

# National Diagnostic Protocol for Detection of *Phytophthora ramorum* (Sudden Oak Death) in Australia



|                 |                        |
|-----------------|------------------------|
| PROTOCOL NUMBER | NDP 5                  |
| VERSION NUMBER  | V1.0                   |
| STATUS          | Endorsed               |
| ISSUE DATE      | 18 February 2010       |
| REVIEW DATE     | December 2012          |
| ISSUED BY       | SPHDS Assessment Panel |



Australian Government  
Department of Agriculture, Fisheries and Forestry

This version of the National Diagnostic Protocol (NDP) for ***Phytophthora ramorum* (Sudden Oak Death)** is current as at the date contained in the version control box on the front of this document.

NDPs are updated every 3 years or before this time if required (i.e. when new techniques become available).

The most current version of this document is available from the SPHDS website [www.daff.gov.au/sphds](http://www.daff.gov.au/sphds)

|       |   |    |
|-------|---|----|
| 1     | Introduction.....   | 1  |
| 1.1   | Host range.....   | 1  |
| 1.2   | Effect on hosts.....  | 1  |
| 1.3   | Lifecycle .....   | 3  |
| 1.4   | Geographic distribution .....   | 4  |
| 1.4.1 | Potential distribution in Australia.....                                  | 5  |
| 1.5   | Transmission .....  | 5  |
| 2     | Taxonomic Information .....   | 6  |
| 3     | Detection .....   | 6  |
| 3.1   | Symptom description.....  | 6  |
| 3.2   | Sampling procedures .....   | 12 |
| 3.2.1 | Plant material .....  | 12 |
| 3.2.2 | Water.....  | 13 |
| 3.2.3 | Soil .....  | 13 |
| 3.3   | Preliminary screening; serological methods .....                          | 13 |
| 3.3.1 | Isolation.....  | 13 |
| 3.3.2 | Media and incubation.....   | 14 |
| 3.3.3 | Specimen Collection, Handling and Preservation .....                      | 15 |
| 3.4   | Diagnostic flow chart .....   | 15 |
| 4     | Identification .....  | 16 |
| 4.1   | Identification summary .....  | 16 |
| 4.2   | Morphological methods .....   | 17 |
| 4.2.1 | Growth characteristics in culture and morphology.....                     | 17 |
| 4.2.2 | Morphology of other <i>Phytophthora</i> species .....                     | 20 |
| 4.3   | Molecular methods.....  | 21 |
| 4.3.1 | Identification at species level by conventional PCR .....                 | 21 |
| 4.3.2 | Identification at species level by real-time PCR.....                     | 23 |
| 4.3.3 | Identification at species level by sequencing part of the ITS region..... | 25 |
| 5     | Contact points for further information .....                              | 27 |
| 6     | Reference material .....  | 27 |
| 7     | Acknowledgements .....  | 27 |
| 8     | References .....  | 28 |
|       | Appendix 1 .....  | 30 |
|       | Appendix 2 .....  | 33 |
|       | Appendix 3 .....  | 35 |
|       | Appendix 4 .....  | 36 |

# 1 Introduction

*Phytophthora ramorum* is a foliage/shoot blight and stem canker. It is an oomycota pathogen and was first described in 2001 as a disease of *Rhododendron* spp., *Viburnum* spp. and *Pieris* spp from Germany and the Netherlands. However, the disease caused by this pathogen had been observed since 1993 (Werres *et al.*, 2001). In Europe, it has since been recorded in Belgium, Denmark, France, Italy, Norway, Poland, Republic of Ireland, Slovenia, Spain, Sweden, Czech Republic and UK including the Channel Islands. It is commonly referred to as Ramorum dieback or Sudden Oak Death.

In the USA, *P. ramorum* has caused the mortality of oaks (mainly tanoak and coast live oak) in native forests and the urban-native forest interface in the coastal fog belts of southern Oregon and northern California since 1994 (Figure 1) ('Sudden Oak Death' Rizzo *et al.*, 2002). The disease is currently in epidemic proportions in coastal California, covering an area that runs approximately 600 km south to north from central California to southern Oregon. In Oregon it is subject to an eradication program. In 2005 a significant spread of the pathogen occurred across the USA through infected nursery plants, although to date this has not been shown to have spread into susceptible hosts in native ecosystems.

## 1.1 Host range

Host pathogenicity testing has shown susceptibility of many plant families, genera and species highlighting the significant risk to forest species in Europe and the USA (Brasier *et al.*, 2004). The wide host range includes a diversity of tree species, shrubs and herbaceous species including members of the Aceraceae, Adiantaceae, Anacardiaceae, Apiaceae, Berberidaceae, Betulaceae, Calycanthaceae, Caprifoliaceae, Ericaceae, Fagaceae, Griselinaceae, Hamamelidaceae, Lauraceae, , Pinaceae, , Hippocastanaceae Leguminosae, Liliaceae, Magnoliaceae, Moraceae, Myrtaceae, Nothofagaceae, Oleaceae, Pieridae, Pittosporaceae, Primulaceae, Rhamnaceae, Rosaceae, Salicaceae, Taxaceae, Taxodiaceae, Theaceae and Winteraceae (Appendix 1).

Of particular concern to Australia are both the field observations and pathogenicity tests that a number of Australian genera from a range of families are highly susceptible to *P. ramorum*. This includes *Eucalyptus gunnii* (Myrtaceae) (Brown unpublished data), *Nothofagus obliqua* (Nothofagaceae, Brown, unpublished data) and *Pittosporum undulatum* (Pittosporaceae, Huberli *et al.*, unpublished data). However, variation in genera between species susceptibility from highly susceptible to resistant has been recorded (Appendix 2).

## 1.2 Effect on hosts

*Phytophthora ramorum* causes mortality of oaks (mainly tanoak and coast live oak) in native forests and the urban-native forest interface in the coastal fog belts of southern Oregon and northern California (Figure 1) ('Sudden Oak Death' Rizzo *et al.*, 2002). Symptoms of the disease on large trees include cankers on the lower trunk that have brown or black discolored outer bark and bleeding sap (Hong 2003). Sunken or flattened cankers may occur beneath bleeding areas which when the outer bark is removed from bleeding cankers, mottled areas of necrotic, dead discoloured inner-bark tissues may be seen. Black 'zone lines' are often present within and around edges of the necrotic areas. On young or thinner trees, a distinct edge between necrotic and healthy tissues may also be visible. These cankers develop before foliar symptoms become evident. However, due to these girdling necroses, the whole crown of affected trees often appears to die rapidly. The name 'Sudden Oak Death' reflects this rapid symptom development (Figure 1).





Figure 1: Sudden Oak Death in Marin County (north of San Francisco) (Images Fire Department Marin County, California, USA, [http://nature.berkeley.edu/comtf/PRED/LoRez\\_11-10-04.ppt](http://nature.berkeley.edu/comtf/PRED/LoRez_11-10-04.ppt) ).



### 1.3 Lifecycle

The lifecycle of *P. ramorum* is similar to that of other aerial Phytophthoras such as *Phytophthora infestans*, the cause of potato blight and the great Irish famine of 1845 (Figure 2, Figure 3).

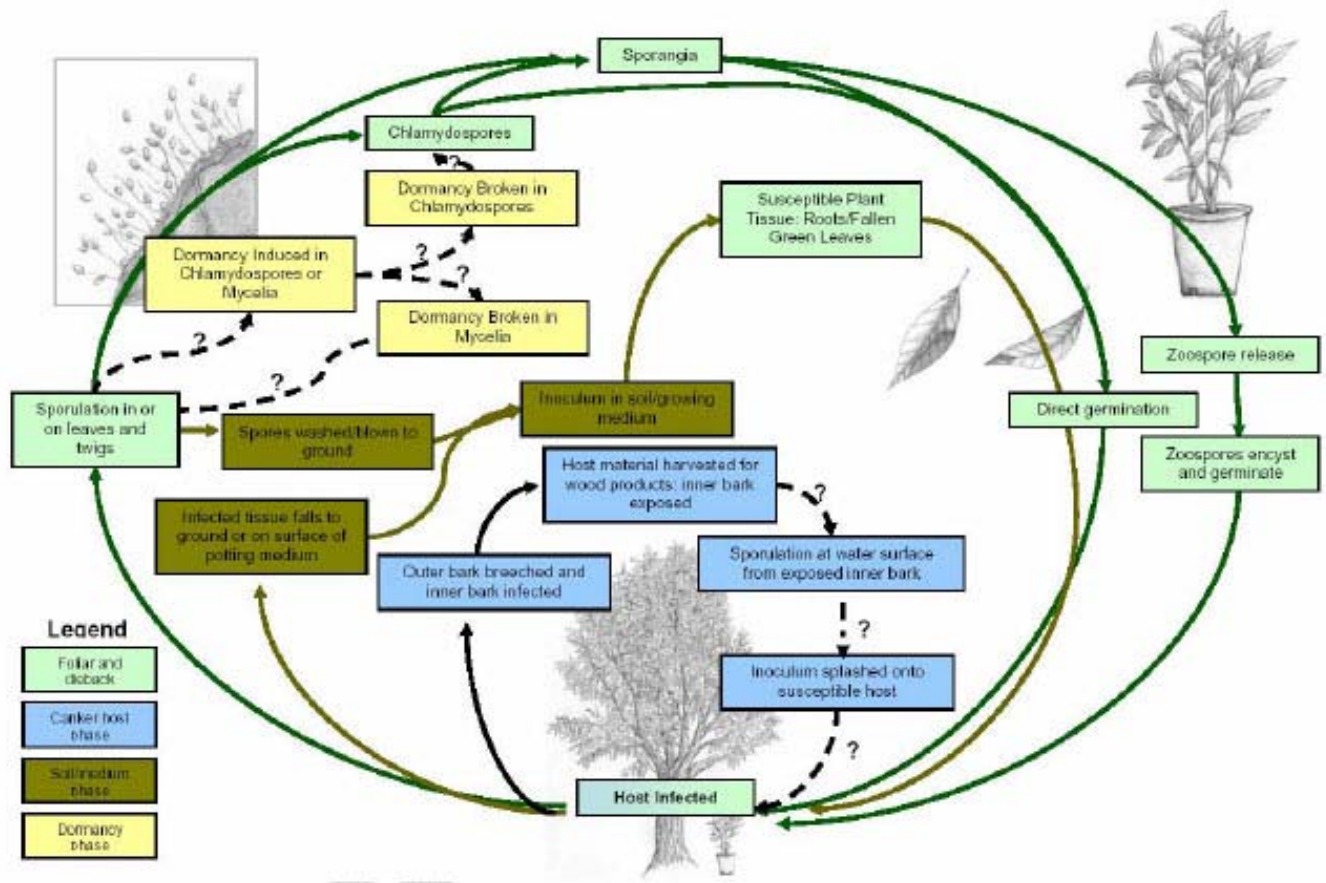


Figure 2: Proposed disease cycle for *Phytophthora* canker (sudden oak death), leaf blight and dieback. Colour is used to designate different hosts and phases. From <http://is.aphis.usda.gov/ppq/ispm/pramorumpamorumppra05-05-05.pdf>



### 1.4.1 Potential distribution in Australia

A recent evaluation in Australia showed that much of Australia's highly productive forests, old growth forests and temperate rainforests fall within the climatic envelope suitable for disease development (Figure 4) (Smith *et al*, unpublished data). If introduced to Australia, *P. ramorum* has the potential to spread rapidly and make it very difficult to control or manage. It is likely to be a major ecological threat to southern Australian forest or woodland ecosystems, amenity trees, horticultural crops and to home gardens in areas with a similar climate to California. Furthermore, the common practice of planting exotic plants (eg rhododendrons) in private gardens adjacent to wet sclerophyll native forests in Australia provides a significant potential pathway for this pathogen to spread should it enter Australia on infected exotic ornamentals. As a comparison, *Chalara australis*, a native pathogen causing wilt of *Nothofagus cunninghamii* in temperate rainforests of Victoria and Tasmania, has similar optimal climatic requirements (Kile *et al* 1989), although with a very limited host range.

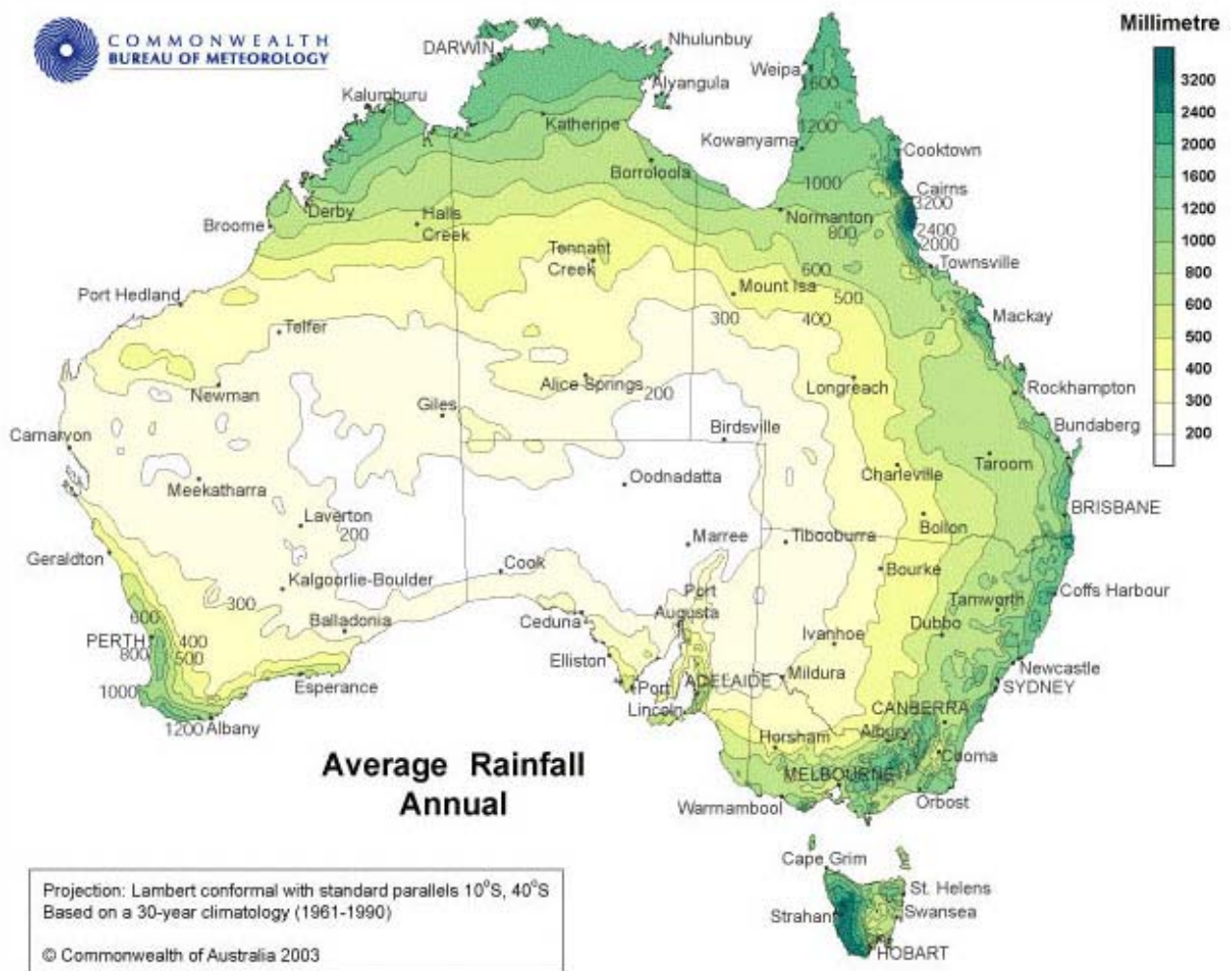


Figure 4: Rainfall map of Australia. In California USA, susceptible species growing in areas above 850 mm are considered highly vulnerable to *Phytophthora ramorum*.

