Phytophthora Species Are Common on Nursery Stock Grown for Restoration and Revegetation Purposes in California

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Abstract

Phytophthora tentaculata was detected for the first time in North America in 2012 in a nursery on sticky monkeyflower plant (*Diplacus aurantiacus*) and again in 2014 on outplanted native plants. At that time, this species was listed as a federally actionable and reportable pathogen by the USDA. As a result of these detections, California native plant nurseries were surveyed to determine the prevalence of *Phytophthora* species on native plant nursery stock. A total of 402 samples were collected from 26 different native plant nurseries in California between 2014 and 2016. Sampling focused on plants with symptoms of root and crown rot. Symptomatic tissue was collected and tested by immunoassay, culture, and molecular techniques (PCR). Identifications were made using sequences from the internal transcribed spacer (ITS) rDNA region, a portion of the trnM-trnP-trnM, or the atp9-nad9 mitochondrial regions. *Phytophthora* was confirmed from 149 of the 402 samples (37%), and from plants in 22 different host families. *P. tentaculata* was the most frequently detected species in our survey, followed by *P. cactorum* and members of the

P. cryptogea complex. Other species include *P. cambivora*, *P. cinnamomi*, *P. citricola*, *P. hedraiaandra*, *P. megasperma*, *P. multivora*, *P. nicotianae*, *P. niederhauserii*, *P. parvispora*, *P. pini*, *P. plurivora*, and *P. riparia*. A few *Phytophthora* sequences generated from mitochondrial regions could not be assigned to a species. Although this survey was limited to a relatively small number of California native plant nurseries, *Phytophthora* species were detected from three quarters of them (77%). In addition to sticky monkeyflower, *P. tentaculata* was detected from seven other hosts, expanding the number of associated hosts. During this survey, *P. parvispora* was detected for the first time in North America from symptomatic crowns and roots of the nonnative Mexican orange blossom (*Choisya ternata*). Pathogenicity of *P. parvispora* and *P. nicotianae* was confirmed on this host. These findings document the widespread occurrence of *Phytophthora* spp. in native plant nurseries and highlight the potential risks associated with outplanting infested nursery-grown stock into residential gardens and wildlands.

California is the most geographically and climatically diverse state in the United States. Geographically, most of the state lies within the California Floristic Province, one of 36 global hot spots for floristic biodiversity and endemism (Baldwin et al. 2017). California's warm to temperate Mediterranean climate over much of its area and its unique topography and geography have influenced its species richness (Baldwin 2014). More than 25% of California's estimated 5,280–6,000 native plant species are endemic to the state and found nowhere else (Baldwin 2014; Baldwin et al. 2012; Jepson Flora Project 2017; Stebbins and Major 1965). In addition to their intrinsic beauty, California native plants are important components of the ecosystem, providing food, shelter, sources of medicines, and other industrial products for people and animals. While California native plants belong to more than 190 families, nearly 40% of native plant species belong to six major families: Asteraceae, Boraginaceae, Brassicaceae, Fabaceae, Poaceae, and Polygonaceae (Baldwin et al. 2012; Jepson Flora Project 2017).

Phytophthora species are Oomycetes, or eukaryotic water molds currently classified within the Stramenopile lineage. Although their growth habit resembles filamentous fungi, they are more closely related to brown algae and diatoms (Kamoun et al. 2015). Most *Phytophthora* species produce infectious propagules including zoospores, chlamydospores, and oospores that can be spread short or long distances by soil, rain, water, or movement of infected plant material.

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In addition to causing important diseases of agricultural crops (Erwin and Ribeiro 1996; Kamoun et al. 2015; Zadoks 2008), *Phytophthora* species can also cause extensive damage to natural ecosystems. In California, sudden oak death and ramorum blight, caused by *Phytophthora ramorum*, has resulted in millions of dead tanoak (*Notholithocarpus densiflorus* [Hook & Arn.] Manos et al.) and coast live oak (*Quercus agrifolia* Née), since first detected in 1995 (Rizzo et al. 2002). Genetic analyses indicate that *P. ramorum* was introduced into California's forests by infected nursery stock and spread throughout North America and Europe in the nursery trade (Goss et al. 2011; Grünwald et al. 2012; Mascheretti et al. 2008, 2009). Recent models predict that slowing the spread of the pathogen in California forests is no longer possible (Cunniffe et al. 2016).

California's native plant nurseries propagate and supply nursery stock of California native plants to the public and for projects to restore and revegetate wildlands. The goals of these projects include restoring watersheds for the improvement of ground and surface water and mitigating the loss of natural habitat caused by human action. There are nearly 100 native plant nurseries in California that are recognized by the California Native Plant Society (CNPS), with other smaller unrecognized operations. Many of them are unlicensed and not subject to annual nursery inspections for pests and diseases by regulatory officials.

