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Diversity of *Phytophthora*, *Pythium*, and *Phytophythium* Species in Recycled Irrigation Water in a Container Nursery

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Accepted for publication 19 November 2018.

ABSTRACT

Recycling of irrigation water increases disease risks due to spread of waterborne oomycete plant pathogens such as *Phytophthora*, *Pythium*, and *Phytophythium*. A comprehensive metabarcoding study was conducted to determine spatial and temporal dynamics of oomycete communities present in irrigation water collected from a creek (main water source), a pond, retention reservoirs, a chlorinated water reservoir, and runoff channels within a commercial container nursery in Oregon over the course of 1 year. Two methods, filtration and leaf baiting, were compared for the detection of oomycete communities. Oomycete communities in recycled irrigation water were less diverse but highly enriched with biologically active plant pathogens as compared with the creek water. The filtration method captured a larger portion of oomycete diversity, while leaf baiting was more selective for plant-associated oomycete species of *Phytophthora* and a few *Pythium* and *Phytophythium* species. Seasonality strongly influenced oomycete diversity in irrigation water and detection with leaf baiting. *Phytophthora* was the major colonizer of leaf baits in winter, while all three

genera were equally abundant on leaf baits in summer. The metabarcoding approach was highly effective in studying oomycete ecology, however, it failed to distinguish some closely related species. We developed a custom oomycete internal transcribed spacer (ITS)1 reference database containing shorter sequences flanked by ITS6 and ITS7 primers used in metabarcoding and used it to assemble a list of indistinguishable species complexes and clusters to improve identification. The predominant bait-colonizing species detected in recycled irrigation water were the *Phytophthora citricola*-complex, *Phytophthora syringae*, *Phytophthora parsiana*-cluster, *Phytophthora chlamydospora*, *Phytophthora gonapodyides*, *Phytophthora irrigata*, *Phytophthora* taxon Oaksoil-cluster, *Phytophthora citrophthora*-cluster, *Phytophthora megasperma*-cluster, *Pythium chondricola*-complex, *Pythium dissotocum*-cluster, and *Phytophythium litorale*.

Keywords: filtration, irrigation water, ITS1, leaf baiting, metabarcoding, *Phytophthora*, *Pythium*, *Phytophythium*.

Members of the oomycete genera *Phytophthora* and *Pythium* commonly cause disease in horticultural nurseries (Jones and Benson 2001). An important source of inoculum is water used for irrigation (Hong and Moorman 2005). A single nursery may draw upon several different sources of irrigation water including

rivers, ponds, and reservoirs, as well water is generally insufficient to meet demand, and municipal water sources too expensive for the large volume required. Nurseries also recapture, remediate, and recycle their irrigation water to reduce depletion of water sources, and minimize the environmental pollution from runoff of nutrients and pesticides (Ferraro et al. 2017). While recycling of irrigation water is fundamental to sustainable water management, recycled water is potentially enriched with soil, plant debris, and plant pathogens, thereby increasing disease risks (Cultice et al. 2016; Hong and Moorman 2005). Growers are encouraged to disinfest irrigation water (Raudales et al. 2014), or to test water for the presence of phytopathogens.

Several methods have been developed to detect *Phytophthora* and to a lesser extent *Pythium* species in water, in plant tissue followed by culture on selective media, filtration followed by plating of the filter, or enzyme-linked immunosorbent assay (ELISA)-based detection (Ali-Shtayeh 1991; Hong et al. 2002;

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Funding: This project was funded by the U.S. Department of Agriculture NIFA Specialty Crop Research Initiative (SCRI) program (grant number 2014-51181-22372).

*The e-Xtra logo stands for “electronic extra” and indicates that five supplementary figures, four supplementary tables, and one supplementary method are published online.

The author(s) declare no conflict of interest.

Hüberli 2015; Ivors 2015; MacDonald et al. 1994; Rollins et al. 2016; Themann et al. 2002). However, no single bait is suitable for capture of all *Phytophthora* and *Pythium* species, nor is there a single set of environmental conditions during baiting that is likely to result in recovery of all species. Filtration and plating of the filters may work well for water populations dominated by a single species (Rollins et al. 2016; Themann et al. 2002), but is problematic for mixed populations, where faster growing colonies reduce the recovery of slower growing colonies. Moreover, culture-based methods often require the extra step of species identification based on morphological features or Sanger sequencing (Eyre and Garbelotto 2015; Winton and Hansen 2001). ELISA-based methods, whether applied to baits or filters (Ivors 2015; MacDonald et al. 1994), are only genus-specific and therefore have a limited resolution.

Not all species of *Phytophthora*, *Pythium*, and *Phytophthium* have been observed to be phytopathogens. Some species found in aquatic habitats, especially *Phytophthora* Clade 6 species, appear to be facultative pathogens or aquatic opportunists that have not been associated with plant disease (Hansen et al. 2012; Jung et al. 2011; Marano et al. 2016; van der Plaats-Niterink 1981). Phytopathogens appear to constitute only a small portion of the total species recovered from streams, rivers, and irrigation water reservoirs (Brazee et al. 2017; Choudhary et al. 2016; Copes et al. 2015; Hong et al. 2012, 2008; Loyd et al. 2014; MacDonald et al. 1994; Parke et al. 2014; Yang 2013; Yang and Hong 2013; Yang et al. 2012, 2014, 2016). Genus-level identification of *Phytophthora*, *Pythium*, and *Phytophthium* is therefore insufficient to assess the risks posed to plants.

There are indications that oomycete species diversity in irrigation water may change with source, location, and time of the year, influencing the survival of individual species, and the risk of plant disease (Bush et al. 2003; Choudhary et al. 2016; Copes et al. 2015; Hong and Moorman 2005; Hong et al. 2008; Loyd et al. 2014; MacDonald et al. 1994; Parke et al. 2014), but the biases of culture-based methods have limited our understanding of how pathogen communities vary in time and space within the nursery irrigation system.

We applied a metabarcoding approach to investigate the spatial and temporal variation of oomycete communities in nursery irrigation water, comparing DNA extracted from direct filtration of water to DNA extracted from baits. We tested the following hypotheses: (i) community diversity and abundance is greatest in the creek water source and is reduced in the nursery irrigation water; (ii) plant-pathogenic oomycete communities are enriched in the nursery irrigation water relative to the creek water source; (iii) oomycete communities, including *Phytophthora*, *Pythium*, and *Phytophthium* communities differ seasonally; and (iv) communities of *Phytophthora*, *Pythium*, and *Phytophthium* detected with baits and filters differ.

MATERIALS AND METHODS

Sampling of irrigation water from nursery. *Research sites.* A nursery located in the Willamette Valley in western Oregon, USA was selected for this study (Fig. 1). The nursery's irrigation water is drawn from a nearby creek (site A), and stored in on-site reservoirs (sites C, K, and L). This water is either chlorinated or pumped to other reservoirs (sites J, I, and G) for chlorination before irrigating plants on the premises. Excess water (runoff) is channeled through drainage ditches (sites B, D, E, and F) for temporary storage in reservoirs (sites G, C, I, H, and K). Recycled water is treated, pumped to "clean" reservoirs, and reused for irrigation. Site M is a natural pond on the property and water is drawn from this pond from late spring to the end of fall for irrigation purposes.