## 1.5 Transmission

Autonomous spread between trees appears to be via spores (sporangia/zoospores) contained in rain splash and wind-driven rain, although the pathogen can also be recovered from rivers downstream from infected hosts providing irrigation water as another possible source of spread. While *P. ramorum* has not been observed to cause symptoms below the soil line in the forest, it can be recovered from soil and leaf litter.



## 2 Taxonomic Information

|                            |   |
|----------------------------|---|
| <b>Scientific Name:</b>    | <i>Phytophthora ramorum</i> Werres, De Cock & Man in't Veld   |
| <b>Common Names:</b>       | Sudden Oak Death, Ramorum shoot dieback, Ramorum leaf blight. |
| <b>Anamorph:</b>           | None  |
| <b>Synonym:</b>            | None  |
| <b>Taxonomic position:</b> | Chromista: Oomycota, Peronosporales, Pythiaceae               |

## 3 Detection

### 3.1 Symptom description

Symptoms caused by *P. ramorum* can be diverse with three distinct disease syndromes observed (Hansen *et al.* 2002):

1. Sudden Oak death, characterised by lethal cankers;
2. "ramorum shoot dieback", which results from foliar infection and/or direct infection of stems;
3. "ramorum leaf blight", which results from foliar infection.

These symptoms are summarised in a depiction of the life cycle of the pathogen (Figure 2).

#### 1. Sudden Oak Death

Diagnostic symptoms of the disease on large trees include cankers on the lower trunk that have brown or black discoloured outer bark and bleeding sap (Hong 2003, Figure 5a). Sunken or flattened cankers may occur beneath bleeding areas which when the outer bark is removed from bleeding cankers, mottled areas of necrotic, dead discoloured inner-bark tissues may be seen (Figure 5b). Black 'zone lines' are often present within and around edges of the necrotic areas. On young or thinner trees, a distinct edge between necrotic and healthy tissues may also be visible. These cankers develop before foliar symptoms become evident. However, due to these girdling necroses, the whole crown of affected trees often appears to die rapidly. The name 'Sudden Oak Death' reflects this rapid symptom development (Figure 1). *Eucalyptus gunnii* and *Nothofagus obliqua* have been shown to also exhibit similar symptoms in the United Kingdom (Brown unpublished data) (Figure 6).

**Similar symptoms:** Bleeding cankers with dark stained wood under the bark, can occur on the trunks of several plant species in Australia caused by other pathogens such as *Botryosphaeria* and other *Phytophthora* species. In particular, the soil-borne root and stem infecting pathogen *P. cinnamomi* can cause bleeding cankers on chestnuts, avocados, plane trees and several species of eucalypts and many other genera. However, *P. ramorum* appears to attack only aerial plant parts. Disease symptoms have not been detected below the soil-line which contrasts with many other diseases caused by *Phytophthora* spp.. Cankers caused by *P. cinnamomi*, *P. citricola*, or *P. cactorum* usually start with root rot then develop into cankers on the main stem and move upwards (Figure 7). This is a useful characteristic for distinguishing cankers caused by *P. ramorum* from those caused by other *Phytophthora* spp. However in the United Kingdom, *P. kernoviae* also produces similar cankers to *P. ramorum*. *Armillaria* species that can also cause bleeding cankers can be easily distinguished by the white mycelial fans under the bark (Figure 8).

Other exotic pathogens yet to reach Australia, that can cause bleeding cankers in eucalypts include *Cryphonectria cubensis* (Figure 9a), *C. parasitica* and *Coniothyrium zuluense* (Figure 9b)<sup>1</sup>.

---

<sup>1</sup> Dr Anna Brown, DEFRA, United Kingdom

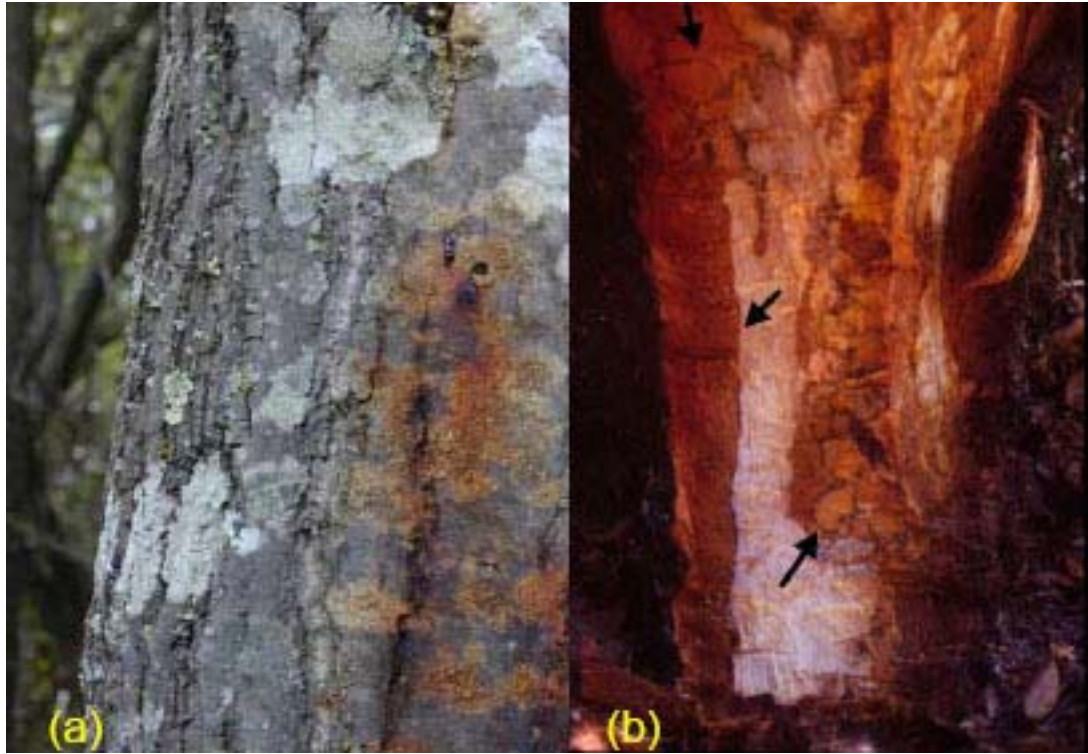


Figure 5 a) Bleeding canker on tanoak infected with *Phytophthora ramorum*, b) bark removed showing mottled areas of necrotic, dead discoloured inner-bark.



Figure 6: a) Bleeding canker on *Nothofagus obliqua* infected with *Phytophthora ramorum* in the United Kingdom. b) bark removed showing mottled areas of necrotic, dead discoloured inner-bark (image courtesy Dr Anna Brown, DEFRA, United Kingdom).





Figure 7: Canker on Chestnut (*Castanea sativa*) caused by *Phytophthora cinnamomi* in Victoria, Australia.



Figure 8 a) Mycelial fans under bark and b) fruiting bodies of *Armillaria* causing cankers on trees.





Figure 9 a) Canker on *Eucalyptus grandis* caused by *Cryphonectria cubensis* (Image Edward L. Barnard, Florida Department of Agriculture and Consumer Services, [www.forestryimages.org](http://www.forestryimages.org)). b) Serious and fatal fungal canker caused by *Coniothyrium zuluense*. *Eucalyptus* sp. Kwazulu, South Africa.  
[http://www.fao.org/documents/show\\_cdr.asp?url\\_file=/docrep/007/y5041e/y5041e09.htm](http://www.fao.org/documents/show_cdr.asp?url_file=/docrep/007/y5041e/y5041e09.htm)

## 2. & 3. Ramorum shoot dieback and leaf blight

The most common symptoms on shrubs such as rhododendron are shoot dieback and leaf blight (Figure 11). Ramorum shoot dieback is characterised by blackened shoots with or without foliage attached (Figure 10a and Figure 11a). Symptoms of Ramorum leaf blight include diffuse brown (dark-brown) spots or blotches with fuzzy margins frequently at the leaf tip (where moisture can accumulate and remain for extended periods encouraging infection (Figure 10 b and c, Figure 11c and Figure 12). However, spots can also form elsewhere (drops with zoospores falling down on the leaf surface cause round, dark-brown patches). Eventually, entire leaves can turn brown to black and may fall prematurely. With the exception of *Viburnum* spp, *P. ramorum* usually does not kill shrub hosts (Figure 11b).

**Similar symptoms:** As with Sudden Oak Death, these symptoms are not unique to Ramorum shoot dieback and leaf blight. *P. nicotianae*, *P. citrophthora* and *P. heveae* also may cause foliar symptoms similar to those of Ramorum dieback. In the United Kingdom *P. kernoviae* also produces similar symptoms to *P. ramorum*. *Colletotrichum*, *Botryosphaeria* and *Botrytis* can also cause similar symptoms so care should be taken with the diagnosis (Figure 13). Abiotic factors such as sunburn may also give similar symptoms although in these cases a defined margin is usually expressed (Figure 14).



Figure 10 a) Shoot dieback of *Rhododendron* infected with *Phytophthora ramorum* (image courtesy Everett Hansen, Oregon State University) b) underside & c) top of leaves infected with *Phytophthora ramorum* (images Bruce Moltzen, Missouri Dept. of Conservation).

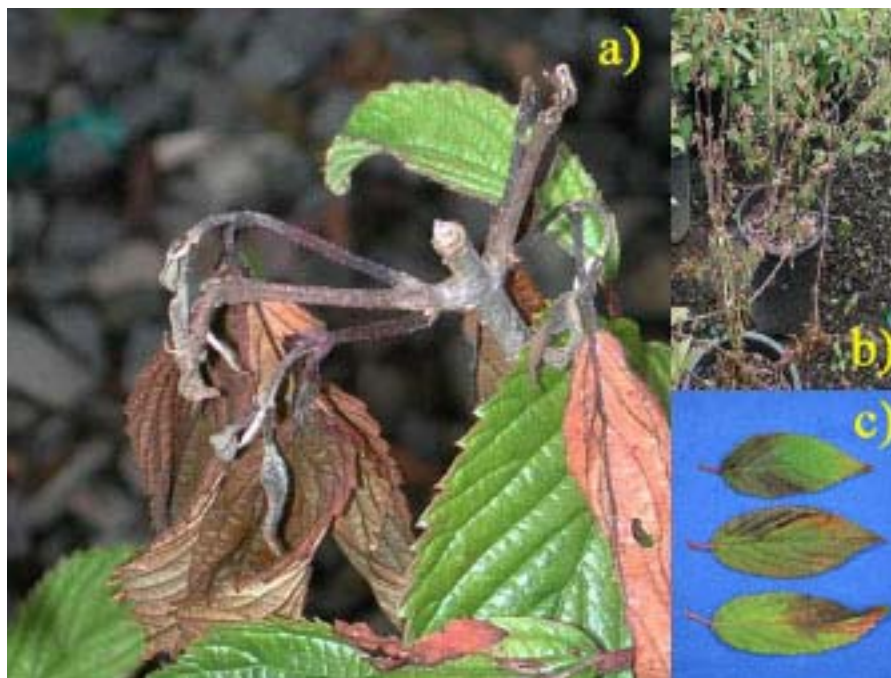




Figure 11 a) Shoot dieback of *Virburnum* sp. infected with *Phytophthora ramorum* (image Oregon Department of Agriculture), b) seedlings in pots killed by *P. ramorum* (image Oregon Department of Agriculture), and c) leaf symptoms (image Jennifer Parke, Oregon State University).



