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Short title: *Phytophthor*a species in streams

Phytophthora species in forest streams in Oregon and Alaska

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Abstract: Eighteen *Phytophthora* species and one species of *Halophytophthora* were identified in 113 forest streams in Alaska, western Oregon and southwestern Oregon that were sampled by baiting or filtration of stream water with isolation on selective media. Species were identified by morphology and DNA characterization with single strand conformational polymorphism, COX spacer sequence and ITS sequence. ITS Clade 6 species were most abundant overall, but only four species, *P. gonapodyides* (37% of all isolates), *P.* taxon Salixsoil, *P.* taxon Oaksoil and *P. pseudosyringae*, were found in all three regions. The species assemblages were similar in the two Oregon regions, but *P.* taxon Pgchlamydo was absent in Alaska and one new species present in Alaska was absent in Oregon streams. The number of *Phytophthora* propagules in Oregon streams varied by season and in SW Oregon, where sampling continued year round, *P.* taxon Salixsoil, *P. nemorosa* and *P. siskiyouensis* were recovered only in some seasons.

Key words: *P. megasperma*, *P. cactorum*, *P. cambivora*, *P. pini*, *P. plurivora*, *P. europaea*, *P. syringae*, *P. ramorum*.

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INTRODUCTION

Phytophthora species are best known as pathogens of agricultural crops or invasive pathogens destroying forests. Little is known about indigenous species, especially in wild ecosystems. Hansen and Delatour (1999), Sutton and Hansen (2002), Hwang et al. (2008a), Greslebin et al. (2005), Ho et al. (2002), Sutton et al. (2009) suggested that *Phytophthora* species are relatively abundant in natural streams in healthy forests, but the species present are poorly characterized, and their ecology is essentially unknown.

Attention to the causes of oak decline in Europe in the 1990s led to a concerted European Union research program (PATHOAK) to characterize *Phytophthora* populations in healthy as well as declining forests, including forest streams (Hansen and Delatour 1999), and to a working party (7.02.09) of the International Union of Forest Research Organizations (IUFRO) focused on "Phytophthoras in forests and wildland ecosystems" (Hansen and Sutton 2000). Recent epidemics of the invasive species *P. alni*, *P. ramorum* and *P. kernoviae* in Europe and North America (Hansen 2007) and the long-standing invasion of *P. cinnamomi* in Australia have triggered systematic sampling of streams for early detection of the pathogens and renewed interest in the assemblage of *Phytophthora* species already resident in streams in natural ecosystems.

Baiting or filtering irrigation water for pathogenic *Phytophthora* species is a useful monitoring tool in agricultural systems, including nurseries (Hong and Moorman 2005). Foliage and fruit of many plants have been used as baits (Erwin and Ribeiro 1996). Klotz and colleagues (1959) used lemon fruit to attract *Phytophthora* species from irrigation canals in citrus orchards. McIntosh (1966) used green pear baits to track *P. cactorum* through irrigation ditches into apple

orchards in British Columbia. Early work in forest streams focused on *P. cinnamomi* (von Broembsen 1984, Kliejunas and Ko 1976).

The primary aim of the present work was to compare methods for the collection, identification and enumeration of *Phytophthora* spp. in forest streams and to apply those methods to the *Phytophthora* assemblage in forest streams in western North America. Three methods of isolate collection from streams were compared (various leaf baits, pear fruit baits and filtration). Species were identified with morphological and growth characters as well as molecular characters, including DNA fingerprint analysis and sequencing two regions of the genome (ITS rDNA and mitochondrial COX spacer region). Results were compiled from sampling forest streams in three regions (western Oregon, southwestern Oregon and south-central Alaska) conducted at different times and with different methods. *Phytophthora* taxa recovered were identified, and intraspecific sequence variation was noted.

MATERIALS AND METHODS

Stream sampling.—Streams were selected as part of other studies in three regions of western North America, the central Coast Range of western Oregon, coastal mountains of southwestern Oregon, and south-central and interior Alaska (TABLE I, FIG. 1). Sampling locations were usually in relatively slow-moving water with good access to the bank and upstream from road crossings.

Western Oregon.—Forest streams were sampled in the central Coast Range near Corvallis. Woods Creek and Oak Creek flow eastward from the forested foothills to Marys River and then Willamette River in Benton County (TABLE I). These streams were selected for intensive sampling to provide baseline information on *Phytophthora* species in healthy forests and nearby agricultural and urban areas and because of their proximity to Oregon State University. Elevations were ca.52–150 m.

One sampling site was established in Woods Creek upstream of residential or agricultural development. Five sites were established in the Oak Creek watershed, as well as downstream rivers. Site 1 was in forest near the headwaters of the stream. Site 2 was about 3 km downstream at the edge of the forest. It and the Woods Creek site were situated similarly. Site 3 was about 4 km further downstream in an area of oak savannah, scattered housing and

mixed agricultural use. Site 4 was in Marys River just below its junction with Oak Creek and about 4 km below site 3, and site 5 was another 1 km downstream in the Willamette River. The Marys and Willamette rivers have large watersheds that include mixed forest, agricultural and residential uses. Upland forests are in the western hemlock forest zone (Franklin and Dyrness 1988), dominated by Douglas-fir (*Pseudotsuga menziesii*). Closer to the Willamette River, oak savannah and upland prairie vegetation types give way to agricultural and residential uses. Riparian tree species present at all sites included Douglas-fir, grand fir (*Abies grandis*), big leaf maple (*Acer macrophyllum*) and red alder (*Alnus rubra*).

To estimate the seasonal variation in density of *Phytophthora* propagules in western Oregon streams water samples were collected and processed by filtration at monthly intervals Dec 1998–Mar 2001 from the site on Woods Creek and from site 2 on Oak Creek (TABLE I). *Phytophthora* isolates were counted but not identified to species at this time. To compare effectiveness of leaf baits, pear baits and filtration and to estimate *Phytophthora* species composition Oak Creek drainage sites 1–5 were sampled biweekly Apr–Jun 2006. During this period stream discharge rates at Oak Creek sites 1–3, estimated from stream cross sectional area and flow rate, were less than 1 m^3s^{-1}. Discharge at downstream sites (http://wdr.water.usgs.gov/wy2006/; USGS sites 14171000 and 14174000) decreased across the sampling period 9–2 m^3s^{-1} at the Marys River site and 371–252 m^3s^{-1} at the Willamette River.

SW Oregon.—Sixty forest streams in the coastal mountains of Curry County were baited systematically for *Phytophthora* spp. as part of the ongoing early detection and containment program of sudden oak death (SOD, caused by *P. ramorum*) (Sutton et al. 2009). Streams were selected for monitoring in and near the perimeter of the SOD quarantine area in watersheds considered at high risk for infestation by *P. ramorum*, including those with concentrations of tanoak (*Lithocarpus densiflorus*). A subset of sample sites was on streams draining known infested areas. Most baiting was in the upper portions of perennial streams where the terrain was steep and strongly dissected. Elevations were near sea level to about 500 m. Some streams could not be sampled during prolonged summer droughts or during winter flooding.

