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[1]Draft Annex to ISPM 27 – *Phytophthora ramorum* (2004-013)

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[21]**Discipline leads history**	[22]Hans DE GRUYTER (NL, Discipline Lead) [23]Robert TAYLOR (NZ, Referee)
[24]**Consultation on technical level**	[25]The first draft of this diagnostic protocol was prepared by: · [26]K.J.D. Hughes (Fera, UK) · [27]M.E. Palm (USDA-APHIS-PPQ Molecular Diagnostic Lab, US) · [28]S.C. Brière (Canadian Food Inspection Agency, CA). [29]It was adapted from the EPPO diagnostic protocol on *P. ramorum,* which was originally drafted by G.C.M. van Leeuwen (Plant Protection Service, NL), C.R. Lane and K.J.D. Hughes (Fera, UK), and S. Werres and S. Wagner (Federal Biological Research Centre for Agriculture and Forestry, DE). A. Schlenzig (Science and Advice for Scottish Agriculture, UK) reviewed the protocol. [30]This current draft is being updated by Patricia Giltrap and Jennifer Tomlinson (Fera, UK), S. C. Briere (Canadian Food Inspection Agency, CA) and Gloria Abad (USDA-APHIS-PPQ-Center of Plant Health Science and Technology, USA). Lynn Laurenson (Fera, UK) has assisted with reviewing molecular comments. [31]In addition, the draft has been subject to expert review and the following experts submitted comments: - [32]Jacqueline Edwards (Department of Economic Development, Jobs, Transport and Resources (Victorian Government), AU) - [33]Nathalie Schenck (Anses, Unit of Mycology of the Plant Health Laboratory (French National Reference Laboratory), FR).
[34]**Main discussion points during development of the diagnostic protocol** [35]**[to be updated throughout DP development]**	- [36]Consider nested PCR to be excluded because of higher risk of false positives - [37]Lateral flow device only for detection, not for identification - [38]State clearly that this is a selection of tests available by the drafting team i.e. most widely used - [39]More information on sensitivity and selectivity and the choice for the tests included to be presented - [40]Consider to include other methods which are more specific, such as loos *et al.*, 2006.
[41]**Notes**	[42]2015-11 Edited [43]This is a draft document.

[44]**Contents [to be added later]**

[45]**Adoption**

[46]This diagnostic protocol was adopted by the Commission on Phytosanitary Measures in 20--.

[47]The annex is a prescriptive part of ISPM 27 (*Diagnostic protocols for regulated pests*).

[48]1. Pest Information

[49]*Phytophthora ramorum* Werres, de Cock & Man in't Veld (Werres *et al*., 2001) is an oomycete pathogen of unknown origin (Brasier *et al.*, 2004). It is considered to have been introduced into western North America and western Europe in the late twentieth century by the ornamental plant trade (Prospero *et al.*, 2007; Mascheretti *et al*., 2008; Goss *et al*., 2011; Grünwald *et al.*, 2012; Van Poucke *et al.*, 2012). *P. ramorum* attacks a wide range of trees and shrubs in nurseries and in the field, causing leaf blight, stem cankers, bleeding stem lesions and dieback.

[50]In North America the pathogen was found in the early 1990s causing mortality of *Quercus* (oak) trees and *Lithocarpus densiflorus* (tanoaks), mainly in California and Oregon (Rizzo *et al*., 2002. Named "Sudden Oak Death" (SOD), the disease has reached epidemic proportions in North America at present. The pathogen was originally considered a woodland disease but since 2003 nursery plants in several states of the United States have been affected. The disease has also been found in Canada.

[51]In Europe *P. ramorum* has been observed in Germany since 1993 causing twig blight of rhododendron in nurseries and on mature bushes in gardens. In the Netherlands it was found in 1998 on diseased *Viburnum* sp. (Werres and Marwitz, 1997; Werres *et al.*, 2001). The pathogen has now been recorded in more than 20 European countries, predominantly on ornamental plants in nurseries and in a few managed gardens. In 2009, however, *P. ramorum* was unexpectedly found infecting and killing large numbers of *Larix kaempferi* (Japanese larch) trees in south-west England. Heavy dieback and mortality of plantation *L. kaempferi* trees in western Britain and Northern Ireland have resulted in the felling of 0.6 million trees (Brasier and Webber, 2010; Webber *et al.*, 2010).

[52]This unexpected finding emphasizes that although many of its hosts are known, the main threat of *P. ramorum* is to tree species and other ecologically important plants such as heathland species. The pathogen is, however, most commonly observed on *Camellia*, *Magnolia*, *Pieris*, *Quercus* (in particular *Q. acuta*, *Q. agrifolia*, *Q. cerris*, *Q. chrysolepis*, *Q. ilex* and *Q. rubra* (red oak) species), *Rhododendron* and *Viburnum.* Recent findings and lists of the known hosts for *P. ramorum* can be found in CABI (n.d.), COMTF (n.d.), Fera (2014a, n.d.) and USDA-APHIS (2014a). Disease symptoms and host plants are listed and regularly updated on websites (COMTF, n.d.; Fera, 2014a).

[53]*P. ramorum* has a complex life cycle and is adapted to cool temperatures, with 20 ºC being optimal. Although *P. ramorum* is soil-borne, deciduous, asexually produced sporangia are formed on the surface of infected leaves or twigs and, depending on environmental conditions, are locally splash-dispersed or spread over long distances by wind and wind-driven rain (Davidson *et al.*, 2005). Rivers, streams and other waterways can also carry the sporangia and thus spread the pathogen (Defra, 2007). Sporangia that land on suitable hosts germinate to produce hyphae. In the presence of water, sporangia will release motile zoospores that encyst on the host surface, germinate and penetrate the host tissue, forming a colony from which more sporangia are produced. These sporangia repeat the cycle and with enough repetitions, under the right environmental conditions, an epidemic can ensue. Different asexual spores, chlamydospores, are produced in abundance within infected plant tissue and allow *P. ramorum* to survive adverse conditions in infected stems and leaves on the plant, in plant debris on the soil surface, or in the soil (Grünwald *et. al*., 2012).

[54]*P. ramorum* is a heterothallic species and may produce sexual oospores, but this requires both mating types. No evidence exists that natural crossing of these mating types has occurred in nature although crossing has been achieved in the laboratory (Brasier and Kirk, 2004). Currently, mating type A1 is the predominant type in Europe while A2 is the predominant type in North America

(Werres and Kaminski, 2005). There are four clonal lineages known, with the first three designated as: NA1 (mating type: A2; distribution: North America; environment: forest and nurseries); NA2 (mating type: A2; distribution: North America; environment: nurseries); and EU1 (mating type: predominantly A1, rarely A2; distribution: Europe and North America; environment: nurseries and gardens) (Grünwald *et al.*, 2009). The fourth, a new lineage designated as EU2, was discovered recently in Northern Ireland and western Scotland and is associated with four host plants, including *L. kaempferi* (Van Poucke *et al.*, 2012).

