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Phytophthora species repeatedly introduced in Northern California through restoration projects can spread into adjacent sites

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Abstract This study investigates whether *Phytophthora* species may have been repeatedly introduced in natural habitats through restoration projects. Six plant species across five research locations in three counties within the San Francisco Bay Area were tested for pathogen infection of stems, roots and for rhizosphere infestation at failing restoration sites. Where possible, the same hosts were evaluated in one neighboring unrestored site disturbed by the presence of culverts, drainages or trails that also intersected the restoration site, and in a naturally regenerated and undisturbed control site. Although native or endemic pathogens were isolated from all three types of sites, *Phytophthora* species were never isolated from control undisturbed sites. Statistical analyses confirmed that percentage of positive *Phytophthora* isolations was significantly higher in restoration sites and adjoining disturbed sites than in control sites. Presence of *Phytophthora* species was correlated with disease symptoms, plant death and lack of regeneration. Furthermore, six of eight *Phytophthora* species isolated in the field had previously been reported from plant production facilities providing stock for habitat restoration. To our knowledge, this is the first controlled survey linking the presence of entire *Phytophthora* species assemblages to failing restoration projects and to the plant production facilities that provide plant stock for restoration, while showing that *Phytophthora* species are absent in neighboring undisturbed sites. This study further proves that these pathogens are spreading from restoration sites through disturbance pathways.

Keywords Failed restorations · Infected plant stock · Soilborne pathogens

Introduction

Biological invasions (Simberloff et al. 2013) are regarded as one of the three major causes for loss of biodiversity at the planetary level, together with climate change (Bellard et al. 2012; Pautasso et al. 2012) and urbanization (DeFries et al. 2010). Microbes often become invasive (Litchman 2010), not unlike plants and animals, and additionally can lead to the emergence of novel plant diseases with additional direct and detrimental effects on the integrity of affected ecosystems (Almeida and Nunney 2015; Desprez-Loustau et al. 2007; Parker and Gilbert

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2004). Although native pathogens may increase their presence due to ecosystem-level alterations such as practices associated with plantation forestry and the planting of exotic hosts (Garbelotto and Gonthier 2013; Hessburg et al. 2001), the majority of microbial invasions of terrestrial ecosystems are caused by exotic plant pathogens (Lovett et al. 2016), many introduced through international trade (Liebhold et al. 2012).

As exemplified by the well known 1:10 rule (Williamson and Fitter 1996), only a fraction of exotic species succeed and become established in novel environments, ecologically and climatically distinct from their native habitats. The factors leading to the success or failure of microbial invasions are not fully understood, but similarity in climate between invaded and region of origin (Santini et al. 2013; Vacher et al. 2008), presence of susceptible hosts (Lovett et al. 2006; Schulze-Lefert and Panstruga 2011), absence of competitors or predators (De Roy et al. 2013), ability to survive in a dormant spore stage or to feed saprobically on dead matter, high reproductive potential (Giordano et al. 2014) and short life cycles along with synchronicity to important host life stage events (Dodd et al. 2008; Pautasso et al. 2012) are often cited among factors known to enhance microbial invasiveness.

One key to understanding microbial invasions is the identification of pathways of introduction. Single "accidental" introductions are less likely to succeed than systematic repeat introductions (Dlugosch and Parker 2008). Likewise, when introductions, even if repeat, occur in artificial ecosystems spatially isolated from natural habitats, e.g. an orchard versus a forest, there may be a significant delay between the establishment of the exotic organism occurring in these artificial environments and its spread in natural ecosystems where it may become invasive and truly endemic (Blitzer et al. 2012). The stronger the separation between artificial and natural habitats, the harder it may be for the organism to successfully move in between them. Although there are several examples of plant pathogens that have succeeded in moving from urban, agricultural and landscaped settings into natural ecosystems (see Anagnostakis 1987, Rizzo and Garbelotto 2003), the introduction of these pathogens directly into natural habitats potentially accelerates the invasion process.

A few well-known cases of such direct introductions into wild ecosystems exist, including *Cronartium ribicola* agent of White Pine Blister Rust introduced multiple times in North America at the beginning of the twentieth century (Geils et al. 2010), and *Phytophthora* × *alni* agent of a lethal Root and Collar Rot of alders, a pathogen that emerged in Europe in the late twentieth century (Brasier et al. 1995). *Cronartium ribicola* and *P.* × *alni* are two particularly interesting examples because their introduction occurred by planting seedlings during large reforestation and restoration programs, respectively. The use of infected plant stock in these programs started outbreaks of infectious diseases that decimated susceptible host populations at the continental level.

Recently, a large number of water and soilborne species belonging to the genus *Phytophthora* have been isolated in and near failing restoration projects in California (reviewed in Garbelotto et al. 2018). Some of these reports have simply identified the presence of these *Phytophthora* species in association with plant disease (Bourret et al. 2018; Rooney-Latham et al. 2017), while other reports have actually been able to provide evidence of the active role played by these microbes as causal agents of the disease and mortality observed (Rooney-Latham et al. 2015; Sims and Garbelotto 2018). The production of infected plant stock in California restoration nurseries has been recently proven (Rooney-Latham et al. 2019, Sims et al. 2018), and at least a couple of studies have provided evidence in support of a presumed pathway of repeated successful introduction of *Phytophthora* spp. in multiple restoration sites through the use of infected plant stock (Rooney-Latham et al. 2015; Sims et al. 2019).

The primary goal of this work was to improve our understanding of the introduction pathways and further spread of soilborne *Phytophthora* species from restorations into wildlands in California. Surveys were conducted in Marin, San Francisco, and San Mateo Counties in the San Francisco Bay Area, targeting sites where high incidence of plant mortality suggested restorations efforts had failed. Immediately neighboring sites that had a comparable plant composition but had not been restored, and sites that were further away from restoration sites while being characterized by the same plant cover, served as controls.

