P. kernoviae*, provided by CSL, gave a CT value of 19 while the positive control of *P. ramorum*, also provided by CSL, gave a CT value of 27.

2.3.3. Statistical analysis

χ^2-square tests of association showed that there was an association between positive results based on morphological criteria and those obtained using molecular criteria (P = 0.641). This means that when samples have been identified as positive for *P. ramorum* based solely on morphological characteristics, these samples are also highly likely to be found positive using molecular analysis.

2.4. DISCUSSION

The null hypothesis that *P. ramorum* is not present in Ireland was rejected. *P. ramorum* has been detected at six locations in Ireland, all on *R. ponticum*. The pathogen was detected by DAFF within five Irish sites and in an additional Irish site (Castletownbere, Co. Cork) as a result of this study. Therefore the null hypothesis that *P. ramorum* is not present at any other locations other than those previously found by DAFF, was also rejected. The null hypothesis that *P. kernoviae* is not present in Ireland, was accepted. Of the 214 *P. ramorum*-negative culture samples tested for *P. kernoviae*, none were found to be positive for the pathogen by RT-PCR. While there is no evidence that *P. kernoviae* has infected Irish flora, it is important to survey for the presence of the pathogen in Irish forests. It is unknown how *P. ramorum* was first introduced into Ireland and therefore there is cause for concern regarding the potential for accidental introduction of *P. kernoviae*.

Over the four-year sampling period *P. ramorum* was found at three locations in the wild: Killarney National Park, and Lauragh, Co. Kerry, and Castletownbere, Co. Cork. The latter site was a new finding for Ireland and it was identified during this study. *P. ramorum* has also been found in the Vee, Co. Waterford, by DAFF. In Killarney National Park, 27 positives were found on rhododendron foliage, and water and soil baits. Throughout the sampling period the number of findings, in relation to sample size, did not decrease. This is a cause for concern as eradication efforts have been ongoing since 2004. The sampling protocol, carried out by DAFF, is that infected areas are cleared, leaving a buffer area of approximately 20 m around the infected host

plant. The infected material is burnt *in-situ*, under quarantine control measures. The stumps are treated with the herbicide, Roundup®, which contains the active ingredient glyphosphate, and a dye, and the surrounding area is raked into the fire to be destroyed. The raking is done to within 2 m of the furthest reaching branch. All re-growth is sprayed for approximately two seasons after clearance (Gerard Cahalane, pers. comm.). During the 2006-2007 sampling period, foliage collection was prohibited due to EC regulations, and signs were in place throughout the park forbidding foliage removal. Despite this, using the soil baiting technique, the number of positive findings remained the same over the four-year period.

The site in Lauragh is a privately owned site and therefore sampling was restricted because access to the site was forbidden. A survey was carried out in 2006 by DAFF and the pathogen was found widespread at this location. Further positives were found in the Derreen Gardens, which are located across the road from the privately owned land. During this study, sampling had to be carried out on a public road adjacent to the privately owned land and positives were found each year, from 2006-2008, with 14 positives in total. Sampling was only carried out by foliage collection, due to the fact that the sampling site was so small and the rhododendron was located in a ditch along the roadside. The policy of containment, in accordance with the legal requirements of findings in the wild, was implemented for the privately owned land and the Derreen Gardens by DAFF (Gerard Cahalane, pers. comm.). The areas of positive findings were demarcated, and removal or harvesting of rhododendron foliage was forbidden. The reason for demarcation and not destruction was due to the fact that both sections of land, the Derreen Gardens and private land, are privately owned, and therefore the cost of removal lies solely with the owners. As of May, 2008, the privately owned land across from the Derreen Gardens was considered to have the highest amount of *P. ramorum* infection in Ireland (Gerard Cahalane, pers. comm.).

During this study a new positive wild site was found in June, 2008 in Dunboy Wood, Castletownbere, Co. Cork. The woodland, which is located approximately 25 km

from Lauragh, is owned by Coillte and is open to the public throughout the year. After the initial finding, DAFF were notified of the new positive and they in turn notified Coillte. Dunboy Wood was surveyed by DAFF later on in June and several positives were found in the wood and in nearby private woodland (Gerard Cahalane, pers. comm.). Signs of rhododendron cutting along the trail through the wood were noted by DAFF (Gerard Cahalane, pers. comm.). Since Coillte owns Dunboy Wood, grant aid will be given in order to remove the infected foliage.

The first finding in Ireland, in the wild, was in the Vee, in October 2003, by DAFF. Since then the pathogen has been found at low levels every year at this site, except for 2004 when very little or no symptoms were present. The dominant landowner is Coillte, and the Vee has one of the highest levels of rhododendron invasion in Ireland, which would cost millions of euros to remove (Gerard Cahalane, pers. comm.). The exact location of the site has not been revealed and despite sampling from 2005-2007 (Table 2.1), I found no positives in this area.

Based on the results of this study, it is evident that *P. ramourm* is either more widespread than initially thought or that it is spreading. So far, it appears to be confined to the south of the country. There are a couple of possible reasons for this; the southwest of the country has the highest level of rhododendron invasion and cutting of rhododendron is carried out intensively in this part of the country. There were signs of rhododendron cutting at all of the positive sites visited during this study, with the exception of Lauragh since the site itself is privately owned and could not be accessed, and it is the belief of the park managers that the cuttings may be for commercial sale. It was not uncommon to return to a sampling site a few months later and find the area completely cleared of rhododendron, some of which would likely have been positive for *P. ramorum*. It is hard to control the removal of rhododendron from the public sites so it is not surprising that the fate of the cuttings is largely unknown. Removal of the cuttings from infected sites could be a method of dispersal for the pathogen, and may explain why it is only found in the southwest of Ireland. The main concern is the equipment

used for cutting rhododendron. Secatures and footwear could infect a new site if they are not sterilized after cutting in an infected area. An area of almost a quarter of a mile long, in Tomies Wood, Killarney National Park, which contained positives, was found to be completely cleared of mature and young rhododendron plants on follow up trips. The people responsible for this removal were believed to be involved in the commercial trade of rhododendron (Per comm. National Parks and Wildlife Services employee 2006). Another cause for concern is that at present there is no information made available to the public regarding the seriousness of the presence of this pathogen in Irish forests. In the UK, information is made available to members of the public describing its symptoms, methods of dispersal, types of hosts, concerns and the locations of positive findings (FERA 2009). In the US, groups such as the California Oak Mortality Task Force educate the public on *P. ramorum* through educational programmes aired on local television stations; provide educational guides to students; and rely on the public for reports of damage caused by the pathogen, such as weakened trees falling down near houses or power lines (COMTF 2009). In addition to an increase in the number of sites, another concern is that the same infection levels of *P. ramorum* are still being found at the positive sites. In Killarney National Park, a group known as Groundwork is a voluntary environmental organisation dedicated to the preservation of some of Ireland's most precious habitats (Groundwork 2009). Most of Groundwork's focus has been on the removal of the invasive *R. ponticum* from Killarney National Park. Their main aim is to clear rhododendron from the park during the summer months (June-September), and their methods of foliage removal and transport should be a cause for concern in the spread of the pathogen throughout the park. At present the group mainly deals with seedling removal, leaving the plant material on site for the NPWS to dispose of it. The group does not sterilise their equipment or footwear at any point during their time in the park (Muireann Debutlier, pers. comm.). As stated previously, the site in Lauragh is privately owned and instead of destruction of *R. ponticum* foliage the site has been demarcated. Until such time that aid is given to the owners for removal of infected plant material, it is unlikely the level of infection of *P. ramorum* will decrease at this site. To date, in the wild, *R. ponticum* is the only known host, but it is noteworthy that in the UK, where infected trees have been reported, infected rhododendrons have always been

found in close proximity (FERA 2009). If efforts to eradicate the pathogen are not increased, it is very likely that it is only a matter of time before infected trees are discovered in Ireland.

Although the aims of this study were carried out to completion, the sampling strategy was not without its faults. For statistical analysis of survey results, systematic sampling, sampling the population according to an ordered method and selecting plants at regular intervals, rather than random sampling, would have been a better strategy. Random collection of leaves did not allow for statistical comparison of individual plants. In future, sampling a host every 100 m, regardless of presence of symptoms or not, is recommended. The reason for sampling asymptomatic plants, as well as symptomatic, is that very often they can still prove positive for the pathogen even though visible symptoms are not present (Sandra Denman, pers. comm.). Also, although it was possible to statistically analyse positive morphological and molecular results, the whole statistical foundation might be flawed when based on the non-rigorous sampling methods employed. It is not surprising that there was a positive association between morphological and PCR results, because when a sample was identified as positive morphologically, it would also be expected to be positive by PCR analysis, provided that DNA protocols were followed correctly. If non-symptomatic leaves had been collected a positive association might not have been detected. Again, it is recommended that more rigorous sampling methods be used, such as systematic sampling mentioned above.

Due to lack of storage facilities, plant material that had been isolated onto P_5ARP agar (Van Leewen et al. 2003) had to be autoclaved for destruction, once DNA extraction had been carried out, instead of freezing the plant material for future analysis. In addition identification of unknown growth on plates was not carried out due to lack of expertise in this area. It would be beneficial to have found out what additional potential pathogens to *P. ramorum* were present at the sites sampled.

Future work on the susceptibility of tree species present at the positive sites would also provide valuable information. Lauragh, in Co. Kerry, is considered to have the highest amount of *P. ramorum* infection in Ireland (Gerard Cahalane, pers. comm.). The predominant tree species at this site is Douglas fir (*Pseudotsuga menziesii*) and analysis of the susceptibility of its stem and foliage would be worthwhile to determine the risk that could be posed by *P. ramorum* to Douglas fir in Ireland. Another species that could benefit from further analysis into stem susceptibility is the strawberry tree (*Arbutus unedo*). It is extremely rare in Ireland, being only found in the wild, in Killarney National Park.

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Microsatellite analysis of Irish wild and nursery isolates of *Phytophthora ramorum*

Abstract

The genetic structure of the Irish wild and nursery populations of *Phytophthora ramorum* was investigated in 93 isolates using eight variable microsatellite loci. All eight loci were non-variant for all isolates tested and only one multilocus genotype, EU1, was identified. No variation was observed within or between forest and nursery populations. A neighbour-joining tree based on the Irish isolate P0023 and nine *P. ramorum* isolates stored on GenBank was created and it showed that P0023 was genetically very close to all nine isolates with pairwise values of 0.000-0.002. The lack of variation between the forest and nursery populations using these loci could be due to the fact that *P. ramorum* is a recent introduction in Ireland. Also, analysis of a larger number of loci might reveal a greater level of polymorphisms within the Irish population of *P. ramorum*.

CHAPTER 3

MICROSATELLITE ANALYSIS OF IRISH WILD AND NURSERY ISOLATES OF *PHYTOPHTHORA RAMORUM*

3.1. INTRODUCTION

The two most widespread lineages of *Phytophthora ramorum* are the EU1 lineage, which occurs in Europe, and the NA1 lineage, which is the dominant lineage in North America (Brasier 2007). These two lineages differ significantly in their colony patterns, their mean growth rate, and in their mean aggressiveness on susceptible hosts (Brasier 2007). Several molecular methods, including amplified fragment length polymorphism (AFLP), microsatellite analyses and sequencing of genes/intragenic regions, have been used to study the molecular genetics of *P. ramorum* populations (Ivors et al. 2004). AFLP analysis amplifies and highlights a large number of molecular markers without the need to build a genomic library, and many *P. ramorum* isolates have been analysed using this technique (Ivors et al. 2004). AFLP analyses generally yields enough polymorphic markers to differentiate individuals within populations (Dutech et al. 2007). Microsatellites are polymorphic loci present in nuclear and organelle DNA that consist of repeating units of 1-6 base pairs (bp) in length (Dutech et al. 2007). They are co-dominant markers that typically display a high level of variability and are therefore frequently used to investigate the genetic structure of populations (Mascheretti et al. 2008). Using the whole genome sequence of *P. ramorum*, microsatellites (simple sequence repeats, SSRs) have been developed and used for fingerprinting large numbers of *P. ramorum* isolates originating from different host species in Europe and the US (Bonants et al. 2005; Ivors et al. 2006; Mascheretti et al. 2008). There are some limits to microsatellite analysis, one being the lack of microsatellite markers developed for studying the population genetics of *P. ramorum*. Ivors et al. (2006) found that microsatellite analysis was less revealing of the genetic diversity within *P. ramorum* populations than was AFLP analysis (Ivors et al. 2004). A common problem with microsatellites is the presence of null alleles; these fail to amplify because of base substitutions or deletions in PCR priming sites flanking microsatellites and thus bias the outocome of population genetic studies (Chapuis and Estoup 2007). Another problem with microsatellite analysis is the possibility of cross-amplification of loci among species of the same genera (Rossetto et al. 2001; Dutech et al. 2007).

There have been several studies carried out on the population genetics of *P. ramorum* in the US. Ivors et al. (2004) examined 67 US isolates, from two states, and 18 European isolates, from five countries, using four primer sets for AFLP fingerprinting, and showed that European and US populations clustered separately within individual clades, each displaying low levels of genetic variation, with a single clonal lineage dominating the North American population and several unique, but closely related, AFLP types represented in the European population. In order to get a better phylogenetic understanding of *P. ramorum* within the genus *Phytophthora* they used gene sequencing of specific mitochondrial genes (portions of the NADH dehydrogenase subunit 5 (*nad* 5) and the cytochrome ocidase subunit II (*cox* II)), and nuclear and ribosomal genes (internal transcribed spacer region of nuclear ribosomal (nr) RNA) to study the genetic structure of the *P. ramorum* populations in the US. The nrRNA gene is chosen in population genetic studies because it is well studied, has multiple copies, and assays that are based on it are generally more sensitive than assays designed for single copy genes (Kelvin Hughes, pers. comm.). They sequenced regions of these genes in *P. ramorum* and additional *Phytophthora* species, and found identical sequences for all *P. ramorum* isolates, and also that *P. ramorum* was most closely related to *P. lateralis* and *P. hibernalis*. Prospero et al. (2004) used 24 microsatellite markers to study the genetic structure of *P. ramorum* in Oregon. Primer sets were tested on 14-30 isolates from Europe and North America. Of the 24 primers used, 14 successfully amplified PCR products of the expected size, but seven were monomorphic across the two mating types, A1 and A2. However, the remaining seven polymorphic loci differentiated between the mating types, in that each was fixed for alternative genotypes at each locus, although there was no variation within mating type.

After finding significant variation between US and European populations by AFLP fingerprinting (Ivors et al. 2004) and microsatellite analysis (Prospero et al. 2004), Ivors et al. (2006) designed 12 new microsatellite markers and tested these on 71 US *P. ramorum* isolates (15 from nurseries and 56 from the wild) and 80 from the EU (79 of which were from nurseries and 1 from the wild). Like previous studies, the results demonstrated significant variation between US and EU isolates. Prospero et al.

(2007) analysed the genetic diversity of the *P. ramorum* population in Oregon, from 2001 to 2004, using 10 microsatellite markers. They analysed 323 isolates, of which 272 were representative of *P. ramorum* populations from 14 forest sites and 51 were from the nursery trade. Oregon forest and nursery populations were significantly differentiated, indicating that there was little gene flow between them. Eight isolates of a single European genotype, PrOR33, were recovered from three nurseries and had different alleles to the NA genotypes at four loci (Pr9C3, PrMS27, PrMS39b, and PrMS43a). Mascheretti et al. (2008) investigated the genetic structure of 292 isolates of *P. ramorum*, from 14 Californian forests and the nursery trade. They used seven variable microsatellite loci, five of which were from Prospero et al. (2007), while the remaining two were from Ivors et al. (2006). In an attempt to discover the origin of the epidemic in California they studied the allelic composition of both forest and nursery populations, from old, intermediate and new *P. ramorum* infestations, and they also studied genetic differentiation among the 14 forest sites in order to gather more information on the spread of the pathogen. They identified 35 multilocus genotypes (MGs) and three common genotypes as the likely cause of the California infestation. The study provided evidence of a link between the nursery and forest populations, as far back as the old isolates, and identified at least two sites in which *P. ramorum* may have been introduced into California.

Sequencing of a partial region of the ITS1 (internal transcribed spacer) 5.8s and a partial sequence of the ITS2 region of the nrRNA gene carried out by Central Science Laboratory (CSL), in the UK, rarely found major sequencing differences for any of the UK, European or US *P. ramorum* isolates, although some isolates have a "sequence wobble" of C or T at 627 bp. Through cloning it has been shown that these isolates have a 50:50 ratio of both gene sequences, possibly recording the very early stages in the evolution to a new species (Kelvin Hughes, pers. comm.). There have been no published reports on a comprehensive study of European populations of *P. ramorum*; although CSL, in the UK, are using microsatellites to investigate isolates from England and Wales their results have not yet been published (Judith Turner, pers. comm.).

The aim of this study was to analyse genetic variation both within and between Irish wild and nursery populations of *P. ramorum*, using eight microsatellite loci. Although 22 loci have been developed for the species, the eight loci used in this study were chosen on the basis of their high polymorphism in European and US isolates (Ivors et al. 2006), and because they have discriminated among North American isolates (Prospero et al. 2007). An analysis of the partial region of the ITS1 5.8s and partial sequence of the ITS2 region of the nrRNA gene was also carried out.

3.2. MATERIALS AND METHODS

3.2.1 Fungal Material

A total of 96 isolates of *P. ramorum* were analysed in this study; 51 were collected between 2005 and 2008, at three field sites in Ireland (Killarney National Park and Lauragh, Co. Kerry and Castletownbere, Co. Cork; see Appendix II tables A4 to A6), six were collected by the Department of Agriculture, Fisheries and Food (DAFF) at a fourth field site and 36 were provided by DAFF from nurseries from nine counties in the Irish Republic (Table 3.1). Ninety-nine percent of the hosts were *Rhododendron* spp., the other 1% were *Viburnum*, *Camellia*, and *Magnolia* species. Three positive controls, originating from different hosts (Table 3.1), were included in this study. *P. ramorum* was identified using the usual morphological criteria and confirmed by molecular analysis according to protocols described in Chapter 2, sections 2.2.4 and 2.2.5.

3.2.2. Microsatellite analysis

Polymerase chain reaction (PCR) amplification of eight polymorphic loci (Table 3.2) of *P. ramorum* (Ivors et al. 2006 and Prospero et al. 2007) was carried out. Each reaction contained 1 X reaction buffer (10 mM Tris-HCL, pH 8.8), 1mM $MgCl_2$, 0.2 mM deoxynucleotidetriphosphates (dNTPs), 0.2 µm of forward and reverse primers, 0.3 units of Taq DNA polymerase, 1 µl of template DNA, made up to a final volume of 10 µl with sterile deionised water (Adapted from Ivors et al. 2006).

Amplification was carried out in a Biometra™ T1 Thermal Cycler. Microsatellite loci 18, 64 and 82 were amplified with an initial denaturation of 95°C for 2 min, followed by 34 cycles of 94°C for 20 s, 55°C for 20 s, and 72°C for 30 s, and a final extension time of 72°C for 10 min. Loci 63 and 65 failed to amplify with this programme, so they were amplified using an initial denaturation of 95°C for 2 min, followed by 35 cycles of 94°C for 20 s, 64°C for 20 s, and 72°C for 30 s, and a final extension time of 72°C for 10 min. PrMS39b and PrMS45 were amplified with an initial denaturation of 92°C for 2 min, followed by 29 cycles of 92°C for 30 s, 58°C (PrMS39b) or 52°C (PrMS45) for 30 s, and 65°C for 30 s, and a final extension time of 65°C for 5 min. PrMS43a was amplified with an initial denaturation of 92°C for 2 min, followed by 34 cycles of 92°C for 30 s, 52°C for 1 min, and 72°C for 1 min, and a final extension time of 72°C for 45 min.

Table 3.1. Summary of Irish *Phytophthora ramorum* isolates used in microsatellite analysis.

Origin	Number of Isolates	Host (numbers)
Killarney	27	*Rhododendron ponticum*
Lauragh	14	*R. ponticum*
Castletownbere	10	*R. ponticum*
The Vee	6	*R. ponticum*
Nursery	36	*Rhododendron* spp. (32), *Camellia* spp. (2), *Viburnum* spp. (1), *Magnolia* spp. (1).
SCRP 954 P1376[$]	1	*Viburnum* spp.
SCRP 955 P1403[$]	1	*Lithocarpus* spp.
CSL *P. ramorum*[€]	1	*Rhododendron* spp.