Sampling scheme. A water sampling pole, fixed with a 1-liter collection bottle, was lowered in the reservoir just below the surface to collect water. A 1-liter sample of water was collected every 2 weeks for the duration of 1 year (April 2015 to May 2016) from 13 different sites within the nursery (Fig. 1). Collection bottles were bleached with 10% sodium hypochlorite, rinsed, and reused. The sampled water was stored at 5°C and processed within 24 h of collection. It was first filtered through nylon screening (75- μ m opening) (M75, Pentair Aquatic Bio-Systems, Apopka, FL) to remove large debris, followed by vacuum filtration using 5- μ m mixed cellulose ester membrane filters (Cat. No. SMWP04700, EMD Millipore, Billerica, MA). The membrane filters were stored at -20°C in tubes containing 950 μ l CTAB buffer with PVP before DNA extraction. On some sampling days, an additional 1-liter water sample was collected in a clean bottle for baiting.

In-vitro baiting. Healthy leaves from *Rhododendron catawbiense* 'Grandiflorum' plants were used as baits. All *Rhododendron* plants originated from rooted cuttings from the same stock plants and they were grown in a greenhouse without using fungicides. One healthy mature (6- to 12-month-old) leaf from these plants was baited in irrigation water for 3 days, followed by incubation of the leaf in a moist chamber for an additional week (Ivors 2015). One to five leaf disks (6 mm diameter) were collected from leaves that developed lesions (positive leaf baits) and stored in silica gel at room temperature before DNA extraction (Ockels et al. 2007). The distribution of processed water samples is shown (Fig. 1B). Negative controls consisted of distilled water that was filtered or baited and treated in the same manner as the samples.

DNA extraction. DNA was extracted from filters using a modified chloroform/phenol extraction method (Burke et al. 2006), and leaf disks were extracted using the Synergy Plant DNA Extraction Kit (Cat. No. SYNP 02-100-02 OPS Diagnostics, Lebanon, NJ). The DNA concentration was measured using a spectrophotometer (NanoDrop 1000, Thermo Fischer Scientific, Inc., Wilmington, DE) and DNA was diluted when necessary to 25 ng μ l⁻¹ and stored at -20°C.

Illumina MiSeq amplicon sequencing. Initial PCR was performed using the primers internal transcribed spacer (ITS)6 (5'GAAGGTGAAGTCGTAACAAGG 3') that binds the small subunit rDNA upstream of the ITS1 region and ITS7 (5'AGCG TTCTTCATCGATGTGC 3') that binds within the 5.8S rDNA (Cooke et al. 2000). Both primers had Illumina MiSeq adapter overhang sequences on their 5' ends (Forward: 5'TCGTCGGC AGCGTCAGATGTGTATAAGAGACAG-ITS6; and Reverse: 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-ITS7). The PCR master mix containing 1 $\times$ reaction buffer, 800 μ M dNTP, 3.5 mM MgCl₂, 0.4 μ M forward and reverse primer each and Platinum Taq polymerase enzyme (Invitrogen Inc., Carlsbad, CA) was added to 50 ng of DNA template. The final reaction volume of 25 μ l was amplified as follows: initial denaturation at 94°C for 2 min, 35 cycles of denaturation at 94°C for 45s, annealing at 60°C for 30s and extension at 72°C for 1 min, followed by final extension at 72°C for 10 min. The amplicons were submitted to the Center for Genome Research and Bio-computing (CGRB) at Oregon State University for amplicon cleanup, indexing and sequencing. Index PCR used Illumina NexteraXT primers to add dual barcodes to amplicon samples followed by 250PE sequencing (Illumina MiSeq v.2 chemistry) with MiSeq sequencer (Illumina, San Diego, CA). The sequencing data were received from CGRB after reads were demultiplexed into individual samples. The sequencing data are available at the NCBI-SRA repository under the accession number PRJNA417859.

Quality control, sequence identification, and post-BLAST analyses. The de-multiplexed sequencing reads were preprocessed using Cutadapt (version 1.10) (Martin 2011): (i) low quality trimming: the bases with quality-score below 35 were trimmed from 5' and 3' ends to get high-quality sequences; (ii) adapter removal: the bases upstream of the ITS6 sequence in read1, and of the reverse complement of ITS7 sequences in read2 were trimmed; (iii) trimming extra bases from read1: an additional 32 bases from the 5' end of read1, which mapped to 18S segment, were trimmed as the oomycete ITS reference database does not include the 18S segment; and (iv) filtering reads: the reads that were less than 100-bp in length were removed for reliable species identification.

A standalone Nucleotide-Nucleotide BLAST+ (version 2.2.29) search (megablast) was performed separately for the preprocessed read1 and read2 data to obtain the best high-scoring segment pair or HSP presenting at least 99% similarity and minimum 100-bp alignment length with the query sequence (Camacho et al. 2009). Manually curated ITS sequences from *Phytophthora-ID* (<http://phytophthora-id.org>) and Robideau et al. (2011) (Grünwald et al. 2011; Robideau et al. 2011) were used as nucleotide reference database for BLAST (Supplementary Methods S1), since the sequence annotations in public DNA repositories are unreliable (Kearse et al. 2012; Nilsson et al. 2006). The BLAST results were compared between all the paired reads as follows: (i) BLAST hits present only in one of the paired reads (i.e., either read1 or read2) were eliminated; (ii) BLAST hits with 100% identity to query reads

were identified as positive-match (Parke et al. 2014); (iii) BLAST hits with sequence similarity ranging between 99% to <100% were identified as closest-match and the suffix “-like” was assigned to the respective taxon (Parke et al. 2014); and (iv) BLAST hits with nonmatching taxa within paired reads were identified and the taxa data for that read pair was modified to represent both lineages (for example taxon1/taxon2). An operational taxonomic unit (OTU) was defined as group of sequences assigned to a given taxon. The OTU tables were made as follows: (i) the total number of query sequences (taxon count) representing a unique taxon was recorded; (ii) the taxon counts for the species difficult to distinguish with the ITS1 region were also added to form one OTU and taxonomy was labeled with the species complex name; (iii) the taxa observed in less than 2 query reads (singletons) were eliminated; and finally (iv) the taxon count table for read1 and read2 were merged. This OTU table was split into two separate tables—one contained only filter-extracted samples, while the other contained only leaf bait samples.

Diversity analyses. Both filter and leaf bait OTU tables were analyzed separately for diversity and taxonomic summary analyses using QIIME (version 1.8.0) (Caporaso et al. 2010). The relative OTU abundance was estimated by dividing absolute OTU abundance by the total of number of sequences per sample (this included both unknown and identified oomycete sequences). The taxa or OTUs present in more than 80% of the samples were identified as core OTUs. The alpha (or within-group) diversity was measured

Fig. 1. Experimental Design. **A**, Schematics of water sampling sites within nursery. This nursery is located in Willamette Valley in western Oregon, U.S.A. Alphabetical letters designate the water sampling sites within the nursery. The natural creek water (site A) is the main source of irrigation for this nursery. The creek water is pumped to three reservoirs: C, L, and K. Water from reservoir C is treated with calcium hypochlorite before use in irrigating the propagation and broadleaf growing area. Runoff from several locations within this growing area is collected at sites B and D. Some water from B is also recycled to C. Runoff water from D encounters vegetation along the channel. Site E is located at the end of vegetation channel, before water exits the nursery premises. Reservoir G receives water from reservoir L, and pond M. Water from reservoir G is treated with sodium hypochlorite before use in irrigating the conifer growing area. Runoff from this area flows into M, and is also recycled to G through a runoff stream (F). Reservoir J is the only reservoir that holds chlorinated water, from reservoirs K and I. This water is again chlorinated before use in irrigating the broadleaf, conifer and perennial growing area. Runoff from this area is collected in K and H. Sites H and I are two ends of the same reservoir. H receives runoff water from the growing area, while I is downstream from H and also receives recycled water from K. Chlorination, and recycling of water was shut down from November to April for cleaning. **B**, Distribution of water samples processed by filtration and leaf baiting. A 1L sample was collected every two weeks from 13 sites (designated as A to M) within the nursery. Numbers 1 to 28 designate the twice-monthly sampling times, and colored bars indicate the respective sampling months and seasons. The gray and green boxes represent samples processed by filtration and leaf baiting respectively. The white blank box indicates the time or sites with no processed samples.

