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The *Phytophthora* species assemblage and diversity in riparian alder ecosystems of western Oregon, USA

Laura Lee Sims[1]

Oregon State University, Department of Botany and Plant Pathology, Corvallis, Oregon 97330, and University of California, Berkeley, Department of Environmental Science, Policy and Management, Berkeley, California 94704

Wendy Sutton
Paul Reeser
Everett M Hansen

Oregon State University, Department of Botany and Plant Pathology, Corvallis, Oregon 97330

Abstract: *Phytophthora* species were systematically sampled, isolated, identified and compared for presence in streams, soil and roots of alder (*Alnus* species) dominated riparian ecosystems in western Oregon. We describe the species assemblage and evaluate *Phytophthora* diversity associated with alder. We recovered 1250 isolates of 20 *Phytophthora* species. Only three species were recovered from all substrates (streams, soil, alder roots): *P. gonapodyides*, the informally described "*P.* taxon Pgchlamydo", and *P. siskiyouensis*. *P. alni* ssp. *uniformis* along with five other species not previously recovered in Oregon forests are included in the assemblage: *P.citricola* s.l., *P. gregata*, *P. gallica*, *P. nicotianae* and *P. parsiana*. *Phytophthora* species diversity was greatest in downstream riparian locations. There was no significant difference in species diversity comparing soil and unwashed roots (the rhizosphere) to stream water. There was a difference between the predominating species from the rhizosphere compared to stream water. The most numerous species was the informally described "*P.* taxon Oaksoil", which was mainly recovered from, and most predominant in, stream water. The most common species from riparian forest soils and alder root systems was *P. gonapodyides*.

Key words: ecology, forest pathogens, hardwoods, microorganisms in streams, oomycetes

Introduction

In western Oregon red alder (*Alnus rubra* Bong. Betulaceae) is the most common hardwood. It is ecologically important and an economically valuable timber species (Harrington et al. 1994). White alder (*Alnus rhombifolia* Nutt.) is less frequent in the same riparian ecosystems (Johnson 1968). In 2009 an unusual amount of alder mortality was observed along the Smith River in Douglas County, Oregon. Alder trees with *Phytophthora*-type cankers and canopy dieback were reported and a preliminary survey of above ground symptoms suggested the dieback might be more widespread (Sims et al. 2014). In Europe (Gibbs et al. 1999, Streito et al. 2002, Webber et al. 2004), in Australia (Smith et al. 2006) and in the United States (Rooney-Latham et al. 2007) alarming new diseases of alder caused by species of *Phytophthora* recently had been reported.

Phytophthora species rank prominently in lists of threats to forest tree health (Hansen 2007). Examples include oak (Hansen and Delatour 1999, Jung et al. 2005) and alder decline (Gibbs 1995, Brasier et al. 2004, Jung and Blaschke 2004) in Europe, oak decline in eastern North America (Hwang et al. 2008) and sudden oak death in the western United States (Werres et al. 2001, Goheen et al. 2002, Rizzo et al. 2002). Nevertheless *Phytophthora* species also may be present, even abundant, without causing significant disease (Hansen et al. 2012a). *Phytophthora* species in Oregon forest streams and in tanoak (*Notholithocarpus densiflorus*) stands in southwestern Oregon have been surveyed (Sutton et al. 2009; Reeser et al. 2011a, b; Hansen et al. 2012b). However, *Phytophthora* species associated with alder in Oregon forests have not been examined and there has been no systematic attempt to relate the *Phytophthora* assemblage in streams to the species present in soil and roots (Hansen 2012a).

Ten lineages and at least 100 species are now recognized within *Phytophthora* (Hansen et al. 2012a, Kroon et al. 2012).The lineages are recognized based on DNA sequence similarity in the ITS (internal transcribed spacer) regions of the nuclear ribosomal genes (Cooke et al. 2000). These lineages may reflect natural relationships because the composition of clades are generally unchanged when additional gene sequences are analyzed to accompany ITS (Blair et al. 2008). The 10-clade system is now widely accepted, and in some cases clades have been associated with particular life histories (Kroon et al. 2012). The mitochondrial chromosome in *Phytophthora*, *Pythium* and many plant species include the cytochrome c oxidase subunit 1 (*Cox* 1) and subunit 2 (*Cox* 2) gene cluster (Martin et al. 2007). The spacer region

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[1] Corresponding author. E-mail: simslaura@berkeley.edu

between *Cox* 1 and *Cox* 2 is useful for sequence-based identification of many *Phytophthora* species (Kang et al. 2010). A rapid species diagnosis can be made with isolate sequence data for this region (Grünwald et al. 2011).

Species from three ITS clades (2, 6, 7) are reported causing alder disease. *Phytophthora* clade 2 includes three species: *P. pini*, *P. plurivora* and *P. siskiyouensis* reported from Oregon streams (Reeser et al. 2011b), two of which have been reported causing disease symptoms in alder. *P. plurivora* is a pathogen of European hardwoods including black alder (*A. glutinosa* L. Gaertn.) (Jung and Burgess 2009). *P. siskiyouensis* was recovered from the trunks of dying black alders in Melbourne Australia (Smith et al. 2006); it causes root collar cankers and kills Italian alders (*Alnus cordata* Desf.) in California (Rooney-Latham et al. 2007, 2009) and most recently was described as the causal agent of alder bark cankers in western Oregon (Sims et al. 2013, Navarro et al. 2015). Clade 6 *Phytophthora* species were associated with forests, streams and riparian areas in multiple studies (Vettraino et al. 2001, Winton and Hansen 2001, Balci and Halmschlager 2003, Brasier et al. 2003, Greslebin et al. 2005, Sutton et al. 2009, Hwang 2009, Remigi et al. 2009, Reeser et al. 2011b, Jung et al. 2011, Randall 2011, Hansen et al. 2012a). Clade 6 *P. gonapodyides* was reported as causing a twig dieback of alder in Denmark (Petersen 1910, Erwin and Ribiero 1999). Clade 7 *Phytophthora* species often are aggressive forest pathogens (Erwin and Ribeiro 1996, Vettraino et al. 2001, Saavedra et al. 2007, Robin et al. 2012). *P. alni* is a clade 7 species and in particular is considered a threat to alder health around the world. It is an emergent hybrid, soil- and waterborne pathogen, causing lethal collar rot of alder in Europe. Spread of the pathogen has been facilitated through streamside planting of contaminated nursery stock (Jung et al. 2009). Once introduced the pathogen spreads naturally with streams, floods and drainage water, and the pathogen negatively affects natural alder stands (Gibbs et al. 1999). The species was divided into three subspecies. The most aggressive is *P. alni* ssp. *alni* Brasier & S.A. Kirk (Brasier et al. 2004). The less aggressive *P. alni* ssp. *uniformis* Brasier & S.A. Kirk recently was found in North America in forest soils in Alaska, where it was not causing any apparent damage to the native thin-leaf alder, *Alnus incana* ssp. *tenuifolia* (Adams et al. 2008). It was not known whether any of the subspecies of *P. alni* were present in western Oregon riparian ecosystems, if they would cause root disease or if red alder would be affected.