To our knowledge, the simultaneous evaluation of infestation levels by plant pathogens in restoration

sites, comparable high-risk neighboring disturbed areas, and control locations has not been conducted previously in California, hence this study represents one of the first proofs of concept that restoration efforts may be responsible for the systematic introduction of pathogens in California, especially in the Wildland Urban Interface (WUI).

Over 25 soilborne *Phytophthora* species have been isolated recently from or near restoration sites (Garbelotto et al. 2018; Rooney-Latham et al. 2017). These findings suggest at least some waterborne and soilborne *Phytophthora* species may be the likely culprits of some of the plant mortality reported in restoration sites, dictating a precise experimental design focusing on plant infection as well as on soil and water infestation.

Direct isolations from symptomatic plant tissues were also performed to provide further evidence in support of a pathogenic role these *Phytophthora* species may have on plants, given that a large number of *Phytophthora* species have been recently identified in plant production facilities that provide stock to be used in restoration projects (Garbelotto et al. 2018; Rooney-Latham and Blomquist 2014, 2015; Sims et al. 2018). Plant species in restoration nurseries with confirmed or suspected *Phytophthora* species infection included *Diplacus aurantiacus, Frangula californica, Ceanothus thyrsiflorus*, and *Heteromeles arbutifolia*, *Aesculus californica*, and *Salix lasiolepis*.

We have previously used molecular and morphological markers to track the spread of the soilborne *Phytophthora crassamura* from infested plant nurseries to multiple wild sites in the WUI of Northern California (Sims et al. 2019). Accordingly, the final part of this study was to verify whether plants in local San Francisco Bay Area production facilities (e.g. restoration nurseries) may also be infected by the same *Phytophthora* species isolated in and adjacent our restoration study sites. Thus, our field sampling included by design two species, namely *Diplacus aurantiacus* and *Frangula californica,* both reported to have high levels of *Phytophthora* infection in plant production facilities (Garbelotto et al. 2018; Rooney-Latham et al. 2015; Sims et al. 2018). Results from this part of the study would provide further evidence corroborating the existence of an introduction pathway that starts from plant production facilities, continues through restoration efforts and ends in the spread of these exotic species in natural ecosystems through the movement of infested soil and water.

Phytophthora cinnamomi has recently been hypothesized to have been introduced through agricultural and horticultural practices (Socorro Serrano et al. 2019), while *P. ramorum* is known to have been introduced through the production and sale of infected ornamental plants by the ornamental plant industry (Croucher et al. 2013). Both are now present in natural ecosystems. The purpose of this study was to provide additional evidence regarding the introduction of pathogens in association with restoration projects. Additionally, we wanted to provide evidence that these pathogens are not limited to restoration sites but are radiating outwards following disturbance pathways along which infested soil and water are moved. A similar introduction pathway has been suggested for *P. lateralis*, a water and soilborne pathogen that was introduced through the use of infected plant stock and then has spread with success thanks to its close association with two hosts present in the forests of Northern California, combined with its ability to infest soil and water (Jules et al. 2002). An alternative hypothesis would be that these species are already present in natural ecosystems and that disease becomes expressed due to the effect of disturbances associated with restoration projects.

Our hypotheses would be confirmed if:

a) Putatively introduced *Phytophthora* species were isolated at higher frequency in restoration and adjacent disturbed unrestored sites, compared to control unrestored and undisturbed sites, disjointed from restorations.
b) Native pathogens were isolated at equal frequency from restoration sites, adjacent disturbed sites and natural control sites.
c) *Phytophthora* species isolated from infected plants in restoration sites and adjacent disturbed sites were at least in part the same reported from "restoration" nurseries providing stock for restoration projects.

This study, even if preliminary, is yet another study further supporting the existence of a pathway for the introduction of *Phytophthora* species in restoration sites (see Frankel et al. 2020a, b). This pathway not only has the potential to result in the systematic failure of restoration projects, but could cause an equally systematic introduction of pathogens in neighboring

sites, possibly reaching adjacent semi-natural habitats. These habitats not only are particularly fragile and vulnerable because located in the WUI, but they are also under significant pressure because of urban sprawl, over use by local human populations, invasion by exotic plants and animals, and climate change.

Materials and methods

Selection of study sites and plant sampling

Field surveys and samplings were conducted between April 2016 and December 2017 to determine plant health and evaluate incidence of plant pathogens including *Phytophthora* species in the San Francisco Bay Area (California, USA). Five study locations were selected: one in San Mateo, three in Marin and one in San Francisco Counties following the finding that restoration nurseries were contaminated with soil-borne *Phytophthora* in these counties (Sims et al. 2018). In Marin and San Mateo Counties, three types of plots were setup up: (1) restored sites with evidence of multiple dying or dead plants often lacking any kind of natural regeneration around them; (2) high risk-disturbed sites, i.e. unrestored sites immediately adjacent to restored sites or, if further away, intersected by the same culvert, drainage ditch, path or road (disturbances) also intersecting the restoration site. It was hypothesized that these sites may have been infested by the movement not only of infected plant material, but also of infested soil and water coming from failing neighboring restoration sites; (3) control sites, i.e. unrestored sites, a few hundred meters from restoration sites or adjacent but uphill from restored or high risk-disturbed sites, characterized by the same vegetation type and without any intersecting disturbance pathway that would have facilitated the movement of infested soil and water. The area surveyed in each plot type was approximately 3000 m^2, and the number of plants surveyed and sampled in each plot ranged between 3 and 10 per plant species, depending on availability. Control unrestored and undisturbed plots could not be set up in San Francisco County, based on the fact that historical disturbances and plantings could not be fully be excluded, so only restored and unrestored but disturbed plots were studied in that County. In summary, evaluations were made in 5 restoration sites, 5 high-risk-annexed ones, and 4 control sites. See Table 1 for number of plants for each species sampled at each research site type in each county.

