

## Identification and Distribution

(Causal Agent, Identification, Hosts, Distribution, Signs & Symptoms, Diagnostics)

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### The Causal Agent

The pathogen *Phytophthora ramorum* Werres, de Cock & Man in't Veld was formally described by Werres and others in 2001. The previously undescribed *Phytophthora* had been observed since 1993 in Germany and the Netherlands associated with diseased rhododendron (*Rhododendron*) and since 1998 on diseased Viburnum (*Viburnum* sp.). Pathogenicity to rhododendrons was demonstrated in 1997 (Werres and Marwitz 1997).

In culture, *P. ramorum* is characterized by the production of large, abundant chlamydospores and elongated, ellipsoidal, deciduous sporangia. Hyaline chlamydospores are produced on hyphal tips, becoming brown with age and when produced on host tissue (Rizzo and others 2002b; Werres and others 2001). Chlamydospores are variously reported to range in size from 40 to 80  $\mu\text{m}$  (Rizzo and others 2002b) and 20 to 91  $\mu\text{m}$  (Werres and others 2001). The sporangia have a mean length of 43.6  $\mu\text{m}$  (20 to 79  $\mu\text{m}$ ) and a mean width of 23.9  $\mu\text{m}$  (12 to 40  $\mu\text{m}$ ) (Werres and Kaminski 2005). Oogonia with amphigynous antheridia are produced by pairings with *P. cryptogea* and other heterothallic *Phytophthora* species representing opposite mating type A1 or A2 (Werres and others 2001, Werres and Kaminski 2005).

The morphology and ITS sequence of California and Oregon isolates are identical to European isolates (Rizzo and others 2002b). The heterothallic pathogen produces two mating types, A1 and A2. The two mating types represented genetically distinct populations (Kroon and others 2004). During initial surveys, only the A2 was found in North America (Rizzo and others 2002a) and only the A1 was found in Europe (Werres and others 2001). However, a few A1 isolates of *P. ramorum* have since been reported from nurseries in North America (Oregon, Washington, and British Columbia; Hansen and others 2003) and isolates were reported on nursery stock from Belgium in Europe (Werres and De Merlier 2003) that were A2 mating type, but otherwise typical of the European population. Recently, the A1 mating type (European population) was reported in a retail nursery in Humboldt County California in March 2007 (unpublished data, CDFA). A draft website (<http://oregonstate.edu/~grunwaln/index.htm>) hosted by Oregon State University provides information on multilocus microsatellite genotypes of *P. ramorum* currently found in North America (Grünwald, personal communication).

A third lineage of *P. ramorum*, distinct from the A1 and A2 lines previously known from European gardens and nurseries and North American forests respectively, was found in west coast nurseries (Ivors and others 2006). The authors reported that U.S. forest and European nursery

isolates clustered into two distinct clades, while one isolate from a U.S. nursery belonged to a third novel clade (a clade is defined as a group of isolates that share a common ancestor, which is not shared by another isolate outside of the clade; a clade is a group of closely related isolates). Microsatellite sequencing and morphological analyses suggested that the three clades represent distinct evolutionary lineages. Some U.S. nurseries contained isolates from all three clades, emphasizing the role of commercial plant trade in the movement of this pathogen. A proposed terminology would label each lineage based on the continent where it was first found; the common European lineage would become the European 1 lineage, or EU1 (almost all isolates are A1 mating type); the predominant North American wildland clade would be designated the North American 1 lineage, or NA1 (all isolates to date are A2 mating type); and the rare, recently identified, third clade from the U.S. nurseries (also A2 mating type) would be designated the North American 2 lineage, or NA2 (Ivors and others 2006).

*Phytophthora ramorum* is classified as belonging to the Stramenopila (formerly in the Kingdom Chromista), a major eukaryotic group that includes diatoms and brown algae, and is distinct from plants, fungi, and animals. In contrast to fungi, stramenopiles are more closely related to plants than to animals. The taxonomy of *P. ramorum* (Alexopoulos and others 1996) is as follows:

Domain:	Eukaryota
Kingdom:	Stramenopila (Chromista)
Phylum:	Heterokontophyta
Class:	Oomycetes
Order:	Peronosporales
Family:	Pythiaceae
Genus:	Phytophthora
Species:	<i>Phytophthora ramorum</i>

The Oomycetes, the Class to which the genus *Phytophthora* belongs, share some superficial morphological and biological characteristics of fungi. Previous taxonomic classifications often placed these two groups together. However, they have since been found to be genetically distinct. Like fungi, Oomycetes exhibit filamentous growth, produce sexual and asexual spores, and can feed on decaying matter or be obligate parasites of plants. However, the nuclei within the Oomycete filaments are diploid, with two sets of genetic information, not haploid or dikaryotic as in fungi. Another difference is that fungal cell walls are made primarily of chitin, while Oomycete cell walls are constructed mostly of cellulose and glucan. In addition, Oomycetes have zoospores with two types of flagella whereas most fungal spores have no flagella (Rossman and Palm 2006).

## Distribution

The native distribution or range of *P. ramorum* is not known. Evidence indicates that the pathogen has been separately introduced into North America and Europe from a third area which is as yet undiscovered (Brasier 2003, Ivors and others 2004, Rizzo and others 2005). The alien distribution of *P. ramorum* is now known to include the United States (specific nurseries in several states; in wildlands only in Oregon and California) and Canada (nurseries only) in North America, and several European countries (nurseries and limited outbreaks in parks and gardens. See details provided below.