The forest is part of the Mixed-Evergreen (*Pseudotsuga*-sclerophyll) Zone (Franklin and Dyrness 1988). Tanoak, often with Douglas-fir, dominates the upland areas near stream sites. Red alder also is common, and Oregon myrtle wood (*Umbellularia californica*) is scattered through the area (Hansen et al. 2008). The forest is a mosaic of vegetation types and ages. About 20% of the area is Douglas-fir plantations less than 40 y old and another 20% is dominated by tanoak stands that succeeded Douglas-fir forests after wildfires and the first timber harvests in the

early 1900s. The remaining area is covered by mixed stands of tanoak, mature Douglas-fir, red alder and big leaf maple (Hansen et al. 2008).

To estimate *Phytophthora* species composition and incidence streams were sampled with leaf baits exchanged at 2 wk intervals Nov 2002–Dec 2004 (TABLE I). Sampling duration varied among streams. Late summer discharge in many streams was less than 1 m^3s^{-1}. Winter high flows exceeded summer low flows by 10 times or more and varied widely among streams.

Alaska.—Work was done in conjunction with a study of the etiology of alder decline in riparian ecosystems, with a focus on *P. alni* (Adams et al. 2008). To estimate *Phytophthora* species composition and incidence 49 streams from the Kenai Peninsula in the south central part of the state to Fairbanks in central Alaska were sampled with leaf baits in a single 2 wk interval in Jun 2008 (TABLE I). Streams varied widely in gradient and discharge rate, from small slow-moving lowland streams to rivers with watersheds up to 18 000 km^2 and discharge during the sample interval averaging 380 m^3s^{-1} (http://wdr.water.usgs.gov/; USGS water year 2008, site 15472000). Elevations were 17–1000 m. All sites had riparian vegetation including species of *Alnus*. Other trees or shrubs included species of *Salix*, *Populus* and *Picea*. Many streams were turbid, at or near peak flow with snow and glacier runoff.

Collecting and enumerating Phytophthora *isolates*.—Leaf baits were placed in open-weave nylon mesh bags, and bags were floated in relatively slow-moving water in streams, anchored to a stake, rock or streamside tree. Pear baits consisted of one firm, green d'Anjou pear in a perforated plastic box with Styrofoam flotation to keep the fruit on the surface. Pears were removed after 3 d. Leaf baits and pear baits were rinsed in tap water and examined for necroses. Leaf petioles and any symptomatic tissues were excised and portions, about 2 mm square, were plated on selective agar (CARP+: cornmeal agar [CMA] with 25 ppm hymexazol [99%], 20 ppm Delvocid [50% natamycin salt], 200 ppm ampicillin sodium salt, 10 ppm rifamycin SV sodium salt and 30 ppm Benlate [benomyl 50WP]). Isolation plates were placed at 20 C in the dark and examined at approximately 3 and 7 d. Colonies characteristic of *Phytophthora* were counted. Representative isolates were transferred to fresh CARP plates (CARP+ without Benlate and hymexazol) to confirm purity and eventually to cornmeal agar with 20 ppm β sitosterol (CMAβ) for characterization, DNA extraction and storage. Colonized agar plugs were stored at room temperature in sterile deionized water with or without hemp seed pieces.

Water was filtered directly from the stream onto 47 mm diam, 5 μm pore, nitrocellulose filters (Millipore SMWP04700, www.millipore.com) with a hand-operated vacuum filtration unit (Nalgene,

http:/nalgenelabware.com). A total of 700 mL was filtered at each sample onto six individual filters with two aliquots each of 50, 100 and 200 mL. Filters were transferred immediately, upside down, to Petri plates containing CARP+ for 24 h when filters were removed. Plates were incubated 3–5 d, then apparent *Phytophthora* colonies were counted and subcultured for purification and identification.

Identifying Phytophthora *isolates*.—Initial grouping and counting of isolates was based on macro- and microscopic appearance on CARP+ isolation plates. If multiple colonies grew on a single plate, representative colonies of each morphological type were chosen for subculture on CMA and DNA extraction from colonized agar plugs with a CTAB buffer with ethanol precipitation protocol, and further grouping based on either single-strand conformational polymorphism (SSCP) or one-way sequence of the mitochondrial COX spacer region.

We modified the SSCP fingerprinting protocol for species of *Phytophthora* of Kong et al. (2003) with fluorescent-labeling chemistry and an additional marker locus to allow quantitative matching of unknown isolates. The ITS1 region of rDNA was amplified with primers ITS6 and ITS7 (Cooke et al. 2000) labeled respectively with fluorescent HEX and FAM, yielding an approximately 300 base pair (bp) product. The mitochondrial COX gene spacer region was amplified according to Martin et al. (2004) with the modified primers FMPh8 and FMPh10 (http://www.ars.usda.gov/Research/docs.htm?docid=8737) labeled respectively with fluorescent HEX and FAM, yielding an approximately 400 bp product. Amplified products were mixed with formamide and ROX 500 marker, heated to 95 C for 3 min and cooled in ice 5 min. Samples were run at 25 C on an ABI 3100 capillary sequencer with a 36 cm array and 4% GeneScan polymer with 10% glycerol and 1× TBE. Fluorescence was analyzed with GeneScan 3.7 (www3.appliedbiosystems.com), and electrophoretic mobility was reported as scan number.

For COX spacer grouping DNA was amplified by PCR with primers FMPh8 and 10 as above and sequenced in one direction on an ABI 3100 capillary sequencer at 25 C, yielding a 400 bp product that subsequently was trimmed to about 300 bases for alignment. These single-strand sequences were aligned with other OSU reference sequences for initial species identification. Isolates not matching a reference isolate were amplified with ITS4 (White et al. 1990) and DC6 (Cooke et al. 2000) primers, and sequenced. ITS sequences were aligned with OSU reference sequences (TABLE II) then BLAST-queried against the GenBank database. Finally isolates were grouped into species units if they were morphologically similar and belonged to the same well supported terminal ITS and COX spacer clades. Names were assigned to isolates based on sequence similarity to validated reference isolates (TABLE II).

RESULTS

Diagnostic methods.—With a few exceptions (e.g. *P. ramorum*) *Phytophthora* species could not be reliably identified in isolation plates. When groups of isolates were transferred at the same time and incubated together on CMA or CMAβ, however they could be arranged in useful groups based on readily observed characters such as growth rate, colony pattern and abundance of aerial hyphae. Generally we initially recognized more colony variants than subsequent molecular characterization supported, but the numbers of isolates subjected to initial molecular comparison were greatly reduced.

The modified SSCP fingerprinting protocol combining amplified ITS and COX spacer regions was sensitive. Isolates of known species were grouped reliably. Small variations in migration rate often could be interpreted by comparisons to validated reference isolates run simultaneously. In general however more SSCP groups were differentiated than subsequent sequence analysis supported. The method is most useful if large numbers of isolates are run together with appropriate reference isolates.

