

Comparing *Phytophthora ramorum* Diagnostic Protocols for the National Sudden Oak Death Stream Monitoring Program¹

W. Sutton,² E.M. Hansen,² P. Reeser,² and A. Kanaskie³

Abstract

Oregon was a participant in the pilot test of the national stream monitoring protocol for SOD. We routinely and continuously monitor about 50 streams in and near the SOD quarantine area in southwest Oregon using foliage baits. For the national protocol, we added six additional streams beyond the area of known infestation, and compared results from different diagnostic tests with results from six streams within the quarantine area. Foliage baits were in streams for two weeks, then assayed by: 1) culture on selective medium; 2) PCR diagnosis using the *P. lat* multiplex primers; 3) nested PCR using the Garbelotto and others protocol; and 4) Real-Time PCR using the CSL protocol. Most samples at most sample times gave consistent results, regardless of diagnostic method. However, occasional positive results from the various molecular protocols were not supported by isolation in culture, and despite intensive surveys near the streams, no plants infected by *P. ramorum* were located. We concluded that culturing from leaf baits was the single most reliable diagnostic method. False positives arose from several sources, including laboratory error, insufficient specificity of primers, and presence of undescribed *Phytophthora* species in the streams.

Key words: *Phytophthora ramorum*, sudden oak death, PCR, stream monitoring.

Introduction

Oregon was a participant in the 2006 pilot survey for *Phytophthora ramorum* in forest streams in the United States, coordinated by Steve Oak of the United States Department of Agriculture (USDA)-Forest Service. The objective of the pilot survey was to develop and modify a standard stream baiting and diagnostic protocol for use across the country. Oregon routinely and continuously monitors about 50 streams in and near the sudden oak death (SOD) quarantine area in southwest Oregon using rhododendron and tanoak leaf baits. For the national pilot survey, we added six additional streams beyond the area of known infestation, and compared results from different diagnostic tests with results from six streams within the quarantine area (fig.1 and table 1). Foliage baits in nylon mesh bags were in streams for 2 weeks, then assayed using four diagnostic techniques.

Isolation on Selective Medium

Lesions and petioles of stream bait leaves were plated on CARP+BH and later examined for *P. ramorum*.

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² Oregon State University, Botany and Plant Pathology, Corvallis, OR 97331 Corresponding author: Everett Hansen, hansene@science.oregonstate.edu.

³ Oregon Department of Forestry, Salem, OR 97310.