The habitat or vegetation type of all study sites can be described as coastal scrub, i.e. heathlands dominated by shrubs with localized presence of small clusters of trees. Information available for each restoration site is provided in the Supplementary Table 1 and in Table 1. Figure 1 shows one of these failing restorations in a coastal scrub site in San Mateo County.

Plant species sampled included *Diplacus aurantiacus* (sticky monkey-flower), *Frangula californica* (California coffeeberry), *Ceanothus thyrsiflorus* (blue-blossum), *Salix lasiolepis* (Arroyo willow), *Heteromeles arbutifolia* (toyon), and *Aesculus californica* (California buckeye), (Table 1). Plant species were selected based on: (1) species that had been previously surveyed in restoration nurseries (Sims et al. 2018), and, (2) availability of at least 3–10 individuals per site. All surviving plants in each site were sampled, and, if there were more than 10 plants, then 10 were randomly selected and sampled zigzagging up and down and across the site. For each sampled plant, roots and soil were collected independent of health status, and symptoms were documented (Table 1, Fig. 2).

Rhizosphere sampling

To sample each plant, two liters of both soil and roots were collected using a sterilized shovel. The organic layer was scraped off, and samples were generally from the upper 25 cm depth. To isolate *Phytophthora* from rhizosphere samples, baiting (Erwin and Ribeiro 1996) was conducted using fruit pieces of *Pyrus communis* of the D'anjou variety (McIntosh 1964), entire leaves or leaf pieces of *Rhododendron* 'Cunningham's White' (Sims et al. 2015), and *Origanum vulgare* leaf and stem pieces used together. Deionized water was added to about 2 cm above the soil line in the plastic bag, and bags were kept at room temperature roughly (22 °C), for 5 days. In the absence of any typical necrotic lesions on baits after the first baiting process, a double-baiting procedure involving a drying out and rewetting step was used (Jeffers and Aldwinckle 1987). Bait pieces displaying typical necrotic lesions or with significant degradation were submerged in selective ½ VARP + medium (V8 based agar amended with 10 ppm Pimaricin,

Table 1 Plant species sampled, where they were collected by county, Marin (M), San Francisco (SF), San Mateo (SM), the number sampled and symptoms observed

Plant species	Area type	Plants sampled by county			Symptoms observed
		M	SF	SM	
Frangula californica	Restoration	9	–	10	Marin-stunted and chlorotic (3), abiotic stress and did not grow away from container space, i.e., roots in shape of container (6); San Mateo—chlorotic, small foliage, extensive dieback 30–90%, some with small aerial branch cankers
	High Risk	9	–	20	Marin-stunted and chlorotic; San Mateo-extensive 30–90% dieback, chlorotc, sparse foliage, root-rot, some with small aerial branch canker
	Control	5	–	10	Marin-none worth noting; San Mateo—plants appear healthy or have small aerial branch canker with callus growth, some branch level dieback, broken branches
Diplacus aurantiacus	Restoration	26	8	10	Marin-50-nearly 100% (only one side of stem with green tissue, no living foliage) dieback, stem cankers near root crown, root rot, some (3) plants healthy with limited dieback 10–20%; San Francisco-not noted; San Mateo—stunted, 20–90% dieback, crown rot
	High Risk	10	–	5	Marin-blackened foliage; San Mateo-limited dieback 10–20%
	Control	24	–	5	Marin-healthy,some with blackened foliage somewhat stunted; San Mateo—wind swept plants on bluff are stunted
Ceanothus thyrsiflorus	Restoration	5	10	–	Marin-small leaves, chlorotic, some grazing; San Francisco—stem cankers on branches near soil, 10–60% dieback, some (3) healthy plants, stunted, one plant with limted gowth due to soil erosion
	High Risk	–	5	–	2 healthy plants, 10–60% dieback
	Control	–	–	–	
Salix lasiolepis	Restoration	5	–	–	Very stunted, root collar cankers, chlorotic, blackened roots subtending from base of stem
	High Risk	5	–	–	Root collar cankers
	Control	–	–	–	
Heteromeles arbutifolia	Restoration	–	3	–	Stunted, 10% dieback, chlorosis
	High Risk	–	–	–	
	Control	3	–	–	Callused canker and foliar spot
Aesculus californica	Restoration	3	–	–	Grazing damage, stunted
	High Risk	–	–	–	
	Control	–	–	–	

200 ppm Ampicillin trihydrate, 10 ppm Rifampicin, 15 ppm Benomyl and 25 ppm Hymexazol [97%]) contained in 100 mm × 15 mm Petri dishes.

Direct isolations from symptomatic tissue

To perform direct isolations from samples, the entire plant was first uprooted and placed in a plastic bag with a moist cloth to avoid drying out the sample, before being placed in cold storage (5 °C) for up to 48 h until sampling occurred.

Direct isolations from symptomatic stems and roots were performed as follows. Symptomatic stem tissue was taken directly from plants showing typical stem canker symptoms. Note that plants had two types of stem cankers: (1) soil associated cankers, present either at the root collar or close to the soil in low hanging branches, (2) aerial cankers present at least 25 cm above the ground. Symptomatic roots were identified instead as necrotic (e.g. blackened) root portions visible after carefully removing the soil from the root ball, rinsing repeatedly the roots under

Fig. 1 Dead and dying coffeeberry (*Frangula californica*) shrubs are clearly visible in a failing restoration in a costal scrub site in San Mateo County, dominated by shrubs with occasional small groups of trees. *Phytophthora crassamura* and *P. megasperma* were both isolated directly from symptomatic tissue and from the rhizosphere (i.e. soil and fine roots) of diseased plants

running tap water until all visible soil was removed, root were sectioned into approximately 3 cm sections, placed in a 0.3% sodium hypochlorite solution for 30 s, re-rinsed, and then placed on a clean paper towel.