In 2012, *Phytophthora tentaculata* Kröber & Marwitz was detected for the first time in the United States in a Monterey County, CA nursery, which produced and sold native and nonnative plants for restoration, commercial, and residential plantings (Rooney-Latham and Blomquist 2014). *P. tentaculata* caused a severe crown and root rot disease of sticky monkeyflower, *Diplacus aurantiacus* (Curtis) Jeps. (= *Mimulus aurantiacus* Curtis), a semiwoody perennial native shrub that is widely planted throughout the state in restoration projects. Significant mortality was seen within the block of plants designated for a large restoration project within the county (Rooney-Latham and Blomquist 2014). The plants had been propagated directly from seed, and the source of the pathogen was never determined. *P. tentaculata* was detected in California again in 2014, on the roots of recently outplanted toyon (*Heteromeles arbutifolia*

[Lindl.] M. Roem.) in a restoration site in Alameda County (Rooney-Latham et al. 2015b). Subsequent sampling at the site showed that many of the newly planted sticky monkeyflower plants that were grown by the same nursery as the infected toyon, but by a different nursery from the original detection in 2012, were also infected with *P. tentaculata* (Rooney-Latham et al. 2015b). *P. tentaculata* remains on the U.S. Regulated Plant Pest List (2018) under the authority of the Plant Protection Act and is considered a quarantined pest for parts of the United States (<https://www.aphis.usda.gov/aphis/ourfocus/planthealth/import-information/rppl>).

Phytophthora species are well known and documented in commercial plant nurseries, causing significant economic losses (Erwin and Ribeiro 1996). A 2-year study on the occurrence of foliar *Phytophthora* species isolated from both native and nonnative hosts in California ornamental nurseries showed that 41% of symptomatic samples tested positive for *Phytophthora* spp. by immunoassay (Yakabe et al. 2009). In total, 13 different *Phytophthora* species were detected in the study, many on hosts with no prior *Phytophthora* associations. Over a 4-year monitoring period, Parke et al. (2014) found 28 *Phytophthora* taxa, including hybrids, in four Oregon ornamental nurseries. Studies in other U.S. states have also found *Phytophthora* infestation in ornamental and commercial landscape nurseries (Bienapfl and Balci 2014; Dart and Chastagner 2007; Donahoo and Lamour 2008; Hoitink and Schmitthenner 1974; Knaus et al. 2015; Patel et al. 2016; Schwingle et al. 2007). A large-scale survey of 732 nurseries in the European Union found more than 49 *Phytophthora* species in 670 nurseries (Jung et al. 2016). Although *Phytophthora* spp. are common in ornamental plant nurseries in the United States and Europe, knowledge about the incidence and diversity of *Phytophthora* species in nurseries that specialize in growing California native plants is limited (Swiecki et al. 2011). The purpose of our survey was to determine whether *Phytophthora* species are present on native stock in California nurseries using a combination of diagnostic testing procedures, and if present, identify these species using PCR sequencing.

Materials and Methods

Collection of samples. Between January 2014 and December 2016, 402 samples from plants exhibiting crown or root rot, and occasionally leaf spots and stem cankers (Supplementary Figs. S1 and S2), were submitted from 26 native plant nurseries in 14 California counties. Most of the nurseries were in northern and/or coastal California counties. Some nurseries were sampled multiple times throughout this period of time. Many of the samples were pre-screened in the field with *Phytophthora* ImmunoStrips (Agdia, Elkhart, IN), which in addition to detecting *Phytophthora*, are known to cross react with *Pythium* and *Phytopythium* spp. (MacDonald et al. 1990). California Department of Food and Agriculture (CDFA) plant pathologists collected most of the samples during scheduled visits to nurseries participating in the Best Management Practices (BMP) Program. In addition, some nursery managers and plant brokers submitted samples directly to the lab as service samples, outside of the BMP program. In some cases, the submitter requested testing before selling the material, while in other cases, the broker or receiver (e.g., land manager) of the plants requested testing prior to finalizing the purchase. Initially, testing for *P. tentaculata*, a federally regulated pathogen, was the primary concern. Soon after the survey began, we began to test for and identify *Phytophthora* species other than *P. tentaculata*, when many plants were found infected with other *Phytophthora* species. In total, 102 different host species, including a few nonnative plant species, were submitted to the CDFA Plant Pest Diagnostics Center (CDFA PPDC) in Sacramento, CA for testing. Samples were packaged in plastic bags and kept at 4°C until they were processed. Samples were collected throughout the year. Although hosts that were known to be susceptible to *P. tentaculata* were often targeted for sampling and testing, no attempt was made to control the numbers, locations, or hosts from which samples were collected.

Isolation and ELISA testing. Root and crown tissues were rinsed thoroughly with tap water to remove soil. Samples were blotted dry

with paper towels before selecting symptomatic tissue for isolation. Once pieces were selected, they were divided into three sets, each third with an equal amount of symptomatic tissue to be used for ELISA, culture plating, and if necessary, PCR (Fig. 1). The first set of tissue pieces (approximately 4–6 pieces 2 mm³ in size) was directly plated into or onto *Phytophthora* semiselective CMA-PARP media (Jeffers and Martin 1986). CMA-PARP plates were kept in the dark at 20–25°C and monitored for hyphal growth for up to 14 days. Suspected *Phytophthora* colonies were subcultured for purity onto CMA-PARP and used for ITS PCR sequencing and identification. No effort was made to sequence and identify isolates that morphologically resembled *Pythium* or *Phytopythium* spp.

The second set of tissue was tested concurrently for *Phytophthora* spp. by immunoassay. Tissue was ground in a FastPrep Lysing Matrix A tube (MP Biomedical, Solon, OH) with 1 ml of GEB2 buffer (Agdia, Elkhart, IN) in a Fast Prep machine (MP Biomedical, Solon, OH). Aliquots of the grindate (100 µl) were tested in duplicate using a *Phytophthora* species-specific ELISA kit (Cat no. SRA 92601; Agdia, Elkhart, IN) according to the manufacturer's instructions. If no *Phytophthora* culture was obtained but the OD₄₀₅ reading of the ELISA was at least two times background level (approximately 0.2 or greater), DNA was extracted and tested for *Phytophthora* using PCR from the third set of tissue, which had been held at 4°C.