## United States, in Forests

Since the disease was first noted in 1995, the pathogen has been confirmed in natural settings, primarily redwood/tanoak and coastal evergreen forests, on various native hosts in 14 coastal California counties (Marin, Santa Cruz, Sonoma, Napa, San Mateo, Monterey, Santa Clara, Mendocino, Solano, Alameda, Contra Costa, San Francisco, Lake, and Humboldt), and in Curry County, Oregon. The infested California counties are contiguous and in central coastal California; Humboldt County is also coastal, and north of and contiguous with Mendocino County, but the known infestation there is approximately 110 miles further north. The known infestation in Humboldt County is relatively isolated. About 130 miles separates the known locations in Humboldt County and Curry County in southern Oregon. See <http://nature.berkeley.edu/comtf/html/maps.html> for current distribution maps of the pathogen.

In Oregon, the pathogen was detected in 2001 via aerial survey (Goheen and others 2002). It is limited to an approximately 25 square-mile area in Curry County near Brookings, Oregon, just north of the California border (Figure 1). An eradication program is underway in Oregon, but the area under quarantine there continues to expand, with a new infestation detected in March 2007, about 2 miles north of the existing area under quarantine (see [http://nature.berkeley.edu/comtf/pdf/Monthly%20Reports/COMTF\\_Report\\_April\\_2007.pdf](http://nature.berkeley.edu/comtf/pdf/Monthly%20Reports/COMTF_Report_April_2007.pdf)).



Figure 1. Tanoak mortality in Oregon. Image: Everett Hansen, Oregon State University.

## United States, in Nurseries

*Phytophthora ramorum* was first recovered from a nursery setting in the U.S. in 2001, when it was isolated from rhododendron container plants in a Santa Cruz nursery. In 2003, 20 additional nurseries were reported infested. In March 2004 infestations of *P. ramorum* were detected in two large southern California nurseries. Those nurseries had shipped potentially infected plants to



Figure 2. *Phytophthora ramorum* infected nursery stock. Image: Jennifer Parke, Oregon State University.

several thousand nurseries throughout most of the U.S. (Figure 2). Subsequently, 20 states and more than 170 nursery-related detections were made (Garbelotto and Rizzo 2005). Infected nursery stock has since been detected and destroyed in the following states with infestations found in 2004: Alabama, Arkansas, Arizona, California, Colorado, Connecticut, Florida, Georgia, Louisiana, Maryland, North Carolina, New Jersey, New Mexico, New York, Oklahoma, Oregon, Pennsylvania, South Carolina, Tennessee, Texas, Virginia and Washington (Cave and others 2005). The number of such detections has continued to decline each year since 2004, which may be a result of implementing the current USDA APHIS federal regulations mandating inspection and eradication of infested stock (Table 1). The large increase in number of positive nurseries in 2004 is due in large part to a Federal Order requiring inspection for the first time of approximately 1400 nurseries that ship host plants or associated plants in California, Oregon and Washington (Jones 2006).

Table 1. Detection of *Phytophthora ramorum* in U.S. nurseries by year.

Year	Number of Positive Nurseries
2000	0
2001	1
2002	0
2003	20
2004	176
2005	99
2006	61



Figure 3. 2006 U.S. *P. ramorum* nursery detections. Image: Jonathan Jones, USDA-APHIS.

In 2006, USDA APHIS reported 61 sites in 11 states as having had nursery-related *P. ramorum* detections (Figure 3). Positive findings by state were: Alabama (1), California (28), Connecticut (1), Florida (2), Georgia (1), Indiana (1), Maine (1), Mississippi (1), Oregon (13), Pennsylvania (1), and Washington (11) (data from [www.suddenoakdeath.org](http://www.suddenoakdeath.org) website, 2006). As of May 2007, there have been 12 positive nursery sites in five states: Washington (3), Oregon (2), California (5), Florida (1) and Mississippi (1) ([http://nature.berkeley.edu/comtf/pdf/Monthly%20Reports/COMTF\\_Report\\_May\\_2007.pdf](http://nature.berkeley.edu/comtf/pdf/Monthly%20Reports/COMTF_Report_May_2007.pdf)). These were identified through inspections required by federal regulations, after state inspections, nursery surveys and other detections (data from [www.suddenoakdeath.org](http://www.suddenoakdeath.org)).

suddenoakdeath.org website). The federal regulations applied by APHIS to the nursery trade to restrict movement of potentially infected plants appear to be effective in reducing the number of new occurrences in nurseries. All known infestations are apparently contained and under eradication.

## Canada

*Phytophthora ramorum* was first reported in Canada in June 2003, when infected rhododendrons were found in a British Columbia nursery (Sabaratnam and Woodske 2006). Additional detections occurred in 2004 and 2005 at a few nurseries and garden centers in British Columbia. Immediate regulatory action by the Canadian Food Inspection Agency (CFIA) has apparently successfully eradicated these occurrences, with the exception of one nursery in Pitt River that remains under regulatory control (Sabaratnam and Woodske 2006). That nursery has recently undergone a control burn followed by disinfection of soil and production areas in an attempt to eradicate the pathogen.

## Europe

Since the first reports in Germany and the Netherlands (Werres and others 2001), *P. ramorum* has been discovered in additional European countries. The pathogen is now known from nurseries and gardens or ornamental plants in Belgium, Denmark, Finland, France, Ireland, Italy, Norway, Poland, Slovenia, Spain, Sweden, Switzerland, and the United Kingdom (De Merlier and others 2003, Delatour and others 2002, Heiniger and others 2004, Herrero and others 2006, Lane and others 2003, Lilja 2007, Moralejo and Werres 2002, Orlikowski and Szkuta 2002, Orlikowski and others, 2004, Swedish Board of Agriculture 2005). One isolated outbreak reported in the Czech Republic has been eradicated (cited in <http://www.forestry.gov.uk/pramorur>).

