Detection of *Phytophthora ramorum* in Nurseries and Forest Lands in California in 2004 to 2009

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Abstract

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From December 2004 through May 2009, samples were collected from California nurseries and wild lands to survey for *Phytophthora ramorum* and comply with federal regulations of nursery stock. Samples were pre-screened by an enzyme-linked immunosorbent assay (ELISA) that detects *Phytophthora* spp. and tested by culture, *P. ramorum*-specific real-time polymerase chain reaction (PCR), and nested PCR. Yearly percentages of infected samples ranged from 0.6 to 2.3%. *Camellia* spp., *Rhododendron* spp., *Magnolia* spp., *Pieris* spp., and *Laurus nobilis* tested positive the most frequently in the nurseries and *Lithocarpus densiflorus*, *Umbellularia californica*, and *Quercus agrifolia* tested positive most often from wild

lands. Of the 118,410 samples isolated onto PARP media, 0.8% was identified as *P. ramorum*. Of 115,056 samples tested by ELISA, 5.9% tested positive for *Phytophthora* spp. Of the 6,520 samples tested by PCR, 12.4% tested positive for *P. ramorum*. The false-negative, positive, and internal control failure rates of the assays are discussed. After removing the seasonal effect of sampling strategy, isolation of the pathogen into culture was found to be seasonally dependent whereas detectability by PCR and ELISA was not. To our knowledge, this is the first evaluation of a regulatory testing program for a plant pathogen on this scale using standardized assays.

Phytophthora ramorum Werres, de Cock & Man in't Veld was described as a new species in 2001 (Werres et al. 2001) and is recognized as the cause of sudden oak death in some oak species and ramorum blight of many woody plant species in California (Davidson et al. 2003). The pathogen now exists in the environment in portions of 15 counties of coastal California and in one county in Oregon. It can infect more than 100 host species from more than 40 genera, mostly woody plant hosts (Grunwald et al. 2008; Rizzo et al. 2005). The discovery of *P. ramorum* in ornamental plant nurseries in Washington, Oregon, and California and the shipment of infected nursery plants from these three states to other states led to regulation by the United States Department of Agriculture Animal Pest Health Inspection Service (USDA-APHIS) with rule 7CFR Ch III 301.92. This rule requires nurseries in California, Oregon, and Washington to be inspected, tested, and found free from *P. ramorum* before they can ship host and associated host plants out of the state in which they were produced. Host species are defined as plant species where *P. ramorum* has been isolated in culture and on which Koch's postulates have been verified; associated hosts are species from which *P. ramorum* has been isolated in culture or detected by polymerase chain reaction (PCR) only and no plant inoculation studies have been completed. Since the initial emergency order was adopted in 2004, *P. ramorum* has been found in nurseries, in limited areas of the natural environment near nurseries, and in waterways outside of California, Oregon, and Washington (Chastagner et al. 2010; Frankel and Hansen 2011).

On 31 March 2014 a new Federal order, DA-2014-02, was adopted which substantially changes the regulation of *P. ramorum* in the nurseries. These regulations require the inspection and testing of host and associated host plants only from nurseries in which *P. ramorum*

has been detected in the last 3 years. Nursery inspections at these previously positive sites involves a more rigorous survey and more extensive sampling of plants and standing water than under previous regulations. These recent regulations now pertain to all nurseries that ship across state lines in the United States, not only those located in Washington, Oregon, and California. Yearly testing of nurseries in the 15 counties in California and 1 county in Oregon where *P. ramorum* is known to occur in the wild lands will continue to be required (Anonymous 2014).

The nursery industry in California had a farm gate value of \$3.78 million in 2009 (Anonymous 2011). Nursery producers sell and ship host, associated, and nonhost plants to customers located out of state. Prior to 2014, if the pathogen was confirmed in a nursery, a destruction block consisting of a continuous block of host plants was delimited and destroyed. The surrounding plants within 10 m of the destruction block were held for inspection over a 90-day period. The economic impact to the plant grower due to plant destruction and plants being held off sale during the 90-day period can be substantial. More than 1 million plants were destroyed when *P. ramorum* was detected in 2004 in a large wholesale nursery in California. Estimated losses, including credits to customers, cost of cleanup, cost of inventory, and lost sales, were \$6.9 million. (J. Keller, personal communication). The economic and environmental impact of introducing the pathogen into a new state or a new susceptible forest environment can have serious environmental consequences. *P. ramorum* has been recently reported causing damage on approximately 0.5 million Japanese plantation larch trees in the United Kingdom (Brasier and Webber 2010; Webber et al. 2010).

