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Recovery of *Phytophthora* species from drainage points and tributaries within two forest stream networks: a preliminary report[†]

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Abstract

To evaluate the number of stream sample sites needed to effectively survey a given stream network for species of *Phytophthora*, two stream networks, Davidson River and Cathey's Creek, in western North Carolina (USA) were studied. One-litre water samples were collected from the terminal drainage points and most of the tributaries in each stream network and filtered through polycarbonate membrane filters with 3-µm pores. Ten taxa of *Phytophthora* were detected in the two stream networks: six species—*P. cinnamomi*, *P. citricola*, *P. citrophthora*, *P. gonapodyides*, *P. heveae*, and *P. pseudosyringae*—and four distinct groups of isolates based on morphological and molecular characters. A total of nine taxa were detected in the Davidson River network on two sample dates in 2007, and five of these taxa eventually were found downstream at the drainage point. In the Cathey's Creek network, a total of seven taxa were found on two sample dates in 2008, and five of these taxa eventually were found at the drainage point. Even though all the taxa found within a stream network were not detected at the terminal drainage point, all of the taxa in the network that represented at least 10% of the total population were detected at the drainage point. More intensive sampling throughout a stream network may be necessary to detect a species with a low population density.

Keywords: filtration; *Phytophthora* species; stream network; survey.

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Introduction

There have been recent epidemics of *Phytophthora ramorum* in several countries in Europe and in the coastal forests of California and southwest Oregon in the United States of America (USA). These, plus the endemic nature of *P. cinnamomi* in Australia, have raised attention about the occurrence and distribution of species of *Phytophthora*—including invasive species—in natural ecosystems (Hansen, 2007). Species of *Phytophthora* are well adapted to aquatic environments and have been detected in natural and agricultural waterways (Hwang et al., 2006, von Broembsen, 1984, Wamishe et al., 2007). The USDA-Forest Service used surveys of terrestrial plants to look for symptomatic trees and shrubs while walking transects through forested areas as the primary tool for their National *P. ramorum* Early Detection Survey in Forests until 2006. However, terrestrial surveys then were replaced by a forest stream survey protocol, in which selected streams are baited with rhododendron leaves because stream surveys proved to be more

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efficient and effective at detecting *P. ramorum* and other species of *Phytophthora* in forests (Oak et al., 2008). In addition, relatively large forest areas can be surveyed by monitoring forest streams. A stream network consists of a main stream, its tributaries, and the terminal drainage point of the main stream. If the diversity of species of *Phytophthora* recovered at the drainage point represents the overall population of *Phytophthora* species within the upstream network, a stream network could be surveyed effectively at the drainage point without sampling the tributaries. To test this hypothesis, two stream networks in western North Carolina, USA were sampled, and both the density and diversity of population of species of *Phytophthora* in each stream network were examined.

Materials and Methods

Sample sites and recovery of *Phytophthora* spp.

Davidson River and Cathey's Creek stream networks in the Pisgah National Forest in western North Carolina were selected for this study. This area is designated as "high risk" for *P. ramorum* based on a favorable climate for disease development, presence of numerous host plants, and ample opportunities for introduction of the pathogen through the nursery industry in this region. The portion of the Davidson River stream network we sampled included seven tributaries as well as the drainage point of the main stream and drained a watershed that covers 35.2 km^2. The stream network was sampled twice; once in September and again in October 2007. The portion of Cathey's Creek stream network we sampled included eight tributaries as well as the drainage point of the main stream and drained a watershed that covers 29.6 km^2. This network also was sampled twice; once in June and again in October 2008. A single 1-L water sample was collected from each sample site, and all samples in a stream network were collected within a 30-min period to minimize temporal variation. Later, an additional single 1-L sample was collected at the drainage point. This was done when water from the tributary farthest upstream was estimated to reach this point based on a real-time stream-flow model: Incident Command Tool for Drinking Water Protection (ICWater) maintained by the US Geological Survey. Water samples were filtered to recover *Phytophthora* spp. as described below. In addition, a plastic mesh bag containing four detached leaves of *Rhododendron maximum* was deployed at each drainage point to compare recovery of *Phytophthora* spp. by filtration and baiting. Two to three weeks after deployment, leaves were brought to the laboratory for isolation.