*Phytophthora ramorum* also occurs on mature trees in the U.K. and the Netherlands (Anonymous 2004a, Anonymous 2004b, Brasier and others 2004, Denman and others 2005a). In November 2003, the first *P. ramorum*-infected tree outside the United States was confirmed on a mature (100 year old) southern red oak (*Quercus falcata* Michx.) tree in Sussex, Great Britain. Infection of several *Quercus rubra* L. trees in the Netherlands was confirmed about the same time. By early December 2005 a range of tree species in Cornwall – including holm oak (*Quercus ilex* L.), turkey oak (*Q. cerris* L.), sessile oak (*Q. petraea* [Matt.] Liebl.), ash (*Fraxinus excelsior* L.), beech (*Fagus sylvatica* L.), horse chestnut (*Aesculus hippocastanum* L.), sweet chestnut (*Castanea sativa* Mill.) and sycamore (*Acer pseudoplatanus* L.) – were found with infections. The foliage of Chinese guger tree (*Schima wallichii* [DC.] Korth.) near Cornwall was reported infected with *P. ramorum* in 2006 ([http://nature.berkeley.edu/comtf/pdf/Nursery\\_Chronology\\_03.07.07.pdf](http://nature.berkeley.edu/comtf/pdf/Nursery_Chronology_03.07.07.pdf)). Infected rhododendrons have been in close proximity to all infected U.K. trees to date.

## Hosts

*Phytophthora ramorum* has a broad and diverse host range that continues to expand. The host list includes both hardwood and conifer trees, shrubs, herbaceous plants and ferns. Some hosts are found in forest situations while others are used widely as landscape and ornamental plants. In coastal California forests, *P. ramorum* infects many different plant species and in some mixed-evergreen forests nearly all woody plants can serve as hosts for *P. ramorum* (Rizzo and others 2002a).

In the U.S., there are, as of June 2007, 45 regulated hosts and 62 associated plants that are regulated as nursery stock only (see link below to APHIS-PPQ list). Plant species are designated as regulated hosts and as associated plants by the USDA. The difference between regulated hosts and associated plants is the demonstration of Koch's postulates. Proven or regulated hosts are hosts for which Koch's postulates have been completed, documented, reviewed, and accepted to confirm they are host plants of *P. ramorum*. All host parts, except the wood of non-bole hosts, are regulated (see *P. ramorum* quarantine and regulations at: <http://nature.berkeley.edu/comtf/pdf/APHIS-2005-0102-0001.pdf>).

Associated plants are plants that have been found to be naturally infected with *P. ramorum*, and *P. ramorum* has been cultured and/or detected using polymerase chain reaction (PCR), but Koch's postulates have not been completed or documented and reviewed, and for which testing has not been completed. Taxa are moved from the Associated Plant List to the Regulated Host List when Koch's postulates are demonstrated and reviewed (Cave and others 2005). USDA APHIS-PPQ maintains an updated list of regulated and associated hosts at [http://www.aphis.usda.gov/plant\\_health/plant\\_pest\\_info/pram/downloads/pdf\\_files/usdaprlist.pdf](http://www.aphis.usda.gov/plant_health/plant_pest_info/pram/downloads/pdf_files/usdaprlist.pdf).

A list of natural hosts of *P. ramorum* in the U.K., Europe and North America is available on the Department of the Environment Food and Rural Affairs (DEFRA) website at <http://www.defra.gov.uk/planth/newsitems/suscept.pdf>. The site includes the origin of the report (the U.K., the rest of the EU, or North America), and also indicates if Koch's postulates have been completed for each host. Further details of known natural hosts, along with experimental data on the susceptibility of potential hosts is available at <http://rapra.csl.gov.uk/>.

A third category, Experimental Hosts, also exists. Experimental Hosts, in contrast to the above two previously described groups where the pathogen is found on naturally infected plants, are those for which pathogenicity was determined by inoculating various plants to predict potential hosts. If the plant becomes infected, it is not added to the Proven Hosts or Associated Plants lists unless found naturally infected. Over 40 plant genera have been susceptible to this pathogen in

inoculation trials. Some of the literature that screened for potential hosts includes Brasier and others (2002), Chastagner and others (2006), Denman and others (2005b), Hansen and others (2005), Park and others 2002a, 2005b), Rizzo and others (2002a), Tooley and Kyde (2003, 2007) and Tooley and others (2004).

The COMTF website ([www.suddenoakdeath.org](http://www.suddenoakdeath.org)) contains the most up-to-date information available on regulated hosts, associated plants and experimental hosts.

Because the lists are being constantly updated, this website and others should be checked for the latest information on hosts.

## Symptoms

Three different diseases – stem or bole canker, twig blight (dieback) and leaf blight – are attributed to *P. ramorum*. Hansen and others (2002) distinguished three distinct disease syndromes: “sudden oak death”, characterized by lethal cankers; “ramorum shoot dieback”, resulting from foliar infection and/or infection of stems; and “ramorum leaf blight”, resulting from foliar infection. Thus, the symptoms produced by *P. ramorum* are diverse and vary from trunk and branch cankers to foliar symptoms including leaf spots, leaf lesions, leaf/twig/stem blights, depending on the host plant and the part of the host affected. The COMTF website contains descriptions and images of symptoms ([http://nature.berkeley.edu/comtf/html/plant\\_symptoms.html](http://nature.berkeley.edu/comtf/html/plant_symptoms.html)). Several diagnostic guides are available at that site with descriptions and pictures of symptoms on many host plants (Davidson and others 2003, Storer and others 2001).

