NEW ZEALAND JOURNAL OF ECOLOGY

RESEARCH

A risk to the forestry industry? Invasive pines as hosts of foliar fungi and potential pathogens

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Published online: 27 January 2022

Abstract: Pathogen accumulation on an invasive plant species can occur over time, through co-invasion, or adaptation of native pathogen species. While accumulated pathogens can reduce the success and spread of an invasive species, they can also spill-over into native plant communities or valuable non-native populations. Transmission of pathogens may be density-dependent, with dense invasive populations creating better opportunities for pathogen spread than scattered individuals. Some pine species (*Pinus*) and some other Pinaceae (including *Pseudotsuga*) are extremely invasive trees in New Zealand but trees in the Pinaceae are also used extensively within the forestry industry. Little is known about the foliar pathogens present on invasive populations and whether they pose a risk to industry. We cultured foliar fungi from needles of *Pseudotsuga menziesii* and *Pinus contorta* found at both low and high densities of invasion. DNA from fungal cultures was extracted and sequenced using Sanger sequencing. We cultured fungi from a greater proportion of *P. menziesii* than *P. contorta* needles and a greater proportion of trees from low versus high densities of invasion. The richness of foliar fungi decreased as a function of density and *P. menziesii* hosted a greater richness of fungi than *P. contorta*. We observed no change in the richness of pathogens between *P. menziesii* and *P. contorta* or between low and high density invasions. However, we did observe a greater proportion of fungi that were potentially pathogenic at high density than at low density. We identified one major widespread pathogen (*Nothophaeocryptopus gaeumannii*) and a number of opportunistic potential pathogens (i.e. *Sydowia polyspora*, *Lophodermium pinastri* and *Alternaria alternata*), indicating the possibility of spill-over into commercial plantations.

Keywords: fungi, pathogen spill-over, plant invasion, Pinaceae, wilding conifers

Introduction

The introduction of invasive plant species into a novel environment may release them from their native enemies, thereby contributing to the success of some invasive species (Colautti et al. 2004; Liu et al. 2006). However, invasive species can also retain the potential to accumulate pathogens from their new environment from co-invasion (Dickie et al. 2010). Invasive plants in a new range often have low genetic diversity which can increase their susceptibility to pathogen infections and may contribute to pathogen accumulation (King et al. 2012; Estoup et al. 2016). While these pathogens have the potential to reduce the success of invasive plants, pathogens could "spill-over" onto native or non-native plants, including plants used in plantation forestry and agriculture (Bufford et al. 2019).

The pine family (Pinaceae; including *Pinus*, *Pseudotsuga*, and *Larix*; hereafter "pines") provides a model system for understanding pathogen communities on invasive plants as they are one of the most invasive plant families globally (Richardson & Rejmánek 2011; Gundale et al. 2014; Sapsford et al. 2020). For example, pines (i.e. unwanted wilding pines excluding planted pine forests) are currently spreading in New Zealand at a rate of approximately 90 000 hectares a year and are set to cover an estimated 20% of the country by 2040 (Richardson & Rejmánek 2011; Ministry for Primary Industries 2014). If uncontrolled, pine invasions will have a myriad of adverse effects such as reducing water availability to agriculture and hydropower, and driving homogenisation of native ecosystems (Dickie et al. 2014; Wyatt 2018).

A further potential effect of invasive pines is the spread of pathogens, which has occurred in other invasive cultivated plants such as wild populations of kiwifruit (*Actinidia* spp.) (Everett et al., 2011). Wild populations of kiwifruit vines provide a reservoir for pathogens, such as the virulent bacterium *Pseudomonas syringae* pv. *actinidiae*, in which the bacterium may evolve and become resistant to agrichemicals used for control in commercial systems (McCann et al. 2017). The spread of diseased kiwifruit vines has led to recurrent pathogen spill-over back into commercial orchards resulting

DOI: https://dx.doi.org/10.20417/nzjecol.46.13

in crop losses (Everett et al. 2011). A similar situation may be occurring in invasive pines: as pines spread, invade across landscapes and increase in density, their potential for disease transmission increases resulting in the eventual spill-over of disease into commercial pine plantations (Burdon & Chilvers 1982; Choudhury et al. 2020). Current knowledge of foliar pathogens in invasive pines in New Zealand is limited, as research has focused largely on the well-known diseases of commercially grown trees (Bulman et al. 2008).

The density of a host can also affect prevalence of pathogens where pathogens with density-dependent transmission increase with population density (Antonovics 2017). Invasive pines vary in density from sparse individuals at the "invasion front" to very high densities in more established stands (Sprague et al. 2019). We expect that dense populations could enable greater transmission of pathogens, potentially supporting more or different pathogens than sparse populations. At higher densities, there would be greater potential for transmission among hosts without the need of long-distance dispersal. Furthermore, pathogens can accumulate over time, potentially increasing the richness of pathogens found on a well-established host in comparison to a newly established host (i.e. at the invasion front) (Carlsson et al. 1990; Diez et al. 2010).

