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ORIGINAL ARTICLE

Rhododendron leaf baiting of coastal California watersheds for *Phytophthora* and *Nothophytophthora*

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Abstract

For nearly two decades, stream baiting of northern and central California coastal streams has been an important tool in the management of sudden oak death, a devastating forest disease caused by the oomycete *Phytophthora ramorum*. *Phytophthora* species are baited with floating rhododendron leaves, serving as an early detection tool for the presence of *P. ramorum* in watersheds across more than 800 km of California coastline. While this long-standing management tool is focused on a single species of *Phytophthora*, other species of *Phytophthora* have been baited alongside *P. ramorum*, and this study documents the presence and distribution of 22 *Phytophthora* and *Nothophytophthora* species across the northern and central coasts of California. Although *P. ramorum* was isolated at the greatest number of sites, several species in subgeneric clade 6 were also abundant and widespread, a common feature of *Phytophthora* stream baiting studies. Clade 3 species *P. nemorosa, P. pluvialis*, and *P. pseudosyringae* were also frequently isolated in northern coastal streams. The species *Nothophytophthora caduca* and the genus *Nothophytophthora* are reported for the first time in North America along with the first report of *P. pluvialis* in California. Two novel species, *Nothophytophthora* sp. *californica* and *P*. sp. *aureomontensis* (a member of the *P. citricola* species complex) are provisionally named. Mitochondrial sequences revealed multiple hybridization events between *P. lacustris* and *P. riparia*. Stream monitoring can serve as an important tool for monitoring ongoing *Phytophthora* invasions as well as establishing baseline pathogen communities, critical data for preventing future invasions.

Keywords *Phytophthora* · *Phytophthora ramorum* · *Nothophytophthora* · Biosurveillance · Rhododendron · Barcode · Hybrid · Species complex

Introduction

The oomycete genus *Phytophthora* contains numerous species pathogenic to plants in agriculture, horticulture, and natural ecosystems. One of the most notorious species in the genus, *P. ramorum*, causes the disease sudden oak death (SOD) in coastal forests of California and Oregon (Rizzo et al. 2002). The pathogen is exotic and is spreading following recent introductions via ornamental nurseries (Mascheretti et al. 2009; Jung et al. 2021). In contrast to many other *Phytophthora* diseases, SOD is primarily foliar and *P. ramorum* is aerially dispersed (Davidson et al. 2005). While the aerial dispersal of the pathogen presents a major management obstacle, it also facilitates a landscape detection tool via stream baiting (Sutton et al. 2009). The pathogen disperses into bodies of water either via airborne sporangia or is washed in on leaf litter, eventually releasing zoospores into streams and lakes where they can be baited with plant material (Aram and Rizzo 2018, 2019). Directly baiting water has been widely used as a way to monitor species of *Phytophthora* on the landscape; when Waterhouse published her survey of the water molds of the Hogsmill River, UK, she claimed it was the first survey of a river, but cited several preceding studies of smaller streams and lentic waters (Waterhouse 1942). Bodies of water used for irrigated agriculture have also been monitored for *Phytophthora* species, and movement of infested water is known to

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be an important means of dispersal for *Phytophthora* and other pathogenic, zoosporic oomycetes (Ghimire et al. 2009; Zappia et al. 2014; Rodríguez-Padrón et al. 2019; Rodríguez et al. 2021).

In recent years, several *Phytophthora* baiting surveys in streams or other water bodies using sequence-based diagnostics have been published; some associated with water in diseased ecosystems, others in landscapes without symptoms of *Phytophthora* disease. While the majority have a baiting, isolation and identification approach, others have used metabarcoding approaches to detect *Phytophthora* directly from filtered water or suspended bait (Català et al. 2015; Redekar et al. 2019, 2020; Browne et al. 2021). The studies have revealed considerable diversity and often encounter undescribed species (Reeser et al. 2011b; Hüberli et al. 2013; Oh et al. 2013; Jung et al. 2017b, 2022). Most strains in the recently described genus *Nothophytophthora* were also discovered from stream baiting surveys (Jung et al. 2017c; O'Hanlon et al. 2021).

Stream baiting studies reported by Reeser et al. (2011a, b) encompassed an area of the North American Pacific coast immediately north of this study, reaching to Alaska. The most commonly-isolated species were in *Phytophthora* clade 6 as well as two species in clade 3, *P. pseudosyringae* and *P. nemorosa*. An additional species in clade 3, *P. pluvialis* was discovered during stream and canopy drip surveys, and has been implicated in emerging diseases of Douglas-fir (*Pseudotsuga menziesii*) in Oregon, and Monterey Pine (*Pinus radiata*) and Douglas-fir in New Zealand (Reeser 2013; Dick et al. 2014; Hansen et al. 2015). The species was recently documented for the first time in Europe infecting Western Hemlock (*Tsuga heterophylla*) (Pérez-Sierra et al. 2022). All three of the tree species *P. pluvialis* is known to infect are indigenous to California, and *Pinus radiata* is a Californian endemic (Jepson Flora Project (eds.) 2020). Analysis of the worldwide population of *P. pluvialis* found the Pacific coast to be the center of origin, while the population in New Zealand appears invasive (Brar et al. 2018; Tabima et al. 2021).

Sampling for *Phytophthora* in natural ecosystems aids in the construction of a list of resident *Phytophthora* species: species that are present but not otherwise associated with landscape disease. As regulatory and management decisions must be made regarding Californian ecosystems, knowledge of which *Phytophthora* species and subspecific lineages may already be resident in Californian natural ecosystems may factor in these important decisions. Because some of the resident species are presumably native (Bourret et al. 2022b), surveys of *Phytophthora* species in natural ecosystems provide the possibility of sampling native pathogens in their native habitats, a relative novelty for a genus that from the beginning has been studied as an invader.

The objectives of this study were twofold: first, to present results showing the movement and distribution of the invasive species *Phytophthora ramorum* across the landscape of susceptible forest watersheds during the ongoing SOD epidemic (the main focus of the ongoing management-based stream monitoring project), and second, to list and characterize other *Phytophthora* species occurring in these natural ecosystems that were isolated alongside *P. ramorum* during the stream monitoring. The latter objective required the use of sequence-based molecular determination techniques and, in some cases, phylogenetic inference. The result is a rare glimpse at the *Phytophthora* communities of natural, forested ecosystems.

Materials and methods

Watersheds sampled

Sampling locations were chosen for the purpose of monitoring watersheds containing coastal forests at risk of *P. ramorum* invasion along the northern and central California coast. The northernmost and southernmost sampling locations are more than 800 km apart, spanning nearly seven degrees of latitude (Figs. 1 and 2, Online Resource 1). The project commenced in 2004. Sampling is focused in the spring, the time when *P. ramorum* propagules are most abundant and sensitivity of this detection method is greatest (Davidson et al. 2005, 2008). Sampling was typically February through June, rarely July. Because the goal of the project was early detection, some of the watersheds sampled changed throughout the course of the project as areas became infested with *P. ramorum* and no longer needed to be monitored, and new watersheds were added as the range of infestation expanded. Limited sampling was performed in some streams draining the Sierra Nevada range; otherwise, monitored watersheds were confined to the westernmost, coastal ranges of California.

Stream baiting

Rhododendron leaves (including *R. catawbiense* "Album," "Boursalt" and "Grandiflorum," cv. Colonel Coen and cv. Cunningham's White) were grown in a pathogen-tested greenhouse and deployed in streams by Rizzo lab members and by cooperating agencies (Fig. 1, Online Resource 1). Two mesh sachets typically holding five leaves each and fitted with PVC pipe floats were placed in streams at each site so that they floated close to the surface and received a continuous flow of water. In the early sampling season, baits were left for as long as 3 weeks; this was reduced to as little as 1 week as the season progressed. When the baiting period was complete, leaves were removed from sachets and washed, patted dry and kept at approximately 4° C during transport and up to 1 month of temporary storage.