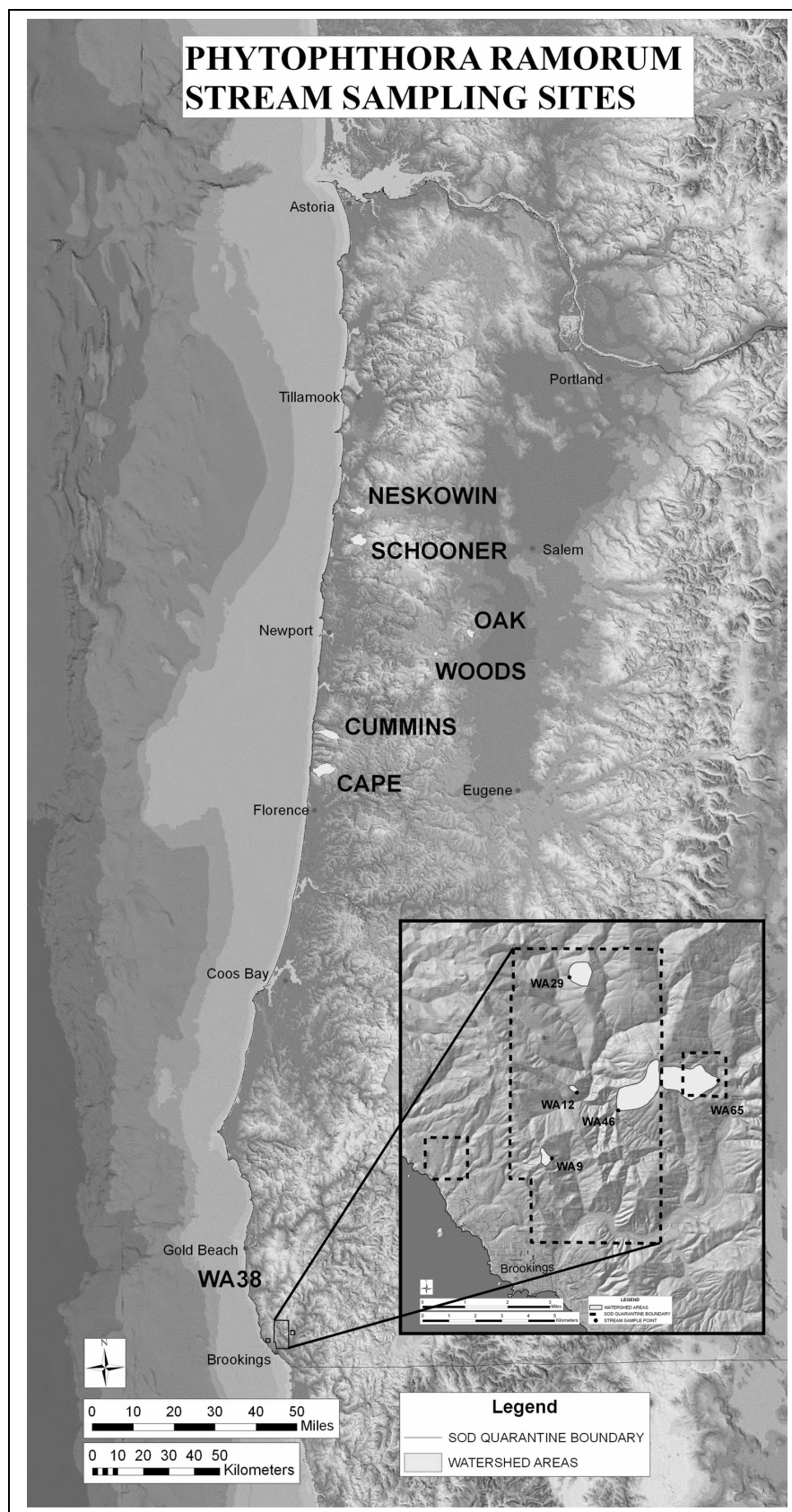


Figure 1—Map of Southwest Oregon emphasizing monitored watersheds and 2006 quarantine boundaries.

Table 1—Site and vegetation information for the 12 Oregon streams selected for the 2006 pilot survey of the National Stream Monitoring Protocol for SOD

STREAM	LOCATION	WATERSHED AREA (hectares)	VEGETATION TYPE	PREDOMINANT TREE SPECIES	DISTANCE TO KNOWN <i>P. RAMORUM</i> OCCURRENCE (Nursery or Forest) (kilometers)	<i>P. RAMORUM</i> HISTORY
WA9	Curry Co.	18	Tanoak-Douglas-fir forest (50%); Douglas-fir (50%)	Tanoak, Douglas-fir	0	Culture and PCR positive since 2002
WA12	Curry Co.	6	Tanoak-Douglas-fir forest	Tanoak, Douglas-fir	0	Culture and PCR positive since 2002
WA38	Curry Co.	16	Tanoak forest (50%); Douglas-fir (50%)	Tanoak, Douglas-fir	17	Ramorum never confirmed
WA46	Curry Co.	169	Tanoak-Douglas-fir forest	Tanoak, Douglas-fir	0	PCR + since 2004, Infected tanoak found in 2005
WA65	Curry Co.	194	Redwood-Douglas-fir-tanoak forest (30%); tanoak-Douglas-fir (70%)	Tanoak, Douglas-fir, redwood	0	Culture and PCR positive since 2005, infected tanoak 2006
WA29-2	Curry Co.	64	Tanoak-Douglas-fir forest	Tanoak	1	Culture and PCR positive in 2006, source plant never found
Cape	Lane Co.	3,094	Douglas-fir-hemlock-red cedar forest (50%); Spruce-hemlock (40%); mixed conifer-deciduous (10%)	Douglas-fir, hemlock, alder	30	Ramorum never confirmed
Cummins	Lane Co.	2,131	Douglas-fir-hemlock-red cedar forest (60%); Spruce-hemlock (30%); mixed conifer-deciduous (10%)	Douglas-fir, hemlock, alder	15	Ramorum never confirmed
Neskowin	Tillamook Co.	1,290	Douglas-fir-hemlock-red cedar forest (70%); mixed conifer-deciduous (30%)	Douglas-fir	38	Ramorum never confirmed
Oak	Benton Co.	773	Douglas-fir-hemlock-red cedar forest (75%); mixed conifer-deciduous (20%)	Douglas-fir	38	Ramorum never confirmed
Schooner Woods	Lincoln Co.	2,121	Douglas-fir-hemlock-red cedar forest (60%); mixed conifer-deciduous (40%)	Douglas-fir, hemlock, alder	25	Ramorum never confirmed
	Benton Co.	1,000	Douglas-fir-hemlock-red cedar forest	Douglas-fir	50	Ramorum never confirmed