Detection of *Phytophthora* spp.

Nine 100-mL aliquots from each 1-L water sample were filtered through polycarbonate membranes (47 mm in diameter) with 3-µm pores (Sterlitech Corp., Kent, WA, USA). Filters were inverted onto PARPH-V8 agar, a medium selective for species of *Phytophthora* (Ferguson & Jeffers, 1999). Isolation plates were kept in the dark at 20 °C for 3 days before filters were removed. The surface of the agar was rinsed under running tap water, and then colony forming units (cfu) of *Phytophthora* spp. were counted. Plates were returned to 20 °C in the dark for up to 10 days and examined for additional colonies of *Phytophthora* spp. *Rhododendron maximum* bait leaves were examined for lesions. Small pieces (approximately 5 mm × 5 mm) taken from the advancing margins of lesions were embedded in PARPH-V8 medium to isolate *Phytophthora* spp. Isolation plates were kept in the dark at 20 °C for up to 3 weeks and examined regularly.

Species identification

The isolates of *Phytophthora* spp. detected on filters and in leaf baits initially were grouped based on morphological characters including mycelium growth habit, oospore formation, and sporangium characters. Representative isolates from each morphological group were grown in 12% pea broth at room temperature (21-24 °C) in the dark for 5 days. Mycelium mats were removed from pea broth and washed with distilled water, and then DNA was extracted from hyphae using a DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). The internal transcribed spacer (ITS) region of the extracted DNA was amplified with the ITS4 and ITS6 primer set. Restriction fragment length polymorphism (RFLP) patterns then were created using restriction enzymes *Alu*I and *Msp*I, and these were compared to published standards (Cooke et al., 2000). Identifications of isolates were confirmed by sequence analysis of the same ITS region of the extracted DNA.

Results and Discussion

Overall, ten taxa of *Phytophthora* were detected in this study. Six of these were distinct species: *P. cinnamomi, P. citricola, P. citrophthora, P. gonapodyides, P. heveae,* and *P. pseudosyringae.* The remaining four taxa were morphologically and genetically distinct groups of isolates, which may not have been described previously. For each stream network, the population densities (i.e. numbers of cfu) varied significantly among sample sites and between months when samples were collected (Tables 1 & 2). Densities were greater in October than the earlier sample month in both stream networks. In contrast, the numbers of taxa detected in each stream network were similar among locations and between sample months (Tables 1 & 2). In the Davidson River stream network, five taxa were identified among 200 colonies recovered in September 2007 and nine taxa were identified among 289 colonies recovered in October 2007 (Table 1). In the Cathey's Creek stream network, 156 and

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TABLE 1: Densities and diversities of populations of *Phytophthora* spp. in seven tributaries and at the drainage point in the Davidson River stream network on two sample dates in 2007.

Sampling location[1]	Density[2]		Diversity[3]	
	September	October	September	October
Shuck Ridge	17	7	2	2
Daniel Ridge	14	1	3	1
Right Fork	10	8	2	3
Laurel Fork	13	21	3	3
No Name Creek	35	37	3	5
Long Branch	45	60	3	7
Cove Creek	31	75	1	5
Drainage point 1[4]	14	34	2	5
Drainage point 2[4]	21	46	3	3
TOTALS	200	289	5	9
Chi-square test[5]	$X^2 = 40.82$ $P < 0.001$		$X^2 = 4.91$ $P = 0.768$	

[1] Tributaries are listed in order based on distance to the drainage point—i.e. the farthest tributary to the drainage point is listed first.
[2] Numbers of colony forming units of *Phytophthora* spp. recovered from 900 mL of stream water collected at each sample location.
[3] Numbers of genetically and morphologically distinct taxa of *Phytophthora* recovered by filtration at each sampling location.
[4] The drainage point of the main stream was sampled twice to examine spatial variation: 1 sampled initially with other tributaries; 2 sampled later in the day.
[5] X^2 = chi square statistic with 8 degrees of freedom; P = probability of a greater chi-square value.