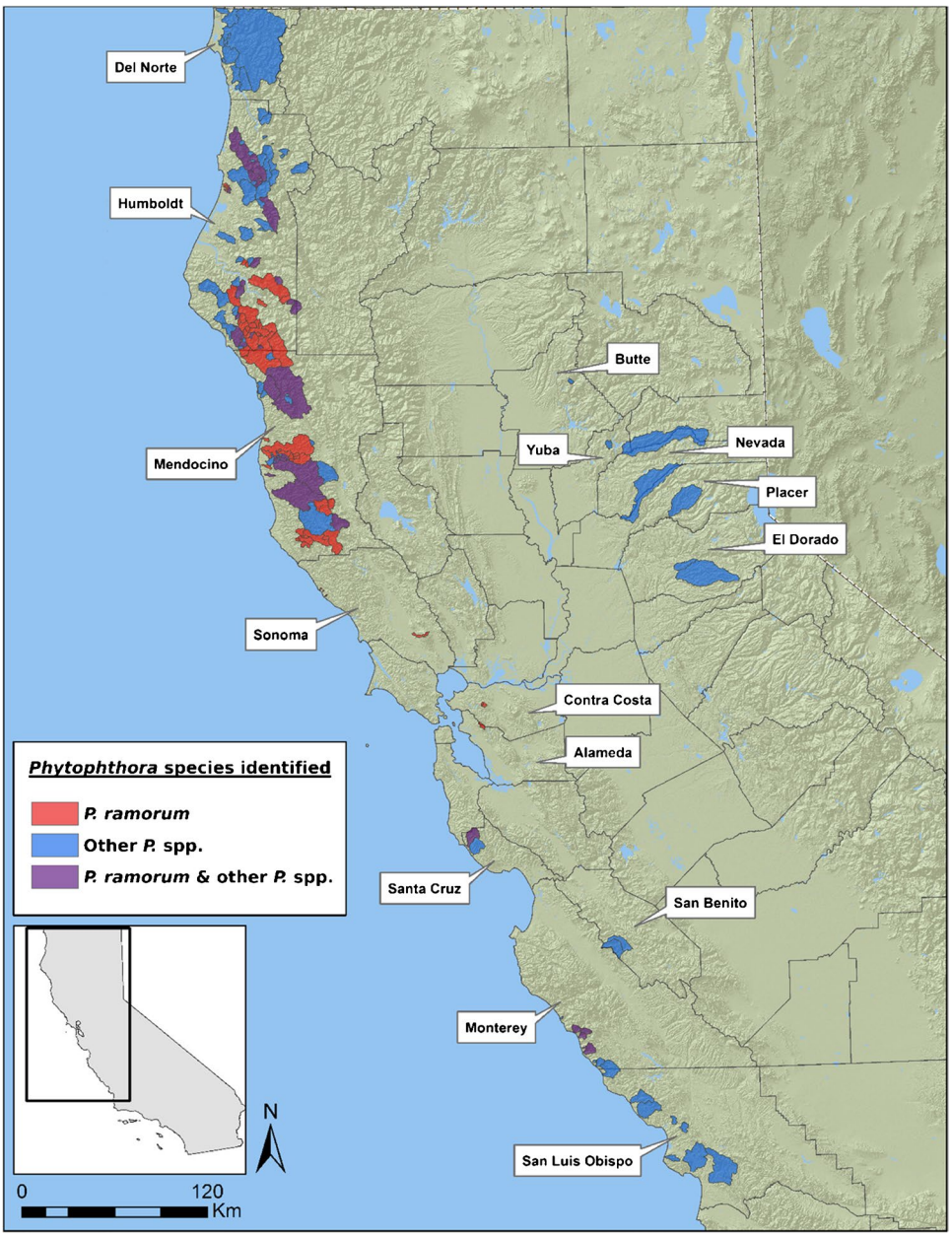

Fig. 1 Map of *Phytophthora*-positive watersheds as of 2015, the final year of this study. Sampling area is within the state of California, USA (grey outline). The names of *Phytophthora*-positive counties are inset. Colored areas represent watersheds. Due to the nature of the project, the San Francisco Bay area (the origin of the *P. ramorum* infestation) was sparsely sampled. The range of *P. ramorum* has continued to expand since the conclusion of this study in 2015, including into Del Norte Co. (Garbelotto et al. 2021)

Isolation and sequencing of isolates

Rhododendron bait leaves were surface sterilized for 1 min in 5% v/v bleach (~ 0.25% w/v NaClO) solution, rinsed and air-dried. Discs (~ 15–30 per leaf) were excised from the margin of lesions (or in a mosaic pattern if leaves were asymptomatic) and submerged in PARPH-low selective media (modified from Erwin and Ribeiro [1996]: 15-g/l corn meal agar [Sigma-Aldrich Co. LLC, St. Louis, MO, USA], 0.025-g/l pentachloronitrobenzene [Sigma-Aldrich Co. LLC, St. Louis, MO, USA], 0.25-g/l ampicillin [Sigma-Aldrich Co. LLC, St. Louis, MO, USA], 0.01-g/l rifampicin [Sigma-Aldrich Co. LLC, St. Louis, MO, USA], 0.01-g/l pimaricin [Sigma-Aldrich Co. LLC, St. Louis, MO, USA], and 0.025-g/l hymexazol [Sankyo Agro. Co., Tokyo, Japan]) and incubated at 18° C for up to 1 month. Isolates growing on the PARPH-low isolation plates were subcultured onto 60-mm PARP (same recipe, but omitting hymexazol) plates. *Phytophthora ramorum* strains were identified at this stage based on a unique combination of morphological features (Werres et al. 2001). From 2004 to 2013, non-ramorum strains, when encountered, were sporadically saved in the Rizzo Lab isolate collection and some were able to be included in this study. From 2013 to 2015, at least one isolate representing each morphologically distinct, non-ramorum morphotype for each sampling location and year was selected for investigation.

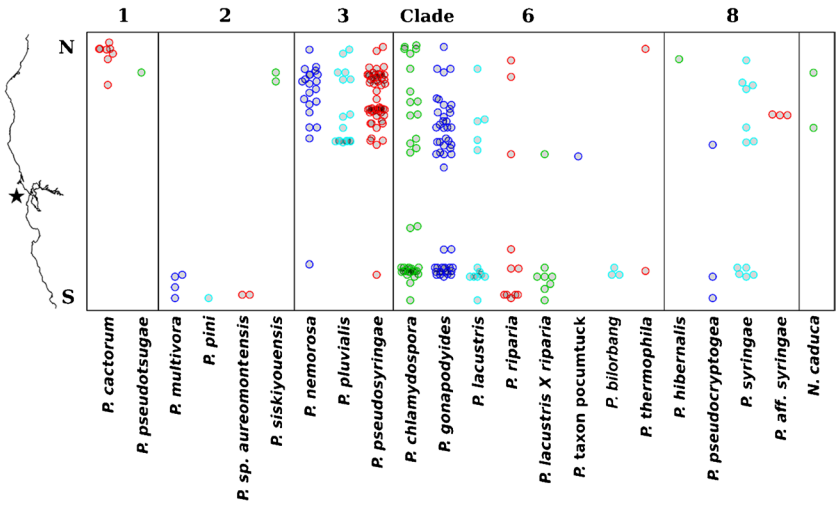

Fig. 2 Beeswarm plot of the latitudinal distribution of stream-baited *Phytophthora* and *Nothophytophthora* isolates. Latitude range is 35.12° N at the southern end (S) to 42.00° N at the northern end (N). Due to the nature of the project, sampling was not equal across the range, and the latitudes occupied by the San Francisco Bay area (black star (★) on the coastal outline and the origin of the *P. ramorum* infestation) were sparsely sampled

Isolates were put into long-term storage using the water vial method: 2-ml cryovials were prepared containing colonized 1/3 strength CV8 agar cubes (modified from Erwin and Ribeiro [1996]: a clarified V8 supernatant was made by centrifuging 14.7 g CaCO3 in 1000-ml V8 [CSC Brands L.P., Camden, NJ, USA] and straining the supernatant through two layers of cheesecloth; 1/3 CV8 media contains 66 ml of supernatant and 17.0 g of agar per liter) in 1-ml sterile ddH_2O. Water vials were stored in duplicate at 20° C and 14° C. Isolates were revived as needed and grown in pea broth for DNA extraction with the PrepMan Ultra kit as previously described (Bourret et al. 2018). The nc ITS rDNA (ITS) locus was amplified and Sanger-sequenced using the FRiz-ITS4TT primer combination as reported by Bourret et al. (2018), except for nine strains of *P. nemorosa* and *P. pseudosyringae* which were preliminarily identified based on morphological characteristics and confirmed using the species-specific primers of Martin et al. (2004) (Online Resource 2). Strains with the same ITS haplotype, site, and date were considered redundant and corresponding to a single positive isolation; none of the 254 strains listed in Online Resource 2 are redundant in that respect. ITS sequences were deposited in GenBank for 243 *Phytophthora* isolates (Online Resource 2).