in terms of species richness (Chao1 index), Simpson's evenness, Simpson's index of diversity ($= 1 - \text{Dominance}$), and Shannon-Wiener diversity index (<http://scikit-bio.org/docs/latest/generated/skbio.diversity.alpha.html>). The beta (or between-group) diversity was calculated from a rarefied OTU table at the sequencing depth of 240 and 37,000 sequences per sample using the Bray-Curtis dissimilarity index for filter-extracted and leaf bait samples, respectively (Bray and Curtis 1957). The alpha- and beta-diversity for the creek (site A) were also analyzed separately at the sequencing depth of 1,900 oomycete sequences per sample for both filters and leaf baits. The Bray-Curtis dissimilarity index was used in the comparison of the statistical significance between sampling locations

and sampling times using *Adonis* (Anderson 2001; Caporaso et al. 2010; Oksanen et al. 2013). To study the seasonal effect on oomycete communities in water, the sampling periods were grouped into seasons: spring (March to May), summer (June to August), fall (September to November), and winter (December to February).

RESULTS

Oomycete species clusters and complexes associated with ITS6/7 metabarcoding. The ITS1 amplicon-sequencing data were comprised of 23,933,858 (79,251 sequences/sample) and 7,489,054 (91,329 sequences/sample) reads originating from 302 filters and 82

TABLE 1
Indistinguishable species complexes based on shorter internal transcribed spacer (ITS)1 region amplified with ITS6 and ITS7 primers

Clade	Member species	Class ^a	Details
<i>Phytophthora</i> genus (Robideau et al. 2011; Yang et al. 2017) (Supplementary Fig. S1)			
1c	<i>P. infestans</i> , <i>P. andina</i> , <i>P. mirabilis</i> , <i>P. ipomoeae</i>	Cluster ^{b,c}	Existing complex with <i>P. ipomoeae</i> addition
1	<i>P. nicotianae</i> , <i>P. tabaci</i>	Complex ^b	
2b	<i>P. capsici</i> , <i>P. glovera</i> , <i>P. mexicana</i>	Cluster ^{b,c}	Existing complex with <i>P. glovera</i> addition
2a	<i>P. citrophthora</i> , <i>P. terminalis</i> , <i>P. occultans</i> , <i>P. botryosa</i> , <i>P. himasilva</i>	Cluster ^b	
2c	<i>P. citricola</i> , <i>P. citricola</i> E, <i>P. citricola</i> III, <i>P. citricola sensu stricto</i> , <i>P. pini</i> , <i>P. acerina</i> , <i>P. plurivora</i> , <i>P. pachypleura</i> , <i>P. multivora</i> , <i>P. inflata</i> , <i>P. capensis</i> , <i>P. taxon emzansi</i>	Complex ^b	<i>P. citricola</i> -complex (Bezuidenhout et al. 2010)
3	<i>P. nemarosa</i> , <i>P. ilicis</i> , <i>P. pseudosyringae</i>	Cluster ^b	
4	<i>P. quercina</i> , <i>P. sp. "ohioensis"</i>	Cluster ^b	
4	<i>P. arecae</i> , <i>P. palmivora</i>	Complex ^b	
4	<i>P. aticola</i> , <i>P. arenaria</i>	Cluster	
5	<i>P. katsurae</i> , <i>P. sp. "novaeguinea"</i>	Cluster	
6a	<i>P. humicola</i> , <i>P. inundata</i>	Cluster ^b	
6b	<i>P. gibbosa</i> , <i>P. gregata</i> , <i>P. taxon Raspberry</i>	Cluster ^b	
6b	<i>P. megasperma</i> , <i>P. gonapodyides</i>	Cluster ^b	
6b	<i>P. riparia</i> , <i>P. lacustris</i>	Cluster ^b	
6b	<i>P. taxon Oaksoil</i> , <i>P. bilorbang</i>	Cluster ^b	
7a	<i>P. alni</i> , <i>P. alni</i> subsp. <i>alni</i> , <i>P. alni</i> subsp. <i>uniformis</i> , <i>P. alni</i> subsp. <i>multiformis</i> , <i>P. cambivora</i>	Complex ^b	
7a	<i>P. fragariae</i> , <i>P. rubi</i>	Complex ^b	
7b	<i>P. asiatica</i> (<i>P. cinnamomi</i> var. <i>robiniae</i>), <i>P. sojae</i>	Cluster	
7b	<i>P. melonis</i> , <i>P. sinesis</i>	Complex	
8a	<i>P. cryptogea</i> , <i>P. erythrosetpica</i> , <i>P. sp. "kelmania"</i> , <i>P. himalayensis</i>	Complex ^b	
8b	<i>P. lactucae</i> , <i>P. pseudolactucae</i>	Complex ^b	ITS6/7 sequence of <i>P. lactucae</i> (KC478768.1) is identical to <i>P. pseudolactucae</i> (AB894385.1)
9a1	<i>P. parsiana</i> , <i>P. hydropathica</i> , <i>P. sp. "thermophilum"</i> , <i>P. sp. "lagoriana"</i> , <i>P. virginiana</i> , <i>P. taxon Zentmyerii</i>	Cluster ^b	Multiple <i>P. parsiana</i> complexes are grouped into one big complex with <i>P. virginiana</i> , <i>P. taxon Zentmyerii</i> additions
9a2	<i>P. richardiae</i> , <i>P. macrochlamydospora</i> , <i>P. quininea</i>	Cluster	
<i>Pythium</i> genus (de Cock et al. 2015; Robideau et al. 2011) (Supplementary Fig. S2)			
A	<i>P. chondricola</i> , <i>P. porphyrae</i>	Complex ^b	

(Continued on next page)

^a Complex is a group of phylogenetically related species that share an identical full-length ITS1 sequence; while a cluster is a group of phylogenetically related species that have an ITS1 sequence identical only between ITS6 and ITS7 priming sites.

^b The cluster or complex was detected in the nursery irrigation water.

^c Some member species within a cluster belonged to a known species complex.

leaf baits, respectively. Only 6% (1,356,499) of the filter sequences and 55% (4,130,310) of the leaf bait sequences were determined to be oomycete taxa with 99 to 100% sequence similarity to known oomycete reference sequences. More than 48 *Phytophthora*, 36 *Pythium*, 6 *Phytopythium*, 7 *Achlya*, 5 *Saprolegnia*, and other rare species were detected on filters, while only 26 *Phytophthora*, 13 *Pythium*, 5 *Phytopythium*, 2 *Achlya*, and 1 *Saprolegnia* species were detected on leaf baits. In several cases, the ITS1 amplicon did not distinguish two or more closely related species. Therefore, we classified every such group of phylogenetically related species either as a “cluster” or a “complex,” depending on extent of ITS1

identity shared between species. Member species within a complex have an ITS1 sequence identical along the full-length (Robideau et al. 2011), whereas those within a cluster have ITS1 sequence identical between ITS6 and ITS7 priming sites. Some of the clusters included members of known species complexes indistinguishable even with full-length ITS1 sequence. The ITS6/7 metabarcoding approach (region flanked by ITS6 and ITS7 priming sites) was unable to resolve 21 species clusters (comprising 13 *Phytophthora*, 6 *Pythium*, 1 *Saprolegnia*, and 1 *Achlya* clusters) and 33 species complexes (comprising 11 *Phytophthora*, 18 *Pythium*, 2 *Phytopythium*, 1 *Saprolegnia*, and 1 *Achlya* complexes) (Table 1).