To assure that all nurseries within the regulated states were being treated uniformly and the samples tested accurately, pathogen testing protocols were standardized and validated with a large number of samples. Under the federal rule, only assays approved by USDA-APHIS may be used for regulatory testing. In this study, we wanted to evaluate the false-positive, negative, and failure rates for each of the assays as they compare with our standard assay. We also wanted to determine which host plant species were most commonly infected in the nurseries. We also hypothesized that there was a seasonal variation in *P. ramorum* infection in the nurseries and in the forest. To our knowledge, this is the first evaluation of a regulatory testing program for a plant pathogen on this scale using standardized assays.

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*The e-Xtra logo stands for "electronic extra" and indicates that four supplementary figures and one supplementary table are published online.

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Materials and Methods

Nursery surveys. Ornamental nurseries from counties in California were surveyed for leaf spots and twig blights as part of the mandated nursery surveys conducted by the California Department of Food and Agriculture (CDFA) and the local county agricultural commissioners to detect *P. ramorum*. From December 2004 through May 2009, nursery samples were collected from wholesale and retail nurseries by trained county agricultural biologists. All samples were collected and processed in accordance with the APHIS survey and sampling protocol that requires all regulated host plants and hosts associated with *P. ramorum* be visually inspected for leaf lesions and twig blights. Although sampling was biased toward these hosts, all other plants showing obvious leaf lesions and twig blights also were sampled. Individual plant samples containing anywhere from 1 to 80 leaves from a single species or variety block were bagged separately in moisture-retaining containers, kept at 4°C, shipped at ambient temperature, and sent to the CDFA laboratory for further processing. For the purposes of this study, a sample lot is defined as a group of samples from one growing ground during one sampling event. Each lot typically contained anywhere from 1 to 50 individual plant samples.

USDA detections by host. Plant samples suspected to be infected with *P. ramorum* collected from nurseries by regulatory officials were sent to the USDA-APHIS Plant Protection and Quarantine (PPQ) testing laboratory in Beltsville, MD for confirmation over the study time period. Samples were tested using the same protocols described below.

Forest sampling. Samples were collected by county agricultural biologists, foresters, homeowners, park managers, and farm advisors from infested and uninfested counties. Trinity County was determined to be an infested county in 2014, after this study was completed. We recommended that samples be submitted from January to August for the best possibility for detection, although we tested samples submitted outside this time frame. *Umbellularia californica* samples were sent in as leaves. *Quercus agrifolia* and *Lithocarpus densiflorus* samples were sent in as bark canker pieces sampled from necrotic margins and isolated onto CMA-PARP, a semiselective medium for *Phytophthora* (Jeffers and Martin 1986) on site, and sent by overnight mail to our laboratory for incubation. Isolation plates were examined for growth from 7 to

14 days after the sample was taken. Occasional leaf and stem samples were also received from *L. densiflorus* and sampled in the lab. No effort was made to control the numbers or locations from which the samples were taken.

Enzyme-linked immunosorbent assay. Five or six pieces, 8 to 12 by 5 to 6 mm in size, were cut from the lesion margins from leaves or stems from each sample bag. The number of leaves sampled from each sample bag ranged from one to six, depending on the symptoms and the number of leaves collected. Each of the pieces was divided into two, each half containing equal amounts of necrotic and healthy tissue. One set of halves were plated directly on CMA-PARP. The other halves of these pieces were ground in 1 ml of GEB2 buffer (Agdia, Elkhart, IN) using a Fast Prep machine (MP Biomedical, Solon, OH) and 100 µl of the grindate was tested in duplicate using a *Phytophthora* spp.-specific enzyme-linked immunosorbent assay (ELISA) kit (catalog number PSA 92600; Agdia). Tests were run according to manufacturer instructions. ELISA tests were considered positive and tested further by PCR if the optical density at 405 nm was at least twice the negative control.

Isolation. Isolation plates were stored at room temperature (20 to 25°C) in the dark and monitored for hyphal growth for up to 14 days. Cultures were identified as *P. ramorum* by distinct colony morphology with coralloid hyphae, chlamydospores, and the occasional presence of semipapillate caudocous sporangia. Sometimes a culture of *P. ramorum* grew from a sample before PCR could be performed. In this case, the sample was considered positive and PCR was not performed to save time and resources. *P. hibernalis* (Erwin and Ribeiro 1996; Waterhouse and Waterston 1964) and *P. foliorum* (Donahoo et al. 2006) were identified by morphological characteristics and confirmed by sequence analysis.

DNA extraction. DNA was extracted from samples that tested ELISA positive using the DNeasy Plant Mini Kit (catalog number 69106; Qiagen, Valencia, CA) using the protocol for plant samples.