[*]Provided by the Department of Agriculture, Fisheries and Food (DAFF), Maynooth, Co. Kildare, Ireland.

[$]Provided by the Scottish Crop Research Institute (SCR), Invergowrie, Dundee, DD2 5DA, Scotland.

[€]Provided by the Central Science Laboratory (CSL), York, Sand Hutton, UK.

Table 3.2. Sequence of the oligonucleotide primers used to amplify eight microsatellite loci in *Phytophthora ramorum*.

Locus ID	Repeat Motif	Primer sequence (5' to 3')[€]
18[*]	$(AC)_{39}$	F: [FAM] TGC CAT CAC AAC ACA AAT CC R: TGT GCT ATC TTT CCT GAA CGG
63[*]	$(CT)_{15}$	F: [FAM] ACA CGT ACA CGT AGG GCT CC R: GCT ATT GCA GTG ACG TGT GC
64[*]	$(CT)_{16}$	F: [FAM] GCG CTA AGA AAG ACA CTC CG R: CAA CAT GTA GCC ATT GCA GG
65[*]	$(CT)_{19}$	F: [HEX] GCA ACA ACA GCA ACA GCA TC R: GTT CTT CGA CGT GTG TGT GG
82[*]	$(GT)_{14}$	F: [HEX] CCA CGT CAT TGG GTG ACT TC R: CGT ACA AGT CAC GAC TCC CC
PrMS39b[$]	$(GA)_4(GATA)_{33}$	F: [HEX] GCA CGG CCA GAG ATT GAT AG R: ATC TGC CGA CGT GAA GAA GT
PrMS43a[$]	$(CAGA)_{71}$	F: [FAM] AAA TAT GCA AAA AGG CAG GA R: CCG CGT AAC CTA GTC TGC TC
PrMS45[$]	$(TCCCG)_{11}$	F: [FAM] CGT GCT GCA TCT GGT GTA GT R: GAA AGT CCG GAT TTG CGT TA

[*]Primers chosen from Ivors et al. (2006).

[$]Primers chosen from Prospero et al. (2007).

[€]F = forward primers; R = reverse primer. Fluorophores (6-carboxyfluorescein [FAM] or hexachloro-6-carboxyfluorescein [HEX]) used for labelling each forward primer are specified within primer sequence.

3.2.2.1 Preparation of acrylamide gel

Glass plates used for gel preparation (25 x 25 cm, 0.5 cm thickness) were cleaned with deionised water and 70% ethanol. A 10% 3-(Trimethoxysilyl) propylmethacrylate solution was spread along the inside on the top of the back glass plate (the area where

the wells would set) and allowed to dry for 10 min. The two glass plates were then placed on top of each other and separated by plastic spacing strips, 0.25 mm thickness; they were then held together by clamps (LI-COR Biosciences, USA). A 10% ammonium persulphate (Sigma, Ireland) solution was freshly prepared before pouring the gel, and 150 µl of this was added to 20 ml of 6.5% gel matrix solution, together with 15 µl of TEMED (Sigma, Ireland). The mixture was then mixed well. Using a 100 ml syringe, 19 ml of the gel solution was quickly poured between the two glass plates and a 48-well, rectangular tooth, plastic comb of 0.25 mm thickness (LI-COR Biosciences, USA) was immediately placed between the top of the two glass plates and the remaining 1 ml of gel solution was poured into the wells. The gel was allowed to set for 2 h.

3.2.2.2. Electrophoretic separation of amplified microsatellites

A tris Borate/EDTA (TBE) 1 X solution was used as electrophoresis buffer. Immediately prior to loading of the gel, 1 µl of PCR product was added to 6 µl of bromophenol blue loading dye and the samples were then denatured at 96°C of 3 min. During the denaturation of the samples, the gel was inserted into the LI-COR 4300 LI-COR Biosciences, USA) and air was removed from each well by placing the tip of a disposable 1 ml pipette into the well and flushing with 1 X TBE buffer. A pre-run was carried out to begin the pre-electrophoresis procedure for the gel. This focuses the laser in the gel and the electrophoresis voltage and temperature are optimized for the run. Once the pre-run was complete the wells were again flushed out with 1 X TBE buffer and the denatured samples were loaded into the wells using a 4200-15 ClickIR Gel Loading Syringe (LI-COR Biosciences, USA). Two molecular weight standards (MWS) were prepared to allow for product sizing. The MWS used were IRDye^{TM} 800 and IRDye^{TM} 700 (LI-COR Biosciences, USA) and the sizes used for each MWS were 50-350 bp and 50-700 bp. Five µl of each MWS were mixed into a 1.5 ml centrifuge tube and vortexed for 5 s, and 0.8 µl was loaded into the first and last two lanes on the acrylamide gel, when loading the samples.

3.2.3 Sequencing and phylogenetic analysis of a partial region of the internal transcribe spacer (ITS) region, of *P. ramorum* Irish isolates.

A partial region of the ITS1 5.8s and partial region of the ITS2 region of the nrRNA gene was amplified from 12 field isolates (three from each of the four field sites sampled), and 36 nursery isolates by PCR using primers PramF1 and PramR1 (Van Leewen at al. 2003). PCR amplification of the 48 isolates produced a 700 bp fragment of partial regions of both the 5.8s ITS region and the ITS 2 region. Products were sequenced by Sequiserve (GmbH Bahnhofstr 30, 85591, Vaterstette, Germany) using primer PramF1. One sequence reaction was carried out for each isolate. The sequence of isolate P0023 was compared with sequence data stored in GenBank (http://www.ncbi.nlm.nih.gov/) using the GenBank BLAST search protocol (Zhang et al. 2000). Sequences were used to determine the similarity coefficient between isolate P0023 and nine other European *P. ramorum* sequences stored within the GenBank database (Table 3.3). Using these similarity coefficients, a neighbour-joining (NJ) tree was generated in MEGA version 4 (Tamura et al. 2007). The robustness of the topology of the phylogenetic tree was assessed by bootstrap analysis (500 replicates) of the data set.

Table 3.3. Origin of nine *Phytophthora ramorum* sequences from GenBank (http://www.ncbi.nlm.nih.gov/).

GenBank accession number	Isolate number	Country of Origin	Genotype
AY785958	CSL 1527	Germany	EU1
EU558515	CC 2568	UK	EU1
DQ873514	CSL 20308642	UK	EU1
EF050516	P3012	Spain	EU1
AY423286	Pr120	USA	US1
AF521569	Pr70	USA	US1
AY423288	BA 12/98	Germany	EU1
AY423289	Phyram1	Spain	EU1
AF521570	Pr71	USA	US1

3.3. RESULTS

3.3.1. Microsatellite analysis

All eight loci were non-variant for all isolates tested (Table 3.4).

3.3.2. Sequencing and phylogenetic analysis

A partial region of the ITS1 5.8s and partial region of the ITS2 region of the nrRNA gene of all of the 48 isolates (12 from field sites and 36 from nurseries) showed 100% homology. The sequence of the partial region of the ITS1 5.8s and partial region of the ITS2 region of the nrRNA gene of *P. ramorum* strain P0023 (isolated from *Rhododendron* spp., and provided by DAFF) was compared with nucleotide sequence data of 101 isolates of *Phytophthora* spp. stored on GenBank, using GenBank BLAST search protocol. The search results showed that 18% of the GenBank sequences were a 100% match to isolate P0023, while 49% were a 99% match, and all were described as *P. ramorum*. Other sequences with substantial homology to the sequence of the partial region of the ITS1 5.8s and partial region of the ITS2 region of the nrRNA gene from *P. ramorum* isolate P0023 were derived from other species of *Phytophthora*.

A NJ tree, based on sequences from isolate P0023 and nine *P. ramorum* isolates from GenBank, showed that sequences from AY785958, EU558515, and DQ873514 were genetically more similar to P0023 (0.000 pairwise distance) than were sequences from EF050516, AY423286, AF521569, AY423288, AY 423289 and AF521570 (0.002 pairwise distance) (Figure 3.1).

Table 3.4. Genotypes for individual microsatellite loci in Irish wild and nursery isolates of *Phytophthora ramorum* and a comparison with results from Ivors et al. (2006) and Prospero et al. (2007). (N/A = loci not analysed in their study).

		Locus							
Multilocus Genotype	**Isolate**	18	63	64	65	82	PrMS39b	PrMS43a	PrMS45
EU1	93 Irish isolates	218/264	155/163	346/392	236/244	112/130/140	136/140	146/146	163/187
EU1[$]	SCRP 954 P1376	218/264	155/163	346/392	236/244	112/130/140	136/140	146/146	163/187
US1[*]	SCRP 955 P1403	220/278	159/165	338/374	234/252	110/112/114	250	377	167/187
EU1	CSL *P. ramorum*	218/264	155/163	346/392	236/244	112/130/140	136/140	146/146	163/187
	Total no. of alleles	2	2	2	2	3	2	1	2
EU1	69 EU isolates (Ivors et al. 2006)	218/264	155/163	346/392	236/244	112/130/140	N/A	N/A	N/A
EU1	8 EU isolates (Prospero et al. 2007)	N/A	N/A	N/A	N/A	N/A	136/140	146/146	163/187

[$]Subculture from *Viburnum*, England, from Clive Brasier Forest Research, Alice Holt, Farnham (David Cooke, pers. comm.).

[*]Subculture from *Lithocarpus*, USA, from Clive Brasier Forest Research, Alice Holt, Farnham (David Cooke, pers. comm.).

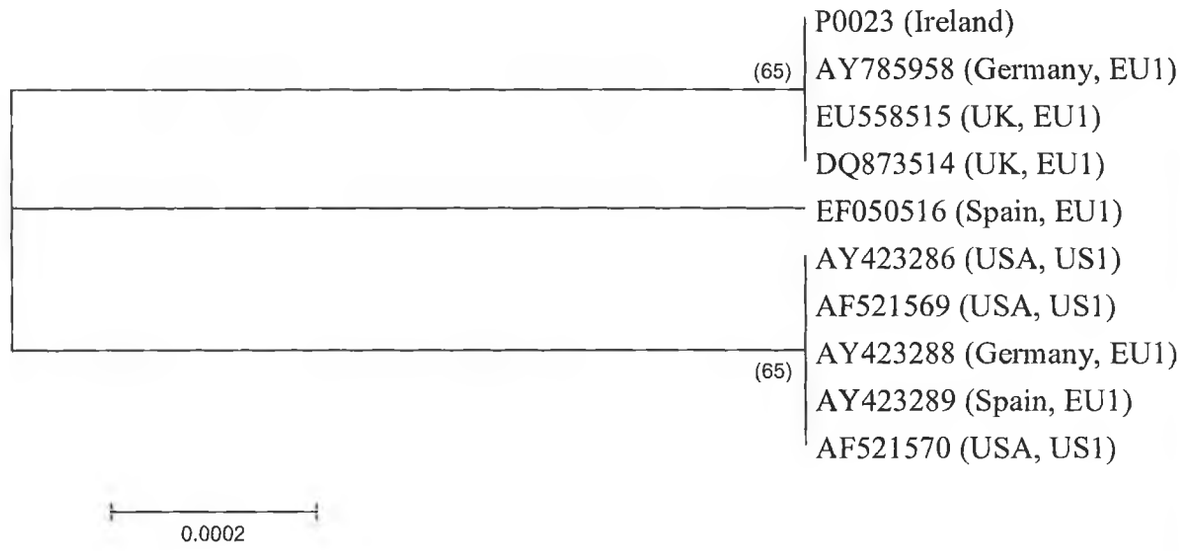

Figure 3.1. Neighbour-joining tree based on the sequence of the partial region of the ITS1 (internal transcribe spacer) 5.8s and partial region of the ITS2 region of the nrRNA gene of *Phytophthora ramorum*, isolate P0023, and nine *Phytophthora ramorum* isolates stored on GenBank inferred from *MEGA* version 4 software. Numbers in parentheses are bootstrap support values over 50% derived from a bootstrap consensus tree obtained through 500 replications.

3.4. DISCUSSION

The null hypothesis that there is no genetic variation either within or between wild and nursery samples was accepted. All of the 93 *P. ramorum* isolates from Irish wild sites and nurseries had the same genotype at each of the eight microsatellite loci; in other words, each isolate showed a similar MG. Five of the eight loci used (loci 18, 63, 64, 65 and 82) were from Ivors et al. (2006). They analysed 71 US *P. ramorum* isolates (15 from nurseries and 56 from the wild) and 80 from the EU (79 of which were from nurseries and 1 from the wild). Using 12 microsatellite loci, two were polymorphic among US isolates (loci 18 and 82), while three were polymorphic

among EU isolates (loci 18, 64, and 82). On this basis loci 18, 64 and 82 were the most informative, as they showed variation within both US and EU populations. They also found that for all 12 loci tested there was significant variation between US and EU populations, but limited variation within them, and there was a higher diversity in US nurseries than in forests. Eleven MGs (over the 12 loci), four in the US (US 1-4) and seven in the EU (EU 1-7) were identified among the total sample collection. The most common MG in EU isolates was EU1, which represented approximately 87% of the isolates tested. The most common US MG was US1; this represented approximately 95% of the US isolates tested. Five of the isolates from US nurseries were identical to the most common EU genotype (EU1), indicating that both EU and US genotypes exist in US nurseries. The 151 isolates clustered into three clades: the US forest and EU nursery isolates clustered into two distinct clades, while one isolate from a US nursery belonged to a third new clade. They concluded that a single genotype (US1) introduction was responsible for almost the entire studied forest population. All of the 93 Irish isolates tested in this study, using loci 18, 63, 64, 65 and 82, showed the same MG as the EU1 isolate from Ivors et al. (2006) (Table 3.4).

Three of the eight loci used in this study (PrMS39b, PrMS43a and PrMS45) were from Prospero et al. (2007). Because Ivors et al. (2004, 2006) reported a low level of genetic variation in Californian isolates of *P. ramorum*, Prospero et al. (2007), for comparison, analysed the genetic diversity of 323 isolates from Oregon's nurseries (N=51) and forests (N=272), from 2001-2004, using 10 microsatellite loci. Isolates from forest sites in Oregon were characterised by low genetic diversity, similar to the levels observed by Ivors et al. (2006) for Californian isolates, and 23 MGs were identified, with a single MG (PrOR1) at frequencies of 60-70%. Prospero et al. (2007) concluded that forest isolates in Oregon belong to the same clonal lineage, i.e. are descendants from a single individual. In contrast, nursery isolates were not dominated by a single MG and one genotype, PrOR33, only recovered from nurseries, belonged to the European lineage of *P. ramorum*.

Prospero et al. (2007) showed that of the 10 loci analysed only four tetranucleotide repeat loci (PrMS39b, PrMS43a, PrMS43b and PrMS45) were

polymorphic, one of which PrMS43b did not produce any amplicon for the EU isolates. Although they speculated that the low diversity they observed could have been due to the microsatellite markers chosen, similar results were also reported by Ivors et al. (2006) for Oregon isolates using different loci. In this study the results from the three loci (PrMS39b, PrMS43a and PrMS45) tested corroborated the results from Prospero et al. (2007); all isolates tested from Irish forest and nurseries had the same MG as the EU lineage sampled in their study (Table 3.4). While alleles at two of the loci (PrMS39b and PrMS43a), were not observed in their US forest or nursery isolates, one allele (187) at PrMS45 was shared between EU and Oregon lineages.

Mascheretti et al. (2008) analysed the genetic structure of 292 isolates of *P. ramorum* from 14 forest sites and the nursery trade, in California. They used seven microsatellite loci, which were previously identified as polymorphic in the North American lineage: PrMS39a, PrMS39b, PrMS43a, PrMS43b and PrMS45 (Prospero et al. 2007) and 18 and 64 (Ivors et al. 2006), five of which were common to this study. They found that loci PrMS39a and PrMS45 were invariant and locus 18 was variable in four individuals, while locus 64 was variable in only one individual. The combination of the five variable loci used in their study identified a total of 35 MGs, out of which none were from the European lineage of *P. ramorum*.

The NJ tree, based on the Irish isolate P0023 and nine *P. ramorum* isolates from GenBank, showed that P0023 was genetically very close to all nine isolates with pairwise values of 0.000-0.002. The Irish isolate along with three EU isolates (AY785958, EU558515, and DQ873514; pairwise distance 0.000) formed one group, while the remaining six isolates (EF050516, AY423286, AF521569, AY423288, AY 423289 and AF521570), which included three USA and three EU isolates, formed two groups, but were still genetically very close to P0023 (0.002 pairwise distance). Although there was no distinct pattern to the phylogenetic tree, the three US isolates clustered together albeit with three EU isolates (Fig.3.1). It is interesting that both the Irish and the two UK isolates also clustered together.

The aim of this study was to analyse genetic variation both within and between Irish wild and nursery populations of *P. ramorum*, using microsatellite loci.

Ivors et al. (2006) found seven MGs within the EU population of *P. ramorum*. The most common MG was EU1 (49%), which was found in both the wild and nursery isolates. The other six MGs had a combined frequency of 7.27%, and were only found in their nursery isolates. The higher diversity in the European nursery population was attributed to the likely introduction of a few closely related genotypes followed by the creation of new genotypes via mitotic recombination and/or mutation. In the Prospero et al. (2007) study, the genotype PrOR33 was recovered from three Oregon nurseries and was of European lineage. None of the genotypes from the forests or nurseries had allele patterns that suggested recombination between PrOR33 and the North American genotype. Analysis of European wild isolates was not carried out so therefore a comparison between the European wild and nursery populations, using these loci, could not be made.

The results of this study indicate that *P. ramorum* in Ireland consists of one MG, which corresponded to EU1 from Ivors et al. (2006) and PrOR33 from Prospero et al. (2007), with no variation exhibited within or between forest and nursery populations. The lack of variation between the forest and nursery populations using these loci could be due to the fact that *P. ramorum* is a recent introduction in Ireland, whereas it is believed that the pathogen has been present in the US since the early 1990s. Also, one cannot rule out the possibility that analysis of a larger number of loci might reveal a greater level of polymorphisms both within and between our isolates. It is regrettable there are no published reports, to date, on analysis of genetic variability in European populations using microsatellite markers developed from European isolates, and therefore it has not been possible to put the results from this study on Irish forest and nursery isolates in the wider context of other European populations.

3.5. REFERENCES

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Susceptibility testing

Abstract

The leaves of four under-storey (*Rhododendron ponticum*, *Fuschia magellanica*, *Hydrangea macrophylla* and *Ilex aquifolium)* and two tree species (*Quercus petraea* and *Arbutus unedo*) important to Irish forests were tested for their susceptibility to the Irish isolate K2, of *Phytophthora ramorum*, using a detached leaf assay. *Rhododendron ponticum* and *I. aquifolium* are among the most common under-storey species found in Irish forests, and along with *H. macrophylla*, are often found in many public and private gardens throughout Ireland. *Quercus petraea* is an important host in Ireland since there are very few remaining native oak woodlands left, and those that remain are heavily invaded by *R. ponticum*. *Arbutus unedo* is one of Ireland's rarest tree species. Wounded and non-wounded leaves were used to determine if wounding the upper leaf surface increased the level of infection. The results of this study showed that all of the under-storey species, with the exception of *H. macrophylla*, were susceptible to P0023, with *R. ponticum* being the most susceptible. Both *Q. petraea* and *A. unedo* showed moderate susceptibility. Wounding the leaves prior to inoculation with *P. ramorum* significantly increased the size of the lesions, compared to non-wounding.

CHAPTER 4
SUSCEPTIBILITY TESTING

4.1. INTRODUCTION

To date, over 120 plant species, from 36 families, have been confirmed as potential hosts of *Phytophthora ramorum* (www.defra.gov.uk/planth/newsitems/suscept.pdf), and the host range is constantly increasing. An important advance in *P. ramorum* research was the recognition that the pathogen can cause very different symptoms on different hosts (Hansen et al. 2005). Symptoms caused by *P. ramorum* depend on the host plant species, and at present three different disease symptoms exist: stem canker, twig and leaf dieback, and leaf necrosis (Davidson et al. 2003). Not all host species are affected to the same extent. *P. ramorum* is only mildly virulent on foliar hosts, but this enables build up of a high inoculum load and assures the long-term survival of the pathogen in most woodland (Garbelotto 2003). Upon infection by *P. ramorum,* such hosts produce non-fatal lesions from which inoculum can be produced and spread to neighbouring hosts (Garbelotto 2003). An example of such a host is Bay laurel (*Umbellularia californica*), which is a foliar host of the pathogen that exhibits a certain level of resistance. Bay laurel is a major host species due to its high abundance in oak woodlands in the US and to the large amount of inoculum that can be produced from its lesions after infection (Anacker et al. 2007). Bay laurel appears to vary in susceptibility to *P. ramorum* but little is known about the causes or extent of this variability (Anacker et al. 2007). Another such example is rhododendron (*Rhododendron ponticum*), which could possibly form a bridge for the pathogen between the ornamental industry and the wild (Garbelotto 2004). *R. ponticum* is likely to be the most significant source of inoculum for UK trees with bark susceptibility, since it is evergreen, possesses both susceptible leaves and stems, appears to have a low inoculum threshold (a small amount of inoculum will cause infection) and can produce significant numbers of infectious spores (Inman 2005).