TABLE 1 (Continued from previous page)

Clade	Member species	Class ^a	Details
B2	<i>P. dissotocum</i> , <i>P. aff. dictyosporum</i> , <i>P. coloratum</i> , <i>P. diclinum</i> , <i>P. lutarium</i>	Cluster ^b	
B2	<i>P. pachycaule</i> , <i>P. oopapillum</i>	Cluster ^b	
B2	<i>P. aquatile</i> , <i>P. sukuense</i>	Complex ^b	
B2	<i>P. flevoense</i> , <i>P. capillosum</i> , <i>P. pectinolyticum</i> , <i>P. caudatum</i> (<i>Lagenidium caudatum</i>)	Complex ^b	(Dick 2001)
B1	<i>P. myriotylum</i> , <i>P. zingiberis</i>	Complex ^b	
B1	<i>P. graminicola</i> , <i>P. peritum</i> , <i>P. tardicrescens</i>	Complex	
B1	<i>P. rhizo-oryzae</i> , <i>P. catenulatum</i> , <i>P. aff. torulosum</i> , <i>P. folliculosum</i>	Cluster ^{b,c}	Two different complexes <i>P. rhizo-oryzae</i> , <i>P. catenulatum</i> and <i>P. aff. torulosum</i> , <i>P. folliculosum</i> are fused together
B1	<i>P. aristosporum</i> , <i>P. arrhenomanes</i> , <i>P. phragmitis</i>	Cluster ^{b,c}	Existing complex with <i>P. phragmitis</i> addition
B1	<i>P. conidiophorum</i> , <i>P. salpingophorum</i>	Complex ^b	
D	<i>P. hydnosporum</i> , <i>P. amasculinum</i> , <i>P. lycopersicum</i> , <i>P. ornamentatum</i> , <i>P. oligandrum</i>	Cluster ^{b,c}	Existing complex with <i>P. oligandrum</i> addition
E1	<i>P. ornacarpum</i> , <i>P. erinaceus</i> , <i>P. echinulatum</i>	Cluster ^{b,c}	Existing complex with <i>P. echinulatum</i> addition
E1	<i>P. acrogynum</i> , <i>P. hypogynum</i>	Complex	
E2	<i>P. minus</i> , <i>P. pleroticum</i>	Complex	
E	<i>P. takayamanum</i> , <i>P. rhizosaccharum</i>	Complex	
H	<i>P. undulatum</i> , <i>P. dimorphum</i>	Complex ^b	
F	<i>P. irregulare</i> , <i>P. cryptoirregulare</i> , <i>P. cylindrosporum</i>	Complex ^b	(Garzon et al. 2007; Spies et al. 2011a)
F	<i>P. sylvaticum</i> , <i>P. terrestris</i>	Complex ^b	
F	<i>P. mamillatum</i> , <i>P. spiculum</i>	Complex ^b	
F	<i>P. spinosum</i> , <i>P. kunmingense</i>	Complex	
F	<i>P. attrantheridium</i> , <i>P. sp. “balticum”</i>	Complex	
I	<i>P. heterothallicum</i> , <i>P. glomeratum</i>	Complex	
I	<i>P. ultimum</i> , <i>P. ultimum</i> var. <i>ultimum</i> , <i>P. ultimum</i> var. <i>sporangiiferum</i>	Complex ^b	(Barr et al. 1996)
J	<i>P. megalacanthum</i> , <i>P. polymastum</i> , <i>P. buismaniae</i>	Complex	
<i>Phytopythium</i> genus (de Cock et al. 2015; Robideau et al. 2011) (Supplementary Fig. S3)			
K	<i>P. megacarpum</i> , <i>P. boreale</i>	Complex	
K	<i>P. vexans</i> , <i>P. sp. “amazonianum”</i> , <i>P. cucurbitacearum</i>	Complex	(Spies et al. 2011b)
<i>Achlya</i> genus (Robideau et al. 2011) (Supplementary Fig. S4)			
	<i>A. ambisexualis</i> , <i>A. bisexualis</i> , <i>A. heterosexualis</i>	Complex ^b	
	<i>A. racemosa</i> , <i>A. sparrowii</i>	Cluster	
<i>Saprolegnia</i> genus (Robideau et al. 2011) (Supplementary Fig. S5)			
	<i>S. diclina</i> , <i>S. ferax</i> , <i>S. lapponica</i> , <i>S. mixta</i> , <i>S. parasitica</i> , <i>S. unispora</i> , <i>S. hypogyna</i> , <i>S. delica</i>	Complex ^b	
	<i>S. unispora</i> , <i>S. monilifera</i>	Cluster	

In this study, we detected 18 clusters and 18 complexes in the nursery irrigation water.

Within the oomycete ITS1 database, the reference sequences of the zero tolerance pathogens *Phytophthora ramorum* and *Phytophthora kernoviae* were distinct from other sequences in the database, meaning these species were not part of any species cluster or complex. However, this fact about *Phytophthora kernoviae* has become invalid after the discovery of a novel *Phytophthora* aff. *kernoviae* species in this study (see details below).

Differences in oomycete community captured with filtration and leaf baiting. The two microbe-capture methods, filtration and leaf baiting, detected different oomycete communities. *Pythium* was the most abundant genus recovered from irrigation water with filtration, whereas *Phytophthora* was the most abundant genus detected with leaf baiting (Fig. 2A). The distribution of oomycete genera and number of species detected with filtration was 56% *Pythium* (36 species), 25% *Phytophthora* (48 species), 14% *Saprolegnia* (5 species), 2% *Achlya* (7 species), 1% *Phytopythium* (6 species), and 2% other; while for leaf baiting, it was 63% *Phytophthora* (26 species), 28% *Pythium* (13 species), 8% *Phytopythium* (5 species), and 1% other (Fig. 2A). Only a small subset of the total oomycete community detected on filters was capable of colonizing leaf baits (Fig. 2B, Table 2). The median richness detected on filter samples (36.3) was more than twice that on leaf bait samples (16.5); the oomycete community on leaf baits was much more diverse (Shannon = 1.71 and Simpson = 0.61) and uniform (Evenness = 0.17) compared with filters (Shannon = 0.39, Simpson = 0.08, and Evenness = 0.03) (Fig. 2B, Table 2).

Spatiotemporal dynamics of oomycete diversity in nursery irrigation water. The spatial and temporal dynamics of the most predominant oomycete taxa detected in recirculating irrigation system suggest that seasonality strongly influences diversity (Fig. 3). The most predominant bait-colonizing taxa detected in irrigation

water, representing at least 1% of the total community detected on leaf baits, were the *Phytophthora citricola*-complex, *Phytophthora syringae*, *Phytophthora parsiana*-cluster, *Phytophthora chlamydospora*, *Phytophthora gonapodyides*, *Phytophthora irrigata*, *Phytophthora* taxon Oaksoil-cluster, *Phytophthora citrophthora*-cluster, *Phytophthora megasperma*-cluster, *Pythium chondricola*-complex, *Pythium dissotocum*-complex, and *Phytopythium litorale* (Fig. 3). For both filters and leaf baits, the beta diversity estimates (Bray-Curtis dissimilarity index) were significantly different for the sampling months, seasons, and locations (Adonis P value < 0.001). Approximately 33 and 22% (for leaf baits) and 21 and 10% (for filters) of the oomycete community dynamics can be explained with month and location of sampling, respectively (Table 2). No single species was ubiquitous in the tested samples; however, the *Pythium dissotocum*-complex was observed in more than 80% of the leaf bait and filter samples (Fig. 3). In addition, the *Phytophthora citricola*-complex, *Pythium flevoense*-complex, and *Saprolegnia delicata*-complex were each also observed in more than 80% of the filter samples. Different runoff channels displayed distinct dominant species on leaf baits, for instance, *Phytophthora citricola*-complex at site B, *Phytopythium litorale* at site D, *Phytophthora gonapodyides* at site E, and *Phytophthora* taxon Oaksoil-cluster at site F (Fig. 3).