Figure 12 *Phytophthora ramorum* infection on the leaves of California bay laurel (*Umbellularia californica*) Joseph O'Brien, USDA-Forest Service <http://www.forestryimages.org/search/action.cfm?q=ramorum&Start=31&results=103>

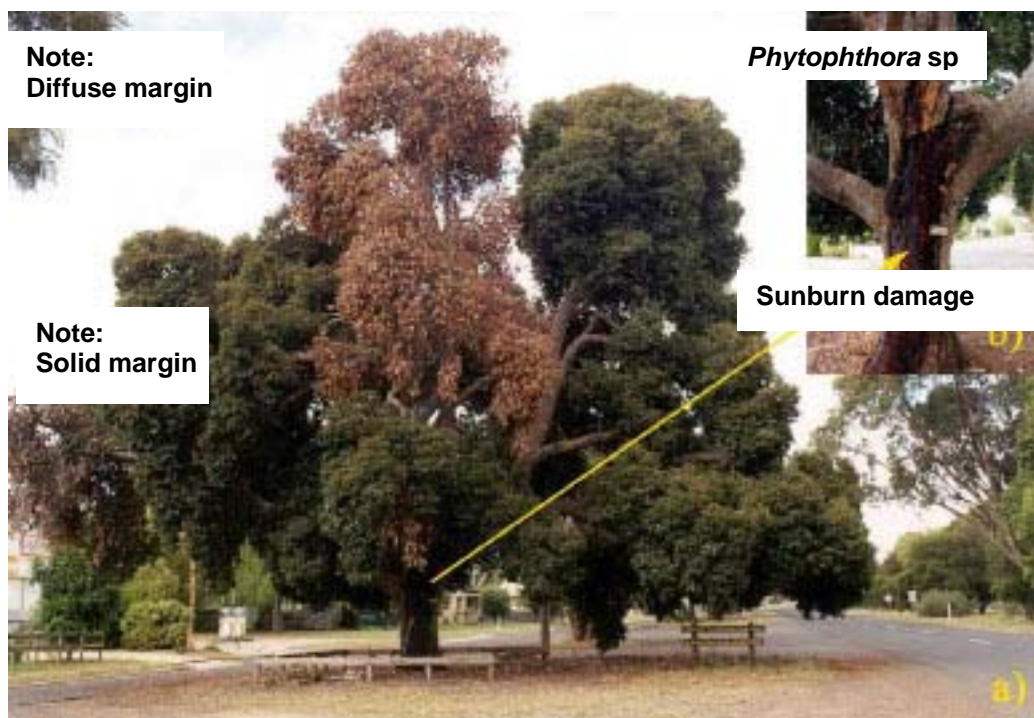




Figure 13 Kino bleeding from the trunk of *Corymbia ficifolia* associated with *Botryosphaeria infection* in Victoria, Australia.

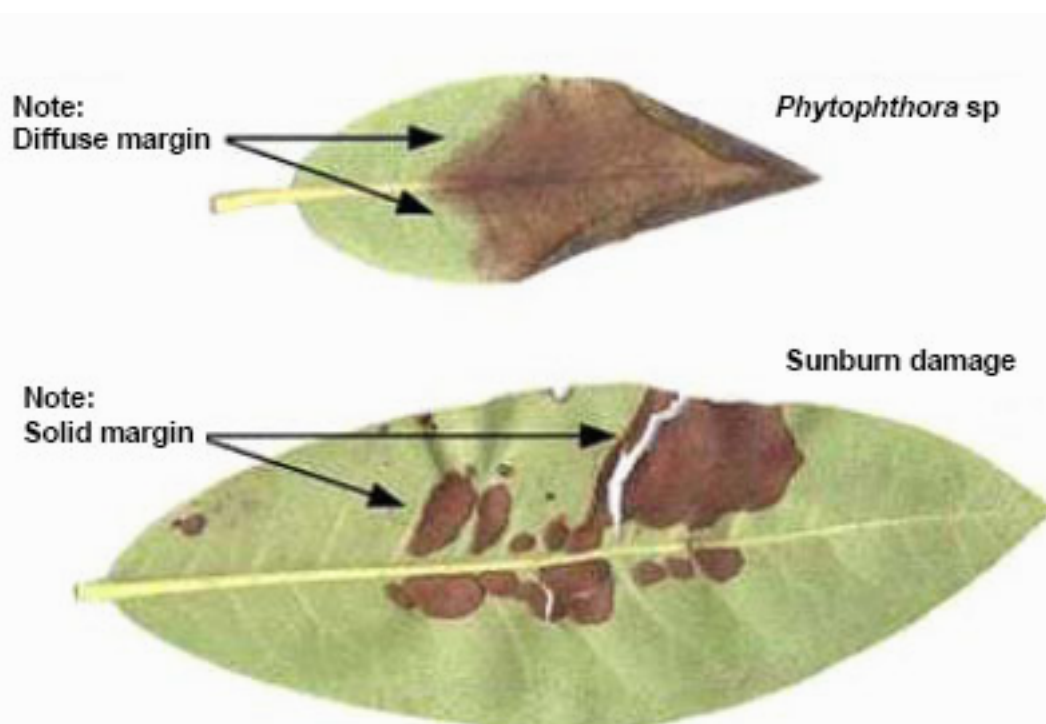


Figure 14 A comparison of leaf lesions caused by *Phytophthora* sp. compared to abiotic factors such as sun scorch. (Tim Tidwell, CA Dept of Food and Agriculture).

## 3.2 Sampling procedures

Different methods are used to sample for the pathogen depending on the type of material to be tested (Rizzo *et al.*, 2002).

### 3.2.1 Plant material

#### 3.2.1.1 Bleeding cankers

The inner bark in the area directly around the oozing sap is cut until a canker margin is evident (Figure 5b, Figure 6b). Pieces of phloem and xylem are removed and placed in a sealed container, or small pieces may also be plated out on different media directly (Rizzo *et al.*, 2002).

#### 3.2.1.2 Shoots/twigs

Leading edge (the junction between diseased and healthy tissue Figure 10a), are located and a piece of stem approximately 15 cm long, with 7.5 cm either side of the leading edge is removed and placed in a sealed plastic bag, with a small piece of damp tissue to prevent desiccation.

#### 3.2.1.3 Leaves

Four to six leaves showing a suitable range of symptoms (Figure 10b and c, Figure 11c), are removed and placed in a sealed plastic bag with a piece of damp tissue. All samples of plant material should be fully labeled and sent to a laboratory to arrive the next day.

Labeling should where possible include:

- Name and postal address of sender,
- Contact phone numbers and email address where available,

- Identification of plant species/parts affected,
- Location of affected plant (where available include GPS reading),
- Symptoms (include image if available).

*Precautions:* Overheating or desiccation of samples prior to despatch should be prevented. Samples may be stored in a fridge (4-10°C) for several weeks if necessary.

### 3.2.2 Water

At least 1000 mL of water including sediment and any floating debris from the surface of the water is skimmed off for testing. It is placed in a strong plastic bottle kept cool (4-10°C) and sent to the laboratory to arrive the next day.

### 3.2.3 Soil

At least 500 g or 200 mL of soil and debris from affected areas is collected and placed in a strong, sealed plastic bag. It should be kept cool (4-10°C), and sent to the laboratory to arrive the next day.

## 3.3 Preliminary screening; serological methods

These can be used to pre-screen the presence of *Phytophthora* spp. but are not specific to *P. ramorum* and false negatives and positives may occur. They are useful for large scale surveys to reduce the number of samples being submitted for further testing, and have been used as an integral component of the initial response to *P. ramorum* outbreaks, especially in the US and UK. To date, these methods have been most successful when applied to leaf lesions and are less effective at diagnosing *P. ramorum* infection from cankers and lesions in wood.

There are two serological methods commercially available: Agdia ELISA test system [www.agdia.com](http://www.agdia.com) and Phytophthora Pocket Diagnostic [www.pocketdiagnostic.com](http://www.pocketdiagnostic.com).

### 3.3.1 Isolation

#### 3.3.1.1 Plant material

Surface sterilisation of plant material should be carried out prior to plating to reduce contamination by surface colonizing microorganisms. There are a number of alternative methods for surface sterilisation or decontamination of plant material that may be used including alcohol treatments or a solution with 0.5 % active sodium hypochlorite for 2-5 min may be used. Plant material should be rinsed with sterile water and dried. The choice of method depends on:

- (1) the type of substrate: thin root or leaf tissue should not be treated with alcohol;
- (2) the type of method to be performed: culturing is prone to contamination by other microorganisms but molecular methods are not;
- (3) the risk for false negatives: water rinses may partially remove contamination with irrelevant organisms but allow the remaining ones to suppress *P. ramorum*, whereas alcohol treatments may remove the contaminants as well as *P. ramorum*. Relevant alternative methods are given for each of the tissue types involved (Appendix 4).

#### 3.3.1.2 Water samples

##### 3.3.1.2.1 Baiting

For all water samples the 'Rhododendron leaf test' should be used (Themann & Werres, 1998; Themann et al., 2002;). Leaves should be taken from plants which have not been sprayed with fungicides previously. Whole, fully formed young leaves are preferable to older leaves and cut leaf pieces as these are more likely to be infected by non-target microbial species.

Small samples: known healthy leaf pieces of Rhododendron, preferably Rhododendron hybrid cv Cunningham's white, are floated on the surface of water samples. Within 3 to 7 days of incubation, suspect leaf areas should be cut out for isolation onto non-selective (e.g. CPA) or selective media (e.g. P<sub>5</sub>ARP[H], for media see Appendix 3) or PCR.

Large samples (e.g. irrigation ponds, streams, etc): 5-10 known healthy leaves of rhododendron, preferably Rhododendron 'hybrid cv. Cunningham's White' are placed into a small piece of muslin (or similar porous material like flywire) and pieces of polystyrene or similar material are added to aid flotation. The material is drawn up into a bag, tied with string and floated on the water for between 1 to 2 weeks (Figure 15). The presence of *P. ramorum* is checked as described for small samples.



Figure 15 Rhododendron leaf baits used in stream monitoring for *Phytophthora ramorum* in the USA. (Murphy et al 2005).

#### 3.3.1.2.2 Filtration

Water may also be filtered to detect the presence of zoospores of *Phytophthora*. In this case water samples of approximately 1000 mL should be collected in a sterile wide mouth bottle, kept at 5 - 10 °C and processed in the lab within 48 hours of collection. The water is vacuum filtered through sterilised 5 µm pore size, nitrocellulose or polycarbonate membrane filters usually at 100 mL at a time. The filter paper is then placed filtrate side down onto selective media (e.g. P<sub>5</sub>ARP[H]).

#### 3.3.1.3 Soil

The Rhododendron leaf test (see above) should be used. The soil is placed in a large plastic box, and sufficient sterile, demineralised water or Petri's mineral solution is added to allow healthy leaf pieces of rhododendron, preferably Rhododendron hybrid 'cv. Cunningham's White', to be floated on the surface. After 3-7 days incubation, the presence of *P. ramorum* is checked as described for the water samples. Other baits known to be effective in the isolation of *P. ramorum* include pears and *Viburnum* leaves (NPDN 2006).

### 3.3.2 Media and incubation

Presence of *P. ramorum* can sometimes be seen directly on the collected material (i.e. presence of typical sporangia). If not, material can be incubated for 3-5 days in a closed box with a small piece of damp tissue on the bottom to promote sporulation. For isolation, a number of media may be used (Appendix 3).

The most commonly used medium is P<sub>5</sub>ARP [H] as this is semi-selective for *Phytophthora* spp. and characteristic features of *P. ramorum* are readily observed. Once samples have been decontaminated using one of the methods outlined above, at least four small pieces (about 2 mm<sup>2</sup>) of tissue are excised with a sterile scalpel and transferred aseptically onto one or more of the agar media mentioned above. In order to permit direct observation under a compound microscope it is advisable to place the pieces approximately 2-3 cm from the edge of the dish. Plates are incubated on the laboratory bench (20-25 °C), or in an incubator between 20-22°C.



### 3.3.3 Specimen Collection, Handling and Preservation

Plant material, cultures and/or supplies used in the examination and isolation of a suspect sample must be destroyed using a biologically monitored autoclave. The autoclave must be set at a minimum of 15 psi, 121°C for 30 minutes. All tools and other equipment must be sanitized and/or sterilized before re-use.

### 3.4 Diagnostic flow chart

The following flowchart (Figure 16) suggests a diagnostic pathway for plant samples suspected of being infected with *Phytophthora ramorum*.

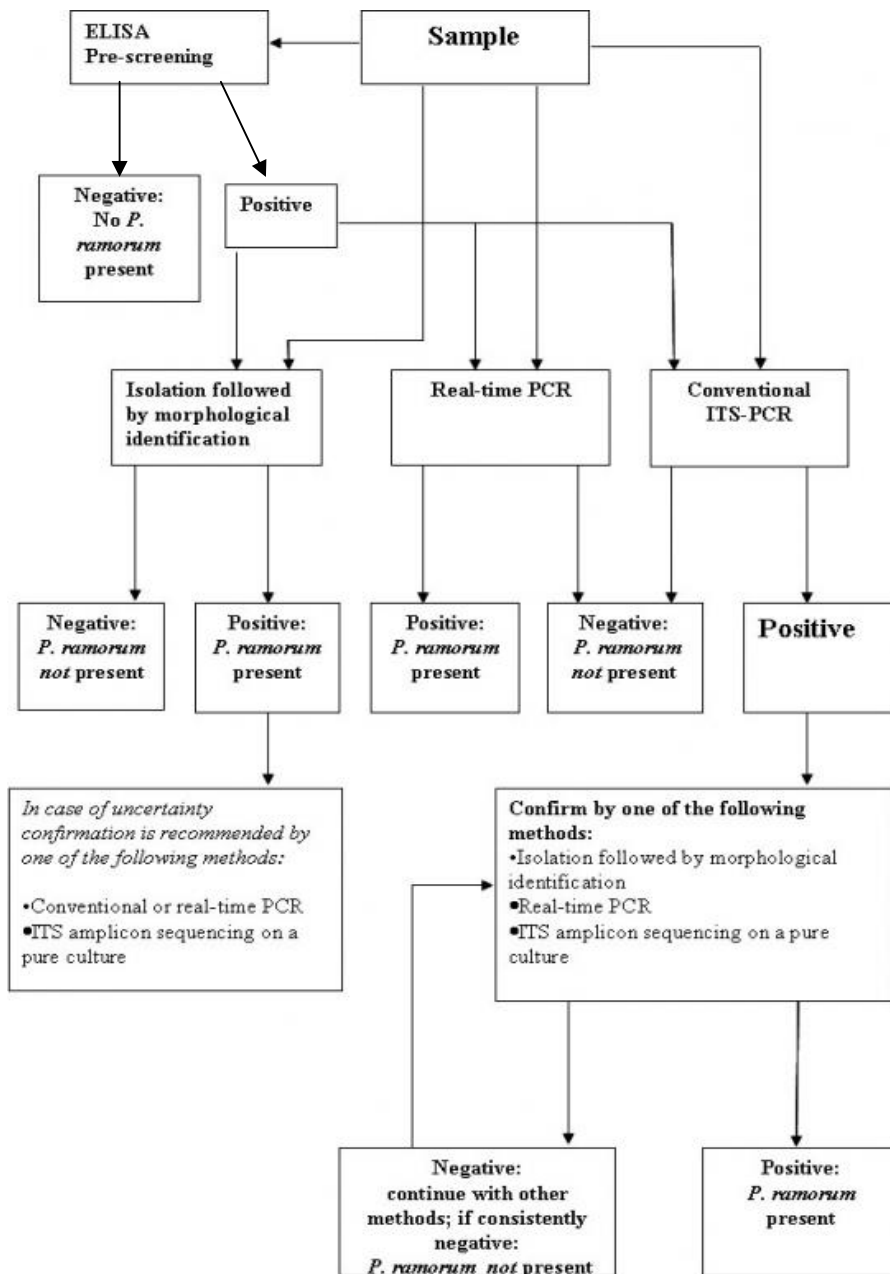


Figure 16 Flow diagram for diagnosis of *Phytophthora ramorum* on plants and plant products.