Molecular species determination

In most cases, ITS data were sufficient to make species-level molecular determinations. Sequences were compared against those available in the NCBI sequence database (GenBank) nucleotide collection using BLAST searches, guided by typification information provided by IDphy (Abad et al. 2019, 2022). Positive determinations were made automatically based on 100% identity or a single mismatch to ex-type ITS sequences (Online Resource 2), provided that type material from other species were sufficiently distinct. If two or more substitutions, insertions/deletions (indels) or single nucleotide variants (SNV) existed between ex-type sequences and the unknown isolate, then BLAST-generated distance trees were consulted to ensure that the determination could be considered unambiguous or whether more study was needed.

Putative hybrids were detected by the presence of multiple SNVs or indels in direct Sanger sequencing results; interspecific hybrids were separated from intraspecific hybrids based on the number of polymorphic sites. No subcloning was performed for these heterozygous nuclear loci; instead, additional sequences from a mitochondrial locus were generated (if possible) from the putative hybrid strains to determine oogonial parentage and ensure the heterozygosity was biological and not due to mixed cultures or micropipetting errors. Eight isolates were determined to represent putative interspecific hybrids and sequences from the intergenic spacer between the mt cox2 and cox1 loci ("cox2-cox1 spacer", Fig. 3) were obtained from seven of the isolates using the primer combination COXFRizC + COXFRizA using methods described by (Bourret et al. 2018, 2022b) (Online Resource 3).

Phylogenetic inference

Additional sequences were obtained for selected isolates to clarify ambiguous phylogenetic relationships or for members of species complexes for which ITS is not sufficient for unambiguous species determination (Online Resource 3). The molecular determination of the subclade 1a isolates, including *P. cactorum* and *P. pseudotsugae* was based on phylogenetic analysis of multiple loci and is described by Bourret et al. (2022b).

Two strains produced ITS sequences that suggested affinity to the *P. citricola* species complex, but could not be placed into one of the previously described species. A three-locus data set composed of ITS, mt cox1 and nc *BTUB* was

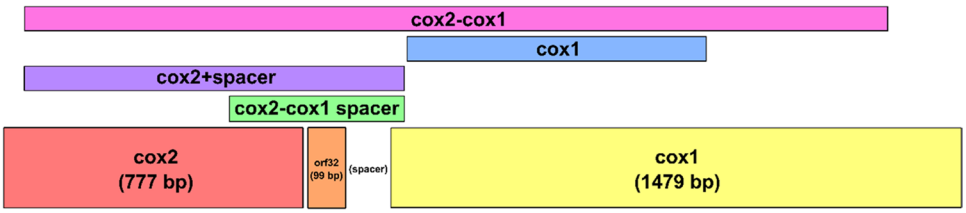

Fig. 3 Names and approximate locations of mitochondrial "cox" loci used in this study. Coding sequences and spacer are drawn to scale based on the *P. sojae* mitochondrion accession NC_009385.1

created featuring all known members of the *P. citricola* complex (i.e., subclade 2c), using *P. mengei* and *P. tropicalis* as outgroups (Online Resource 3). *BTUB* loci and the ~ 2200 bp mitochondrial "cox2-cox1" (Fig. 3), containing the partial cox2 coding sequence, the cox2-cox1 intergenic spacer and the partial cox1 coding sequence were obtained as described by (Bourret et al. 2018, 2022b). The two strains produced identical ITS and cox2-cox1 sequences, and a single isolate was selected for the phylogenetic analysis.

A "cox2-cox1 spacer" (Fig. 3) alignment containing a subset of *Phytophthora* clade 6 was assembled to illustrate the mitochondrial parentage of putative hybrids between *P. lacustris* and *P. riparia*, which comprise subclade 6d. Sequences from five additional hybrid isolates, baited by collaborators from waterways in Santa Clara County, CA, were also included. This data set was also used to investigate the relationship between the provisional species *P.* taxon pocumtuck (Brazee et al. 2016) and *P. borealis* (subclade 6b). *Phytophthora humicola* and *P. inundata* (subclade 6a) were included as outgroups.

While several isolates were unambiguously determined to be *P. syringae*, the ITS sequence of four isolates differed from the more common ITS haplotype (Online Resource 2). The mt rps10 (rps10) sequences of the four genetically distinct isolates, as well as nine other *P. syringae* isolates was obtained, confirmed the phylogenetically distinct, ambiguous status of the four "*P.* aff. *syringae*" isolates. The relationships between the four "aff." isolates and *P. syringae* were also investigated with ITS, mt cox2 plus the cox-spacer ("cox2 + spacer", Fig. 3), and cox1 phylogenies, sampling the *P. syringae* relatives *P. austrocedri* and *P. obscura* (i.e., subclade 8c), and using *P. sansomeana* and *P. foliorum* as outgroups.

Nothophytophthora ITS sequences are greater than 1000 bp, which caused weak amplification until PCR extension time was increased from 30 s to 1 min. Once preliminarily identified as *Nothophytophthora*, *BTUB*, nc LSU rDNA ("LSU") and cox1 sequences were also obtained using methods described by Bourret et al. (2018). A third Californian *Nothophytophthora* isolate (LD268) was also included in these studies; this isolate was obtained from an analogous, contemporaneous stream-baiting project employing locally collected California bay (*Umbellularia californica*) leaves as bait (Aram and Rizzo 2017). ITS, LSU, *BTUB*, and cox1 *Nothophytophthora* alignments were assembled using *P. heveae* and *P. humicola* as outgroups. The three nuclear loci were combined for phylogenetic inference, but due to apparent discordance, mitochondrial cox1 trees were inferred separately.

All data sets were aligned using MAFFT L-ins-I (Katoh and Standley 2013) within AliView (Larsson 2014), with the exception of the *Nothophytophthora* ITS alignment, for which MAFFT X-ins-I was employed (Katoh and Toh 2008). Phylogenetic trees were inferred using maximum likelihood (ML) methods with the program IQ-TREE 2 (Chernomor et al. 2016; Kalyaanamoorthy et al. 2017; Minh et al. 2020) and using Bayesian inference with MrBayes (Ronquist et al. 2012) as described by Bourret et al. (2022b). Protein-coding sections were partitioned by codon, and the ITS was partitioned into ITS1, 5.8S rDNA, and ITS2 subsets. For the ML inference, binary gap data generated with FastGap (Borchsenius 2009) was added to the rDNA alignments and the two alignments containing the cox2-cox1 spacer. Because all alignments were relatively small, the partitioned mode of IQ-TREE 2 was used with branch lengths both linked and unlinked across subsets (options p and Q); the trees inferred with unlinked branch lengths were selected in all cases except the two alignments containing the cox2-cox1 spacer and the clade 8c ITS alignment. The ultrafast bootstrap approximation was used to generate support values (Hoang et al. 2018) for the ML inference. Trees were visualized and Bayesian posterior probabilities were applied to ML trees with TreeGraph2 (Stöver and Müller 2010). Inkscape (www.inkscape.org) was used to add annotations. Partitioning schemata, sequence alignments, and output trees from the phylogenetic inference are compiled in Online Resource 4.

Range mapping

When positive isolations were documented, the infested watershed upstream of the sampling location was highlighted on a shaded relief map of Northern California using ArcGIS (ESRI, Redlands, CA, USA). Watersheds were colored based on whether the positive *Phytophthora* isolations had been *P. ramorum*–only (red), a non-ramorum species–only (blue), or both types (purple). Watersheds from which no *Phytophthora* had been isolated were not indicated on the map. The relatively linear nature of the sampling area was exploited to illustrate the range of individual non-ramorum species by showing the latitudinal distribution of the locations of

positive isolations. In order to summarize these results in a purely descriptive way, a beeswarm plot was created using the beeswarm package of R (www.R-project.org) and annotated using GIMP (www.gimp.org).