[55]2. Taxonomic Information

[56]**Name:** *Phytophthora ramorum* Werres, de Cock & Man in 't Veld, 2001

[57]**Synonyms**: None

[58]**Taxonomic position:** Chromista, Oomycota, Oomycetes, Pythiales, Pythiaceae

[59]**Common names:** Sudden oak death (SOD), ramorum leaf blight, ramorum shoot dieback and sudden larch death

[60]**Reference:** Mycobank MB474485

[61]3. Detection

[62]Laboratory studies have shown that the time between foliage infection and visible disease expression is typically between 3 and 14 days, depending on host and temperature. However, the period may be longer in the field and on different plant parts (Defra, 2007). Leaves selected at random can be checked for surface contamination or latent infection by baiting or molecular methods (section 3.6). The use of fungicides can make it more difficult to detect infected plant material by culture (Hamelin *et al.*, 2000; Shishkoff, 2014). Fungicides may suppress symptom development as well as the viability of the pathogen, which may lead to false negative test results.

[63]This diagnostic protocol describes well-established methods for the detection and identification of *P. ramorum.* It is not a comprehensive review of all methods available for the diagnosis of *P. ramorum*. Detection of *P. ramorum* can be achieved using the serological, biological and molecular methods shown in Figure 1. Serological methods are often used first as a screening test, for the presence of *Phytophthora* spp but may yield false negative or false positive results (Kox *et al.*, 2007). When a *Phytophthora* species has been detected by a serological method, the identity of the species must be confirmed by isolation and morphological identification or polymerase chain reaction (PCR) methods. PCR methods can also be used for detection but the presence of some other *Phytophthora* species may lead to false positive results. Therefore, detection by serological methods can be confirmed by PCR, but the latter must be followed by sequencing or by isolation for morphological identification. If identification of *P. ramorum* represents the first finding for a country, the laboratory may wish to have the diagnosis confirmed by another laboratory.

[64]3.1 Symptoms

[65]Several disease syndromes caused by *P. ramorum* have been described. The symptoms within each syndrome can vary widely depending on the host. The most commonly observed host symptoms are described below and are illustrated in Figures 2 to 6. Additional disease symptoms can be found on several websites (USDA-APHIS, 2009; COMTF, n.d.; EPPO, 2014; Fera, n.d.).

[66]3.1.1 Tree dieback

[67]Despite the name sudden oak death, which is the most common name used for tree dieback caused by *P. ramorum* (McPherson *et al.*, 2001), the following symptoms can be observed on many tree species and can take several years to completely kill mature trees. Typically, symptoms include lethal cankers around the lower trunks of infected trees from which dark red to black sap may ooze (called bleeding cankers or tarry spots) (Figure 2). Removing the outer bark under and around oozing

areas often reveals dead and discoloured inner bark with a black zone line around the edge of the necrosis. The foliage of infected trees may die prematurely, with leaves remaining on the branches after death. Trees that show these symptoms may suddenly completely die. It should be noted that these symptoms are not restricted to an infection caused by *P. ramorum*; they may also be hastened by other plant pathogens (including other *Phytophthora* species) or be associated with non-pathogenic disorders or insect pests.

[68]3.1.2 Shoot dieback

[69]On *Rhododendron* spp. diseased twigs often have brown to black lesions that usually begin at the tip and move towards the base (Figure 3). Mid-stem lesions can also be found. The cambial tissue of diseased twigs is often discoloured. Shoots and stems may have cankers near ground level, resulting in rapid wilting of shoots and causing the leaves, which remain attached, to hang down (Figure 4). Infection on *Viburnum* spp. usually occurs at the base of the stem causing plants to wilt and collapse very quickly (Figure 5). Brown necrosis can often be seen spreading into stems and twigs and leaf spots may also be observed (Figure 6). Infection on *Pieris* spp. tends to cause petiole blackening, leading to stem cankers and aerial dieback.

[70]3.1.3 Leaf blight

[71]On *Rhododendron*, *Camellia*, *Kalmia* and *Pieris* species black–brown lesions occur on leaves, usually at the tip but often at the petiole end. Disease develops across infected leaves often following the midrib, eventually leading to premature leaf fall. On *Magnolia* spp. multiple small spots can also be observed, eventually merging into larger necrotic areas.

[72]3.1.4 Symptoms on Coniferae

[73]*P. ramorum* causes needle blight and dieback of young shoots of the conifers *Pseudotsuga menziesii* (Douglas fir), *Sequoia sempervirens* (coastal redwood) and *Abies grandis* (grand fir). Typical symptoms observed on larches are needle infections, shoot dieback, and branch and trunk cankers. Infected shoot tips wither and wilt and infected needles appear blackened. Early needle abscission of infected needles also occurs.

[74]3.2 Sampling and sample preparation

[75]Different techniques for sampling and sample preparation as described below are recommended depending on the material being tested. Samples should be kept cool and sent to the diagnostic laboratory in strong closed plastic bags or containers, or double-bagged for next day isolation, as prolonged transit times or raised temperatures can reduce the likelihood of successful isolation and detection. Placing a small amount of damp tissue with the plant material will reduce sample desiccation and may increase the chance of isolation. However, in sealed self-closing plastic bags, excessive moisture can hasten tissue degradation and saprophytic activity. Storage at 4 °C is highly recommended to prolong sample life but storage for longer than seven days reduces the ease of isolation.

[76]3.2.1 Plant material

[77]When sampling bleeding cankers from trees, the outer bark around the canker should be removed to reveal the inner bark and the margin of necrosis. Pieces of phloem and xylem can then be excised from across the leading edge and sent for testing. Symptomatic shoots and twig samples approximately 15 cm long, spanning the leading edge of an infection, should be taken while for leaves, several, showing a range of typical symptoms, should be taken.

[78]Non-symptomatic plants can be sampled by taking leaves at random following statistical norms. The leaves sampled are bagged together and submitted for testing.

[79]3.2.2 Water

[80]Water samples should be at least 1 litre in volume and be taken from the surface of the area being tested, preferably where the water is flowing and is not below 4 °C or deeper than 15 cm. The water samples should be kept cool (5–20 °C) during storage and transport and tested within 48 h of collection. Water bait bags sometimes called "bobs" (muslin bags containing leaves for baiting) are an alternative, very effective method of on-site testing for water (Defra, 2007; USDA-APHIS, 2014b). They consist of cut leaves of rhododendron (*Rhododendron. catawbiense* "Grandiflorum", *R* "Cunningham's White" or *R. ponticum*) in muslin bags containing polystyrene to aid flotation. They have been used extensively in field situations to check water sources, including streams and irrigation ponds, for *P. ramorum* (Defra, 2007). Bait bags are best deployed where the water is flowing, however slow, rather than still. Bait bags can be used when the water to be tested is above 4 °C (Defra, 2007).

[81]3.2.3 Soil or plant debris

[82]About 500 g of soil or plant debris should be taken from the sampling sites. This should be placed in a sealed container or bag. Alternatively, cut rhododendron leaves in bait bags (section 3.2.2) (without the polystyrene) can be buried in the soil or the plant debris for later collection, provided it will remain moist.

[83]3.3 Detection by serological methods

[84]Serological methods may be used only to pre-screen samples for the presence of *Phytophthora* spp. A low level of false negative and false positive results may occur (Kox *et al.*, 2007). Different formats are available, including lateral flow devices (Forsite Diagnostics[1]) and ImmunoStrip Tests (Agdia[2]), which are both suitable for field use, primarily to screen out negative samples. Larger format enzyme-linked immunosorbent assays (ELISA) are also available (from Neogen[3], Lexington[4] or Agdia[5]), and are more suitable for laboratory use.

[90]3.4 Isolation and culture from symptomatic or asymptomatic material

[91]3.4.1Isolation from plant samples

[92]Symptomatic samples can be washed with water to remove loose surface contamination. At least four 1 cm^2 pieces should then be excised from the leading edge of infection on each sample and plated on one of the semi-selective isolation media described in section 3.5.