In Europe, *P. ramorum* has been reported in 15 member states, including the UK and Ireland, as either outbreaks, interceptions or on imported hosts (http://rapra.csl.gov.uk/background/index.cfm). The pathogen was first found in the south of the UK in 2002 and since then around 718 outbreaks have been reported in nurseries, garden centres, and in the wild (www.defra.gov.uk/planth/pramorum3.htm. In Ireland, *P. ramorum* has been found at six locations in the wild, on *R. ponticum* (Gerard Cahalane, pers. comm.) and there were 44 positives reported in nurseries and

garden centres from November, 2007 to September, 2008 (Matthew Clarke, pers. comm.). Results from surveys carried out from 2004-2007 in nurseries and retail premises in England and Wales showed that 96% of the findings of *P. ramorum* were on *Rhododendron* (47%), *Viburnum* (41%), and *Camellia* (8%) (Slawson et al. 2007).

Examining patterns of host susceptibility can be an important component of understanding and predicting disease spread (Anacker et al. 2007). There have been many susceptibility tests carried out on ornamental foliar hosts and tree hosts in the US and Europe. While establishing a standard pathogenicity test for *P. ramorum*, the Central Science Laboratory (CSL) in the UK determined the susceptibility of several wounded broad-leaved ornamental and under-storey species (Inman 2005). Results showed that 31 of the 59 plant taxa tested showed some level of susceptibility, using wounded leaves and mycelial plugs. Also in the UK, Denman et al. (2005) carried out *in vitro* leaf inoculation studies to test tree foliage susceptibility of 11 conifer hosts, using summer (leaves) and winter (twigs) host material, and 23 broad-leaved hosts, using summer host material only. Two European isolates and two US isolates were tested on wounded and non-wounded leaves, using the zoospore dipping method. Their results showed no significant difference in the pathogenicity among the *P. ramorum* isolates for either conifers or broad-leaf hosts; most conifer hosts, with the exception of one, showed a greater amount of necrosis for summer inoculations; all of the conifer and broad-leaf hosts showed some level of disease incidence on the wounded leaves, while for one species from each host type there was no evidence of disease on non-wounded leaves. In Spain Moralejo and Hernandez (2002) carried out inoculation trials on wounded leaves of plants of Mediterranean evergreen oak forest and maquis-type vegetation. Out of the 12 species tested, 10 proved to be susceptible and there were differences in lesion expansion. Numerous results from susceptibility studies carried out throughout Europe and North America can be found on the website, Risk Analysis for *P. ramorum* (RAPRA) (http://rapra.csl.gov.uk).

To date, no susceptibility tests have been carried out using Irish isolates of *P. ramorum*. While it is unlikely that the host range specificity of Irish isolates of *P.*

ramorum will differ significantly from that of other European and of US isolates, it is important to find out the level of susceptibility for Irish under-storey hosts that could potentially act as a source of inoculum for tree infection. The aim of this study was to test the susceptibility of four under-storey species and two tree species to *P. ramorum* using one Irish strain of the pathogen. Under-story hosts studied were *R. ponticum*, fuschia (*Fuschia magellanica*), hydrangea (*Hydrangea macrophylla*) and holly (*Ilex aquifolium*), and the two tree species studied were sessile oak (*Quercus petraea*) and the strawberry tree (*Arbutus unedo*). Rhododendron is likely to be the most important foliar host, in Ireland, since it is the most common understorey species in oak woodlands; to date, *P. ramorum* has only been found in the wild on rhododendron (Gerard Cahalane, pers. comm.). Rhododendron, fuschia, and hydrangea are also used as hedging in many public and private gardens throughout Ireland. Very often the public take clippings from the wild and replant them in their gardens, without knowing what diseases they could potentially be carrying. Next to rhododendron, holly is the most abundant under-storey host in Irish woodlands. It is found very often in oak woodlands, mixed in with rhododendron. Even though sessile oak is only mildly susceptible to *P. ramorum*, there are very few remaining native oak woodlands left in Ireland, and those that remain are heavily invaded by rhododendron. The strawberry tree is one of Ireland's rarest tree species and can only be found, growing naturally in the wild, at a few locations in Killarney, SW Ireland (http://homepage.eircom.net/~knp/species/index.htm). If trees were to become infected in Ireland by *P. ramorum* it could have devastating effects on our native stands. *Rhododendron ponticum* is reported as a natural host in three countries on the RAPRA website (http://rapra.csl.gov.uk) and has also been confirmed as a natural host in Ireland (see Chapter 2, section 2.3). *Ilex aquifolium* is not listed as a natural host in any country; it has only been determined as a potential host through trials carried out in the laboratory in three countries in the EU and the USA. *Fuschia magellanica* has not been reported as a natural host in any country; like *I. aquifolium*, it has only been listed as a potential host in the UK. *Quercus petraea* is listed as a natural host in the UK (http://rapra.csl.gov.uk/objectives/wp1/potentialHostSearchResult.cfm). In Spain the *Arbutus unedo* has been found to be a natural host in nurseries, and has been tested as a potential host in Italy. Although there is no record of susceptibility tests having

been carried out on *Hydrangea macrophylla* the species has been reported as non-susceptible to *P. ramorum* (http://www.hionisgreenhouses.com). As part of this study, the influence of leaf wounding, prior to pathogen inoculation on different hosts, was also determined.

4.2. MATERIALS AND METHODS

4.2.1. Plant material

Leaves of rhododendron (*Rhododendron ponticum*), fuschia (*Fuschia magellanica),* hydrangea (*Hydrangea macrophylla),* and holly (*Ilex aquifolium)* and sessile oak (*Quercus petraea)* were collected from Connemara National Park, during the month of May, 2008, and the strawberry tree (*Arbutus unedo)* was collected from a private garden in Co. Galway, also during the month of May. Five leaves were collected from two plants of each species, except in the case of *Arbutus unedo* where ten leaves were collected from one tree, as only one tree was available for sampling.

4.2.2. Susceptibility testing

Mycelial plugs of the *P. ramorum* isolate K2, originating from Killarney National Park (Co. Kerry), were used to test the susceptibility of the four under-storey species and the two tree species, using the methods adapted from Inman (2005) and Denman (2005). Only one isolate was chosen because it was shown in Chapter 3 (section 3.3.1) that all Irish isolates of *P. ramorum* collected during this study were genetically non-variant, consisting of just one multilocus genotype (MG). The isolate was cultured on Pimaricin-ampicillin-rifampicin-pentachloronitrobenzene (P_5ARP) (Van Leewen et al. 2003) according to protocols described in Chapter 2, section 2.2.3. Plant material was divided into two categories, wounded and non-wounded treatments. For each category, susceptibility testing was carried out on the leaves collected from two plants from each species, with five replicates per plant of detached, non-symptomatic, mature leaf material. Prior to inoculation, leaves were surface rinsed with sterile distilled water (SDW) and placed air-dried on sterile paper towels. Leaves were wounded on the middle of the upper leaf surface with a sterile scalpel. A mycelial plug was taken from the edge of the colony growth, and then placed on either the top of the wound or on the centre of non-wounded leaves. A drop of SDW was placed on the plug to prevent it from drying out. Leaves were then

placed on thoroughly moist paper towels, which lined the bottom of clear plastic storage boxes. The boxes were sealed with cling film and incubated at approximately 18-20°C in a fume cupboard. The moist chambers were opened daily and the sides were sprayed down with SDW.

Lesion development was assessed after a seven-day incubation period. Lesion diameter was measured (mm) for each of the five replicates in the two categories and the mean lesion diameter was calculated. The level of susceptibility was determined using the lesion index rating from Inman (2005) (see Table 4.1): 0 = no lesion; 1 = lesion not extending beyond the wound or plug (<9mm); 2 = lesion extending 1-2 mm beyond the plug (~10-13 mm diameter); 3 = lesion extending 3-7 mm beyond the plug (~14-23 mm diameter); 4 = lesion extending >8 mm beyond the plug (>24 mm diameter). P_5ARP agar plugs were used as negative control in treatments.

After the incubation period re-isolation was carried out to confirm that lesions were caused by *P. ramorum* and not by another pathogen. Four pieces of plant material, per leaf, were cut from the leading edge of the lesion and were plated on PARP agar and identification of *P. ramorum* from the leaf material was carried out using the standard morphological criteria and identity was confirmed by molecular analysis according to protocols described in Chapter 2, sections 2.2.4 and 2.2.5. Re-isolation was confirmed when one of the four pieces of leaf material was positive for *P. ramorum*. The scores for re-isolation were determined as described in Denman et al. (2005). Zero = pathogen not re-isolated; low = 1-39% of re-isolations yielded the pathogen; moderate = 40-74% of re-isolations yielded the pathogen; high = *P. ramorum* obtained in > 75% of re-isolations.

4.2.3. Statistical Analysis

To assess the effects of wounding and non-wounding on leaf susceptibility one-way ANOVA was carried out using Minitab 15.1. Data were first tested for normality and homogeneity of variances, using Minitab 15.1.

4.3. RESULTS

Wounded and non-wounded detached leaves of four under-storey species and two tree species were tested for susceptibility to disease caused by the Irish *P. ramorum* isolate K2. In previous studies Rhododendron plants have been used as positive controls to confirm the pathogenicity of the isolates of *P. ramorum* (Denman et al. 2005 and Inman 2005). All *R. ponticum* leaves, from both the wounded and non-wounded categories, showed 100% incidence of necrosis, which confirmed the virulence of the *P. ramorum* isolate, K2.

Results for the leaves of under-storey species in the wounded test showed that *R. ponticum* was highly susceptible, *F. magellanica* was slightly susceptible, *I. aquifolium* was resistant and *H. macrophylla* was virtually immune. The leaves of the tree species showed that both *Q. petraea* and *A. unedo* were moderately susceptible. Results from ANOVA showed that wounding significantly increased the size of the lesions compared to non-wounding ($F = 8.99$, $DF = 1$, $P < 0.05$). This was evident in the case of *I. aquifolium* and *F. magellanica*. In the absence of wounding both under-storey plant species were virtually immune to *P. ramorum*, whereas, in the presence of wounding, *I. aquifolium* became resistant and *F. magellanica* was slightly susceptibility. Based on their mean lesion index, on wounded plant material, plant species were assigned to one of the five disease susceptibility categories of Inman (2005) (Table 4.1).

P. ramorum was not isolated from any of the leaves for the negative controls, and, as will be discussed below, isolation efficacy from inoculated plant leaves depended on plant species.

Table 4.1. Susceptibility categories for broad-leaved plant species tested using wounded leaves and mycelial plugs of *Phytophthora ramorum*. After Inman (2005).

Susceptibility category	Mean lesion index (MLI)	Leaf lesion development (diameter)
0: Virtually Immune	0.00 – 0.49	No necrosis or necrosis only in damaged tissue
1: Resistant	0.50 – 1.49	Lesion not extending much beyond wound (<9mm)
2: Slightly susceptible	1.50 – 2.49	Lesion extension slight (~10-13 mm in 7 days)
3: Moderately susceptible	2.50 – 3.49	Lesion well developed (~14-23 mm in 7 days)
4: Highly susceptible	3.50 – 4.00	Lesions very extensive (~24 mm in 7 days)

Table 4.2. Levels of re-isolation of *Phytophthora ramorum*, Irish isolate K2, from inoculated leaves of four under-storey species and two tree species. Adapted from Denman et al. (2005).

Host species	Total % of re-isolation of *P. ramorum* from tested leaves	Infection potential (re-isolation)
Rhododendron ponticum	**100**	**High**
Ilex aquifolium	**50**	**Moderate**
Fuschia magellanica	**80**	**High**
Hydrangea macrophylla	**0**	**Zero**
Quercus petraea	**100**	**High**
Arbutus unedo	**71**	**Moderate**

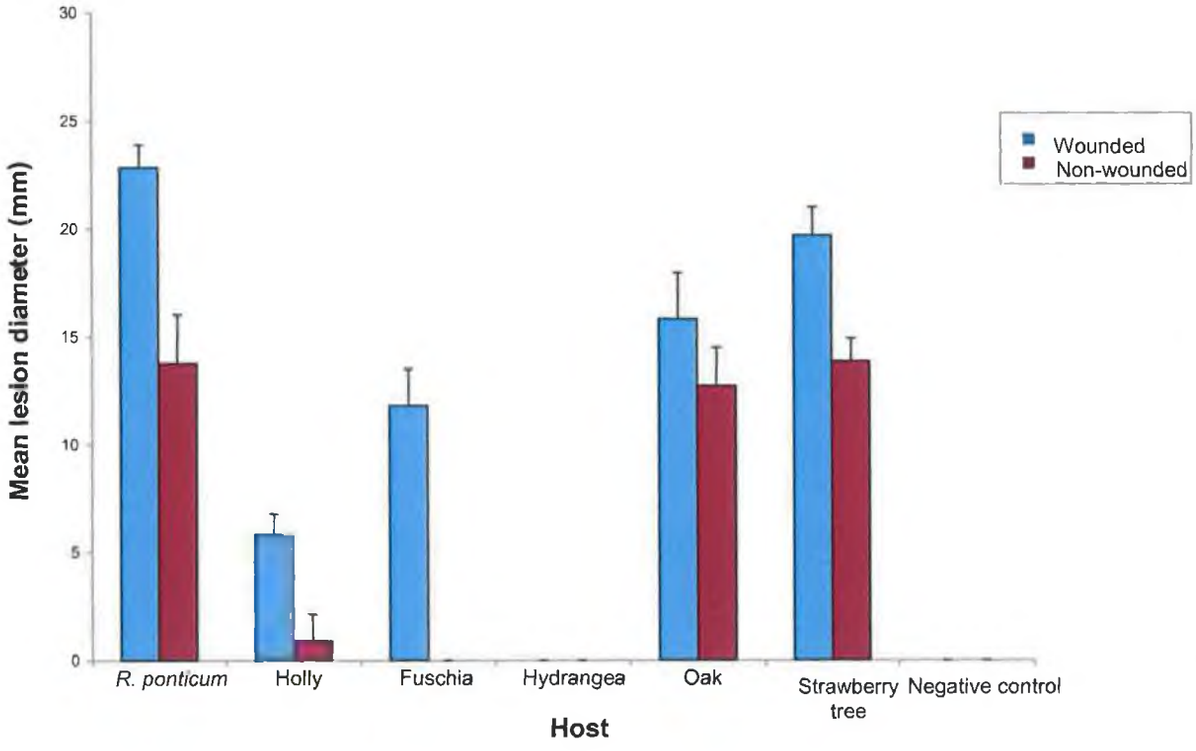

Figure 4.1. Mean lesion diameter (mm) on plant leaves seven days post-inoculation with mycelial plugs of *Phytophthora ramorum* isolate K2.

4.4. DISCUSSION

The null hypothesis that the leaves of four under-storey and two tree species are not susceptible to *P. ramorum* was rejected in the case of all plant species tested, with the exception of *H. macrophylla*. The null hypothesis that wounding the leaves on the upper surface does not increase the level of infection caused by *P. ramorum* was also rejected. Wounding the leaves prior to inoculation with *P. ramorum* significantly increased the size of the lesions compared to non-wounding.

The results for *R. ponticum* in this study showed a significant difference in susceptibility between wounded and non-wounded leaves, the former showing high susceptibility and the latter moderate susceptibility. When Inman (2005) carried out mycelial plug inoculation studies on wounded leaves of *R. ponticum* he found extensive lesion development, indicative of high susceptibility. The same test was

not carried out on non-wounded leaves. Denman et al. (2005) used rhododendron as a positive control in inoculation studies on wounded and non-wounded leaves and found a 100% incidence of necrosis for both treatments. Re-isolation of *P. ramorum* from *R. ponticum* was high, with 100% of the leaves showing necrosis.

I. aquifolium was resistant in the wounded test and virtually immune in the non-wounded test. Inman (2005) also tested *I. aquifolium* by the mycelial plug inoculum method on wounded, but not non-wounded leaves, and found it to be resistant. Denman et al. (2005) used the zoospore dipping method and found that *I. aquifolium* had low susceptibility on non-wounded leaves and high susceptibility on wounded leaves. The fact that their results are different to the results of this study could be due to the difference in the methods used. The zoospore dipping method, which is used to determine the potential for hosts to have natural leaf infection of *P. ramorum*, provides a better prediction of susceptibility to the range of diseases caused by *P. ramorum*, than mycelial plugs (Hansen et al. 2005). This is possibly because artificial wounding is not required and it best mimics the field susceptibility of the same species (Hansen et al. 2005). In the case of *I. aquifolium*, the pathogen was re-isolated from 50% of the leaves, which indicates a moderate infection potential (Table 4.2), which was consistent with results from Denman et al. (2005).

F. magellanica was found to be slightly susceptible in the wounded test and virtually immune in the non-wounded test. Inman (2005) found this species to be moderately susceptible using wounded leaves. The difference in results from the wounded test compared to Inman (2005) can be explained by the fact that although *F. magellanica* was found to be slightly susceptible in this study (a mean lesion diameter of 11.5 mm), it was close to the moderately susceptible category. *P. ramorum* was re-isolated from 80% of *F. magellanica* leaves (Table 4.2), which were all from the wounded category, as non-wounded leaves were resistant to *P. ramorum*.

Wounded leaves of *Q. petraea* were moderately susceptible, while the non-wounded leaves were slightly susceptible. Denman et al. (2005), using the same species of oak as in this study, tested detached non-wounded leaves with the zoospore dipping method and found a high proportion of leaf necrosis, and a high

level of infection potential, that rated oak as moderately susceptible. This differs from our results, which found *Q. petraea* in the non-wounded test to be only slightly susceptible, but again this could be due to the putative sensitivity of their method (Hansen et al. 2005). The susceptibility of *Q. petraea* was also tested for a Forestry Commission UK report in 2005 (http://www.forestresearch.gov.uk/forestry/INFD-6RWL44), using detached foliage and, in contrast to our results, moderate susceptibility was found. As the methods used were not specified in the report it is unclear as to the reason for these differences. Re-isolation frequency of *P. ramorum* from *Q. petraea* was high, which is similar to results reported by Denman et al. (2005).

The *A. unedo* was moderately susceptible in both wounded and non-wounded leaf tests and re-isolation levels were moderate (Table 4.2). Moralejo and Hernandez (2002) carried out zoospore dipping on detached non-wounded leaves of *A. unedo* and found the leaves to be moderately to highly susceptible, with necrotic lesions apparent 72 hours after inoculation with extensive blight developing in nine days. Tests were also carried out in Italy using the detached leaf zoospore dipping method on non-wounded leaves; the results indicated moderate susceptibility (http://rapra.csl.gov.uk/objectives/wp1/potentialHostSearchResult.cfm).

There are several possible reasons for the differences in results compared to previous studies; type of inoculum used, leaf age, number of isolates used, and isolate effects. As stated previously, the zoospore dipping method, which was used in Denman et al. (2005) and Hansen et al. (2005), may be slightly more robust to infection than mycelial plugs. The test used in this study was the mycelial plug assay on detached stems, after Inman et al. (2005). In comparative evaluations with the alternative zoospore-dipping method, Inman et al. (2005) found that the mycelial plug method performed equally well when tested over 15 plant species and gave more consistent results. The mycelial plug method has shown to be a relatively good predictor of natural susceptibility, it is easier to use, and allows quantification of infection (Inman et al. 2005). All plant material collected for this study was collected from mature healthy trees. Physiological conditions of host tissue associated with leaf age affects disease symptoms (Denman et al. 2005). In that

study they also collected leaf material from mature healthy trees and found that leaf necrosis was more severe in the young tissue used in initial experiments and decreased when slightly older leaves were used. Hansen et al. (2005) also pointed out that leaf age affects susceptibility when using the detached leaf assay.