In 2010 the Forest Health Monitoring Program of the USDA Forest Service, Pacific Northwest Region, initiated an examination of alder trees exhibiting dieback along streams in western Oregon. Bole cankers, similar to those caused by *P. alni* in Europe, were observed and were associated with canopy dieback. *P. siskiyouensis* was isolated several times from this type of canker (Sims et al. 2015) and was identified as the causal agent of bark cankers on alder in western Oregon (Sims et al. 2013, Navarro et al. 2015). The following year a subset of the 2010 transects was revisited focusing on *Phytophthora* species from surface-sterilized diseased red alder roots. Here we examine the soil, roots and stream water within riparian alder ecosystems to describe the assemblage and diversity of *Phytophthora* species and clades from 88 riparian forest transects. The study focused on *Phytophthora* species in stream water and from soil and unwashed roots (the alder rhizosphere). We were ecologically motivated to answer questions about *Phytophthora* species in riparian alder ecosystems of western Oregon: (i) is the *Phytophthora* species assemblage widespread or limited? (ii) how many *Phytophthora* species will we find? and (iii) are there particular substrate (stream water, soil, roots) determinants of species composition? (iv) does diversity change along streams or across substrates? Finally we hoped to discuss how the assemblage and diversity compared to that found in similar studies. We hypothesized that (i) the *Phytophthora* species assemblage from diseased alder stands would be widespread, because forests have evolved with pathogens including *Phytophthora* species (Hansen et al. 2015); (ii) that diversity and species richness would change along streams and across substrates because these environs differs so much themselves; (iii) that *Phytophthora alni* would be recovered from the soil, roots and streams around diseased alder because this continual association with all aspects of the substrates surrounding alder is what is found for the alder pathogen *P. alni* across Europe. This work provides a baseline of information on *Phytophthora* species in alder-dominated riparian ecosystems in western Oregon and furthers our understanding of the determinants of the composition of the *Phytophthora* community in forests. Establishing this baseline will provide a backdrop of the species expected from alder dominated riparian ecosystems in western Oregon for studies that might find exotic *Phytophthora* species.

MATERIALS AND METHODS

Survey design.—Riparian alder stands in western Oregon (west side of the Cascade Mountains to the Pacific Ocean) were surveyed 2 Jun 2010–19 Oct 2010, along 88, 100 m long by 10 m wide transects (Sims 2014) oriented lengthwise along streams. Transects were at least 0.5 km apart.

Transects were selected to include alder trees exhibiting canopy dieback, and with ready access. To assure reasonably uniform sampling across all of western Oregon, transects were divided among three subregions: the Willamette Valley subregion with streams draining into the Willamette River (33 transects); the southern subregion in SW Oregon (25 transects); and the coastal subregion with streams draining into the Pacific Ocean (30 transects). All transects were in riparian alder stands, but the amount of human disturbance in the surrounding landscapes differed and disturbance generally increased downstream. Three transects were placed adjacent to each of the 21 streams in the Willamette and coastal subregions with transects upstream, midstream and downstream. Initial sampling was from streams and the rhizosphere (soil and unwashed alder roots).

A subset of 18 of the established 88 transects was revisited Dec 2011–Mar 2012 to follow up on tree symptoms, especially bleeding cankers and root cankers. Above-ground methods and results are presented in Sims et al. (2015), below-ground are presented herein.

Sampling the assemblage.—Two 1 L water samples, five 1 L root samples and two 1 L soil samples were systematically collected per transect. Stream water was collected near alder roots that extended into the water where these were present and/or beneath an alder canopy, with one sample from near each end of each transect. Alder root samples were collected near the base of symptomatic alder trees (one sample/tree). Loose dirt was removed from roots by gentle shaking. Alder roots were identified by presence of root nodules or roots were traced from the tree base. A soil sample was collected from two locations within each transect, each beneath an alder tree with disease symptoms. Water, root, and soil samples were kept in a cooler after collection and processed within 72 h. Each sample was processed separately. Water samples were filtered, and root and soil samples were baited with Rhododendron leaves. Details are provided below.

Each water sample was divided into one 10 mL, two 50 mL and two 100 mL subsamples and each subsample was vacuum-filtered onto a 47 mm diam with a 5 uM pore size nitrocellulose Millipore© filter. Filters were inverted onto *Phytophthora* selective VARP+ medium (15 g Bactoagar, 50 mL V8 stock, 10 ppm rifamycin SV sodium salt, 20 ppm Delvocid [50% natamycin salt], 200 ppm ampicillin sodium salt, 30 ppm Benlate [benomyl 50WP], 50 ppm hymexazol). Filters were incubated on the medium surface for 72 h at 20 C. Filters were removed and plates were examined for *Phytophthora* colonies with a 20–60 × variable magnification, transillumination, dissecting microscope.

For isolation, plates with 1–10 distinct colonies visible after a 72 h incubation were selected. Plates with overlapping colonies were discarded. Visibly separable *Phytophthora* colonies were subcultured onto CARP medium (cornmeal agar, 10 ppm rifamycin SV sodium salt, 20 ppm Delvocid [50% natamycin salt], 200 ppm ampicillin sodium salt) (Reeser et al. 2011b). After an additional 48–96 h, filter plates were checked again for slow-emerging *Phytophthora* colonies and then again after 10 or more d.