Given both interest in pathogen interactions with invasive species and the risk that pathogens represent for commercial forestry, we aimed to characterise the foliar fungal and pathogen community associated with invasive pines in terms of diversity and composition. We hypothesised that the prevalence and richness of foliar pathogens would increase with pine density. We also aimed to determine if *Pseudotsuga menziesii* supported a higher or lower diversity of foliar fungi and pathogens than *Pinus contorta. Pseudotsuga menziesii* is known to support a broader range of root-associated ectomycorrhizal fungi (Moeller et al. 2015) than *Pinus contorta* (Dickie et al. 2010), but there is no prior knowledge of how these species differ in foliar fungal diversity in New Zealand.

Methods

Sample collection and culturing

Our study site was located in Molesworth, Canterbury, New Zealand. The short tussock grasslands of Molesworth have slowly been replaced by native shrublands, e.g. mānuka (*Leptospermum scoparium*) and matagouri (*Discaria toumatou*). Remnant mountain beech (*Fuscospora cliffortioides*) is present in some areas (Husheer 2018). However, invasive species such as *Pinus* spp., *P. menziesii*, and Scotch broom (*Cytisus scoparius*) are slowly spreading throughout the area.

We collected needles from a range of self-established invasive pine species (*Pseudotsuga menziesii* n = 15; *Pinus contorta* n = 19; *Pinus nigra* n = 6; *Pinus muricata* n = 3; *Pinus radiata* n = 2). The cultures from *P. nigra*, *P. muricata*, and *P. radiata* were not included in the statistical analysis due to their small sample size. Therefore, from *P. menziesii* and *P. contorta*, we sampled 11 trees at high (7 × *P. contorta*; 4 × *P. menziesii*) and 23 trees at low densities (12 × *P. contorta*; 11 × *P. menziesii*; Fig. 1). The age of individuals ranged from 5 to 27 years with diameter at breast height (DBH)

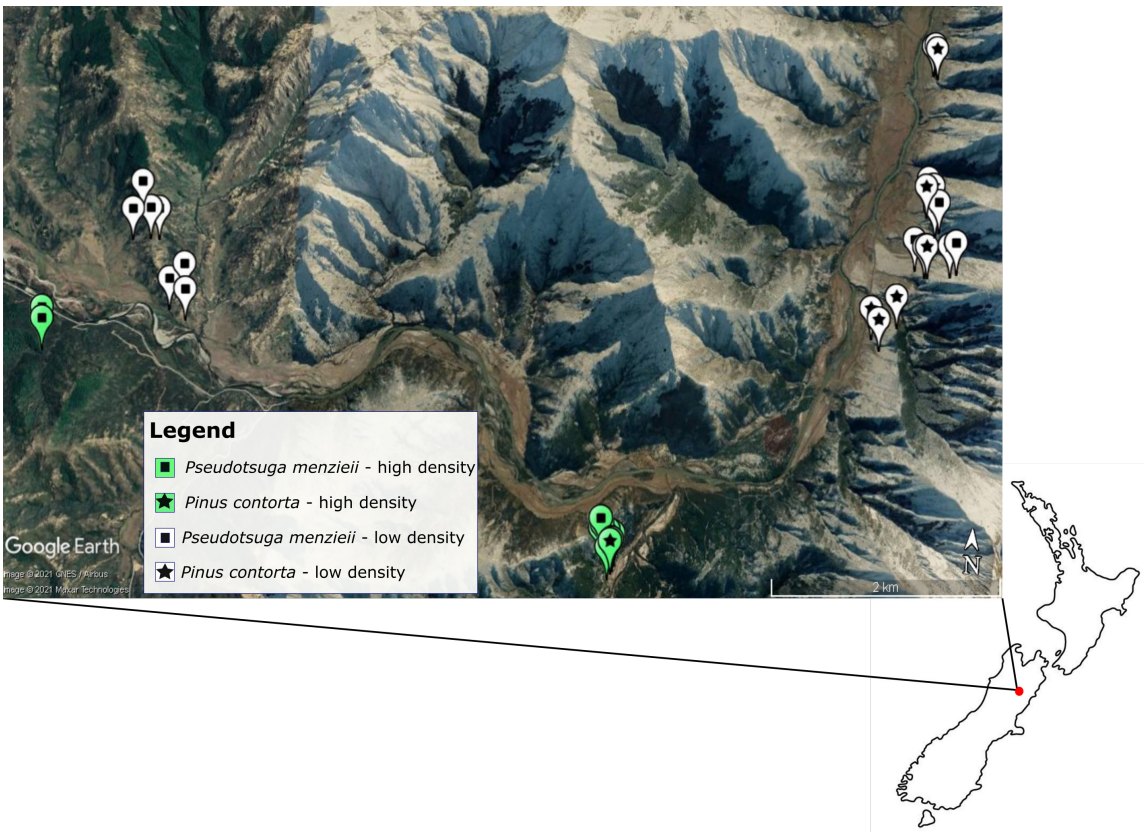

Figure 1. Map of Molesworth, Canterbury, New Zealand showing the locations of high (7 *Pinus contorta*, 4 *Pseudotsuga menziesii*) and low (12 *P. contorta*, 11 *P. menziesii*) density pines sampled for foliar fungi and pathogens. Green points with stars indicate locations of *P. contorta* at high density and green points with squares indicate locations of *P. menziesii* at high density. White points with stars indicate locations of *P. contorta* at low density and white points with squares indicate locations of *P. menziesii* at low density.