[93]Where no semi-selective medium is used, surface sterilization is recommended. The 1 cm^2 pieces are dipped in an aqueous solution of bleach (1% active sodium hypochlorite) for 2–5 min depending on the thickness of the material (e.g. if they are leaves or stems) or 70% ethanol for 30 s, then rinsed in sterile distilled water and dried. The stem sections are split lengthwise before plating to aid culture growth.

[94]Non-symptomatic plants may be tested by baiting (section 3.4.2).

[95]3.4.2Isolation from water samples

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[85][1] In this diagnostic protocol, methods (including reference to brand names) are described as published, as these defined the original level of sensitivity, specificity and/or reproducibility achieved. The use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

[86][2] See footnote 1.

[87][3] See footnote 1.

[88][4] See footnote 1.

[89][5] See footnote 1.

[96]In the laboratory, water samples are placed in a 1 litre sterilised container with a large surface area (such as a Ziploc[6] 946 ml square disposable plastic box wiped with 50% ethanol and dried before use). To promote infection from zoospores, a sterilized metal screen or cheese cloth may be used in the box to keep floating debris from touching the leaf baits. At least four 1 cm^2 pieces of healthy rhododendron leaf that has not been treated with fungicide are placed on the water surface. Alternatively, small young rhododendron leaves that have been cut several times on the leaf margin with a sterile scalpel can be used. *Rhododendron* "Cunningham's White" is recommended because it is highly susceptible to *P. ramorum*; however, many other rhododendron species are as susceptible (De Dobbelaere *et al.*, 2010).

[98]The box is sealed and incubated on the laboratory bench at room temperature (18–25°C). Within three to seven days symptoms of *P. ramorum* infection usually develop if the pathogen is present; however, the lack of symptoms is not conclusive that *P. ramorum* is absent. The bait leaves should be plated as described in section 3.5 or used directly for DNA extraction. Alternatively, whole or partial leaf baits can be slipped under the selective media with the aid of a sterile spatula to help discourage bacterial contamination and allow the suspect *Phytophthora* to grow through the media. It can then be excised from the surface and transferred to a non-selective medium. Where bait bags have been used, the rhododendron leaves are retrieved after three to seven days, and washed and plated (section 3.4.1) or used directly for DNA extraction.

[99]Baiting with rhododendron can detect *P. ramorum* at sporangial concentrations of 1 to 40 000 per litre of water (Defra, 2007). Other baiting substrates have been described, such as *Pyrus communis* (pear fruit) (Themann *et al.*, 2002), but rhododendron leaves have been used most commonly, work very well and are easy to handle.

[100]Baiting is not specific to *P. ramorum* and may pick up other *Phytophthora* species, as well as *Pythium* species. Using selective media when plating out helps reduce the growth of other fungi, making morphological identification of *P. ramorum* easier.

[101]3.4.3 Isolation from soil or plant debris samples

[102]Approximately 250 g soil to be tested is placed in a large plastic box, covered with about 500 ml Petri's mineral solution (1 litre distilled water with $CaNO_3$ 0.4 g; $MgSO_4.7H_2O$ 0.15 g; KH_2PO_4 0.15 g; and KCl 0.06 g), and cut rhododendron leaves are placed as baits on the surface of the solution, as described in section 3.4.2. Plant debris can be treated in the same manner. The box is incubated for three to seven days then the sample is checked for the presence of *P. ramorum* by plating or molecular methods (section 3.6). Where bait bags have been used, these are treated as for water samples.

[103]3.5 Isolation media

[104]For isolation, P5ARP(H) (pimaricin, ampicillin, rifampicin, pentachloronitrobenzene, hymexazol) culture medium (Jeffers and Martin, 1986) is recommended, as this is semi-selective for *Phytophthora* spp. and on it, characteristic features of *P. ramorum* are readily observed. Hymexazol is included in this medium to suppress *Pythium* spp. and can be particularly useful when working with soil and water. Hymexazol has been shown to slow the growth of certain *Phytophthora* spp., including *P. ramorum*; however, adding up to 25 mg/litre hymexazol has been shown to have minimal effects on *P. ramorum* (Murphy *et al.*, 2007).

[105]P5ARP(H) medium is made by adding 17 g cornmeal agar to 1 litre distilled water, stirring thoroughly, then autoclaving at 121 °C for 15 min before cooling to 50 °C in a water bath (EPPO, 2012, validation 2009-03-31–F16_S08). Additions, where necessary, are prepared by suspending them in 10 ml sterile distilled water or dissolving them in ethanol before adding to the medium. For 1 litre P5ARP(H) medium, 5 mg pimaricin, 250 mg ampicillin (sodium salt), 10 mg rifampicin

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[97][6] See footnote 1.

(dissolved in 1 ml of 95% ethanol), 100 mg pentachloronitrobenzene and 75 mg (final concentration: 22.5 parts per million (p.p.m.)) hymexazol (30% active substance) are added to the cooled (50 °C) medium, stirred thoroughly and poured onto plates. The plates should be stored at 4 °C in the dark (rifampicin is inactivated by light) and used before five to seven days have elapsed since they were made (Jeffers and Martin, 1986).

[106]The final concentration of hymexazol should be considered when making any amended medium. When isolating the pathogen from leaves or woody tissue, hymexazol can be considered optional. Another selective medium including hymexazol and similar bactericides is PARP-V8 (Fergusson and Jeffers, 1999).

[107]Other media that can be used include cherry decoction agar (CHA). Cherry juice is made by boiling 1 kg cherries, free of stones and petioles, in 1 litre tap water for approximately 2 h. The juice is filtered through cheesecloth, poured into bottles, sterilized at 110 °C for 30 min, adjusted to pH 4.5 with 1 N KOH or 1 N HCl, and stored until use. In a bottle containing 0.8 litre distilled water, 20 g Technical Agar No. 3 is added and the mixture is sterilized at 121 °C for 15 min. Immediately after sterilization, 0.2 litre sterilized cherry extract is added, mixed well and sterilized at 102 °C for 5 min (Gams *et al*., 1998).

[108]The plant material to be tested should be decontaminated as described in section 3.4.1, then at least four small pieces (about 2 cm^2) of tissue from each sample should be aseptically transferred onto either of the semi-selective media. As much of each section as practically possible should be slid under the media to force any *Phytophthora* present to grow through the media. A maximum of ten leaf sections should be placed on each plate. Leaf sections from different sites (e.g. nurseries) or different subsamples (hosts or locations) within a site should be placed on different plates. Sporangia are formed more readily on unsealed plates (T. Giltrap, personal communication, 2014). The plates are incubated in daylight or in the dark (the dark favours chlamydospore production) between 18 and 25°C, and examined for *Phytophthora* growth after three to seven days. Samples plated onto media containing rifampicin should be incubated in the dark because rifampicin is inactivated by light. Growth should occur within ten days but morphological features can be seen after three days in some cases.

[109]For extended culturing, isolates should be transferred to carrot piece agar, made by first finely grating 50 g carrots. Twenty-two grams of Technical Agar No. 3 is dissolved in 1 litre water in a 2 litre beaker, and stirred thoroughly before adding the grated carrots and stirring again. When the contents are thoroughly mixed the beaker is covered with foil and placed into a steamer for 1 h. Before removing from the steamer, thorough stirring of the medium is recommended. The medium is then transferred to bottles, ensuring that the carrot pieces are divided equally between them. The bottles are autoclaved at 121 °C for 15 min before the medium is poured onto plates, which are stored at room temperature (Gams *et al*., 1998).