Six stem or root pieces were excised, approximately 10 mm × 5 mm × 2.5 mm in size for stems and about 5 mm in length for root sections, then, each piece containing healthy and diseased tissue was plated into a selective oomycete ½ VARP medium (above recipe without Hymexazol) and on PDA to isolate fungal plant pathogens.

For *Phytophthora* or *Pythium* identification, isolates were transferred on to non-selective V8 medium and evaluated for morphological characteristics (Erwin and Ribeiro 1996). For fungal identification, morphology was analyzed directly on the PDA plates used for the original isolation. DNA amplification and sequencing was done from cultures as described in Sims et al. (2018). The primers and amplified region were as follows: the ribosomal region spanning the internal transcribed spacer (i.e., ITS; ITS1-5.8S-ITS2), amplified with DC6 (Cooke et al. 2000) and ITS4 (White et al. 1990) primers. PCR products were prepped for sequencing using the amplification primers and PCR clean-up and Sanger sequencing was conducted by the University of California, Berkeley, DNA Sequencing Facility. For fungal pathogens all steps were as above, except that the forward PCR primer ITS1f (Gardes and Bruns 1993) was used instead of DC6.

Analyses

Bernoulli trials were used to evaluate whether differences in *Phytophthora* detection were significant in ad hoc comparisons in which each sample plant was considered a trial. Comparisons included: (1) sites in each of the three counties (189 trials); (2) restored

Fig. 2 Upper left- healthy (no Phytophthora), versus upper right-unhealthy (Phytophthora positive) *Diplacus aurantiacus* plants and a close up of a canker (bottom image from Sims et al. 2019) from which *Phytophthora megasperma* was isolated

versus control sites (136 trials); (3) high risk-disturbed versus control sites (100 trials); (4) restored versus high risk-disturbed sites (142 trials); (5) upland versus lowland plants (189 trials), and; (6) *Diplacus aurantiacus* versus *Frangula californica* plants (150 trials), given the two were the most abundant species sampled in this study. We also used the same statistical analysis to compare (7) the frequency of putatively native non-*Phytophthora* plant pathogens among restored, high risk-disturbed sites, and control sites (189 trials). For this comparison we considered all isolates as belonging to the single category of putative native non-*Phytophthora* pathogens, independent of species designation.

For data analysis, Pearson's chi-square (X^2) with Yates continuity correction for any deviance from the chi-square distribution was used to evaluate hypotheses and plant species associations. Fisher's exact tests

were used in place of chi-squares where expected cell counts were < five (Agresti 2007; Fisher et al. 1943). Tests were conducted for each hypothesis in *R* (R core team 2020). The Pearson's chi-squared statistic was used for testing H_0, with each set of trials in an $I \times J$ table (Agresti 2007) for independence where n_{ij} row i column j, for $i = 1,\ldots, I$, $j = 1,\ldots, J$) was the cell counts in the table of healthy plant (success) outcomes versus *Phytophthora* detection outcomes, and where π denotes the probability of success for a given trial and the values $u_{ij} = n_{\pi_{ij}}$ were the expected frequencies. The test statistic used here:

$$X^2_{\text{Yates}} = \sum \frac{\left(\left|n_{ij} - u_{ij}\right| - 0.5\right)}{u_{ij}}$$

If comparisons displayed dependency based on chi-squared then they were further evaluated, in terms of health outcomes, for the odds ratio (Agresti 2007) of achieving a healthy sample from the comparative factors. Where the odds ratio (θ) equals:

$$\theta = \frac{\pi_1/(1-\pi_1)}{\pi_2/(1-\pi_2)}$$

Fisher's exact tests were used to compute *p*-values and 95% confidence intervals around the odds ratio outcomes. Further, if expected cell counts were zero, as in the case of controls a small number (five) were added to each cell count in the comparison to normalize the odds ratio outcomes and 95% confidence interval and to calculate expected outcomes.

Finally, the list of *Phytophthora* species recently isolated from "restoration" nurseries, i.e., production facilities providing plant stock for restoration projects in the greater San Francisco Bay Area (Rooney-Latham et al. 2019; Sims et al. 2018) was compared to the list of *Phytophthora* species identified from restoration sites in this study, to determine if and how many species may be present both in restorations and in plant production facilities.

We also calculated how many of the total number of *Phytophthora* isolates obtained in this study belonged to species previously identified in local plant production facilities.

Results

Detailed results by county, treatment (restored vs. disturbed vs. control sites or upland vs. lowland sites) and plant host are provided in Table 2. In brief, no *Phytophthora* species were isolated from any of the 47 samples in control sites. For restoration and high risk sites, 33 of 89 (38%) and 21 of 53 (39.6%) plants were positive, respectively. Thirty-six of 149 (24%) samples in upland sites were positive versus 19 of 40 (47.5%) positive samples in lowland sites.

For the two most commonly sampled plant species, 17 of 88 (19%) *D. aurantiacus* and 21 of 62 (34%) *F. californica* were *Phytophthora* positive. For the lesser sampled plant species, 11 of 20 (55%) *C. thyrsiflorus*, 6 of 10 (60%) *S. lasiolepis*, 0 of 6 *H. arbutifolia,* and 0 of 3 *A. californica* were *Phytophthora* positive.