ranging from 7 cm to 70cm (Appendix S1 in Supplementary Materials). Individual trees were classified as being within a low density population if fewer than five pines above 2.5 cm DBH were within a 20 m radius of the target tree (i.e. the tree from which needles were collected). Low density pines were also at least 50 m or more from the nearest established pine stand. Individual trees were classified as being within a high density population if greater than 5 trees above 2.5 cm DBH were within a 20 m radius of the target tree. We were specifically interested in isolated trees at the invasion front, so we deliberately looked for these individuals from the road before sampling. This was a more deliberate strategy than setting up transects which would have been limited in their ability to sample very low density populations.

We walked around each individual tree and collected needles from various parts of the tree indiscriminately which allowed for a mixture of symptomatic and asymptomatic needles. From this collection, six needles were used for culturing and fungi were cultured within 24 hours of collection. Needles were surface sterilised by submerging whole needles in 95% ethanol for 1 minute, 5% sodium hypochlorite solution for 2 minutes, and 70% ethanol for 2 minutes (U'Ren et al. 2014). Needles were cut into c. 1 cm long pieces, and segments from the base, middle, and tip of each needle were placed onto a low-nutrient medium (i.e. water agar) containing 5 g agar per 1 L distilled water. This low nutrient medium was used to enrich a wide range of fungi by preventing fast-growers from out-competing slower growers. All mycelial outgrowths were then sub-cultured onto a high-nutrient medium, potato dextrose agar (PDA), containing 5 g agar and 7.5 g PDA per 1 L of distilled water, to enhance their growth (Fig. 2).

DNA extraction and Sanger sequencing

DNA was extracted using the REDExtract-N-Amp^{TM} Plant PCR Kit (Sigma Aldrich) as per the manufacturers protocol. Mycelia were scraped from all isolated cultures using a sterile pipette tip and placed into a microtube. To each tube, 50 µl of extraction solution was added, the sample crushed, mixed, and left to stand for 15 minutes. Samples were heated to 95°C for 10 min, diluted with 50 µl of dilution solution, centrifuged, and stored at −80°C. The REDExtract-N-Amp^{TM} Plant PCR Kit from Sigma Aldrich was used to amplify the extracted DNA using 50 mM of the forward primer ITS1-F (5′-CTTGGTCATTTAGAGGAAGTAA -3′) and the reverse primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990). PCR amplifications were run on the Eppendorf Mastercycler pro PCR system (Thermo Fisher Scientific). The parameters for this protocol were as follows: 1 min 25 s at 94°C, 13 cycles of 35 s at 95°C, 55 s at 55°C and 45 s at 72°C, 14 cycles of 35 s at 95°C, 55 s at 55°C and 2 minutes at 72°C, and 9 cycles of 35 s at 95°C, 55 s at 55°C and 3 minutes at 72°C, and 10 minutes at 72°C, before cooling to 4°C. PCR product was purified using QIAquick PCR Purification Kit (QIAGEN) following the manufacturer's instructions and sequenced using the Sanger chain termination method (Sanger et al. 1977).