The creek A exhibited the greatest oomycete richness (filter: 52.4; leaf bait: 23.1); however, only 44% (estimated as follows: $23.1/52.4 = 44.08\%$) of the total oomycete population detected on filters colonized leaf baits (Table 2). This was expected due to a potentially greater proportion of saprophytes or nonplant infecting oomycetes in the creek water. Compared with runoff and retention water, the creek water also showed the highest *Phytophthora* mean richness (filter: 21.58; leaf bait: 14.72) and diversity (filter: Shannon = 3.13, Simpson = 0.82; leaf bait: Shannon = 2.27, Simpson = 0.73) (Supplementary Table S1). The next highest mean oomycete richness (filter: 41.5, leaf bait: 17.9) and diversity (filter: Shannon = 0.53, Simpson = 0.12; leaf bait: Shannon = 1.87, Simpson = 0.63)

Fig. 2. Pairwise comparisons in oomycete diversity detected using filtration and leaf baiting techniques. Differences in the filtration and the leaf baiting approaches are represented as **A**, pie chart distribution of oomycetes community composition and **B**, alpha diversity in terms of (a) richness (Chao1 index), (b) Shannon diversity, (c) Simpson diversity, and (d) evenness (Simpsons' evenness index). The alpha diversity estimates for filter and leaf bait were significantly different when evaluated using analysis of variance (P value of <0.0001).

was found in the runoff water (Table 2); however, a greater proportion (54.83%) of biologically active plant pathogens was found in the retention water as compared with runoff water (43.13%) or creek water (44.08%) (Table 2). The term “biologically active plant pathogen” is defined here as capable of causing lesions on leaf baits. For example, the proportion of biologically active plant pathogens in retention water was estimated as follows: (bait richness)/(filter richness) = 15.3/27.9 = 54.83% (Table 2). The recycling of irrigation water within the nursery results in enrichment of biologically active plant pathogens.

The communities detected with filtration and bait-colonization in irrigation water also varied greatly across seasons. Species richness on both filters and leaf baits appeared to be greater during winter (particularly in December), and reduced in summer (particularly in July) (Table 2). *Phytophthora* was the major colonizer of leaf baits in winter, with only trace amounts of *Phytophthora litorale* and *Pythium dissotocum*-complex detected (Fig. 3). In contrast, all three genera were detected equally on leaf baits in summer (Fig. 3). We also noticed species-selectivity bias and increased species abundance on leaf baits as compared with filters (Fig. 4). This species selectivity bias in leaf baiting may not only be due to seasonality, but also due to the competition of two or more pathogenic species to colonize baits. For example, the *Pythium dissotocum*-complex was highly abundant in all sites (detected on filters), and although it was capable of colonizing baits, it was not detected on leaf baits from F and L sites (Fig. 4). Instead, leaf baits from F and L sites were dominated by *Phytophthora* taxon Oaksoil-cluster and *Pythium chondricola*-complex, respectively (Fig. 3), perhaps due to the exclusion of the *Pythium dissotocum*-complex.

Seasonality of *Phytophthora* species in natural creek water.

The creek is the main source of irrigation water in the nursery. The creek water is regularly drawn and stored in reservoirs on the premises. Oomycetes inhabiting the creek water can enter the nursery, and runoff from the nursery also flows back into the creek, potentially carrying plant pathogens with it. It is therefore necessary to monitor the oomycete species from incoming creek water and runoff. The oomycete diversity differed across seasons on both filters and leaf baits (Fig. 5A). With filtration, *Pythium* was the most abundant (43%) oomycete genus found in creek water, followed by *Phytophthora* (29%), *Saprolegnia* (13%), *Achlya* (7%), and *Phytophthora* (2%) (Fig. 5B). Primarily, *Phytophthora* (83%) species infected leaf baits, followed by *Pythium* (14%), and *Phytophthora* (3%) (Fig. 5B). The oomycete richness and proportion of *Phytophthora* were lowest on filters and leaf baits in summer (estimates based on sequencing depth of 1,900 sequences per sample) (Fig. 5C). The oomycete richness was highest in winter on filters and spring on leaf baits. The highest proportion of *Phytophthora* species was detected in winter and spring on leaf baits (Fig. 5C). There was a lack of correlation between OTU abundance on filters and occurrence on leaf baits (Fig. 5C). For example, abundance of *Phytophthora citricola*-complex was greater than for the *Phytophthora parsiana*-cluster or *Phytophthora irrigata* on filters in summer, but it did not grow on leaf baits (Fig. 5C). Moreover, the abundance of *Phytophthora parsiana*-cluster was greater than *Phytophthora irrigata* on filters, but more *Phytophthora irrigata* than *Phytophthora parsiana*-cluster was detected on leaf baits in summer (Fig. 5C). In addition to the taxa represented in Figure 5C, *Phytophthora* aff. *kernoviae* (positive match 1.4% and closest match 2.3%) were also detected on filters in fall, and *Phytophthora megasperma*-like cluster (1.2%) was detected on leaf baits in summer (not shown). All other taxa detected on filters and leaf baits had very low (<1%) abundance. *Phytophthora irrigata* dominated on leaf baits in summer and fall; while the *Phytophthora riparia*-

cluster and *Phytophthora chlamydospora* dominated in spring and winter, respectively (Fig. 5C). The *Phytophthora citricola*-complex was observed on filters in every season, but only in spring it colonized the leaf baits (Fig. 5C). The *Phytophthora parsiana*-cluster was only identified in summer and spring; while *Phytophthora* taxon Oaksoil-cluster was only identified in fall and winter (Fig. 5C).

***Phytophthora* species in the treated water reservoir.** The main reservoir, J, is the only clean water reservoir on the nursery premises that contains chlorinated recycled water from reservoirs K and L. The chlorination treatment (target concentration 2 ppm, contact time 10 min) was expected to eliminate zoospore plant pathogens from the recycled water (Raudales et al. 2014). The chlorination system at reservoir J is active most of the year, except winter (November to early March), when it is shut down for cleaning and to prevent freeze-damage. As expected, no species were detected with the leaf baits in this reservoir during the chlorination treatment period (Fig. 6). When the chlorination system was shut down in winter, the total *Phytophthora* increased up to 85% on leaf baits from December 2015 and January 2016 water samples (Fig. 6). *Phytophthora chlamydospora* (21% in December 2015 and 51% January 2016) and *Phytophthora syringae* (38% in December 2015) were the dominant bait-colonizing species detected in winter (not shown). Surprisingly, we also detected *Pythium dissotocum*-complex (61%) on leaf baits in June 2015 when the chlorination system was in operation (not shown). Peaks in *Phytophthora* and *Pythium* abundance (in winter) and *Pythium* abundance (in June 2015) were also reflected on filters (Fig. 6). *Phytophthora* levels started to increase on filters in September, and did not drop until March when chlorination had started. *Pythium*, on the other hand, showed several peaks during the year on filters, but no species were detected with baiting except in winter and June 2015 (Fig. 6).