With regard to native or endemic non-*Phytophthora* species we present the result by site type, e.g. restored, high risk-disturbed, and control [Table 2(7)]. *Fusarium lateritium*, and *Pythium heterothallicum* were isolated from all three types of sites. Specifically, 8 out of 89 (9%) samples, 5 out of 53 (9%) samples, and 5 out of 47 (10.6%) samples were positive for native or endemic non-*Phytophthora* pathogens in restored, high risk-disturbed, and control sites, respectively.

Direct isolations from symptomatic plant tissue were successful for the following pathogen × host species combinations: *D. aurantiacus* × *P. megasperma*, *C. thyrsiflorus* × *P. pseudocryptogea*, *C. thyrsiflorus* × *P. multivora*, *H. arbutifolia* × *P. psuedocryptogea, S. lasiolepis* × *P.* "taxon raspberry", *S. lasiolepis* × *P. pseudocryptogea, Frangula californica* × *Fusarium lateritium* (Table S2). Only *Phytophthora* species were successfully isolated from basal and soil associated stem cankers, while only *F. lateritium* was isolated from small aerial stem cankers.

Statistical analyses did not provide support for H_0, i.e. meaning that we had to reject the H_0 that *Phytophthora* incidence was undistinguishable, for the following comparisons, numbered as per the materials and methods section: (2) restored versus control sites (p-value < 0.0001 from Fisher's exact test; Table 2(2)); (3) high risk-disturbed versus control sites (p-value < 0.0001 from Fisher's exact test; Table 2(3)); (5) lowland versus upland sites (X^2 = 7.2326, p-value = 0.0072; Table 2(5)). Fisher's tests further indicated that *Phytophthora* incidence

Table 2 $I \times J$ tables showing comparisons for healthy plant versus Phytophthora outcomes in Bernoulli trials (observed n_{ij} versus expected u_{ij}) by county (1), restoration versus control (2), annexed areas versus control (3), movement from restored to annexed areas (4), accumulation from upland to lowland areas (5), plant species (6), and finally, (7), the isolation of suspected native non-*Phytophthora* pathogen species from the three site types

County	Healthy		*Phytophthora*		Total plants	Proportion healthy
	n_{ij}	u_{ij}	n_{ij}	u_{ij}		
(1)						
Marin	76	73	27	29.9	103	0.738
San Francisco	17	17.5	9	7.6	26	0.654
San Mateo	41	42.5	19	18.4	60	0.683
X-squared = 0.9908, df = 2, p-value = 0.6093						
Movement	Healthy		*Phytophthora*		Total plants	Proportion healthy
	n_{ij}	u_{ij}	n_{ij}	u_{ij}		
(2)						
Restoration	55	66.1	34	22.9	89	0.618
Control	47	35.9	0	11.1	47	1
Fisher's exact test p-value < 0.0001						
(3)						
Annexed	32	41.7	21	11.3	53	0.604
Control	47	37.3	0	9.7	47	1
Fisher's exact test p-value < 0.0001						
(4)						
Restoration	55	54.5	34	34.5	89	0.618
Annexed	32	32.5	21	20.5	53	0.604
X-squared = 9.8632e-31, df = 1, p-value = 1						
Accumulation	Healthy		*Phytophthora*		Total plants	Proportion healthy
	n_{ij}	u_{ij}	n_{ij}	u_{ij}		
(5)						
Upland	113	105.6	36	43.4	149	0.758
Lowland	21	28.3	19	11.6	40	0.525
X-squared = 7.2326, df = 1, p-value = 0.0072						
Plant species	Healthy		*Phytophthora*		Total plants	Proportion healthy
	n_{ij}	u_{ij}	n_{ij}	u_{ij}		
(6)						
D. aurantiacus	71	65.7	17	22.3	88	0.807
F. californica	41	46.3	21	15.7	62	0.661
X-squared = 3.3394, df = 1, p-value = 0.0676						

Site types (non Phytophthoras analysis)	Healthy		Natives		Total plants	Proportion healthy
	n_{ij}	u_{ij}	n_{ij}	u_{ij}		
(7)						
Restoration	81	80.5	8	8.5	89	0.910
Annexed	48	47.9	5	5.0	53	0.906

Table 2 continued

Site types (non Phytophthoras analysis)	Healthy		Natives		Total plants	Proportion healthy
	n_{ij}	u_{ij}	n_{ij}	u_{ij}		
Control	42	42.5	5	4.4	47	0.894
X-squared = 0.0978, df = 2, p-value = 0.9523						

The proportion of healthy plants for each comparison and X^2 or Fisher's exact test (in the cases where control expected values were zero) statistics are also provided

was significantly larger in restored (Odds 6.69 in favor of restored *Phytophthora* incidence, 95% confidence interval: 2.39, 23.35) and high-risk (7.19 odds in favor of high-risk *Phytophthora* incidence, 95% confidence interval: 2.42, 26.23) disturbed sites than in control sites where incidence of *Phytophthora* was nil. Likewise, *Phytophthora* incidence was higher in lowland than in upland sites (Odds 2.82 in favor of lowland *Phytophthora* incidence; 95% confidence interval: 1.28, 6.22).

Statistical analyses provided support for H_0, i.e. meaning that we could not reject the H_0 that *Phytophthora* incidence was undistinguishable, for the following comparisons, numbered as per the materials and methods section: (1) incidence by county (X^2 = 0.9908, p-value = 0.6093; Table 2(1)); (4) high risk-disturbed versus restored sites (X^2 = 0, p-value = 1; Table 2(4)); (6) *D. aurantiacus* versus *F. californica* (X^2 = 3.3394, p-value = 0.0676; Table 2(6)); and (7) incidence of non-*Phytophthora* pathogens when comparing restored, high risk-disturbed and control sites (X^2 = 0.0978; p-value = 0.9523; Table 2(7)).