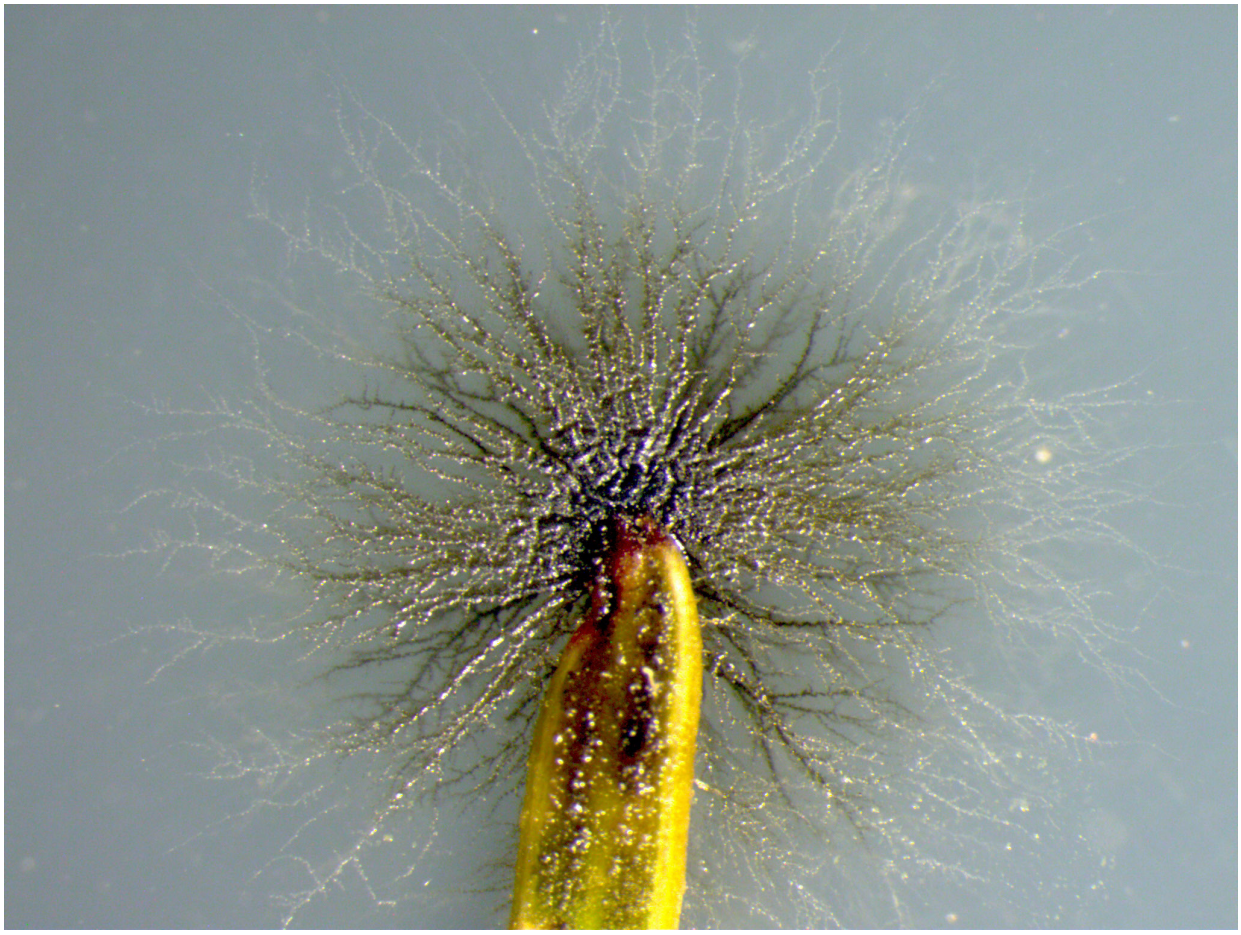

Figure 2. Mycelia of a *Biscogniauxia* sp. (OTU7) emerging from a *Pseudotsuga menziesii* needle plated onto water agar (5 g agar per 1 L H_20) and grown at 20°C.

Bioinformatics

Sequence quality control and clustering was carried out in R 4.0.2 (R Core Team 2020). Sequences were trimmed, excluding the lower quality ends of sequence reads based on a mean sequence quality across a four-base-call sliding window of less than 20 phred quality score. Sequences were clustered into operational taxonomic units (OTUs) based on > 95% sequence similarity and a cluster overlap of 0.95. Sequences were blasted against the NCBI nucleotide collection (nr/nt) database to identify closely related taxa at the lowest taxonomic resolution possible (Altschul et al. 1990). Ecological information of each OTU was based primarily on FUNGuild (https://github.com/UMNFuN/FUNGuild) following the user's manual where assignments were made on ranks of "probable" and "highly probable" (Nguyen et al. 2016). Where there was no match or an OTU was ranked as "possible", literature searches were conducted and OTUs assigned to a guild based on published literature. If no guild could be assigned, we classified these unknown OTUs in an undefined guild. One fungal culture, with a DNA sequence matching *Tranzschiella hypodytes* (98.71% identity), was discarded, as this Ustilaginales (smut) fungus is believed to be specific to grass. Grasses were being handled in the lab at around the same time as our experiment, and hence we cannot reject the possibility that this may have been a contaminant.

Statistical Analyses

All data were analysed in program R version 4.0.2 (R Core Team 2020). We first determined whether age of trees between low and high density were different using a linear model to account for any correlations and found no difference in age (t value = −1.34, p = 0.19). To determine prevalence of fungi in needles (i.e. success of culturing fungi from a needle, out of six needles) as a function of tree density (i.e. low or high density) and species (i.e. *Pinus contorta* and *Pseudotsuga menziesii*), we used a binomial linear model with function *glm*. Prevalence of fungi was the response variable, and species and tree density were the predictor variables. To determine if fungal OTU richness (i.e. average number of fungal species found on each individual tree cultured from six needles) differed between tree species and between low and high density, we used a generalised linear model with function *glm* with family Poisson. The above two models were repeated to determine both prevalence of putative pathogens and pathogen richness. Species accumulation curves were produced using function *specaccum* in package vegan (Oksanen et al., 2015). Model assumptions were verified by inspecting residuals for assumptions of normality and homoscedasticity (Zuur & Ieno 2016).

Results

Culturing

We isolated 143 fungal cultures of which 126 were successfully sequenced and submitted to GenBank (accessions MW842782–MW842907). These sequences were clustered into 41 operational taxonomic units (OTUs; Table 1; Appendix S2). Of the 41 OTUs (mean OTU per tree = 2.84), 29.3% were saprotrophs, 22% were plant pathogens, 22% were classified as endophytes/saprotrophs/plant pathogens (due to not being able to identify a sequence past order or family), 2.4% (one OTU) was classified as an endophyte or plant pathogen, 2.4% (one OTU) was classified as an endophyte or saprotroph and the remaining 22% had unknown or undefined functions (due to not being able to identify a sequence past order or family).