PCR identification. *Phytophthora* spp. isolates. DNA from *Phytophthora* cultures obtained from direct isolation was extracted from 5- to 10-day-old colonies. For each isolate, a 2 mm³ agar piece was excised from colonies and DNA was extracted following the Qiagen DNeasy Plant Mini Kit protocol (Qiagen, Valencia, CA). Between January 2014 and July 2016, rDNA of the internal transcribed spacer (ITS) region, including the 5.8S gene and portions of 18S and 28S genes were amplified with primers ITS1 and ITS4 (White et al. 1990) using a Bio-Rad C1000 Touch Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). PCR reactions were performed using the following parameters: an initial denaturation for 5 min at 94°C, followed by 34 cycles of 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C. A final extension of 72°C was done for 5 min. In 2016, rDNA of the ITS region was amplified and sequenced using primers PHY.OO18S.F and PHY.OO.28S.R from G. Abad (*personal communication*). PCR reactions were mixed in a total volume of 25 µl containing 1× Takara Premix Ex Taq DNA Polymerase (Takara Bio Inc., Otsu, Japan), 400 nM of forward and reverse primers, and 2 µl of DNA template. Each sample was tested undiluted and at a 1:100 dilution. Molecular grade water was added to obtain a final volume of 25 µl. PCR reactions included an initial denaturation for 1.5 min at 95°C, followed by 35 cycles of 55 s at 94°C, 30 s at 64°C, and 1 min at 72°C, with a final extension of 72°C for 10 min.

All products were purified using the Qiagen QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and directly sequenced by GeneWiz (South Plainfield, NJ) using the amplification primers. Complimentary forward and reverse DNA strands were aligned with the software program Sequencer 5.3 (Gene Codes Corp., Inc., Ann Arbor, MI). Consensus sequences were compared with those from voucher specimens deposited to the Phytophthora database website (www.phytophthoradb.org, accessed Jan. 2014–Dec. 2016). Taxa were assigned to isolates with sequence identities above 99% (Jung et al. 2017; Junker et al. 2016). Identification of some samples could not be resolved using the single ITS locus, and these samples were assigned to a *Phytophthora* species complex if appropriate.

Directly from root tissue. Root samples which tested positive by ELISA with an OD₄₀₅ of at least two times background, but from which no *Phytophthora* species grew in culture, were also tested using *Phytophthora* species-specific PCR primers. Between January 2014 and July 2016, 0.075 g of washed root tissue from each sample was extracted using the Qiagen DNeasy Plant Mini Kit as described above, and tested for *Phytophthora* spp. using a real-time PCR (qPCR) assay specific for *Phytophthora*. Two different mitochondrial loci, trnM-trnP-trnM (trnM) and atp9-nad9 (nad9) (Bilodeau et al. 2014), were targeted to identify *Phytophthora* spp. present in DNA root extracts. The nad9 locus was preferentially used for

sequencing and species identification. For samples from which a *Phytophthora* species could not clearly be identified using the nad9 region sequences, the trnM region was amplified and sequenced.

The Cepheid Smart Cycler II thermal cycler (Cepheid, Sunnyvale, CA) was used for all qPCR reactions amplifying mitochondrial DNA. DNA was amplified from the nad9 region using 1× RealMasterMix without Rox (5Prime Inc., Gaithersburg, MD), 0.5 μM PhyG-ATP9/R6, 6.0 mM MgCl₂, 0.05 μM ATP9-PhyG2 probeR, labeled with FAM fluorophore and 2 μl of DNA template, in a total volume of 25 μl (Bilodeau et al. 2014). Each sample was tested undiluted and at a 1:10 dilution. PCR reaction parameters included an initial denaturation for 120 s at 95°C, followed by 50 cycles of 95°C for 15 s and 57°C for 90 s. PCR reactions amplifying the trnM region included 1× RealMasterMix without Rox, 0.5 μM PhyG-F2/Rb, 6.0 mM MgCl₂, and 0.1 μM TrnM PhyG probe2, labeled with FAM fluorophore, in a total volume of 25 μl (Bilodeau et al. 2014). Initial denaturation was at 95°C for 120 s, followed by 50 cycles of 95°C for 15 s and 62°C for 90 s. Primers FMP12b and FMP13b (0.0325 μM) and probe Plant CAL-Red (0.026 μM) were used as an internal control to amplify plant DNA (Bilodeau et al. 2014). Samples with a valid internal control TxRed Ct value were determined to be *Phytophthora* positive if they had FAM Ct values between 15.00 and 38.00, and negative if they had values of 0. An additional qPCR was performed on the

positive samples, without the internal plant primers and probes, for species identification. The resulting amplicon, of either 340 bp (nad9) or 206 bp (trnM), was purified using the QIAquick PCR Purification Kit, sequenced by GeneWiz, and compared with a bank of known *Phytophthora* mitochondrial sequences.

DNA from samples with an OD₄₀₅ of at least 8 times background, but that did not amplify a *Phytophthora* spp. by qPCR were amplified and sequenced using PCR primers DC6 and ITS4 (Cooke et al. 2000; White et al. 1990) to confirm that a *Phytophthora* was not present. PCR products were purified and sequenced as described above and consensus sequences were compared with those deposited online on The Phytophthora Database.