Soil and root samples (1 L each) were collected in separate 2 L bags and transported to the lab in coolers. Root and soil samples were flooded with deionized water to approximately 3 cm above the soil/root line. Flooding and baiting took place within 24 h of collection. Baits were made from mature *Rhododendron* leaves grown outdoors specifically for the purpose of baiting in an Oregon State University enclosed grow area. Leaves from these plants were baited as negative controls with only DI water to ensure they were not *Phytophthora* positive before experiments; also none of the leaves had any symptoms of *Phytophthora* infection. To make the baits, 1 cm wide strips, centered on the midvein, were cut from clean, healthy, mature green leaves. Strips then were cut across the midvein into 2 mm wide pieces. Six bait pieces were placed in tea bag paper with a polystyrene-foam packing peanut to serve as a float, stapled shut, and placed on the surface of the water over the flooded soil or roots. Baits remained in place for 72 h. Bait bags were removed and opened with sterile forceps. The contents of one bait bag were placed on a clean paper towel and blotted dry. Loose dirt was removed by wiping on the paper towel (Reeser et al. 2011b). All clean, dry leaf pieces within each bait bag were partially submerged into H/2 medium (cornmeal agar, 10 ppm rifamycin SV sodium salt, 20 ppm Delvocid [50% natamycin salt], 200 ppm ampicillin sodium salt, 25 ppm hymexazol). Clean paper towels were used for processing each bait bag, and forceps were dipped in 95% ETOH and flamed between each sample. Plates were checked again at 10 or more days for *P. alni*-like oogonia. The soil and root samples were processed separately but analyzed together as "the rhizosphere".

From the subset (18 of the 88) five root samples were systematically collected per transect. Samples were brought to the lab in a cooler and processed. In the lab root samples were washed to remove soil, soaked under running water for 3 h and drained. Then 500 mL 10% chlorine bleach (0.6 % NaOCl) was added to the root sample in a bag and shaken by hand for 30 s. Root samples were rinsed with running tap water to remove bleach, and soaked under running water for an additional 10 min. Root samples were sorted into fine and woody pieces. Woody roots were defined as those with a corky outer bark layer. Fine roots were those with a thin outer epidermal layer and were <2 mm diam. Each root type was examined for cankers, necrotic spots and/or water-soaked lesions. Symptomatic pieces of roots of each root type were directly plated onto H/2 media.

Phytophthora *species identification.*—Morphological characterization to genus was based on culture morphology. *Phytophthora* was distinguished from other fungi growing on the plates by rate of growth, branching pattern and greater refraction of light by hyphae in media compared to *Pythium* species. Occasionally isolates were ambiguous (it was uncertain if they were *Phytophthora* or another oomycete such as *Pythium*). These were grown in water culture to examine zoosporogenesis. If vesicles were observed to form from sporangia before zoospore release then the isolate was classified "not *Phytophthora*" and discarded. If zoospores were observed to develop within sporangia, typical of *Phytophthora* zoosporogenesis, isolates were grouped with *Phytophthora*. For a few isolates zoosporogenesis did not

occur and these isolates were classed as uncertain identity and set aside for DNA extraction.

DNA was extracted from all putative *Phytophthora* isolates and those of uncertain identity with the aid of a DNeasy Tissue Extraction Kit (QIAGEN Inc., Valencia, California). To amplify the DNA, the *Cox* spacer primers FMPh-8 (AAGGTGTTTTTTATGGACAATGTA) and FMPh-10 (GCA AAAGCACTAAAAATTAAATATAAA) were used in a PCR cocktail and sourced from http://www.ars.usda.gov/Research/docs.htm?docid=8737. The cocktail contained 10 uL ddH20, 5.5 uL dNTPs (2.5 mM), 4.4 uL MgCl2 (25 mM), 3.4 uL 10× buffer (Sigma), 2.8 uL primer FMPH8(10 uM), 2.8 uL primer FMPH 10 (10 uM), RedTaq (Sigma), 1.7 uL (1U/uL) along with 1–5 iL extracted template DNA. The PCR cycling parameters contained eight steps set to (step 1) 94 for 2 min, (step 2) 94 for 30 s, (step 3) 52 for 30 s, (step 4) 72 for 40 seconds, (step 5) return to step 2 34 times, (step 6) 72 for 7 min, (step 7) 24 for 30 s and (step 8) end program. The PCR product was prepped for sequencing in the mitochondrial *Cox* spacer region flanking the *Cox*1 and *Cox*2 genes and then submitted for Sanger sequencing at the Oregon State University Center for Genome Research and Biocomputing sequencing center. If an isolate Cox spacer sequence was not 100% identical to any reference sequence, the internal transcribed spacer region (ITS) DNA were amplified with primers DC6 and ITS4 (Cooke et al. 2000). Amplified products were sequenced with DC6, ITS2, ITS3 and ITS4 as sequencing primers (White et al. 1990). All raw sequence traces were quality-control processed with PreGap4, which is part of the Staden package (1996). Then contiguous stretches were assembled with the Gap4 program; overlapping sequences were manually visualized to ensure quality and edit double peaks (Staden 1996). Edited sequences were aligned in Clustal X 2.1 (Larkin et al. 2007) with reference sequences of forest *Phytophthora* species available for comparison at HPLOSU. If an isolate's sequences still did not match any reference then it was grouped with the closest reference species based on clustering in a neighbor-joining phylogeny and subjected to manual examination of base pair differences. Morphology also was reexamined to determine whether the isolate conformed to a known species description. Isolates were identified to species when they grouped with a known isolate in a terminal clade. Unique sequences and reference sequences were submitted to GenBank, and accession numbers are reported in the results.