Amplification and one-way sequencing of the relatively short COX spacer region also provided a quick and useful grouping of isolates. The approach was handicapped by the low number of available reference sequences, but as the database grew (http://phytophthora-id.org/) this provided an increasingly useful initial determination.

Final identification of species and species units required careful ITS sequencing. Polymorphisms often were present among isolates of a species but usually were confined to only one or two bases or appeared only as double peaks at specific positions on the electropherogram, indicating simultaneous presence of two bases. COX spacer sequences generally were more variable than ITS sequences within species but provided useful verification of species. Isolates

were considered to be part of the same species unit when they clustered in a terminal clade in both ITS and COX spacer phylogenetic analyses.

Phytophthora *diversity*.—Eighteen *Phytophthora* "species" and one *Halophytophthora* species (TABLE II) were distinguished among the 1318 isolates identified from streams. These included 12 formally named species and three species that have been recognized previously and partially described but not formally named. These identifications were supported by ITS sequence and morphology similar to published references. Variation among the isolates we collected and differences from the published descriptions are described below. In addition multiple isolates in the collection represented three undescribed species. Also a small number of isolates were characterized but could not be identified to species for various reasons. These included one group of nine isolates from SW Oregon streams that were morphologically similar to *Phytophthora* but had an unusually large ITS region that could not be aligned with known species.

Isolates in ITS Clade 6 (Cooke et al. 2000) were most frequently recovered in all geographic regions (83% of all isolates), and *P. gonapodyides* (Brasier et al. 1993) was the most frequently identified species overall (37% of all isolates). It was identified from most streams in all regions. The isolates examined closely included three different ITS sequences, distinguished by single nucleotide polymorphisms at two positions. COX spacer sequences were similarly uniform, with three variants identified, differing at three nucleotide positions.

P. taxon Pgchlamydo (Brasier et al. 2003) also was abundant in SW Oregon and western Oregon streams but was not recovered in Alaska. This species is similar in appearance to *P. gonapodyides*, but most isolates form chlamydospores in agar, especially at higher temperatures

(28 C). ITS sequences of all isolates examined were identical, except for combinations of double peaks at four bases. Two COX spacer sequence patterns differed at 10 bases.

P. taxon Salixsoil, originally described from Europe (Brasier et al. 2003), was recovered most abundantly from western Oregon streams but also from SW Oregon and Alaska streams. Some isolates from Alaska and western Oregon exactly matched ITS sequence of the European reference isolate, and others differed by double peaks at one or three nucleotide positions. Most isolates from Oregon and Alaska had identical COX spacer sequences, but a few had polymorphisms at one or two nucleotides.

Two groups of isolates represented previously unrecognized species in ITS Clade 6. New species 1 was recovered frequently from streams in Alaska but not from Oregon. It had ITS sequence closest to *P*. taxon Pgchlamydo but differed from it at eight bases. ITS sequences of the Alaska isolates examined in detail were identical except for double peaks at two bases; COX spacer sequences were identical among the isolates but fell within the range of sequence variation observed in *P*. taxon Pgchlamydo.

New species 2 is related to but distinct from *P*. taxon Salixsoil. It was identified most frequently in Alaska but also was present in western Oregon streams (TABLE II)**.** All isolates had identical ITS sequences that differed from the reference *P*. taxon Salixsoil isolate at five nucleotides plus one in/del position. Four clusters of isolates were distinguished based on COX spacer sequences, although differences were small (a single nucleotide polymorphism and single nucleotide in/dels at two positions). COX spacer sequences of New species 2 differed from *P*. taxon Salixsoil at 10 nucleotides.

Other Clade 6 taxa identified in our collection were *P*. taxon Oaksoil and *P*. *megasperma*. *P*. taxon Oaksoil first was described as a single unique isolate from soil in northeastern France

(Hansen and Delatour 1999) and subsequently considered in an examination of taxonomic variation in ITS Clade 6 (Brasier et al. 2003). In the current work it was identified at low frequency in SW Oregon and western Oregon streams but was not identified in Alaska (TABLE II). Little variation in ITS sequence was noted. Most isolates have identical sequence to the French isolate, but several have double peaks at two locations. COX spacer sequences are nearly identical in all isolates, although one group has a 30-base deletion of a region of contiguous "A" and "T" bases. Both COX spacer groups are found in western Oregon and SW Oregon.

P. megasperma sensu stricto (Hansen and Maxwell 1991) was identified infrequently in each of the regions; only a single isolate was from Alaska. This remains a variable species despite continuing segregation of unrelated taxa and sister species (Hansen et al. 2009). Isolates identified here shared a common morphology but varied among themselves and from the reference isolate in ITS sequence by up to four base substitutions with double peaks at one to three loci. COX spacer sequences of stream isolates were identical to the reference isolate or differed by one to five bases.

ITS Clade 1 was represented only by *P. Cactorum*, and it was recovered only once, from Alaska. This isolate differed at a single base from the reference isolate used by Cooke et al. (2000). Three species from ITS Clade 2 were identified. *P. siskiyouensis* was present only in SW Oregon samples. Isolates obtained from streams were identical in ITS and COX spacer sequences to the sequences of the type isolate (Reeser et al. 2007) from the same region.

Two species of the "*P. citricola* complex" were present. *P. plurivora* was recovered several times from western Oregon and SW Oregon. ITS sequences of these isolates were more variable than other species identified, but all fell within the range of variation reported in the species description (Jung and Burgess 2009). Additional isolates corresponding to *P.* "cit I"

(Kong et al. 2003, Jung and Burgess 2009) were identified in western Oregon streams. Isolates differed from the reference ITS sequence (originally identified as *P. pini* Leonian) only by double peaks at two bases. Nomenclature of the species in the "*P. citricola* complex" is in flux, but it seems likely that the name, *P. pini*, is appropriate for this group of isolates (Hong pers comm).

Three species from ITS Clade 3 were recorded. *P. nemorosa* was commonly identified in SW Oregon streams but not elsewhere. Sequence variation was not detected. *P. pseudosyringae* (Jung et al. 2003) first was described from soils in Europe and subsequently reported from mixed evergreen forests in California and Oregon, often associated with *P. ramorum* and *P. nemorosa* on leaves of *Umbellularia californica* (Hansen et al. 2006). In our study it was identified from several streams in all three regions. ITS sequences were identical to the type isolate at all bases except one, where our isolates contained either an "A" or a "G" or a double peak combining the two. COX spacer sequences showed polymorphisms or in/dels at 14 loci.

New species 3 was distinguished from *P. ilicis* and *P. pseudosyringae*, the species with nearest sequence similarity, by both morphology and DNA sequence. It is homothallic with amphigynous antheridia and weakly deciduous sporangia. All isolates had identical ITS sequences that differed from *P. ilicis*, the closest validated match in GenBank, at five bases, and from *P. pseudosyringae* by six bases. Isolates of New sp. 3 were polymorphic at three COX spacer loci, and differed from *P. pseudosyringae* at 17 bases.