[110]It should be noted that *P. ramorum* isolation from woody tissue is difficult and can lead to false negative results. Therefore, for woody tissue, more than one method of detection is advisable. Isolation is as for soil or plant debris (section 3.4.3) – covering the woody material in Petri's mineral solution and using cut rhododendron leaves as bait, which are then plated or tested by molecular methods.

[111]Molecular tests can be used directly on plant material. The methods for these tests are described in section 3.6.

[112]3.6 Detection by molecular methods

[113]Molecular tests have been developed to identify *P. ramorum* from culture or *in planta* using conventional or real-time PCR. Many of these methods were compared by Kox *et al*. (2007) and Martin *et al.* (2009). Four methods are described below and were selected because of the experience obtained by laboratories with them and the availability of validation data. However, other PCR

methods can be used. PCR-based methods will detect non-viable *P. ramorum* in infected plant material, which would not be detected by isolation and culture (Bilodeau *et al.*, 2007). Real-time PCR may be preferred for high throughput, routine testing as the closed-tube format reduces the risk of carrying over contamination due to processing of amplification products (e.g. for nested PCR or gel electrophoresis).

[114]3.6.1 Preparation of material

[115]DNA for PCR can be extracted from plant material or from cultures using various commercially available kits and other methods. When testing symptomatic plant material it may be beneficial to sample from the leading edge of the lesion. Depending on the sample matrix (leaves or stems or soil), different methods may be used for homogenization or disruption of the tissue. Plant tissue (from leaves) or mycelium (from cultures) may be disrupted using a tissue pulverizer or bead beater. Pre-freezing in liquid nitrogen can be beneficial for disruption. Various grinding methods can be used, providing they produce a homogenously ground sample; for example, mortar and pestle with liquid nitrogen (for leaves and cut stems), bead mills, or the Homex grinder (Bioreba[7]) (for cultures and tough woody tissue).

[117]3.6.2 DNA extraction

[118]DNA extraction can be performed using commercial kits (e.g. the NucleoSpin Plant II Extraction Kit (Macherey-Nagel[8]) or the DNeasy Plant Mini Kit (Qiagen[9]) following the manufacturers' instructions. For DNA extraction from cultured isolates, the same kits can be used. DNA should be stored at –20 °C until use. Refer to the source papers in the following sections for the extraction methods originally used; however, laboratories may find that alternative extraction techniques work equally well.

[121]3.6.3 Conventional PCR

[122]There are several conventional PCR methods described in the literature. Two of these are described below.

[123]3.6.3.1 Conventional PCR of Kox *et al.* (2002)

[124]The primers Phyto 1 (forward) and Phyto 4 (reverse) from the internal transcribed spacer (ITS) ribosomal (r)DNA were developed by M. Garbelotto (Hayden *et al.*, 2004) and used for the detection of *P. ramorum* by conventional PCR (Kox *et al.*, 2007) as described below and in Table 1.

[125]Phyto 1: 5′-CATGGCGAGCGCTTGA-3′

[126]Phyto 4: 5′-GAAGCCGCCAACACAAG-3′

[127]**Table 1.** Master mix composition, cycling parameters and amplicons for conventional PCR with primers Phyto 1 and Phyto 4

[128]Reagent	[129]Final concentration	[130]Master mix for 25 µl final reaction volume (µl)
[131]PCR-grade water	[132]–	[133]17.70
[134]10× PCR buffer	[135]1×	[136]2.50
[137]$MgCl_2$	[138]1.5 mM	[139]0.75
[140]dNTPs	[141]200 µM	[142]0.50
[143]Primer Phyto 1	[144]0.2 µM	[145]0.50

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[116][7] See footnote 1.

[119][8] See footnote 1.

[120][9] See footnote 1.

[146]Primer Phyto 4	[147]0.2 µM	[148]0.50
[149]DNA polymerase	[150]0.5 U	[151]0.05
[152]DNA (quantity/volume)	[153]5 µl	[154]2.50
[155]**Cycling parameters**		
[156]Initial denaturation	[157]95 °C for 15 min	
[158]Number of cycles	[159]35	
[160]Denaturation	[161]94 °C for 15 s	
[162]Annealing	[163]62 °C for 1 min	
[164]Elongation	[165]72 °C for 45 s	
	[166]Repeat 35	
[167]Final elongation	[168]72 °C for 10 min	
	[169]4 °C pause	
[170]**Expected amplicons**		
[171]Size	[172]687 base pairs	

[173]3.6.3.2 Conventional PCR of Ioos *et al.* (2006)

[174]This PCR is based on the amplification of DNA from intronic regions using two pairs of specific primers: GPA-PRAM-F (forward) and GPA-PRAM-R (reverse) from intron GPA1, and TRP-PRAM-F (forward) and TRP-PRAM-R (reverse) from intron TRP1. The primers TRP-PRAM-F/R can be used for detection and GPAPRAM-F/R for confirmation, and have been fully validated and characterized (Ioos *et al.*, 2006). They are listed below, and the details for the PCR are in Table 2.

[175]GPA-PRAM-F: 5′-TAAGGAACAAGGTACCAAAG-3′

[176]GPA-PRAM-R: 5′-CTCAGGAATTCACTCTCACG-3′

[177]TRP-PRAM-F: 5′-GAGTAGAAACTTCGGGAATG-3′

[178]TRP-PRAM-R: 5′-GTTCGGCACATTAACGCAG-3′

[179]**Table 2.** Master mix composition, cycling parameters and amplicons for conventional PCR with primers GPA-PRAM-F/GPA-PRAM-R and TRP-PRAM-F/TRP-PRAM-R

[180]**Reagent**	[181]**Final concentration**	[182]**Master mix for 25 µl final reaction volume (µl)**
[183]PCR-grade water	[184]–	[185]13.20
[186]10× PCR buffer	[187]1×	[188]2.50
[189]$MgCl_2$	[190]2 mM	[191]1.00
[192]dNTPs	[193]200 µM	[194]0.30
[195]Bovine serum albumin	[196]0.60 µg/µl	[197]2.25
[198]Primer GPA-PRAM-F or TRP-PRAM-F	[199]0.45 µM	[200]2.25
[201]Primer GPA-PRAM-R or TRP-PRAM-R	[202]0.45 µM	[203]0.50
[204]DNA polymerase	[205]0.5 U	[206]1.00
[207]DNA (quantity/volume)	[208]2 µl (30–80 ng)	[209]2.00
[210]**Cycling parameters[1]**		
[211]Initial denaturation	[212]95 °C for 3 min	

[213]Number of cycles	[214]35
[215]Denaturation	[216]94 °C for 30 s
[217]Annealing	[218]58 °C for 30 s
[219]Elongation	[220]72 °C for 1 min
[221]Final elongation	[222]72 °C for 7 min
[223]**Expected amplicons**	
[224]Size	[225]527 base pairs (bp) for TRP primers
	[226]248 bp for GPA primers

[227][1] The maximum temperature ramping rate should be used between steps.

[228]3.6.4 TaqMan real-time PCR

[229]There are several real-time PCR methods described in the literature. Two of these are described below.