Diversity measures.—To measure diversity (Hill 1973) the following equations were defined as:

$$H = -\sum_{i=1}^{G} p_i \ln p_i \qquad \text{Shannon (1)}$$

$$D_1 = 1 - \sum_{i=1}^{G} p_i^2 \qquad \text{Simpson (2)}$$

$$D_2 = \frac{1}{\sum_{i=1}^{G} p_i^2} \qquad \text{inverse Simpson (3)}$$

where G is the number of species or clades and p_i is the proportion of species or clade i so that $\sum_{i=1}^{G} p_i = 1$. Diversity, evenness and rarefied richness were calculated with the vegan package (Oksanen et al. 2013) with the statistical program R (R core team 2013). Rarefied richness (Hurlbert 1971) was included because richness increases due to the number of individuals sampled, so rarefied richness was calculated by examining the same number of individuals (220 for substrate and 250 for location comparisons) across groups and is based on the number of total individuals recovered and accumulation curves (data not shown). Total richness also was reported and referred to simply as "richness". All diversity measures were calculated both for species and clades, and diversity was compared for different locations along stream transects and for rhizosphere vs. stream water. Simulated quantitative null models were generated with a randomization algorithm (Patefield 1981), to study the significance of community patterns of richness, evenness, and diversity measures. The location comparisons were made between upstream (null) to midstream locations, upstream (null) to downstream locations and substrate rhizosphere (null) to substrate stream. The (nsimul = 5000) *P* values were estimated with a function (function[x] diff [diversity{x}]) with the *oecosimu* wrapper in vegan with method = *r2dtable* (Oksanen et al. 2013). The simulated *P* values were computed by assuming no difference from the null distribution for either the locations or the substrates.

RESULTS

The Phytophthora *species assemblage.*—*Phytophthora* species were recovered in association with alder trees from 83 of the 88 transects sampled in western Oregon riparian ecosystems. In total 1250 *Phytophthora* isolates representing 20 species from eight of the 10 *Phytophthora* clades were recovered (TABLE I). Ten species were recovered fewer than 10 times (range 1–5). Six species were recovered more than 10 but less than 100 times (range 14–79) and four species were recovered more than 100 times (range 114–506).

An average of 14 isolates (range 0–62) representing three species (range 0–8) were identified from the combined substrate samples from each transect. Fourteen of the 20 species recovered were from three clades: clades 2 (four species), 6 (six species) and 7 (four species). The remaining six species were from several clades (1, 3, 8, 9, 10).

Two *Phytophthora* species were mainly from transects with white alder. *Phytophthora lacustris* was mostly recovered from locations with white alder (97 of 137

TABLE I. *Phytophthora* species from riparian alder ecosystems in western Oregon, by ITS clade and substrate

Species	Clade	Substrate (No. of isolates)				Total isolates
		Woody roots	Fine roots	Rhizo-sphere	Water	
P. nicotianae Breda de Haan	1	—	—	—	2	2
P. citricola Sawada	2	—	—	—	1	1
P. pini (Leonian) Gallegly, Hong, Richardson & Kong	2	—	—	—	16	16
P. plurivora T. Jung & T.I. Burgess	2	—	—	1	19	20
P. siskiyouensis Reeser & E.M. Hansen	2	1	4	5	12	22
P. pluvialis Reeser, Sutton and Hansen	3	—	—	—	1	1
P. pseudosyringae T. Jung & Delatour	3	—	3	—	111	114
P. gonapodyides (H.E. Petersen) Buisman	6	12	16	122	145	295
P. gregata T. Jung, M.J.C. Stukely & T. Burgess	6	—	—	1	2	3
P. lacustris Brasier, Cacciola, Nechw., Jung & Bakonyi	6	—	1	51	85	137
P. riparia Reeser, W. Sutton & E.M. Hansen	6	—	—	—	2	2
"*P.* taxon Oaksoil"	6	—	—	20	486	506
"*P.* taxon Pgchlamydo"	6	5	11	24	39	79
P. alni ssp. *uniformis* Brasier & S.A. Kirk	7	2	—	—	—	2[a]
P. cambivora (Petri) Buisman	7	2	2	10	—	14
P. cinnamomi Rands	7	—	—	4	—	4
P. europaea E.M. Hansen & T. Jung	7	1	—	—	—	1
P. cryptogea Pethybr. & Laff.	8	—	—	1	—	1
P. parsiana Mostowfizadeh, D.E. Cooke & Banihashemi	9	—	—	5	20	25
P. gallica T. Jung & J. Nechwatal	10	—	—	3	2	5
Total species		6	6	12	15	20
Total clades		3	4	6	6	8
Total isolates		23	37	247	943	1250

[a] Two additional isolates were recovered in a pre-survey assessment.

isolates, 71%, were from transects with white alder). It comprised 37% of the total *Phytophthora* species isolated from transects with white alder. *Phytophthora gallica* was recovered infrequently (TABLE I) but only from transects with white alder.

Incidence of Phytophthora alni.—*Phytophthora alni* ssp. *uniformis* was recovered four times (isolates 110-R-1N.1, 118-R-1K.1, 118-R-1J.3, 118-R-1101711.4) from necrotic lesions on surface-sterilized woody roots of two red alder trees. Two were recovered from a pre-survey assessment. Recovery was from two transects, one on the Smith River in Douglas County and one on Cape Creek in Lane County (only the two isolates recovered in the survey are included in TABLE I). In addition, an isolate from Smith River, identified as *P. cambivora* based on ITS sequence and morphology matched *P. alni* ssp. *alni* in the *Cox* spacer region. This *P. cambivora* isolate (112-R-1O.2) and an isolate matching *P. europaea* (112-R-1O.3) but with unique morphology, were recovered from the same alder tree root system and downstream from a tree that yielded *P. alni* ssp. *uniformis*. *P. alni* was not recovered from any rhizosphere or stream water sample.

Variability within species.—Intraspecific sequence variability was detected for half of the 20 species with 42 different sequence types recognized (TABLE II). The morphology of unique isolate sequence types within a species conformed to species descriptions, except in one case where the isolate identified by sequence as *P. europaea* differed from the original species description in having abundant amphigynous antheridia as well as paragynous antheridia. *P. europaea* was described with only paragynous attachment (Jung et al. 2002).

Twenty-five isolate (sequence) types were an exact match (100%) to an identified sequence (TABLE II).