Results

Diversity and distribution of *Phytophthora* and *Nothophytophthora*

The spread of *Phytophthora ramorum* from the San Francisco Bay area across coastal Californian forests, both northwards and southwards, was documented in part by the stream monitoring project, helping to identify the northern and southern limits of the invasion as of 2015 (Fig. 1). As much SOD-susceptible forest still remains uninfested by *P. ramorum* (Meentemeyer et al. 2004), the project (in its early detection context) continues as of 2023. From an additional 254 non-ramorum *Phytophthora*-like strains isolated during the project, twenty-one species of *Phytophthora* and one species of *Nothophytophthora* were identified (Table 1, Fig. 2, Online Resource 2). During the more intensive sampling between 2013 and 2015, up to four *Phytophthora* species were isolated from a single sampling location during a single month (Online Resource 2). Although the management-based methods of the study did not allow for direct characterization and comparison of relative abundances, *P. ramorum*, the target of the survey, was the most frequently isolated and found at the most sites (Table 1). Members of clades 3 and 6 were also widely distributed and although not isolated from as many sites, some species encompassed a greater latitudinal range than *P. ramorum* (Table 1, Online Resource 2). Besides *P. ramorum*, *Phytophthora pseudosyringae* (clade 3) was baited from the most sites and was represented by the most

Table 1 List of *Phytophthora* and *Nothophytophthora* species isolated

Clade	Species	Total sites	Number of isolates								ITS haplotypes
			Total isolates	North coast counties			Central coast counties		Interior counties	Other coastal counties	
				DNO	HUM	MEN	MON	SLO			
1	*P. cactorum*	6	8	7	1						2
1	*P. pseudotsugae*	1	1		1						1
2	*P. multivora*	4	4				1	3			1
2	*P. pini*	1	1					1			1
2	*P. siskiyouensis*	2	2		2						1
2	*P.* sp. *aureomontensis*	1	2					2			1
3	*P. nemorosa*	18	21	1	17	3	1				1
3	*P. pluvialis*	14	17	2	8	7					1
3	*P. pseudosyringae*	39	54	2	41	8	2				4
6	*P. bilorbang*	2	3				3				2
6	*P. chlamydospora*	25	36	5	6	16	3	3	1 (YUB)	2 (SCR)	9
6	*P. gonapodyides*	35	49	1	12	13	15	1	5[Δ]	2 (SBE)	22
6	*P. lacustris*	10	13		2	1	3	5	2 (NEV, PLA)		10
6	*P. riparia*	7	11	1	1		2	5	1 (PLA)	1 (SBE)	8
6	*P. lacustris* × *riparia*	6	8				1	6	1 (PLA)		8
6	*P. thermophila*	2	2	1			1				1
6	*P.* taxon pocumtuck	1	1			1					1
8	*P. hibernalis*	1	1	1							1
8	*P. pseudocryptogea*	3	3			1		2			2
8	*P. ramorum*	95	95[§]	*	46	32	5			4[§]	N/A
8	*P. syringae*	11	11	1	2	3	4	1			3
8	*P.* aff. *syringae*	2	4		4						3
	N. caduca	2	2		1	1					N/A

DNO, Del Norte Co.; *HUM*, Humboldt Co.; *MEN*, Mendocino Co.; *MON*, Monterey Co.; *NEV*, Nevada Co.; *PLA*, Placer Co.; *SBE*, San Benito Co.; *SCR*, Santa Cruz Co.; *SLO*, San Luis Obispo Co.; *YUB*, Yuba Co. [Δ]*P. gonapodyides* was baited twice from El Dorado and Placer Co. and once from Butte Co. [§]*P. ramorum* was also baited from Alameda, Contra Costa, Santa Cruz, and Sonoma Co. *After this study concluded in 2015, *P. ramorum* was baited from DNO; the pathogen remains undetected in SLO or any of the interior counties.

isolates, followed closely by *P. gonapodyides*, then *P. chlamydospora* (both clade 6).

Four species, *P. chlamydospora*, *P. gonapodyides*, *P. nemorosa*, and *P. riparia*, were baited at least once in each of the 6th months between February and July during which sampling occurred (Table 2). Within the abundant clade 3, the psychrophilic *P. nemorosa* was most commonly isolated in February, tapering off afterwards, *P. pluvialis* was most common in March, and *P. pseudosyringae* was most commonly isolated in June. The common clade 6 species were more temporally consistent. The single *P.* taxon pocumtuck strain was baited from February, the earliest month, while the single *P. pseudotsugae* strain was baited in July, the latest and least-sampled month.

Species determination

We attempted a molecular species determination with a total of 254 isolates. The species of nine isolates was determined using species-specific primers. Three strains (the two *P.* sp. *aureomontensis* isolates and the single *P.* taxon pocumtuck) were placed into provisional species. This left 242 isolates with ITS sequences that could be compared against ex-type sequences. Of these, 110 had ITS sequences identical to the ex-type, 62 differed from ex-type ITS sequence by a single difference, 45 by two differences, 12 by three, four by four, and sequences from a single *P. lacustris* isolate differed from the ex-type by five differences (Online Resource 2). The eight *P. lacustris* × *riparia* isolates exhibited at least five ITS differences from the ex-type strains of either species. In most cases, ITS sequences were sufficient for molecular determination to species, even within the *P. citricola* and *P. cryptogea* species complexes.

The *P. citricola* complex (subclade 2c) was represented in this survey by *P. multivora*, *P. pini*, and a phylogenetically distinct taxon we provisionally name as *P.* sp. *aureomontensis* (Latin: of the golden mountain) (Fig. 4), so named because both strains were baited from Islay Creek, a watershed almost completely within Montaña de Oro State Park. Although the species can be distinguished, the *P. citricola* species complex is not well-resolved with either ITS or *BTUB* sequences alone (Online Resources 5 and 6), and is better-resolved with cox1 (Online Resource 7). The three loci combined resolved the species complex with modest support, demonstrating the distinct placement of *P.* sp. *aureomontensis* as sister to *P. plurivora* (Fig. 3). The ITS sequences of *P.* sp. *aureomontensis* is distinct from all other members of the complex, and matched an accession from a Oregon stream isolate deposited as *P. citricola*, strain III_5100B1F (KJ666721.1) (Sims et al. 2015). The cox2-cox1 spacer sequence from both Californian strains was also identical to strain III_5100B1F (KJ666721.1).

Eight isolates were identified as the nothospecies *P. riparia* × *lacustris* based on ITS heterozygosity.

Table 2 Baiting frequency of *Nothophytophthora* and non-ramorum *Phytophthora* species by month Sampling was not equal across time periods

Clade	Species	February	March	April	May	June	July
1	*P. cactorum*	2	1	3	2		
1	*P. pseudotsugae*						1
2	*P. multivora*	2		1	1		
2	*P. pini*			1			
2	*P. siskiyouensis*		1	1			
2	*P.* sp. *aureomontensis*	1			1		
3	*P. nemorosa*	10	3	3	3	1	1
3	*P. pluvialis*	4	9	1	2	1	
3	*P. pseudosyringae*	8	11	6	9	19	
6	*P. bilorbang*			1		1	
6	*P. chlamydospora*	9	7	8	5	6	1
6	*P. gonapodyides*	10	11	11	6	10	1
6	*P. lacustris*	1	2	4	6		
6	*P. riparia*	2	1	1	4	2	1
6	*P. lacustris* × *riparia*		2	2	2	2	
6	*P. thermophila*	1			1		
6	*P.* taxon pocumtuck	1					
8	*P. hibernalis*			1			
8	*P. pseudocryptogea*	2				1	
8	*P. syringae*	4	3	2	1	1	
8	*P.* aff. *syringae*		2	1			
	N. caduca			1		1	

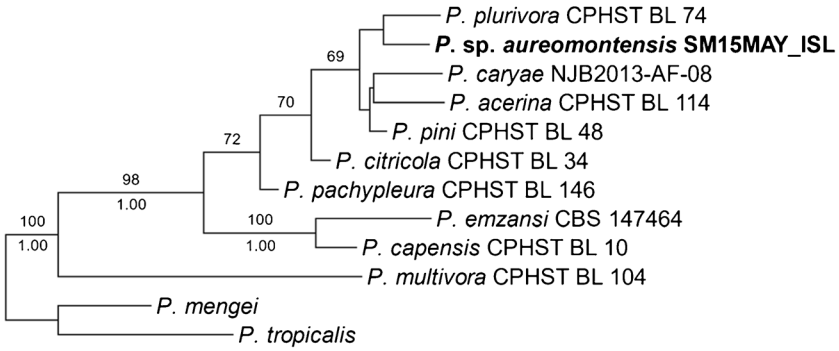

Fig. 4 Maximum likelihood tree of the *Phytophthora citricola* species complex (*Phytophthora* subclade 2c). Tree inferred with IQ-TREE 2 from a combined nc ITS rDNA + mt cox1 + nc *BTUB* alignment. Support values above branches are ultrafast bootstrap approximations ≥ 50 and below are posterior probabilities ≥ 0.90 from an analysis with MrBayes. Sequence accessions available in Online Resource 3. All described species are represented by ex-type or holotype strains

Phylogenetic analysis of cox2-cox1 spacer sequences from seven of the isolates plus five other Californian strains found three different *P. lacustris* genotypes among 11 of the 12 isolates, including one that was identical to the *P. lacustris* ex-type (Fig. 5). The single *P. riparia* × *lacustris* isolate from the interior Placer County exhibited a unique cox2-cox1 spacer sequence that was phylogenetically placed within *P. riparia*.