[230]3.6.4.1 Real-time PCR of Hughes *et al.* (2006) targeting *P. ramorum*

[231]The primers and probe described by Hughes *et al*. (2006) target the ITS-1 region of the nuclear ribosomal (nr) RNA gene. Primer and probe sets have been developed that target other genes such as for cytochrome oxidase subunit I (COXI) (Tooley *et al*., 2006), beta-tubulin and elicitin (Bilodeau *et al*., 2007) and *Ypt*1 (Schena *et al*., 2008).

[232]Hughes *et al*. (2006) reported a limit of detection of 10 pg genomic DNA, and no cross-reactivity with 29 species of non-target *Phytophthora*, with the exception of *P. lateralis*, which was detected at or above concentrations of approximately 10 ng per 25 µl reaction. For a full list of species used for the assessment of specificity, see Hughes *et al*. (2006).

[233]The primers and probe are listed below, and the details for the PCR are in Table 3.

[234]Pram 114-FC: 5′-TCA TGG CGA GCG CTG GA-3′

[235]Pram 190R: 5′-AGT ATA TTC AGT ATT TAG GAA TGG GTT TAA AAA GT-3′

[236]Probe Pram 134-T: 6-FAM 5′-TTC GGG TCT GAG CTA GTA G-3′ TAMRA

[237]**Table 3.** Master mix composition, cycling parameters and amplicons for real-time PCR with primers Pram 114-FC/Pram 190R and probe Pram 134-T

[238]**Reagent**	[239]**Final concentration**	[240]**Master mix for 25 µl final reaction volume (µl)**
[241]PCR-grade water	[242]–	[243]12.20
[244]10× PCR buffer	[245]1×	[246]2.50
[247]$MgCl_2$ (50 mM)	[248]6.0 mM	[249]3.00
[250]dNTPs (10 mM each)	[251]240 µM	[252]0.60
[253]Primer Pram 114-FC (2 µM)	[254]120 nM	[255]1.50
[256]Primer Pram 190R (2 µM)	[257]120 nM	[258]1.50
[259]Probe Pram 134-T	[260]120 nM	[261]1.50
[262]DNA polymerase	[263]5 U/µl	[264]0.20
[265]DNA (quantity/volume)	[266]1 (20–100 ng)	[267]2.00
[268]**Cycling parameters**		
[269]Initial denaturation	[270]50 °C for 2 min	

	[271]95 °C for 10 min
[272]Number of cycles	[273]40
[274]Denaturation	[275]95 °C for 15 s
[276]Annealing	[277]–
[278]Elongation	[279]60 °C for 1 min
[280]Final elongation	[281]–
[282]**Expected amplicons**	
[283]Size	[284]Range of cycles detection for positive samples 13–36 cycle threshold

[285]Real-time PCR carried out by Hughes *et al.* (2006) used TaqMan Universal PCR Master Mix (Applied Biosytems[10]). The Real-time PCR was carried out in 96 well plates in 25 µl reactions containing 1 x Master Mix, 300 nM each primer, 100 nM probe and 1µl sample or control DNA. Reactions were subjected to generic thermal cycling conditions (10 min at 95 °C and 40 cycles of 15 s at 95 °C and 1 min at 60 °C) on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems[11]), using real-time data collection. The cycle threshold (Ct) value was assessed using a default threshold setting of 0.2 ΔRn (fluorescence units).

[288]Under the Hughes *et al*. (2006) conditions, samples with Ct values less than 36 may be considered positive for *P. ramorum*. Ct values between 36 and 40 may be a result of aerosol contamination or cross-reaction with non-target DNA at high concentrations (e.g. *P. foliorum* or *P. lateralis*, see section 4). Samples giving these results should be re-sampled or re-tested and if the result is still in doubt, the presence of *P. ramorum* confirmed by another means. Samples with Ct values of 40 or more are considered negative.

[289]3.6.4.2 Real-time PCR of Hughes *et al.* (2006) targeting *P. ramorum* and host

[290]Hughes *et al*. (2006) carried out real-time PCR for *P. ramorum* in parallel with an internal control assay for detection of DNA (the COXI gene) from the host plant (section 3.6.4). This test can be used with the same reaction conditions as described in section 3.6.4.1 or in a duplex reaction with the *P. ramorum* primers and probe listed below, with final concentrations of 240 nM COX-F (forward primer), 240 nM COX-RW (reverse primer) and 120 nM COX-P (probe). It is recommended to run the *P. ramorum* suspect samples as both undiluted and diluted 1:10. See Table 4 for the PCR details.

[291]COX-F: 5′-CGTCGCATTCCAGATTATCCA-3′

[292]COX-RW: 5′-CAACTACGGATATATAAGRRCCRRAACTG-3′

[293]Probe COX-P: VIC 5′-AGGGCATTCCATCCAGCGTAAGCA-3′ TAMRA

[294]**Table 4.** Master mix composition, cycling parameters and amplicons for real-time PCR with primers Pram 114-FC/Pram 190R and probe Pram 134-T and primers COX-F/COX-RW and probe COX-P

[295]**Reagent**	[296]**Final concentration**	[297]**Master mix for 25 µl final reaction volume (µl)**
[298]PCR-grade water	[299]–	[300]7.70
[301]10× PCR buffer	[302]1×	[303]2.50
[304]$MgCl_2$ (50 mM)	[305]6.0 mM	[306]3.00
[307]dNTPs (10 mM each)	[308]240 µM	[309]0.60

[]

[286][10] See footnote 1.

[287][11] See footnote 1.

[310]Primer Pram 114-FC (2 µM)	[311]240 nM	[312]1.50
[313]Primer Pram 190 R (2 µM)	[314]240 nM	[315]1.50
[316]Probe Pram 134-T	[317]120 nM	[318]1.50
[319]Primers COX-F and COX-RW (2 µM mix)	[320]240 nM	[321]3.00
[322]Probe COX-P (2 µM)	[323]120 nM	[324]1.50

[325]Platinum Taq (5 U/µl) (Invitrogen[12])	[327]1 U	[328]0.20
[329]DNA (quantity/volume)	[330]1 (20–100 ng)	[331]2.00 µl
[332]**Cycling parameters (Cepheid SmartCycler[13] II settings)**		
[335]Initial denaturation	[336]95 ºC for 20 s	
	[337]Optics OFF	
[338]Number of cycles	[339]46	
[340]Denaturation	[341]95 °C for 1 s	
	[342]Optics OFF	
[343]Annealing	[344]–	
[345]Elongation	[346]58 °C for 40 s	
	[347]Optics ON	
[348]Final elongation	[349]–	
[350]**Expected amplicons**		
[351]Size	[352]Range of cycles detection for positive samples 13–36 cycle threshold	

[353]Note: This protocol has been validated at the USDA-APHIS-CPHST Beltsville Laboratory, United States, for Cepheid SmartCycler II (Z.G. Abad, personal communication, 2015).

[354]3.6.5 Controls for molecular tests

[355]For the test result obtained to be considered reliable, appropriate controls – which will depend on the type of test used and the level of certainty required – should be considered for each series of nucleic acid isolation and amplification of the target pest or target nucleic acid. For PCR a positive nucleic acid control and a negative amplification control (no template control) are the minimum controls that should be used. The use of an internal control assay for the detection of host plant DNA, to be used in multiplex with the pathogen-specific assay or in parallel singleplex reactions, or in parallel tests for conventional and real-time PCR, can assist in the interpretation of P. ramorum-negative results. The use of an internal control plant is highly recommended to confirm the quality of the extracted DNA, especially for the test described in section 3.6.4.2.