The species list compiled includes where they were found in terms of county, restoration or annexed area, upland or lowland area, and plant species (Table 3). The greatest number of *Phytophthora* species was found in Marin County (six), restorations (seven), lowlands (seven) and from *F. californica* (six).

When comparing the list of 19 *Phytophthora* species recently isolated from a similar list of plant species in approximately 30 "restoration" nurseries (Sims et al. 2018; Rooney-Latham et al. 2019), six of which provided plant stock that was outplanted in our restoration study sites (Sims et al. 2018), and the list of eight *Phytophthora* species isolated from restorations and adjacent high risk-disturbed sites herein, six of them (75%) were found in restoration nurseries (Table 4). The two species not isolated from any "restoration" nurseries (*P. inundata* and unknown *Phytophthora* species) were also rarely isolated in the field in this study (once and twice respectively; Table 3). In total, 94.5% of 55 *Phytophthora* isolates collected in this study were previously reported in "restoration" nurseries.

Discussion

Important work has been done around the world in the past 20 years regarding the presence of *Phytophthora* species in forests and natural ecosystems in which unexplained plant mortality and disease have emerged (Braiser et al. 1995 and 2004; Cahill et al. 2008; Fitchner et al. 2010; Frankel et al. 2020a, b; Garbelotto et al. 2001, 2006; Goheen et al. 2002; Hansen and Delatour 1999; Hansen et al. 2000; Jung and Blaschke 1996; Jung et al. 2005; Navarro et al. 2015; Rizzo and Garbelotto 2003; Rizzo et al. 2002; Sims et al. 2015; Swiecki et al. 2003; Webber et al. 2004). In California and Oregon, in addition to the well known cases of Sudden Oak Death caused by the aerially splash-dispersed and exotic *Phytophthora ramorum* (Goheen et al. 2002; Rizzo et al. 2002; 2003) and of Port-Orford Cedar Root Rot caused by the soil and waterborne *P. lateralis* (Hansen et al. 2000), many other less known wild infestations are caused by exotic water and soilborne *Phytophthora* species. One notable case is that of *Phytophthora cinnamomi* causing widespread manzanita mortality in the Sierra Foothills and in the coast range, mortality of clusters of madrone and California bay laurel on the coast, and predisposing some oak species to drought-induced mortality in Southern California (Garbelotto et al. 2006; Fitchner et al. 2010; Swiecki et al. 2003). Even the highly damaging and exotic *P. ramorum*, has now become

endemic around the greater San Francisco Bay Area and is causing unprecedented mortality of several rare and endangered Manzanita species (Garbelotto et al. 2020; Rooney-Latham et al. 2020), in addition to causing large scale oak and tanoak mortality.

The inadvertent introduction of *Phytophthora* species in restoration sites and their spread into adjacent natural ecosystems will surely have long-term environmental and economic impacts (Brasier et al. 1995; Grünwald et al. 2012; Hansen 2008; Jung et al. 2016; Pimentel et al. 2005; Shor et al. 2017). The discovery presented here that these introduced pathogens are spreading outwards from restoration sites is likely to generate much needed discussion and research regarding the identification of the exact pathway for the introduction of these microbes and the long-term effects they may have, once introduced. Restoration efforts, in fact, are complex, costly, involve many stakeholders, and -above all-are meant to restore the integrity of disturbed habitats by reversing land degradation, increasing the resilience of biodiversity, and delivering important ecosystem services (Aerts and Honnay 2011; Wortley et al. 2013).

Where plant stock infected by plant pathogenic *Phytophthora* species is used, further damage occurs in already disturbed ecosystems, rather than restoring their integrity (Frankel et al. 2020a, b). Thus, restorations become a further pathway of introduction of invasive species to be listed among the many introduction pathways caused by humans (Vitousek et al. 1997). Based on a recently published review (Garbelotto et al. 2018), the number of putatively exotic *Phytophthora* species introduced and the number of introductions associated with restoration projects in Northern California is significantly greater than that reported here. Recent reports suggest a high incidence of *Phytophthora* species in restoration nurseries of Southern California as well, suggesting this is a large-scale and not a regional problem (Fajardo et al. 2020; Rooney-Latham et al. 2019) with restoration nurseries representing one of the main introduction sources of these *Phytophthora* species (Rooney-Latham et al. 2019; Sims et al. 2018). In the case of *P. crassamura*, reasonable evidence has been provided for multiple introductions in Northern California associated with the use of infected plant stock in restoration efforts (Sims et al. 2019), while the introduction of *P. tentaculata* in multiple restoration sites has been documented by Rooney-Latham et al. (2015).

Comparing incidence and species composition of *Phytophthora* isolations from restored, control sites and "restoration" nurseries provides additional evidence that the introduction and establishment of multiple *Phytophthora* species in California is indeed associated with restoration projects. Alternatively, restorations could simply increase the population size of *Phytophthora* species already present in these ecosystems because of the disturbances they cause. In spite of our limited sample study, we believe that the consistency of the results here presented suggests these *Phytophthora* species are being repeatedly introduced through restoration projects and are not ubiquitous. The consistent isolation of two species of non-*Phytophthora* pathogens from all three types of study sites (restorations, high risk-disturbed and controls) provides instead an example of the very different outcome expected for pathogens that are not introduced but are native or may have become endemic with time.