Disease symptoms are reviewed, and well-illustrated, in many other publications, including – Garbelotto and others (2002a), McPherson and others (2002), Parke and others (2003, 2004), Storer and others (2002), and Tjosvold and others (2004). McPherson and others (2002) contains descriptions of symptoms by host family. The RAPRA website (<http://rapra.csl.gov.uk/background/hosts.cfm>) contains links to images of symptoms on various hosts. The DEFRA website at <http://www.defra.gov.uk/plant/pestnote/newram.pdf> also contains images of symptoms on various hosts, including some of the tree hosts in the U.K.

The pathogen has also been isolated from asymptomatic root tissue of infected tanoak seedlings (Parke and others 2006).

## Look-alikes

Signs and symptoms produced by *P. ramorum* are variable, not unique, and often indistinguishable from those caused by other pathogens, insect problems or abiotic injuries encountered. Other organisms and injuries can produce symptoms very similar to *P. ramorum*. Diagnosis of *P. ramorum* based on visual symptoms is a judgment rather than a positive determination, and the presence of the pathogen can only be confirmed through laboratory diagnosis.

Look-alike diseases, insect damage or injuries are described and illustrated in numerous publications. Some of the most comprehensive for look-alikes on oaks and tanoak include descriptions on the COMTF website ([http://nature.berkeley.edu/comtf/html/look-alikes\\_\\_\\_misdiagnosis.html](http://nature.berkeley.edu/comtf/html/look-alikes___misdiagnosis.html)); the western and eastern and pest alerts (Frankel 2002, O'Brien and others 2002) and the University of California Pest Alert number 6 (Storer and others 2002).

## Diagnostics

For reasons stated above, diagnosis of *P. ramorum* based on symptoms alone is unreliable at best. Methods for detecting plant pathogens in host tissue – plating on selective media (isolation and culture), and molecular techniques/assays based on either proteins (ELISA) or DNA (PCR-based methods) – have been used for *P. ramorum*. Culturing or molecular diagnostics are necessary for confident identification of *P. ramorum*.

### Plating on selective media (isolation and culture)

Traditional isolation techniques used for other *Phytophthora* species have been successful for recovery of *P. ramorum* from host tissue. Plating host material taken from the leading edge of a canker or lesion on media semi-selective for *Phytophthora*, such as pimarinic acid-ampicillin-rifampicin-pentachloronitrobenzene (PARP) (Erwin and Ribeiro 1996), incubating the plates in the dark at 20 to 22°C, and examining within 2 to 5 days has been commonly used (Davidson and others 2003). Once *P. ramorum* is growing in culture, its identity is confirmed by microscopic examination for its unique morphological characteristics. The USDA APHIS-PPQ protocol for cultural isolation is found at [http://www.aphis.usda.gov/plant\\_health/plant\\_pest\\_info/pram/downloads/pdf\\_files/cultureprotocol6-07.pdf](http://www.aphis.usda.gov/plant_health/plant_pest_info/pram/downloads/pdf_files/cultureprotocol6-07.pdf).

Although successful, several issues are associated with the traditional isolation-based diagnostics. Culturing of the pathogen from symptomatic plant material is time-consuming, and under some circumstances its success may be dependent on the species of the host or the environmental conditions from which the sample was taken (Martin and others 2004). Isolation success is ex-

tremely variable based on plant substrate and time of year (Davidson and others 2005, Garbelotto 2003). The fungicides and antibiotics in the selective media may sometimes suppress the development of hyphae from plant tissue and/or the development of *Phytophthora* propagules (Davidson and others 2003). Furthermore, despite *P. ramorum*'s distinctive morphological characteristics, the identification of an unknown culture solely on the basis of morphology does require training and experience. Because of these limitations, molecular diagnostic approaches have been developed and are used to augment identification of the pathogen.

## ELISA

ELISA (enzyme-linked immunosorbent assay) is a serological test used to detect the presence or absence of proteins in a plant produced by all *Phytophthora* species (Figure 4). The procedure is used as a quick pre-screen that will detect many species of *Phytophthora*. If a large number of samples are to be processed for *P. ramorum*, ELISA is used as a pre-screen to reduce the number of samples that will need to be processed for subsequent tests. An ELISA test that is specific to *P. ramorum* is not available.



Figure 4. ELISA plate. Image: Cheryl Blomquist, California Department of Food and Agriculture.

ELISA tests are available in prepackaged kit form. Single test, self contained ELISA kits are commercially available; the one produced by Agdia Inc. (Elkhart, Indiana) is approved by APHIS. Another kit, developed in Great Britain and marketed as a “lateral flow device (LFD)” (Forsite Diagnostics Ltd, York) for *Phytophthora* species, was evaluated by Lane and others (2007). Though false-positives were common, their assay was simple to use, and provided results comparable to laboratory methods (isolation and real-time PCR) in a few minutes. Results suggested that the use of LFD at the time of inspection may be a useful primary screen for selecting samples for subsequent laboratory testing to determine species.