P. europaea (Clade 7) first was described from oak forest soils in France and Germany (Hansen and Delatour 1999, Jung et al. 2002) and later was reported from eastern USA (Balci et al. 2006). In the present survey isolates with similar molecular sequences were recovered from Oak Creek in western Oregon but not from streams in SW Oregon or Alaska. While similar in

general morphology to the European isolates, those from Oregon lacked the characteristic funnel-shaped oogonial attachment of the type isolate, and ITS sequences differed at three bases from the type isolate. COX spacer sequences were different at three bases from the sequence of the type isolate.

P. cambivora causes serious collar rot on chinquapin trees in Oregon and has been isolated sporadically from forest soils in the state (Saavedra et al. 2007). It was recovered infrequently from three streams in SW Oregon and from two forested stream sites on Oak Creek in western Oregon but not from downstream sites in rural and urban areas. It was not found in Alaska. Stream isolates exhibited polymorphism at one ITS base pair as well as double peaks in some isolates at five bases. COX spacer sequences differed at only one base among the isolates. Our reference isolate differed from the Cooke et al. (2000) reference by a polymorphism at one base and double peaks at three additional locations.

P. syringae (Clade 8) was recovered at low frequency from a stream at a forest site and two rural agricultural sites in western Oregon. ITS sequences varied at a single base from the reference isolate, and COX spacer sequences had one variable site and three sites with in/dels. *P. ramorum*, also Clade 8, was recovered multiple times from 11 streams within the established sudden oak death quarantine area in Curry County but never from streams outside that area. It is an exotic, invasive pathogen subject to state and federal quarantine and an intensive disease containment effort in SW Oregon. Streams where *P. ramorum* was identified either drained known areas of disease occurrence or SOD was subsequently discovered in the watershed (Sutton et al. 2009).

A *Halophytophthora* (sometimes classified in *Phytophthora*) species was recovered on six occasions from streams in SW Oregon. This is perhaps the first report of this genus from

freshwater. Isolate sequences were most similar to *H. avicenniae* (Gerr.-Corn. & J.A. Simpson) H.H. Ho & S.C. Jong but our isolates differed from that species both in morphology and in ITS sequence.

Diversity by region.—Alaska. Isolates of *Phytophthora* were recovered from 28 of the 49 Alaska streams (57%) that were successfully baited in the single 2 wk sampling period including streams from the Kenai Peninsula in the south and streams north of Fairbanks in the interior. *P. gonadopyides* and other Clade 6 species were most abundant (267/274 isolates, 97%). Seven species were identified, including two undescribed species in ITS Clade 6. Both new species were recovered with both bait plant species (TABLE III) and were found in multiple streams in the southern and northern parts of the sampled area.

Western Oregon. Oak Creek, Marys River and Willamette River were sampled six times Apr–Jun 2006 at five locations. *Phytophthora* species were recovered at all locations at all sampling times (TABLE IV). Although 12 species were identified, only the most numerous species, *P. gonapodyides* and *P.* taxon Pgchlamydo, were identified at all sites at all times. Site 1, with low flow near the origin of Oak Creek, and site 5, in Willamette River, had no other species in common. *P. europaea* and *P. cambivora* were identified only from the upper two forested sample sites, and *P. megasperma*, *P. pini*, New species 2 and *P.* taxon Oaksoil were recovered only from the three downstream sites (TABLE VII).

SW Oregon. Streams were sampled in 36, 2 wk intervals Nov 2002–Nov 2004. *P. ramorum* specific diagnostic PCR (Winton and Hansen 2001), with no isolations, was done in alternating sample periods during a portion of the time. Those PCR results are not included here. *Phytophthora* spp. were recovered from all 60 streams that were baited and at all sampling intervals, although the number of colonies recovered varied by season, with higher overall

frequency in summer and fall than in winter and spring (data not shown). Twelve species were identified and species in Clade 6 were most frequent (67% of isolates), with *P*. taxon Pgchlamydo most numerous (TABLE V, FIG. 3). An average of 2.6 species (max six) were identified in individual streams.

The most abundant species (*P*. *gonapodyides* and *P*. taxon Pgchlamydo) were identified in most streams at most sample times (TABLE V). *P*. taxon Salixsoil was widely distributed but was (66 of 67 isolates) found primarily in July, August and September samples. *P*. *nemorosa* and *P*. *siskiyouensis* also were widely distributed, but most isolates were recovered February–May (52/56 isolates and 17/18 isolates respectively).

Comparison of recovery methods from streams.—In all three regions we used at least two bait species for isolation of *Phytophthora* from streams. The plant species used for baits did not affect the diversity of *Phytophthora* species recovered although it did affect the number of isolates. *Phytophthora* species usually were associated with black necroses on bait leaves, but we occasionally did recover isolates from green tissues, especially leaf petioles. When selecting leaf pieces for plating necrotic areas were preferred, but the petiole, necrotic or not, was included. For enumeration and identification we took isolates only from discrete lesions. Individual lesions sometimes coalesced on large bait leaves and only a single isolate was saved for identification. Because more leaves of small-leafed species, such as *Arctostaphylos uva ursi* in Alaska, were included in bait bags more distinct isolates were possible (TABLE III).

In Oak Creek and downstream sites *Phytophthora* propagules were captured on filters as well as three different foliage baits and pear fruit. No differences were noticed between the foliage bait plants (*Chamaecyparis*, *Rhododendron* and *Lithocarpus*), but the assemblage of species recovered from pears was different than from filters or foliage baits. *P*. *gonapodyides* and

its allies in ITS Clade 6 numerically comprised 95% of isolates recovered from filters and 91% of isolates from foliage baits, but only 77% of isolates from pear fruits. Eight different *Phytophthora* species were recovered from pears, compared to six species from filters and from leaf baits, even though in total only one-third as many isolates were recovered from pears as from filters (TABLE VI).

Sample time and location.—The numbers of *Phytophthora* propagules and the diversity of species varied by sample time and location within and among streams in each region. Data for western Oregon, where *Phytophthora* species were recovered by filtering stream water, are most complete. *P. syringae* was recovered from three locations but only at one time (TABLE IV). *P. cambivora* and *P. europaea* came only from sample sites 1 and 2 (TABLE VII). In contrast *P. pini* was recovered only from downstream sites late in the season while *P. megasperma* came from downstream sites early in the sampling period. Clade 6 groups (*P. gonapodyides*, *P.* taxon Pgchlamydo and *P.* taxon Salixsoil) tended to dominate at all locations and sample times (FIG. 3).

The number of *Phytophthora* propagules (colony forming units, cfu) per liter water was estimated at single sample locations in Oak and Woods creeks by filtration at monthly intervals Dec 1998–Mar 2001 (FIG. 2). All *Phytophthora* species were combined in this tally. CFU varied 0–185/L water across this period. The population in the two streams followed a similar pattern over time. Counts were highest in summer and fall and lowest in winter and spring. A similar seasonal pattern in recovery frequency by baiting was noted in SW Oregon streams for *P. ramorum* (Sutton et al. 2009) and all *Phytophthora* species (data not shown).