In August 2016, a conventional PCR using oomycete-specific primers PHY.OO.18S.F and PHYOO.28S.R (G. Abad, *personal communication*) was used in place of the real-time PCR for identification of *Phytophthora* sp. from root extracts. DNA was extracted as previously described for fungal cultures using the Qiagen DNeasy Plant Mini Kit protocol except instead of extracting from fungal hyphae in agar plugs, DNA was extracted from approximately 0.075 g of root tissue. PCR reactions, amplicon purification, and sequencing were performed as described above using primer pairs PHY.OO.18S.F and PHYOO.28S.R on *Phytophthora* cultures.

Pathogenicity tests of new *Phytophthora* host associations. Pathogenicity of *P. nicotianae* and *P. parvispora* was tested on

Fig. 1. Flow chart for testing of plant samples for *Phytophthora* spp. at the California Department of Food and Agriculture Laboratory. Ten 2-mm² samples from root and crown tissue were selected and divided. Five of the pieces were placed on PARP semiselective media and the other five were tested by an Agdia *Phytophthora* enzyme-linked immunosorbent assay (ELISA). If the optical density (OD) at 405 nm from ELISA assay measured more than twice the background, but no *Phytophthora* was obtained in culture, 0.075 g of root and crown tissue was tested for *Phytophthora* by polymerase chain reaction (PCR). Between January 2014 and July 2016, DNA from root extracts was tested by real-time PCR (qPCR) using primers that target two different mitochondrial loci of *Phytophthora*, trnM-trnP-trnM (trnM) and atp9-nad9 (nad9). In August 2016, an oomycete-specific conventional PCR targeting rDNA of the internal transcribed spacer (ITS) region, including the 5.8S gene and portions of 18S and 28S genes was used. If a *Phytophthora* culture was obtained during the original plating process, DNA was extracted directly from the culture and PCR of the ITS region was done, regardless of the ELISA OD value. All PCR amplicons were sequenced and compared to known reference strains for *Phytophthora* confirmation and species identification.

Mexican orange blossom (*Choisya ternata* ‘Sundance’) by inoculating plants grown in 15.24-cm-diameter pots. To ensure plants were healthy before inoculation, they were kept in isolation in a shade house for 12 weeks and monitored for any symptoms of root and crown disease. After this period, three plants were inoculated with *P. nicotianae* and three were inoculated with *P. parvispora*. One 3-mm colonized agar plug taken from cultures grown on V8 agar for 7 days was placed into a wedge-shaped wound made into the vascular tissue of the crown, near the soil line, and secured with Parafilm. Plugs of uncolonized V8 agar were placed onto wounds of two control plants. Plants were placed in a 23°C growth chamber with a 12-h photoperiod. Vascular discoloration was measured after 18 days for the plants inoculated with *P. nicotianae* and after 42 days for those inoculated with *P. parvispora*. The vasculature near the inoculation point was assessed on one control plant after 18 days and the other after 42 days. Isolations from symptomatic tissue were made on CMA-PARP, and cultures were monitored regularly for growth of *Phytophthora*. DNA from suspected *Phytophthora* colonies was extracted, and confirmation of species was made by ITS PCR sequencing as described above. The experiment was performed once.

Results

Isolation and ELISA testing. In this study, 370 (92%) of the 402 total samples tested had an immunoassay OD₄₀₅ reading of 0.2 (average of twice the background) or greater and required further testing. Of these 370 samples, 195 had OD₄₀₅ readings at least 8 times background (0.8), and 149 (40%) tested positive for *Phytophthora* sp. by culture or PCR. *Phytophthora* sp. was detected from only 3 (1.7%) of the 175 samples with OD₄₀₅ readings between 0.2 and 0.79.

Pythium and *Phytopythium*, two related oomycete genera that are known to cross-react with the *Phytophthora* immunoassay, were confirmed by PCR from some of the samples that did not yield a *Phytophthora* sp. Of the samples with immunoassay OD₄₀₅ readings between 0.2 and 0.8, *Pythium irregulare*, *Phytopythium litorale*, and *Phytopythium helicoides* were repeatedly confirmed from root extracts by PCR sequencing using primers DC6/ITS4. Additional samples yielded *Pythium* spp. sequences that could not be identified, either because infections were mixed or sequence queries found no matches in GenBank. Twelve out of the 44 samples with an OD₄₀₅ greater than 0.8, that did not yield a *Phytophthora* in culture or by PCR, were *Pythium dissotocum* and *Pythium catenulatum*. The average OD₄₀₅ readings were 2.2 for *Pythium dissotocum* and 2.9 for *Pythium catenulatum*.

PCR identification. A *Phytophthora* sp. was cultured from 92 (61.7%) of the 149 samples that were infected with a *Phytophthora* species. *Phytophthora* was confirmed from the remaining 58 samples by direct PCR from DNA extracted from plant tissue. Two *Phytophthora* species were cultured from one of the samples. Although no effort was made to sequence and identify isolates that morphologically resembled *Pythium* or *Phytopythium* spp., some of the sequences matched species of these genera (data not shown).

Phytophthora species in native plant nurseries. Of the 14 California counties in which samples were collected, *Phytophthora* was detected from 11 of them (Butte, Contra Costa, Monterey, Orange, Placer, Sacramento, San Diego, San Francisco, San Mateo, Santa Clara, and Santa Cruz). One or more *Phytophthora* species was detected from 20 of the 26 (77%) nurseries that were surveyed. *Phytophthora* was confirmed from 149 of the 402 total samples (37.3%), including one sample that was infected with two different species.