***Phytophthora lateralis* Multiplex PCR Assay**

The *P. lateralis* (*P.lat*) multiplex primers were developed as a species specific diagnostic for *P. lateralis* before *P. ramorum* was discovered (Winton and Hansen 2001). The assay has one set of primers for *P. lateralis* (amplification indicating the presence of *P. lateralis*), and a second set that amplifies plant DNA (amplification indicating a successful extraction and PCR reaction). Because of the similarity of the *P. lateralis* and *P. ramorum* sequences in the target region, the *P. lateralis* primers also consistently amplify *P. ramorum* DNA.

Nested PCR Assay

The nested protocol, published by Garbelotto and others (2002)., was developed for *P. ramorum* and has the advantage of two amplification steps, aiding in the detection of low DNA copy samples. In round one the first set of primers is *P. ramorum* specific, bracketing 5.8S, and amplifying portions of both ITS1 and ITS2. The round two reaction primers target a portion of ITS2 that falls within the round one amplicon and uses the diluted product of round one for template.

CSL Real-time PCR Assay

The CSL protocol was developed as a real-time *P. ramorum* specific PCR assay (Tomlinson and others 2005, Hughes 2003). *Phytophthora ramorum* specific primers and labeled probe, along with primers and probe designed to amplify plant DNA, are run together. Using this technique eliminates the need to visualize results on a gel.

Materials and Methods

Rhododendron and tanoak leaf baits were washed and examined for lesions or other evidence of infection after a two week baiting period. Petioles and lesions were excised and each piece was cut in two, half being plated for isolation onCARP+BH and half being extracted with a CTAB – modified Qiagen DNeasy Tissue Kit extraction. The same DNA extract was used for all three PCR assays.

The selective medium CARP+BH was made using 17grams of BBL Corn Meal Agar and 1 liter of de-ionized water. After autoclaving and cooling, the medium was amended with 20 ppm Delocid (50 percent natamycin salt), 200 ppm Ampicillin

sodium salt, 10 ppm Rifamycin SV sodium salt, 30 ppm Benlate (benomyl 50WP) and 25 ppm Hymexazol (99 percent).

Isolation plates were incubated at 20°C. Plates were examined at approximately days 3 and 7 for *P. ramorum* and other *Phytophthora* species. All *Phytophthoras* were transferred to CARP for further clean up and eventually to corn meal agar augmented with beta sitosterol.

The *P. lat* multiplex PCR was performed in 15 µL reactions of 1X enzyme buffer, 200 µM dNTP, 0.4 µM PLITS87F and PLITS786R (*P. lateralis* primers), 0.07 µM NS1 and NS2 (universal primers), 0.15 µL 5 percent blocking powder, 0.8 U RedTaq DNA polymerase, and 2 µL DNA template. The thermal cycler conditions were 2 minutes at 94°C, 35 cycles of 94°C for 30 seconds, 52°C for 30 seconds and 72°C for 1 minute, followed by 72°C for 4 minutes and 24°C for 1 minute. The products were visualized with ethidium bromide under UV light in a 1.5 percent agarose gel.