In 2006 *Phytophthora* species were counted in filtered water (cfu/L) at six times April–June from five sites in the Oak Creek/Willamette River system. Site 2 in 2006 was the same

location as the Oak Creek site where samples were taken 1998–2001. The mean for all species combined were 7.5–19.8 cfu/L (average 12.6) in these months. Average recovery was lowest at the headwaters site (1.8 cfu/L) followed by the Willamette River site (7.5 cfu/L; TABLE VII).

DISCUSSION

Phytophthora species are best known as root or foliage pathogens of cultivated plants. Water is considered central to their lives but primarily as a reservoir of inoculum and a medium for zoospore dispersal not as a habitat in its own right. It is increasingly clear however that some *Phytophthora* species, especially those in ITS Clade 6, are abundant in streams beyond agricultural areas and are seemingly resident there. This and similar studies are beginning to provide some hints to their ecology in these aquatic environments.

The methodology for sampling *Phytophthora* species in streams remains limiting. This work suggests that the choice of plant for foliar baits does not influence the assortment of *Phytophthora* species recovered, although differences among baits in seasonal availability, durability and handling might be important. The list of *Phytophthora* species from foliage baits ranked by recovery frequency differs from the water filtration list only among species infrequently recovered and might be a simple consequence of sampling intensity. Green pear fruit did yield a lower proportion of isolates of common Clade 6 species, perhaps increasing the opportunity for identification of less abundant species. It is possible that slow growing species and those with particular growth requirements are underrepresented, particularly when the most abundant species are relatively fast growing. It was observed that the density (cfu/L) of *Phytophthora* propagules estimated from a single filter was inversely proportional to the volume of water filtered (data not shown). This suggests that abundant fast growing *Phytophthora* species and *Pythium* species that could grow on hymexazol-amended medium interfered with

identification of slower growing colonies. Therefore estimates of species diversity might be enhanced by dividing water samples onto several filters.

Sequence based phylogenetic analysis has greatly aided our ability to distinguish *Phytophthora* species and provided analytical tools to test emerging biological species concepts for this large group of organisms. ITS sequence is sufficient for identification of *Phytophthora* species in nearly all instances although another DNA region, such as the mitochondrial COX spacer in this study, can add confidence to phylogenetic analyses. Isolates clustering in terminal clades based on ITS sequences provided a reasonably objective standard for delimiting species in this work. A similar standard was used in a recent analysis of *Phytophthora* species in Australian forests (Burgess et al. 2009).

The variability in number of ITS polymorphisms observed among the species highlights the futility of using any fixed number of polymorphisms to define species. Certainly a species standard that accepts 99% similarity will lump many biologically distinct taxa. Statistical interpretation of insertions and deletions and especially double peaks remains challenging. Careful alignments of isolate sequences, with polymorphisms highlighted, allow a useful visual sense of relatedness.

Use of the term "assemblage" to describe the group of *Phytophthora* species in a stream emphasizes our limited ecological understanding of these organisms. The more familiar ecological term "community" implies ecological interaction among taxa in a particular environment. We do not know how, or whether, these species are interacting with each other or with the other organisms in the ecosystem, although it seems likely that they are. They are remarkably numerous, even given the limitations of our sampling. *Phytophthora* species were

recovered in most sampling intervals and in most streams in all three regions. Both the number of individuals and the diversity of species were roughly proportional to the sampling intensity.

In western Oregon streams where filtration allowed quantification we usually isolated 5–160 *Phytophthora* colonies per liter of water. Preliminary reports of baiting and filtering for *Phytophthora* in forest streams in North Carolina and neighboring states in southeastern USA by Hwang and colleagues (Hwang et al. 2007, 2008a, b) suggested comparable results. In their work propagule numbers were 5–50 cfu/L water, with nine taxa identified in total (Hwang et al. 2008b). *P. pseudosyringae* frequently was found and comprised about 3% of all isolates (Hwang et al. 2007). *P. gonapodyides* (87% of all isolates), *P. pseudosyringae* and *P.* "*citricola*" were the only species found in all streams. As in SW Oregon streams, recovery of *Phytophthora* spp. was highest in July and lowest in winter months (Hwang et al. 2008a). Water temperatures vary relatively little between seasons in Oregon forest streams (Sutton et al. 2009); most streams are always cool to cold, but volume changes by orders of magnitude. Perhaps the sporulation rate is relatively constant, but propagules are diluted in a greater or lesser volume of water. This hypothesis is supported by the recovery of fewer *Phytophthora* from Willamette River than from its tributaries, Oak Creek and Marys River.

Focus on characterizing the *Phytophthora* populations in forest streams coupled with new molecular techniques to improve species identification have led to recognition of the common occurrence of *P. gonapodyides* and other representatives of ITS Clade 6 (Cooke et al. 2000) in waterways. In addition to phylogenetic relatedness these taxa are morphologically similar, homothallic or sterile and usually associated with water or wet soils (Brasier et al. 2003). In western North America, at least, the ambient temperature of the streams they inhabit is well

below their growth optima. They might be opportunistic pathogens but often are encountered in ecological situations without apparent disease on nearby riparian vegetation.

Several taxa have been recognized in the literature but have not yet been formally described as species. Brasier et al. (2003) described 12 taxa in ITS Clade 6. We found five of these in western North American streams and added two more. Almost all are new; only the homothallic pathogen *P. megasperma* and the most abundant *P. gonapodyides* were recognized before molecular techniques allowed their distinction. Recent work in Western Australia (Burgess et al. 2009) also identified a diverse array of Clade 6 taxa, including several that previously were unknown. The undescribed Australian species are readily distinguished from our new species by ITS sequences (data not shown). The two new Clade 6 species (and New species 3 from Clade 3) reported here will be described formally in subsequent publications.

Apparent regional differences in *Phytophthora* diversity must be interpreted cautiously, given the opportunistic nature of the sampling that resulted in different scales and durations of sampling. Only two species were identified in all three regions, the abundant *P. gonapodyides* and the rare *P. megasperma*. It is difficult to interpret species that are recovered infrequently, but the ubiquity of *P. gonapodyides* cannot be doubted. The absence of *P.* taxon Pgchlamydo from the Alaska sample is the more striking because of its abundance in SW Oregon and western Oregon streams. It is not likely that this was the result of bait bias because rhododendron leaves were used in all three regions; likewise it is unlikely this is the result of the limited sampling season in Alaska because *P.* taxon Pgchlamydo was recovered throughout the year in Oregon streams. New species 1, also in ITS Clade 6, was identified only in Alaska. It was relatively numerous and found in several streams. It will be interesting to see whether this species is present in other boreal areas.