## 4 Identification

*Phytophthora ramorum* may be identified at species level by either its growth characteristics in culture and morphology or by appropriate molecular methods. A flow diagram indicating equivalence of (combinations of) methods is given in Figure 16.

### 4.1 Identification summary

It is likely that an incursion of *P. ramorum* will begin with the identification of a small number of samples. These will probably be in pure culture, isolated from diseased plant material. In this case, the most appropriate method for identification is to obtain ribosomal DNA Internal Transcribed Spacer (ITS) sequence data.

ITS sequencing is unequivocal and should be applied during an emergency response to confirm a *P. ramorum* detection in a new location or on a significant new host.

For routine testing, during surveillance or an emergency response, one of the three methods should be used:

1. Morphological examination;

OR

2. Real-time PCR;

OR

3. Conventional PCR with positives checked by ITS sequencing.

When choosing a molecular method to use, both the real-time PCR's are equivalent, but the conventional PCR's are listed in order of preference.

Mating type may be determined using A1 and A2 tester strains (Werres et al., 2001; Werres and Zielke, 2003; Brasier & Kirk, 2004). Molecular biotypes may be identified by PCR-RFLP analysis or sequencing of the mitochondrial cytochrome oxidase (Cox)-gene (Kroon et al., 2004).

## 4.2 Morphological methods

### 4.2.1 Growth characteristics in culture and morphology

The growth characteristics on agar and morphological features are described in Werres et al. (2001). The most essential features on a selective and a non-selective medium are given in Table 1. Generally, the unique morphological features described make *P. ramorum* a relatively easy organism to identify in culture. Possible confusion in morphology/cultural characteristics may occur with *P. palmivora*. *Phytophthora ramorum* has a nearly unique set of morphological characteristics and a unique molecular sequence at the ITS region.

The most distinguishing feature of *P. ramorum* is the presence of large (22 to 72 µm) chlamydospores that are mainly terminal (Figure 17c). Chlamydospores change from hyaline to cinnamon brown as they mature. This change is especially pronounced in media with host material.

Sporangia are semi-papillate, caducous (Figure 18a) and will form in clusters on agar flooded with soil water. Soil water is prepared by shaking 400g of sandy soil in 1L water and filtering and autoclaving the extract. They are highly deciduous in soil water at 20°C. *P. ramorum* has highly branching hyphae on PARP that becomes septate in older cultures.

This pathogen appears to be heterothallic. Mating in vitro has been achieved through crosses with other *Phytophthora* species (Werres et al 2003, Figure 18b). Pairings of European and American isolates in vitro on carrot agar (C. M. Brasier and S. Kirk, REF unpublished data) and in vivo within rhododendron twigs (Werres & Zielke 2003) have led to production of oospores (Davidson et al 2003).



Table 1 Growth characteristics on a selective and non-selective medium

| Character                            | P <sub>5</sub> ARP(H) *   | Carrot piece agar *  |
|--------------------------------------|---|--|
| Colony<br>Figure 17a & b             | relatively slow growing, approximately 2 mm per day   | weak rosette-like pattern, pronounced concentric rings, growth rate approximately 3 mm per day   |
| Mycelium<br>Figure 17 c              | weakly coralloid, growing within the agar with little superficial growth, no hyphal swellings   | aerial mycelium sparse, no hyphal swellings  |
| Sporangia<br>Figures 17 e & f & 18 a | produced abundantly on the agar surface, semi-papillate, caducous with short or no stalk. Size: 20-32 x 40-80 µm, average 24 x 52 µm; average length/width ratio 2.16   |  |
|                                      | ellipsoid, frequently in small clusters and relatively narrow, initial sporangium commonly producing secondary, smaller sporangia   | ellipsoid, spindle-shaped or elongated-ovoid, single or in clusters  |
| Chlamydospores<br>Figures 17 c & d   | more common in older colonies (7-10 days), very large (up to 80 µm diameter), hyaline to pale brown to brown  | after 3 days incubation in the dark, in the older parts but very often also in the young parts of the colony, thin-walled, hyaline to pale brown up to 88 µm |
| Sexual structures<br>Figure 18b      | can be observed on carrot piece agar after pairing with the opposite mating type, e.g. that of <i>P. cryptogea</i> (Werres and Zielke, 2003). <i>P. ramorum</i> x <i>P. ramorum</i> pairing is also possible <i>in vitro</i> (not with all isolates) (Brasier, pers. comm.) and in Rhododendron twigs (Werres and Zielke, 2003) |  |

\* On P<sub>5</sub>ARP(H) characters can be observed after 4-6 days incubation on the bench at 20°C, 12h light/12h dark. On carrot piece agar this is after 3-5 days incubation at 20°C in darkness.

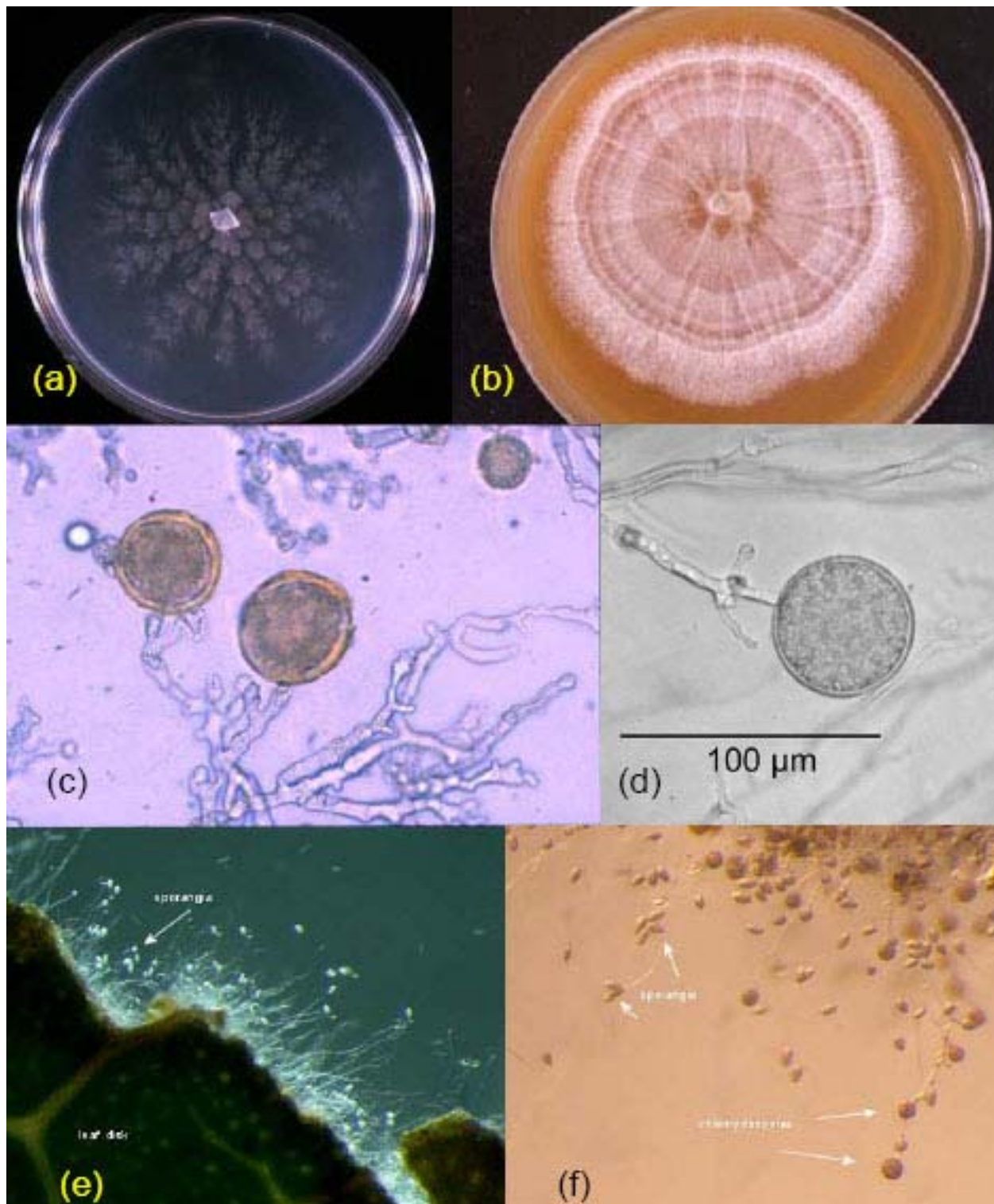


Figure 17 a) *Phytophthora ramorum* a) water agar and b) 15 day-old culture on V8 juice agar (USDA 2005), c) & d) Mycelium and chlamydospores of *Phytophthora ramorum* (UC Davis and UC Berkeley), e) & f) Sporangia and chlamydospores of *Phytophthora ramorum* on the edge of a bay leaf disk, e) 20 hours and f) 7 days after inoculation. J. L. Parke, Oregon State University, Corvallis, OR 97331 [http://nature.berkeley.edu/comtf/photos/sporulation\\_photos\\_0.html](http://nature.berkeley.edu/comtf/photos/sporulation_photos_0.html)

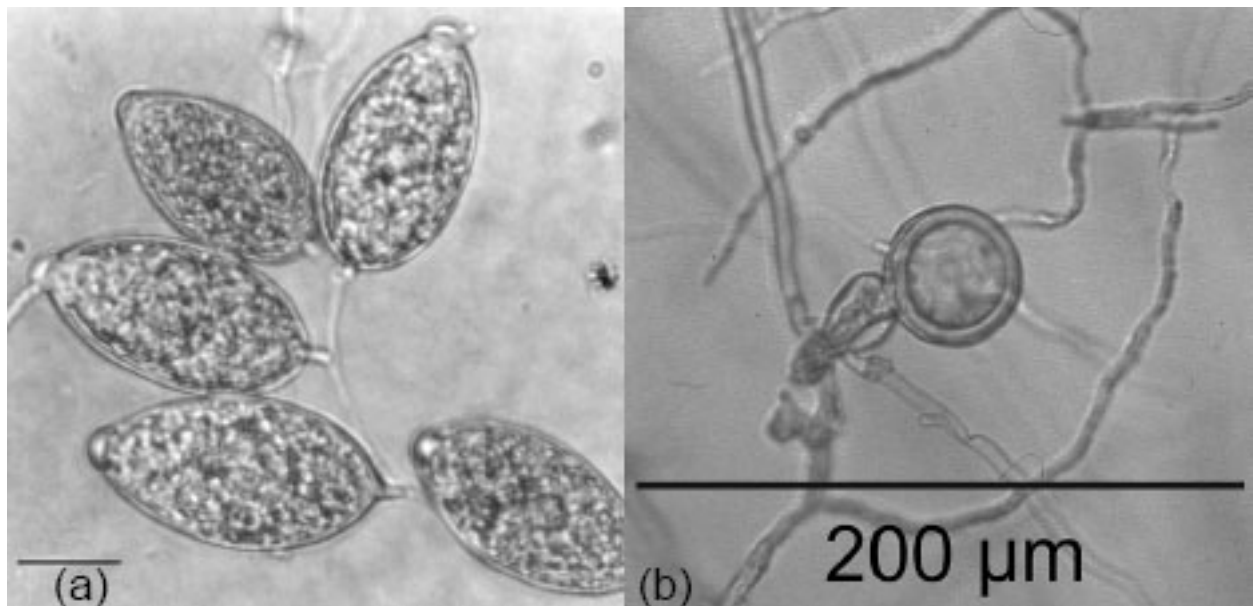


Figure 18 a) Sporangia (Werres et al 2003). b) Oospore of *Phytophthora ramorum*, Davidson et al (2003) Sudden Oak Death and Associated Diseases Caused and by *Phytophthora ramorum*. Plant Management Network.  
<http://www.plantmanagementnetwork.org/php/shared/sod/>

#### 4.2.2 Morphology of other *Phytophthora* species

<http://www.invasivespecies.net/database/species/SimilarSpecies.asp?si=563&fr=1&sts=>

*Phytophthora cinnamomi* has straighter, non-dendritic hyphae, but have ballooned swellings at tips and clusters of chlamydospores which are produced laterally on the hyphae. Sporangia of *P. cinnamomi* are non-papillate and persistent, not deciduous.

*Phytophthora ramorum* is closely related to *P. lateralis*. *P. lateralis* causes Port-Orford cedar root and has lateral chlamydospores whereas *P. ramorum* has terminal chlamydospores.

*Phytophthora nemorosa* resembles *P. ilicis* with homothallic, amphigynous antheridia and deciduous and semi-papillate sporangia. It causes symptoms similar to *P. ramorum*, as well as having a similar host range (Hansen et al., 2003).

*Phytophthora kernoviae* also causes similar symptoms and has caducous sporangia but as with *P. nemorosa* is homothallic rather than heterothallic. *Phytophthora kernoviae* has plerotic oogonia, often with distinctly tapered stalks and amphigynous antheridia. It produces papillate sporangia, sometimes markedly asymmetric with medium length pedicels (Brasier et al 2005).