Nested PCR. PCR was performed according to the USDA-APHIS protocol. The nested PCR (nPCR) test specific for *P. ramorum* is a variation of the nPCR assay originally developed by Hayden et al. (2004). A sample is considered positive when, in a two-step reaction, an amplicon of 291 bp is produced. The control reaction for this test, which is performed in parallel to the nPCR, uses the PCR test for *P. lateralis* (Winton and Hansen 2001), which includes the ribosomal DNA (rDNA) universal primers NS1 and NS2 as internal controls. The detection primers in the Winton and Hansen test will amplify *P. ramorum*, *P. lateralis*, and *P. hibernalis* DNA but, due to reduced sensitivity in amplification of *P. ramorum* compared with the nPCR test, its results are not used for final determination. If an amplicon of approximately 550 bp is not produced by the internal control primers, the results are scored as an internal control failure for the nPCR test.

Real-time PCR. The real-time PCR (qPCR) test was implemented after January 2007 and is a variation of Hughes et al. (2006). The multiplex qPCR test uses primers which amplify a portion of the rDNA gene of *P. ramorum* and another set of primers which amplify a portion of the plant *cox* gene which serves as an internal control. After January 2007, when the qPCR test became the primary test, if the FAM cycle threshold (Ct) was 0 and the internal control Ct was <36, the sample was considered negative. If the FAM Ct in the qPCR test was ≤36 and the internal control Ct was <36, the sample was considered positive. If the Ct for the qPCR test was >36, the DNA sample was tested further by the nPCR test (Fig. 1). If the subsequent nPCR tests were positive, the sample was positive. If a sample tested negative by nPCR (no amplicon was produced or none of the right size), the sample was considered negative (Fig. 1). An internal control was considered a failure if the Ct for the *cox* gene test was ≥36 after one test.

HaeIII digestion. The nPCR test sometimes produced an amplicon when *P. hibernalis* or *P. foliorum* was present due to cross

Fig. 1. Flow chart for testing of nursery samples for *Phytophthora ramorum* at the California Department of Food and Agriculture Laboratory. Five 4-mm² lesion margins from symptomatic plants or the same size pieces from healthy plants are selected and divided. Half of each of the five pieces were placed on PARP semiselective media and half were tested by an Agdia *Phytophthora* spp. enzyme-linked immunosorbent assay (ELISA). If the optical density at 405 nm from the ELISA test measures more than twice the background, the lesion margins are resampled from the original plant sample and DNA is extracted. Before January 2007, the DNA extract was tested by nested polymerase chain reaction (nPCR). After January 2007, the DNA extract was tested by a real-time PCR (qPCR). If the FAM cycle threshold (Ct) ≤ 36, the sample is considered positive. If the sample FAM Ct ≥ 36, the DNA extract is tested by nPCR. If a band is produced in the nPCR test, the test is positive. The amplicon from the nPCR test is later digested by HaeIII to confirm the identity of the product.

amplification (Blomquist et al. 2005). To distinguish between these species and *P. ramorum*, a restriction fragment length polymorphism (RFLP) was developed using the restriction enzyme *Hae*III. On digestion of the nPCR product with *Hae*III, *P. ramorum* produces three distinct bands, *P. foliorum* produces a doublet, and *P. hibernalis* produces a single band (Blomquist et al. 2005).

Time series analysis. As described above, the complete diagnostic process included several components (ELISA, PCR, and isolation of the pathogen into culture), each of which contributed to the final determination of pathogen status and, hence, to the data for that category. One of the important practical issues that arose during the pathogen response period was whether these different diagnostic approaches, and the final determination, were affected by seasonal variation. A time series analysis approach was used to examine this question and to compare the temporal patterns of positive diagnoses (for each diagnostic technique)

for forest and nursery samples. The time series for forest and nursery samples were analyzed to study their separate autocorrelation structures and their cross-correlation structure (i.e., the correlation between the two series at different lag times). The presence of significant autocorrelation in a time series indicates two important properties. First, it may indicate the presence of periodicity, or seasonality, in the data, so that observations separated by particular intervals tend to be similar to each other. Second, it may suggest the presence of a process which causes observations at later time points to be dependent upon or, to some extent, predictable from earlier ones (Crawley 2013; Royama 1992). The process and rationale of the analysis was as follows.

The raw data series of numbers of confirmed positive cases of *P. ramorum* (for each of the diagnostic tests) and their autocorrelation functions (ACF) are presented in the e-Xtra material but are

Fig. 2. Pictorial map of the location by California counties where nurseries were sampled from June 2004 to May 2009 for *Phytophthora ramorum* are marked with a star. Total Farmgate value of nursery and floral culture in thousands of dollars from 2007 is labeled on each county. The 15 counties where *P. ramorum* is known to exist in the wild lands are shaded. *P. ramorum* was found in Trinity County after this study was completed.