Sampling in the western Oregon streams was limited, but the number of identified isolates was comparable to the other two regions. The 2006 sample design allowed comparison of sample times, locations downstream from forest sites and recovery methods, but the qualitative nature of results from baiting precludes statistical analysis. A total of 12 species was recovered, but the maximum number of species from all samples (baits + filters) collected at all locations at any one time was eight. In Alaska, with only one sample time, one stream yielded four of the seven species identified in the state; other streams had zero, one or two species identified. Even in SW Oregon, where individual streams were sampled repeatedly in all seasons, no single stream yielded more than six of the 13 total species identified, and at any one sample time the most species recovered from a single stream was two. This, plus evidence for seasonal as well as geographic differences in the assemblages of species of *Phytophthora* present, indicates that sampling will be necessary in all seasons and from multiple locations to get a complete census of the assemblage of species in an area. In SW Oregon, with 60 streams sampled by foliage baiting every 2 wk, the species list did not plateau until the 33rd sampling interval. Multiple samples (or larger sample sizes) are necessary to collect the most numerous species. In western Oregon the three species most frequently identified represent 86% of all isolates, yet only 10 of 30 filter samples (five locations and six sample times) contained all three. In SW Oregon the three most abundant species represented 68% of all isolates. The third most abundant species (*P*. taxon Salixsoil) first was identified from the eighth sampling interval.

Some *Phytophthora* species were notable for their absence. The Alaska sampling effort was justified by a search for *P. alni*. This invasive pathogen is destructive of riparian alder trees along European streams (Brasier et al. 1995) and recently was isolated from alder rhizosphere soil in Alaska, although it has not yet been associated with disease on Alaska alders (Adams et

al. 2008). Alaskan streams sampled here included those adjacent to areas where *P. alni* had been recovered from soil. Similar results have been obtained in Europe, where *P. alni* was more readily isolated from diseased trees and soil than from adjacent watercourses. Absent from the SW Oregon sample was *P. lateralis*. This emphasizes the host specificity to Port Orford cedar of this invasive species. Although this tree is native to SW Oregon and northern California, it has a spotty distribution and is not known from the specific watersheds sampled in this study (Sutton et al. 2009).

In summary three ecological groupings of *Phytophthora* species, defined by their behavior as pathogenic opportunists, foliar pathogens or root pathogens, can be distinguished in this work. The stream resident, opportunistically pathogenic Clade 6 group is discussed above. In SW Oregon streams a second group of *Phytophthora* species also was encountered regularly. *P. nemorosa* and *P. siskiyouensis* are known as foliar and occasional stem canker pathogens of evergreen hardwood trees (Hansen et al. 2003; Reeser et al. 2007, 2008). The distribution and behavior of these species suggest that they are endemic to this region. *P. pseudosyringae* also is associated with these trees in SW Oregon and California (Reeser et al. 2008), but it apparently has a larger ecological amplitude on a global scale. In this study it was recovered from Alaska as well, and first reports were from soil in European forests (Jung et al. 2003). It is also relatively frequent (3% of isolates) in streams in SE USA (Hwang et al. 2007). The exotic and invasive *P. ramorum* has similar distribution and host relationships in SW Oregon. In our area species that are pathogenic on foliage might be accidental in streams, deposited on leaves falling from the overstory. Finally a group of more widely known soilborne Phytophthoras was less frequently encountered. These species, including *P. cactorum*, *P. pini* and *P. plurivora*, *P. cambivora*, *P. syringae*, *P. megasperma* and *P. europaea* in this study, often are associated with root and crown

rots of woody plants but also are encountered in forest soils and streams in the absence of dramatic disease. The distribution and behavior of these soilborne species in forests remains poorly understood.

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LEGENDS

FIG. 1. Regions in Oregon and Alaska where streams were sampled for species of *Phytophthora*.

FIG. 2. Estimated densities of *Phytophthora* species (colony forming units cfu/L) recovered by filtration of stream water from Oak and Woods creeks in western Oregon.

FIG. 3. Frequency of the most commonly identified species of *Phytophthora* found in forest streams in Alaska, western Oregon and southwestern Oregon.