Of the species listed, only *P. cinnamomi* has been identified in Australia and the remainder (*P. lateralis*, *P. ilicis*, *P. kernoviae* and *P. nemorosa*) are not known to be present in Australia at this time and are therefore less likely to be encountered. However, there are numerous other species of *Phytophthora* present that may be encountered. A list of these, their known distribution and host species are provided in Irwin et al (1995) and Burgess et al (2009).

## 4.3 Molecular methods

Molecular methods have been developed to identify *P. ramorum* from culture plates as well as direct in planta using either conventional PCR with primers designed by M. Garbelotto (USA) (Method A, Kox et al., 2002; Method B, Wagner & Werres, 2003) or with K. Hughes's primers (UK) (Method C, Lane et al., 2003b) or Real-time PCR (Method A, Hughes et al., 2005); and Method B, Hayden et al., 2004). In general, the conventional PCR primers are quite specific for *P. ramorum* with a small number of cross-reactions. The conventional PCR using Garbelotto's primers may show cross-reaction with *P. lateralis* (L.F.F. Kox, pers.comm.), a fungus that is not native to Europe and does not occur on hosts of *P. ramorum*, and *P. cambivora* at high DNA concentrations (Davidson et al., 2003). The UK Real-time PCR primers are specific to *P. ramorum* (Hughes, pers. comm.). Further identification methods include sequencing of the ITS region to permit identification to species.

### 4.3.1 Identification at species level by conventional PCR

#### 4.3.1.1 Conventional PCR: Method A (Kox et al., 2002)

*Primers:* a primer pair (Phyto 1 and Phyto 4) has been developed by M. Garbelotto (Hayden *et al.*, 2004) for the detection of *P. ramorum* by conventional PCR. The primer sequences are :

Phyto 1 : 5'-CAT GGC GAG CGC TTG A-3' and

Phyto 4 : 5'-GAA GCC GCC AAC ACA AG-3'

##### 4.3.1.1.1 Sample handling

Plant material (leaves, twigs, stems, stem base) is disinfected superficially with a tissue using 1% sodium hypochlorite. Small pieces of material (0.5-1 cm<sup>2</sup> each) are cut from the edge of symptomatic tissue with a sharp knife. In general 6-10 pieces are collected and washed in a laminar flow cabinet according to the following procedure : tap water (10 s), alcohol 50 % (10 s), tap water (10 s), 1% sodium hypochlorite (10 s), and finally tap water (10 s). The material is then dried on sterile filter paper for 20-30 s. The pieces are cut into very small parts (each approximately 1 mm<sup>2</sup>) with a sterile scalpel, and placed in a clean 1.5 mL screw cap tube. When not immediately processed, the tubes are stored with material in a freezer (- 20 °C). Cultures can also be used as starting material for extraction. A piece of approximately 1 cm<sup>2</sup> from the agar (taking as little agar as possible) is cut, and put it into a 1.5 mL screw cap tube.

##### 4.3.1.1.2 DNA extraction

500 µL extraction buffer (0.02 M PBS, 0.05 % Tween T25, 2 % polyvinylpyrrolidone, 0.2% bovine serum albumin) and beads are added to the tube which is beaten for 80 s at speed 5 (5m/s) (Hybaid Ribolyzer). The tube is centrifuged for 5 s in a microcentrifuge at maximum speed (16100 g) and 75 µL of the supernatant is transferred to a new 1.5 mL tube.

##### 4.3.1.1.3 DNA isolation

DNA for PCR is isolated using the Dneasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions (Anonymous, 2000), and is eluted with 100 µl AE buffer. It is further purified using polyvinylpyrrolidone (PVPP) columns. These columns are prepared by filling a Micro Bio-Spin chromatography column (Biorad, cat no: 732-6204) with 0,5 cm PVPP, and placed in an empty tube. Then 250 µL RNase- and DNase free water are applied to the PVPP column which is centrifuged for 5 min at 4000 g in a microcentrifuge. This last step is repeated. The columns are put on a clean tube, and DNA is applied to the column which is centrifuged for 5 min at 4000 g.

##### 4.3.1.1.4 Amplification and analysis

The reaction mixture (25 µL) should contain: 5 µL DNA suspension; 2.5 µL of 10x-concentrated reaction buffer containing 15 mM MgCl<sub>2</sub> (Qiagen); 0.5 µL 10 mM dNTPs; 0.1 µL 100 µM of each primer Phyto 1 and Phyto 4; 0.1 µL HotStarTaq Taq DNA polymerase (5 U/ul; Qiagen), and 16.7 µL DNase and RNase free water to give a final volume 25 µL. The amplification is performed in thin-walled 200µL PCR tubes in a Peltier type thermal cycler with a heated lid using the following conditions : 15 min at 95°C; then 35 cycles of 15 s at 94°C, 60 s at 62°C, 45 s at 72°C. One cycle for 10 min at 72°C should be conducted after the 35 cycles, and



finally 1 s at 20°C. After amplification, 10 µL of the reaction mixture is loaded onto a 1.0 % agarose gel in 0.5x TBE buffer, separated by electrophoresis, stained with ethidium bromide, and viewed and photographed under UV light. A negative control (DNase- and RNase-free water) should be included in every experiment to test for contamination as well as a positive control (DNA from a reference strain of the pathogen) to monitor the performance of the PCR. The positive control should yield an amplicon of 687 bp, but not the negative control. Strains yielding an amplicon of this size are positive for *P. ramorum*. Samples not yielding such an amplicon can be considered negative for *P. ramorum*. If either the negative or positive control fails the electrophoresis and/or the PCR should be repeated. To monitor for false negatives resulting from inhibition of the PCR reaction, duplicate reactions are spiked with *P. ramorum* DNA. If the spiked reaction gives a negative result, the PCR should be repeated with diluted DNA extract.

#### **4.3.1.2 Conventional PCR: Method B (Wagner and Werres, 2003)**

For Garbelotto's primers see above.

##### **4.3.1.2.1 Sampling and sample preparation**

If possible at least five twigs or leaves with disease symptoms are taken per plant. About five tissue pieces per twig or leaf (each 0.5 cm<sup>2</sup>, per sample about 100-200 mg total weight) are cut out from the discoloured tissue using a sterile knife.

##### **4.3.1.2.2 DNA extraction**

500 µL CTAB-buffer (2 %) and 50 mg of sterile quartz sand are added to the sample, which is then homogenised with a pestle and mortar for 2 min. This preparation is transferred into a 2 mL tube and vortex for 20 s. It is then frozen at -20°C defrosted at 75°C. The operation is repeated two times for two min., and a third time for 30min. 350 µL of phenol/chloroform/isoamyl alcohol (25:24:1) is added and the preparation is vortexed for 1 min at maximum speed. It is then centrifuged for 15 min in a microcentrifuge at maximum speed (16100 g), and the supernatant is removed and pipetted into a new sterile 1.5 mL tube.

##### **4.3.1.2.3 DNA isolation**

The DNA is purified with GeneClean Turbo Kit (Nucleic Acid Purification Kit: QBIOWE).

##### **4.3.1.2.4 Amplification and analysis**

The reaction mixture (25 µL) should contain : 6.25 µL DNA suspension; 2.5 µL of 10x-concentrated reaction buffer containing 7.5 mM MgCl<sub>2</sub> (INVITROGEN); 2.5 µL 2 mM dNTPs; 0.25 µL 50 µM of each primer Phyto 1 and Phyto 4; 0.25 µL Taq DNA polymerase (INVITROGEN, 5U/ ul), and 12.25 µL DNase and RNase free water.

Thermocycler conditions should be as follows: 1.25 min at 94°C; then 34 cycles of 35 sec at 93°C, 55 sec at 62°C, 50 sec at 72°C adding 5 sec at each cycle. One cycle for 10 min at 72°C should be conducted after the 34 cycles. Separate the PCR product after amplification. Load 10 µL of the reaction mixture onto a 1.0 % agarose gel in 1 x TAE buffer, separate by electrophoresis, stain with ethidium bromide, and view and photograph under UV light.

A negative and a positive control should be included in every PCR. For the negative control 6.25 µL DNase- and RNase free water and 18.75 µL PCR mix are taken. For the positive control DNA from the type strain is used. The DNA of the type strain should yield an amplicon of 687 bp. Samples yielding an amplicon of this size should be identified as *P. ramorum*, assuming that the controls have reacted properly.

#### **4.3.1.3 Conventional PCR: Method C (Lane et al., 2003b)**

The following protocol is for the conventional PCR identification of *P. ramorum* from cultures and plant material.

##### **4.3.1.3.1 Primers**

A primer pair (Pram F1 and Pram R1) has been developed by Hughes (Lane et al., 2003b) for conventional PCR. The primer sequences are :

Pram F1 : 5' CTA TCA TGG CGA GCG CTT GA 3' and

Pram R1 : 5' GAA GCC GCC AAC ACA AG 3'.

#### **4.3.1.3.2 Sample handling**

A 0.5 cm x 1 cm sample from a test culture is cut aseptically, or several small pieces of tissue from the leading infection edge of suspect plant material are removed and placed in a thick-walled plastic bag.

#### **4.3.1.3.3 DNA extraction**

The bagged sample is placed in liquid nitrogen. Once the sample is frozen, the bagged sample is put on the laboratory bench and ground by rolling the bag with a wallpaper seam roller or similar device. Alternatively, samples may be ground up by cutting them into small pieces and placing these in a 2 mL centrifuge tube containing approximately 150 mg siliconised 0.5 mm glass beads (Biospec products, Bartlesville, USA). The tube is closed with a screw-fitting lid containing an o-ring and the tube is oscillated in a Mini-Beadbeater (Biospec products) at full power for at least 20 s.

#### **4.3.1.3.4 DNA isolation**

DNA is extracted from ground-up samples using a suitable kit such as the NucleoSpin plant extraction kit (Macherey-Nagel, Düren, DE, Cat. ref. 740 570.250) or a more traditional method such as described in Hughes *et al.* (2000). Extracted (neat) DNA is stored at 4°C for immediate use or at -20°C if testing is not to be performed on the same day.

#### **4.3.1.3.5 Amplification and analysis**

Extracted DNA is defrosted if necessary and a ten-fold dilution of each extract is prepared in sterile molecular grade water. Then in an area dedicated for PCR work and using dedicated pipettes with filtered tips, enough reaction mix for testing at least two replicates of the neat and ten-fold dilution for each extract is prepared. For each PCR run positive control reactions of master mix plus *P. ramorum* DNA and negative control reactions of reaction mix loaded with water rather than DNA are included.

The reaction mixture (25 µL) should contain: 1.0 µL DNA suspension; 2.5 µL 10 X reaction buffer containing 15 mM MgCl<sub>2</sub> (Applied Biosystems); 2.0 µL 10 mM dNTPs; 2.5 µL 5 µM of each primer Pram F1 and Pram R1; 0.125 µL AmpliTaq (Applied Biosystems) (5 U/µl), and 14.375 µL sterile molecular grade water to give a final volume of 25 µL.

Amplification is performed in thin-walled PCR tubes in a PCR thermocycler programmed as follows: 2 min at 94°C; then 30 cycles of 30 s at 94°C, 30 s at 57°C, 30 s at 72°C. One cycle for 10 min at 72°C should be conducted after the 30 cycles. After amplification, 10 µL from the cycled reactions is mixed with 2 µL of loading dye (25 µg bromophenol blue and 25 µg xylene cyanol FF in 10 mL 50 % glycerol) and amplification products are resolved by electrophoresis on a 1.5 % agarose gel made with 1X TBE buffer at pH 8.0 (9.0 mM Tris, 8.9 mM boric acid and 2.5 mM EDTA). At least one replicate of a 100 base pair (bp) marker is added to each gel for amplicon size determination. Following electrophoresis, stain the gel for 30 min with ethidium bromide [0.5 µg/mL] then wash off excess stain and view the gel on a UV transilluminator.

#### **4.3.1.3.6 Assessment of PCR**

Reactions containing amplifiable DNA from *P. ramorum* produce a single c. 700 bp amplicon while no bands should be produced for the negative controls. Following extensive testing, some isolates of other *Phytophthora* species simultaneously amplify two bands, one between 100 and 500 bp and the second at c. 700 bp. Samples should only be considered positive for *P. ramorum* if a single 700 bp band is amplified. As DNA concentration can affect PCR amplification it may be that only one concentration of positive test samples is amplified, this is normal and the reason why two concentrations of test DNA are tested. If neither concentration is amplified, the DNA should be tested with the universal ITS primers ITS1 and ITS4 (White *et al.*, 1990), and their cycling conditions are described below ('Identification by sequencing part of the ITS-region'). Amplification with these primers shows that the test DNA is of an amplifiable quality and that a true negative for *P. ramorum* has occurred. However, if amplification is still not produced, fresh DNA should then be extracted and retested.

### **4.3.2 Identification at species level by real-time PCR**

The following two equivalent and validated methods may be used for TaqMan®-PCR identification of *P. ramorum* from cultures and plant material.

#### 4.3.2.1 RT-PCR: Method A (Hughes et al., 2006)

Primers/ TaqMan®- probe: the primer sequences are:

Pram 114-FC: 5' TCA TGG CGA GCG CTG GA 3',

Pram 1527-190-R: 5' AGT ATA TTC AGT ATT TAG GAA TGG GTT TAA AAA GT 3',

and the TaqMan®- probe is :

Pram 1527-134-T: 5' TTC GGG TCT GAG CTA GTA G 3'.

The TaqMan®-probe is labelled at the 5' end with the fluorescent reporter dye 6- carboxyfluorescein (FAM) and at the 3' end with the quencher dye, 6-carboxytetramethyl- rhodamine (TAMRA).

##### 4.3.2.1.1 Sample handling / DNA extraction / DNA isolation

DNA from samples is prepared at approximately 20-100 ng/μL as described above for the conventional PCR-method C.