The distance between the *P. borealis* ex-type and *P.* taxon pocumtuck isolate SM08FEB_FLZ1, despite forming a moderately-supported clade, suggests the two taxa are not conspecific. This confirms the species determination of

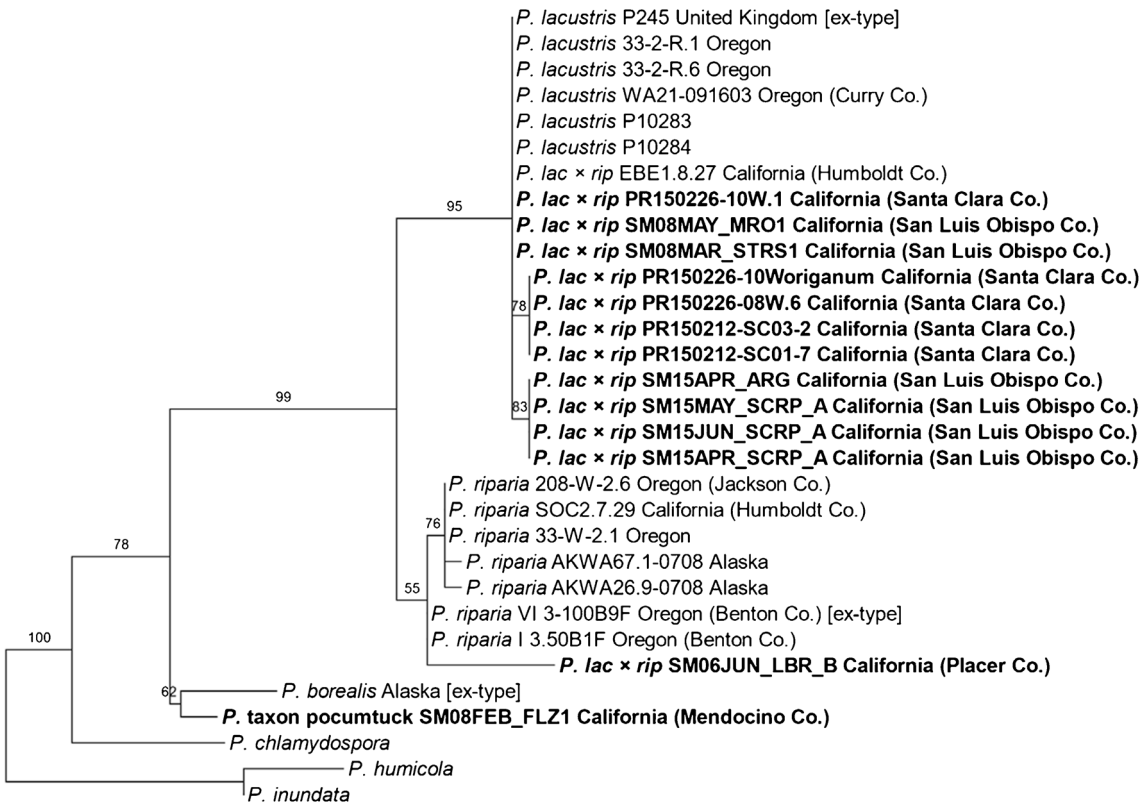

Fig. 5 Maximum likelihood tree of a subset of *Phytophthora* clade 6. Tree inferred with IQ-TREE 2 from a mt cox2 spacer alignment. Support values above branches are ultrafast bootstrap approximations ≥ 50 and below are posterior probabilities ≥ 0.90 from an analysis with MrBayes. Isolates in bold are from this study. Sequence accessions available in Online Resource 3. Co., County; *lac* × *rip*, *lacustris* × *riparia*

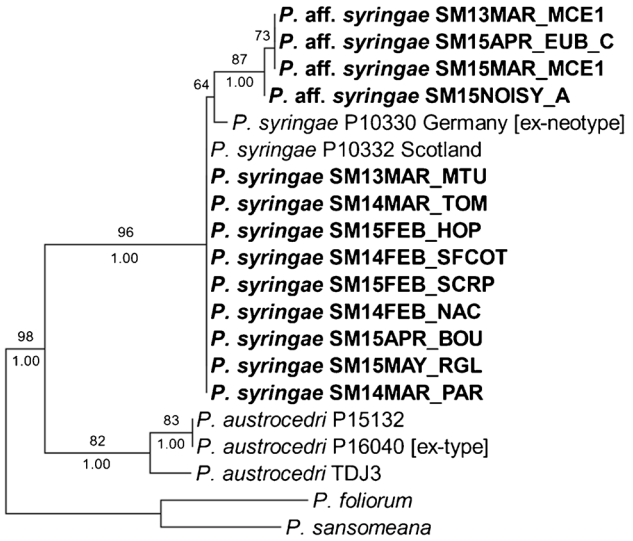

Fig. 6 Maximum likelihood tree of *Phytophthora* subclade 8c. Tree inferred with IQ-TREE 2 from a mt rps10 alignment. Support values above branches are ultrafast bootstrap approximations ≥ 50 and below are posterior probabilities ≥ 0.90 from an analysis with MrBayes. Isolates in bold are from this study. Sequence accessions available in Online Resource 3

SM08FEB_FLZ1 as the provisional *P.* taxon pocumtuck (Fig. 5).

The phylogenetic analyses conducted to investigate the relationships of the four *P.* aff. *syringae* isolates could not unambiguously determine if they represent a novel species or should be considered a new intraspecific lineage. ITS trees placed the isolates in a monophyletic *P. syringae*, with three of the "aff." strains forming a well-supported clade (Online Resource 8). In both the rps10 and cox1 trees all other *P. syringae* isolates (*P. syringae* s.s.) are paraphyletic with respect to the four "aff." isolates, which are on a relatively long branch (Figs. 6 and 7). The cox2 + spacer tree had the four "aff." isolates forming a sister clade to *P. syringae* s.s. (Fig. 8).

The ITS, *BTUB* and LSU sequences derived from the two Californian *Nothophytophthora caduca* isolates were identical to all 11 other isolates of *N. caduca* (including the holotype), all of which were baited from Chilean streams (Fig. 9); all other accessions had less than 99% identity. However, the cox1 sequences derived from the two Californian isolates were at most 97.7% identical to other *N. caduca* strains. The Californian *N. caduca* isolates formed a well-supported sister clade to six of the *N. caduca* strains, including the holotype, while the other five *N. caduca* strains were placed in a moderately supported clade with *N. intricata* and *N. vietnamensis* (Fig. 10).

Isolate LD268, obtained from a similar study and included in the phylogenetic analysis, was sister to *N. caduca* in the combined and single-locus nuclear trees (Fig. 9, Online Resources 9–11), but appeared in a separate clade with the discordant group of *N. caduca* isolates in the cox1 tree (Fig. 10). Based on its phylogenetic distinction from all described species of *Nothophytophthora* in both nuclear and mitochondrial trees, LD268 was determined to belong to an undescribed species, provisionally named here as *N.* sp. *californica*.