Although only one isolate was used in this study, it was confirmed in chapter 3, section 3.3.1, that all isolates of *P. ramorum* collected during this study were genetically identical, consisting of just one multilocus genotype. The use of more than one isolate might have yielded different results, although Denman et al. (2005) used four isolates of *P. ramorum* and found no significant differences among the isolates for any of the parameters measured (incidence of necrosis and infection based on absence or presence of re-isolation). This merely indicated that the populations of *P. ramorum* used in that study did not behave differently on the various hosts. No conclusions could be drawn about the relative aggressiveness of sub-populations from the data presented in that study (Denman et al. 2005). However, it has previously been shown that sub-populations of *P. ramorum* have demonstrated differences in aggressiveness when tested on logs (Brasier 2003).

The susceptibility studies confirmed that the Irish isolate of *P. ramorum*, K2, can infect the leaves of *R. ponticum*, *F. magellanica, I. aquifolium, Q. petraea,* and *A. unedo*. *H. macrophylla* is not considered as a host of *P. ramorum* (http://www.hionisgreenhouses.com), and this was the case in our study, although no other records of susceptibility tests on the species have appeared in the literature to date. A study into the environmental factors that affect the establishment of *P. ramorum* in the field, i.e. leaf age and position of leaves in the canopy, would be useful to those currently monitoring the spread of the pathogen in Ireland. Further work comparing the mycelial plug assay and the zoospore-dipping method and using more isolates of *P. ramorum*, would be useful in confirming the results of this study, and for studying the susceptibility of further important ornamental and tree species in Ireland to the pathogen.

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Temperature and pH sensitivity testing

Abstract

The chlamydospores of *Phytophthora ramorum* are considered to play a role in the survival of the pathogen, acting as resting structures, which allow the pathogen to survive during unfavourable climates or when susceptible hosts are not present. The survival potential of the chlamydospores was determined by examining the effects of temperature and pH on chlamydospores. Four temperature regimes (-21, 4, 20 and 40°C) were investigated over a 24-h period, and six pH regimes (2, 3, 4, 5, 7 and 9) were investigated over a 6-h period. Statistical analysis showed that the percent germination differed significantly among the temperature treatments ($P < 0.001$). Temperatures of 4°C and 20°C did not affect the survival of chlamydospores, as compared to the other treatments tested. At a temperature of -21°C all chlamydospores were killed by 5 h and at 40°C 90% of chlamydospores were dead by 6 h. Statistical analysis also showed that the percent germination differed significantly among the pH treatments ($P < 0.001$). Exposure to pH 3, 4, 5, 7 and 9 did not affect germination of chlamydospores over the 6-h period, while at pH 2 there was only 1% survival at 4 h and no chlamydospores survived the 6-h period.

CHAPTER 5
TEMPERATURE AND PH SENSITIVITY TESTING

5.1. INTRODUCTION

Phytophthora ramorum spreads mostly aerially and usually infects trees and plants above the soil line. The pathogen also exhibits the soil-borne and water-borne characteristics of its close *Phytophthora* relatives (Garbelotto 2004). In general, *Phytophthora* species that infect aerial parts of plants spread through a cycle of production of sexual, or more often, asexual spores, movement of spores, and infection of new host tissue (Davidson and Shaw 2003). Production, movement, and survival of spores of *P. ramorum* are likely to be highly affected by seasonal climatic changes in temperature and moisture (Davidson et al. 2001). Two different types of asexual spores are formed in *P. ramorum*: zoospores and chlamydospores (Davidson et al. 2001). The pathogen readily forms zoospores within sporangia; sporangia are highly deciduous and adapted for aerial dispersal, which is thought to be the main mode of dissemination. The high number of sporangia produced by *P. ramorum* is key to driving the epidemic (Denman et al. 2007). Most sporangia production tends to occur during the rainy season (Garbelotto 2004). During wet weather, the sporangia on the susceptible host release a large number of zoospores, which then infect the leaf of the plant and cause leaf blight and dieback of the host species. Since sporangia production is the stage responsible for dispersal and infection, sporangia and zoospores are the primary propagules that drive *P. ramorum* epidemics (Davidson et al. 2005). *P. ramorum* sporangia can be found in abundance on the foliage and branches of a variety of tree and shrub species, without lethal consequences. This form of infection may allow the pathogen to sustain its population indefinitely in infested forests, and appears to play a critical role in disease spread (Rizzo and Garbelotto 2003, and Denman et al. 2007). *P. ramorum* readily produces chlamydospores (asexual spores) in culture and on some foliar hosts (Davidson and Shaw 2003). The chlamydospores are considered to play a role in the survival of the pathogen, acting as resting structures, which allow the pathogen to survive during unfavourable climates, or when susceptible hosts are not present (Garbelotto 2004). When environmental conditions are conducive for vegetative growth the chlamydospores can germinate and create new colonies either vegetatively, or through the production of sporangia and subsequent release of zoospores (Smith and Hansen 2007).

Information on the effects of temperature on the spores of *P. ramorum* may provide useful information for future experiments investigating host range and conditions conducive to infection and survival of the pathogen (Englander et al. 2006). Werres et al. (2001) reported on some of the basic biology of *P. ramorum,* and Englander et al. (2006) carried out further tests on the growth and sporulation of *P. ramorum* in response to temperature and light. Tests were also carried out by the Central Science Laboratory (CSL) as part of a DEFRA project on the long-term survival potential of *P. ramorum* in relation to UK climatic conditions (DEFRA and CSL 2004). Climate plays a crucial role in the establishment and development of the disease caused by *P. ramorum* (Webber 2007). Production and survival of spores is likely to be highly affected by seasonal changes in temperature and moisture, and abundance of *P. ramorum* spores may be highly seasonal and coincide with the winter rain (Davidson et al. 2005). *Phytophthora* spores may also survive adverse summer conditions and the dormant resting spores, chlamydospores, may reside in the host tissue (Davidson et al. 2005).

In Ireland a small number of isolates of *P. ramorum* were first discovered in the wild in 2003, in the Vee. In 2004, the area was cleared and infected material was burnt on site under quarantine measures. As there were no positives at this location in 2005 the pathogen was regarded as having been eradicated. Unfortunately, in 2006 a positive was found in re-growth from stumps from a previously cleared area (Pers. comm. Gerard Cahalane 2007). According to Englander et al. (2006), establishing the temperature extremes, beyond which *P. ramorum* does not grow, might help explain why it is difficult to isolate the pathogen during certain seasons of the year. Although the symptoms might not be evident on host material the chlamydospores might still be surviving in the soil and cause infection in subsequent years. Hayden et al (2004) reported lower detection frequencies for the pathogen in California in Nov–Feb (rainy, cool) and Jul–Aug (dry, warm) each year compared with Mar–Jun (moderate moisture, temperature). Finding out the range of temperatures at which the spores can survive may help to explain why *P. ramorum* is being found in rhododendron-cleared areas. To date, no tests have been carried out on Irish isolates of *P. ramorum* in relation to survival of the chlamydospores in Irish conditions.

The aim of this study was to ascertain the survival potential of chlamydospores of *P. ramorum*, by examining the effects of temperature and pH on chlamydospores (DEFRA and CSL 2004 protocol), the spores associated with the long-term survival of *P. ramorum*.

5.2. MATERIALS AND METHODS

5.2.1. Isolation and identification of *Phytophthora ramorum*

Symptomatic tissue was collected from Killarney National Park, wrapped in tissue to prevent the sample from drying out and stored in a labelled ziplock bag for later isolation and molecular analysis. Isolation was carried out on *Phytophthora*-selective medium, Pimaricin-ampicillin-rifampicin-pentachloronitrobenzene (P_5ARP) (Van Leewen et al. 2003). Sample plates were incubated for 5–10 days at 20°C, away from direct sunlight. *P. ramorum* was identified using the morphological criteria outlined in Chapter 2, section 2.2.3, and confirmed by molecular analysis according to PCR protocols described in Chapter 2, sections 2.2.4 and 2.2.5.

5.2.2. Temperature sensitivity testing

The effects of temperature on chlamydospore survival were investigated using four temperature regimes (-21, 4, 20, and 40°C) and five exposure periods (0, 2, 4, 6 and 24 h). These temperatures were modified from those used in DEFRA (2004). Chlamydospores were produced by inoculating three thinly poured P_5ARP agar plates for isolate K2 (from *R. ponticum*, Killarney National Park). Three thinly poured uninoculated P_5ARP agar plates were used as negative controls. Plates were then sealed and incubated at 20°C for 10 days in the dark. Spores were harvested by blending the three agar plates for 2 min in 100 ml sterile deionised water (SDIW) and then extracted by centrifugation at 300 rpm for 3 min. The resulting pellet was washed twice with SDIW and then re-suspended in SDIW and divided into forty 1.5 ml aliquots (two for each treatment and placed under different temperature regimes.

A sample was taken immediately (T_0) and 100 µL of the chlamydospore suspension was aseptically spread onto two P_5ARP agar plates, using a bent sterile plastic rod, and incubated at 20°C for five days in the dark. This process was repeated for each temperature treatment and at the end of each exposure period. Germination was assessed by examination of 50 chlamydospores per plate. Spores were considered to have germinated when the length of the germ tube was greater than the diameter of the chlamydospore (DEFRA 2004). This experiment was repeated once.

5.2.3. pH sensitivity testing

The effect of pH on chlamydospore survival was investigated under six pH regimes (2, 3, 4, 5, 7 and 9) and four exposure periods (0, 2, 4, and 6 h) (DEFRA, 2004). Chlamydospores and negative controls were produced and harvested as described above, in section 5.2.2. The resultant chlamydospore suspension was divided into forty-eight 1.5 ml aliquots (two for each pH regime and time period). Each aliquot was centrifuged at 400 rpm for 3 min, the supernatant was removed and the pellets were re-suspended in 1 ml of the pH buffer solutions. A sample was taken immediately (T_0) and 100 µL of the chlamydospore suspension was spread inoculated onto two P_5ARP agar plates. This process was repeated for each pH treatment and at the end of each exposure period. Plates were incubated at 20°C for five days in the dark and chlamydospores were considered to have germinated when the length of the germ tube was greater than the diameter of the chlamydospore. This experiment was repeated once.

5.2.4. Statistical analysis

Percent chlamydospore germination was determined [= Number of germinated chlamydospores/(Number of germinated chlamydospores + Number of intact non-germinated chlamydospores)] (Smith, 2007). To assess the effects of different treatments on germination, percentage chlamydospore germination data were first tested for normality and homogeneity of variances using Minitab 15.1 and as none of the data were normally distributed, although variances were equal, the Kruskal-Wallis test was carried out using the same program. Where significant differences were detected they were further interrogated using the Mann-Whitney test.

5.3. RESULTS

5.3.1. Temperature sensitivity testing

Exposure to temperatures of 4°C and 20°C did not affect survival of chlamydospores, as compared to the other temperatures tested. Approximately 80% of the spores were found to have germinated after a 24-h period. At a temperature of -21°C, 96% of chlamydospores were killed by 2 h and all were dead by 5 h. At 40°C the number of germinated chlamydospores decreased dramatically from 70% by 4 h, to just 10% by 6 h and none survived the 24-h period (Fig. 5.1).

The Kruskal-Wallis test showed that the % germination differed significantly among treatments ($H = 39.18$, $DF = 3$, $P < 0.001$). The Mann-Whitney test was carried out to determine which treatments differed from one another (Table 5.1). The results showed that the % germination differed significantly between -21 and 4°C ($P < 0.05$), and between -21 and 20°C ($P < 0.05$) but not between -21 and 40°C ($P=0.29$). There was no significant difference in germination between 4 and 20°C ($P = 0.21$) with both temperatures showing ~80% germination after the 24-h period (Fig. 5.1). There was also a significant difference in % germination between 20 and 40°C ($P < 0.05$). The negative controls showed no growth at any temperature.

Table 5.1. A 2-sample rank test of individual % germination means of chlamydospores of *Phytophthora ramorum*, for a range of temperatures, as determined using the Mann-Whitney test.

Temperatures		4°C	20°C	40°C
-21°	Adjusted P-value	<0.05	<0.05	0.29
4°	Adjusted P-value		0.21	0.09
20°C	Adjusted P-value			<0.05

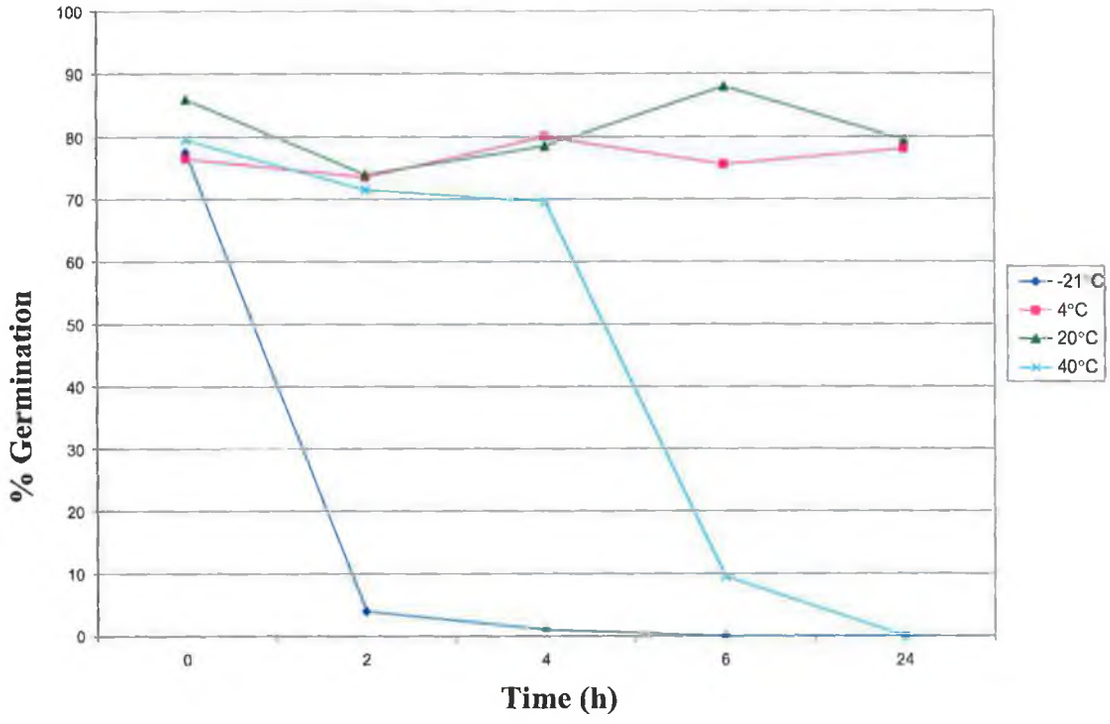

Figure 5.1. The effect of temperature on the germination of chlamydospores of *Phytophthora ramorum*, isolate K2, over a 24-h period.

5.3.2. pH sensitivity testing

Exposure to pH 3, 4, 5, 7, and 9 did not affect germination of chlamydospores over a 6-h period. However, at pH 2 from 0-2 h the % germination dropped from 48% to 24%, with a further drop to 1% at 4 h, and no spores survived at 6 h (Fig. 5.2).

The Kruskal-Wallis test showed that the % germination differed significantly among treatments ($H = 49.39$, $DF = 5$, $P < 0.001$). Mann-Whitney test showed that there was significantly lower % germination between pH 2 and pH 4, 7 and 9 ($P < 0.05$; Table 5.2), although the test did not show a significant difference in % germination between pH 2 and pH 3 and 5 (Table 5.2). There was a significantly lower % germination between pH 3 and pH 7 and 9 ($P < 0.05$; Table 5.2). At pH 3, there was a continuously slow decrease in % germination over the 6-h period, going from 57% at T_0 to 43% after 6 h, whereas at pH 2 there was a dramatic decrease in %

germination after 2 h. No difference in germination was observed between pH 4, 5, 7, and 9. The negative controls showed no growth at any pH.

Table 5.2. A 2-sample rank test of individual % germination means of chlamydospores of *Phytophthora ramorum*, for a range of pHs, determined using the Mann-Whitney test.

pH		3	4	5	7	9
2	Adjusted P-value	0.15	0.05	0.09	0.05	<0.05
3	Adjusted P-value		0.07	0.12	<0.05	<0.05
4	Adjusted P-value			0.99	0.66	0.77
5	Adjusted P-value				0.30	0.38
7	Adjusted P-value					0.88

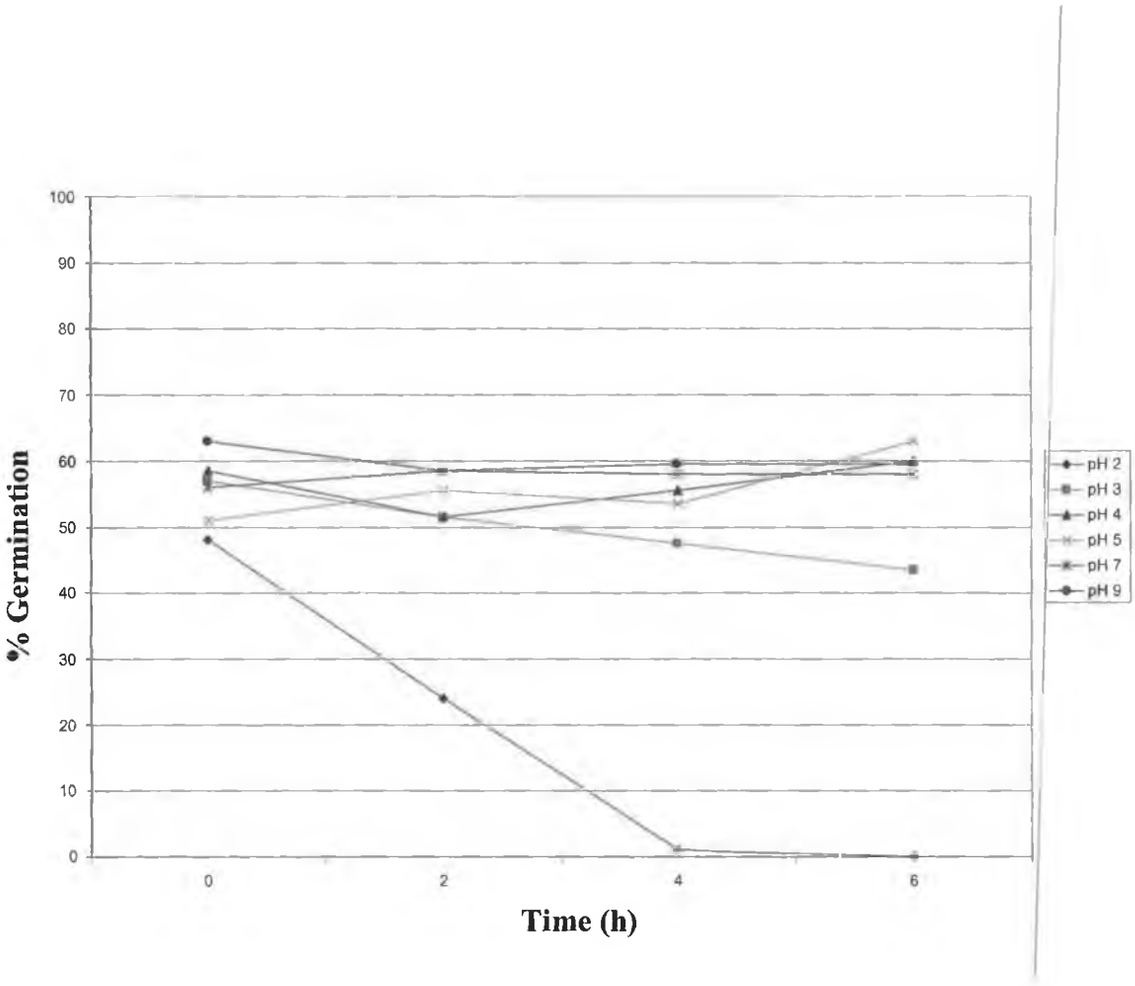

Figure 5.2. The effect of pH on the germination of chlamydospores of *Phytophthora ramorum*, isolate K2, over a 6-h period.