TABLE II. *Phytophthora* species, variant sequence types isolated, matching reference isolates and GenBank accession numbers

Species	Host	Substrate	Isolate	HPLOSU reference match	GenBank accession Nos.			ITS base similarity (isolate/alternate reference)
					Cox spacer	ITS	Alternate reference ITS	
P. alni spp. *uniformis*	*Alnus rubra*	Roots	118-R-1101711.4	—	KJ666717	KJ666754	EU371545	830/831
P. cambivora	*A. rubra*	Roots	112-R-1O.2	4048.2	KJ666718	EF486693		
P. cambivora	*A. rubra*	Roots	111-R-4O.1	WA18.1-111003	KJ666719	KJ666756		
P. cambivora	*A. rubra*	Roots	112-R-2O.1	—	KJ666719	KJ666755	EF486693	1210/1210
P. cinnamomi	*A. rubra*	Rhizosphere	223-2-R.1	9641.1	KJ666720	KJ666757		
P. citricola sl	Stream	Water	15-W-1.5	III 5-100B1F	KJ666721	KJ666758		
P. cryptogea	*Alnus rhombifolia*	Rhizosphere	33-2-S.2	MRW2.3.11A	KJ666722	KJ666759		
P. europaea	*A. rubra*	Roots	112-R-1O.1	VI 1-2P	KJ666723	HM004226		
P. gallica	*A. rhombifolia*	Rhizosphere	33-14-S.1	—	KJ666725	KJ666761	KF286894	855/855
P. gallica	*A. rhombifolia*	Rhizosphere	33-4-R.1	—	KJ666724	KJ666760	KF286894	855/855
P. gonapodyides	*A. rubra*	Rhizosphere	31-1-S.2	I 2B4L	KJ666726	HM004231		
P. gregata	*A. rubra*	Rhizosphere	11-3-R.1	—	KJ666727	KJ666762	HQ012938	818/819
P. lacustris	Stream	Water	107-W-2.8	WA21-091603	KJ666730	HM004219		
P. lacustris	*A. rhombifolia*	Rhizosphere	33-2-R.1	—	KJ666728	KJ666763	JF804803	813/817
P. lacustris	*A. rhombifolia*	Rhizosphere	33-2-R.6	—	KJ666729	KJ666734	JF804803	812/817
P. nicotianae	Stream	Water	207-W-2.4	—	KJ666731	KJ666735	JX978446	849/851
P. parsiana	Stream	Water	207-W-2.6	RWC2.7.8B	KJ666701	—		
P. parsiana	*A. rubra*	Rhizosphere	111-2-R.1	WA23.3-081803	KJ666702	KJ666742		
P. parsiana	*A. rhombifolia*	Rhizosphere	33-2-R.5	—	KJ666700	KJ666741	AY659736	748/751
P. pini	Stream	Water	112-W-1.1	V 4-3P	KJ666706	HM004227		
P. plurivora	Stream	Water	3-W-1.34	151.77	KJ666708	KJ666744		
P. plurivora	Stream	Water	121-W-1.12	—	KJ666707	KJ666745	HM004223	1136/1138
P. pluvialis	Stream	Water	19-W-2.3	WA28-022404	KC853447	HM004217		
P. pseudosyringae	Stream	Water	120-W-1.11	33-2-3.1-1102	KJ666709	KJ666747		
P. pseudosyringae	Stream	Water	102-W-1.1	WA11-111302	KJ666712	KJ666749		
P. pseudosyringae	Stream	Water	125-W-2.12	WA64.2-080304	KJ666713	KJ666746		
P. pseudosyringae	Stream	Water	113-W-1.12	WA1.2-021903	KJ666711	KJ666748		
P. pseudosyringae	Stream	Water	117-W-2.8	—	KJ666710	KJ666750	HM004228	1165/1166
P. riparia	Stream	Water	208-W-2.6	208-W-2.6	JQ626581	JQ626594		
P. riparia	Stream	Water	104-W-1.16	VI_3.100B9	JQ626580	HM004225		
P. riparia	Stream	Water	33-W-2.1	—	KJ666714	KJ666751	JQ626594	1191/1193
P. siskiyouensis	*A. rubra*	Canker	222-29-B.1	33-2-3.2-1102	KJ666716	KJ666752		
P. siskiyouensis	*A. rubra*	Roots	118-R-1081011.5	—	KJ666715	KJ666753	EF490682	1131/1131
"*P.* taxon Oaksoil"	Stream	Water	101-W-1.3	WA46.3-101804	KJ666697	HM004234		
"*P.* taxon Oaksoil"	Stream	Water	101-W-1.1	VI 5-100B1F	KJ666732	KJ666736		

TABLE II Continued

Species	Host	Substrate	Isolate	HPLOSU reference match	GenBank accession Nos.			ITS base similarity (isolate/alternate reference)
					Cox spacer	ITS	Alternate reference ITS	
"*P.* taxon Oaksoil"	Stream	Water	108-W-2.3	—	KJ666733	KJ666737	HM004233	1193/1193
"*P.* taxon Oaksoil"	Stream	Water	219-W-1.1	VI 5-100B1F	KJ666732	KJ666740		
"*P.* taxon Oaksoil"	Stream	Water	122-W-2.13	—	KJ666696	KJ666738	HM004233	1191/1193
"*P.* taxon Oaksoil"	Stream	Water	123-W-1.2	—	KJ666699	KJ666739	HM004234	1191/1193
"*P.* taxon Pgchlamydo"	Stream	Water	104-W-1.14	133	KJ666703	AF541902		
"*P.* taxon Pgchlamydo"	Stream	Water	113-W-1.16	WA5.1-072003	KJ666704	HM004224		
"*P.* taxon Pgchlamydo"	*A. rubra*	Rhizosphere	102-2-R.1	WA46.3-100404	KJ666705	KJ666743		

One isolate (208-W-2.6) represented new variability in *P. riparia* and was added to the HPLOSU reference collection, and the other 16 isolate types were close (99% similar) or exact match to alternative existing published ITS species references available in GenBank (TABLE II: Alternative reference column). *P. pseudosyringae* and "*P.* taxon Oaksoil" were especially variable (five and six sequence types, respectively, TABLE II). Sequences for a representative isolate of each isolate sequence type were deposited in GenBank (accession numbers in TABLE II).

All substrates.—Three *Phytophthora* species were recovered from soil (rhizosphere), roots (both fine root and woody roots) and water: *P. gonapodyides* (clade 6), "*P.* taxon Pgchlamydo" (clade 6), and *P. siskiyouensis* (clade 2).