Control areas near restorations were devoid of any *Phytophthora*, and six of eight *Phytophthora* species or 94.5% of 55 isolates found in restoration sites were also found in or belonged to species found in local nurseries which had provided the plant stock, suggesting infected plant stock may represent the actual introduction pathway of these *Phytophthora* species. However, *Phytophthora* species associated with plant species that were not sampled may easily have been missed, or mortality may have been compounded by other ecological or soil-related factors. Likewise, this preliminary study does not prove per se that infected plant stock is responsible for all of the observed introductions, nor that all introduced *Phytophthora* species are exotic. The potential roles as introduction pathways played by infested soil or mulch, by contaminated equipment or by infested water and soil from neighboring ornamental plantings all need to be investigated. Finally, the native range of *Phytophthora* taxa introduced in restoration sites needs to be identified.

The role of these *Phytophthora* species as agents of disease on infected plants found herein has not been proven through Koch's postulates for most of the *Phytophthora* species isolated in this study on these particular hosts, however direct isolations from cankered stems were successfully made for several

Table 3 The Phytophthora species, quantity isolated and locations where they were found including: restoration or annexed-high-risk area, upland and lowland locations and hosts

Phytophthora species	Quantity isolated	Restoration/annexed	Upland/lowland	Host(s)**
County: Marin n = 6, 27, 103*				
P. inundata	1	A	L	FRCA
P. megasperma	4	R	U/L	DIAU/FRCA
P. pseudocryptogea	11	A/R	U/L	DIAU/FRCA/CETH/SALA
P. taxon 'kelmania'	8	A/R	U/L	DIAU/FRCA/SALA
P. taxon 'raspberry'	2	R	L	SALA
Unknown *Phytophthora* species	1	R	L	SALA
County: San Francisco n = 3, 9, 26				
P. multivora	5	A/R	U	CETH
P. pseudocryptogea	2	A	U	CETH
P. taxon 'kelmania'	2	R	U	DIAU
County: San Mateo n = 4, 19, 60				
P. crassamura	10	A/R	U/L	DIAU/FRCA
P. megasperma	1	R	L	FRCA
P. multivora	7	A	U	FRCA
Unknown *Phytophthora* species	1	R	U	DIAU

Phytophthora species	Quantity isolated
Restoration n = 7, 34, 89	
P. crassamura	6
P. megasperma	5
P. multivora	4
P. pseudocryptogea	8
P. taxon 'kelmania'	7
P. taxon 'raspberry'	2
Unknown *Phytophthora* species	2
Annexed n = 5, 21, 53	
P. crassamura	4
P. inundata	1
P. multivora	8
P. pseudocryptogea	5
P. taxon 'kelmania'	3
Upland n = 6, 36, 149	
Phytophthora crassamura	9
Phytophthora megasperma	3
Phytophthora multivora	12
Phytophthora pseudocryptogea	6
Phytophthora taxon 'kelmania'	5
Unknown *Phytophthora* species	1
Lowland n = 7, 19, 40	
P. crassamura	1
Phytophthora inundata	1
P. megasperma	2
P. pseudocryptogea	7

Table 3 continued

Phytophthora species	Quantity isolated
P. taxon 'kelmania'	5
P. taxon 'raspberry'	2
Unknown *Phytophthora* species	1
Plant species: Diplacus aurantiacus n = 5, 17, 88	
P. crassamura	3
P. megasperma	3
P. pseudocryptogea	4
P. taxon 'kelmania'	6
Unknown *Phytophthora* species	1
Plant species: Frangula californica n = 6, 21, 62	
P. crassamura	7
P. inundata	1
P. megasperma	2
P. multivora	7
P. pseudocryptogea	1
P. taxon 'kelmania'	3
Plant species: Ceanothus thrysiflorus n = 2, 11, 20	
P. multivora	5
P. pseudocryptogea	6
Plant species: Salix lasiolepis n = 4, 6, 10	
P. pseudocryptogea	2
P. taxon 'kelmania'	1
P. taxon 'raspberry'	2
Unknown *Phytophthora* species	1

All data is shown together in the upper portion of the table and expanded out based on quantity for each hypothesis tested

*n = total *Phytophthora* species, total Phytophthora isolates, total plants sampled **FRCA = *Frangula californica*, DIAU = *Diplacus aurantiacus*, CETH = *Ceantothus thyrsiflorus*, SALA = *Salix lasiolepis*

species × pathogen combinations: *P. multivora* and *P. pseudocryptogea* × *C. thyrsiflorus*, *P. megasperma* and *P. crassamura* × *D. aurantiacus* and *P. pseudocryptogea* × *S. lasiolepis*. In addition, symptoms always matched those expected for Phytophthora disease (Erwin and Ribeiro 1996). Natural recruitment and regeneration of the hosts studied here was basically nil in proximity of dead or dying individuals from which *Phytophthora* was isolated (data not shown). Although this aspect was not one of the main aims of this study, our observations possibly indicate a likely further negative effect associated with the introduction of *Phytophthora* species. Nonetheless, the true ecological role of these *Phytophthora* species, including their pathogenicity, virulence, infectivity and persistence has yet to be determined at the species level and other biotic and abiotic factors causes of mortality cannot simply be discarded without in depth studies.