5.4. DISCUSSION

The null hypothesis that temperature (-21, 4, 20 and 40°C) or pH (2, 3, 4, 5, 7, 9) does not effect survival of the chlamydospores of *P. ramorum* was rejected. The results indicate that chlamydospores are capable of surviving at temperatures between 4 and 20°C but show a dramatic decrease in survival at -21 and 40°C after just a 2-4 h exposure period. The results corroborate those for four isolates of *P. ramorum* tested under UK climatic conditions (DEFRA and CSL 2004). The effect of a range of temperature (-25, -2, 0, 5, 20, 25, and 40°C) on chlamydospore survival was consistent for all four isolates. Their results showed that while exposure to temperatures between 0 and 20°C for a period of up to 24 h did not affect overall survival, survival was affected at -25, -2 and 40°C, with no spores surviving after a 24-h period. They found that at -25°C there was an immediate decrease in spore survival after T_0, which is similar to our results for -21°C. They also found that at temperatures of 5 and 20°C the % germination after the 24-h period was between 80 and 85%, which is comparable to our germination rate of ~80% for 4 and 20°C.

Our results and those of DEFRA and CSL (2004) are similar to those reported by Englander et al. (2006) on US and EU isolates of *P. ramorum*. Although Englander et al. (2006) did not assess the survival of chlamydospores they did test for chlamydospore production at a range of temperatures (6, 10, 18, 20, 22, 26, and 30°C). They found that both US and European isolates produced chlamydospores at temperatures between 6 and 26°C, with optimal production at temperatures between 18 and 26°C. They observed no growth at temperatures above 30°C for either the US or European isolates and did not test for temperatures below 6°C. In a more recent study, Tooley and Browning (2007) showed that chlamydospores of US isolates can survive at temperature from -10 to 40°C. Their results showed almost 100% survival at 0 and 20°C, high levels of germination at 30°C, near zero survival at -10 and -20°C, and no growth at 40°C. Although Tooley and Browning (2007) did observe high levels of germination at 30°C, they observed this over a 2 and 4-day period. After just one day the 30°C treatment showed a significant reduction in chlamydospore germination compared to the 20°C treatment.

All three studies (present study, DEFRA and CSL 2004, Tooley and Browning 2007) indicated that chlamydospores of *P. ramorum* can survive temperatures from 0 to 30°C. The optimum temperature for chlamydospores germination is between 18 and 30°C. Temperatures of -21 to 0°C and 40°C are not suitable for the production and germination of chlamydospores, as there is either very little production of spores or no growth of *P. ramorum* at all. Results of this study indicate that the chlamydospores of the Irish isolate, K2, of *P. ramorum* are capable of surviving at temperatures between 4–20°C. Extreme temperatures of -21 and 40°C are not suitable for the long-term survival of the pathogen. The mean temperature in the southwest of Ireland in February (the coldest month) 2007, and for August (the warmest month), were 7.9 and 15.2°C, respectively (www.met.ie/climate/monthly-data.asp?Num=53). The mean soil temperatures for those months were 7.4 and 17.7°C, respectively.

The results for pH treatments showed that chlamydospores were able to survive at pH 3–9, with 44–63% germination. At pH 3 there was a difference in % germination compared to pH 4–9, although germination was still relatively high (44%) after the 6-h period. However, at pH 2 there was a significant decrease in spore survival after just 2 h. This is similar to the results obtained by DEFRA and CSL (2004) where they tested spore survival over a pH range of 2–9 and found that pH 2 had a significant effect on chlamydospore survival, with no chlamydospore germination observed after 4 h. Also, they found that pH treatments 4–9 did not have an overall affect on spore survival in any of the four isolates tested, with approximately 60% germination after 6 h. Our results showed a significant difference between pH 3 and pH 7–9, whereas their results showed no difference between pH 3–9. Results of this study indicate that the chlamydospores of the Irish isolate, K2, of *P. ramorum* are capable of surviving in a pH range of 3–9, while pH 2 is not suitable for the long term survival of the pathogen. Most of the woods in Killarney National Park occur on old red sandstone and are under the influence of the oceanic climate, which forms an infertile, stony podzolic soil, with a pH between 4.0 and 4.5 (Cross 1981). Killarney National Park therefore has ideal temperature and pH conditions for survival of *P. ramorum* chlamydospores in the soil throughout the entire year in Ireland.

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CHAPTER 6

CONCLUDING REMARKS

6.1. CONCLUDING REMARKS

At present, *Phytophthora ramorum* has been found at six locations in the wild in Ireland. Official surveys carried out in Ireland since 2003, by the Department of Agriculture, Fisheries and Food (DAFF), have confirmed the presence of *P. ramorum* at five locations in the wild; The Vee, Co. Tipperary, Killarney National Park, Co. Kerry, Lauragh, Co. Kerry, and two privately owned areas of land, location unknown (Gerard Cahalane, pers. comm.). The sixth positive wild site, Castletownbere, Co. Cork, was a new finding for Ireland and it was identified during the course of this study, in June 2008. To date, there have been no findings of *P. ramorum* on trees in Ireland. There has also been no report of *Phytophthora kernoviae* in Ireland.

This study detailed the first finding of the pathogen in an Irish water sample: in 2006, it was detected within a still body of water located in Killarney National Park. However, additional water baits along a five-mile stretch of water, yielded no further positives. Since the initial positive was found next to a large pile of cut rhododendron, which had been cleared from other parts of the park, it is suspected that this may have been the cause of the positive finding. This, however, does not reduce the importance of the positive water finding since water sources have the potential to become infected from wind driven rain, atmospheric fall out, and from infected host plants on the river banks (Davidson et al. 2001, 2003). Analysis of water at positive sites needs to be carried out in the future. Were *P. ramorum* to infect rivers at positive sites there is the strong possibility of the spread of infection to unaffected sites.

Eight microsatellite loci, which were previously used in the US to study both US and European isolates of *P. ramorum*, were used to study the wild and nursery populations of *P. ramorum* in Ireland. The results indicated that *P. ramorum* in Ireland consists of one multilocus genotype (MG), with no variation exhibited within or between forest and nursery populations. The lack of variation between the forest and nursery populations using these loci could have been due to the fact that *P. ramorum* is a recent introduction in Ireland, whereas it is believed that the pathogen has been present in the US since the early 1990s. In order to obtain a clearer

understanding of the population genetics of the Irish population of *P. ramorum* analysis of a larger number of loci should be carried out and this might reveal a greater level of polymorphisms both within and between our isolates. A recent study was carried out in Belgium, to test for sexual reproduction within their *P. ramorum* nursery population and to determine the population structure, evolution and spread of the pathogen (Vercauteren et al. 2009). They analysed 411 isolates collected over a 7year period using 11 microsatellite loci, three of which were the same loci used during this study. They concluded that all isolates were from EU1 lineage and that the most common genotype, EU1MG1, was the ancestral genotype. They also suggested that less variant genotypes can be removed from populations through eradication efforts, random genetic drift and unfavourable conditions, such as unsuitable weather and absence of hosts. These could also explain why only one MG was found during the course of this study.

The susceptibility studies confirmed that the Irish isolate of *P. ramorum*, K2, can infect the leaves of *R. ponticum*, *F. magellanica, I. aquifolium, Q. petraea,* and *A. unedo*. *H. macrophylla* was not found to be susceptible to *P. ramorum*. All the positive sites have *Rhododendron ponticum* as an under-storey, and although sessile oak (*Quercus petraea*) is not the dominant tree species at all the positive sites, there are other susceptible tree species present, such as douglas fir (*Pseudotsuga menziesii*) and sitka spruce (*Picea sitchensis*). Although susceptibility tests have already been carried out on *Q. petraea*, *P. menziesii*, and *P. sitchensis* in the UK and US, log inoculation tests carried out on these tree species in Ireland along with the strawberry tree (*Arbutus unedo*) would be important. Even though *Q. petraea* has been shown to be less susceptible than other oak spp., such as Shreves's oak (*Quercus parvula* var. *shrevei*), Northern red oak (*Quercus rubra*), and Southern red oak (*Quercus falcata*), what few oak stands we have in Ireland are very exposed, very vulnerable, and infection of just one tree with *P. ramorum* could have devastating effects. In the case of *P. menziesii* and *P. sitchensis*, Lauragh, in Co. Kerry, is considered to have the highest amount of *P. ramorum* infection in Ireland (Gerard Cahalane, pers. comm.) and this site is predominantly populated by *P. menziesii*, along with a few *P. sitchensis*. Analysis of the susceptibility of stem and foliage of these two tree species would be worthwhile in order to determine the risk that could be posed by *P.*

ramorum to this small area. Another species that would benefit from further analysis into stem susceptibility is the strawberry tree (*Arbutus unedo*). Even though *R. ponticum* has been found to be the key under storey component, in the UK, where infected trees have been found (Webber 2007), *A unedo* has the potential to support moderate to high levels of sporulation (Morelejo et al. 2006). The strawberry tree is extremely rare in Ireland, being only found in the wild, in Killarney National Park (http://homepage.eircom.net).

In relation to the survival of the chlamydospores of *P. ramorum*, which are spores associated with the long-term survival of the pathogen (Garbelotto 2004), temperature studies indicated that chlamydospores can survive temperatures from 0 to 30°C, with the optimum temperature for germination being between 18 and 30°C. Also, pH studies showed that chlamydospores were able to survive at pH 3-9, with pH 2 not suitable for germination of chlamydospores. The southwest of Ireland has ideal temperature conditions for survival of *P. ramorum* chlamydospores in the soil throughout the entire year. In regards to pH, in Killarney National Park most of the woods occur on old red sandstone and are under the influence of the oceanic climate, which forms an infertile, stony podzolic soil, with a pH between 4.0 and 4.5 (Cross 1981). Killarney National Park therefore has ideal temperature and pH conditions for survival of *P. ramorum* chlamydospores in the soil throughout the entire year in Ireland. A future study into the long-term survival of *P. ramorum* in the soil, along with the survival of sporangia and zoospores, is recommended. Since sporangia production is the stage responsible for dispersal and infection, sporangia and zoospores are the primary propagules that drive *P. ramorum* epidemics (Davidson et al. 2005). Ideal sampling sites would be those that are currently undergoing eradication programmes of *P. ramorum* infected rhododendron. This might explain why the pathogen is found in areas where *P. ramorum* was considered to be already eradicated.

Apart from the combination of trees with susceptible stems growing close to foliar hosts infected by *P. ramorum*, climate plays a crucial role in disease establishment and development (Webber 2007). In nature, *P. ramorum* readily produces sporangia, zoospores and chlamydospores for dispersal and survival.

Climatic factors affect the dispersal and survival of the pathogen (Cohen and Venette, 2005). In 2003, comparisons were made by the Central Science Laboratory (CSL) in the UK using a climate matching model (CLIMEX) to compare southern Oregon with Europe, to determine which parts of Europe could be at greatest risk from *P. ramorum*, based on climate alone. The comparison was then revised based on Meentemeyer et. al (2004) and this identified the west of the UK, Ireland and northwest parts of France, Spain and Portugal as regions with the closest eco-climate matching in relation to *P. ramorum* (Webber 2007). Since then, findings of *P. ramorum* in the wild have been most common in south-western parts of England and Wales, where the combination of abundant under storey rhododendron and mild, often wet climate, appears to have provided a near perfect environment for the pathogen (Webber 2007). According to the CLIMEX map, provided by Richard Baker (CSL, UK) the south west of Ireland and parts of the south, west and north west, have a CLIMEX match index of 0.71-0.75, which is the closest eco-climate matching in relation to *P. ramorum* (Webber 2007). Efforts should be made to survey more intensively in parts of Ireland, where these conditions are met. The principal influence on Ireland's climate is the Atlantic Ocean. As a result, Ireland does not suffer from the extremes of temperature experienced by many other countries at similar latitude. MET Eireann, the leading provider of weather information for Ireland (www.met.ie/about/default.asp), provides 30year average reports from 14 weather stations in Ireland. According to the report for the south of the country, where *P. ramorum* has been found at four locations, the mean annually temperature is 10°C, while the mean annually rainfall is 1191mm (www.met.ie/climate/30year-averages.asp). The average annual temperature this year is ~ 9°C (www.met.ie/climate/climate-of-ireland.asp), which is an ideal temperature for the survival of *P. ramorum*. The pathogen has an optimum growth temperature of 20°C and a minimum and maximum temperature for growth of 2 and 30°C, respectively. It prefers areas with wet climate and constant mild temperatures for optimal disease development (Sansford et al. 2003).

It was initially intended, as part of this study, to carry out a Geographic Information System (GIS) study to highlight potentially susceptible sites that should be sampled, and to possibly predict the directionality of the spread of *P. ramorum* in

Ireland. The only GIS information made available was the number of oak stands in Ireland, which was provided by Coillte, a commercial company operating in forestry and land based businesses. Because there was no information on the scale of rhododendron growth in these stands it was difficult to accurately choose susceptible sites. Efforts were made to contact individual forest managers to determine the scale of rhododendron invasion in forests throughout the country but again the contact information for the managers was not available. The most recent map of rhododendron distribution in Ireland is from 1980 (John Cross, pers. comm.). John Cross carried out the surveys that created these maps and he suggested getting in touch with DAFF as personnel there are very familiar with the rhododendron problem in Irish forests. However, rhododendron is considered an irrelevant part of the inventories carried out in Ireland and so data are not collected (Gerard Cahalane, pers. comm.).

The sampling strategy used in this study was not without its faults. For statistical analysis of survey results, systematic sampling, rather than random sampling, would have been a better strategy. Unfortunately, random collection of leaves did not allow for statistical comparison of individual plants. In future, sampling a host every 100 m, regardless of presence of symptoms or not, is recommended. Also, although it was possible to statistically analyse results from samples that were positive using morphological and molecular criteria, the whole statistical foundation might be faulty when based on the non-rigorous sampling methods employed. The majority of sampling, with the exception of Lauragh, Co. Kerry and Killballyboy Wood, Co. Waterford, was carried out along the paths at the various sampling sites. The use of climbing gear and additional people would have facilitated the survey of trees and plants off the beaten track. Only sampling along the path does not give a complete picture of the presence of *P. ramorum* in these areas, and limits the number of hosts sampled. A more random selection of sites would also be recommended for future studies. Choosing sites without the presence of *R. ponticum* and *Q. petraea* might have limited the discovery of new hosts and positive sites.

The results from this study indicate that *P. ramorum* is present just in the

south of Ireland, and only found on *Rhododendron ponticum* in the wild. In 2007, when only three positive wild sites had been identified, it appeared that the level of infection was low in Ireland. However, by the following year the number of positive wild sites had increased to six. There is no indication, at least for Killarney National Park and Lauragh, sampled from 2005–2008, that there has been a decrease in the number of positives at these sites over this time span. The privately owned land in Lauragh has shown no signs of eradication efforts, a likely reason being that the owner is responsible for the cost of removal of rhododendron (Gerard Cahalane, pers. comm.). The National Parks and Wildlife Service (NPWS) handle the eradication efforts of both *R. ponticum* and *P. ramorum* in Killarney National Park. From sampling throughout the park over the four-year period it was obvious that burning *in-situ* and spraying chemicals on the stumps of *R. ponticum* were the treatments used. Despite these efforts *P. ramorum* is still being found at these locations.

Phytophthora ramorum has already had major economic implications for forests and commercial forestry in the US. Where it has become established in California, *P. ramorum* adversely affects ecosystem functions, and increases fire and safety hazards. The US, as well as the European Union, has imposed regulations for host plants and associated soil from infested areas. These regulations restrict shipment of rhododendron and other horticultural host plants from areas where the pathogen is found. Coillte owns two of the positive sites sampled during this study, and owns over one million acres of land, most of which is forested. Some of the company's forestry businesses include log sales, farm forestry services, and plant sales. They grow over 18 species of conifers and 12 species of broadleaves, some of which include species that are susceptible to *P. ramorum*, such as Sessile oak, Douglas fir, and Sitka spruce (www.coillte.ie/coillteforest). Although damage on these species appears to be limited to foliage and small branches, regulatory actions has already had an impact on the redwood and douglas-fir industry in California at an estimated $50 million a year (www.docstoc.com/docs/39899328/Plant-Diseases-Caused-by-Phytophthora-ramorum). If sessile oak, Douglas fir and Sitka spruce were to be found infected with *P. ramorum* in Ireland it could have a devastating affect on the commercial forestry industry. The pathogen has already started to

affect how Coillte manage their land. *Rhododendron ponticum* is a major problem in most of their forests. The presence of *P. ramorum* has restricted their efforts to eradicate *R. ponticum* due to a ban on cutting of rhododendron in the certain areas, imposed by DAFF (www.coillte.ie/?id=128&no_cache=1).

Since the first findings of *P. ramorum* in the UK in 2002, there have been continuing efforts to eradicate the pathogen. Research on host susceptibility studies, potential reasons for spread, and various other studies have been carried out in order to eradicate the pathogen or reduce the level of inoculum to epidemiologically insignificant levels. Despite all these efforts, the disease has continued to spread, although slowly and mainly confined to the southern and western parts of the UK. A new five-year programme has been drawn up by the Food and Environment Research Agency (FERA), in the UK, on the start date scheduled for 1st April, 2009. The plan includes research and development, an awareness programme, and disease control through funding clearance of host (http://www.fera.defra.gov.uk).

Although Ireland does not have the same level of infection as the UK, the same risks apply. As stated previously, what few natural oak stands and rare species we have need to be protected.

6.2 REFERENCES

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CHAPTER 7

APPENDICES

7.0. APPENDICES

Appendix I

Site descriptions

Site 1. *Glenveagh National Park, Co. Donegal*

Glenveagh National Park is situated 24 km from Letterkenny, Co. Donegal. The area is generally mountainous, with Atlantic blanket bog as the dominant habitat (http://www.glenveaghnationalpark.ie/wildlife.html). The park is very isolated from surrounding developments and housing. No rhododendron or oak trees are found near the park except in the park estate. The road leading up to the castle, the main visitor attraction, is 3 km long and the shrub boarder along it is rhododendron. The largest area of semi-natural deciduous woodland is in Mullangore Wood on the south-eastern side of the park. This woodland is on the shore of Loch Beagh, and many streams run through the wood down into the lake. The dominant trees present are *Q. petraea* and birch (*Betula alba*). Holly (*Ilex aquifolium*) occurs in the under-storey and *R. ponticum* has invaded much of the woodland. Visitors are free to walk within the grounds along several paths and within the woods. Although work has started on eradication of rhododendron it is still at an early stage. Sampling was carried out along the pathway that runs from Glenveagh Castle to Mullangore Wood, and along the lake shore (Fig A1; Table A1).

Site 2. *Brackloon Woods, Co. Mayo*

Brackloon woods are situated 7 km from Westport, Co. Mayo and are owned by Coillte. In 1999, the wood was designated as a proposed SAC (Special Area of Conservation) under EU legislation. The woods cover an area of approximately 74 hectares (http://www.ucd.ie/ferg/Research/Sites/Brackloon.html). A river forms the eastern boundary of the woodland, and a small number of streams run through the wood forming small patches of marshy ground. The wood is species-rich, including *Q. petraea*, ash (*Fraxinus excelsior*), and elm (*Ulmus glabra*). The under-storey consists mainly of *I. aquifolium* along with *R. ponticum* scattered throughout the wood. Sampling was carried out along a path in the wood next to a river (Fig. A2; Table A2), but since very little rhododendron was present, sampling was restricted to just two sampling dates.

Figure A1. Ordnance Survey map of Glenveagh National Park, Co. Donegal; sampling area outlined in red (X: 201964.81, Y: 420793.97).

Figure A2. Ordnance Survey map of Brackloon Woods, Co. Mayo; sampling area outlined in red (X: 97100.77, Y: 280186.60).

Site 3. *Connemara National Park, Co. Galway*

Connemara National Park covers over 2 957 hectares of rugged quartzite and schist terrain in north Connemara. Western blanket bog and heathland are the main vegetation types (http://www.connemaranationalpark.ie). Due to the fact that blanket bog is the predominant vegetation through the park, visitors tend not to wander off the wooden and stone pathways because of unsuitable walking ground. Rhododendron is found mainly along the border of the park, along the roadside. Sampling was carried out at the entrance to the park in the car park, and along the roadside next to the park (Fig. A4; Table A3). The pathway leading through the park did not have any rhododendron growing nearby as the predominant soil type is blanket bog.