[356]*Positive nucleic acid control.* This control is used to monitor the efficiency of the test method (apart from the extraction). Pre-prepared (stored) genomic DNA, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product) may be used. A good positive control for *P. ramorum* is DNA extracted from a host plant (e.g. *Rhododendron*) infected with *P. ramorum* with $20.00 \leq$ FAM Ct ≤ 23 and $20.00 \leq$ TxR Ct (COX) ≤ 25. These are suggested Ct values (Z.G. Abad, personal communication, 2015).

[]

[326][12] See footnote 1.
[333][13] See footnote 1.

[334]

[357]*Negative amplification control (no template control).* This control is necessary for conventional and real-time PCR to rule out false positives due to contamination during preparation of the reaction mixture. PCR-grade water that was used to prepare the reaction mixture is added at the amplification stage.

[358]*Internal control.* For testing plant samples by real-time PCR, plant internal controls (e.g. COXI as used by Hughes *et al*. (2006)) should be incorporated into the protocol to eliminate the possibility of PCR false negatives due to extraction failure, nucleic acid degradation or the presence of PCR inhibitors.

[359]The internal control primers can be used in a multiplex reaction with the pathogen-specific primers or they can be used in parallel singleplex reactions. Performing the reactions in singleplex may help to avoid a reduction in the sensitivity of detection of *P. ramorum*. Laboratories may choose to establish a cut-off Ct value to be used to identify samples for which extraction or amplification has not failed but was suboptimal (which could lead to false negative results). The appropriate cut-off may need to be determined for each sample type (host, tissue, etc.). Samples with failed internal controls should be plated onto selective media to try to derive a culture for DNA extraction and subsequent PCR.

[360]Alternative internal controls may be used. For example, Hayden *et al*. (2006) describe a universal primer and probe set targeting a conserved region of the small subunit of the rDNA gene to detect any eukaryote.

[361]3.6.5.1 Additional controls

[362]*Positive extraction control.* This control is used to ensure that target nucleic acid extracted is of sufficient quantity and quality for PCR and that the target is detected. Nucleic acid is extracted from infected host tissue or healthy plant tissue that has been spiked with the target can be used if suitable infected material is not available. It is recommended that plant samples be tested at a minimum of two concentrations – out of undiluted, 1:5 and 1:10 – to overcome effects caused by inhibitors, which can occur with many of the host plant species that infect *P. ramorum*.

[363]*Negative extraction control.* This control is used to monitor contamination during nucleic acid extraction and/or cross-reaction with the host tissue. The control comprises nucleic acid that is extracted from uninfected host tissue and subsequently amplified. Multiple controls are recommended to be included when large numbers of positives are expected.

[364]Alternatively, extraction blanks can be processed with the samples to be tested if sufficient uninfected host tissue is not available. This will allow contamination of extraction reagents and/or cross-contamination between samples to be identified (but will not confirm the efficacy of nucleic acid extraction).

[365]4. Identification

[366]*P. ramorum* may be identified either by its growth characteristics and morphology in culture or by sequence analysis.

[367]Possible confusion in morphology and cultural characteristics is most likely to occur with *P. palmivora* while *P. lateralis* and *P. foliorum* may give a cross-reaction in a PCR test (section 4.3).

[368]A flow diagram for the diagnosis of *P. ramorum* on symptomatic plant material is given in Figure 1. A positive diagnosis can be based on morphology; however, experience with the identification of *Phytophthora* species is required. Further PCR or sequencing is recommended.

[369]A very low percentage of cross reactivity has been observed with ITS real-time PCR when *P. foliorum* or *P. hibernalis* are present in very high concentration. The Ct values are usually over 36 Ct and for those cases morphological or sequencing studies of pure cultures are needed for a final identification.

[370]4.1 Morphological identification

[371]4.1.1 Cultural characteristics and morphology

[372]The growth characteristics and morphological features of *P. ramorum* on agar, described in Werres *et al*. (2001), can be affected by the type of agar, substrate or host plant (T. Giltrap, personal communication, 2014). Colonies on carrot piece agar, PARP-V8 agar and cornmeal agar are submerged, showing pronounced (PARP-V8 agar) or weak (carrot piece and cornmeal agar) concentric rings. On CHA, colonies have an appressed aerial mycelium with weak rosette-like patterns. Sporangia are ellipsoid, elongate-ovoid, caducous, often with short a pedicel, semipapillate, hyaline, 45.6–65 × 21–28.3 µm, single but in clusters; chlamydospores are numerous, thin-walled, globose, hyaline to pale brown, mostly 46–60 µm, terminal or intercalary. Generally, characteristic chlamydospores allow accurate identification of *P. ramorum* in culture. Possible confusion in morphology and cultural characteristics is most likely to occur with *P. palmivora*. The key characteristics are illustrated in Figures 7, 8, 9 and 10. The most essential features formed on examples of selective and non-selective media are given in Table 5.

[373]If no sporangia are produced on agar, sporulation can be encouraged by cutting 6 mm plugs from four day old colonies and placing these in a sterile Petri dish, mycelium side up, along with enough sterile tap water or Petri's mineral solution to be level with the top of the plugs but not covering the mycelium. Unsterile pond water or soil extract water can be used provided contamination with *P. ramorum* has been ruled out. The dishes are placed in the dark at 18 °C or cooler for 24–48 h. This should encourage sporangia to form on the edge of the plugs. Clusters of *P. ramorum* sporangia may be seen also in the water, having broken away from the agar plug.

[374]**Table 5.** Growth characteristics of *Phytophthora ramorum* on selective and non-selective media

[375]**Characteristic**	[376]**P5ARP(H)[1] (selective)**	[377]**Carrot piece agar[1, 2] (non-selective)**
[378]Colonies	[379]Relatively slow growing, approximately 2 mm per day	[380]Weak rosette-like pattern, pronounced concentric rings, growth rate approximately 3 mm per day
[381]Mycelia	[382]Weakly coralloid, growing within the agar with little superficial growth, no hyphal swellings. Superficial, fluffy growth may be observed when growing out of plant material and coralloid appearance can differ according to the host the mycelium is growing out of[3].	[383]Aerial mycelium sparse, no hyphal swellings
[384]Sporangia	[385]Produced abundantly on the agar surface, semipapillate, caducous with short (5 µm) or no stalk. Size: 20–32 × 40–80 µm, average 24 × 52 µm; average length/width ratio: 2.16.	
	[387]Ellipsoid, frequently in small clusters and relatively narrow, initial sporangium commonly producing secondary, smaller sporangia. When growing out of plant material, can appear papillate when about to germinate. Sporangia with constrictions (central or at pedicel end) have been observed[3], particularly when growing out of plant material.	[388]Ellipsoid, spindle-shaped or elongated ovoid, single or in clusters
[389]Chlamydospores	[390]More common in older colonies (seven to ten days) unless growing out of plant material. Very large (up to 80 µm diameter), hyaline to pale brown to brown. Hyphal swellings present.	[391]After three days' incubation in the dark, in the older parts but very often also in the young parts of the colony. Up to 88 µm diameter, thin-walled, hyaline to pale brown.

[392]Source: Werres *et al*. (2001).

[393][1] On P5ARP(H) characteristics can be observed after four to six days' incubation in daylight at 20 °C, 12 h light/12 h dark. On carrot piece agar, characteristics can be observed after three to five days incubation at 20 °C in darkness.