***Phytophthora ramorum* and an unidentified *Phytophthora* aff. *kernoviae* species in irrigation samples.** The ITS1 sequence identical to *Phytophthora ramorum* and *Phytophthora kernoviae* were detected in both filters and leaf baits related to water sampling in spring, summer or winter from the reservoirs C, D, G, H, J, L, and M (Table 3). This was unexpected, since *Phytophthora ramorum* had never been detected at this nursery during multiple inspections over several years by the Oregon Department of Agriculture (Osterbauer et al. 2014), and *Phytophthora kernoviae* has never been reported from North America. With quantitative real-time PCR and a *Phytophthora kernoviae* species-specific probe targeting the ATP-NAD9 region (Bilodeau et al. 2014), we confirmed that the samples showing *Phytophthora kernoviae* OTUs were negative. This provided evidence for presence of an unknown close relative of *Phytophthora kernoviae* in United States, with a matching ITS1 sequence henceforth called *Phytophthora* aff. *kernoviae*. Surprisingly, 6 out of 15 filter samples showing *Phytophthora ramorum* OTUs were confirmed positive using *Phytophthora ramorum*-specific probes (Bilodeau et al. 2014). This included a sample from February 2016 (site-period: J-20) and five samples from March 2016 (H-22, M-22, C-23, L-23, and M-23) (Table 3). All the leaf-bait samples tested for *Phytophthora ramorum* were negative.

Since the majority of *Phytophthora ramorum* positives detected using qPCR were related to sampling in March, we retested irrigation water from sites C, D, G, H, J, L, and M in March 2017 with an intention of isolating and/or confirming the presence of *Phytophthora ramorum*, and the unknown *Phytophthora* aff. *kernoviae* species. *Phytophthora* species detected in resampled irrigation water are summarized in Supplementary Table S2. The resampled irrigation water was baited with pears, *Rhododendron* and *Viburnum* leaves. Lesions developed on (i) pears baited with water from sites G, H, and J; (ii) *Viburnum* leaves baited with water from sites C, G, and H; and (iii) *Rhododendron* leaves baited with

TABLE 2
Diversity estimates for the total oomycete community detected on filters and leaf baits

	Filter				Leaf bait			
Group	Richness ^a	Shannon ^a	Simpson ^a	Evenness ^a	Richness ^a	Shannon ^a	Simpson ^a	Evenness ^a
Overall								
Minimum	4.0	0.00	0.00	0.01	7.0	0.10	0.01	0.06
Median	36.3	0.39	0.08	0.03	16.5	1.71	0.61	0.17
Mean	35.7	0.47	0.10	0.04	18.1	1.70	0.57	0.17
Maximum	83.6	3.03	0.84	0.30	34.0	3.26	0.85	0.33
Month ^b	Adonis $P < 0.001$; $R^2 = 0.2048$				Adonis $P < 0.001$; $R^2 = 0.3280$			
Apr. 2015	32.7	0.51	0.12	0.04	–	–	–	–
May 2015	34.6	0.63	0.16	0.04	19.9	2.01	0.64	0.17
Jun. 2015	28.9	0.34	0.07	0.05	17.3	1.72	0.61	0.18
Jul. 2015	28.7	0.22	0.04	0.05	13.0	1.36	0.52	0.21
Aug. 2015	33.5	0.22	0.04	0.04	17.6	1.54	0.57	0.16
Sep. 2015	34.2	0.37	0.08	0.04	19.7	1.64	0.52	0.13
Oct. 2015	44.0	0.66	0.15	0.03	14.7	1.28	0.41	0.17
Nov. 2015	38.0	0.90	0.21	0.04	–	–	–	–
Dec. 2015	44.5	1.04	0.25	0.05	22.6	2.19	0.72	0.18
Jan. 2016	38.9	0.64	0.14	0.04	20.6	1.51	0.50	0.13
Feb. 2016	35.8	0.37	0.08	0.04	21.4	2.26	0.73	0.18
Mar. 2016	33.7	0.29	0.06	0.05	–	–	–	–
Apr. 2016	34.1	0.34	0.07	0.04	–	–	–	–
May 2016	35.0	0.32	0.06	0.04	–	–	–	–
Location ^b	Adonis $P < 0.001$; $R^2 = 0.0968$				Adonis $P < 0.001$; $R^2 = 0.2185$			
Creek water								
A	52.4	0.53	0.10	0.02	23.1	2.16	0.68	0.16
Runoff water	41.5	0.53	0.12	0.04	17.9	1.87	0.63	0.18
B	41.0	0.60	0.14	0.04	19.6	2.14	0.70	0.21
D	40.5	0.41	0.09	0.03	15.4	1.69	0.51	0.18
E	46.4	0.34	0.07	0.03	18.1	2.13	0.71	0.20
F	24.8	0.56	0.14	0.07	15.3	1.47	0.61	0.17
H	48.2	0.57	0.12	0.03	20.7	1.81	0.57	0.16
I	47.9	0.70	0.16	0.03	18.3	1.98	0.68	0.21
Retention water	27.9	0.43	0.10	0.05	15.3	1.26	0.46	0.15
C	20.9	0.29	0.07	0.06	15.9	1.14	0.43	0.13
G	30.3	0.48	0.11	0.04	13.7	1.34	0.49	0.17
J	32.7	0.42	0.09	0.04	19.1	1.57	0.58	0.15
K	39.0	0.75	0.18	0.04	16.9	1.77	0.62	0.18
L	16.5	0.22	0.05	0.08	10.7	0.47	0.19	0.13
Pond water								
M	25.3	0.20	0.04	0.05	15.8	0.95	0.34	0.12
Season ^b	Adonis $P < 0.001$; $R^2 = 0.06718$				Adonis $P < 0.001$; $R^2 = 0.2543$			
Spring	34.1	0.39	0.09	0.04	19.9	2.01	0.64	0.17
Summer	30.4	0.26	0.05	0.05	16.8	1.66	0.59	0.18
Fall	39.6	0.61	0.14	0.04	16.3	1.40	0.45	0.16
Winter	39.5	0.67	0.15	0.04	21.6	1.94	0.64	0.16

^a Refer to the following webpage for information on diversity estimates: <http://scikit-bio.org/docs/latest/generated/skbio.diversity.alpha.html>.

^b The diversity estimates for sampling month, location, and season represent group averages.

Fig. 3. Spatiotemporal dynamics of oomycetes-detected in the nursery irrigation water. Relative abundance heatmap of oomycete operational taxonomic units detected in irrigation water using filtration and leaf baiting across 14 sampling months and 13 sampling locations within the nursery. Genera are abbreviated as follows: P = *Phytophthora*, Py = *Pythium*, Ppy = *Phytopythium*, and Sap = *Saprolegnia*.

water from sites G and H. Lesioned tissue was plated on *Phytophthora*-selective PARPH media. Neither *Phytophthora ramorum* nor the *Phytophthora* aff. *kernoviae* species were detected on baits although *Phytophthora* aff. *kernoviae* was abundant on filters.