FOOTNOTES

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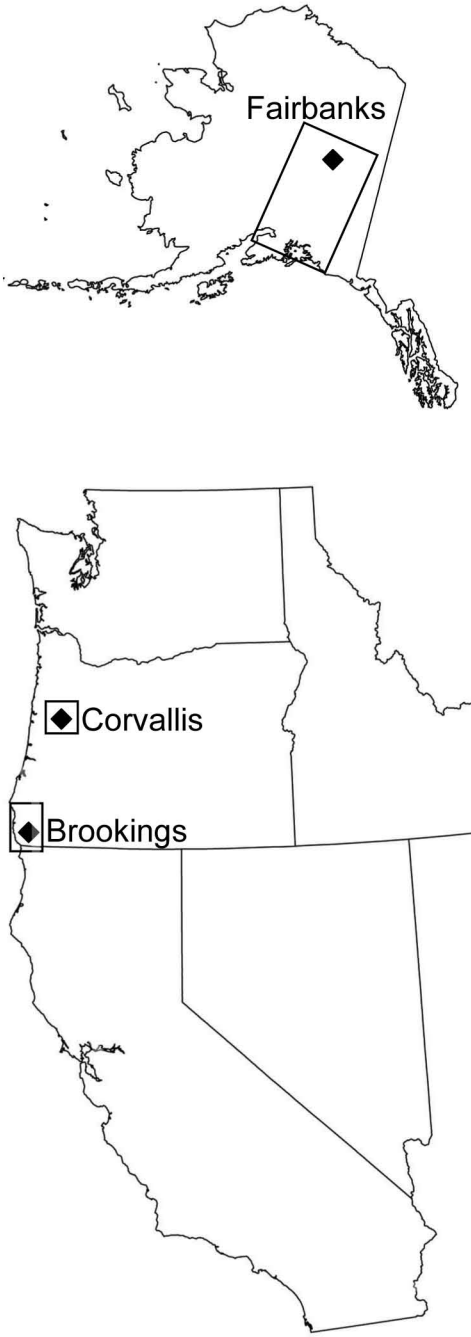
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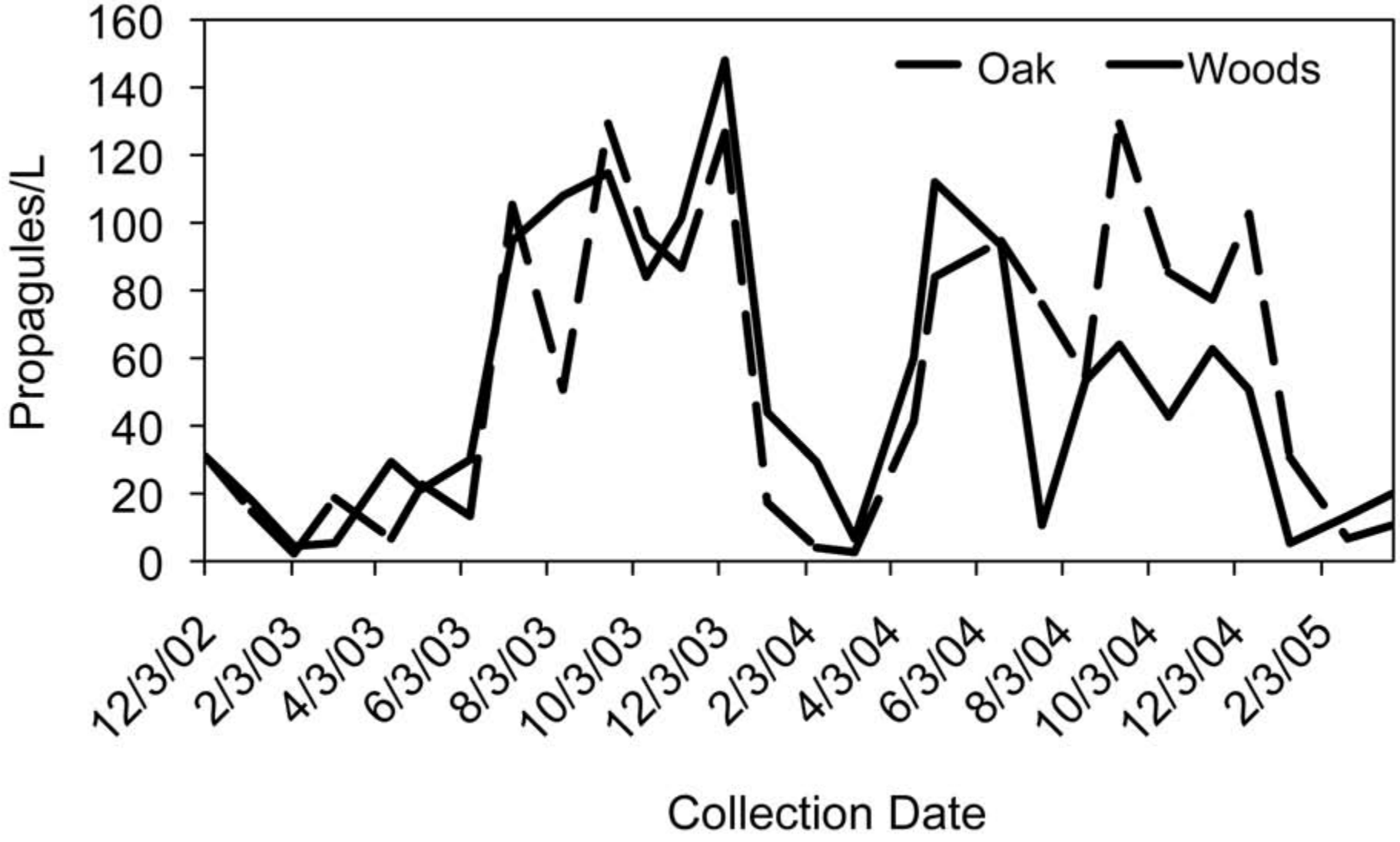
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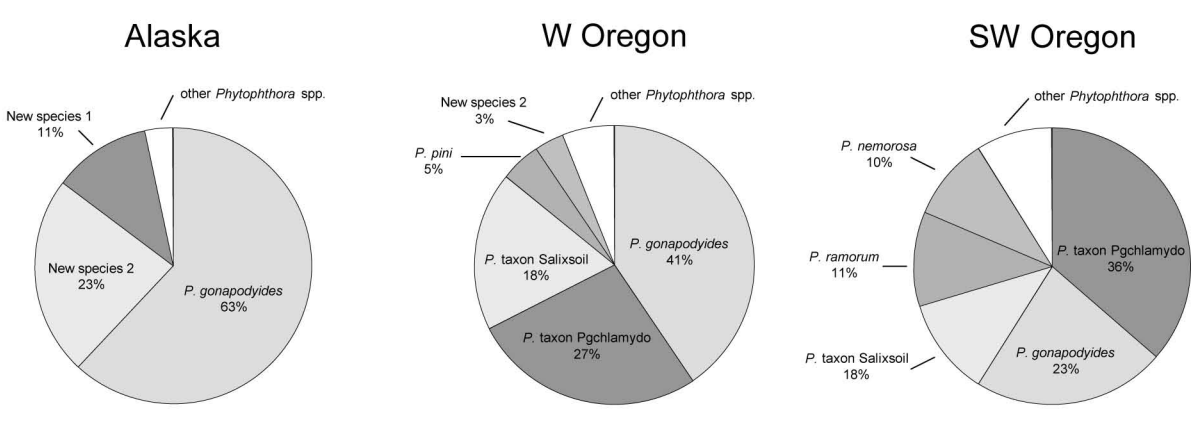
Alaska
other Phytophthora spp.
New species 1
11%
New species 2
23%
P. gonapodyides
63%
W Oregon
New species 2
3%
other Phytophthora spp.
P. pini
5%
P. taxon Salixsoil
18%
P. gonapodyides
41%
P. taxon Pgchlamydo
27%
SW Oregon
other Phytophthora spp.
P. nemorosa
10%
P. ramorum
11%
P. taxon Salixsoil
18%
P. taxon Pgchlamydo
36%
P. gonapodyides
23%

TABLE I. Locations, sampling periods, sampling intensities and methods used for recovery of *Phytophthora* species from forest streams in western North America

Region	Latitude	Number of Streams	Sample Period	Method		Number of *Phytophthora* Isolates Identified
				Filtration [c]	Bait species	
Western Oregon[a]	45°N	2	Dec 1998–Mar 2001	Monthly (Count only)	Not Done	N/A[d]
Western Oregon[b]	45°N	3	Apr–Jun 2006	Biweekly (Count and Identify)	*Chamaecyparis lawsoniana* *R. macrophyllum* *Lithocarpus densiflorus* D'Anjou pear *(Pyrus communus)*	451
Southwest Oregon	42°N	60	Nov 2002–Dec 2004	Not Done	*R. macrophyllum* *L. densiflorus*	606
Alaska	61°N to 64.5°N	49	June 2008	Not Done	*Rhododendron catawbiense* *Arctostaphylos uva-ursi*	274

[a] Oak Creek and Woods Creek, one sampling site each.
[b] Oak Creek drainage, three sampling sites on Oak Creek, one site on Marys River, one site on Willamette River.
[c] Count only: *Phytophthora* colonies were counted, but not identified to species. Count and identify: *Phytophthora* colonies were counted, and representative types were identified to species.
[d] Not Applicable. *Phytophthora* colonies on isolation plates were counted, but not identified to species.