Site 4. *Killarney National Park, Co. Kerry*

Killarney National Park covers up to 10 236 hectares of mountains, lakes, and woodland. The park was designated as a Biosphere Reserve in 1981 by the United Nations Educational, Scientific and Cultural Organisation (UNESCO), part of a world network of natural areas that has conservation, research, education and training as its major objectives (http://homepage.eircom.net/~knp/intro/index.htm). The underlying geology of the wooded areas in Killarney is mainly old red sandstone (Cross 1981). The oak woodlands occur mainly around the Killarney lakes and are the habitat for which the area is best known. They form the most extensive area of native woodland remaining in Ireland (http://homepage.eircom.net/~knp/intro/index.htm). The woods are typically dominated by *Q. petraea*, with an under-storey of *I. aquifolium*, if it has not been replaced by *R. ponticum*. The park contains the only sizeable Yew woodland left in Ireland, with some of the trees almost 200 years old (http://homepage.eircom.net/~knp/intro/index.htm). Situated in Killarney National Park is Muckross house and gardens. It is one of the popular visitor attractions in Killarney with its well-known collection of rhododendrons, a sunken garden and an arboretum containing many trees from the southern hemisphere (http://www.muckross-house.ie/intro.htm).

Rhododendron was introduced into the Killarney area of SW Ireland during the 19th century, and has subsequently spread by means of large numbers of very small, easily dispersed seeds, throughout oak woods (Cross 1981). The National

Parks and Wildlife Service (NPWS) oversee the management of Killarney National Park and control *R. ponticum* by herbicide application, cutting and burning (Fig. A3.A). Signs were erected throughout the park (Fig. A3.B) from 2006-2007 and were removed in 2008 even though the pathogen was still present at several locations in the park (personal observation). Despite eradication efforts, of burning in-situ and treating the stumps with Roundup® and a dye, *R. ponticum* continues to invade approximately 650 hectares of the woodlands (http://www.muckross-house.ie/intro.htm).

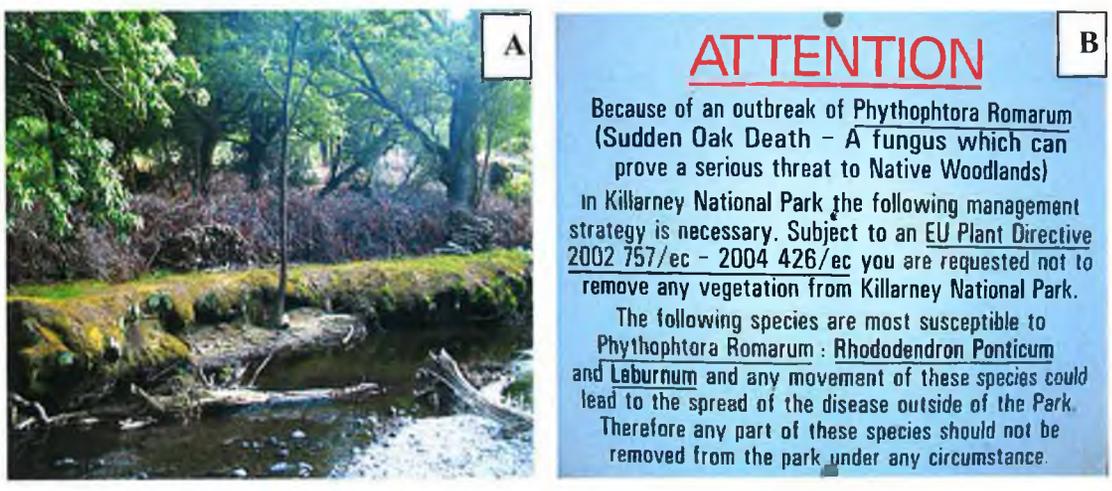

Figure A3. A: An area of rhododendron eradication in Killarney National Park; B: Signs erected to prevent the removal of foliage from the park.

Killarney National Park appeals to tourists due to its beautiful scenery, the network of paths throughout the woodlands and gardens, boat tours and numerous other recreational activities. The disturbance of the woods by humans and grazing animals continues to aide the spread of *R. ponticum* (Cross 1981).

Sampling was carried out on several pathways throughout the park's woodlands, and within the woodland when it was accessible. The four sampling locations were Muckross Gardens, along a pony trekking path, an unnamed wood on the south of Muckross Lake, and Tomies Wood, located on the west of the park (Fig. A5; Table A4).

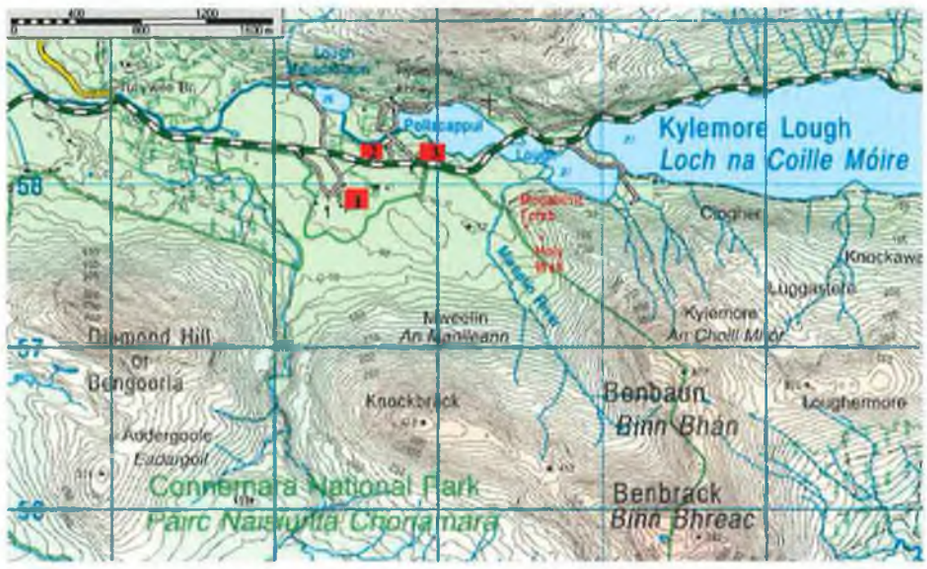

Figure A4. Ordnance Survey map of Connemara National Park, Co. Galway; sampling areas indicated by red rectangles (1. X: 74394.39, Y: 257925.43; 2. X: 74445.20, Y: 258224.64; 3. X: 74953.29, Y: 258190.76).

Figure A5. Ordnance Survey map of Killarney National Park; sampling sites indicated by red rectangles (1. X: 97298.68, Y: 86520.42; 2. X: 97524.44, Y: 85850.13; 3. X: 95441.66, Y: 84579.49; 4. X: 91447.79, Y: 88364.61).

Site 5. *Lauragh, Co. Kerry*

Lauragh is a small town situated on the well-travelled Ring of Beara Peninsula. The woodlands in Lauragh are species-rich with sitka spruce (*Picea sitchensis*), Douglas fir (*Pseudotsuga menziesii*) and a very small number of *Q. petraea*, with an under-storey consisting mainly of rhododendron. The site in Lauragh is privately owned and rhododendron is completely invasive (Gerard Cahalane, Pers. comm.). Although the site is not accessible to tourists there are several roads leading to it with rhododendron growing along the roadsides. Sampling was carried out along the roadside next to an area of private woodland, on an unmarked side road, which was across from the Derreen Gardens. The sampling area consisted of a quarter-mile stretch of road (Fig. A6; Table A5). The Derreen Gardens are woodland gardens that are famous for their tree ferns (*Dicksonia antarctica*). The gardens are open to the public from August to October and are also well known for the various different rhododendrons planted there, including original plantings of *Rhododendron arboretum* (http://www.irelandseye.com/aarticles/travel/attractions/gardens/derreen.shtm).

Figure A6. Ordnance Survey map of Lauragh, Co. Kerry; sampling area indicated by the red rectangle (X: 77441.96, Y: 58990.75).

Site 6. *Dunboy Wood, Castletownbere, Co. Cork*

Dunboy Wood is about 3.5 km from the town of Castletownbere, in the southwest of Ireland, in Co. Cork. The wood is owned by Coillte, a commercial company operating in forestry and land-based businesses. The main tree species is *P. sitchensis*, with *R. ponticum* and *I. aquifolium* as the predominant under-storey species. The wood is situated next to a saltwater inlet (Fig. A7) and has facilities for picnics and fishing. Sampling was carried out in June 2008 (Table A6) along a path that runs through the woodland and around the parking and picnic areas.

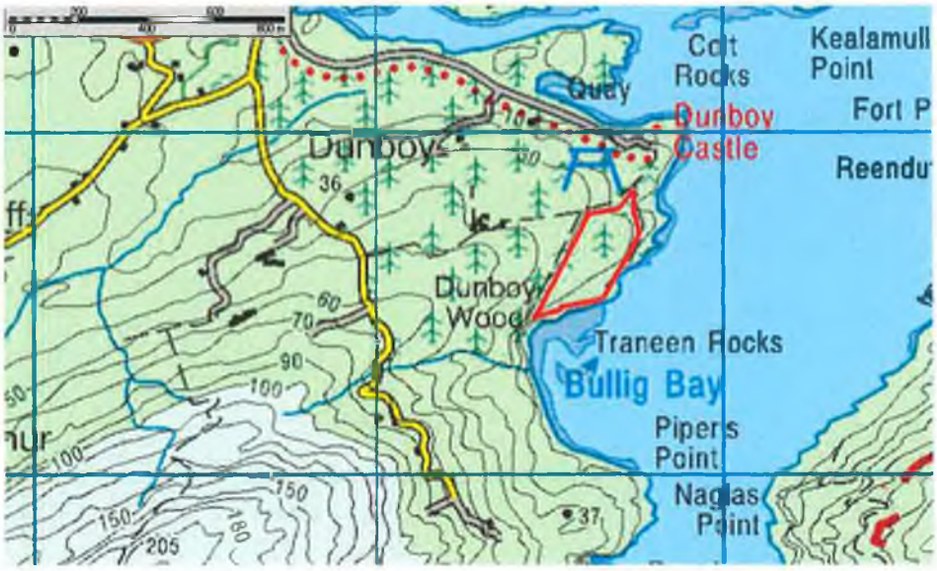

Figure A7. Ordnance Survey map of Dunboy wood, Castletownbere, Co. Cork; sampling area indicated by red rectangle (X: 66736.96, Y: 43840.75).

Site 7. *Glengarriff, Co. Kerry*

Glengarriff Woods Nature Reserve covers up to 300 hectares of mature old *Q. petraea* woodland and young woodland, which is regenerating in areas that have been cleared of other trees. The reserve lies in the heart of the old Glengarriff valley and is managed by the NPWS (URL 7). The under-storey consists mainly of *R. ponticum*, along with a small number of *I. aquifolium*. The woods contain many roads, paths and several lookout points within easy reach of the main road. Perhaps the most well known of these is Lady Bantry's Lookout, which gives a panoramic view across

Bantry Bay and Glengarriff Harbour. Glengarriff is visited by thousands of visitors each year on their travels around the Beara Peninsula, walking up to Lady Bantry's Lookout for the spectacular views (http://europeforvisitors.com/europe/countries/ireland/walking-in-ireland-glengarriff.htm). Over the last four years, there has been an ongoing and sustained programme of eradicating the large rhododendron problem in the area, yet despite these efforts regeneration seems inevitable. Sampling was carried out mainly along the roadside, which leads right through the reserve, on both sides of the path leading up to Lady Bantry's Lookout, and in several fields along the roadside (Fig. A8; Table A7).

Figure A8. OS map of Glengarriff, Co. Cork; red rectangles 1 & 3 indicate beginning and end of road sampling and rectangle 2 indicates Lady Bantry's Lookout (1. X: 92173.77, Y: 56161.94; 2. X: 92132.18, Y: 56311.20; 3. X: 90923.25, Y: 57124.41).

Sites 8 and 9. *Lismore and Killballyboy Wood, The Vee, Co. Waterford*

The Vee is known locally as the road through the Knockmealdown Mountains of Waterford and Tipperary (http://www.ireland.ie/things_2_do_results_single.asp?Sid=45357). The R668 road descends down into the valley into deeply wooded areas,

and stretches over 22 km (Fig. A9). Starting out at the highest point on the road is the town of Lismore. Sampling was carried along the roadside just outside the town, going north along the road to Clogheen (Fig. A10; Table A8). The landscape is predominantly grassland, which had been completely invaded by rhododendron. The use of the land is for grazing sheep and cattle.

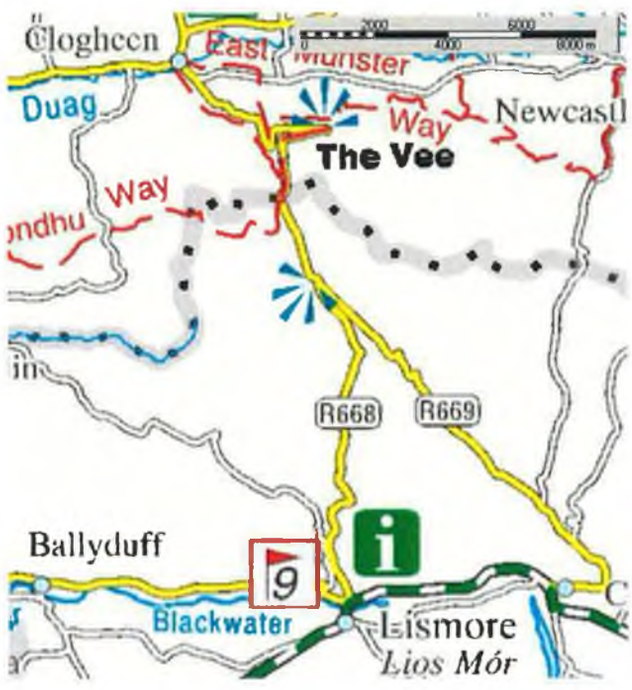

Figure A9. Ordnance Survey map of the Vee, Co. Waterford.

The second sampling point along the road was in Killballyboy Wood, which is located north of the first sampling point, 2.5 km from the town of Clogheen (Fig. A.11). The route is well known for its spectacular views and in the summer one can see miles and miles of what resembles pink fields when *R. ponticum* flowers are out. Killballyboy Wood is mainly planted with scots pine (*Pinus sylvestris*), some *P. menziesii* and *P. sitchensis*. *R. ponticum* has completely invaded the wood and the landscape along the drive through the valley. Although the entrance to the wood is hidden on a bend along the road, it is well known locally for its picturesque walks and picnic areas, and is visited regularly throughout the year by tourists and locals

(http://www.ireland.ie/things_2_do_results_single.asp?sID=45357). Sampling was carried out along the two paths that lead through the woods (Fig. A11; Table A9).

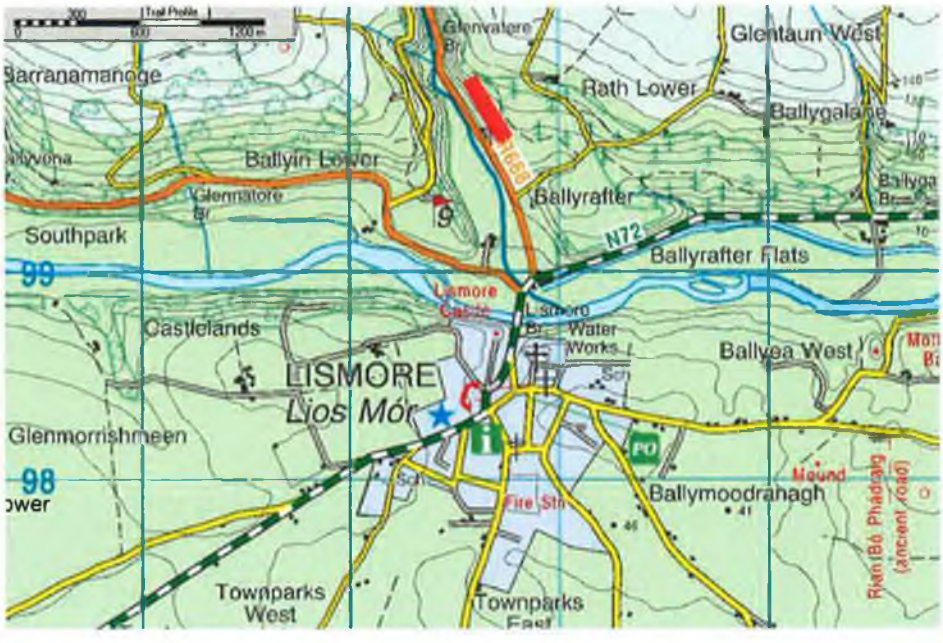

Figure A10. Ordnance Survey map of Lismore, Co. Waterford; sampling area indicated by red rectangle (X: 204721.82, Y: 99480.46).

Site 10. *John F. Kennedy Arboretum, Co. Wexford*

John F. Kennedy Arboretum covers up to 252 hectares on the southern slopes and summit of Slieve Coillte. It contains 4 500 types of trees and shrubs from all temperate regions of the world. With 200 forest plots and over 500 different types of rhododendron, the park attracts thousands of visitors each year (http://www.heritageireland.ie/en/South-East/TheJohnFKennedyArboretum). There is a lake situated within the park and a road that travels throughout, allowing visitors easy access to all parts of the park. Sampling was carried out along the paths within the park and also within the forest plots, which contained very few rhododendrons (Fig. A12; Table A10). The park personnel thoroughly manage rhododendron, by frequent cutting, since the park contains over 500 species of rhododendron.

Figure A11. Ordinacne Survey map of Killballyboy Wood, Co. Waterford; sampling area indicated by red rectangle (X: 2019.95, 112552.34).

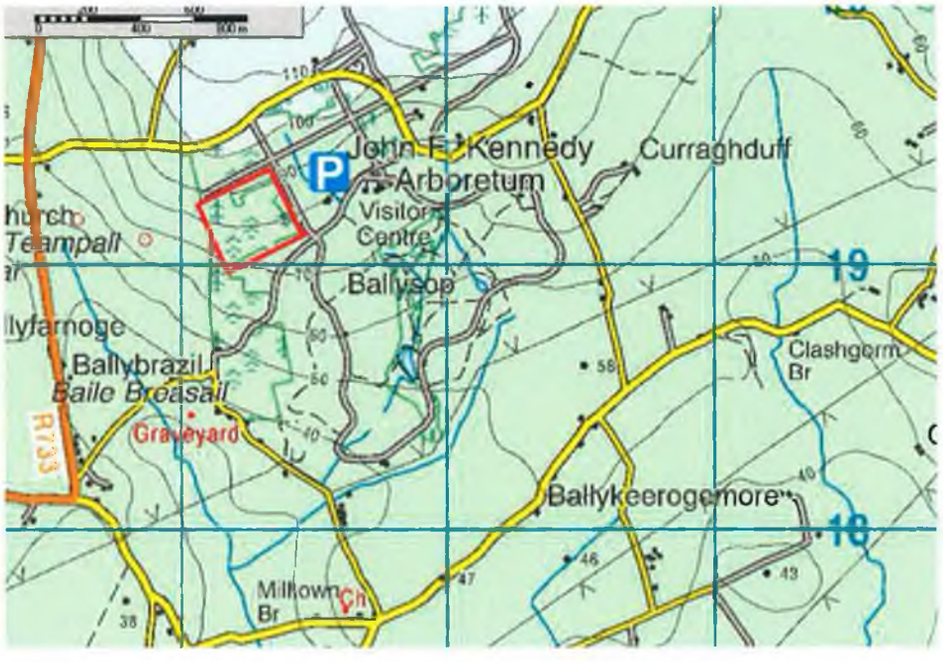

Figure A12. Ordnance Survey map of John F. Kennedy Arboretum, Co. Wexford; sampling area indicated by red rectangle (X: 270514.56, Y: 124200.47).

Site 11. *Tomnafinnoge Wood, Co. Wicklow*

This old oak woodland is dominated by mature, widely spaced *Q. petraea* along with beech (*Fagus sylvatica*) and *P. sylvestris*. The under-storey is rich in *I. aquifolium,* hazel (*Corylus avellana*), and young oak, with only a few *R. ponticum* scattered throughout the wood. A river flows through the wood with a few streams breaking off from the river and creating some marshy ground. Sampling was carried out mainly along the paths, which run throughout the wood, and along the river bank (Fig. A13; Table A11).