Table 1. Frequency of *Phytophthora ramorum* positive-determinations collected from forest and nursery locations from 2004 to 2009 by county and submitted to the California Department of Food and Agriculture Laboratory^a

County ^a	Frequency of <i>P. ramorum</i> -positive determinations ^a			Total samples collected	<i>P. ramorum</i> positive (%)
	Forest	Nursery	Total		
Alameda	46	92	138	839	16.45
Amador	0	0	0	132	0.00
Butte	0	0	0	1,288	0.00
Calaveras	0	0	0	248	0.00
Contra Costa	18	14	32	2,282	1.40
Del Norte	0	0	0	1,022	0.00
El Dorado	0	6	6	73	8.22
Fresno	0	2	2	2,131	0.09
Glenn	0	0	0	1	0.00
Humboldt	114	13	127	3,459	3.67
Imperial	0	0	0	44	0.00
Kern	0	0	0	2,257	0.00
Kings	0	0	0	7	0.00
Lake	0	0	0	62	0.00
Los Angeles	1	42	43	19,190	0.21
Madera	0	1	1	337	0.30
Marin	164	2	166	399	41.60
Mendocino	59	15	74	1,541	4.80
Merced	0	0	0	50	0.00
Monterey	87	2	87	974	9.14
Napa	10	3	13	125	10.40
Nevada	0	20	20	156	12.82
Orange	0	4	4	5,058	0.08
Placer	0	8	8	1,493	0.54
Plumas	0	0	0	1	0.00
Riverside	0	6	6	23,621	0.03
Sacramento	0	65	65	3,412	1.90
San Benito	0	0	0	192	0.00
San Bernardino	0	0	0	2,557	0.00
San Diego	0	17	18	22,680	0.08
San Francisco	10	0	10	25	40.00
San Joaquin	0	11	11	1,180	0.93
San Luis Obispo	0	2	2	3,785	0.05
San Mateo	246	33	276	746	37.40
Santa Barbara	0	4	4	1,632	0.25
Santa Clara	95	32	127	2,307	5.50
Santa Cruz	41	41	82	3,018	2.63
Shasta	0	5	5	516	0.97
Siskiyou	0	0	0	20	0.00
Solano	24	2	26	1,125	2.31
Sonoma	109	30	136	598	23.24
Stanislaus	0	5	5	2,149	0.22
Tehama	0	0	0	23	0.00
Tulare	1	0	1	1,309	0.08
Tuolumne	0	0	0	6	0.00
Ventura	0	5	5	4,325	0.11
Yolo	0	0	0	8	0.00
Yuba	0	0	0	7	0.00

^a Counties where *P. ramorum* had been detected in the forest are in bold.

Table 2. Frequency of *Phytophthora ramorum* detections from nursery and forest plant samples by year submitted to the California Department of Food and Agriculture Laboratory from June 2004 to May 2009

Sample year	Months	<i>P. ramorum</i> -positive samples	Total samples tested	<i>P. ramorum</i> positive (%)
2004	June to December	91	7,460	1.2
2005	January to December	522	22,569	2.3
2006	January to December	374	22,324	1.7
2007	January to December	259	24,707	1.0
2008	January to December	145	22,371	0.6
2009	January to May	112	18,959	0.6
Total	...	1,503	118,410	1.3

not analyzed in detail. The raw data series are subject to a seasonal effect directly related to the fluctuations in monthly sampling intensity (see forest sampling, above). This systematic variation in sampling effort would be likely to induce a corresponding periodicity in the data for numbers of positive diagnoses in each of the tests, and any estimate of a true seasonal variation in the performance of each of the tests needs to be made in the absence of this seasonal sampling effect.

The sampling effect was removed from the data as follows. For each of the series of positives results for each test and the series for “final determination”, a simple linear regression was fitted with the number of negative samples, specific to each test, in each month as the explanatory variable. The residuals from these regressions were then used as the data series to study the ACF of the nursery and forest time series. The rationale for this approach is that the negative samples and positive samples together form the total sample size each month, with the negatives in the majority. Therefore, the series for the negative results are a good indicator for the seasonal fluctuation in sampling effort. By regressing the positive cases on negative samples, the variation in the positive results explainable by the seasonal fluctuation in sampling effort is removed from the data for positive cases. The resulting residuals contain any remaining autocorrelation in the data series combined with unmeasured random effects. Following the removal of the seasonal effect, the ACF for time series for the forest and nursery confirmed cases were calculated for each of the tests. The default parameters in R were used in all cases.

Results

Sample distribution within the state. An average of 1,380 nursery growing grounds were tested for *P. ramorum* every year from June 2004 to May 2009 in our laboratory. Samples were received from 48 of 58 California counties (Fig. 2). The majority of the nursery samples (57.8%) were sent from three southern counties: San Diego, Riverside, and Los Angeles. Samples from the forest were sent from the 14 infested counties, with two exceptions (Table 1). Over the time of this study, *P. ramorum* was detected at least once in each of 30 counties, 17 times from counties without the pathogen in the wild lands.