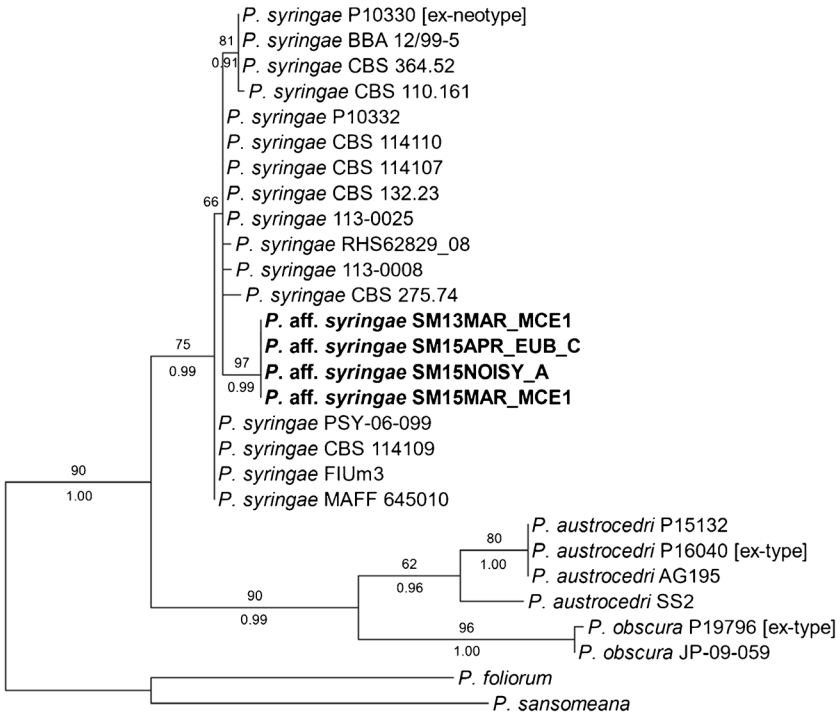

Fig. 7 Maximum likelihood tree of *Phytophthora* subclade 8c. Tree inferred with IQ-TREE 2 from a mt cox1 alignment. Support values above branches are ultrafast bootstrap approximations ≥ 50 and below are posterior probabilities ≥ 0.90 from an analysis with MrBayes. Isolates in bold are from this study. Sequence accessions available in Online Resource 3

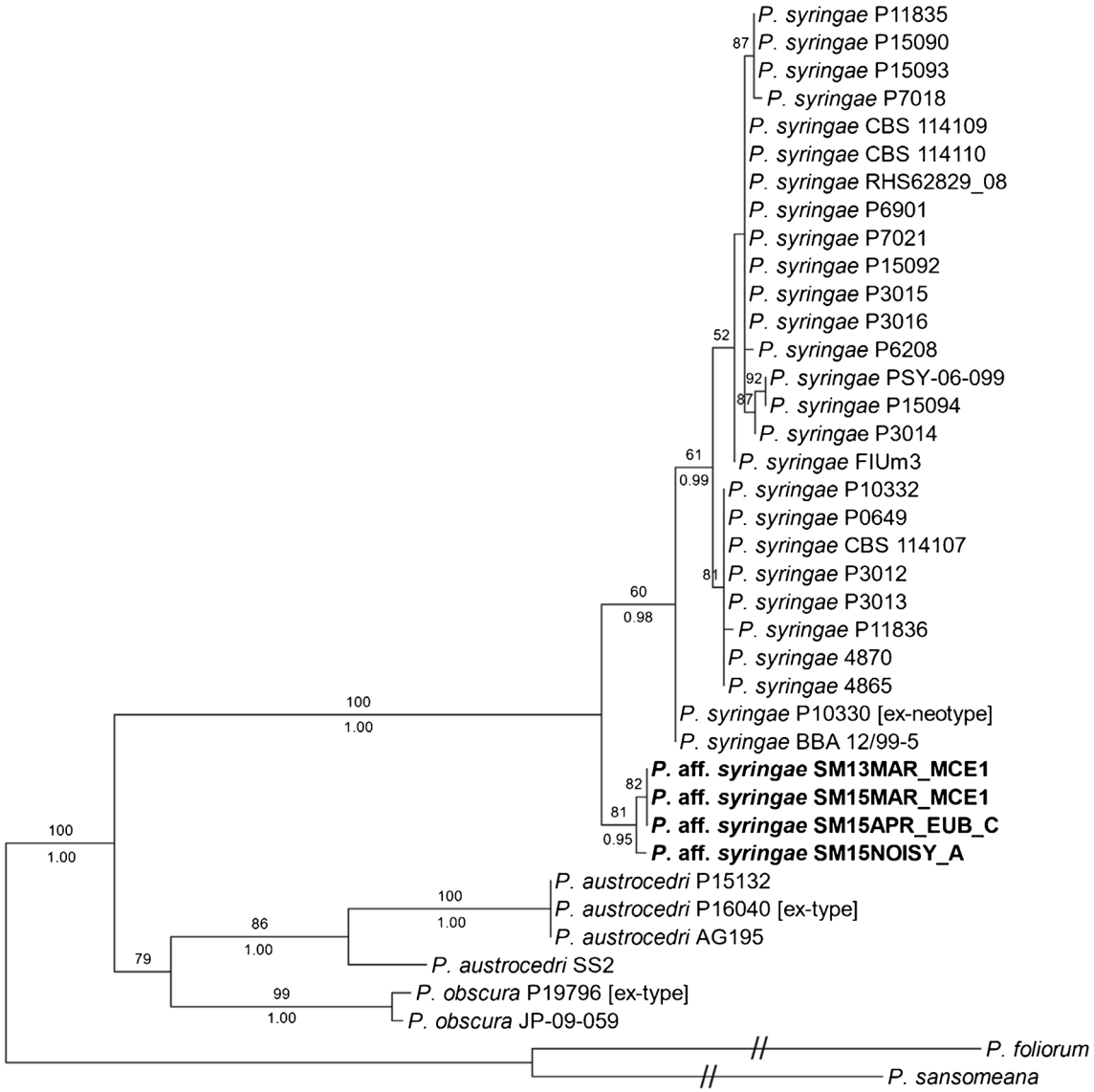

Fig. 8 Maximum likelihood tree of *Phytophthora* subclade 8c. Tree inferred with IQ-TREE 2 from a mt cox2 + spacer alignment. Support values above branches are ultrafast bootstrap approximations ≥ 50 and below are posterior probabilities ≥ 0.90 from an analysis with MrBayes. Isolates in bold are from this study. Sequence accessions available in Online Resource 3. Slashes indicate half-length branches for display purposes

Discussion

Distribution of *Phytophthora* species

Over the course of the survey, *Phytophthora* species were isolated from 178 different baiting sites (Online Resource 1). While *P. ramorum* was the main target of the survey, it is still no surprise that the exotic, invasive pathogen was isolated from the greatest number of sites (Table 1), as it is an epidemic pathogen. Thanks in large part to the ongoing epidemic and measures to prevent the spread of *P. ramorum*, the west coast of North America is becoming a well-studied stretch of coastline when it comes to *Phytophthora* species occurring in lotic ecosystems.

Phytophthora pluvialis, *P. nemorosa*, and *P. pseudosyringae* were well-represented in the Oregon stream surveys (Reeser et al. 2011a, b; Sims et al. 2015) and were also widely distributed in the three northernmost California counties surveyed (Del Norte, Humboldt and Mendocino). However, abundances of all three clade 3 species were dramatically reduced in the southern section south of the SF Bay area and *P. pluvialis* was never isolated south of Mendocino Co. It may be that central California represents the southern limits of these species' ranges, but if latitude affects

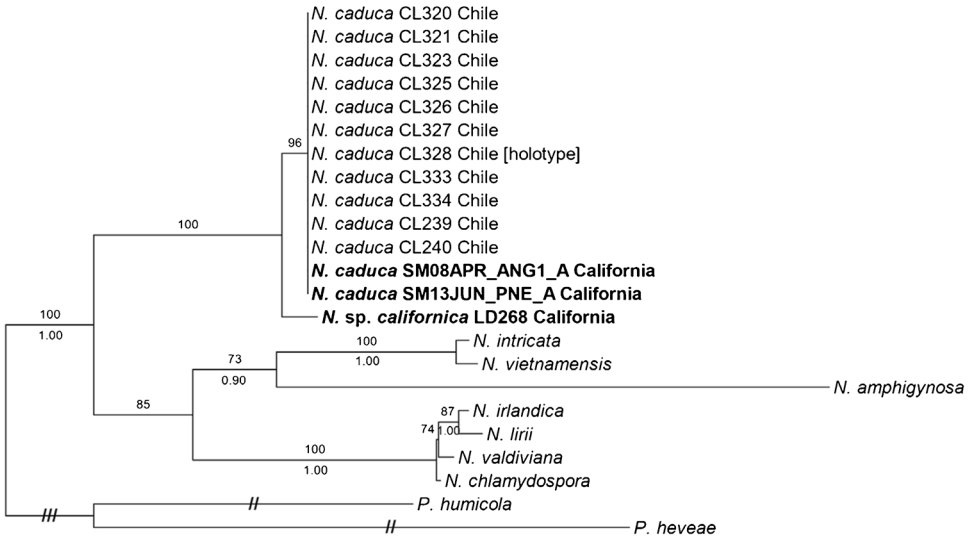

Fig. 9 Maximum likelihood tree of *Nothophytophthora*. Tree inferred with IQ-TREE 2 from a nuclear-only, three-locus (ITS rDNA, LSU rDNA, and *BTUB*) alignment. Support values above branches are ultrafast bootstrap approximations ≥ 50, and below are posterior probabilities ≥ 0.90 from an analysis with MrBayes. Isolates in bold are from this study. Sequence accessions available in Online Resource 3. The length of branches with double slashes were halved for display purposes, and the branch connecting the outgroups is 5% original length

the active season of these species, the lower abundances may also be a result of the sampling window starting too late. The more abundant clade 6 species, such as *P. gonapodyides* and *P. chlamydospora* were distributed across the entire sampling area and period.