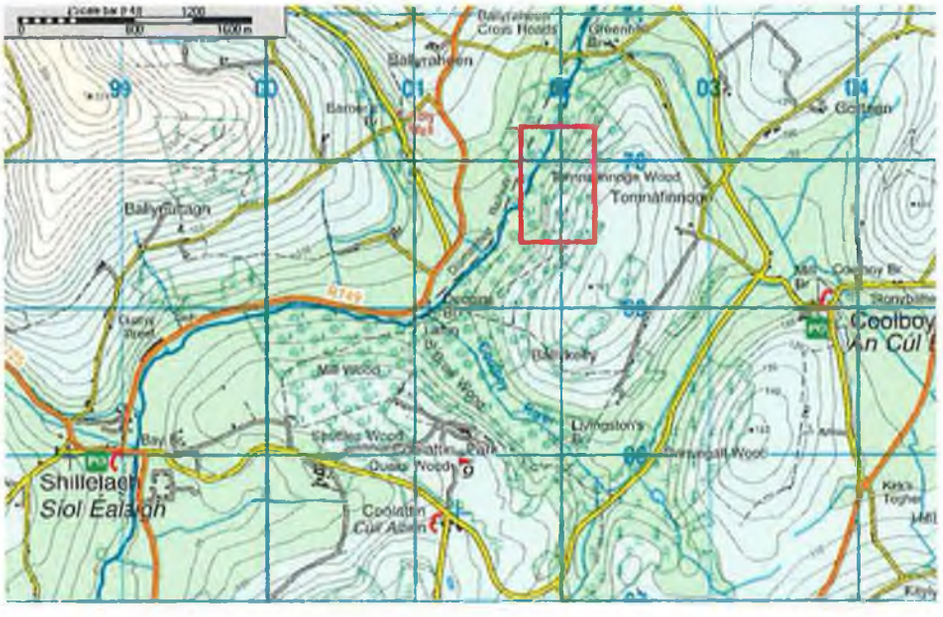

Figure A13. OS map of Tomnafinnoge Wood, Co. Wicklow; sampling area indicated by red rectangle (X: 302015.38, Y: 169881.47).

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Appendix II

Table A1. Sampling results for Glenveagh National Park, Co. Donegal, from 2005-2007.

Sample Number	Host plant	Location	County	Date sampled	Number of leaves sampled	Morphological No. of +ve leaves	Growth	No. of DNA extractions	Molecular No. of +ve PCR
GV001(WB)	*Rhododendron ponticum*	National Park	Donegal	07.12.2005	N/A	0	None	0	0
GV001	Soil	National Park	Donegal	07.12.2005	N/A	0	Unknown	1	0
GV001	*R. ponticum*	National Park	Donegal	07.12.2005	3	0	Unknown	7	0
GV002	Soil	National Park	Donegal	07.12.2005	N/A	0	None	0	0
GV002	*R. ponticum*	National Park	Donegal	07.12.2005	3	0	Unknown	1	0
GV003(WB)	*R. ponticum*	National Park	Donegal	07.12.2005	N/A	0	Unknown	3	0
GV003	Soil	National Park	Donegal	07.12.2005	N/A	0	Unknown	1	0
GV003	*R. ponticum*	National Park	Donegal	07.12.2005	2	0	Unknown	1	0
GV004(WB)	*R. ponticum*	National Park	Donegal	07.12.2005	N/A	0	Unknown	1	0
GV004	Soil	National Park	Donegal	07.12.2005	N/A	0	Unknown	2	0
GV004	*R. ponticum*	National Park	Donegal	07.12.2005	5	0	Unknown	11	0
GV005	Soil	National Park	Donegal	07.12.2005	N/A	0	Unknown	2	0
GV005	*R. ponticum*	National Park	Donegal	07.12.2005	5	0	Unknown	3	0
GV006	Soil	National Park	Donegal	07.12.2005	N/A	0	Unknown	6	0
GV006	*R. ponticum*	National Park	Donegal	07.12.2005	5	0	None	0	0
GV007	*R. ponticum*	National Park	Donegal	07.12.2005	4	0	Unknown	6	0
GV008	*R. ponticum*	National Park	Donegal	07.12.2005	5	0	Unknown	2	0
GV009	*R. ponticum*	National Park	Donegal	12.07.2006	5	0	Unknown	1	0

GV0010	*R. ponticum*	National Park	Donegal	12.07.2006
GV0011	*R. ponticum*	National Park	Donegal	12.07.2006
GV0012	*R. ponticum*	National Park	Donegal	12.07.2006
GV0013	*R. ponticum*	National Park	Donegal	12.07.2006
GV0014	*R. ponticum*	National Park	Donegal	12.07.2006
GV0015	*R. ponticum*	National Park	Donegal	10.08.2007
GV0016	*R. ponticum*	National Park	Donegal	10.08.2007
GV0017	*R. ponticum*	National Park	Donegal	10.08.2007
GV0018	*R. ponticum*	National Park	Donegal	10.08.2007
GV0019	*R. ponticum*	National Park	Donegal	10.08.2007
GV0020	*R. ponticum*	National Park	Donegal	10.08.2007
GV0021	*R. ponticum*	National Park	Donegal	10.08.2007
GV0022	*R. ponticum*	National Park	Donegal	10.08.2007
GV0023	*R. ponticum*	National Park	Donegal	10.08.2007
GV0024	*R. ponticum*	National Park	Donegal	10.08.2007

5	0	Unknown	1	0
5	0	Unknown	2	0
3	0	Unknown	1	0
3	0	Unknown	2	0
4	0	Unknown	5	0
5	0	Unknown	7	0
4	0	Unknown	4	0
3	0	Unknown	2	0
5	0	Unknown	1	0
5	0	Unknown	1	0
5	0	None	0	0
3	0	Unknown	3	0
5	0	Unknown	2	0
4	0	Unknown	8	0
3	0	Unknown	1	0

Table A2. Sampling results for Brackloon woods, Co. Mayo, from 2006-2007.

Sample Number	Host plant	Location	County	Date sampled	Number of leaves sampled	Morphological No. of +ve leaves	Growth	No. of DNA extractions	Molecular No. of +ve PCR
M001	*Rhododendron ponticum*	Brackloon Forest	Mayo	14.08.2006	5	0	Unknown	1	0
M002	*R. ponticum*	Brackloon Forest	Mayo	14.08.2006	3	0	Unknown	1	0
M003	*R. ponticum*	Brackloon Forest	Mayo	14.08.2006	5	0	Unknown	3	0
M004	*R. ponticum*	Brackloon Forest	Mayo	14.08.2006	5	0	Unknown	1	0
M005	*R. ponticum*	Brackloon Forest	Mayo	09.08.2007	4	0	Unknown	11	0
M006	*R. ponticum*	Brackloon Forest	Mayo	09.08.2007	4	0	Unknown	7	0
M007	*R. ponticum*	Brackloon Forest	Mayo	09.08.2007	4	0	Unknown	1	0
M008	*R. ponticum*	Brackloon Forest	Mayo	09.08.2007	4	0	Unknown	2	0
M009	*R. ponticum*	Brackloon Forest	Mayo	09.08.2007	3	0	Unknown	1	0
M010	*R. ponticum*	Brackloon Forest	Mayo	09.08.2007	3	0	None	0	0
M011	*R. ponticum*	Brackloon Forest	Mayo	09.08.2007	5	0	Unknown	1	0
M012	*R. ponticum*	Brackloon Forest	Mayo	09.08.2007	3	0	Unknown	1	0

Table A3. Sampling results for Connemara National Park, Co. Galway, from 2005-2007.

Sample Number	Host plant	Location	County	Date sampled	Number of leaves sampled	Morphological No. of +ve leaves	Growth	No. of DNA extractions	Molecular No. of +ve PCR
G001	*Rhododendron ponticum*	National Park	Galway	24.11.2005	3	0	Unknown	1	0
G002	*R. ponticum*	National Park	Galway	24.11.2005	3	0	Unknown	7	0
G003	*R. ponticum*	National Park	Galway	24.11.2005	3	0	Unknown	2	0
G004	*R. ponticum*	National Park	Galway	24.11.2005	5	0	None	0	0
G005	*Soil*	National Park	Galway	24.11.2005	5	0	Unknown	2	0
G006	*Soil*	National Park	Galway	24.11.2005	5	0	Unknown	2	0
G007	*R. ponticum*	National Park	Galway	16.04.2006	5	0	Unknown	4	0
G008	*R. ponticum*	National Park	Galway	16.04.2006	5	0	Unknown	13	0
G009	*R. ponticum*	National Park	Galway	16.04.2006	5	0	Unknown	10	0
G010	*R. ponticum*	National Park	Galway	30.03.2007	4	0	Unknown	6	0
G011	*R. ponticum*	National Park	Galway	30.03.2007	3	0	Unknown	1	0
G012	*R. ponticum*	National Park	Galway	30.03.2007	3	0	Unknown	1	0
G013	*R. ponticum*	National Park	Galway	30.03.2007	3	0	Unknown	2	0
G014	*R. ponticum*	National Park	Galway	30.03.2007	4	0	Unknown	1	0
G015	*R. ponticum*	National Park	Galway	30.03.2007	5	0	Unknown	3	0
G016	*R. ponticum*	National Park	Galway	30.03.2007	4	0	Unknown	1	0

Table A4. Sampling results for Killarney National Park, Co. Kerry, from 2005-2008.

Sample Number	Host plant	Location	County	Date sampled	Number of leaves sampled	Morphological No. of +ve leaves	Growth	No. of DNA extractions	Molecular No. of +ve PCR
K001	*Rhododendron ponticum*	National Park	Kerry	30.05.2005	3	0	Unknown	1	0
K002	*R. ponticum*	National Park	Kerry	30.05.2005	5	3	Unknown	7	5
K003	*R. ponticum*	National Park	Kerry	30.05.2005	5	0	Unknown	2	0
K004	*R. ponticum*	National Park	Kerry	30.05.2005	4	0	None	0	0
K005	*R. ponticum*	National Park	Kerry	30.05.2005	3	0	Unknown	4	0
K006	*R. ponticum*	National Park	Kerry	30.05.2005	5	0	None	0	0
K007	*R. ponticum*	National Park	Kerry	28.06.2005	5	2	*P. ramorum*	4	4
K008	*R. ponticum*	National Park	Kerry	28.06.2005	5	3	*P. ramorum*	5	5
K009	*R. ponticum*	National Park	Kerry	28.06.2005	5	3	*P. ramorum*	7	5
K010	*R. ponticum*	National Park	Kerry	28.06.2005	5	1	*P. ramorum*	2	2
K011	*Quercus laevis*	Unknown	Kerry	02.08.2005	N/A	0	Unknown	3	0
K012	*Q. laevis*	Unknown	Kerry	02.08.2005	N/A	0	Unknown	4	0
K013	*Q. laevis*	Unknown	Kerry	02.08.2005	N/A	0	Unknown	4	0
K014	*Q. laevis*	Unknown	Kerry	02.08.2005	N/A	0	Unknown	2	0
K015	*Q. laevis*	Unknown	Kerry	02.08.2005	N/A	0	Unknown	2	0
K016	*Q. laevis*	Unknown	Kerry	02.08.2005	N/A	0	Unknown	2	0
K017	*Q. laevis*	Unknown	Kerry	02.08.2005	N/A	0	Unknown	2	0
K018(WB)	Water	National Park	Kerry	23.02.2006	N/A	5	*P. ramorum*	15	10

K019	Soil	National Park	Kerry	23.02.2006
K020	Soil	National Park	Kerry	23.02.2006
K021	Soil	National Park	Kerry	23.02.2006
K022(WB)	Water	National Park	Kerry	23.02.2006
K023(WB)	Water	National Park	Kerry	23.02.2006
K024	Soil	National Park	Kerry	23.02.2006
K025(WB)	Water	National Park	Kerry	23.02.2006
K026	Soil	National Park	Kerry	23.02.2006
K027(WB)	Water	National Park	Kerry	24.06.2006
K028(WB)	Water	National Park	Kerry	24.06.2006
K029(WB)	Water	National Park	Kerry	24.06.2006
K030(WB)	Water	National Park	Kerry	24.06.2006
K031(WB)	Water	National Park	Kerry	24.06.2006
K032(WB)	Water	National Park	Kerry	24.06.2006
K033(WB)	Water	National Park	Kerry	24.06.2006
K034(WB)	Water	National Park	Kerry	24.06.2006
K035(WB)	Water	National Park	Kerry	24.06.2006
K036(WB)	Water	National Park	Kerry	24.06.2006
K037	*R. ponticum*	National Park	Kerry	24.06.2006
K038	*R. ponticum*	National Park	Kerry	24.06.2006
K039	*R. ponticum*	National Park	Kerry	24.06.2006
K040	*R. ponticum*	National Park	Kerry	24.06.2006
K041	*R. ponticum*	National Park	Kerry	24.06.2006
K042	*R. ponticum*	National Park	Kerry	24.06.2006
K043	*R. ponticum*	National Park	Kerry	24.06.2006
K044	*R. ponticum*	National Park	Kerry	24.06.2006

N/A	3	*P. ramorum*	8	8
N/A	0	None	0	0
N/A	4	*P ramorum*	4	2
N/A	0	Unknown	4	0
N/A	0	None	0	0
N/A	0	Unknown	3	0
N/A	0	None	0	0
N/A	0	None	0	0
N/A	0	Unknown	2	0
N/A	0	Unknown	2	0
N/A	0	Unknown	1	0
N/A	0	Unknown	2	0
N/A	0	Unknown	1	0
N/A	0	Unknown	3	0
N/A	0	Unknown	2	0
N/A	0	Unknown	4	0
N/A	0	Unknown	2	0
N/A	0	Unknown	3	0
5	0	Unknown	1	0
4	0	Unknown	6	0
5	2	*P. ramorum*	6	5
3	0	Unknown	10	0
3	0	Unknown	2	0
3	0	Unknown	2	0
4	0	Unknown	1	0
5	3	*P. ramorum*	7	6

K045	*R. ponticum*	National Park	Kerry	24.06.2006
K046	*R. ponticum*	National Park	Kerry	24.06.2006
K001_TW	*R. ponticum*	National Park	Kerry	24.06.2006
K002_TW	*R. ponticum*	National Park	Kerry	24.06.2006
K003_TW	*R. ponticum*	National Park	Kerry	24.06.2006
K004_TW	*R. ponticum*	National Park	Kerry	24.06.2006
K005_TW	*R. ponticum*	National Park	Kerry	24.06.2006
K006_TW	*R. ponticum*	National Park	Kerry	24.06.2006
K007_TW	*R. ponticum*	National Park	Kerry	24.06.2006
K008_TW	*R. ponticum*	National Park	Kerry	24.06.2006
K009_TW	*R. ponticum*	National Park	Kerry	24.06.2006
K010_TW	*R. ponticum*	National Park	Kerry	24.06.2006
K047	*R. ponticum*	National Park	Kerry	01.07.2007
K048	*R. ponticum*	National Park	Kerry	01.07.2007
K049	*R. ponticum*	National Park	Kerry	01.07.2007
K050	*R. ponticum*	National Park	Kerry	01.07.2007
K051	*R. ponticum*	National Park	Kerry	01.07.2007
K052	*R. ponticum*	National Park	Kerry	01.07.2007
K011_TW	*R. ponticum*	National Park	Kerry	01.07.2007
K012_TW	*R. ponticum*	National Park	Kerry	01.07.2007
K013_TW	*R. ponticum*	National Park	Kerry	01.07.2007
K014_TW	*R. ponticum*	National Park	Kerry	01.07.2007
K015_TW	*R. ponticum*	National Park	Kerry	01.07.2007
K016_TW	*R. ponticum*	National Park	Kerry	01.07.2007
K017_TW	*R. ponticum*	National Park	Kerry	01.07.2007
K018_TW	*R. ponticum*	National Park	Kerry	01.07.2007

4	1	*P. ramorum*	2	2
5	0	Unknown	8	0
5	0	Unknown	3	0
4	2	*P. ramorum*	3	3
4	0	Unknown	3	0
4	0	Unknown	2	0
4	1	*P. ramorum*	2	2
3	0	None	0	0
3	0	Unknown	1	0
3	0	Unknown	7	0
4	0	Unknown	5	0
4	0	None	0	0
5	0	Unknown	3	0
4	2	*P. ramorum*	6	6
5	4	*P. ramorum*	4	4
4	0	Unknown	6	0
5	0	Unknown	3	0
5	4	*P. ramorum*	12	12
3	0	Unknown	1	0
3	0	Unknown	2	0
3	0	Unknown	6	0
4	0	None	0	0
3	0	Unknown	7	0
4	0	Unknown	3	0
5	0	Unknown	8	0
4	0	Unknown	7	0

K019_TW	*R. ponticum*	National Park	Kerry	01.07.2007	4	0	Unknown	3	0
K020_TW	*R. ponticum*	National Park	Kerry	01.07.2007	4	0	Unknown	3	0
K053	*R. ponticum*	National Park	Kerry	09.05.2007	3	3	*P. ramorum*	10	8
K054	*R. ponticum*	National Park	Kerry	09.05.2007	4	2	*P. ramorum*	3	3
K055	*R. ponticum*	National Park	Kerry	09.05.2007	4	0	None	0	0
K_M1	*R. ponticum*	National Park	Kerry	09.05.2007	4	4	*P. ramorum*	8	7
K_M2	*R. ponticum*	National Park	Kerry	09.05.2007	4	0	Unknown	5	0
K_M3	*R. ponticum*	National Park	Kerry	09.05.2007	5	0	Unknown	3	0
K_M4	*R. ponticum*	National Park	Kerry	09.05.2007	5	0	Unknown	1	0
K_M5	*R. ponticum*	National Park	Kerry	09.05.2007	3	1	*P. ramorum*	2	1
K_M6	*R. ponticum*	National Park	Kerry	09.05.2007	5	0	Unknown	11	0
K056	*R. ponticum*	National Park	Kerry	17.05.2008	5	0	Unknown	1	0
K057	*R. ponticum*	National Park	Kerry	17.05.2008	3	0	Unknown	2	0
K058	*R. ponticum*	National Park	Kerry	17.05.2008	5	2	*P. ramorum*	5	5
K059	*R. ponticum*	National Park	Kerry	17.05.2008	4	2	*P. ramorum*	3	3
K060	*R. ponticum*	National Park	Kerry	17.05.2008	4	4	*P. ramorum*	12	11
K_M6	*R. ponticum*	National Park	Kerry	17.05.2008	5	3	*P. ramorum*	7	4
K_M7	*R. ponticum*	National Park	Kerry	17.05.2008	5	4	*P. ramorum*	1	1
K_M8	*R. ponticum*	National Park	Kerry	17.05.2008	5	0	None	0	0
K_M9	*R. ponticum*	National Park	Kerry	17.05.2008	5	0	Unknown	1	0
K001_h	*R. ponticum*	National Park	Kerry	24.06.2008	4	0	Unknown	3	0
K002_h	*R. ponticum*	National Park	Kerry	24.06.2008	5	3	*P. ramorum*	12	8
K003_h	*R. ponticum*	National Park	Kerry	24.06.2008	5	2	*P. ramorum*	4	4
K004_h	*R. ponticum*	National Park	Kerry	24.06.2008	5	0	Unknown	6	0
K005_h	*R. ponticum*	National Park	Kerry	24.06.2008	5	0	Unknown	2	0

K006_h	*R. ponticum*	National Park	Kerry	24.06.2008	5	0	Unknown	1	0
K007_h	*R. ponticum*	National Park	Kerry	24.06.2008	5	0	Unknown	3	0
K008_h	*R. ponticum*	National Park	Kerry	24.06.2008	5	0	Unknown	1	0

Red font indicates positive finding for *P. ramorum*

Table A5. Sampling results for Lauragh, Co. Kerry, from 2005-2008.