From June 2004 to May of 2009, 118,410 samples were tested for *P. ramorum*. Of these, 1.3% tested positive. Percentages of infected samples ranged from a high of 2.3% in 2005 to 0.6% in 2008 and 2009 (Table 2). *Camellia* spp., *Rhododendron* spp., *Magnolia* spp., *Pieris* spp., and *Laurus nobilis* were the species that tested positive the most frequently in the nurseries and *Lithocarpus densiflorus*, *U. californica*, and *Q. agrifolia* were the species that tested positive most often in samples taken from the forest (Table 3). Overall, 68% samples that tested positive for *P. ramorum* were submitted from the forests of infested counties (Table 3) even though this represented approximately 3% of the total samples submitted (data not shown). *Rhododendron* spp. accounted for 51% of the positive detections by the USDA testing lab, with *Rhododendron*, *Camellia*, *Viburnum*, and *Pieris* spp. accounting for 82% of the hosts which tested positive for *P. ramorum* (Fig. 3).

Test results: ELISA, isolation, and PCR. Of 115,056 samples tested by ELISA, 5.9% tested positive for *Phytophthora* spp. Of the 118,410 isolated onto PARP media, 0.8% was identified as *P. ramorum*. Of the 6,520 samples tested by PCR, 12.4% tested positive for *P. ramorum* (Table 4). The overall accuracy of the ELISA test in predicting *P. ramorum* was 6.1%. That is, 6.1% of the samples that tested positive in the ELISA test, tested positive by either culture or PCR for *P. ramorum*.

FAM > 36. Of the 237 samples in which the Ct fell between 36 and 45 for the *P. ramorum*-specific primers in the qPCR test, 77 tested positive by nPCR. There was no correlation ($P > 0.05$) between the actual Ct values and whether the sample tested positive by nPCR (data not shown). For example, samples with Ct values closer to 36 were no more or less likely to actually test

positive for *P. ramorum* in the nPCR test than those with Ct values closer to 44. If a sample was submitted from the forest and the Ct > 36, it was 85% likely to be *P. ramorum*. If a sample was tested from a nursery and the Ct > 36, it was only 15% likely to be *P. ramorum* (data not shown). *P. hibernalis* and *P. foliorum* grew on isolation plates from some of the samples which tested negative.

False negatives. *P. ramorum* grew on culture plates from 7 of 5,711 samples that had tested negative by PCR and positive by ELISA.

False positives. Of 498 samples which initially tested positive by nPCR, 92 were *P. foliorum* and 21 were *P. hibernalis* by RFLP analysis. After January 2007, when the qPCR test was introduced, only five samples tested falsely positive for *P. ramorum* by nPCR and were *P. foliorum* or *P. hibernalis*.

Internal control failures. Amplification of the internal control primers failed 95 times, 24 with the NS1, NS2 primers and 71 times with the *cox* primers in the qPCR test. The largest numbers of failures were with extracted DNA from *Sequoia* and *Arctostaphylos* spp., although extracts from 24 other genera also failed at least once in one of the PCR tests (Table 5).

Time series analysis. A strong regular seasonal effect in sampling, particularly in the nursery samples, resulted from the sampling

Table 3. Frequency of *Phytophthora ramorum* determinations by host species from nursery and forest samples in California from 2004 until 2009

Host	Frequency of <i>Phytophthora ramorum</i> -positive determinations ^a		
	Nursery	Forest	Total
<i>Abies concolor</i> ^b	6	0	6
<i>Arbutus unedo</i>	3	0	3
<i>Arctostaphylos</i> spp.	2	1	3
<i>Camellia japonica</i>	129	0	129
<i>C. sasanqua</i>	101	0	101
<i>C. sinensis</i>	1	0	1
<i>Camellia</i> spp.	70	0	70
<i>Cinnamomum camphora</i>	1	0	1
<i>Heteromeles arbutifolia</i>	5	1	6
<i>Laurus nobilis</i>	9	0	9
<i>Leucothoe fantanesiana</i>	1	0	1
<i>Lithocarpus densiflorus</i>	1	51	52
<i>Loropetalum chinense</i>	1	0	1
<i>Magnolia grandiflora</i>	7	0	7
<i>Magnolia</i> sp.	2	0	2
<i>Olea europa</i>	1	0	1
<i>Nerium oleander</i>	1	0	1
<i>Osmanthus fragrans</i>	5	0	5
<i>O. heterophyllus</i>	2	0	2
<i>Pieris formosa</i>	1	0	1
<i>P. japonica</i>	6	0	6
<i>Pieris</i> spp.	4	0	4
<i>Pittosporum tobira</i>	1	0	1
<i>P. undulatum</i>	7	0	7
<i>Quercus agrifolia</i>	0	83	83
<i>Quercus</i> sp.	0	3	3
<i>Rhamnus</i> sp.	1	2	3
<i>Rhododendron 'azalea'</i>	1	0	1
<i>Rhododendron</i> sp.	88	6	94
<i>Sequoia sempervirens</i>	0	2	2
<i>Syringa vulgaris</i>	1	0	1
Unknown species	5	0	5
<i>Umbellularia californica</i>	14	872	886
<i>Vancouveria</i> spp.	1	1	2
<i>Viburnum tinus</i>	3	0	3