[394][2] Sexual structures can be observed on carrot piece agar after pairing with an opposite mating type; for example, *Phytophthora cryptogea* (Werres and Kaminski, 2005). A *P. ramorum* × *P. ramorum* pairing is also possible *in vitro* (not with all isolates) (Brasier and Kirk, 2004) and in rhododendron twigs (Werres and Zielke, 2003).

[395][3] T. Giltrap, personal communication, 2014.

[396]A positive morphological identification would be recorded if caducous, semipapillate sporangia in the correct size range and shape with short pedicels (5 µm) were observed along with the characteristic chlamydospores.

[397]4.2 Biological identification

[398]4.2.1 Pathogenicity tests

[399]Koch's postulates have to be performed if *P. ramorum* has been found on a new host species.

[400]4.3 Molecular identification

[401]The following tests are recommended for identification of species, including *P. ramorum*, from clean cultures. The conventional PCR and real-time PCR methods described in section 3.6 for *in planta* detection of *P. ramorum* are species-specific and are used for detection of the pathogen in infected material or in cultures. Molecular diagnostic tests detect DNA, not the viable organism, and cross-reaction with closely related species, including *P. lateralis*, *P. hibernalis* and *P. foliorum*, is possible at high DNA concentrations. In addition, environmental samples (infected samples) that have very low titre can yield negative results, so care should be taken in the interpretation of results when testing DNA extracts from cultures, which may be at a higher concentration than extracts from plant material. ITS sequencing as described in section 4.3.1 may be used for species level identification.

[402]4.3.1 ITS sequencing for species level identification using the primers of White *et al.* (1990)

[403]The identity of *P. ramorum* isolated in culture can be confirmed by sequencing the ITS-1 and ITS-2 regions of the nrRNA gene as described below and in Table 6. These primers can be used to generate amplification products for sequencing from all species of *Phytophthora*.

[404]ITS-5 F: 5′-GGA AGT AAA AGT CGT AAC AAG G-3′

[405]ITS-4 R: 5′-TCC TCC GCT TAT TGA TAT GC-3′

[406]**Table 6.** Master mix composition, cycling parameters and amplicons for conventional PCR with primers ITS-5 F and ITS-4 R

[407]Reagent	[408]Final concentration	[409]Master mix for 25 µl final reaction volume (µl)
[410]PCR-grade water	[411]–	[412]14.50
[413]10× PCR buffer	[414]1×	[415]2.50
[416]$MgCl_2$ (50 mM)	[417]1.5 mM	[418]1.25
[419]dNTPs (10 mM each)	[420]200 µM	[421]0.50
[422]Primer ITS-5 F (10 µM)	[423]0.2 µM	[424]2.50
[425]Primer ITS-4 R (10 µM)	[426]0.2 µM	[427]2.50
[428]DNA polymerase	[429]0.5 U	[430]0.25
[431]DNA (quantity/volume)	[432]1 (500-50 pg)	[433]1.00

[434]**Cycling parameters**	
[435]Initial denaturation	[436]95 °C for 1 min 25 s
[437]Number of cycles	[438]34
[439]Denaturation	[440]92 °C for 35 s
[441]Annealing	[442]62 °C for 55 s
[443]Elongation	[444]72 °C for 50 s
[445]Final elongation	[446]72 °C for 10 min
	[447]4 °C pause
[448]**Expected amplicons**	
[449]Size	[450]800-900 base pairs

[451]Amplification products may be visualized by agarose gel electrophoresis: a single amplicon of 800–900 base pairs (bp) is produced by DNA from *Phytophthora* spp. The remaining amplification product can be purified using a suitable PCR purification kit following the manufacturer's instructions and the purified amplicon can be two-way sequenced with ITS-5 F (forward) and ITS-4 R (reverse) primers. The quality of the resulting sequence should be checked by visual assessment of the electropherograms. Consensus sequences may be built from the forward and reverse reads and compared with published sequences using the Basic Local Alignment Search Tool (BLAST) (National Center for Biotechnology Information (NCBI), United States; http://www.ncbi.nlm.nih.gov/). In order to make a correct identification of the generated sequences to *Phytophthora* species level, use of the GenBank accession number that corresponds to the ex-type of *P. ramorum* P10103 (WPC) NCBI is recommended, which is FJ801269.

[452]The following steps are suggested for processing sequences by BLAST http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&BLAST_SPEC=blast2seq&LINK_LOC=align2seq Select "Align two or more sequences using BLAST" (under Specialized BLAST)

[453]Paste the FASTA sequence in the first box

[454]Paste the accession number (FJ801269) in the second box

[455]Select "Highly similar sequences (megablast)"

[456]Click in BLAST.

[457]In the absence of a 100% or 99% match to *P. ramorum*, phylogenetic trees may be compiled to assess intraspecific and interspecific variation in order to make the identification.

[458]4.3.2 Controls for molecular tests

[459]The required controls are a negative amplification control and a positive nucleic acid control for the PCR. See section 3.6.5 for more details on controls for molecular tests.

[460]5. Records

[461]Records and evidence should be retained as described in section 2.5 of ISPM 27 (*Diagnostic protocols for regulated pests*).

[462]Cultures of *P. ramorum* can be stored on carrot piece or oatmeal agar slopes at room temperature or in sterile distilled water at 5 ºC. DNA can be stored at –80 °C.

[463]6. Contact Points for Further Information

[464]Further information on this protocol can be obtained from:

[465]Fera Science Ltd. (Fera), Sand Hutton, York YO41 1LZ, United Kingdom (Ann Barnes; email ann.barnes@fera.co.uk; tel.: +44 (0) 1904 462494 or Jennifer Tomlinson; e-mail: jenny.tomlinson@fera.co.uk; tel.: +44 (0) 1904 462000 extension 3207).

[466]A request for a revision to a diagnostic protocol may be submitted by national plant protection organizations (NPPOs), regional plant protection organizations (RPPOs) or Commission on Phytosanitary Measures (CPM) subsidiary bodies through the IPPC Secretariat (ippc@fao.org), which will in turn forward it to the Technical Panel on Diagnostic Protocols (TPDP).

[467]7. Acknowledgements

[468]The first draft of this protocol was written by K.J.D Hughes (Fera, United Kingdom), M. E. Palm, (USDA-APHIS-PPQ-Molecular Diagnostic Lab, United States) and S. C. Brière (Canadian Food Inspection Agency, Canada). It was adapted from the European and Mediterranean Plant Protection Organization (EPPO) diagnostic protocol on *P. ramorum*, which was drafted by G.C.M. van Leeuwen (Plant Protection Service, Netherlands), C.R. Lane and K.J.D. Hughes (Fera, United Kingdom), and S. Werres and S. Wagner (Federal Biological Research Centre for Agriculture and Forestry, Germany). Alexandra Schlenzig (Science and Advice for Scottish Agriculture, United Kingdom) reviewed the first draft of this present diagnostic protocol.

[469]The current draft of this protocol has been updated by P. Giltrap and J. Tomlinson (Fera Science Ltd, United Kingdom), S. C. Brière (Canadian Food Inspection Agency, Canada) and Z.G. Abad (USDA-APHIS-PPQ-Center of Plant Health Science and Technology, United States).