246 colonies were recovered in June and October 2008, respectively, and seven taxa were found at each sample date (Table 2). The lower three tributaries in the Davidson River network had a higher mean density of *Phytophthora* spp. (47 cfu/900 mL) than that in the upper four tributaries (11 cfu/900 mL). However, the upper four tributaries in the Cathey's Creek network had a higher mean density of *Phytophthora* spp. (28 cfu/900 mL) than that in the lower four tributaries (14 cfu/900 mL). Previously, seasonal and spatial variation in populations of taxa of *Phytophthora* had been reported in a study done in the same area (Hwang et al., 2009). Based on an assumption that some of the taxa found in forest streams originated from infected plants or infested soils, density and diversity of these taxa in streams may be affected by differences in vegetation composition and environmental conditions.

Not all the taxa recovered within a stream network were detected at the terminal drainage point of the main stream using the filtration method (Tables 1 & 2). In the Davidson River stream network, a maximum of three and five taxa were recovered at the drainage point in September and October 2007, respectively. In the Cathey's Creek stream network, a maximum of three taxa were recovered at the drainage point in June and four taxa were found in October 2008. Samples collected additionally later in the day at the drainage points yielded more propagules of *Phytophthora* compared to those collected initially when other tributaries were sampled. Over two sample months and two stream networks, density of *Phytophthora* spp. from samples collected later was 40% higher than the samples collected initially. However, diversity of *Phytophthora* spp. recovered from the samples collected initially did not differ from those collected later (Tables 1 and 2). More studies are needed to examine daily rhythmic activity of *Phytophthora* spp. in forest streams. With the leaf-bait method, three taxa were detected at the drainage point of the Davidson River stream network during each sample period. In the Cathey's Creek stream network, only two taxa were detected with leaf baits at the drainage point during each sample period. All of these taxa detected with leaf baits were detected also by filtration. Once again, we have demonstrated that the filtration method is more efficient than the rhododendron leaf-bait method for estimating the diversity of taxa of *Phytophthora* in natural waterways (Hwang et al., 2009). Similar results were reported by Reeser et al. (2011) with comparison of leaf baits, pears, and filtration as recovery methods for *Phytophthora* spp. in Oregon streams.

Even though not all the taxa found within a stream network were recovered at the terminal drainage point, the taxa not recovered at the drainage point were present at relatively low population densities in the samples from tributaries. Each of the taxa in the network that represented at least 10% of the total population was detected at the drainage point. These results suggest that recovery of taxa of *Phytophthora* at the terminal drainage point of a forest stream

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TABLE 2: Densities and diversities of populations of *Phytophthor*a spp. in eight tributaries and at the drainage point in the Cathey's Creek stream network on two sample dates in 2008.

Sampling location[1]	Density[2]		Diversity[3]	
	June	October	June	October
Charles Creek	37	50	4	3
Dunn Creek	42	36	5	3
Tarkiln Branch	5	18	2	3
Cedar Rock Creek	7	27	4	3
Kagle Branch	11	7	2	1
N. Prong	7	13	2	2
Walnut Cove	4	12	2	1
Kuykendall Creek	17	39	2	2
Drainage point 1[4]	8	21	2	4
Drainage point 2[4]	18	23	3	3
TOTALS	156	246	7	7
Chi-square test[5]	X^2 = 24.36 P = 0.004		X^2 = 2.16 P = 0.989	

[1] Tributaries are listed in order based on distance to the drainage point—i.e. the farthest tributary to the drainage point is listed first.
[2] Numbers of colony forming units of *Phytophthora* spp. recovered from 900 mL of stream water collected at each sample location.
[3] Numbers of genetically and morphologically distinct taxa of *Phytophthora* recovered by filtration at each sampling location.
[4] The drainage point of the main stream was sampled twice to examine spatial variation: 1 sampled initially with other tributaries; 2 sampled later in the day.
[5] X^2 = chi square statistic with 8 degrees of freedom; P = probability of a greater chi-square value.

network is dependent upon population density, and detection of a taxon with a low population density may require more intensive sampling throughout the stream network, including sampling the tributaries.

Conclusion

Spatial and temporal variations in populations of taxa of *Phytophthora* within forest stream networks were observed. Sampling at the terminal drainage point of the main stream in a forest stream network detected all of the more common taxa present throughout the network; however, some taxa present at low population densities in the overall stream network were not detected at the drainage point.

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