^a Samples were determined to be positive for *P. ramorum* by culture and/or DNA-based diagnostic. The sampling period was from June 2004 through May 2009 ($n = 118,410$ samples).

^b *Abies concolor* samples were taken from a Christmas tree farm

guidelines issued to sample collectors (Supplementary Figs. 1 and 2). This seasonal effect resulted in significant ACF values in the nursery and forest autocorrelograms at lags corresponding to an annual cycle; again, the effect was pronounced in the nursery data series (Supplementary Figs. 3 and 4).

Removal of seasonal sampling effect from the data. Regressing the number of positive cases on the number of negative samples produced statistically significant estimates for the explanatory variable for both the forest and nursery samples for each of the tests and the final determination data. Summary statistics for these analyses are given in Supplementary Table 1. The percent variance accounted for by the regressions ranged from 10.4% in the case of the PCR data from forest samples to 58.2% in the case of ELISA data from nursery samples. These values give an indication of the extent to which the seasonal fluctuations in sampling effort were correlated with fluctuations in the numbers of positive test results in the raw data.

Time series analysis of seasonally-adjusted data. After removal of the seasonal sampling effect from the data, differences were apparent between the biochemical diagnostic tests (PCR and ELISA) and culture isolation for both the forest and nursery time series (Figs. 4 to 7). For the PCR and ELISA test results for both nursery and forest samples, removal of the seasonal sampling effect removed any significant ACF values except at lag 1 or lag 2 (Figs. 4 and 5). In contrast, for the isolation by culture test, significant autocorrelations were present at lags 11 (nursery only) and 12 (both forest and nursery) (Fig. 6). These results indicate that, independent of the seasonal sampling intensity, there were predictable, periodic fluctuations in the probability of isolating live cultures of *P. ramorum* from the samples submitted to the diagnostic laboratory for evaluation.

An illustration of how the information from different tests was combined to produce a final determination (positive or negative) for *P. ramorum* from each sample is shown in Figure 7. Not surprisingly, the seasonally adjusted data for the final determinations were intermediate between those for the biochemical diagnostic tests and the isolation by culture procedure.

Particularly in the case of the nursery samples (Fig. 7B and D), there were positive autocorrelations at lags 1, 12, and 13 that approached the upper Bartlett band (i.e., 95% confidence interval for a zero autocorrelation), indicating that samples collected at equivalent months in successive years tended to have similar status with respect to final determination of *P. ramorum* status regardless of sampling intensity.

Discussion

Even though there are more than 100 species listed as hosts of *P. ramorum*, relatively few species were detected with the pathogen in the nurseries in California or throughout the United States. The most frequently detected host genera and species found infected with *P. ramorum* in California nurseries from 2004 to 2009 were *Camellia*, *Rhododendron*, *Magnolia*, *Pieris*, and *Laurus nobilis*. This contrasts with the most frequently detected host genera found United States wide: *Rhododendron*, *Camellia*, *Viburnum*, *Pieris*, and *Magnolia*. The differences may be partially explained by the fact that most of the *Rhododendron* spp. that are grown in California are evergreen azaleas which are rarely infected with *P. ramorum*. This is in contrast to the larger landscape rhododendrons grown widely in Oregon and Washington. *L. nobilis* appears on the California list because it is a very

Table 4. Results for individual tests of plant samples for *Phytophthora ramorum* at the California Department of Food and Agriculture Laboratory from June 2004 to May 2009

Assay ^a	Number positive	Total number tested	Per cent positive
<i>Phytophthora</i> spp., ELISA	6,844	115,056	5.9
<i>P. ramorum</i> culture	977	118,410	0.8
PCR	809	6,520	12.4

^a Enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR).

Fig. 3. Percent nursery hosts plant samples sent to the United States Department of Agriculture diagnostic lab for analysis which were confirmed infected with *Phytophthora ramorum*.

popular landscape plant and widely grown in frost-free areas of California but not grown through the winter in most parts of the United States.