Several species isolated in this study, including most of the clade 2 and many of the clade 6 species were only baited in the southern part of the sampling area, leading to the potential conclusion that their range does not extend into Northern California. Yet several of these species, including *P.* sp. *aureomontensis* were isolated in Oregon streams by Sims et al. (2015). The seasonality of the studies may underlie these apparently disjunct distributions. This study sampled between February and June, rarely into July, while Sims et al. (2015) surveyed streams between June and October. Supporting this, most of the southern clade 2 isolates in this study were isolated in the later sampling months (Table 2). The lack of sampling during the warmest months may also account for why no member of *Phytophthora* clade 9 was baited in this survey. Many clade 9 species are relative thermophiles, have been documented in water bodies in California, and can be baited with rhododendron leaves (Yang et al. 2014; Bourret et al. 2022a). The seasonality of detection of many species, including *P. ramorum* and *P. pluvialis* varies greatly with season, largely associated with precipitation levels (Davidson et al. 2005, 2008; Eyre and Garbelotto 2015; Fraser et al. 2020). Because this project had a geographically biased sampling procedure and did not sample year-round, resulting species distributions (Fig. 2) may resemble a springtime snapshot rather than a thorough survey, and should be interpreted accordingly.

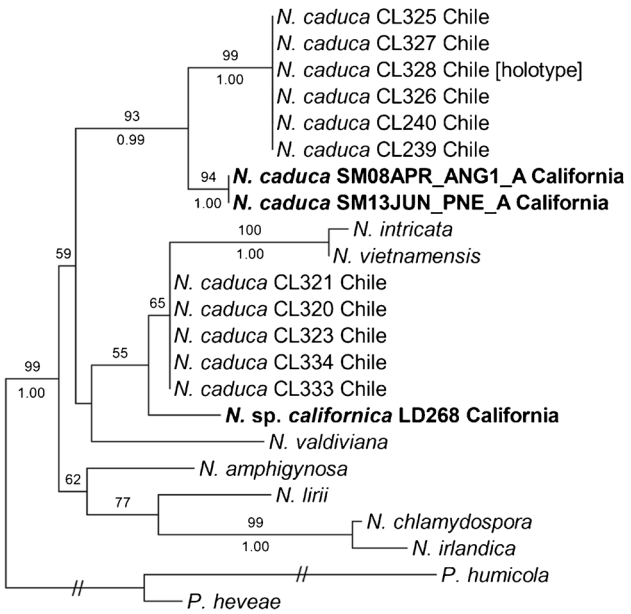

Fig. 10 Maximum likelihood tree of *Nothophytophthora.* Tree inferred with IQ-TREE 2 from a mitochondrial cox1 alignment. Support values above branches are ultrafast bootstrap approximations ≥ 50, and below are posterior probabilities ≥ 0.90 from an analysis with MrBayes. Sequence accessions available in Online Resource 3. Isolates in bold are from this study. The length of branches with double slashes were halved for display purposes

When the results of this survey were compared to 22 contemporary stream baiting projects across 20 worldwide locations, the *P. citricola* species complex (taken as a whole) was the most widely distributed "species," present in 15 of the 20 locations (Table 3). Following the *P. citricola* complex were *P. chlamydospora* and *P. gonapodyides* at 13 and 12 of the locations, respectively. Mexico and Western Australia were the only two locations where neither *P. chlamydospora* nor *P. gonapodyides* was baited. Notably, *P. gonapodyides* was not isolated by any of the southern hemisphere studies. Although *P. pseudosyringae* was detected in North Carolina and Chile, the relative prevalence of clade 3 species seems

Table 3 Worldwide distribution of the three most common *Phytophthora* species and clade 3 across 22 stream-baiting studies

Hemisphere	Continent	Country (state, province)	*P. citricola* species complex	*P. chlamydospora*	*P. gonapodyides*	Clade 3	Study
Northern	Asia	Xinjiang Province, China	+	+	+	-	(Xu et al. 2019)
Northern	Asia	Yunnan Province, China	+	+	+	-	(Huai, W.-x. et al. 2013)
Northern	Asia	Vietnam	+	+	-	-	(Jung et al. 2020)
Northern	Asia	Taiwan	+	+	-	-	(Jung et al. 2017a)
Northern	Europe	Sicily, Italy	+	-	+	-	(Jung et al. 2019)
Northern	Europe	Ukraine	-	-	+	-	(Matsiakh et al. 2016)
Northern	Europe	Bulgaria	+	+	+	-	(Christova 2022)
Northern	North America	Alaska, USA	-	-	+	+	(Reeser et al. 2011b)
Northern	North America	Oregon, USA	+	+	+	+	(Reeser et al. 2011b)
Northern	North America	Oregon, USA	+	+	+	+	(Reeser et al. 2011a)
Northern	North America	Oregon, USA	+	+	+	+	(Sims et al. 2015)
Northern	North America	California, USA	+	+	+	+	This study
Northern	North America	Interior SW, USA	-	-	+	-	(Stamler et al. 2016)
Northern	North America	Connecticut, USA	+	+	+	-	(Brazee et al. 2016)
Northern	North America	Mississippi, USA	-	+	-	-	(Bily et al. 2018)
Northern	North America	North Carolina, USA	+	-	+	+	(Hwang et al. 2011)
Northern	North America	Tennessee, USA	+	+	+	-	(Shrestha et al. 2013)
Northern	North America	Mexico	-	-	-	-	(Rodríguez et al. 2021)
Southern	Africa	South Africa	+	+	-	-	(Nagel et al. 2015)
Southern	Africa	South Africa	+	+	-	-	(Oh et al. 2013)
Southern	Australia	Western Australia	+	-	-	-	(Hüberli et al. 2013)
Southern	Australia	Victoria	+	+	-	-	(Dunstan et al. 2016)
Southern	South America	Chile	+	+	-	+	(Jung et al. 2018)

to be restricted only to riparian studies from western North America.

Detection of *Phytophthora* species

Baiting for *Phytophthora* with clean plant material provides a powerful method for detecting and isolating microbes that are typically rare, particularly in soil communities. But there is no single plant bait that will detect all *Phytophthora* species (Burgess et al. 2021), nor can surveys that rely solely on baiting hope to comprehensively sample the genus (La Spada et al. 2022). Nevertheless, rhododendron leaves may represent an optimal choice if only a single bait is able to be used (Ghimire et al. 2009). This study and others demonstrate that stream baiting represents an efficient way of detecting local *Phytophthora* species on the landscape, particularly compared to soil-baiting. However, most soil-borne species are unlikely to be detected in streams. While some soil-borne species such as members of the *P. cryptogea* or *P. alni/cambivora* species complexes are well known to attack roots in riparian habitats and are commonly encountered in infested waterways, others, such as *P. cinnamomi*, are rarely encountered in stream surveys, even in infested areas (Hüberli et al. 2013; Sims et al. 2015). Some *Phytophthora* species, such as *P. pseudosyringae* and *P. syringae*, are well known to infect both underground, root-and-stem tissue as well as foliar plant tissue (Erwin and Ribeiro 1996; Hansen et al. 2017) and were consistently detected in this survey. Other species, such as *P. gonapodyides*, *P. riparia*, and *P. lacustris*, appear to be true residents of aquatic habitats, and may be primarily saprotrophic (Marano et al. 2016; Aram and Rizzo 2018, 2019). It is clear that because of both physical distributions and methodological shortcomings, a hypothetical isolation-based survey of the entire *Phytophthora* community in a particular region would employ aquatic ecosystem monitoring, soil, and root baiting with a variety of plant baits and direct isolation from aboveground symptomatic material, using both isolation-based and eDNA-based detection methods. Climate change is also likely affecting the optimal ranges of many *Phytophthora* species and their plant hosts. To catch seasonal diversity, year-to-year fluctuations, and climate-induced range alterations, sampling should be year-round and span multiple years.