Stream water.—The collection from filtered stream water included 943 isolates and 16 species representing eight clades. Clade 6 species composed 81% of the isolates from stream water. The most numerous was "*P.* taxon Oaksoil", a clade 6 species, (52% of all isolates recovered from streams). Other clade 6 species included *P. gonapodyides* (16%), *P. lacustris* (9%) and "*P.* taxon Pgchlamydo" (4%). *P. pseudosyringae* (clade 3) was the third most abundant species in water (12% of isolates). Two isolates of *P. nicotianae* (clade 1) were identified and; two isolates each of *P. gregata* and *P. riparia* were identified. Four species from clade 2 (*P. citricola*, *P. pini, P. plurivora,* and *P. siskiyouensis*) collectively comprised 5% of the recovered isolates.

Rhizosphere.—The rhizosphere sampling included isolates baited from both soil and unwashed roots. There were no obvious or statistically meaningful differences (data not shown) in species composition between soil and root baits, so they were combined for analysis as rhizosphere sampling. A total of 247 *Phytophthora* isolates, representing 12 species and six clades, came from the rhizosphere. Isolates in clade 6 were most numerous (88%). *P. gonapodyides* accounted for 49% of the recovered rhizosphere isolates. Other clade 6 species recovered were *P. lacustris* (21%), "*P.* taxon Pgchlamydo" (10%) and "*P.* taxon Oaksoil" (8%). A single isolate of *P. gregata* was identified. clade 2 *P. siskiyouensis* comprised 2% of the recovered isolates from the rhizosphere with one isolate of *P. plurivora.* Clade 7 species (*P. cambivora*, *P. cinnamomi*) comprised 6% of the recovered isolates, and clade 9 *P. parsiana* comprised 2% of the rhizosphere isolates. Clade 10 *P. gallica* was represented by just three isolates. The only isolate of *P. cryptogea* (clade 8) recovered in the survey was from the rhizosphere.

FIG. 1. Alder root piece infected with *Phytophthora* species. The root has both fine and woody root necrosis. (F) Fine root necrosis, (W) woody root necrosis.

Stream water and rhizosphere comparisons.—Clade 6 species predominated in both stream water and the rhizosphere (81% and 88%, respectively). Clade 6 species recovered from both substrates included *P. gonapodyides*, *P. lacustris*, "*P.* taxon Oaksoil" and "*P.* taxon Pgchlamydo". Different clade 6 species predominated in each substrate, however, "*P.* taxon Oaksoil" comprised the majority of isolates from stream water and *P. gonapodyides* was the most abundant species from the rhizosphere. No clade 7 or clade 8 species were identified from stream water. In contrast, clade 3 species *P. pseudosyringae* and *P. pluvialis* (clade 3) were recovered only from streams.

Phytophthora *species from red alder roots.*—Six *Phytophthora* species (37 isolates) in four clades were recovered from fine roots (FIG. 1). Most isolates were members of clade 6 (76%), including *P. gonapodyides* (43% of all fine root isolates), "*P.* taxon Pgchlamydo" (30%), and a single isolate of *P. lacustris*. *P. siskiyouensis* (11%, clade 2), *P. pseudosyringae* (8%, clade 3) and *P. cambivora* (5%, clade 7) also were isolated. Six species (23 isolates) in three clades were recovered from necrotic lesions on woody surface-sterilized red alder roots (FIG. 1). Again most isolates (74%) were clade 6 species including two species, *P. gonapodyides* (52%), and "*P.* taxon Pgchlamydo" (22%). Three species from clade 7 were recovered: *P. alni* (two isolates), *P. cambivora* (two isolates), and *P. europaea* (one isolate). One isolate of *P. siskiyouensis* also was recovered. Clade 6 *P. gonapodyides* and "*P.* taxon Pgchlamydo" comprised the majority of isolates recovered from both fine and woody roots. Clade 2 *P. siskiyouensis* was recovered from both root types but was recovered less frequently than *P. gonapodyides* and "*P.* taxon Pgchlamydo". *P. alni* ssp. *uniformis* and *P. europaea* were recovered only from alder roots in this study.

Diversity.—*Phytophthora* species diversity was compared at different locations along streams, using the upstream sites as a means of comparison to midstream and downstream. There was statistically greater species diversity, greater richness (including rarefied richness) and evenness in downstream transects compared to upstream transects (TABLE III). There was not statistical support for a difference in species diversity when comparing midstream to upstream. Clade diversity was variable and there was some evidence for location differences, although not all diversity measures were significantly different. Clade richness was greatest midstream and downstream and evenness was greatest upstream. No evidence supported a statistically significant difference in species diversity from streams compared to the rhizosphere (TABLE III), evenness and rarefied species richness were similar as well. Differences in clade diversity from streams compared to the rhizosphere was statistically supported, but richness was the same.

DISCUSSION

The assemblage of Phytophthora *species.*—*Phytophthora* species in riparian alder ecosystems were widespread and were composed of many different species from a variety of clades. However, only half of the species composed 98% of the isolate collection, suggesting that we were isolating the rare species within the assemblage. Only one of these isolates could not be ascribed comfortably to a described species, because it belongs in the still incompletely characterized *P. citricola* species complex. However, the most common species was "*P.* taxon Oaksoil" and has been described only informally (Brasier et al. 2003). The large number of isolates of this species most likely were overlooked previously, because the alder associated assemblage of *Phytophthora* species had not been studied before in western Oregon. "*P.* taxon Oaksoil" is a sterile *Phytophthora* "species" first identified in 1999 from leaf debris from a healthy forest in France (Hansen and Delatour 1999). At the time it was recognized as a novel, sterile *Phytophthora* and was given the informal name "*P.* taxon Oaksoil" (Brasier et al 2003). It subsequently was identified in Poland and Denmark (Belbahri et al. unpubl; GenBank EU810387) and in a stream in western Oregon (Remigi et al. 2009). *Phytophthora bilorbang* is a closely related (based on ITS sequence data) but fertile species, described from Western Australia in 2012, associated with the death of wild blackberries

TABLE III. *Phytophthora* diversity comparison along streams (top) and from the rhizosphere compared to streams (lower half)