All of the hosts of *P. ramorum* found infected in the nurseries of California are evergreen plants. Even though deciduous members of the genera *Magnolia* and *Viburnum* are grown in the state, the deciduous forms are almost never infected. It is possible that we see little infection of *Viburnum* spp. in comparison with the rest of the country because the deciduous *Viburnum* spp. generally leaf out in California after spring rains have ended for the year, when inoculum pressure presumably is lower. In other parts of the United States and Canada which have different rain patterns, deciduous species such as witch hazel, dogwood, and *Prunus* spp. have been infected with *P. ramorum*.

In general, California's climate is coastal or interior. Coastal areas have mild temperatures year round, with summer fog and winter rain. The interior areas have hot dry summers and cool winters with rain. Large wholesale nurseries in California are located in both coastal and interior areas, with the majority located in interior areas in Southern California. Most all are irrigated using overhead sprinklers, with many plant species such as *Camellia japonica* and *Rhododendron* spp. grown under shade cloth. Our data suggest that even, though *P. ramorum* is relatively uncommon in the nurseries of California, these microenvironments appear to be conducive to the survival of *P. ramorum* over the summer months.

The general *Phytophthora* spp. ELISA screened out large numbers of *Phytophthora*-negative samples quickly and inexpensively. Testing by ELISA reduced the number of samples tested by PCR by 94%. In 2004, we set the cutoff value at twice background below a single culture-positive *Camellia* sample which tested 2.5 times background in the ELISA test. Since then, Canadian and U.S. regulations have adopted a similar cutoff.

Culture isolation is not required by APHIS regulations in the initial testing procedure and has advantages and disadvantages. With some training, *P. ramorum* can be fairly quickly and easily identified in culture. In addition, if isolates grow in culture, all *Phytophthora* spp. present in a sample can be identified, not just *P. ramorum*. Observations of what organisms grow in culture can aide in the investigation of possible false-positive PCR tests. Cultures can be genotyped easily, stored in a culture collection, and shared with other investigators. When *P. ramorum* is present in very small, invisible lesions, the pieces of tissue placed in culture become another sample taken and another chance that *P. ramorum* is detected. This extra sampling probably explains why a few of the samples tested PCR negative and culture positive. We detected *P. ramorum* on *Loropetalum chinense* in 2007 for the first time in exactly this scenario (Blomquist et al. 2012).

Unsurprisingly, our results show that culture isolation is not as sensitive a detection method as PCR. Of the 810 samples that tested positive by PCR, 26.54% were culture negative. *P. ramorum*'s ability to grow in culture may be affected by environmental and host conditions in the nursery or forest where the sample was collected. These conditions could include high temperatures, drought stress, or a previous fungicide treatment. A positive PCR test, which depends on intact extractable DNA, is presumably less affected by these factors. In a group of 300 *Camellia* plants tested from an infected Southern California nursery, 76% of the plants were nPCR positive whereas only 42% were culture positive (Bullock et al. 2006). In an early study in Oregon, PCR was not found to be more sensitive than culture but this was possibly because only one punch per leaf was tested for each sample, with a total sample size of 24 (Osterbauer and Trippe 2005). Unfortunately, we did not obtain PCR and culture results for every *P. ramorum*-positive sample for comparison. If a sample was culture positive, resources were not always used to determine whether the sample was also PCR positive.

False positives in the nPCR test, as measured by *Hae*III digestion of the nPCR amplicon, were much more common before the

use of qPCR in 2007. This may be because the qPCR is incorrect less often than the nPCR, or because there was no obvious alternate method to evaluate whether the qPCR-tested samples were truly positive. The latter seems more likely because *P. hibernalis* and *P. foliorum* tested falsely positive if present in large amounts that were tested in the positive Ct range from 33 to 36 in qPCR performed during a ring test (Martin et al. 2009). A test using *P. ramorum*-specific primers to a portion of the elicitor gene (Bilodeau et al. 2007) is now available as an additional confirmatory test which does not cross amplify with *P. hibernalis* or *P. foliorum*.

DNA extracted from certain host genera such as *Sequoia* and *Arctostaphylos* failed to amplify using control primers more frequently than those from other genera. The reason for this is unknown, although polysaccharides and complex phenolic compounds such as humic acids present in plants and decayed plant material have been identified as causes of PCR inhibition in other systems (Rädström et al. 2004; Tebbe and Vahjen 1993). In addition, Taq polymerases vary in sensitivity to inhibitory compounds, with Taq polymerases from some suppliers up to eight times more resistant to inhibitors in one study (Abu and Radstrom 1998). In our laboratory, we are usually able to obtain amplifiable DNA from a failed plant sample by diluting the initial DNA extract, reextracting the plant sample, or extracting a new replacement sample from the same location.