The nested PCR round one was performed in 25 µL reactions of 1X enzyme buffer, 200 µM dNTP, 0.4 µM Phyto1 and Phyto4, 0.5 µL 5 percent blocking powder, 1.25 U RedTaq DNA polymerase, and 2 µL DNA template. Round two used the same reaction cocktail replacing Phyto1 and 4 with Phyto2 and Phyto3, and adding 2 µL of round one PCR product diluted 1:10. The thermal cycler conditions were 85 seconds at 94°C, 34 cycles of 93°C for 35 seconds, 62°C for 55 seconds and 72°C for 50 seconds + 5 seconds/cycle, followed by 72°C for 10 minutes. The products were visualized with ethidium bromide under UV light in a 1.5 percent agarose gel.

The CSL real-time PCR was performed in 25 µL reactions of 1X Taqman Universal master mix, 0.0 5uM Pram 114-FC, 0.2 µM Pram 1527-190R 0.1 µM Probe 1527-134T (for *P. ramorum*), 0.1 µM COX-F, COX-RW and Probe COX (universal), 2.375 µL 25 percent Trehalose and 2 µL DNA template. The thermal cycler conditions were 10 minutes at 94°C followed by 45 cycles of 94°C for 15 seconds, 60°C for 1 minute, 72°C for 1 minute and a plate read. The threshold was set at 10 standard deviations above the mean fluorescence of cycles 3 to 15. A Ct value of less than 40 cycles was considered positive, 40 thru 45 inconclusive and anything not crossing the threshold negative.

Results and Discussion

The three molecular diagnostic assays gave consistent, positive results (table 2) in known infested streams from which *P. ramorum* is regularly cultured (WA9, WA12 and WA29-2). *P. lat* multiplex PCR and CSL real-time assays also gave consistent negative results for the streams beyond the known range of SOD in Oregon forests and from which *P. ramorum* has never been cultured (Cape, Cummins, Neskowin, Oak, Schooner, Woods and WA38).

Results from *P. lat* multiplex PCR matched epidemiological evidence in all 12 streams.

Nested PCR matched 9 of 12 streams, giving round two positives in three streams not believed to be *P. ramorum* infested, possibly due to lowered specificity in the second round reaction. CSL real-time matched for 10 of 12 streams, although these results vary from sample to sample in recently infested streams with low inoculum loads.