##### 4.3.2.1.2 Amplification and analysis

In optical quality reaction tubes/plates (Applied Biosystems) at least two replicate reactions for each test sample and control samples of known *P. ramorum* DNA (positive control) and water (negative control) are prepared.

The reaction mixture (25 μL) should contain: 12.5 μL of 2 X Taqman Universal master mix (Applied Biosystems); 1.5 μL 5 μM primer Pram 114-FC; 1.5 μL 5 μM primer Pram 1527-190-R; 0.5 μL 5 μM probe Pram 1527-134-T; 1.0 μL c. 20-100 ng DNA test suspension; 8.0 μL sterile molecular grade water.

Test reactions are cycled in a suitable instrument for detection of reporter fluorescence, for example an ABI Prism 7700 or 7900 Sequence Detection System (Applied Biosystems) using the following conditions: 10 min at 94°C; then 40 cycles of 15 s at 94°C and 60 s at 60°C.

##### 4.3.2.1.3 Assessment of PCR

Data from the TaqMan® run are analyzed as per manufacturer's instructions. Samples with cycle threshold (Ct) values less than 36 are considered as positive for *P. ramorum*, typically Ct values are between 25 and 35. A Ct value of 36 indicates a negative result. Internal control primers should be used when plant material is tested directly; this is not necessary when using pure cultures. The internal primers and probe are based on sequences by Weller *et al.* (2000) and amplify plant DNA present in the test extracts. Their use confirms that amplifiable DNA is present in test extracts from plants which are PCR- negative for *P. ramorum*.

Internal control primers: the primer sequences are:

COX-F 5' CGT CGC ATT CCA GAT TAT CCA 3', and

COX-RW 5' CAA CTA CGG ATA TAT AAG RRC CRR AAC TG 3'

N.B. Primer COX-RW contains degenerative nucleotides indicated by the IUPAC code R, indicating that both adenine and guanine are inserted at these positions in equal amounts.

Internal control TaqMan®- probe:

COX-P 5' AGG GCA TTC CAT CCA GCG TAA GCA 3'

The TaqMan®-probe is labelled at the 5' end with the fluorescent reporter dye VIC (Applied Biosystems) and at the 3' end with the quencher dye TAMRA.

##### 4.3.2.1.4 Amplification and analysis

Test reactions and positive/ negative controls are prepared using the master mix as described below, and each sample is cycled as described above for testing cultures by TaqMan® PCR.

The reaction mixture (25 μL) should contain: 12.5 μL 2 X Taqman Universal master mix (Applied Biosystems); 1.5 μL 5 μM primer Pram 114-FC; 1.5 μL 5 μM primer Pram 1527-190-R; 0.5 μL 5 μM probe Pram 1527-134-T; 1.0 μL 5 μM primer COX-F; 1.0 μL 5 μM primer COX-RW; 0.5 μL 5 μM probe COX-P; 1.0 μL c. 20-100 ng DNA test suspension; 5.5 μL sterile molecular grade water.

#### 4.3.2.1.5 Assessment of PCR

Samples containing amplifiable *P. ramorum* DNA produce FAM fluorescence as recorded by Ct FAM values of < 40. These samples may also produce VIC fluorescence as recorded by Ct values of < 40 as should all other samples NOT containing *P. ramorum* DNA. VIC fluorescence indicates that the COX primer/probe set has amplified viable DNA present in the test sample. If neither FAM or VIC fluorescence is recorded this indicates that the sample contains no amplifiable DNA and that sample should be re-extracted and tested again.

#### 4.3.2.2 RT-PCR: Method B (Hayden et al., 2004)

Primers/ TaqMan®- probe: the primer sequences are:

Pram-5: 5' TTA GCT TCG GCT GAA CAA TG 3',

Pram-6: 5' CAG CTA CGG TTC ACC AGT CA 3',

and the TaqMan®- probe is :

Pram-7 : 5' ATG CTT TTT CTG CTG TGG CGG TAA 3'.

The hybridization oligonucleotide is labeled with 6-FAM at the 5' end (reporter) and TAMRA at the 3' end (quencher).

##### 4.3.2.2.1 Sample handling / DNA extraction / DNA isolation

DNA from samples are prepared as described in section 'Identification at species level by conventional PCR', Method A).

##### 4.3.2.2.2 Amplification and analysis

The reaction mixture (15 µL) should contain: 1 X Taqman Universal PCR Master Mix (Applied Biosystems part no. 4324018); 0.2 µM of each primer; 0.2 µM of probe; 5.0 µL template DNA.

Cycle test reactions should be performed in a suitable instrument, for instance ABI Prism 7700 or 7900 Sequence Detection System (Applied Biosystems) under the following conditions: 1 cycle at 95 °C for 3 min; then 35 cycles at 95°C for 15 s, 60.5 °C for 1 min. Store end products between 4 and -20 °C.

A negative control (DNase- and RNase-free water) should be included in every experiment to test for contamination as well as a positive control (DNA from a reference strain of the pathogen) to monitor the performance of the PCR. If either the negative or positive control does not give the proper result, the PCR should be repeated. To monitor for false negatives resulting from inhibition of the PCR, duplicate reactions are spiked with *P. ramorum* DNA. If the spiked reaction gives a negative result, the PCR should be repeated with diluted DNA extract.

##### 4.3.2.2.3 Assessment of PCR

Samples containing amplifiable *P. ramorum* DNA produce FAM fluorescence as recorded by Ct FAM values of < 35.

### 4.3.3 Identification at species level by sequencing part of the ITS region

The identity of *P. ramorum* isolates from new hosts or isolates that do not morphologically match published descriptions can be confirmed by sequencing. Only DNA from pure isolates can be tested using this method, otherwise sequences from multiple organisms may be amplified in the same reaction. The primer sequences are

ITS 1 : 5' TCC GTA GGT GAA CCT GCG G 3' and

ITS 4 : 5' TCC TCC GCT TAT TGA TAT GC 3' (White *et al.*, 1990).

#### 4.3.3.1 Amplification and analysis

The reaction mixture should contain: 1.0 µL DNA suspension; 10.0 µL 10 X reaction buffer containing 15 mM MgCl<sub>2</sub> (Applied Biosystems); 8.0 µL 10 mM dNTPs; 10 µL 5 µM of each primer ITS 1 and ITS 4; 0.5 µL Taq



polymerase (Applied Biosystems) (5 U/μL), and 60.5 μL sterile molecular grade water to give a final volume of 100 μL.

Amplification is performed in thin-walled PCR tubes in a PCR thermocycler programmed as follows: 2 min at 94°C; then 30 cycles of 1 min at 94°C, 1 min at 53°C, 1.5 min at 72°C. One cycle for 10 min at 72°C must be conducted after the 30 cycles. Samples are resolved on a 1.5 % agarose gel as previously described. Using this method samples containing *Phytophthora* DNA produce single amplicons of c. 900 bp in size.

#### **4.3.3.2 Sequencing of amplicons**

The remaining product from positive test reactions is purified using a suitable PCR purification kit such as QIAquick PCR purification kit (Qiagen, Crawley, GB, Cat. ref. 28106) following the manufacturers instructions. Send samples for two-way sequencing with forward primer ITS1 and reverse primer ITS4. Finally, consensus sequences are compared for test samples with those on GenBank ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). The sequence should differ by no more than 2 bases from authentic sequences those on GenBank.

## 5 Contact points for further information

James Cunningham  
Department of Primary Industries- Knoxfield  
Private Bag 15  
Ferntree Gully Delivery Centre  
Vic., 3156, Australia  
James.Cunnington@dpi.vic.gov.au

Further information can be obtained from :

Mycology Section, Plant Protection Service, P.O. Box 9102, 6700 HC Wageningen, The Netherlands (fax: 31.317.421701, tel: 31.317.496111, e-mail: g.c.m.van.leeuwen@minlnv.nl)

Central Science Laboratory, YO41 1LZ York, England, GB (fax: 44 1904 462111, tel: 44 1904 462000 , e-mail: c.lane@csl.gov.uk, or k.hughes@csl.gov.uk)

BBA, Federal Biological Research Centre for Agriculture and Forestry, Institute for Plant Protection in Horticulture, Messeweg 11/12, D- 38104, Braunschweig, DE. (fax: 49 531 299 3009, tel: 49531 299 4407, e-mail: S.Werres@bba.de)

## 6 Reference material

### Reference cultures (Not available in Australia)

Type strain of *P. ramorum*: BBA 9/95 (A1) = CBS 101553. Available from BBABraunschweig, DE (Federal Biological Research Centre for Agriculture and Forestry, Institute for Plant Protection in Horticulture, Messeweg 11/12, D- 38104, Braunschweig, DE)), or from CBS, Utrecht, the Netherlands.

For DNA repository see: <http://www.biosecuritybank.com/>

## 7 Acknowledgements

The information displayed on these Sudden Oak Death webpages was sourced from the Diagnostic protocol for detection of *Phytophthora ramorum* in Australia (Smith, I. and Cunningham, J. (2006)), and the Sudden Oak Death Pest Risk Review (2006). These documents were kindly provided by Office of the Chief Plant Protection Officer and Plant Health Australia. This diagnostic protocol was developed from existing best practise protocols currently in use in the USA and Europe and adapted for Australian conditions. It incorporates sampling, isolation and morphological and molecular identification techniques needed to maximise the probability of detection, and laboratories able to undertake these tests.

The EPPO protocol was originally drafted by G.C.M. van Leeuwen, Plant Protection Service, Wageningen (NL); C.R. Lane and K.J.D. Hughes, Central Science Laboratory, York (GB); S. Werres and S. Wagner, Federal Biological Research Centre for Agriculture and Forestry, Braunschweig (DE). It was produced with the assistance of members of the European Mycological Network who have refereed its contents.

Authors:

Ian W. Smith,  
School of Forest and Ecosystem Science  
123 Brown Street, Heidelberg, Victoria, 3084, Australia

James Cunningham  
Department of Primary Industries, Victoria

## 8 References

Websites with background information are provided below:

<http://www.suddenoakdeath.org/>

<http://www.aphis.usda.gov/ppq/ispm/sod/>

<http://cemar.ucdavis.edu/index2.html>

<http://www.oda.state.or.us/plant/ppd/path/SOD/index.html>

<http://www.cnr.berkeley.edu/garbelotto/downloads/davidson2003.pdf>

<http://www.ceris.purdue.edu/napis/pests/sod/page1.html>

<http://nature.berkeley.edu/comtf/>

APHIS (2005) List of hosts and plants associated with *Phytophthora ramorum* (Nov 2005)

<http://www.aphis.usda.gov/ppq/ispm/pramorum>

- Brasier CM, Beales, P.A., Kirk, S.A., Denman S and Rose J. (2005) *Phytophthora kernoviae* sp. nov., an invasive pathogen causing bleeding stem lesions on forest trees and foliar necrosis of ornamentals in the UK. *Mycological Research* 109, 853-859.
- Brasier CM, Denman S, Rose J, Kirk, SA, Hughes, KJD, Griffin, RL, Lane CR, Inman AJ & Webber, JF (2004) First report of ramorum bleeding canker on *Quercus falcata*, caused by *Phytophthora ramorum*. *New Disease Reports*, [www.bspp.org.uk/ndr/volume9.htm](http://www.bspp.org.uk/ndr/volume9.htm).
- Brasier C & Kirk S (2004) Production of gametangia by *Phytophthora ramorum* in vitro. *Mycological Research* 108, 823-827.
- Burgess TI, Webster JL, Ciampini JA, White D, Hardy GES, Stukely MJC (2009) Reevaluation of *Phytophthora* species isolated during 30 years of vegetation health surveys in Western Australia using molecular techniques. *Plant Disease* 93 (3): 215-223.
- COMTF (2006) March 2006 Monthly report of the California Oak Mortality Task Force.
- Davidson, J., Werres, S., Garbelotto, M., Hansen, E. M., Rizzo, D. M. (2003) Sudden Oak Death and Associated Diseases caused by *Phytophthora ramorum*. Online journal 'Plant Management Network'. [www.plantmanagementnetwork.org/pub/php/diagnosticguide/2003/SOD](http://www.plantmanagementnetwork.org/pub/php/diagnosticguide/2003/SOD)
- Gruyter J de, Baayen RP, Meffert J, Bonants PJM & van Kuik AJ (2002) Comparison of pathogenicity of *Phytophthora ramorum* isolates from Europe and California. Abstract in proceedings of "Sudden Oak Death, a Science Symposium", December 2002, Monterey, CA (US) pp. 28-29.
- Hansen E, Sutton W, Parke J & Linderman R (2002) *Phytophthora ramorum* and Oregon forest trees- One pathogen, three diseases. Abstract in proceedings of "Sudden Oak Death, a Science Symposium", December 2002, Monterey, CA (US) pp. 78.
- Hayden KJ, Rizzo D, Tse J & Garbelotto M (2004) Detection and quantification of *Phytophthora ramorum* from California forests using a real-time polymerase chain reaction assay. *Phytopathology* 94, 1075-1083.
- Hong, C. (2003) Sudden Oak Death. Virginia Cooperative Extension Publication Number 450-801. <http://www.ext.vt.edu/pubs/plantdiseases/450-801/450-801.html>
- Hughes KJD, Inman AJ & Cooke DEL (2000) Comparative testing of nested PCR-based methods with bait-plant tests for detecting *Phytophthora fragariae* var. *fragariae* in infected strawberry roots from fruit crops in the UK. *Bulletin OEPP/EPPO Bulletin* 30, 533-538.
- Hughes KJD, Tomlinson JA, Griffin RL, Boonham N, Inman AJ & Lane CR (2006) Development of a One-Step Real-Time Polymerase Chain Reaction Assay for Diagnosis of *Phytophthora ramorum*. *Phytopathology* 96: 975-981.
- Irwin JAG, Cahill DM and Drenth A (1995) *Phytophthora* in Australia. *Australian Journal of Agricultural Research*, 46 1311-1337.
- Jeffers SN & Martin SB (1986) Comparison of two media selective for *Phytophthora* and *Pythium* species. *Plant Disease* 70, 1038-1043
- Kox L, de Gruyter J, Garbelotto M, van Brouwershaven I, Admiraal J & Baayen RP (2002) Validation of a PCR method for detection and identification of *Phytophthora ramorum*. Abstract in proceedings of "Sudden Oak Death, a Science Symposium", December 2002, Monterey, CA (US) pp. 57-58.