We identified OTUs with high sequence similarity to the following taxa listed as plant pathogens in FUNGuild: *Sydowia polyspora* (OTU2; 99% match), *Nothophaeocryptopus gaeumannii* (OTU35; 97.99% match), *Neocatenulostroma germanicum* (*Neocatenulostroma* species complex; OTU18; 99% match), *Alternaria alternata* (OTU45; 99% match), *Ramularia eucalypti* (OTU10; 99% match) and *Lophodermium pinastri* (OTU31; 99% match) (Fig. 3). We also found a close match to a pathogen of *Eucalyptus* spp., *Austroafricana parva* (OTU44; 99.61% match) and close matches to pathogens of vegetable and fruit crops: a *Didymella* sp. (OTU14; 98.07% match) and *Parastagonospora nodorum* (OTU13; 100% match) (Appendix S2).

Prevalence of fungi

We successfully cultured fungi from a greater proportion of *Pseudotsuga menziesii* needles than *Pinus contorta* needles (mean proportion of the six needles per tree producing a culture; *z-value* = 2.241, $p = 0.025$; Fig. 3). The density of trees significantly influenced the success of culturing fungi, with a greater proportion of fungi cultured from needles from low density stands than from high density stands (mean proportions per tree; *z-value* = 2.883, $p = 0.004$; Fig. 3).

Fungal richness was also significantly higher on individual *Pseudotsuga menziesii* trees (*z-value* = 3.433, $p < 0.001$) than *Pinus contorta* trees (Fig. 4a). The richness of fungal OTUs was higher in low density pine stands (*z-value* = 4.119, $p < 0.001$) than in high density pine stands (Fig. 4a). Species rarefaction curves showed that the curves for high and low density never overlapped, with total species richness always higher for an equal sampling effort of low density trees compared to high density trees (Fig. 4b).

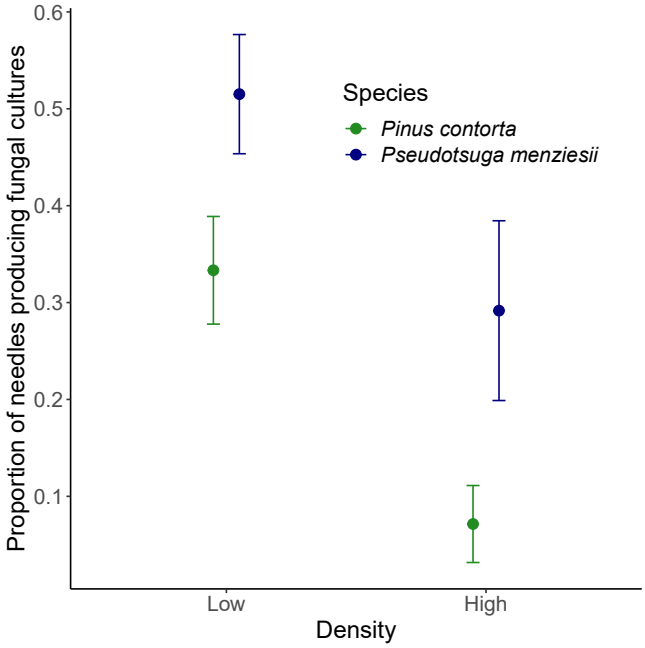

Figure 3. Mean (± standard error) proportion of needles producing fungal cultures from *Pinus contorta* (green) and *Pseudotsuga menziesii* (blue) at low and high density, based on six needles sampled per tree. Means are fitted values from the best fit model.

Table 1. Result of clustering of sequences from fungal culture isolated from *Pinus* spp. and *Pseudotsugo menziesii*. We sequenced 41 OTUs for which we assigned functional guilds (FUNGuild) and listed the closest match from GenBank.