Group	Location	Shannon	*P* value*	Simpson	*P* value	Inverse Simpson	*P* value	Evenness (Pielou)	Richness	Rarefied richness (n = 250)
Species	Upstream	1.36	—	0.65	—	2.87	—	0.65	8, n = 266	7.9 ± 0.3
Species	Midstream	1.43	0.461	0.65	0.909	2.84	0.930	0.62	10, n = 339	9.4 ± 0.7
Species	Downstream	1.75	<0.001	0.75	<0.001	4.03	0.002	0.70	12, n = 268	11.8 ± 0.4
Clade	Upstream	0.67	—	0.39	—	1.63	—	0.61	3, n = 266	3.0 ± 0.0
Clade	Midstream	0.61	0.127	0.28	0.020	1.39	0.020	0.38	5, n = 339	4.9 ± 0.3
Clade	Downstream	0.72	0.419	0.36	0.090	1.56	0.120	0.45	5, n = 268	4.9 ± 0.3

Rhizosphere and stream water comparison

Group	Substrate	Shannon	*P* value	Simpson	*P* value	Inverse Simpson	*P* value	Evenness (Pielou)	Richness	Rarefied richness (n = 220)
Species	Rhizosphere	1.58	—	0.69	—	3.30	—	0.64	12, n = 247	11.7 ± 0.5
Species	Streamwater	1.58	0.804	0.69	0.763	3.20	0.643	0.58	15, n = 943	11.1 ± 1.2
Clade	Rhizosphere	0.52	—	0.22	—	1.28	—	0.29	6, n = 247	5.9 ± 0.3
Clade	Water	0.69	0.048	0.33	0.005	1.50	0.003	0.38	6, n = 943	4.8 ± 0.7

* *P* values were computed with the assumption of no difference between the locations and the substrates (location = upstream and substrate = rhizosphere were used to set the null values so *P* values are not included).

(*Rubus anglocandicans*, Aghighi et al. 2012). We think the Oregon isolates of "*P.* taxon Oaksoil" are not causing important damage to alder and that the abundance of this organism is related to the accumulation of alder leaf debris in streams (Sims and Hansen 2012). A formal description of this taxon is being prepared.

P. pseudosyringae was abundant and one of the most genetically variable species with five different isolate types recovered (TABLE III). In spite of its abundance in streams, it was recovered only rarely from alder roots and was not isolated from riparian soils. Its relationship to alder from these ecosystems is not clear at this time, but it is probably not causing serious disease for a significant number of alder trees.

Only three of the 20 species (*P. siskiyouensis*, "*P.* taxon Pgchlamydo" and *P. gonapodyides*; TABLE I) were identified from every substrates sampled: diseased root pieces (both woody and fine), the rhizosphere around diseased trees and from streams associated with alder trees. This is what we expected to find from *Phytophthora alni* if it had been the causal agent based on the European alder disease system, where the pathogen is described as moving in streams, to infect and eventually kill alder trees along stream banks. However, based on the evidence presented here and elsewhere (Sims 2014, Navarro et al. 2015, Sims et al. 2015), *P. siskiyouensis*, "*P.* taxon Pgchlamydo" and *P. gonapodyides* are the important species in alder root and stem disease in western Oregon. Perhaps the disease described herein also extends south into California riparian forests and north into Washington forests. The western Oregon alder disease system described here may extend along the west coast of North America, although the geographic extent has yet to be explored.

A different species from clade 6 predominated the assemblage depending on the substrate examined. Similar to other studies in Oregon forests (Hansen et al. 2012a), clade 6 *Phytophthora* species dominated the streams, however, in this study Clade 6 also dominated the rhizosphere of riparian soils. Although clade 6 was similar, one species appeared to be mostly limited from entering the riparian soil environment: "*P.* taxon Oaksoil" was the most prevalent species in streams but was comparably limited in the rhizosphere and absent from roots. *P. gonapodyides* had a similar but inverse association in that it was the most common single species from the rhizosphere and *P. gonapodyides* dominated diseased alder roots but was less common from streams compared to "*P.* taxon Oaksoil". The ability of *P. gonapodyides* to cause disease in alder (Navarro et al. 2015) could explain the frequent recovery from land (roots and rhizosphere) compared to "*P.* taxon Oaksoil" that was unable to cause lesions significantly larger than controls (Navarro et al. 2015), despite its prominence in streams.

P. alni *and* P. siskiyouensis. *Phytophthora alni* ssp. *uniformis* was recovered from necrotic red alder roots of two trees but not from any other trees or substrates and was not consistently associated with diseased alder trees. Initial concern that the invasive species *P. alni* was causing decline disease of alder in western Oregon riparian ecosystems does not appear to be warranted. We did not recover *P. alni* ssp. *uniformis* from *Phytophthora* cankers on the boles of alder (Sims 2014) or from surrounding diseased alder; and *P. alni* ssp. *uniformis* was only infrequently recovered from diseased alder roots. However, we did recover *P. siskiyouensis* from alder bole cankers (Sims et al. 2015), surrounding diseased alder, and diseased alder roots. *P. siskiyouensis* is the species responsible for *Phytophthora* bole cankers in western Oregon (Sims et al. 2013, Navarro et al. 2015). *P. siskiyouensis* is probably also responsible for alder root disease along with other *Phytophthora* species such as *P. gonapodyides*, "*P.* taxon Pgchlamydo" and *P. alni* ssp. *uniformis* (Navarro et al. 2015).

New and unusual species from Oregon forests; comparing the assemblage to other studies.—The list of *Phytophthora* species from this survey was similar to results from earlier surveys in western Oregon (Reeser et al. 2011a, b). However, we did identify five species not previously reported from Oregon forests in addition to *P. alni* ssp. *uniformis*: *P. citricola* s.l., *P. gallica, P. gregata, P. nicotianae* and *P. parsiana*. Each of these was recovered only from transects at lower elevations in the watersheds, in areas with surrounding human disturbance. *P. nicotianae* and *P. parsiana* are reported from horticultural nurseries in Oregon and elsewhere (Parke et al. 2014, Parke and Grünwald pers comm); they may be introduced to riparian ecosystems from horticultural plantings.