For making regulatory determinations, USDA APHIS-PPQ encourages the use of a procedure that incorporates Agdia's ELISA test for pre-screening of all samples ([http://www.aphis.usda.gov/plant\\_health/plant\\_pest\\_info/pram/downloads/pdf\\_files/ELISA.pdf](http://www.aphis.usda.gov/plant_health/plant_pest_info/pram/downloads/pdf_files/ELISA.pdf)). The procedures used by the USDA to determine the presence or absence of *P. ramorum* in plant samples, summarized by Berger (Berger 2006), is based on a combination of tests. The Agdia-based ELISA is encouraged by the USDA-APHIS-PPQ so that further screening on *Phytophthora* negative samples is avoided. ELISA negative samples are considered negative for *P. ramorum* by the

USDA. Samples that are positive on the basis of ELISA are then subjected to further testing. Laboratories have the option, either before or after ELISA testing, to try to culture the organism. Presumptive positive cultures are then sent to the National Mycologist in Beltsville, MD for confirmation. If cultural isolation is confirmed, the sample is considered positive, even in rare cases where PCR (if done) is negative. For samples that are ELISA positive, but from which *P. ramorum* was not isolated, or that are ELISA positive and no culture was attempted, DNA is extracted and the DNA sent to the National Identification Service (NIS) (Beltsville, MD), for PCR analysis.

The complete and rather involved details of the APHIS-PPQ system for sample submission from provisionally approved laboratories, referred to as the “Potentially Actionable Suspect (PASS) System”, are available at [http://www.aphis.usda.gov/plant\\_health/plant\\_pest\\_info/pram/downloads/pdf\\_files/passpolicy4-06.pdf](http://www.aphis.usda.gov/plant_health/plant_pest_info/pram/downloads/pdf_files/passpolicy4-06.pdf).

## DNA-based techniques/assays

Several molecular techniques (PCR of the ITS region or other regions of the genome, Real-Time PCR, AFLP, ISSR, microsatellites, sequence analysis of several genes and PCR-RFLP for example) have been developed for detection and characterization of *P. ramorum* (Bonants and others 2005).

ITS PCR: Amplifying unique DNA sequences, such as the internal transcribed spacer (ITS) region, by using species-specific primers, has been used as a method for screening of other *Phytophthora* spp. (Bonants and others 1997, Cook and others 2000b, Kong and others 2003). Because the ITS region is conserved across *Phytophthora* spp., but differs among species within the genus, and the ITS sequence in that region is known for most *Phytophthora* spp. (Cooke and others 2000a, Cooke and others 2000b, Lee and Taylor 1992), ITS PCR has been used successfully for detection of specific species of *Phytophthora*.

Based upon ITS sequence analysis, a species-specific PCR detection method for *P. ramorum* was developed and validated using stem and leaf material from plants infected with *P. ramorum* (Garbelotto and others 2002b, Kox and others 2002). The primers developed may show cross reaction with *P. lateralis* (Kox and others 2002) and with *P. cambivora* at high concentrations (Davidson and others 2003). Primers developed by Lane and others (2003) are specific to *P. ramorum*. A PCR-based molecular diagnostic assay initially developed for *P. lateralis* (Winton and Hansen 2001) is also effective for *P. ramorum*, and remains the primary molecular diagnostic test used in Oregon for diagnosis of *P. ramorum* from the forest.

Nested PCR may be required to detect low levels of infection when small amounts of the pathogen are present (Bonants and others 1997, Gunderson and others 1996, Hayden and others 2004, Martin and others 2004). Nested PCR is basically the same as regular ITS PCR using species-specific primers, but the PCR reaction is run twice, with different combinations of primers. The test is thus able to detect much smaller amounts of DNA in a sample. However, nested PCR is much more prone to false positives resulting from laboratory contamination and other factors, including the presence of inhibitors. To be used with confidence, it requires dedicated laboratories with strict procedures and numerous internal controls. In a comparison between successful diagnoses in California forests using a nested PCR approach and traditional isolation techniques, molecular diagnosis showed the greater sensitivity (Hayden and others 2004). However, PCR was not always successful in detecting the pathogen and was highly dependent on which plant part (i.e., wood vs. leaves) or plant species was tested (Hayden and others 2004). The most promising results in forest surveys were obtained from traditional isolation combined with ITS PCR-based assays (Hayden and others 2004).

Real-time PCR (SYBR®-Green, TaqMan® and molecular beacons): Real time PCR assays, especially those based on TaqMan® chemistry (Holland and others 1991), are increasingly preferred to gel-based PCR because they are quicker, less labor-intensive, and are less prone to post-PCR contamination because subsequent manipulation of amplified DNA is avoided (Schaad and Fredrick 2002).

A nested real-time PCR assay, based on SYBR®-Green technology, was developed by Hayden and others (2004). These authors (Hayden and others 2004) developed and tested a real-time, nested PCR assay that was sensitive and host-specific for *P. ramorum*. The technique allowed the confirmation of *P. ramorum* in symptomatic plants and facilitated the expansion of the host range by 10 hosts and four California counties far sooner than if identification had been based on pathogen isolation alone.

A real-time quantitative PCR method that measures PCR product accumulation through a dual-labeled fluorogenic probe (TaqMan® probe) was developed in 1996 (Heid and others 1996). Real-time PCR methods based on TaqMan® chemistry do not require certain pre-amplification steps and therefore reduce the risk of cross-contamination. A TaqMan® probe has been developed for *P. ramorum* based upon the ITS sequence (Ivors and Garbelotto 2002).