The most frequently detected *Phytophthora* species in our survey was *P. tentaculata* (41), followed by *P. cactorum* (34), species in the *P. cryptogea* complex (20), *P. nicotianae* (11), *P. niederhauserii* (8), *P. hedraiaandra* (8), *P. cambivora* (7), and *P. multivora* (5). Species with fewer than 5 detections included *P. cinnamomi*, *P. citricola*, *P. megasperma*, *P. parvispora*, *P. pini*, *P. plurivora*, and *P. riparia* (Table 1). Detections of *P. tentaculata* accounted for 41 (27%) of the total *Phytophthora* detections. Of the samples determined to be positive for *P. tentaculata*, it was successfully cultured from 66% while the remaining 34% were confirmed by PCR amplification

and sequencing from DNA extracted from root tissue. The rate of success obtaining *P. tentaculata* in culture was similar to our rate of other *Phytophthora* spp. in the study. In total, *P. tentaculata* was detected from seven different hosts. In addition to *Diplacus aurantiacus* (Phrymaceae), many species in the Asteraceae and Lamiaceae were infected with *P. tentaculata*. Species of *Artemisia* L. may be especially susceptible to *P. tentaculata*; it was detected from eight different samples, including all four of the *Artemisia* taxa that were submitted (Table 2).

P. cactorum was the second most frequently detected species, and accounted for 23% of the overall *Phytophthora* detections. Most of the detections were from plant species with known associations of *P. cactorum*, including coffeeberry (*Frangula californica* [Eschsch.] A. Gray), toyon (*Heteromeles arbutifolia* [Lindl.] M. Roem), blueblossom (*Ceanothus thyrsiflorus* Eschsch.), Pacific madrone (*Arbutus menziesii* Pursh.), and wild strawberry (*Fragaria vesca* L.) (Farr and Rossman 2017). Detections from plant species not known to be hosts of *P. cactorum* included sticky monkeyflower (*Diplacus aurantiacus* [Curtis] Jeps.), Monterey manzanita (*Arctostaphylos montereyensis* Hoover), dune manzanita (*Arctostaphylos pumila* Nutt.), Monterey ceanothus (*Ceanothus rigidus* Nutt.), chamise (*Adenostoma fasciculatum* Hook. & Arn.), mountain mahogany (*Cercocarpus betuloides* Nutt.), sticky cinquefoil (*Drymocallis glandulosa* [Lindl.] Rydb.), California wild rose (*Rosa californica* Cham. & Schltdl.), and common verbena (*Verbena lasiostachys* Link.). Isolates matched the vouchers specimens with 99–100% similarity. Samples infected with *Phytophthora* species in the *P. cryptogea* complex, which included close matches to *P. kelmania*, *P. cryptogea*, and *P. drechsleri*, accounted for the third most detections, while *P. nicotianae* was the fourth most detected species (Table 1).

Of the 102 different host species that were tested, at least one *Phytophthora* species was detected from 51 of them (Table 2). Most of the hosts were members of the plant families Asteraceae, Ericaceae, Phrymaceae, Rhamnaceae, and Rosaceae. Sticky monkeyflower (*Diplacus aurantiacus*), including its various cultivars, was the only detection in the Phrymaceae (Table 2). Thirty of the 36 *Phytophthora* detections on sticky monkeyflower and its cultivars were *P. tentaculata*. Other species detected on sticky monkeyflower were *P. cactorum*, *P. cryptogea*, and *P. nicotianae* (Table 2). Prior to this study, no *Phytophthora* associations other than *P. tentaculata* were known on sticky monkeyflower (Farr and Rossman 2017).

The number of samples collected in each nursery and the number of *Phytophthora* species detected in those nurseries varied widely. Ten of the 26 nurseries that were surveyed provided 80% of the total

Table 1. Frequency of *Phytophthora* spp. detections in California native plant nurseries by either culture or PCR

	<i>Phytophthora</i> detections		
	Culture	PCR	Total
<i>P. cactorum</i> and c.f. <i>cactorum</i>	19	15	34
<i>P. cambivora</i>	4	3	7
<i>P. cinnamomi</i>	1	1	2
<i>P. citricola</i>	2	0	2
<i>P. cryptogea</i> complex	12	8	20
<i>P. hedraiaandra</i>	4	4	8
<i>P. megasperma</i>	0	1	1
<i>P. multivora</i>	3	2	5
<i>P. nicotianae</i>	9	2	11
<i>P. niederhauserii</i>	8	0	8
<i>P. parvispora</i>	1	0	1
<i>P. pini</i>	1	1	2
<i>P. plurivora</i>	1	1	2
<i>P. riparia</i>	0	1	1
<i>P. tentaculata</i>	27	14	41
Other <i>Phytophthora</i> sp. or Unresolved	0	5	5
<i>Phytophthora</i> spp.			
Total	92	58	150
		(61.3%)	(38.7%)

samples, while a few nurseries provided very few. One nursery submitted 92 samples for *Phytophthora* testing, and we detected *Phytophthora* from 59% of these samples. Thirty-four different plant species and cultivar selections at this site tested positive for one or more *Phytophthora* species, and 12 of the 15 *Phytophthora* species that were detected in this study were found at this infested nursery.