TABLE II. *Phytophthora* species identified from forest streams in western North America, with GenBank accession numbers of new species and reference isolates and numbers of isolates by region

	OSU reference		Most similar GenBank reference					
Species[a]	**Isolate**	**GenBank accession number**	**Species**	**GenBank accession number**	**Base similarity**	**ITS clade[b]**	**Region**	**Number of isolates**
P. gonapodyides	I 2B4L	HM004231	*P. gonapodyides*	AF541890	819/819	6	Alaska	170
							West OR	183
							SW OR	133
P. taxon Pgchlamydo	WA5.1-072003	HM004224	*P.* taxon Pgchlamydo	AF541901	818/819	6	Alaska	0
							West OR	122
							SW OR	214
New species 1	AKWA58.1-0708	HM004232	*P.* taxon Pgchlamydo	AF541901	811/819	6	Alaska	31
							West OR	0
							SW OR	0
New species 2	VI 3-100B9F	HM004225	*P.* taxon Salixsoil	AF541909	810/817	6	Alaska	64
							West OR	14
							SW OR	0
P. taxon Oaksoil	WA46.3-101804	HM004234	*P.* taxon Oaksoil	AF541906	816/816	6	Alaska	0
							West OR	3
							SW OR	6
P. taxon Salixsoil	WA21-091603	HM004219	*P.* taxon Salixsoil	AF541909	816/817	6	Alaska	1
							West OR	82
							SW OR	67
P. megasperma	I 5-200BF	HM004230	*P. megasperma*	AF541898	813/813	6	Alaska	1
							West OR	5
							SW OR	2
P. cactorum	AKWA40.1-0708	HM004233	*P. cactorum*	AF266772	791/792	1	Alaska	1
							West OR	0
							SW OR	0
P. siskiyouensis	WA5-030403	EF523386	*P. siskiyouensis*	EF523386	1131/1131	2	Alaska	0
							West OR	0
							SW OR	18

P. plurivora	WA5.1-101403	HM004223	*P. plurivora*	FJ665225	759/761	2	Alaska West OR SW OR	0 1 3
P. pini	V 4-3P	HM004227	*P. pini*	GQ324989	759/761	2	Alaska West OR SW OR	0 22 0
P. nemorosa	WA9.3-030804	HM004222	*P. nemorosa*	AY332651	817/817	3	Alaska West OR SW OR	0 0 56
P. pseudosyringae	III 2-1P	HM004228	*P. pseudosyringae*	AY230190	789/789	3	Alaska West OR SW OR	6 1 11
New species 3	WA28-022404	HM004217	*P. ilicis*	AY423297	757/762	3	Alaska West OR SW OR	0 0 1
P. europaea	VI 1-2P	HM004226	*P. europaea*	AF449493	857/860	7	Alaska West OR SW OR	0 3 0
P. cambivora	WA18.1-111003	HM004220	*P. cambivora*	AF266763	827/831	7	Alaska West OR SW OR	0 10 12
P. syringae	II 2-4P	HM004229	*P. syringae*	AF266803	810/813	8	Alaska West OR SW OR	0 4 0
P. ramorum	WA12-012604	HM004221	*P. ramorum*	AY369368	798/798	8	Alaska West OR SW OR	0 0 65[c]
Halophytophthora	WA26-091603	HM004218	*H. avicenniae*	AY598668	851/856		Alaska West OR SW OR	0 0 6

[a] Species listed in order of presentation in RESULTS.
[b] Cooke et al. 2000.

[c] Number of isolates of *P. ramorum* represents number of sample times and streams that were positive for *P. ramorum*. Additional isolates were identified but not counted.

TABLE III. Recovery of *Phytophthora* species from streams in Alaska using two leaf bait species in a single two-week sampling period Jun 2008

Species[a]	Number of streams (n = 49)	Number of isolates	
		Rhododendron catawbiense	*Arctostaphylos uva-ursi*
P. gonapodyides	25	45	125
New Species 1	6	12	19
New species 2	10	22	42
P. pseudosyringae	2	6	0

[a] Only *Phytophthora* species representing 1% or more of the total *Phytophthora* isolates identified are included.

TABLE IV. Incidence of *Phytophthora* species recovered from five sampling sites in the Oak Creek drainage in Western Oregon during six sampling intervals, Apr–Jun 2006

Species[a]	Incidence	
	Sampling sites[b] (n = 5)	Sampling intervals[c] (n = 6)
P. gonapodyides	5	6
P. taxon Pgchlamydo	5	6
P. taxon Oaksoil	2	3
P. taxon Salixsoil	4	6
New species 2	3	3
P. megasperma	3	3
P. pini	3	3
P. europaea	1	3
P. cambivora	2	3
P. syringae	3	1

[a] Only *Phytophthora* species representing 1% or more of the total *Phytophthora* isolates identified are included.
[b] Three sites on Oak Creek, one site on Marys River and one site on the Willamette River.
[e] Two-week intervals ended 4 Apr, 21 Apr, 5 May, 19 May, 2 Jun and 16 Jun 2006.

TABLE V. Incidence of *Phytophthora* species recovered from 60 streams in SW Oregon by leaf baiting in 38 2 wk sampling intervals Nov 2002–Dec 2004

	Incidence	
Species[a]	Streams (n = 60)	Intervals (n = 38)
P. gonapodyides	45	26
P. taxon Pgchlamydo	36	34
P. taxon Oaksoil	6	4
P. taxon Salixsoil	11	6
P. siskiyouensis	11	9
P. nemorosa	30	10
P. pseudosyringae	8	5
P. cambivora	3	6
P. ramorum	11	50

[b] Only species representing 1% or more of the total *Phytophthora* isolates identified are included.

TABLE VI. Recovery of *Phytophthora* species from the Oak Creek drainage in western Oregon with three sampling methods. Counts are combined over five sampling sites[a] and six sampling times in 2006[b].

	Number of isolates			
Phytophthora species	Filter	Leaf baits	Pear baits	Total
P. cambivora	0	4	6	10
P. plurivora	1	0	0	1
P. europaea	2	0	2	4
P. gonapodyides	89	74	20	183
P. megasperma	5	0	0	5
New species 2	10	4	0	14
P. taxon Oaksoil	3	0	0	3
P. taxon Pgchlamydo	45	69	8	122
P. pini	6	11	5	22
P. taxon Salixsoil	38	14	30	82
P. syringae	0	1	3	4
P. pseudosyringae	0	0	1	1
Total	199	177	75	451

[a] Sites 1, 2 and 3 on Oak Creek; 4 on Marys River; and 5 on the Willamette River.

[b] Sampling times: 4 Apr, 21 Apr, 5 May, 19 May, 2 Jun, and 16 Jun 2006.

TABLE VII. Recovery of *Phytophthora* species from five sampling sites in the Oak Creek drainage in western Oreegon. Recovery from filters, leaf baits and pears is combined over six sampling times in 2006[a].

	Number of isolates					
Phytophthora species	Site 1[b]	Site 2	Site 3	Site 4	Site 5	Total
P. cambivora	8	2	0	0	0	10
P. plurivora	0	0	0	0	1	1
P. europaea	4	0	0	0	0	4
P. gonapodyides	35	54	53	31	10	183
P. megasperma	0	0	2	2	1	5
New species 2	0	0	7	1	6	14
P. taxon Oaksoil	0	0	2	0	1	3
P. taxon Pgchlamydo	4	56	27	29	6	122
P. pini	0	0	4	17	1	22
P. taxon Salixsoil	0	12	32	24	14	82
P. syringae	0	1	1	2	0	4
P. pseudosyringae	0	1	0	0	0	1
Total	51	126	128	106	40	451

[a] Sampling times: 4 Apr, 21 Apr, 5 May, 19 May, 2 Jun and 16 Jun 2006.
[b] Sites 1, 2 and 3 on Oak Creek; 4 on Marys River and 5 on the Willamette River.