A second important result of our study consists in proving, possibly for the first time, that *Phytophthora* species are not only being introduced in sites that are the target of restoration efforts, but are spreading from these introduction sites. Given that most of the *Phytophthora* species identified in association with restoration sites are waterborne or soilborne, our experiment was designed to track their spread by following possible pathways of soil and water movement such as drainage draws, culverts, trails or dirt roads intersecting unrestored sites adjacent to restored

Table 4 *Phytophthora* species found in (a) both nurseries (Sims et al. 2018) and restoration sites in this study; (b) *Phytophthora* species found in only nurseries (Sims et al. 2018); (c) *Phytophthora* species found only in restoration sites from this study

(a)
Phytophthora crassamura
Phytophthora multivora
Phytophthora pseudocryptogea
P. "taxon kelmania"
P. "taxon raspberry"
*Phytophthora megasperma**
(b)
Phytophthora cactorum
Phytophthora hedraiandra
Various (five) *P.* × hybrids
P. occultans
P. "citricola-pini complex"
P. thermophila
Phytophthora niederhauserii
P. "close to niederhauserii"
P. cryptogea
P. "taxon kelmania" type 2
(c)
Phytophthora inundata
Unknown *Phytophthora* species

*Collected in restoration nurseries from Rooney-Latham et al. (2019) and not Sims et al. (2018)

sites. In addition, by design, we selected to compare incidence of *Phytophthora* species isolation from lowlands, where soil and water run off would accumulate, with that of uplands, less subject to capture soil and water moving from infested locations. Results clearly showed that *Phytophthora* species incidence in these disturbed sites was comparable to that measured in restored sites, with the implications that disturbed sites were likely to have received infested soil and water from restoration sites through drainages, culverts, trails or roads.

Likewise, the incidence of *Phytophthora* species was significantly higher in lowlands compared to uplands. Given the biology of these organisms, this result was expected and further corroborates these *Phytophthora* species are actively being moved by the movement of infested soil and water that accumulates in such lowlands. Combined, results from the sampling of disturbed sites and lowlands corroborate the role of the movement of infested soil and water as a pathway for the further spread of exotic *Phytophthora* species from restoration sites into wildlands. Although spread via soil and water can be assumed to be intrinsically much slower than that of airborne pathogen species such as *Phytophthora ramorum*, the rate of spread by water and soilborne pathogens can be exponentially increased by further disturbances such as rural or grazing activities (Cardillo et al. 2020), mining operations (Shearer and Tippett 1989), or by contamination of waterways (Hansen et al. 2000).

The major outcome of this study is the discovery that the pathway of pathogen introduction reported in the literature as being associated to restoration efforts and restoration nurseries (see Garbelotto et al. 2018) may not be accidental or limited to a few isolated events, but may have the potential to be systematic and may result in the further spread of these pathogens in neighboring habitats. *Phytophthora* species were isolated in all five restoration sites studied, in all three counties, and on four different plant hosts. This is a pattern clearly indicative that we are dealing with a generalized process dictated by one or more breaches of security in the chain of operations, possibly including the production of infected plant sock in production facilities, compounded by the generalism, i.e. the broad host range, of most *Phytophthora* species introduced, and by their common ability to disperse through infected plants, as well as infested soil and water (Garbelotto et al. 2018). Because a systematic pathway of introduction results in multiple introduction events, the potential for landscape-level changes in the ecosystems affected is great. Historically, multiple pathogen introductions have resulted in continental or regional-level plant epidemics (Dlugosch and Parker 2008). Examples with confirmed multiple introductions of other *Phytophthora* forest pathogens include, *Phytophthora alni* causing Alder Root and Crown Rot in riparian ecosystems of all of Europe (Brasier et al. 1995, 2004; Ioos et al. 2006), and *Phytophthora ramorum* causing Sudden Oak Death in California and Oregon (Croucher et al. 2013).

We further believe that the scale of the problem may be even greater than that exposed by this study for two reasons. First, positive *Phytophthora* isolations may be more difficult to obtain from older restoration sites. Second, if infected before they are planted, seedlings may die rapidly, making selection of sampling sites even more difficult.

The systematic introduction of these exotic pathogens is even more problematic because they occur in proximity of natural ecosystems. Thus, it should be no surprise our study detected successful spread of these exotic pathogens in each and all five high risk-disturbed sites that were studied. Conversely, pathogens that have been systematically introduced in systems that are separate from natural ecosystems, for instance in agricultural settings, may take a substantial longer amount of time to spill over to natural ecosystems (Blitzer et al. 2012). For instance, this may have been the case for *P. cinnamomi* originally introduced in agricultural settings in California (Zentmyer 1976), and only recently reported from natural settings (see Socorro Serrano et al. 2019).

Conclusions

Based on this and on an increasing number of other studies (Frankel et al. 2020a, b; Garbelotto et al. 2018; Hunter et al. 2018; Rooney-Latham et al. 2015, 2017, 2019; Sims et al. 2018, 2019), a likely pathway for the introduction of *Phytophthora* species in restorations may be identified in the use of plant stock becoming infected in plant production facilities. Although this pathway may be only one of several, this conclusion can be looked at with concern and optimism at the same time. Concern, because recent evidence has indicated that *Phytophthora* strains that pass through production facilities may be selected for mutations increasing aggressiveness and resistance to fungicides (Hunter et al. 2018; Sims and Garbelotto 2018; Sims et al. 2019), making them more formidable foes than their natural counterparts. A further limitation is caused by our incomplete understanding of which *Phytophthora* species may be more persistent and infectious in natural settings: acquiring this knowledge may be key in the development of appropriate diagnostic tools. Optimism, because it has been recently shown that the adoption of best management practices (BMPs) in plant production facilities result in the production of plant stock that is free of *Phytophthora*, independent of the host or of the *Phytophthora* species in question (Sims et al. 2018). Likewise, new diagnostic approaches, including the use of dogs are becoming available and should be pursued to ensure plants used in restorations are free of *Phytophthora* or other pathogens (Gottwald et al. 2020; Swiecki et al. 2018).

Any cost associated with the implementation of BMPs and novel diagnostic approaches would be offset by the benefits provided by increasing the success rate of expensive restoration projects, avoiding the introduction of lethal pathogens that may be difficult to eradicated and have been shown to spread from restored sites to natural habitats, and preventing increased erosion and run off associated with failed restoration efforts, as clearly observed (data not shown) in one of our study sites.

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Availability of data and material Data presented in Tables.

Code availability Formulas used are presented in the text.

Declarations

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