Isolates									
Low Density (*n* from 30 trees)	**High Density (*n* from 15 trees)**	**Order**	**Family**	**Genus**	**Determined Identity**	**Guild**	**Best matching GenBank Accession (Accession #)**	**% Identity**	**Length of Identity match (bp)**
24	1	Xylariales	Xylariaceae	*Biscogniauxia*	*Biscogniauxia* sp.	Saprotroph	*Biscogniauxia* sp. (MW054258)	99.49	714
21	2	Xylariales	Xylariaceae	*Biscogniauxia*	*Biscogniauxia* sp.	Saprotroph	*Biscogniauxia* sp. (JN225898)	97.99	717
7	0	Coniochaetales	Coniochaetaceae		Coniochaetaceae sp.	Endophyte - Saprotroph - Plant pathogen	*Coniochaeta lignicola* (MH855438)	95.24	421
7	0	Xylariales	Xylariaceae	*Anthostomella*	*Anthostomella* sp.	Endophyte-Saprotroph	*Anthostomella pinea* (HQ599578)	98.88	532
6	1	Eurotiales	Aspergillaceae	*Penicillium*	*Penicillium* sp.	Saprotroph	*Penicillium chrysogenum* (MK881028)	100.00	561
5	0	Dothideales	Dothioraceae	*Sydowia*	*Sydowia* sp.	Saprotroph	*Sydowia polyspora* (KP152486)	99.65	569
5	0				Dothidiomycetes sp.	Undefined	*Perusta inequalis* (NR_144958)	99.80	504
2	1	Rhytismatales	Rhytismataceae	*Lophodermium*	*Lophodermium* sp.	Plant pathogen	*Lophodermium pinastri* (KY742588)	99.86	689
0	3	Phacidiales			*Phacidiales* sp.	Undefined	*Pragmopora* cf. *piceae* (MN547971)	97.88	509
1	1	Coniochaetales	Coniochaetaceae	*Coniochaeta*	*Coniochaeta* sp.	Endophyte - Saprotroph - Plant pathogen	*Coniochaeta luteoruba* (MH865901)	97.08	532
1	1	Xylariales	Xylariaceae		Xylariaceae sp.	Endophyte - Saprotroph - Plant pathogen	*Xylaria eucalypti* (NR_166326)	89.35	386
2	0	Mycosphaerellales	Mycosphaerellaceae	*Ramularia*	*Ramularia* sp.	Plant pathogen	*Ramularia eucalypti* (KJ504791)	99.80	508
1	1	Pleosporales	Didymellaceae	*Didymella*	*Didymella* sp.	Plant pathogen	*Didymella americana* (MK495981)	99.08	508
2	0	Xylariales	Xylariaceae	Xylaria	*Xylaria* sp.	Saprotroph	*Xylaria eucalypti* (NR_166326)	97.52	512
2	0	Pezizales	Pyronemataceae		Pyronemataceae sp.	Saprotroph	*Tricharina gilva* (NR_160170)	96.73	533

Table 1. Continued.

Isolates									
Low Density (*n* from 30 trees)	**High Density (*n* from 15 trees)**	**Order**	**Family**	**Genus**	**Determined Identity**	**Guild**	**Best matching GenBank Accession (Accession #)**	**% Identity**	**Length of Identity match (bp)**
2	0	Eurotiales	Aspergillaceae	*Aspergillus*	*Aspergillus* sp.	Saprotroph	*Aspergillus rugulosus* (NR_131290)	100.00	525
2	0	Pleosporales	Sporormiaceae	*Preussia*	*Preussia* sp.	Saprotroph	*Preussia minima* (MH859242)	98.07	509
2	0				Leotiomycetes sp.	Undefined	*Pragmopora* cf. *piceae* (MN547971)	99.88	845
1	0	Xylariales	Xylariaceae		Xylariaceae sp.	Endophyte - Saprotroph - Plant pathogen	*Biscogniauxia* sp. (MH410020)	83.22	625
1	0	Xylariales	Xylariaceae		Xylariaceae sp.	Endophyte - Saprotroph - Plant pathogen	*Muscodor suthepensis* (MN844407)	92.18	542
1	0	Coniochaetales	Coniochaetaceae		Coniochaetaceae sp.	Endophyte - Saprotroph - Plant pathogen	*Coniochaeta canina* (NR_120211)	90.84	248
1	0	Coniochaetales	Coniochaetaceae	*Coniochaeta*	*Coniochaeta* sp.	Endophyte - Saprotroph - Plant pathogen	*Coniochaeta canina* (NR_120211)	97.96	528
0	1	Phaeomoniellales			Phaeomoniellales sp.	Endophyte - Saprotroph - Plant pathogen	*Phaeomoniella* sp. (KT264593)	99.64	553
1	0	Pleosporales	Pleosporaceae	*Alternaria*	*Alternaria* sp.	Endophyte - Saprotroph - Plant pathogen	*Alternaria alternata* (MN596828)	100.00	546
1	0	Diaporthales	Diaporthaceae	*Diaporthe*	*Diaporthe* sp.	Endophyte-Plant pathogen	*Diaporthe nothofagi* (NR_137105)	98.58	554
1	0	Pleosporales	Pleosporaceae	*Pyrenophora*	*Pyrenophora* sp.	Plant pathogen	*Pyrenophora biseptata* (MK539974)	100.00	621
1	0	Mycosphaerellales	Teratosphaeriaceae	*Neocatenulostroma*	*Neocantenulostroma* species complex	Plant pathogen	*Neocatenulostroma germanicum* (MH862143)	99.43	519
0	1	Mycosphaerellales	Mycosphaerellaceae	*Nothophaeocryptopus*	*Nothophaeocryptopus* sp.	Plant pathogen	*Nothophaeocryptopus gaeumannii* (MF951336)	97.99	292

Table 1. Continued.