Pathogenicity tests of two new *Phytophthora* host associations. The detection of *Phytophthora parvispora* on *Choisya ternata* in September 2016 was the first detection of this species in North America. Original symptoms on submitted plants included yellow foliage, stunted growth, and brown necrotic roots and root collars. Pathogenicity experiments were performed on separate healthy *C. ternata* ‘Sundance’ plants with both *P. parvispora* and *P. nicotianae* to complete Koch’s postulates. After 14 days, the foliage of *C. ternata* plants inoculated with *P. nicotianae* began to wilt, and dark brown, sunken cankers were seen at the point of inoculation. Plants inoculated with *P. parvispora* began to wilt after 32 days, and brown lesions were seen at the inoculation points. After 18 days, stem lesions averaged 70 mm for plants inoculated with *P. nicotianae*, while they averaged 35 mm after 42 days for plants inoculated with *P. parvispora*. No vascular discoloration or wilting was observed on the control plants. *P. parvispora* and *P. nicotianae* were isolated from stem cankers of all inoculated plants, while no *Phytophthora* spp. grew from the control plants. Species confirmation was made by ITS PCR amplification and sequencing of the ITS region from recovered isolates. Sequences of both species matched sequences of

vouchered specimens of *P. parvispora* and *P. nicotianae* with 100% similarity and were deposited into GenBank (MH375345 *P. nicotianae*; MH375344 *P. parvispora*).

Discussion

Phytophthora species appear to be widespread and common in native plant nurseries in California. Results from this survey of native plant nurseries over a period of 35 months confirmed the presence of *Phytophthora* spp. in 20 out of the 26. A *Phytophthora* species was detected by culture or PCR from approximately 37% of the total samples collected. Confirmations were made with positive cultures from 23% of the total samples collected and by direct PCR from 15% of the samples. Overall, ELISA testing of roots and crowns was a useful prescreen for *Phytophthora*, with *Phytophthora* confirmed in more than 75% of the samples with OD₄₀₅ readings of 0.8 or greater. The remaining 25% were likely *Pythium* and *Phytophthora* spp. that are known to cross react with the immunoassay. This was evident in our study, especially with samples with OD₄₀₅ values between 2 and 8 times background. Two species of *Pythium*, *Py. dissotocum* and *Py. catenulatum*, cross-reacted strongly with OD₄₀₅ readings near 20 times background, highlighting the potential for false positives if the immunoassay alone is used for diagnosis. Although both *Py. dissotocum* and *Py. catenulatum* can cause damping-off and root rots on plant hosts in other parts of the world (Farr and Rossman 2017), and *Py. dissotocum* was recently found to be a pathogen on hydroponically grown lettuce (McGehee and Raudales

Table 2. Frequency of *Phytophthora* spp. detections by host during a 2014–2016 survey of California native plant nurseries^a

	<i>P. cac</i>	<i>P. cam</i>	<i>P. cin</i>	<i>P. cit</i>	<i>P. cry</i>	<i>P. hed</i>	<i>P. meg</i>	<i>P. mul</i>	<i>P. nic</i>	<i>P. nie</i>	<i>P. par</i>	<i>P. pin</i>	<i>P. plu</i>	<i>P. rip</i>	<i>P. ten</i>	<i>P. spp.</i>	Total
Agavaceae																	
<i>Hesperoyucca whipplei</i>					2												2
Asteraceae																	
<i>Artemisia californica</i>							1								2		3
<i>Artemisia dracunculoides</i>															2		2
<i>Artemisia douglasiana</i>															2		2
<i>Artemisia palmeri</i>															1		1
<i>Artemisia</i> ‘Powis Castle’					1												1
<i>Baccharis pilularis</i>								1									1
<i>Ericameria ericoides</i>					1												1
<i>Eriophyllum confertiflorum</i>					1												1
<i>Lessingia</i> sp.																1	1
Betulaceae																	
<i>Corylus cornuta</i>																1	1
Calycanthaceae																	
<i>Calycanthus occidentalis</i>									1								1
Caprifoliaceae																	
<i>Lonicera hispidula</i>												1					1
Cistaceae																	
<i>Crocanthemum scoparium</i>										2							2
Ericaceae																	
<i>Arbutus menziesii</i>	3																3
<i>Arctostaphylos glandulosa</i>					1				1								2
<i>Arctostaphylos monteyensis</i>	1	3						1									5
<i>Arctostaphylos pumila</i>	1	2				8		1									12
<i>Rhododendron occidentale</i>																1	1
Fagaceae																	
<i>Notholithocarpus densiflorus</i>			1														1
<i>Quercus agrifolia</i>					1												1
Grossulariaceae																	
<i>Ribes divaricatum</i>					1												1
Lauraceae																	
<i>Umbellularia californica</i>			1											1			2

(Continued on next page)

^a **Key to *Phytophthora* species:** *P. cac* - *Phytophthora cactorum*/P. cf. *cactorum*, *P. cam* - *Phytophthora cambivora*, *P. cin* - *Phytophthora cinnamomi*, *P. cit* - *Phytophthora citricola*, *P. cry* - *Phytophthora cryptogea* complex, *P. hed* - *Phytophthora hedraiaandra*, *P. meg* - *Phytophthora megasperma*, *P. mul* - *Phytophthora multivora*, *P. nic* - *Phytophthora nicotianae*, *P. nie* - *Phytophthora niederhauserii*, *P. par* - *Phytophthora parvispora*, *P. pin* - *Phytophthora pini*, *P. plu* - *Phytophthora plurivora*, *P. rip* - *Phytophthora riparia*, *P. ten* - *Phytophthora tentaculata*, *P. spp.* - other *Phytophthora* or unable to speciate.

2018), their pathogenicity on California natives is not known and was not investigated in this work.