Sample Number	Host plant	Location	County	Date sampled	Number of leaves sampled	Morphological No. of +ve leaves	Growth	No. of DNA extractions	Molecular No. of +ve PCR
LH001	*Rhododendron ponticum*	Roadside	Kerry	28.07.2005	5	0	None	0	0
LH002	*R. ponticum*	Roadside	Kerry	28.07.2005	5	0	Unknown	1	0
LH003	*R. ponticum*	Roadside	Kerry	28.07.2005	4	0	Unknown	2	0
LH004	*R. ponticum*	Roadside	Kerry	28.07.2005	5	0	Unknown	7	0
LH005	*R. ponticum*	Roadside	Kerry	28.07.2005	5	0	None	0	0
LH006	*R. ponticum*	Roadside	Kerry	28.07.2005	3	0	Unknown	2	0
LH007	*R. ponticum*	Roadside	Kerry	28.07.2005	3	0	Unknown	3	0
LH008	*R. ponticum*	Roadside	Kerry	28.07.2005	3	0	Unknown	3	0
LH009	*R. ponticum*	Roadside	Kerry	28.07.2005	5	0	Unknown	9	0
LH010	*R. ponticum*	Roadside	Kerry	28.07.2005	5	0	Unknown	8	0
LH011	*R. ponticum*	Roadside	Kerry	28.07.2005	3	0	None	0	0
LH012	*R. ponticum*	Roadside	Kerry	26.02.2006	5	0	Unknown	2	0

LH013	*R. ponticum*	Roadside	Kerry	26.02.2006	5
LH014	*R. ponticum*	Roadside	Kerry	26.06.2006	5
LH015	*R. ponticum*	Roadside	Kerry	26.06.2006	5
LH016	*R. ponticum*	Roadside	Kerry	26.06.2006	5
LH017	*R. ponticum*	Roadside	Kerry	26.06.2006	5
LH018	*R. ponticum*	Roadside	Kerry	26.06.2006	4
LH019	*R. ponticum*	Roadside	Kerry	26.06.2006	5
LH020	*R. ponticum*	Roadside	Kerry	02.07.2006	4
LH021	*R. ponticum*	Roadside	Kerry	02.07.2006	5
LH022	*R. ponticum*	Roadside	Kerry	02.07.2006	3
LH023	*R. ponticum*	Roadside	Kerry	02.07.2006	5
LH024	*R. ponticum*	Roadside	Kerry	02.07.2006	4
LH025	*R. ponticum*	Field	Kerry	02.07.2006	4
LH026	*R. ponticum*	Field	Kerry	02.07.2006	3
LH027	*Quercus petraea*	Field	Kerry	02.07.2006	5
LH028	*Q. petraea*	Field	Kerry	02.07.2006	5
LH029	*Q. petraea*	Field	Kerry	02.07.2006	5
LH030	*R. ponticum*	Roadside	Kerry	08.05.2007	3
LH031	*R. ponticum*	Roadside	Kerry	08.05.2007	3
LH032	*R. ponticum*	Roadside	Kerry	08.05.2007	3
LH033	*R. ponticum*	Roadside	Kerry	08.05.2007	3
LH034	*R. ponticum*	Roadside	Kerry	08.05.2007	5
LH035	*R. ponticum*	Roadside	Kerry	08.05.2007	5
LH036	*R. ponticum*	Roadside	Kerry	08.05.2007	5

0	Unknown	3	0
0	Unknown	1	0
1	*P. ramorum*	2	2
3	*P. ramorum*	9	9
0	None	0	0
0	Unknown	2	0
0	Unknown	2	0
0	Unknown	9	0
2	*P. ramorum*	7	7
1	*P. ramorum*	3	3
1	*P. ramorum*	2	2
0	None	0	0
0	Unknown	5	0
0	Unknown	1	0
0	Unknown	2	0
0	Unknown	2	0
0	Unknown	1	0
0	Unknown	1	0
0	Unknown	2	0
1	*P. ramorum*	1	1
0	Unknown	3	0
3	*P. ramorum*	2	2
2	*P. ramorum*	1	1
0	Unknown	1	0

LH037	*Urtica dioica*	Roadside	Kerry	08.05.2007
LH038	*U. dioica*	Roadside	Kerry	08.05.2007
LH039	*R. ponticum*	Roadside	Kerry	19.05.2008
LH040	*R. ponticum*	Roadside	Kerry	19.05.2008
LH041	*R. ponticum*	Roadside	Kerry	19.05.2008
LH042	*R. ponticum*	Roadside	Kerry	19.05.2008
LH043	*R. ponticum*	Roadside	Kerry	19.05.2008
LH044	*R. ponticum*	Roadside	Kerry	19.05.2008
LH045	*R. ponticum*	Roadside	Kerry	19.05.2008
LH046	*R. ponticum*	Roadside	Kerry	19.05.2008
LH047	*R. ponticum*	Roadside	Kerry	19.05.2008
LH048	*R. ponticum*	Roadside	Kerry	19.05.2008
LH049	*R. ponticum*	Roadside	Kerry	19.05.2008
LH050	*R. ponticum*	Roadside	Kerry	19.05.2008

3	0	None	0	0
4	0	None	0	0
5	0	Unknown	2	0
5	2	*P. ramorum*	4	4
5	3	*P. ramorum*	7	3
3	2	*P. ramorum*	3	2
5	4	*P. ramorum*	7	7
4	0	Unknown	6	0
5	0	Unknown	11	0
3	0	Unknown	4	0
3	1	*P. ramorum*	1	1
4	1	*P. ramorum*	2	2
5	0	Unknown	3	0
5	0	Unknown	3	0

Table A6. Sampling results for Dunboy wood, Castletownbere, Co. Cork, during 2008.

Sample Number	Host plant	Location	County	Date sampled	Number of leaves sampled	Morphological No. of +ve leaves	Growth	No. of DNA extractions	Molecular No. of +ve PCR
D001	*Rhododendron ponticum*	Coillte wood	Cork	05.06.08	5	3	*P. ramorum*	9	6
D002	*R. ponticum*	Coillte wood	Cork	05.06.08	4	3	*P. ramorum*	4	4
D003	*R. ponticum*	Coillte wood	Cork	05.06.08	5	0	Unknown	7	0
D004	*R. ponticum*	Coillte wood	Cork	05.06.08	5	0	Unknown	2	0
D005	*R. ponticum*	Coillte wood	Cork	05.06.08	5	0	Unknown	8	0
D006	*R. ponticum*	Coillte wood	Cork	05.06.08	5	2	*P. ramorum*	3	3
D007	*R. ponticum*	Coillte wood	Cork	05.06.08	3	2	*P. ramorum*	6	5
D008	*R. ponticum*	Coillte wood	Cork	05.06.08	4	1	*P. ramorum*	2	2
D009	*R. ponticum*	Coillte wood	Cork	05.06.08	5	4	*P. ramorum*	2	2
D010	*R. ponticum*	Coillte wood	Cork	15.06.08	5	5	*P. ramorum*	12	4
D011	*R. ponticum*	Coillte wood	Cork	15.06.08	4	2	*P. ramorum*	2	2
D012	*R. ponticum*	Coillte wood	Cork	15.06.08	4	0	Unknown	3	0
D013	*R. ponticum*	Coillte wood	Cork	15.06.08	4	0	Unknown	7	0
D014	*R. ponticum*	Coillte wood	Cork	15.06.08	4	2	*P. ramorum*	6	6
D015	*R. ponticum*	Coillte wood	Cork	15.06.08	3	0	Unknown	1	0
D016	*R. ponticum*	Coillte wood	Cork	15.06.08	5	2	*P. ramorum*	1	1

Table A7. Sampling results for Glengarriff, Co. Cork, from 2005-2008.

Sample Number	Host plant	Location	County	Date sampled	Number of leaves sampled	Morphological No. of +ve leaves	Growth	No. of DNA extractions	Molecular No. of +ve PCR
G001	*Rhododendron ponticum*	Nature Reserve	Cork	31.05.2005	5	0	Unknown	3	0
G002	*R. ponticum*	Nature Reserve	Cork	31.05.2005	5	0	Unknown	4	0
G003	*R. ponticum*	Nature Reserve	Cork	25.02.2006	5	0	Unknown	2	0
G004	*R. ponticum*	Nature Reserve	Cork	25.02.2006	4	0	Unknown	1	0
G005	*R. ponticum*	Nature Reserve	Cork	25.02.2006	4	0	Unknown	3	0
G006	*R. ponticum*	Nature Reserve	Cork	25.02.2006	5	0	Unknown	1	0
G007	*R. ponticum*	Nature Reserve	Cork	25.02.2006	3	0	Unknown	1	0
Glengarriff	**No samples, woodland cleared**			26.06.2006	0	0	0	0	0
G008	*R. ponticum*	Nature Reserve	Cork	02.07.2006	0	0	None	0	0
G009	*R. ponticum*	Nature Reserve	Cork	02.07.2006	4	0	Unknown	1	0
G010	*R. ponticum*	Nature Reserve	Cork	02.07.2006	4	0	Unknown	2	0
G011	*R. ponticum*	Nature Reserve	Cork	02.07.2006	0	0	None	0	0
G012	*R. ponticum*	Nature Reserve	Cork	02.07.2006	5	0	Unknown	5	0
G013	*R. ponticum*	Nature Reserve	Cork	02.07.2006	5	0	Unknown	7	0
G014	*R. ponticum*	Nature Reserve	Cork	08.05.2007	3	0	Unknown	7	0
G015	*R. ponticum*	Nature Reserve	Cork	08.05.2007	3	0	Unknown	3	0
G016	*R. ponticum*	Nature Reserve	Cork	08.05.2007	4	0	Unknown	6	0
G017	*R. ponticum*	Nature Reserve	Cork	08.05.2007	4	0	Unknown	5	0
G018	*R. ponticum*	Nature Reserve	Cork	08.05.2007	4	0	Unknown	3	0

G019	*R. ponticum*	Nature Reserve	Cork	08.05.2007
G020	*R. ponticum*	Nature Reserve	Cork	08.05.2007
G021	*R. ponticum*	Nature Reserve	Cork	08.05.2007
G022	*R. ponticum*	Nature Reserve	Cork	08.05.2007
G023	*R. ponticum*	Nature Reserve	Cork	17.05.2008
G024	*R. ponticum*	Nature Reserve	Cork	17.05.2008
G025	*R. ponticum*	Nature Reserve	Cork	17.05.2008
G026	*R. ponticum*	Nature Reserve	Cork	17.05.2008
G027	*R. ponticum*	Nature Reserve	Cork	17.05.2008
G028	*R. ponticum*	Nature Reserve	Cork	17.05.2008
G029	*R. ponticum*	Nature Reserve	Cork	17.05.2008
G030	*R. ponticum*	Nature Reserve	Cork	17.05.2008
G031	*R. ponticum*	Nature Reserve	Cork	17.05.2008
G032	*R. ponticum*	Nature Reserve	Cork	17.05.2008

0	0	None	0	0
4	0	Unknown	3	0
3	0	Unknown	1	0
3	0	Unknown	1	0
3	0	Unknown	2	0
5	0	Unknown	12	0
3	0	Unknown	8	0
3	0	Unknown	7	0
3	0	Unknown	2	0
0	0	None	0	0
0	0	None	0	0
3	0	Unknown	2	0
4	0	Unknown	1	0
4	0	None	0	0

Table A8. Sampling results for Lismore, the Vee, Co. Waterford, from 2005-2007.

Sample Number	Host plant	Location	County	Date sampled	Number of leaves sampled	Morphological No. of +ve leaves	Growth	No. of DNA extractions	Molecular No. of +ve PCR
L001	*Rhododendron ponticum*	Roadside	Waterford	31.05.2005	3	0	Unknown	1	0
L002	*R. ponticum*	Roadside	Waterford	31.05.2005	5	0	Unknown	1	0
L003	*R. ponticum*	Roadside	Waterford	27.06.2006	5	0	Unknown	3	0
L004	*R. ponticum*	Roadside	Waterford	27.06.2006	3	0	Unknown	3	0
L005	*R. ponticum*	Roadside	Waterford	27.06.2006	4	0	None	0	0
L006	*R. ponticum*	Roadside	Waterford	03.07.2007	5	0	Unknown	1	0
L007	*R. ponticum*	Roadside	Waterford	03.07.2007	5	0	None	0	0
L008	*R. ponticum*	Roadside	Waterford	03.07.2007	3	0	Unknown	2	0
L009	*R. ponticum*	Roadside	Waterford	03.07.2007	5	0	None	0	0

Table A9. Sampling results for Killballyboy wood, the Vee, Co. Waterford, from 2005-2007.

Sample Number	Host plant	Location	County	Date sampled	Number of leaves sampled	Morphological No. of +ve leaves	Growth	No. of DNA extractions	Molecular No. of +ve PCR
TV001	*Rhododendron ponticum*	Private Woodland	Waterford	31.05.2005	5	0	Unknown	2	0
TV002	*R. ponticum*	Private Woodland	Waterford	31.05.2005	5	0	Unknown	2	0
TV003	*R. ponticum*	Private Woodland	Waterford	27.06.2006	5	0	Unknown	4	0
TV004	*R. ponticum*	Private Woodland	Waterford	27.06.2006	5	0	None	0	0
TV005	*R. ponticum*	Private Woodland	Waterford	27.06.2006	5	0	Unknown	2	0
TV006	*R. ponticum*	Private Woodland	Waterford	27.06.2006	4	0	Unknown	2	0
TV007	*R. ponticum*	Private Woodland	Waterford	27.06.2006	3	0	None	0	0
TV008	*R. ponticum*	Private Woodland	Waterford	27.06.2006	5	0	Unknown	4	0
TV009	*R. ponticum*	Private Woodland	Waterford	27.06.2006	5	0	None	0	0
TV010	*R. ponticum*	Private Woodland	Waterford	27.06.2006	3	0	None	0	0
TV011	*R. ponticum*	Private Woodland	Waterford	27.06.2006	3	0	Unknown	1	0
TV012	*R. ponticum*	Private Woodland	Waterford	03.07.2007	5	0	Unknown	2	0
TV013	*R. ponticum*	Private Woodland	Waterford	03.07.2007	4	0	None	0	0
TV014	*R. ponticum*	Private Woodland	Waterford	03.07.2007	3	0	None	0	0
TV015	*R. ponticum*	Private Woodland	Waterford	03.07.2007	3	0	Unknown	2	0
TV016	*R. ponticum*	Private Woodland	Waterford	03.07.2007	3	0	Unknown	4	0
P0415*	*R. ponticum*	Unknown	Waterford	30.11.2007	N/A	N/A	*P. ramorum*	7	7
P0417*	*R. ponticum*	Unknown	Waterford	30.11.2007	N/A	N/A	*P. ramorum*	8	7
P0419*	*R. ponticum*	Unknown	Waterford	30.11.2007	N/A	N/A	*P. ramorum*	4	4

P0420*	*R. ponticum*	Unknown	Waterford	30.11.2007	N/A	N/A	*P. ramorum*	6	2
P0421*	*R. ponticum*	Unknown	Waterford	30.11.2007	N/A	N/A	*P. ramorum*	6	6

* Indicates samples supplied by the Department of Agriculture, Fisheries and Food (DAFF) for molecular analyses, not collected in the field during the course of this study.

Table A10. Sampling results for John F. Kennedy Arboretum, Co. Wexford, from 2006-2007.

Sample Number	Host plant	Location	County	Date sampled	Number of leaves sampled	Morphological No. of +ve leaves	Growth	No. of DNA extractions	Molecular No. of +ve PCR
WX001	*Rhododendron ponticum*	National Park	Wexford	27.09.2006	3	0	Unknown	2	0
WX002	*R. ponticum*	National Park	Wexford	27.09.2006	3	0	Unknown	1	0
WX003	*R. ponticum*	National Park	Wexford	27.09.2006	5	0	Unknown	3	0
WX004	*R. ponticum*	National Park	Wexford	27.09.2006	5	0	Unknown	1	0
WX005	*R. ponticum*	National Park	Wexford	27.09.2006	4	0	Unknown	1	0
WX006	*R. ponticum*	National Park	Wexford	04.07.2007	5	0	Unknown	1	0
WX007	*R. ponticum*	National Park	Wexford	04.07.2007	5	0	Unknown	7	0
WX008	*R. ponticum*	National Park	Wexford	04.07.2007	4	0	Unknown	3	0
WX009	*R. ponticum*	National Park	Wexford	04.07.2007	3	0	None	0	0
WX010	*R. ponticum*	National Park	Wexford	04.07.2007	3	0	None	0	0
WX011	*R. ponticum*	National Park	Wexford	04.07.2007	3	0	Unknown	1	0
WX012	*R. ponticum*	National Park	Wexford	04.07.2007	3	0	Unknown	1	0

Table A11. Sampling results for Tomnafinnoge Wood, Co. Wicklow, from 2006-2007.

Sample Number	Host plant	Location	County	Date sampled	Number of leaves sampled	Morphological No. of +ve leaves	Growth	No. of DNA extractions	Molecular No. of +ve PCR
WK001	*Rhododendron ponticum*	National Park	Wicklow	26.09.2006	3	0	Unknown	2	0
WK002	*R. ponticum*	National Park	Wicklow	26.09.2006	5	0	Unknown	8	0
WK003	*R. ponticum*	National Park	Wicklow	26.09.2006	3	0	None	0	0
WK004	*R. ponticum*	National Park	Wicklow	26.09.2006	3	0	Unknown	5	0
WK005	*R. ponticum*	National Park	Wicklow	26.09.2006	3	0	Unknown	1	0
WK006	*R. ponticum*	National Park	Wicklow	26.09.2006	3	0	Unknown	1	0
WK007	*R. ponticum*	National Park	Wicklow	04.07.2007	3	0	Unknown	2	0
WK008	*R. ponticum*	National Park	Wicklow	04.07.2007	3	0	None	0	0
WK009	*R. ponticum*	National Park	Wicklow	04.07.2007	3	0	None	0	0
WK010	*R. ponticum*	National Park	Wicklow	04.07.2007	4	0	Unknown	3	0
WK011	*R. ponticum*	National Park	Wicklow	04.07.2007	3	0	Unknown	3	0
WK012	*R. ponticum*	National Park	Wicklow	04.07.2007	3	0	Unknown	2	0
WK013	*R. ponticum*	National Park	Wicklow	04.07.2007	3	0	Unknown	3	0
WK014	*R. ponticum*	National Park	Wicklow	04.07.2007	2	0	Unknown	4	0
WK015	*R. ponticum*	National Park	Wicklow	04.07.2007	3	0	Unknown	1	0

Appendix III

Table A1. Concentrations for PCR mastermix for *Phytophthora ramorum*.

Chemicals	**x1 (µl)**
Distilled, sterile water	14.875
10 x reaction buffer containing $MgCl_2$ (15 mM)*	2.5
dNTPs* (10 mM)	0.5
Forward Pram Primer$ (5 µM)	2.5
Reverse Pram Primer$ (5 µM)	2.5
AmpliTaq* (5 U/µl)	0.125
Total	**23.0**

* Chemical produced by Promega

$ MWG Biotech AG

Table A2. *Phytophthora ramorum* PCR Protocol from Beales et al. (2003).

Incubator files	**Temperature (°C)**	**Time**
Time delay file (Denaturing)	94	2 min
Step cycle file (Denaturing)	94	30 s
(Annealing)	57	30 s
(Primer extension)	72	30 s
Final extension time delay file	72	10 min
Soak file	-1	~

Table A3. Concentrations for real-time PCR reaction mix for *Phytophthora kernoviae* and *Phytophthora ramorum*.

Chemicals	Concentration	x1 (µl)
Taqman PCR Master Mix*	1x	22.5
IPC Mix*	1x	4.5
IPC DNA*	1x	0.9
60F Primer$	0.3 µM	1.35
121R Primer$	0.3 µM	1.35
84T Probe$	0.1µM	0.45
Deionised, sterile water	-	13.95
Total		**45**

* Applied Biosystems

$ MWG Biotech AG

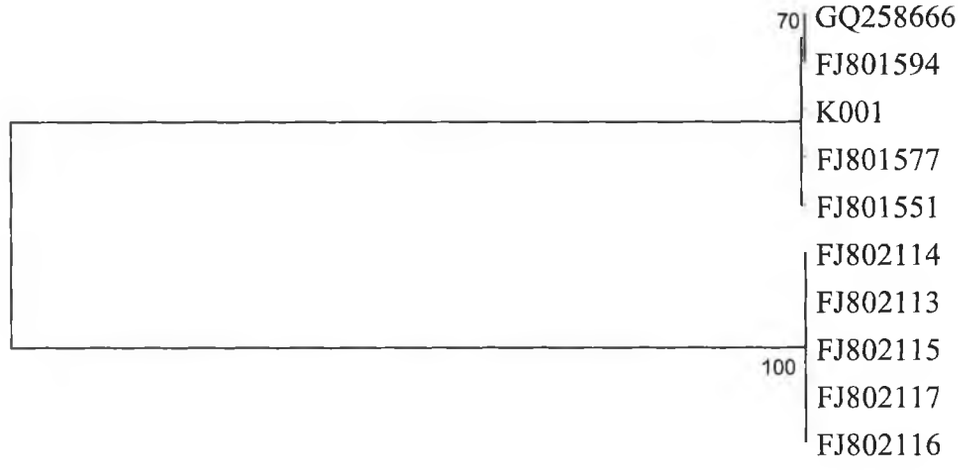

Figure A1. Neighbour-joining tree based on the sequence of the partial region of the ITS1 (internal transcribe spacer) 5.8s and partial region of the ITS2 region of the nrRNA gene of *Phytophthora ramorum*, isolate P0023, and nine *Phytophthora ramorum* isolates stored on GenBank inferred from *MEGA* version 4 software. Numbers in parentheses are bootstrap support values over 50% derived from a bootstrap consensus tree obtained through 500 replications.