Hayden and others (2006) developed and tested a nested TaqMan® assay that included a first round amplification step. The assay was as sensitive as the real-time PCR assay developed by Hayden and others (2004) that used SYBR-Green® for detection. The nested TaqMan® method successfully detected *P. ramorum* in field-collected samples (Hayden and others 2006). Sensitivity of the assay was markedly increased with the nested protocol vs. single-round, with

detection rates increasing more than twofold (Hayden and others 2006). Their technique provided for both detection and quantification of *P. ramorum* in plant material, even in the presence of inhibitors and low concentrations of *P. ramorum* DNA. However, because the assay requires two reactions and the moving of the product to a new tube, it has a risk of contamination.

When comparing isolation of *P. ramorum* using PARPH medium (cultural isolation using a variation of the PARP medium previously described) with a real-time PCR TaqMan® assay developed by the Central Science Laboratory (York, U.K.), the two techniques were equally reliable and robust for diagnosis of *P. ramorum* from the U.K. plant material tested (Hughes and others 2005a).

A single-round, real-time TaqMan® PCR assay for the detection of *P. ramorum*, involving no post amplification steps or multiple rounds of PCR, has been developed (Hughes and others 2006). The single-step protocol eliminates possible contamination introduced between the first and second round of PCR when using nested PCR. The assay detected *P. ramorum* in plant material containing as little as one percent infected material by weight. The real-time protocol gave results comparable with a traditional isolation technique for diagnosis of *P. ramorum* in plant material from common U.K. hosts (Hughes and others 2006). This assay is routinely used at the Central Science Laboratory (CSL) in the U. K., in conjunction with isolation techniques, for the detection of *P. ramorum* in symptomatic plant material in the laboratory.

On-site real-time PCR techniques: Tests that can be reliably used immediately at the point of sampling rather than sending samples to a central laboratory for testing and waiting for results would be useful in certain instances. For example, availability of on-site testing techniques would permit the targeted testing of known *P. ramorum* hosts, such as imported nursery stock, at points of entry with minimal disruption to trade. In addition, on-site testing would reduce the need to hold suspect material while waiting for a laboratory test result. If an on-site test is suitably sensitive, samples that test positive in the field can then be sent to a diagnostic laboratory for confirmation. Because the current methods for the molecular detection of fungal pathogens in plant material requires the extraction of DNA (Schaad and Frederick 2002), on-site molecular testing requires a portable real-time PCR platform and a suitable assay, as well as a simple and robust method for extracting DNA in the field.

Tomlinson and others (2005) developed a rapid and simple method for DNA extraction from symptomatic foliage and stems in the field, followed by a real-time PCR (TaqMan®) assay using a portable real-time PCR platform (Cepheid SmartCycler II) for accurate on-site detection of *P. ramorum* within 2 hours. The combination of an extraction method, real-time PCR assay, and lyophilized, field-stable reagents, all optimized for use in the field, allowed the detection of *P. ramorum* in naturally infected material at the point of sampling, with comparable results to those

of real-time PCR testing in the laboratory. A variation of that method was successfully evaluated under U.S. conditions using leaf and stem samples from 20 plant species collected from five sites around San Francisco, CA (Hughes and others 2005b).

Tomlinson and others (2007) developed a number of assays based on their earlier method (Tomlinson and others 2005) that had various advantages for use in the field. A variation of a real-time PCR assay called “scorpion runs”, was twice as fast as TaqMan®, and allowed the detection of *P. ramorum* in less than 30 minutes. They also designed a loop-mediated isothermal amplification (LAMP) assay, which allowed sensitive and specific detection of *P. ramorum* (as indicated by a color change visible to the naked eye) in 45 minutes using only a heated block to maintain a single constant temperature.

**Multiplex PCR:** Amplification of more than one region of DNA simultaneously (multiplex PCR) was first used as a diagnostic test for *Phytophthora* by Winton and Hansen (2001). Their technique amplifies a very highly conserved region of DNA as well as the piece of ITS DNA specific to *Phytophthora lateralis* and *P. ramorum*, resulting in a built-in check on the reaction (if the conserved region, found in all living organisms, does not amplify, then something is wrong with the test; Figure 5). Multiplex PCR, which can be used for simultaneous (multiplex) detection of more than one pathogen species in a sample of plant tissue and is a next step from single species detection, provides benefits to programs such as national surveys where large numbers of samples are involved. In addition, multiplex PCR can be used to incorporate a variety of control reactions to measure the quantity and integrity of the DNA tested. Various modifications of existing TaqMan® assays and other new approaches are being developed for parallel testing. For example, Tooley and others (2006) used a real-time PCR method based on TaqMan® in a three multiplex format to simultaneously detect *P. ramorum*, *P. pseudosyringae* and plant DNA in a single tube. Schena and others (2006) developed a real-time multiplex PCR assay based on TaqMan® PCR to simultaneously identify and detect *P. ramorum*, *P. kernoviae*, *P. quercina* and *P. citricola* within the same plant extract.