Although the host sampling in our study was somewhat biased toward *Diplacus* sp., other potentially new hosts associations of *P. tentaculata* were also identified. Prior to this work, *P. tentaculata* had primarily been detected on common ornamental woody perennials and a limited number of agricultural hosts in Europe and China, including *Apium* L., *Aucklandia* Falc., *Chrysanthemum* L., *Cichorium* L., *Gazania* Gaertn., *Origanum* L., *Santolina* L., and *Verbena* L. spp. (Farr and Rossman 2017; Rooney-Latham et al. 2015a). To date, in the United States, *P. tentaculata* has been detected only on California native species, not on agricultural hosts. Our study confirmed that California natives are associated with other *Phytophthora* species (Table 2). The detection of *P. parvispora* in this survey and subsequent completion of Koch's postulates on *C. ternata* confirmed the presence of a new pathogenic *Phytophthora* species in the United States. Genetically, *P. parvispora* is very closely related to *P. cinnamomi* and was originally considered a variety of *P. cinnamomi* when it was first detected causing stem rot of *Beaucarnea* spp. in Germany (Kröber and Marwitz 1993). It was also recently found to cause severe dieback including root and collar rot on *Arbutus unedo* L. in Italy (Scanu et al. 2014). In our study, *P. parvispora* was found with *P. nicotianae* causing crown rot of a nonnative host, *C. ternata*. Although pathogenicity experiments confirmed that *P. nicotianae* is more virulent on *C. ternata*, *P. parvispora* was also able to induce wilting and stem lesions. The host range of *P. parvispora* is still unknown, but if it has a similar host range (more than 3,000 species) as

P. cinnamomi (Hardham 2005), there is cause for concern. *P. parvispora* and *P. tentaculata* remain on the U.S. Regulated Plant Pest List (2018) and are considered quarantined pests in parts of the United States (<https://www.aphis.usda.gov/aphis/ourfocus/planthealth/import-information/rppl>).

Although relatively few *Phytophthora* associations with native species have been documented, the susceptibility of California natives to *Phytophthora* is not surprising. The climate of most of California, including the coastal areas, Central Valley, and Sierra Nevada foothills, is regarded as Mediterranean (moderately wet winters and hot to warm, dry summers) (Köppen 1884; Rubel and Kottek 2011). Whether pathogenic *Phytophthora* species are native soil inhabitants in California wildlands is unknown. However, native plants often exhibit little to no resistance to nonnative pathogens like *P. tentaculata*. Unlike plants in the wildlands, container grown nursery stock requires summer irrigation for survival. If a *Phytophthora* sp. is present in the roots of a few plants in a nursery, watering in the warmer months, when many *Phytophthora* species are in active growth, can cause the release of zoospores into water films out of the pots and into adjacent susceptible host plants, spreading the pathogen.

Although culturing was a successful method for detecting *Phytophthora* in our study, direct PCR amplification and sequencing from infected roots was a useful tool when a culture could not be obtained from infected plants. Prior to August 2016, we used two different real-time PCR assays that target different loci of *Phytophthora* for our testing (Bilodeau et al. 2014). Both trnM and nad9 are highly

Table 2. (Continued from previous page)

	<i>P. cac</i>	<i>P. cam</i>	<i>P. cin</i>	<i>P. cit</i>	<i>P. cry</i>	<i>P. hed</i>	<i>P. meg</i>	<i>P. mul</i>	<i>P. nic</i>	<i>P. nie</i>	<i>P. par</i>	<i>P. pin</i>	<i>P. plu</i>	<i>P. rip</i>	<i>P. ten</i>	<i>P. spp.</i>	Total
Lamiaceae																	
<i>Salvia leucophylla</i>					2												2
<i>Salvia mellifera</i>					1				1								2
<i>Salvia</i> sp.										1					1		2
<i>Monardella villosa</i>															2		2
Phrymaceae																	
<i>Diplacus aurantiacus</i>	1				1				3						22		27
<i>Diplacus aurantiacus</i> cvs.					1										8		9
Plantaginaceae																	
<i>Penstemon centranthifolius</i>					2												2
Platanaceae																	
<i>Platanus racemosa</i>					1												1
Polygonaceae																	
<i>Eriogonum latifolium</i>					1												1
Rhamnaceae																	
<i>Ceanothus</i> 'Julia Phelps'									1								1
<i>Ceanothus cuneatus</i>									1						1		2
<i>Ceanothus rigidus</i>	2																2
<i>Ceanothus thyrsiflorus</i>	1							1	1								3
<i>Frangula californica</i>	6			2				1		3						1	13
<i>Ceanothus</i> sp.	2																2
Rosaceae																	
<i>Adenostoma fasciculatum</i>	1									2		1					4
<i>Cercocarpus betuloides</i>	1	1															2
<i>Dryocallis glandulosa</i>	1				2												3
<i>Fragaria vesca</i>	1																1
<i>Heteromeles arbutifolia</i>	9				1												10
<i>Prunus ilicifolia</i>		1															1
<i>Rosa californica</i>	3																3
Rutaceae																	
<i>Choisya ternata</i>									1		1						2
Salicaceae																	
<i>Salix laevigata</i>													1				1
Sapindaceae																	
<i>Acer macrophyllum</i>													1				1
<i>Aesculus californica</i>																1	1
Scrophulariaceae																	
<i>Scrophularia californica</i>									1								1
Verbenaceae																	
<i>Verbena lasiostachys</i>	1																1
Total	34	7	2	2	20	8	1	5	11	8	1	2	2	1	41	5	150

conserved in the gene order of *Phytophthora* and do not amplify *Pythium* or *Phytophythium* spp. Of the two loci, nad9 was preferentially used since it is more variable in closely related *Phytophthora* species, including the loci of the closely related *P. cactorum* and *P. hedraiaandra*. However, a major drawback we found with the nad9 primers was that they did not always amplify California isolates of *P. tentaculata* in infected roots, resulting in some false negatives, if used alone. We were able to confirm these plants were infected with *P. tentaculata* by amplifying and sequencing either the trnM or ITS region. We suspect the inability of these primers to amplify all isolates may be due to single nucleotide polymorphisms (SNPs) in the California population of *P. tentaculata*, although it was not confirmed in this study.