P. gregata first was described from isolates recovered from declining vegetation in Australia (Jung et al. 2011). This appears to be the first report of *P. gregata* in the United States. One of the three isolates identified as *P. gregata* appears to have a hybrid history with "*P.* taxon Oaksoil". The putative hybrid was sequenced in the β-tubulin region, and the isolate had 20 double peaks, each corresponding exactly to one of the two supposed parent species (data not shown). The higher peak at each base location always corresponded to a *P. gregata* isolate from Australia (GenBank JN547605), and the lower peak always corresponded with "*P.* taxon Oaksoil".

P. gallica first was identified from forest and riparian soils in Europe (Jung and Nechwatal 2008).

Our isolates matched an isolate from Australia (GenBank DQ286726, Jung et al. 2011), and to our knowledge this is the first report of *P. gallica* in USA outside Alaska (Adams et al. 2010).

P. europaea recovered in this study matched another GenBank isolate from Oregon (HM004226, Reeser et al. 2011b) in the ITS gene region and was similar to European isolates (AF449493, Jung et al. 2002) and isolates recovered in eastern/north-central USA (DQ313222, Balci et al. 2006). *P. europaea* was isolated only once in this study, but it was from a necrotic lesion on a diseased alder root.

The diversity of Phytophthora *species.*—Capturing the diversity of *Phytophthora* species in a complex ecosystem is challenging. Presence of numerous rare *Phytophthora* species required us to collect a large number of samples. Recovery of some species requires sampling for species from different locations within ecosystems such as streams, soil and roots, which adds to the difficulty. There was little to no difference in diversity measures between the upstream and midstream transects, but diversity was most likely the same for different reasons; the midstream transects were more species rich and upstream had slightly greater evenness. There was a clear difference in diversity, evenness and species richness, including rarefied richness, between upstream and downstream; this phenomena could be due to increased disturbance downstream that creates an environment more favorable to and supportive of *Phytophthora* species that would generally not be captured in less disturbed environments. Clade diversity was more variable, and there was a small amount of evidence for location differences between upstream and midstream. Clade diversity from different locations along a stream most likely was similar because clade richness was greatest midstream and downstream and evenness was greatest upstream.

The picture that emerges both challenges and enhances our notions of *Phytophthora* ecology. The plant species and riparian nature of the surrounding habitat are likely to influence the *Phytophthora* species composition. For example, *P. gonapodyides* is often associated with forest streams in Oregon especially (Hansen et al. 2012a) and is relatively less frequent from land in forests (upland forest soils is the main source of soil samples in Hansen et al. 2012a). Here we found *P. gonapodyides* was the predominating species from forest soils (our samples were riparian), which contrasts with the finding of Hansen et al. (2012a) that rarely found *P. gonapodyides* from their (mostly upland) forest soils. The contrast suggests that riparian soil assemblage differs from upland forest soils. Studies regarding *Phytophthora* diversity could evaluate both upland and riparian areas to examine whether *Phytophthora* such as *P. gonapodyides* in western Oregon are indicator species of riparian plant communities in the region. An indicator species analysis would be a method for the evaluation.

Comparing the diversity to other studies.—This study provides a record of the diversity of *Phytophthora* species in a riparian forest ecosystem. Two other recent large-scale ecosystem-based studies provide interesting contrast. Reeser et al. (2011a) collected soil, water and diseased plant samples in several years from multiple sites in the tanoak forest ecosystem in SW Oregon. Parke et al. (2014) sampled soil, water and plants in horticultural nurseries in western Oregon. The two forest studies, this one focused on alder and the previous one on tanoak, produced similar results. In the current alder survey five of the most frequent species composed 90% of all isolates. Four of the five most frequently identified species were ITS clade 6. In the tanoak survey, 16 species were identified (not counting the locally introduced *P. ramorum*) among 924 isolates, of which the five most frequent composed 92% of isolates. Again four of the top five were clade 6. In the nursery analysis, by contrast, 23 species (not counting apparent hybrid isolates) were identified among 602 isolates. The five most frequent species included only 66% of the isolates, and only one of the five most abundant species, "*P.* taxon Pgchlamydo", was clade 6. The latter was the only species that also was important in the forest surveys. *P. plurivora* and *P. cinnamomi*, the most frequently encountered species in the nurseries, were infrequently encountered in forest surveys. *P. cinnamomi* in particular is only locally established in Oregon forests.

How does riparian *Phytophthora* species diversity compare to nursery diversity in Oregon? The combined evidence from this study and Parke et al. (2014) suggests forest streams were less diverse than recycled water from nurseries. The largest values of *Phytophthora* species diversity (Shannon diversity 1.75) and evenness (0.7) found from Oregon forest stream (TABLE III) were smaller than the minimum values of *Phytophthora* species diversity (Shannon diversity 2.23) and evenness (0.8) from Oregon nurseries that used recycled water (Parke et al. 2014). In addition, the diversity and evenness values (1.58, 0.64, respectively) we found from the rhizosphere in Oregon forests were smaller than the values (2.16, 1.78, 1.73, 1.20 and 0.90, 0.90, 0.89, 0.67) from soil/gravel in three of the four nurseries examined (Parke et al. 2014). This suggests that in many cases nursery soil/gravel has a more diverse *Phytophthora* assemblage than occurs in forests.

Conclusions.—The *Phytophthora* assemblage from and near diseased alder in alder-dominated western

Oregon riparian ecosystems is widespread, predominantly composed of clade 6 species, but also characterized by species such as *P. siskiyouensis* which causes alder bole cankers in these ecosystems (Sims et al. 2013, Navarro et al. 2015). Species diversity changes along streams increasing in downstream habitats, perhaps from disturbed downstream habitats, influenced by horticultural planting, that favor increased pathogen diversity. There was no convincing evidence for species diversity changes across substrates and there were no rigid substrate determinates of species composition, but a few, relatively rare species were detected only in one or another substrate and a couple were mostly from streams. The substrates examined are all components of the riparian system, and many of the *Phytophthora* species seem to traverse substrate boundaries within the riparian system to some extent. Still there appeared to be a limitation for some species in their ability to move through waterways and soil, which could explain, in particular, the unexpected finding that *P. alni* spp. *uniformis* did not appear to be an important component in alder disease in western Oregon.

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