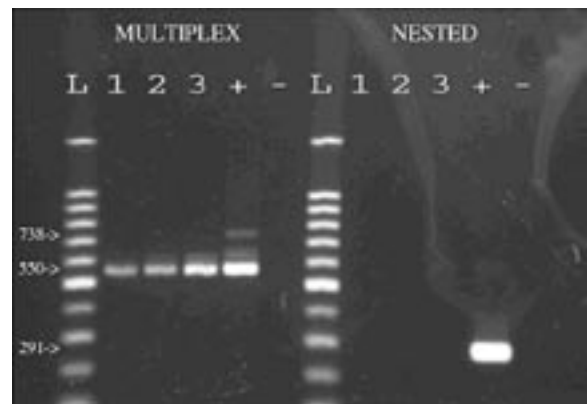


Figure 5. Multiplex and nested PCR amplification products from testing three nursery samples for *P. ramorum*. A 550-bp amplicon in multiplex PCR indicates DNA was successfully extracted from the samples. A 738-bp amplicon in multiplex PCR indicates *P. ramorum*, *P. lateralis*, or *P. hibernalis* DNA was present in the sample. A 291-bp amplicon in nested PCR indicates *P. ramorum* DNA was present in the sample. For all three nursery samples, DNA was successfully extracted, but no *P. ramorum* was present. The positive (*P. ramorum*) and negative (no DNA template) controls behaved as expected, indicating the PCR reactions were run correctly. Image: Nancy Osterbauer, Oregon Department of Agriculture.

**Single Strand Conformation Polymorphism:** Single strand conformation polymorphism (SSCP) analysis is an effective molecular fingerprinting technique for species differentiation in the genus *Phytophthora*, and *P. ramorum* shows a unique pattern of ribosomal DNA internal transcribed

spacer 1 when analyzed (Kong and others 2003c, Kong and others 2003a, Kong and others 2004). SSCP separates species based on the PCR-amplified ITS DNA sequence, but indirectly. Differences in sequence between species cause the strands of DNA to assume different shapes (conformations) and thus to move at different speeds through an electrophoresis gel. SSCP analysis reported by Kong and others (2004) was rapid and efficient, and SSCP profiles easily distinguished *P. ramorum* from other described *Phytophthora* species. The authors suggested that SSCP analysis may be a superior technique to other existing molecular fingerprinting methods such as restriction fragment length polymorphism (RFLP). However, because each *Phytophthora* species is identified by a profile of three bands, presence of multiple species in same sample may be confusing; in addition, SSCP from environmental rather than cultural samples may result in spurious bands (Garbelotto 2003). A modification of the technique using fluorescent-labeling chemistry and an additional marker locus was successfully used to allow quantitative matching of *Phytophthora* isolates from streams, soil and plants with reference species (Hansen and others 2005a). Kong and others (2005) reported a modification of their 2003 (Kong and others 2003c) technique that provided reliable diagnoses of *P. ramorum*, whether it is a single infection or dual infection (a second *Phytophthora* species involved). The technique also provided accurate diagnoses of diseases caused by 12 other species of *Phytophthora* without additional work.

Population differentiation/determination of molecular biotype: Microsatellite techniques and amplified fragment length polymorphism (AFLP) have been successfully used to differentiate between the North American and European lineages of *P. ramorum* (Hansen and others 2003). AFLP DNA fingerprinting showed significant differences between EU and U.S. populations (Bonants and others 2002, Ivors and others 2002). Using sequence differences between EU and U.S. isolates of *P. ramorum* in the mitochondrial Cytochrome c oxidase subunit 1 (cox1) gene, Kroon and others (2004) developed a single-nucleotide polymorphism (SNP) protocol to distinguish between isolates of *P. ramorum* originating in Europe and those originating in the United States. All isolates could be consistently and correctly allocated to either the European or the U.S. populations using the SNP protocol.

Microsatellite analysis of *P. ramorum* was first developed in the Hansen lab at Oregon State University. Large numbers of *P. ramorum* isolates have been analyzed with AFLP (Ivors and others 2004). Results show two clusters, with EU isolates and U.S. isolates grouping separately. A1 mating type isolates from the U.S. and the EU A2 mating type isolate cluster within the EU clade. Using the recently assembled whole genome sequence of *P. ramorum*, Simple Sequence Repeat (SSR) techniques were used to fingerprint large numbers of *P. ramorum* isolates originating from different host species within Europe and the United States (Ivors and others 2005). Many loci showed variation between the EU and U.S. populations. Minor variation was found within the EU populations and even less variation within the U.S. population.

## APHIS PPQ Protocol

The current (as of June 5 2007) diagnostic protocol used by the Animal and Plant Health Inspection Service, Plant Protection and Quarantine (APHIS PPQ) for samples that have regulatory significance states that all samples that are ELISA positive and/or culture negative must be assayed using the validated nested PCR method by USDA or at authorized USDA laboratories. DNA is extracted from ELISA positive samples (see [http://www.aphis.usda.gov/plant\\_health/plant\\_pest\\_info/pram/downloads/pdf\\_files/pcrprotocol4.pdf](http://www.aphis.usda.gov/plant_health/plant_pest_info/pram/downloads/pdf_files/pcrprotocol4.pdf)), and these DNA extracts are forwarded to the National Identification Services (NIS) Molecular Diagnostic Lab (which at present is the only authorized laboratory) as described in the protocol. If the diagnostic laboratory has not performed the ELISA test, then culture negative samples need to have DNA extracted and these extracts sent to the NIS Molecular Diagnostic Lab for analysis. Several labs outside of USDA authorized labs have been Provisionally Approved to process diagnostic samples using the USDA validated protocols. For the majority of regulatory samples, these labs provide final determinations. Only “Potentially Actionable Suspect Samples (PASS)” need to be forwarded for USDA confirmation. This laboratory approval program has several quality assurance measures in place to ensure participating laboratory capabilities and proficiency.