DISCUSSION

The oomycete community detected in irrigation water was primarily populated with species from three pathogen genera: *Pythium* (containing plant, animal, and fungal pathogens), *Phytophthora* (plant pathogens), and *Saprolegnia* (pathogens of fish and other aquatic organisms) (Beakes et al. 2012; Hong and Moorman 2005). The filtration method traps all microbes >5 µm present in water. Filters captured a greater spectrum of oomycete species, whereas leaf baits attracted a subset of oomycetes that were alive, active, and capable of colonizing and causing lesions on detached rhododendron leaves. We

anticipated that baiting should increase the detection sensitivity for species more commonly associated with plant disease relative to detection with filters. As expected, only a subset of the total oomycete population detected with filtering was detected on leaf baits. For instance, of the *Phytophthora* and *Pythium* species detected on filters only 54 and 36% of those were detected on leaf baits, respectively. However, the abundance of individual species detected on leaf baits was considerably higher than that detected on filters. Baiting appeared to enrich for plant pathogenic species, but also detected species considered being aquatic opportunists or saprophytes. This is not surprising because many *Phytophthora* species, especially members of Clades 6 and 9, are known primarily as saprophytes on leaves in fresh water (Brasier et al. 2003; Hansen et al. 2012; Jung et al. 2011; Marano et al. 2016). Interestingly, we also detected fish pathogens like *Saprolegnia parasitica* and *S. diclina* on leaf baits. These saprophytic species flourishing on leaf baits may scavenge the nutrients released

Fig. 4. The relative distribution of bait colonizing oomycete species across sampling sites. **A**, Filters and **B**, leaf baits. Solid colors indicate a positive match operational taxonomic unit (OTU) with 100% sequence identity to a known species, while colored stripes indicate closest match OTU with 99% sequence similarity to a known species. Genera are abbreviated as follows: P = *Phytophthora*, Py = *Pythium*, and Ppy = *Phytophythium*.

when baits are colonized by phytopathogens. Several studies have captured *Phytophthora* and *Pythium* species in water from rivers, streams, and nursery irrigation ponds using baiting and culture-based approaches, but none reported members of Saprolegniaceae (Bush et al. 2003; Copes et al. 2015; Parke et al. 2014; Sims et al. 2015; Stamler et al. 2016; Yang et al. 2016).

The recycled irrigation water exhibited considerable spatio-temporal dynamics of oomycetes, with enrichment of biologically active phytopathogens disseminating through recycled irrigation water within the nursery. Surface sources of water such as the creek in this study harbor highly diverse oomycete communities, and utilizing these sources for irrigation can introduce new plant pathogenic species to the nursery. Oomycete diversity in the creek water appears to be greater in winter than in summer. The ability of pathogenic species to colonize *Rhododendron* leaf baits is also seasonally biased. The proportion of *Phytophthora* colonizing baits increased from summer to spring. *Phytophthora*, *Pythium*, and *Phytophythium* colonized baits in summer, while *Phytophthora* was the main colonizer of baits in winter. Directing the runoff water into the retention reservoirs can facilitate dissemination of phytopathogens, and is equally concerning as harnessing surface sources of water for irrigation. The oomycete community was more diverse and rich in the runoff water as compare with the retention water, however the biologically active phytopathogens capable of infecting baits were greater in the retention water. Chlorination was observed to be an

effective water disinfection method in eliminating these pathogens from the retention water. A chlorination system failure could expose hosts to these pathogens, increasing disease risk. These findings highlight the importance of testing irrigation water for pathogens.

Chlorination was an effective method of disinfection during most of the growing season, except once (June 2015), when the chlorination failed. We detected several pathogens in treated water during this time similar to when the chlorination system was inactive. The effectiveness of sodium hypochlorite in killing *Phytophthora* and *Pythium* species is well established (Cayanan et al. 2009; Hong et al. 2003). However, it largely depends on free chlorine concentration, contact time, suspended solids, organic matter, pH, oxidation-reduction potential, organic nitrogen, and ammonium ions in water (Raudales et al. 2014).

While this study is the first to examine the total community of waterborne oomycetes, comparisons with previous studies in Oregon waterways and nursery irrigation systems are possible for the genus *Phytophthora*. The number of *Phytophthora* taxa (>48, including 11 species clusters and 7 species complexes) detected in the current study far exceeds the 28 species (Parke et al. 2014) or 20 species (Sims et al. 2015) reported in previous studies of waterborne *Phytophthora* communities in western Oregon that relied on culture-based approaches. The greater richness detected with the metabarcoding approach likely reflects a more comprehensive picture of the *Phytophthora* community than is possible with studies that rely on colonization of bait and/or growth on selective media

Fig. 5. Oomycete diversity in creek water used as the main irrigation water source in the nursery. **A**, Beta-diversity observed in creek water samples is represented as principal component analysis plot. **B**, Overall distribution of oomycetes on filters and leaf baits. **C**, Seasonal changes in relative abundance of dominant *Phytophthora* species detected in creek water. Positive-match and closest-match operational taxonomic units (OTUs) correspond to the sequences with 100% identity and more than 99% similarity to the known oomycetes reference sequences, respectively. A closest-match OTU is placed next to its respective positive-match OTU. The median richness estimates (Chao1) and proportion of *Phytophthora* genus representing the total oomycete community is based on oomycete community analysis at the sequencing depth of 1,900 sequences per sample. *P* = *Phytophthora*.

and also offers the advantage of analyzing mixed populations in environmental samples. Differences in *Phytophthora* diversity among the three studies were also observed. Diversity in creek water (Sims et al. 2015) was less than that detected in the creek water A by high-throughput sequencing of filters in the current study (Supplementary Table S1). The *Phytophthora* community detected in Sims et al. (2015) represented only a subset (live and cultivable) of the total *Phytophthora* community present in the stream water; hence less diversity as compared with filters in the current study. Interestingly, the *Phytophthora* community diversity detected with in-situ baiting in Parke et al. (2014) was greater than that detected with the off-site baiting of irrigation water in the current study (Supplementary Table S4). Off-site baiting allows capture of *Phytophthora* from a larger volume of water, in contrast to off-site baiting of 1 liter of irrigation water in this study.

The current study detected all *Phytophthora* species found by Parke et al. (2014) (except *Phytophthora* taxon Walnut and hybrids) and Sims et al. (2015) (except *Phytophthora siskiyouensis*) as well as additional species. By considering the *Phytophthora* taxa that were repeatedly observed in stream/irrigation water in western Oregon (Parke et al. 2014; Sims et al. 2015), we have some clues as to which members of the indistinguishable species clusters are more likely to exist in nursery irrigation water in the current study. This includes *Phytophthora citricola*, *Phytophthora pini*, and *Phytophthora plurivora* from the *Phytophthora citricola*-complex, *Phytophthora citrophthora*, *Phytophthora cryptogea*, *Phytophthora nicotianae*, and *Phytophthora parsiana* from their respective clusters; *Phytophthora pluvialis* and *Phytophthora pseudosyringae* from *Phytophthora nemorosa*-cluster, and all members from *Phytophthora riparia*-cluster, *Phytophthora* taxon Raspberry-complex, and *Phytophthora* taxon Oaksoil-cluster.

The seasonal trends in the *Phytophthora* population contrast with previous studies. We found that the largest proportion of *Phytophthora* was detected on baits in winter (December to February), whereas in Virginia, *Phytophthora* recovery from the runoff water reservoir was greatest in November and declined considerably as winter progressed (Ghimire et al. 2011). The *Phytophthora* population was also reported to decline in rivers and streams from western Oregon during winter (Hansen et al. 2012; Reeser et al.

2011), but this could be due to dilution of propagules as a result of increasing water level.