Isolates									
Low Density (*n* from 30 trees)	**High Density (*n* from 15 trees)**	**Order**	**Family**	**Genus**	**Determined Identity**	**Guild**	**Best matching GenBank Accession (Accession #)**	**% Identity**	**Length of Identity match (bp)**
1	0	Phaeomoniellales	Phaeomoniellaceae	*Phaeomoniella*	*Phaeomoniella* sp.	Plant pathogen	*Phaeomoniella* sp. (JN225891)	97.85	501
0	1	Mycosphaerellales	Teratosphaeriaceae	*Austroafricana*	*Austroafricana* sp.	Plant pathogen	*Austroafricana parva* (EU707874)	99.61	505
1	0	Pleosporales	Phaeosphaeriaceae	*Parastagonospora*	*Parastagonospora* sp.	Saprotroph	*Parastagonospora nodorum* (KY090647)	100.00	541
1	0	Phaeotrichales	Phaeotrichaceae	*Phaeotrichum*	*Phaeotrichum* sp.	Saprotroph	*Phaeotrichum benjaminii* (NR_160140)	96.56	534
1	0	Pleosporales	Sporormiaceae	*Preussia*	*Preussia/Sporomiella* species complex	Saprotroph	*Preussia australis* (AY943052)	90.14	448
1	0	Xylariales	Xylariaceae	*Biscogniauxia*	*Biscogniauxia* sp.	Saprotroph	*Biscogniauxia* sp. (MW054258)	99.49	589
1	0	Xylariales			*Xylariales* sp.	Undefined	*Xylaria longipes* (MT502422)	89.94	474
1	0	Pleosporales			Pleosporales sp.	Undefined	*Comoclathris* sp. (KY940776)	87.15	407
1	0	Helotiales	Mollisiaceae		Mollisiaceae sp.	Undefined	*Phialocephala amethystea* (MT026388)	94.36	803
1	0	Coniochaetales			*Coniochaetales* sp.	Undefined	*Coniochaeta hoffmannii* (MH859265)	89.55	514
1	0	Coniochaetales	Coniochaetaceae		Coniochaetaceae sp.	Undefined	*Coniochaeta hoffmannii* (NR_167688)	92.83	531
1	0	Leotiomycete			Leotiomycetes sp.	Undefined	*Pragomora* cf. *pini* (MN547972)	99.88	845