Until recently, APHIS PPQ utilized two PCR tests for *P. ramorum* determination (Berger 2006). The first is the multiplex PCR developed by Oregon State University (OSU) (Winton and Hansen 2001). Although it is not as sensitive as the main diagnostic test and can produce positive results with a few other closely related *Phytophthora* species, the OSU test is used as a quality assurance procedure to ensure that DNA received by PPQ is of sufficient quantity and quality to be amplifiable in the nested PCR test. Any DNA samples that do not meet these criteria are not tested further, and a new sample is requested. The second assay, assuming that amplifiable DNA is present in the sample, is a validated nested PCR test, modified slightly from the technique developed at the University of California at Berkeley, and described in [http://www.aphis.usda.gov/plant\\_health/plant\\_pest\\_info/pram/downloads/pdf\\_files/pcrprotocol4.pdf](http://www.aphis.usda.gov/plant_health/plant_pest_info/pram/downloads/pdf_files/pcrprotocol4.pdf). Any sample DNA that passed amplification quality control, and that reacted to produce either a Nested PCR or a *Phytophthora*-specific Multiplex PCR product, or both PCR products, are retested using both conventional PCR assays, and is tested in the Real-time PCR assay. Results of different replications of assays on individual samples are compared to confirm that the results were both repeatable, and robust. If the results of different replications of the assays for any sample were not all conclusive and compatible, DNA sequences are obtained for conventional PCR products, and these sequences are compared to those of reference sequences contained in public and local databases to aid in completing a diagnosis. Although a rare occurrence, if a diagnosis cannot then be obtained, a new sample is requested (Berger, PPQ, personal communication).

APHIS also has validated an additional Real-time PCR assay for *P. ramorum*. This test is based on the test currently used in the U. K. and developed by the Central Science Laboratory (York, U.K.). It is robust and sensitive, incorporates DNA quality controls into the single reaction, and has higher throughput than current APHIS methods. Comparative analysis with the primary PCR diagnostic demonstrated that this real-time PCR protocol is less sensitive than the original test, so some samples that cannot be confirmed with this test due to low concentration of *P. ramorum* need additional testing for confirmation. Recent research has provided a comparative analysis to determine the correlation between ELISA, culture, real-time and conventional PCR detection and is described below (Bulluck and others 2006). In addition, collaborative work involving several United States, Canadian and United Kingdom laboratories has occurred to help identify the most promising tests for entrance into the validation process.

## **EPPO Protocol**

The European and Mediterranean Plant Protection Organization (EPPO) publishes standards on diagnostic protocols for regulated pests of the EPPO region, including the EU. The provisional diagnostics protocol for *P. ramorum*, approved September 2005, was published as PM (Phytosanitary Measures) 7/66(1) in 2006 (OEPP/EPPO 2006). The publication contains a summary of sampling procedures for plants, water, and soil; methods for isolation from plants water, and soil; and a section on identification of the pathogen, including growth and morphology in culture, biochemical methods, and molecular methods.

## **Relative sensitivity/specificity of diagnostic techniques**

In addition to those already mentioned, several research efforts designed to obtain information on the relative sensitivity and specificity of the diagnostic tests used for *P. ramorum* identification – including culturing, ELISA, nested PCR, and real-time PCR – have been conducted or are underway.

In a comparison of diagnostic techniques within a nursery setting (Bulluck and others 2006), a block of 300 camellia plants within a California nursery known to be infested with *P. ramorum* was simultaneously assayed for visual symptoms, growth medium pH, and moss presence. Host plant leaf tissue or leaf bait from the growth medium extracts were either plated on PARP-V8, tested using ELISA, and subjected to nested and real-time PCR analysis. Diagnostic sensitivity and specificity of the assays were determined to compare the performance of each method for diagnosis of *Phytophthora* spp. or *P. ramorum* in camellia tissues and associated potting medium. All diagnostic assays were highly correlated with one another and disease symptoms, with nested

PCR having the best correlation with symptoms, followed by real-time PCR, ELISA, and then culture. A second research effort underway at UC Berkeley is designed to obtain similar data, but also investigate the influence of different hosts, environmental conditions, and other factors on the ability to accurately detect and identify the pathogen.

Using three different regions of the nuclear genome of *P. ramorum* (ITS,  $\beta$ -tubulin and elicitin gene regions) to construct species-specific markers, Bilodeau and others (2005, 2007) evaluated three different real-time PCR technologies (molecular beacons, TaqMan® probes and SYBR®-Green assays) for sensitivity and specificity. The best performing system (TaqMan® probes) was also used to compare the three DNA regions. Overall, TaqMan® assays with ITS or elicitin had the best combination of sensitivity and specificity.

A PCR-based mitochondrial DNA detection system for determining if a *Phytophthora* species was present in symptomatic plant tissue and clarifying if it was *P. ramorum*, *P. nemorosa*, or *P. pseudosyringae* was developed by Martin and others (2004). Results obtained with their system correlated well with the results obtained at the California Department of Food and Agriculture (CDFA) diagnostics laboratory for pathogen recovery from symptomatic tissue, and with PCR amplification for *P. ramorum*, using the rDNA ITS *P. ramorum*-specific primers of Garbelotto and others (2002b). For *P. ramorum*, no differences in results were obtained; all samples that scored positive for *P. ramorum* in the CDFA laboratory also were positive with the mitochondrial markers. The results for four of these samples were validated by sequence analysis of the *Phytophthora* genus-specific amplicon and comparison with data from purified cultures of this pathogen. The mitochondrial marker system also identified 16 additional samples infected with other *Phytophthora* spp. that could not be identified with the ITS marker system (Martin and others 2004).

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