The proportion of oomycetes detected in the current study (filter: 6%, leaf bait: 55%) was much lower as compared with other metabarcoding studies (25 to 79%) that examined oomycete or *Phytophthora* diversity in soil and water (Català et al. 2015; Cerri et al. 2017; Prigigallo et al. 2016; Riit et al. 2016; Sapkota and Nicolaisen 2015; Vannini et al. 2013). However, this could be due to differences in the total number of sequences available for metabarcoding. The total oomycetes sequences detected in the current study (>31 million total sequences with 79,251 and 91,329 sequences per sample for filter and leaf bait, respectively) was greater than those detected in other metabarcoding studies (maximum number of reads ranging from 1,727 to 18,199 per sample) (Català et al. 2015; Cerri et al. 2017; Prigigallo et al. 2016; Riit et al. 2016; Sapkota and Nicolaisen 2015; Vannini et al. 2013). The primer set used for metabarcoding can greatly influence the community detections. The ITS6 and ITS7 primers used in the current study can amplify DNA from fungi, diatoms, and other species (Coince et al. 2013; Sapkota and Nicolaisen 2015), contributing toward metabarcoding sequences. It is also possible that some portion of the unidentified sequences in the current study originate from *Phytophthora* and *Pythium* intraspecific hybrids that exist in nature (Burgess 2015; Goss et al. 2011; Husson et al. 2015; Nechwatal and Mendgen 2009); however, there is no way of knowing as the current metabarcoding cannot determine sequences originating from intraspecific hybrids.

The shorter metabarcoding sequences cannot differentiate phylogenetically close microbial species resulting in indistinguishable species complexes. Despite increasing the sequence matching thresholds from 97% (standard) to 100% to improve accuracy of species identification, the metabarcoding approach fails to achieve identification of closely related species to single taxa within a species complex (Khaliq et al. 2018; Prigigallo et al. 2016; Vannini et al. 2013; Vettraino et al. 2012). The term “species complex” is highly relative, as different target genes or loci will give rise to different sets of species complexes. For instance, oomycete species complexes such as *Phytophthora boreale*/*Phytophthora megacarpum*, *Pythium mamillatum*/*Pythium spiculum*, and *Phytophthora*

Fig. 6. *Phytophthora* and *Pythium* abundance in treated irrigation water reservoir. Relative operational taxonomic unit abundance scale for filter (left) and leaf bait (right) are represented separately. Shaded area indicates no chlorination phase.

cambivora/Phytophthora alni can be differentiated with COI gene, but not with ITS1 (Robideau et al. 2011). Several *Pythium* and *Phytophthora* species complexes have been defined based on ITS phylogenetic data (Robideau et al. 2011). The current study highlights the differences in the species complexes resulting from shorter ITS6/7 amplicons as opposed to those described in Robideau et al. (2011), but resolving these complexes is beyond scope of this study.

Unresolved species complexes are a major concern for both pathologists and ecologists, especially if the member species include pathogens of regulatory concern. Fortunately, quarantine pathogen *Phytophthora ramorum* is not part of any indistinguishable species complex identified in this study. But many complexes are comprised of two or more species with what appear to be divergent ecologies. For example, *Phytophthora megasperma* (phytopathogen with broad

TABLE 3
Samples tested for the presence of *Phytophthora ramorum* and *Phytophthora kernoviae*

Year	Month	Period	Location	Sample type	Abundance ^a	qPCR results (ATP-NAD9 amplicon) ^b
<i>Phytophthora ramorum</i>						
2015	Apr.	1	C	Filter	3.93%	Unknown
	May	2	M	Filter	4.55%	Negative
	Jun.	4	G	Leaf bait	16.97%	Not tested
			L	Filter	27.85%	Negative
	Jul.	5	C	Leaf bait	3.30%	Negative
		6	C	Filter	8.45%	Negative
			H	Filter	2.42%	Unknown
		7	L	Filter	32.21%	Not tested
2016	Jan.	18	J	Filter	20.67%	Negative
	Feb.	20	J	Filter	9.36%	Positive
	Mar.	22	G	Filter	5.15%	Negative
			H	Filter	57.54%	Positive
			M	Filter	50.60%	Positive
		23	C	Filter	32.63%	Positive
			J	Filter	4.41%	Negative
			L	Filter	2.40%	Positive
			M	Filter	4.44%	Positive
	May	28	H	Filter	1.57%	Not tested
<i>Phytophthora kernoviae</i>						
2015	Jun.	10	G	Leaf bait ^c	10.26%	Negative
	Aug.	15	B	Filter ^d	8.26%	Negative
			J	Filter ^d	3.69%	Negative
	Sep.	16	L	Leaf bait ^c	4.94%	Negative
		17	J	Filter ^d	2.17%	Negative
	Oct.	18	C	Leaf bait ^c	2.46%	Negative
			J	Filter ^d	1.20%	Negative
		19	L	Filter ^d	8.90%	Negative
		20	C	Filter ^d	8.42%	Negative
	Nov.	21	M	Leaf bait ^c	9.15%	Negative
			J	Filter ^d	3.60%	Negative
			C	Filter ^d	9.34%	Negative
2016	Jan.	24	C	Leaf bait ^c	13.21%	Negative
			D	Leaf bait ^c	4.60%	Negative
			J	Filter ^d	7.97%	Negative
	Feb.	26	J	Filter ^d	7.79%	Negative

^a Abundance (%) calculated as the fraction of *Phytophthora ramorum* or *Phytophthora kernoviae* operational taxonomic unit in the total oomycetes community detected × 100.

^b Results summarized from two independent qPCR runs of each template. Unknown = failed qPCR reactions, Negative = no amplification, Positive = amplification, and Not tested = DNA extract was unavailable for qPCR reaction.

^c DNA from specified leaf bait were pooled for qPCR reaction.

^d DNA from specified filter samples were pooled for qPCR reaction.

host range) forms a complex with *Phytophthora gonapodyides* (a saprophyte or opportunistic pathogen) (Erwin and Ribeiro 1996; Navarro et al. 2015). The complexes that include one or more plant-pathogenic member species were conservatively designated as pathogenic.

Despite these limitations, the high-throughput metabarcoding approach continues to be the most effective and sensitive method to study oomycete ecology at the genus and species levels. The metabarcoding approach can detect both noncellular, freely floating DNA, inactive propagules and living organisms; as opposed to the culture-based approaches that can only detect those organisms can grow on selective media used to isolate species from baits (Erwin and Ribeiro 1996; Khaliq et al. 2018; Rollins et al. 2016). Detection of noncellular DNA in metabarcoding can provide insights on microbial fossil from past, however this could skew the ecological observations. Additional tests are required to separate signals from live propagules from dead ones. For example, *Phytophthora ramorum* and *Phytophthora kernoviae* OTUs were detected on filters in the current study. Further investigation with species-specific probes also revealed that sequences might have originated from noncellular DNA or dead *Phytophthora ramorum* and a novel *Phytophthora* aff. *kernoviae*, respectively. The *Phytophthora ramorum* OTU was not detected on baits indicating that *Phytophthora ramorum* inoculum was dead. The noncellular DNA of dead *Phytophthora ramorum* was previously detected in soils of Italian chestnut groves using metabarcoding (Vannini et al. 2013). The nursery in the current study receives some planting stock from other nurseries and it is possible that a very small amount of residual DNA from dead *Phytophthora ramorum* was temporarily present.

The metabarcoding approach used to examine oomycete communities, particularly *Phytophthora*, *Pythium*, and *Phytopythium* in nursery irrigation water has been very effective and sensitive for species-level identification compared with traditional culture-based approaches. Further improvements in the high throughput sequencing platforms, taxonomic resolutions of oomycetes, availability of reference sequences of newly described oomycete species and their newly emerging hybrids could substantially reduce current limitations of this approach in studying oomycete ecology.

ACKNOWLEDGMENTS

We thank the anonymous nursery for their cooperation during the course of this study.

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