Overall, the major advantage to both the nad9 and the trnM PCR assays was their specificity to *Phytophthora* and their inability to cross-react with *Pythium* and *Phytophythium* spp. However, the need to perform additional PCR tests that target other genes when the nad9 PCR failed was laborious. Furthermore, we found it difficult to resolve species in complexes like *P. cryptogea* and *P. cactorum* with the limited databases available for these two loci, a problem which has been previously documented for other databases (Kang et al. 2010). Hybrid species may also not be represented in more limited databases and may explain our inability to resolve all our PCR *Phytophthora* detections. Since mitochondrial regions are inherited uniparentally, identification of hybrid species is not always possible without confirmation using an additional nuclear region, such as the ITS.

Since ITS sequences are overall more readily available on multiple sequence databases and for newly described taxa, we began using a conventional PCR protocol with the oomycete primers (PHYOO) in August 2016. Although these primers cross-react with *Pythium* and *Phytophythium* species, they consistently amplify an ITS product that can be sequenced easily and compared with sequences in larger and more diverse database than those available for mitochondrial based sequences. For this reason, most *Phytophthora* surveys over the last 15 years have targeted the ITS region for species identification (Jung et al. 2016; Yakabe et al. 2009). However, the multicopy nature of the ITS region makes it difficult to recognize potential hybrid species (Jung et al. 2017) if used alone. Many previous *Phytophthora* surveys have focused only on cultural methods. Our multitool approach using an immunoassay prescreen, culturing, and PCR amplification likely resulted in more *Phytophthora* detections. Without the use of PCR, we would have missed nearly 40% of the *Phytophthora* infections in our survey.

The detection of *P. tentaculata* from containerized native plant material that had been planted out into the wildlands raised significant concerns among California's native plant groups and regulatory agencies (Frankel et al. 2015). Invasive *Phytophthora* species can cause severe and long-lasting damage to native plant communities. In addition to *P. ramorum*, introductions of *P. cinnamomi* have caused significant mortality throughout the world. In California, *P. cinnamomi* has threatened multiple species of already endangered manzanita, including *Arctostaphylos pallida* Eastw. and *A. myrtifolia* Parry (Swiecki et al. 2011). *P. cinnamomi* has also caused widespread damage in Europe and Australia, where it is continuing to threaten some of the world's most species-rich and complex ecosystems (Brasier 2008). The detection of *P. cinnamomi*, as well as at least 14 other *Phytophthora* species on native plant nursery stock in our study, is concerning.

In addition to fears about the movement of infected plant material into native habitats, concerns over the outplanting of asymptomatic but infected plant material have also been raised (Dart and Chastagner 2007; Migliorini et al. 2015). In response to this concern, there has been an increased interest in using biological baits such as green pears to screen roots, media, and leachate in native plant nurseries for the presence of any *Phytophthora*. While this is a more exhaustive detection method and allows for the nondestructive sampling of larger lots of plants, this technique was not used in our survey. Since CDFA is a regulatory agency and our positive detections of *Phytophthora* in container stock often results in the destruction of large blocks of nursery stock, our work focused on active

Phytophthora infections in roots, crowns, and stems. A much greater number and diversity of *Phytophthora* species would likely have been detected if the root and crown samples had been baited with pears instead of cultured from or extracted for DNA directly from the plant tissue.

Although the presence of *Phytophthora* species in commercial nurseries in the United States and Europe is well documented (Bienapfl and Balci 2014; Dart and Chastagner 2007; Junker et al. 2016; Knaus et al. 2015; Parke et al. 2014; Patel et al. 2016; Schwingle et al. 2007; Yakabe et al. 2009), this limited survey of 26 native plant nurseries in California showed that most have *Phytophthora* spp. With the exception of *P. tentaculata* and *P. parvispora*, all the *Phytophthora* species identified in this study are widely known in the nursery trade in California and worldwide. Nursery workers can spread *Phytophthora* species by receiving and brokering material from other native plant nurseries or outside commercial nurseries. They can also, as we learned during this study, reuse pots, often from other outside sources, and potting mix without any prior treatment to eliminate plant pathogens. These practices have likely contributed to the spread of *Phytophthora* species, especially *P. tentaculata*, within the community of native plant nurseries. For nurseries to produce cleaner, healthier plants, safeguards must be taken during the production process to reduce and eliminate the potential pathways for *Phytophthora* infection. At that time, most native plant nursery workers were unfamiliar with the symptoms of soilborne diseases and had not been exposed to nursery best management practice (BMP) programs, which identify critical control points for *Phytophthora* introduction and spread. The unintended introduction of plant pathogens on infected nursery stock into natural habitats that may threaten native plant species is contrary to the mission and goals of the California Department of Water Resources, CDFA, local municipal water districts, and the California native plant industry. These groups work to protect, enhance, and restore environments, and in some cases, include perpetual commitments to responsibly manage wildlands and native plant species. Discussions are ongoing in California to establish a clean stock accreditation program to ensure the outplanting of disease-free native plants into the wildlands.

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