- Kroon LPNM, Verstappen ECP, Kox LFF, Flier WG & Bonants PJM (2004). A rapid diagnostic test to distinguish between American and European populations of *Phytophthora ramorum*. *Phytopathology* 94, 613-620.
- Lane CR, Beales PA, Hughes KJD, Griffin RL, Munro D, Brasier CM, & Webber JF (2003a). First outbreak of *Phytophthora ramorum* in England, on *Viburnum tinus*. *Plant Pathology* 52, 414.
- Lane CR, Barnes AV, Beales PA, Griffin RL, Hughes KJD, Inman AJ, Townend, VC, Brasier CM & Webber JF (2003b) First report of *Phytophthora ramorum* in the UK. ICPP Paper 19.35, 8th International Congress of Plant Pathology, Christchurch, New Zealand, 2-7th February 2003. ISBN 086476 151-1.
- Man in't Veld WA, de Gruyter J & Haas AM (2002a) *Phytophthora ramorum* : een bedreiging voor inheemse bomen en struiken. *Gewasbescherming* 33, 145-149.
- Man in't Veld WA, de Gruyter J, Bonants PJM & Baayen RP (2002b) Isozyme genotyping of *Phytophthora ramorum* reveals a remarkable genetic homogeneity. Abstract in proceedings of "Sudden Oak Death, a Science Symposium", December 2002, Monterey, CA (US) pp. 58-59.
- Man in't Veld WA, Veenbaas-Rijks WJ, Ilieva E, de Cock AWAM, Bonants PJM & Pieters, R (1998) Natural hybrids of *Phytophthora nicotianae* and *Phytophthora cactorum* demonstrated by isozyme analysis and random amplified polymorphic DNA. *Phytopathology* 88, 922-929.
- Murphy. S.K., Lee. C, Valachovic. Y, Bienapfl. J, Patterson. H.A., Falk. K.F. and Rizzo. D.M. (2005) 'Monitoring *Phytophthora ramorum* Distribution in Streams within Coastal California Watersheds', Department of Plant Pathology. University of California. USA.  
<http://nature.berkeley.edu/comtf/pdf/MurphyStream%20Monitoring%20Poster.pdf>
- NPDN (2006) National Plant Diagnostic Network. Standard Operating Procedure for Plant Diagnostic Laboratories *Phytophthora ramorum*. Draft 5.5. <http://spdn.ifas.ufl.edu/Phytophthora%20ramorum-Draft%205.5.doc>.
- RAPRA (2006) Risk Analysis for *Phytophthora ramorum*.  
<http://rapra.csl.gov.uk/objectives/wp1/naturalhostresults.cfm>
- Rizzo DM, Garbelotto M, Davidson, JM, Slaughter, GW & Koike ST (2002) *Phytophthora ramorum* as the cause of extensive mortality of *Quercus* spp. and *Lithocarpus densiflorus* in California. *Plant Disease* 86, 205-214.
- Themann K & Werres S (1998) Verwendung von Rhododendronblättern zum Nachweis von *Phytophthora*-Arten in Wurzel- und Bodenproben. *Nachrichtenblatt des Deutschen Pflanzenschutzdienstes* 50, 37-45.
- Themann K & Werres S (2006) Guidelines for the handling of the Rhododendron leaf test to detect *Phytophthora* spp. in root, soil and water samples. [www.bba.de/phytoph/diagn\\_r.htm](http://www.bba.de/phytoph/diagn_r.htm)
- Themann K, Werres S, Diener H-A & Lüttmann, R (2002) Comparison of different methods to detect *Phytophthora* spp. in recycling water from nurseries. *Journal for Plant Pathology*, 84, 41-50.
- Wagner S & Werres S (2003) Diagnostmöglichkeiten für *Phytophthora ramorum*. *Nachrichtenblatt des Deutschen Pflanzenschutzdienstes* 55, 245-257.
- Weller SA, Elphinstone JG, Smith NC, Boonham N & Stead DE (2000). Detection of *Ralstonia solanacearum* strains with a quantitative, multiplex, real-time fluorescent PCR (TaqMan) assay. *Applied and Environmental Microbiology* 66, 2853-2858.
- Werres S (2002) *Phytophthora ramorum* - Erste Ergebnisse zum Wirtspflanzenkreis in Deutschland. *Deutsche Baumschule* 7, 46.
- Werres S & Kamanski K (2005). Characterisation of European and North American *Phytophthora ramorum* isolates due to their morphology and mating behaviour in vitro with heterothallic *Phytophthora* species. *Mycol. Res.* 109 (8): 860–871.
- Werres S & Marwitz R (1997). Unbekannte *Phytophthora*. *Deutscher Gartenbau* 21, 1166-1168.
- Werres S & Zielke B (2003). First studies on the pairing of *Phytophthora ramorum*. *Journal of Plant Diseases and Protection* 110, 129-130.
- Werres S, Marwitz R, Man in 't Veld WA, de Cock AWAM, Bonants PJM, De Weerd M, Themann K, Ilieva E & Baayen RP (2001) *Phytophthora ramorum* sp. nov., a new pathogen on Rhododendron and Viburnum. *Mycological Research* 105, 1155-1165.
- White TJ, Bruns T, Lee S. & Taylor J (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In : PCR Protocols, a guide to methods and applications. Eds. Innis M.A., Gelfand D.H., Sninsky J.J. & White T.J. Academic Press, London (GB). pp 315-322.



# Appendix 1

List of hosts and plants associated with *Phytophthora ramorum*, (APHIS 2006), COMTF report (September 2006), RAPRA (2006) and from personal communication with Dr. Anna Brown, DEFRA, UK\*

| Scientific Name                  | Common Name                                | Plant Family     |
|----------------------------------|--|------------------|
| <i>Abies concolor</i>            | White fir                                  | Pinaceae         |
| <i>Abies grandis</i>             | Grand fir                                  | Pinaceae         |
| <i>Abies magnifica</i>           | Red Fir                                    | Pinaceae         |
| <i>Acer circinatum</i>           | Vine Maple                                 | Aceraceae        |
| <i>Acer davidii</i>              | Striped bark maple                         | Aceraceae        |
| <i>Acer laevigatum</i>           | Evergreen Maple                            | Aceraceae        |
| <i>Acer macrophyllum</i>         | Bigleaf maple                              | Aceraceae        |
| <i>Acer pseudoplatanus</i>       | Planetree maple                            | Aceraceae        |
| <i>Adiantum aleuticum</i>        | Western maidenhair fern                    | Adiantaceae      |
| <i>Adiantum jordanii</i>         | California maidenhair fern                 | Adiantaceae      |
| <i>Aesculus californica</i>      | California buckeye                         | Hippocastanaceae |
| <i>Aesculus hippocastanum</i>    | Horse chestnut                             | Hippocastanaceae |
| <i>Arbutus menziesii</i>         | Madrone                                    | Ericaceae        |
| <i>Arbutus unedo</i>             | Strawberry tree                            | Ericaceae        |
| <i>Arctostaphylos columbiana</i> | Hairy Manzanita                            | Ericaceae        |
| <i>Arctostaphylos manzanita</i>  | Manzanita                                  | Ericaceae        |
| <i>Ardisia japonica</i>          | Ardisia                                    | Myrsinaceae      |
| <i>Calluna vulgaris</i>          | Scotch heather                             | Ericaceae        |
| <i>Calycanthus occidentalis</i>  | Spicebush                                  | Calycanthaceae   |
| <i>Camellia spp.</i>             | Camellia - all species, hybrids, cultivars | Theaceae         |
| <i>Castanea sativa</i>           | Sweet chestnut                             | Fagaceae         |
| <i>Castanopsis chryophylla</i>   | giant chinquapin,                          | Fagaceae         |
| <i>Castanopsis orthacantha</i>   | Castanopsis,                               | Fagaceae         |
| <i>Ceanothus impressus</i>       | Californian lilac                          | Rhamnaceae       |
| <i>Ceratonia siliqua</i>         | carob                                      | Leguminosae      |
| <i>Clintonia andrewsiana</i>     | Andrew's clintonia bead lily               | Liliaceae        |
| <i>Corylus cornuta</i>           | California hazelnut                        | Betulaceae       |
| <i>Drimys winteri</i>            | Winter's bark                              | Winteraceae      |
| <i>Dryopteris arguta</i>         | California wood fern                       | Moraceae         |
| <i>Eucalyptus gunnii</i>         | Cider Gum*                                 | Myrtaceae        |
| <i>Eucalyptus haemastoma</i>     | Scribbly Gum                               | Myrtaceae        |
| <i>Euonymus kiautschovicus</i>   | Spreading euonymus                         | Celastraceae     |
| <i>Fagus sylvatica</i>           | European beech                             | Fagaceae         |
| <i>Frangula californica</i>      | California coffeeberry                     | Rhamnaceae       |
| <i>Frangula purshiana</i>        | Cascara                                    | Rhamnaceae       |
| <i>Fraxinus excelsior</i>        | European ash                               | Oleaceae         |
| <i>Fraxinus latifolia</i>        | Oregon ash                                 | Oleaceae         |
| <i>Griselinia littoralis</i>     | Griselinia                                 | Griselinaceae    |

|  |  |                |
|--|--|----------------|
| <i>Gaultheria shallon</i>  | Salal, Oregon wintergreen  | Ericaceae      |
| <i>Hamamelis x intermedia</i>                                    | Hybrid witch-hazel   | Hamamelidaceae |
| <i>Hamamelis mollis</i>  | Chinese witch-hazel  | Hamamelidaceae |
| <i>Hamamelis virginiana</i>                                      | Witch hazel  | Hamamelidaceae |
| <i>Heteromeles arbutifolia</i>                                   | Toyon  | Rosaceae       |
| <i>Kalmia latifolia</i>  | Mountain laurel  | Ericaceae      |
| <i>Laurus nobilis</i>  | Bay laurel   | Lauraceae      |
| <i>Leucothoe axillaris</i>                                       | Fetterbush, dog hobble   | Ericaceae      |
| <i>Leucothoe fontanesiana</i>                                    | Drooping leucothoe   | Ericaceae      |
| <i>Lithocarpus densiflorus</i>                                   | Tanoak   | Fagaceae       |
| <i>Lonicera hispidula</i>  | California honeysuckle   | Caprifoliaceae |
| <i>Magnolia grandiflora</i>                                      | Southern magnolia  | Magnoliaceae   |
| <i>Magnolia stellata</i>   | Star magnolia  | Magnoliaceae   |
| <i>Magnolia x loebneri</i>                                       | Loebner magnolia   | Magnoliaceae   |
| <i>Magnolia x soulangeana</i>                                    | Saucer magnolia  | Magnoliaceae   |
| <i>Maianthemum racemosum</i>                                     | False Solomon's seal   | Liliaceae      |
| <i>Michelia doltsopa</i>   | Michelia   | Magnoliaceae   |
| <i>Michelia maudiae</i>  | Michelia   | Magnoliaceae   |
| <i>Michelia wilsonii</i>   | Michelia   | Magnoliaceae   |
| <i>Nothofagus obliqua</i>  | Roble beech  | Nothofagaceae  |
| <i>Osmorhiza berteroi</i>  | Sweet cicely   | Apiaceae       |
| <i>Osmorhiza decorus</i>   | Osmanthus  | Apiaceae       |
| <i>Parrotia persica</i>  | Persian ironwood   | Hamamelidaceae |
| <i>Photinia fraseri</i>  | Red tip photinia   | Rosaceae       |
| <i>Pieris floribunda</i> and <i>Pieris floribunda x japonica</i> | Mountain Andromeda and all cultivars of the hybrid with Japanese Pieris  | Pieridae       |
| <i>Pieris formosa</i> and <i>P. formosa x japonica</i>           | Himalaya Andromeda, and all cultivars of the hybrid with Japanese Pieris | Pieridae       |
| <i>Pieris japonica</i>   | Japanese Pieris  | Pieridae       |
| <i>Pittosporum undulatum</i>                                     | Sweet pittosporum, Victorian box   | Pittosporaceae |
| <i>Prunus lusitanica</i>   | Portuguese laurel cherry   | Rosaceae       |
| <i>Pseudotsuga menziesii</i>                                     | Douglas-fir  | Pinaceae       |
| <i>Pyracantha koidzumii</i>                                      | Formosa firethorn  | Rosaceae       |
| <i>Quercus agrifolia</i>   | Coast live oak   | Fagaceae       |
| <i>Quercus cerris</i>  | European turkey oak  | Fagaceae       |
| <i>Quercus chrysolepis</i>                                       | Canyon live oak  | Fagaceae       |
| <i>Quercus falcata</i>   | Southern red oak   | Fagaceae       |
| <i>Quercus ilex</i>  | Holm oak   | Fagaceae       |
| <i>Quercus kelloggii</i>   | California black oak   | Fagaceae       |
| <i>Quercus parvula</i> var. <i>shrevei</i>                       | Shreve's oak and all nursery grown <i>Q. parvula</i>                     | Fagaceae       |
| <i>Quercus petraea</i>   | Sessile oak  | Fagaceae       |
| <i>Quercus rubra</i>   | Northern red oak   | Fagaceae       |