Intraspecific diversity of *Phytophthora* and *Nothophytophthora* isolates

Nothophytophthora was previously documented from forest streams (Jung et al. 2017c) and has been isolated in Europe,

Asia, and South America (Jung et al. 2017c; O'Hanlon et al. 2021); to our knowledge, the Californian stream–baiting isolates represent the first documentation of the species *N. caduca* and the genus *Nothophytophthora* in North America. With two species now documented in California, either multiple species have been introduced to the state, or California (and North America) is within the natural geographic range of *Nothophytopthora*. If *N. caduca* is indeed native to both Chile and California, more study is needed to determine if the species has a continuous or disjunct distribution and understand how the nuclear genomes appear so similar between Californian and Chilean isolates, yet mitogenomes so distinct. Anthropogenic movement and/or hybridization may also explain the patterns observed.

We report the emerging pathogen *P. pluvialis* for the first time in California, to our knowledge. The Californian *P. pluvialis* isolates were baited in this study concurrently with the description of the species and its implication as an emerging conifer pathogen around the world (Reeser 2013; Dick et al. 2014; Hansen et al. 2015). All 17 *P. pluvialis* strains isolated in the current study were shared with researchers in Oregon and New Zealand, helping to demonstrate that the New Zealand population appears imported from a native Pacific coast population, providing valuable information to Californian regulators and land managers (Brar et al. 2018; Tabima et al. 2021). Although some watersheds within the native range of *Pinus radiata* were stream-baited in this study, none of the *P. pluvialis* isolates came from these watersheds. It has been proposed that the most or all of *Phytophthora* clade 3 is native to the west coast of North America (Hansen et al. 2017; Bourret et al. 2020). Besides the population genetic results from *P. pluvialis*, the documentation of distinct, Californian *P. pseudosyringae* ITS haplotypes lends some additional evidence to this theory.

The incongruence observed between the *P. syringae* topology in the cox2 + spacer tree and the other two mitochondrial loci (Figs. 6, 7, and 8) is unexpected, inasmuch as sections of the mitogenome are not expected to evolve separately. Although more study is needed, the evidence concerning the four *P*. aff. *syringae* isolates leans towards them being conspecific with *P. syringae* rather than a distinct species. Based on this interpretation, the four isolates represent substantial, novel intraspecific diversity within *P. syringae*, suggesting California may be within the native range of the species.

Sequencing of the cox2-cox1 region of the *P. cactorum* isolates revealed considerable intraspecific diversity, with seven of the eight strains forming a distinct intraspecific lineage deemed the "Northern California forest lineage" (Bourret et al. 2022b). Isolate SM15APR_WNS (from the northernmost site in the study) was found to possess an ancestral cox2-cox1 sequence according to network analysis, and the study concluded that California appeared to be within the native range of both *P. cactorum* and *P. pseudotsugae*. While population genetic evidence has suggested that species such as *P. cactorum* and *P. pluvialis* are indigenous to California, *P. multivora*, the most common clade 2 species in the survey, was found to be native to the southern hemisphere (Tsykun et al. 2022), strongly suggesting it is an invasive species in California. Thus, while monitoring the spread of one invasive pathogen (*P. ramorum*) across the landscape, we were also tracking another invasive species (*P. multivora*). In California, *P. multivora* appears to be the most commonly isolated member of the *P. citricola* species complex, where it has been identified as *P. citricola* until recent years (Bourret et al. 2022a). Regardless of whether they determine *Phytophthora* species to be native or non-native, population genetic studies provide valuable evolutionary, ecological, and biogeographic insights for researchers and regulators.

The *Phytophthora* species in clade 6 often exhibited polymorphic positions (SNVs and indels) in their direct-sequenced ITS traces, and the diversity of these polymorphisms contributed in large part to the great diversity of ITS haplotypes exhibited by many of these clade 6 species (Table 1, Online Resource 2). *P. chlamydospora* was represented by nine haplotypes, three of which were not previously in GenBank when deposited, and *P. riparia* was also represented by eight haplotypes, five of which were new to GenBank. *P. lacustris* had seven unique out of ten total and *P. gonapodyides* eighteen unique out of twenty-two total (Table 1, Online Resource 2). The large diversity of ITS haplotypes recovered for these species lends evidence that they are native or long-established in the area, but it is also possible that the "sterile" nature of some of the clade 6 species contributes to this ITS diversity. The concerted evolution by which most eukaryotic rDNA tandem repeats are homogenized depends on crossing-over events that may only take place during meiosis (Cluster et al. 1996; Naidoo et al. 2013; Xu et al. 2017). The lack of meiosis in truly sterile, non-homothallic, asexual *Phytophthora* strains might allow the accumulation of mutations across the rDNA tandem repeats that would normally be purged or homogenized. This would be visualized during direct Sanger sequencing as a greater number of heterozygous positions (SNVs and indels) and result in the relatively rapid accumulation of diverse ITS haplotypes. It is also common for these clade 6 species to exhibit exceedingly noisy sequence reads, even when other species in a batch were otherwise clean — another possible sign of the breakdown of concerted evolution as mutations are not able to be purged from the tandem repeats. Sterility, or rare and cryptic sex, appears to be a hallmark of some clade 6 species (Brasier et al. 2003), but it may be possible for any strain to go through prolonged asexual cycles without meiosis, especially in aquatic environments.

Hybridization is another source of ITS haplotype diversity in the form of SNVs, as some interspecific hybrids (such

as the *P. lacustris* × *riparia* isolates from this study) exhibit numerous heterozygous ITS positions due to the presence of both parental ITS haplotypes. Hybridization is well-characterized in clade 6 (Nagel et al. 2013; Oh et al. 2013; Burgess 2015; Burgess et al. 2018), and it is not clear what portion of the SNVs common in clade 6 ITS sequences are the result of breakdown of concerted evolution, intragenomic allelic diversity from either recent or past intraspecific hybridization, or other possibilities (Cluster et al. 1996). More work is needed to characterize the way rDNA evolves in oomycetes and how this might affect ITS-based barcoding and phylogenetic inference.

P. lacustris × *P. riparia*

Strains that appeared to be interspecific hybrids between *P. lacustris* and *P. riparia* were isolated along with both parental species. The hybridization between the species is well-documented, and the original description of *P. riparia* identified a hybrid isolate from Northern California (Hansen et al. 2012). While *P. lacustris* is undoubtedly one of most widespread species of *Phytophthora* on the planet according to sequence depositions, the geographic range of *P. riparia* is restricted. The mitochondrial sequences obtained in this study demonstrated that among the collection of putative hybrid stream isolates are strains with both species as mitochondrial parents, strongly suggesting that at least two hybridization events have taken place. All three species were also present in the stream surveys of Stamler et al. (2016) in interior southwestern US states. That study also found strong evidence for diversity of *P. lacustris* × *riparia* isolates and multiple hybridization events using subcloning methods. Brazee et al. (2016) baited *P. lacustris* and *P. lacustris* × *riparia* from the Connecticut River valley in the northeast US, but did not find *P. riparia*. Additional work is needed to discover whether the existence of *P. lacustris* × *riparia* is an ongoing natural process, or one driven by recent human activities.

Conclusion

Stream monitoring of *Phytophthora* species has served as an important early detection tool for *P. ramorum* in natural ecosystems for nearly two decades, and is used across all three west-coast US states. While sampling for *P. ramorum*, many other species of *Phytophthora* can be isolated and studied, adding greatly to our knowledge of baseline, resident communities of this fascinating and troublesome genus.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1007/s11557-023-01910-8.

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Author contribution All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Kamyar Aram, Tyler Bourret, and Heather Mehl. The first draft of the manuscript was written by Tyler Bourret, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability Sequence accessions employed for all phylogenetic inference can be found in Online Resource 3. Sequence alignments and phylogenetic inference output files (including partitioning and model schemata and trees) from this study can be found in Online Resource 4.

Declarations

Competing interests The authors declare no competing interests.

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