Sites	Collection	Culture	PJat multiplex	Nested round 1	Nested round 2	CSL - Real-time
Cape	080906	Phsp	neg	neg	neg	neg
Cape	082106	Phsp	neg	neg	neg	neg
Cape	090706	Phsp	neg	neg	neg	neg
Cape	092106	Phsp	neg	neg	neg	neg
Cape	100506	Phsp	neg	neg	neg	neg
Cape	101806	Phsp	neg	neg	neg	neg
Cape	110206	Phsp	neg	neg	neg	neg
Cummins	080906	M	M	M	M	M
Cummins	082106	Phsp	neg	neg	neg	failed
Cummins	090706	Phsp	neg	neg	neg	neg
Cummins	092106	Phsp	neg	neg	neg	neg
Cummins	100506	Phsp	neg	neg	neg	neg
Cummins	101806	Phsp	neg	neg	neg	neg
Cummins	110206	Phsp	neg	neg	neg	neg
Neskowin	080906	Phsp	neg	neg	neg	neg
Neskowin	082106	Phsp	neg	neg	neg	neg
Neskowin	090706	Phsp	neg	neg	neg	neg
Neskowin	092106	Phsp	neg	neg	neg	neg
Neskowin	100506	Phsp	neg	neg	neg	neg
Neskowin	101806	Phsp	neg	neg	neg	neg
Neskowin	110206	Phsp	neg	neg	neg	neg
Oak	080906	Phsp	neg	neg	neg	neg
Oak	082106	Phsp	neg	neg	neg	neg
Oak	090706	Phsp	neg	neg	neg	neg
Oak	092106	Phsp	neg	neg	neg	neg
Oak	100506	Phsp	neg	neg	neg	neg
Oak	101806	Phsp	neg	neg	neg	neg
Oak	110206	Phsp	neg	neg	neg	neg
Schooner	080906	Phsp	neg	neg	neg	neg
Schooner	082106	Phsp	neg	neg	neg	neg
Schooner	090706	Phsp	neg	neg	neg	neg
Schooner	092106	Phsp	neg	neg	neg	neg
Schooner	100506	Phsp	neg	neg	neg	neg
Schooner	101806	Phsp	neg	neg	neg	neg
Schooner	110206	Phsp	neg	neg	neg	neg
Woods	080906	Phsp	neg	neg	neg	neg
Woods	082106	Phsp	neg	neg	neg	neg
Woods	090706	Phsp	neg	neg	neg	neg
Woods	092106	Phsp	neg	neg	neg	neg
Woods	100506	Phsp	neg	neg	neg	neg
Woods	101806	Phsp	neg	neg	neg	neg
Woods	110206	Phsp	neg	neg	neg	neg
WA9	080706	M				
WA9	082106	M				neg
WA9	090506					40.03
WA9	091806					
WA9	100206	phsp				
WA9	101506	phsp				
WA9	102906					
WA12	080706	M	neg	neg	neg	neg
WA12	082106	M				
WA12	090506					
WA12	091806					
WA12	100206	phsp				neg
WA12	101506					
WA12	102906					
WA29-2	080706					
WA29-2	082106	phsp				
WA29-2	090506	phsp				
WA29-2	091806	phsp				
WA29-2	100206	phsp				43.7
WA29-2	101506	phsp				neg
WA29-2	102906	phsp				
WA38	080706	phsp	neg	neg	neg	neg
WA38	082106	phsp	neg	neg	neg	neg
WA38	090506	phsp	neg	neg	neg	neg
WA38	091806	phsp	neg	neg	neg	neg
WA38	100206	phsp	neg	neg	neg	neg
WA38	101506	phsp	neg	neg	neg	neg
WA38	102906	phsp	neg	neg	neg	neg
WA46	080706	phsp	neg	neg		neg
WA46	082106	phsp	neg	neg		neg
WA46	090506	phsp	neg	neg		neg
WA46	091806	phsp	neg	neg	neg	neg
WA46	100206	phsp	neg	neg	neg	neg
WA46	101506	phsp	neg	neg		neg
WA46	102906	phsp				42.36
WA65	080706	M				40.52
WA65	082106	M	neg	neg	neg	neg
WA65	090506	phsp	neg	neg		neg
WA65	091806	phsp	neg	neg		neg
WA65	100206	phsp				neg
WA65	101506	phsp				neg
WA65	102906	phsp				neg

Table 2—Results for seven baiting periods on 12 streams. Streams WA9, WA12, WA29-2 and WA65 were all culture positive sites previously and/or during this monitoring period. WA38 has been PCR positive three times over the course of 3 years and WA46 is frequently PCR positive with a known inoculum source although we have not yet cultured it from stream baits. Black = tested positive, neg = tested negative, phsp = other *Phytophthora* species were cultured, M and failed = missing data and gray = Ct values >40 which are inconclusive.

The advantage of a culture positive is it is an unambiguous result. The disadvantage is that in attempting to isolate from a stream or soil sample, even with a selective medium, there can be an abundance of other *Phytophthora*-*Pythium* species that may out compete and/or interfere with the growth of *P. ramorum*. It is also possible that *P. ramorum* could be dormant or nonviable.

P. lat multiplex PCR was robust, with no ambiguity in streams outside of the known infested area, or known positive streams. However, it would be less reliable in areas of *P. lateralis* infestation.

The nested protocol's round one results were consistent with the other tests. Round two results however were problematic. Many of the additional positives were consistent with stream history and so can be attributed to the added sensitivity of the second round amplification. Some of the additional positives were apparent false positives, coming from streams well outside the known *P. ramorum* infestation, that have not been found positive with any other assay.

The CSL assay results closely matched the results of the other tests, although on several occasions it gave false negatives. Adhering to the suggested strict cycle threshold it failed to detect *P. ramorum* in infested streams with a low inoculum load (WA46 and WA65).

While a *P. ramorum* culture is the most trusted positive result, it is clearly not the most sensitive assay. PCR assays have the advantage of being able to detect *P. ramorum* even if it is no longer viable. On the other hand, with the continuing discovery of new *Phytophthoras* it is difficult to know if *P. ramorum* specific primers truly are species specific.

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