|  |  |                |
|--|--|----------------|
| <i>Rhododendron</i> spp.                         | Rhododendrons (including azalea)                 | Ericaceae      |
| <i>Rhus diversiloba</i>                          | poison oak                                       | Anacardiaceae  |
| <i>Rosa gymnocarpa</i>                           | Wood rose  | Rosaceae       |
| <i>Rosa</i> "Meidiland"                          | Hybrid rose                                      | Rosaceae       |
| <i>Rosa rugosa</i>                               | Rugosa rose                                      | Rosaceae       |
| <i>Rubus spectabilis</i>                         | Salmonberry                                      | Rosaceae       |
| <i>Salix caprea</i>                              | Goat willow                                      | Salicaceae     |
| <i>Schima (yunnanensis)</i> spp                  | Schima   | Theaceae       |
| <i>Sequoia sempervirens</i>                      | Coast redwood                                    | Taxodiaceae    |
| <i>Syringa vulgaris</i>                          | Lilac  | Oleaceae       |
| <i>Taxus baccata</i>                             | European yew                                     | Taxaceae       |
| <i>Taxus brevifolia</i>                          | Pacific yew                                      | Taxaceae       |
| <i>Taxus x media</i>                             | Yew  | Taxaceae       |
| <i>Torreya californica</i>                       | California nutmeg                                | Taxaceae       |
| <i>Toxicodendron diversilobum</i>                | Poison oak                                       | Anacardiaceae  |
| <i>Trientalis latifolia</i>                      | Western starflower                               | Primulaceae    |
| <i>Umbellularia californica</i>                  | California bay laurel, pepperwood, Oregon myrtle | Lauraceae      |
| <i>Vaccinium ovatum</i>                          | Evergreen huckleberry                            | Ericaceae      |
| <i>Vancouveria planipetala</i>                   | Redwood ivy                                      | Berberidaceae  |
| <i>Viburnum bodnantense</i>                      | Arrowwood  | Caprifoliaceae |
| <i>Viburnum davidii</i>                          | David Viburnum                                   | Caprifoliaceae |
| <i>Viburnum farreri</i> (= <i>V. fragrans</i> )  | Fragrant Viburnum                                | Caprifoliaceae |
| <i>Viburnum lantana</i>                          | Wayfaringtree Viburnum                           | Caprifoliaceae |
| <i>Viburnum opulus</i> (= <i>V. trilobum</i> )   | European & American cranberrybush                | Caprifoliaceae |
| <i>Viburnum plicatum</i>                         | Doublefile Viburnum                              | Caprifoliaceae |
| <i>Viburnum tinus</i>                            | Laurustinus                                      | Caprifoliaceae |
| <i>Viburnum x bodnantense</i>                    | Bodnant Viburnum                                 | Caprifoliaceae |
| <i>Viburnum x burkwoodii</i>                     | Burkwood Viburnum                                | Caprifoliaceae |
| <i>Viburnum x carlcephalum</i> x <i>V. utile</i> | Viburnum   | Caprifoliaceae |
| <i>Viburnum x pragense</i>                       | Prague Viburnum                                  | Caprifoliaceae |
| <i>Viburnum x rhytidophylloides</i>              | Viburnum   | Caprifoliaceae |
| <i>Viburnum tinus</i>                            | Alleghany or Willowood Viburnum                  | Caprifoliaceae |

**Confirmed Host:** naturally infected plants upon which Koch's postulates have been confirmed.

**Other:** Associated plants are those reported found naturally infected and from which *P. ramorum* has been cultured and/or detected using PCR (Polymerase Chain Reaction). For each of these, traditional Koch's postulates have not yet been completed or documented and reviewed.

## Appendix 2

Potential host susceptibility based on leaf tests using mini-inoculators on wounded leaves. From Inman *et al.*: [www.defra.gov.uk/plant/ramorum/meeting/europe.pdf](http://www.defra.gov.uk/plant/ramorum/meeting/europe.pdf)

| Susceptibility category   | Plant species   | Plant Family   |
|---|---|----------------|
| <b>HIGHLY SUSCEPTIBLE</b><br>Mean lesion index 3.50-4.00<br>Leaf lesions extensive (c. >25 mm diameter in 7 days)         | <i>Sambucus racemosa</i> (ornamental red-berried elder) | Caprifoliaceae |
|   | <i>Sambucus nigra</i> (common elder)                    | Caprifoliaceae |
|   | <i>Rhododendron ponticum</i> (wild UK rhododendron)**   | Ericaceae      |
|   | <i>Rhododendron</i> (control cultivar) **               | Ericaceae      |
|   | <i>Leucothoe</i> sp. (**, unconfirmed)                  | Ericaceae      |
|   | <i>Pieris japonica</i> **                               | Ericaceae      |
|   | <i>Eucalyptus</i> sp.                                   | Myrtaceae      |
|   | <i>Syringa</i> (lilac)                                  | Oleaceae       |
|   | <i>Camellia japonica</i>                                | Theaceae       |
| <b>MODERATELY SUSCEPTIBLE</b><br>Mean lesion index 2.50-3.49<br>Leaf lesions well developed (c. 15-24 mm diam. in 7 days) | <i>Symphoricarpos albus</i> (snowberry)                 | Caprifoliaceae |
|   | <i>Viburnum tinus</i> **                                | Caprifoliaceae |
|   | <i>Arctostaphylos uva-ursi</i> (common bearberry) **    | Ericaceae      |
|   | <i>Fraxinus</i> † (ash)                                 | Oleaceae       |
|   | <i>Fuchsia</i>  | Onagraceae     |
|   | <i>Photinia</i> (Christmas berry)                       | Rosaceae       |
|   | <i>Tilia</i> (lime) †                                   | Tiliaceae      |
|   | <i>Ulmus glabra</i> (wych elm)                          | Ulmaceae       |
|   | <i>Ulmus</i> (ornamental yellow-leaved Scottish elm)    | Ulmaceae       |
|   |   |                |
| <b>SLIGHTLY SUSCEPTIBLE</b><br>Mean lesion index 1.50-2.49<br>Lesions extension slow (c. 10-14 mm diam. in 7 days)        | <i>Acer pseudoplatanus</i> (sycamore) † **              | Aceraceae      |
|   | <i>Viburnum davidii</i> **                              | Caprifoliaceae |
|   | <i>Rhododendron</i> (Azalea l) **                       | Ericaceae      |
|   | <i>Gaultheria x Wisleyensis</i>                         | Ericaceae      |
|   | <i>Malus</i> (crab apple)                               | Rosaceae       |
|   | <i>Prunus laurocerasus</i> (cherry laurel)              | Rosaceae       |
|   | <i>Prunus lusitanica</i> (Portugal laurel)              | Rosaceae       |
|   | <i>Prunus persica</i> (nectarine)                       | Rosaceae       |
|   | <i>Prunus</i> sp. (ornamental cherry)                   | Rosaceae       |



|   |   |                            |
|---|---|----------------------------|
| <b>RESISTANT</b><br>Mean lesion index 0.50-1.49<br>Lesions not extending much beyond wound                  | <i>Acer campestre</i> (field or hedge maple) **           | Aceraceae                  |
|   | <i>Ilex aquifolium</i> (Holly)                            | Aquifoliaceae              |
|   | <i>Alnus</i> (alder)                                      | Betulaceae                 |
|   | <i>Lonicera</i> ( <i>periclymenus</i> ?) (honeysuckle) ** | Caprifoliaceae             |
|   | <i>Viburnum opulus</i> (guelder rose)†**                  | Caprifoliaceae             |
|   | <i>Cornus alba</i>  | Cornaceae                  |
|   | <i>Corylus avellana</i> (hazel)                           | Betulaceae<br>(Corylaceae) |
|   | <i>Rhododendron japonicum</i> (azalea II)**               | Ericaceae                  |
|   | <i>Erica cinerea</i> (native heather)                     | Ericaceae                  |
|   | <i>Rhododendron simmsii</i> **                            | Ericaceae                  |
|   | <i>Vaccinium corymbosum</i> (blueberry) **                | Ericaceae                  |
|   | <i>Ligustrum</i> (hedge privet)                           | Oleaceae                   |
|   | <i>Clematis montana</i>                                   | Ranunculaceae              |
|   | <i>Crataegus monogyna</i> (hawthorn)                      | Rosaceae                   |
| <b>VIRTUALLY IMMUNE</b><br>Mean lesion index 0.00-0.49<br>No necrosis, or necrosis only in the wounded area | <i>Humulus lupulus</i> (golden hop)                       | Cannabidaceae              |
|   | <i>Weigela japonica</i>                                   | Caprifoliaceae             |
|   | <i>Aucuba japonica</i>                                    | Cornaceae                  |
|   | <i>Calluna</i> sp. (heath)                                | Ericaceae                  |
|   | <i>Rhododendron</i> (Azalea III) **                       | Ericaceae                  |
|   | <i>Laurus nobilis</i> (English bay laurel)                | Lauraceae                  |
|   | <i>Buddleja davidii</i>                                   | Loganiaceae                |
|   | <i>Lavatera</i> (tree mallow)                             | Malvaceae                  |
|   | <i>Morus</i> (mulberry)                                   | Moraceae                   |
|   | <i>Forsythia</i>  | Oleaceae                   |
|   | <i>Rubus fruticosus</i> aggr. (bramble)                   | Rosaceae                   |
|   | <i>Spiraea japonica</i>                                   | Rosaceae                   |
|   | <i>Choisya ternata</i> (Mexican orange blossom)           | Rutaceae                   |
|   | <i>Skimmia japonica</i>                                   | Rutaceae                   |
|   | <i>Vitis vinifera</i> (grapevine)                         | Vitaceae                   |

†Being repeated. \*\* Genus recorded as natural host. (A. Inman\*, P. Beales\*, C. Lane\* & C. Brasier, Pathogenicity of European & American Isolates of *Phytophthora ramorum* to Ornamental, Hedgerow and Woodland Under-storey Plants in the UK)

## Appendix 3

### Media for culturing *Phytophthora ramorum*

**Vegetable juice agar (V8):** vegetable juice 250 mL; CaCO<sub>3</sub> 5 g; agar 15 g; distilled water 1000 mL. Add CaCO<sub>3</sub> to the vegetable juice and stir firmly during 15 min. Centrifuge the mixture for 20 min at 5000 rpm, and pour off the supernatant. Make up the resultant to 1 L with distilled water, and autoclave at 120°C for 20 min.

**P<sub>5</sub>ARP[H]** (Jeffers & Martin, 1986): cornmeal agar 17 g; distilled water 1000 mL. Autoclave, then cool to 50°C in a water bath. Then prepare pimarinic acid 5 mg; ampicillin (Na salt) 250 mg; rifampicin (dissolved in 1 mL 95% Ethanol) 10 mg; PCNB 100 mg; hymexazol 22.5 mg and dissolve all in 10 mL sterile distilled water. Add to cooled media, pour, store at 4°C in the dark, use within 5 days.

**P<sub>5</sub>ARP** If hymexazol is unavailable, then PARP is still very useful.

**PARB [H]** (Robin *et al.*, 1998): cornmeal agar 17 g; distilled water 1000 mL. Autoclave, then cool to 50°C in a water bath. Then prepare pimarinic acid 10 mg; ampicillin 250 mg; rifampicin 10 mg; benomyl 15 mg; hymexazol 50 mg.

**Carrot Piece Agar** (Werres *et al.*, 2001): agar 22 g, carrot pieces 50 g, distilled water 1000 mL.

**Carrot Juice Agar 5%** (Kröber, 1985): agar 15-22 g; carrot juice (without honey) 50 mL; distilled water 950 mL.

**CSL Dark Carrot Agar (DCA):** carrots 200 g; agar Oxoid No.3 15 g; distilled water 1000 mL.

Slice the fresh carrots and comminute in a blender with 500 mLs of distilled water for 1 min at high speed. Filter through four layers of cheesecloth and squeeze out the juice from the residue. Make up the resultant filtrate to 1 L and add the agar. Heat to dissolve the agar, pour into bottles and autoclave at 121°C for 15 min.

**Cherry decoction Agar (CHA):** agar 60 g; distilled water 3600 mL; cherry juice 400 mL. Filter the cherry juice, and adjust the pH to 4.4 with KOH. Dissolve the agar thoroughly first, then add cherry juice. Autoclave at 102°C for 5 min. Snyder and Nash Agar (SNA): KH<sub>2</sub>PO<sub>4</sub> 1 g; KNO<sub>3</sub> 1 g; MgSO<sub>4</sub>·7H<sub>2</sub>O 0.5 g; KCl 0.5 g; glucose 0.2 g; saccharose 0.2 g; agar 15 g; distilled water 1000 mL. Autoclave for 15 min at 120°C.

## Appendix 4

### Disinfection techniques for aerial plant parts

#### Sodium hypochlorite dipping and rinsing

Plant material is disinfected superficially with a tissue soaked in a 1% sodium hypochlorite solution. Excise small pieces of material (0.5-1 cm<sup>2</sup> each) from the leading edge then wash in tap water (10 s), ethanol 50 % (10 s) and then finally tap water (10 s).

#### Alcohol swabbing

Quickly wipe the surface of selected plant tissue with cotton wool impregnated with 70% ethanol, then excise small pieces of material and transfer aseptically to selective medium. For stems, remove the bark prior to plating out.

#### Rinsing in water

Either select appropriate plant parts, place in a large plastic bag, add about 20-30 mL of distilled water and allow to soak for several minutes, agitate the sample within the bag for 10 seconds, drain off the water, repeat washing, then remove the plant material for isolation (wash water should be treated as for contaminated waste, e.g. autoclaving); or place in approximately 0.5 % active sodium hypochlorite solution for 2-5 min in a laminar flow cabinet, wash in sterile distilled water twice, dry carefully (on filter paper), then transfer aseptically to one of the media listed below in 'Media and incubation'. For twigs : cut out at least three pieces per twig, one from the dark brown area, one from the edge of the lesion and one from the healthy looking tissue just under the lesion. For leaves: cut out little pieces (circa 0.5 x 0.5 cm) from the edge of a necrosis or spot.

#### Stem base material/roots, or heavily contaminated samples

##### *i) Flushing with water*

Excise suitable pieces of tissue, place in a 250 mL side arm conical flask and over the neck place a porous cover (e.g. muslin, fine wire mesh, Parafilm with small holes pierced in it). Connect the flask to the tap and flush with water for at least 2 hours. Wash water should be sterilised, e.g. by autoclaving. Following surface decontamination aseptically transfer at least four pieces of tissue to an appropriate medium.

##### *ii) Alcohol treatment*

Cut out small pieces from the cambium area and stick them (in the field) directly on carrot piece agar or selective medium. Or cut out large pieces (minimum 10 x 10 cm) to send to the laboratory. In the laboratory, dip these pieces into 98 % alcohol let them dry and cut out little pieces to stick into agar medium.