Examining the time series of data for confirmed cases indicated that there is no seasonal component in detection of *P. ramorum* either by ELISA or PCR testing. Under the expectation that the pathogen is easier to detect in samples collected from January through August, a higher proportion of samples was collected each year during this period. However, after removing the seasonal effect of sampling strategy from both the forest and nursery data sets, there was no strong evidence of an underlying seasonal effect in detectability of the pathogen by PCR and ELISA. However, isolation of the pathogen into culture was found to be seasonally dependent and, consequently, so were the data for final determination of pathogen

Table 5. Frequency plant host species failures to amplify the internal control primers in either the conventional or real-time polymerase chain reaction (PCR) test for *Phytophthora ramorum*

Host	Conventional PCR	Real-time PCR	Total
<i>Sequoia</i> spp.	3	14	17
<i>Arctostaphylos</i> spp.	5	11	16
<i>Camellia</i> spp.	2	7	9
<i>Heteromeles arbutifolia</i>	0	8	8
<i>Arbutus unedo</i>	3	4	7
<i>Rhododendron</i> spp.	3	4	7
<i>Umbellularia californica</i>	3	1	4
<i>Photinia fraseri</i>	0	3	3
<i>Quercus</i> spp.	0	3	3
<i>Podocarpus</i> spp.	0	2	2
<i>Rhus integrifolia</i>	0	2	2
Unknown	1	1	2
<i>Viburnum</i> sp.	1	1	2
<i>Morus nigra</i>	1	0	1
<i>Pittosporum undulatum</i>	1	0	1
<i>Melaleuca</i> sp.	1	0	1
<i>Begonia</i> sp.	0	1	1
<i>Abies</i> sp.	0	1	1
<i>Adiantum aleuticum</i>	0	1	1
<i>Hemerocallis</i> sp.	0	1	1
<i>Ilex</i> sp.	0	1	1
<i>Leucothoe fontanesiana</i>	0	1	1
<i>Loropetalum chinense</i>	0	1	1
<i>Malosma laurina</i>	0	1	1
<i>Pelargonium</i> sp.	0	1	1
<i>Rumohra adiantiformis</i>	0	1	1

Fig. 4. Seasonally adjusted time series and corresponding autocorrelations for **A** and **C**, polymerase chain reaction (PCR) and **B** and **D**, enzyme-linked immunosorbent assay (ELISA) positive samples from nurseries. Horizontal lines in the autocorrelograms indicate the Bartlett band 95% confidence interval for a zero correlation. Bars crossing the Bartlett band thresholds indicate the presence of significant autocorrelations between samples collected at the interval indicated on the horizontal axis. For example, in the nursery samples, pathogen status as indicated by ELISA was significantly correlated between adjacent months (i.e., at lag 1) in the data series.

Fig. 5. Seasonally adjusted time series and corresponding autocorrelations for **A** and **C**, polymerase chain reaction (PCR) and **B** and **D**, enzyme-linked immunosorbent assay (ELISA) positive samples from forests. Horizontal lines in the autocorrelograms indicate the Bartlett band 95% confidence interval for a zero correlation. Bars crossing the Bartlett band thresholds indicate the presence of significant autocorrelations between samples collected at the interval indicated on the horizontal axis. ELISA samples show significant correlation between adjacent months in the series.

Fig. 6. Seasonally adjusted time series and corresponding autocorrelations for isolation into culture for **A** and **C**, forest and **B** and **D**, nursery positive samples. Horizontal lines in the autocorrelograms indicate the Bartlett band 95% confidence interval for a zero correlation. Nursery samples show significant autocorrelation at lags 1, 12, and 13. Forest samples show significant autocorrelation at lags 1 and 12.

Fig. 7. Seasonally adjusted time series and corresponding autocorrelations for final determination of pathogen status for **A** and **C**, forest and **B** and **D**, nursery positive samples. Nursery samples show significant autocorrelation at lags 1, 12, and 13. Forest samples show significant autocorrelation at lag 2.

presence, which include information from all three of the diagnostic procedures.

The data set collected during this study is a unique resource of information on the dynamics of *P. ramorum* in California, and the analysis presented here was intended only to examine one question about those dynamics, focused on the issue of detectability. More detailed analysis and statistical modeling of the data set is clearly possible, particularly by connecting it with corresponding weather data, but such analyses are beyond the scope of this report. To our knowledge, this is the first analysis of using time series analysis on a large plant diagnostic data set spanning a 5-year period. These data are derived from samples collected from wild lands and California nurseries as part of a regulatory response to *P. ramorum*. Further validation and standardization of diagnostic protocols by individual laboratories, the National Plant Diagnostic Network, and the USDA-APHIS-PPQ, together with increased computational capabilities within electronic diagnostic repositories, will provide additional and robust analyses of diagnostic data records like the ones evaluated in this study.

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