[470]Photographs of symptoms were taken at Fera by P. Beales and D. Crossley, and at USDA-APHIS-CPHST Beltsville Laboratory by J. Bienapfl. In vitro photographs were taken at USDA-APHIS-CPHST Beltsville Laboratory by Z.G. Abad.

[471]Lynn Laurenson (Fera Science Ltd., United Kingdom) has assisted with reviewing molecular comments.

[472]8. References

[473]The present annex refers to ISPMs. ISPMs are available on the International Phytosanitary Portal (IPP) at https://www.ippc.int/core-activities/standards-setting/ispms.

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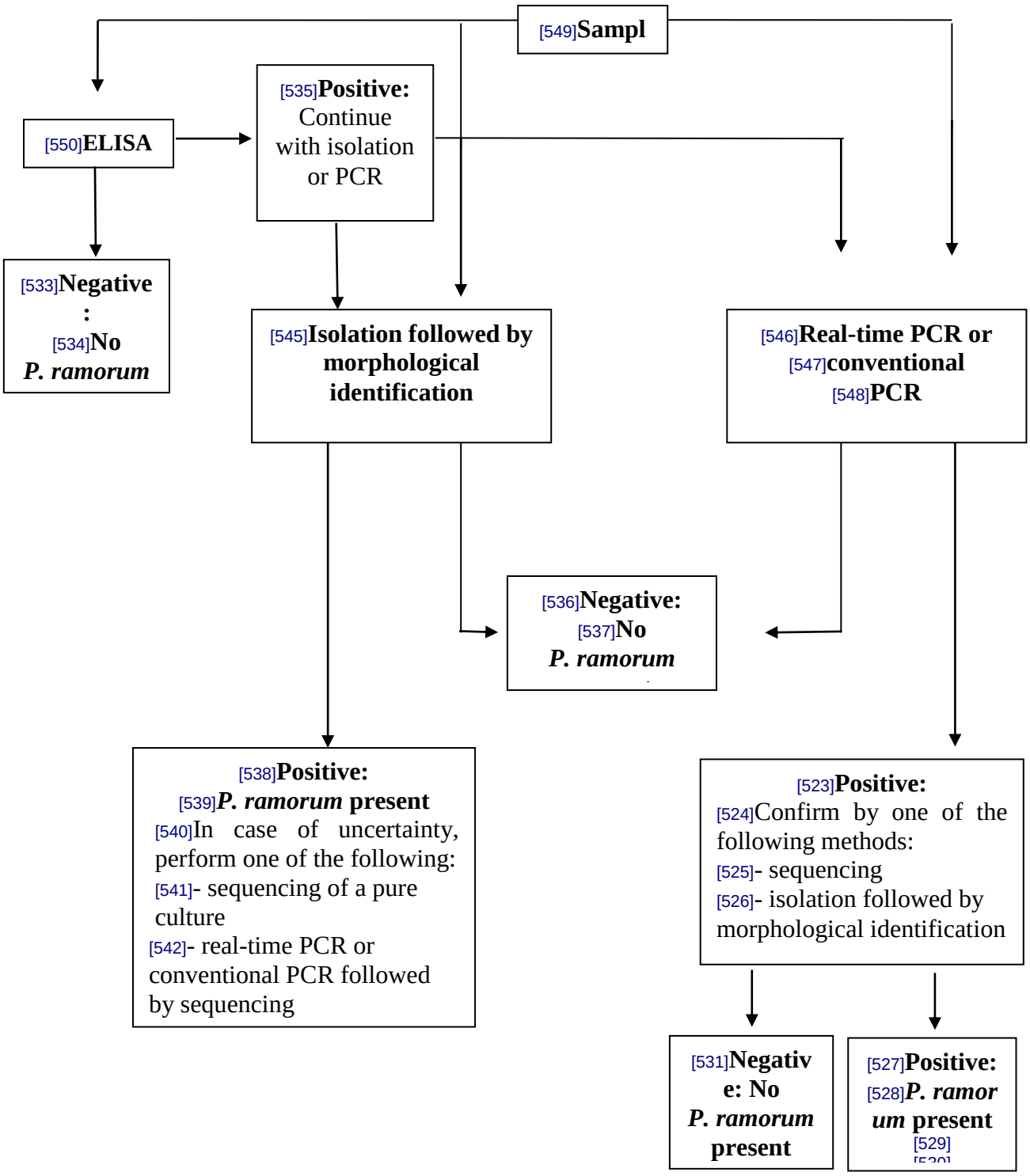
[521]

9. Figures



[522]**Figure 1.** Flow diagram for the diagnosis of *Phytophthora ramorum* on symptomatic plant material.
[551]ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction.

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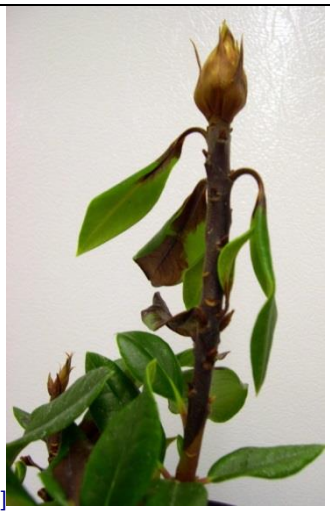

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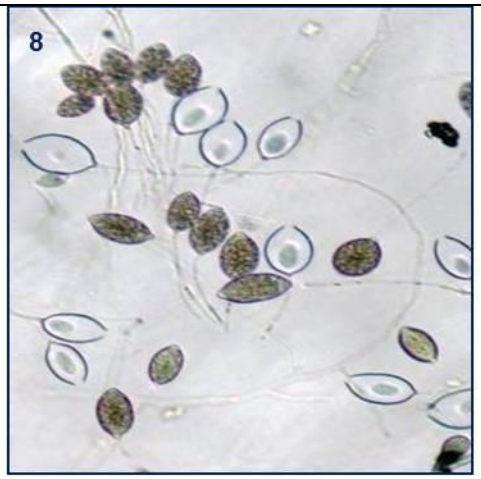
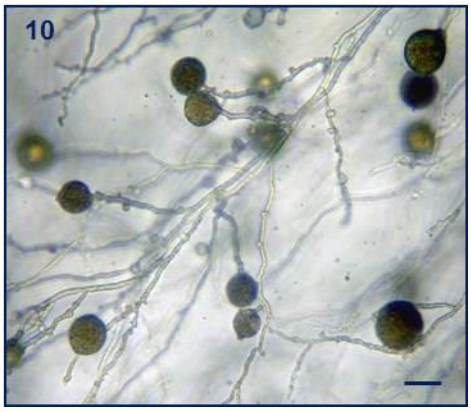
[573]**6B**

[574]**Figures 2–6.** *Phytophthora ramorum* symptoms on different hosts: **2** *Quercus*, bleeding canker; **3** *Rhododendron*, shoot dieback; **4** *Rhododendron*, shoot tip wilt; **5** *Viburnum*, stem base discoloration; **6**(A) *Rhododendron*, leaf blight; and **6**(B) *Camellia*, leaf blight.





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[576]**Figures 7-10.** Typical morphological features of the asexual phase of *Phytophthora ramorum* on P5ARP (H) isolation medium (section 3.5): **7** coralloid mycelium and sporangia; **8** sporangia attached to sporangiophores; **9** sporangium semipapillated, caducous, with short pedicel (scale bar: 10 µm); and **10** characteristic chlamydospores (scale bar: 30 µm). (Photos by Z. G. Abad, USDA-APHIS-CPHST Beltsville Laboratory).