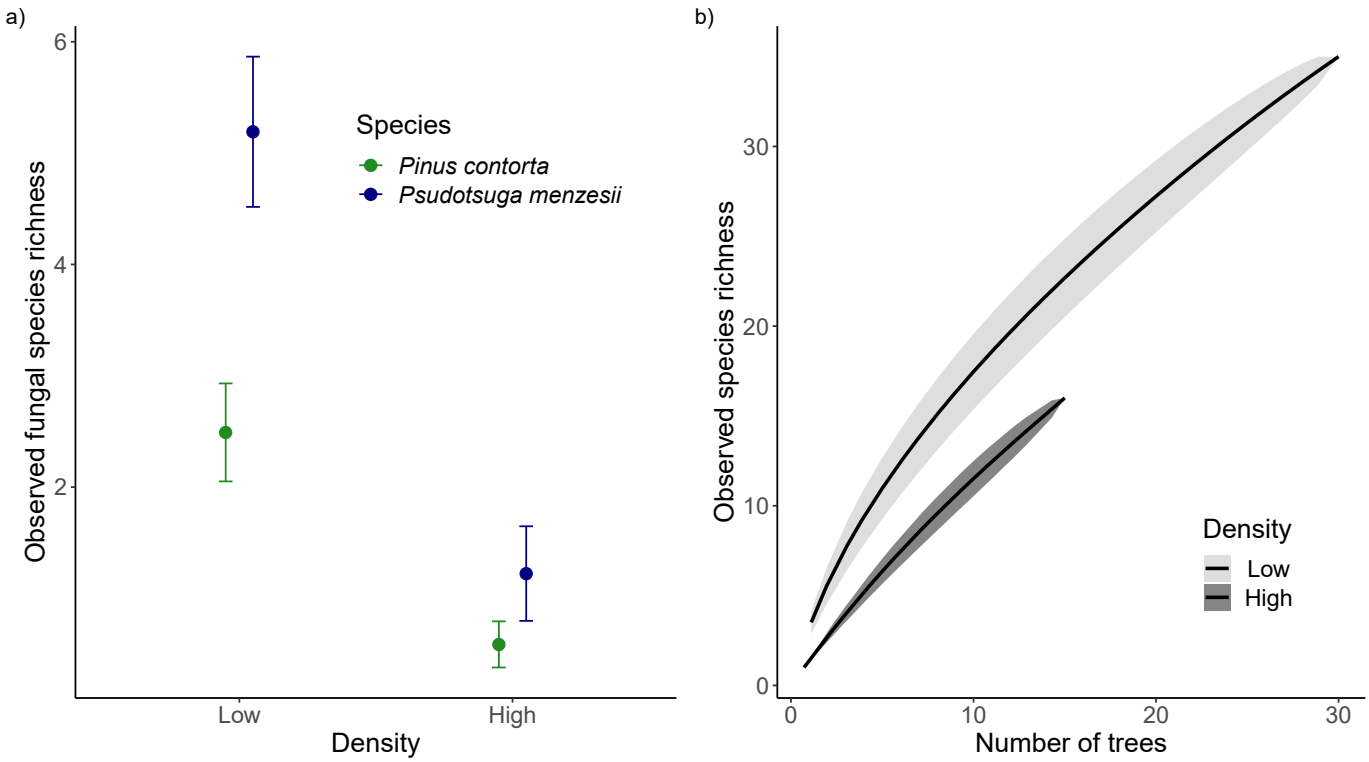

Figure 4. (a) Observed foliar fungal species richness on *Pinus contorta* and *Pseudotsuga menziesii*. Individual trees were classified as being within a low density population if fewer than five pines above 2.5 cm diameter at breast height (DBH) were within a 20 m radius of the target tree (i.e. the tree from which needles were collected). Low density pines were also at least 50 m or more from the nearest established pine stand. Individual trees were classified as being within a high-density population if greater than 5 trees above 2.5cm DBH were within a 20 m radius of the sampled tree. Lines indicate mean fitted values extracted from the best fit model with standard errors about the means. (b) Species accumulation curves of fungal species at low and high density.

Of the 33 fungal species found on both *P. contorta* and *P. menziesii*, only 5 species were shared between the two hosts (Fig. 5). Six species were unique to *P. contorta* and 22 were unique to *P. menziesii* (Fig. 5).

Plant pathogens

The proportion of isolated foliar fungi that were putative pathogens (i.e. OTUs classified as "plant pathogens" in FUNGuild) did not significantly differ between *Pseudotsuga menziesii* and *Pinus contorta* (*z-value* = −1.384, p = 0.166; Fig. 6). However, the proportion of pathogens did significantly differ between low and high density (*z-value* = −3.329, p = 0.0009; Fig. 6). The richness of putative foliar pathogens did not differ between *Pseudotsuga menziesii* and *Pinus contorta* (*z-value* = 0.454, p = 0.650) nor between low and high density (*z-value* = −1.089, p = 0.276).

Discussion

Fungal cultures

Our results show that wilding pines support a diverse foliar fungal community, including close DNA matches to species that can be pathogens: *Sydowia polyspora*, *Nothophaeocryptopus gaeumannii*, the *Neocatenulostroma* species complex, *Alternaria alternata*, *Ramularia eucalypti*, and *Lophodermium pinastri*. The foliar communities of invasive pines have not been previously documented and may represent a threat of pathogen spill-over onto commercial forestry species or native plants.

A number of the species we identified may be occurring primarily as endophytes, and only *Nothophaeocryptopus gaeumannii* is considered to currently be causing significant economic impact (Kimberley et al. 2010). Although international fungal databases suggest the potential for species to be pathogens (Nguyen et al. 2016), the actual expression of disease depends on plant host, environment, and time (Stevens 1960). Knowing that these species are present is important for two reasons. First, species that are currently considered primarily endophytic may become pathogenic under stressful conditions, such as drought or other environmental changes with climate change (Sturrock et al. 2011). Second, our results are unequivocal evidence that wilding pines support a diverse community of foliar fungi, regardless of any current economic effects. Thus, the results demonstrate the potential for wilding pines to serve as a reservoir for pathogen establishment and evolutionary adaptation to New Zealand conditions.

Sydowia polyspora is both a foliar endophyte and opportunistic pathogen which has been found in the seed of a range of pines including *P. radiata*, *P. mugo*, *P. pinaster*, *P. sylvestris*, *P. strobus*, and *P. pinea* (Cleary et al. 2019). Under biotic and abiotic pressures, *S. polyspora* can become pathogenic (Busby et al. 2016), causing the disease "current season needle necrosis" on pines (Talgø et al. 2010). Additionally, *S. polyspora* can enhance disease expression when co-inhabiting needles with other pathogens such as *Dothistroma septosporum* (Ridout & Newcombe 2015). This interaction is cause for concern as Dothistroma needle blight is one of the major foliar diseases of plantation pines in New Zealand, and costs c. $20 million in control measures

Figure 5. Fungal OTUs found on *Pinus contorta* and *Pseudotsuga menziesii* (*Pinus radiata* and *Pinus nigra* are also included). Filled green boxes indicate which fungal species was present on the host.

annually (Bulman et al. 2008; Watt el al. 2011; Baillie et al. 2017). This type of pathogen-pathogen interaction is likely to be altered due to climate change; depending on the region, conditions may become more or less favourable for pathogens, affecting host susceptibility to infection (Woods et al. 2005; Wakelin et al. 2018).

Nothophaeocryptopus gaeumannii is a foliar pathogen that exclusively infects *P. menziesii* and is the causal agent of Swiss needle cast (Watt et al. 2010; Bennett et al. 2019). *Nothophaeocryptopus gaeumannii* is already widespread in *P. menziesii* plantations across the North and South Islands (Bennett et al. 2019), which comprise c. 6% of NZ forest plantations (Ministry for Primary Industries 2020).

The *Neocatenulostroma* species complex expands the pathogen-endophyte-saprobe spectrum (Markovskaja et al. 2016). We identified a sequence (OTU18) that closely matches three *Neocatenulostroma* spp.: *N. abietis*, *N. microsporum*, and *N. germanicum*. All three species in the *Neocatenulostroma* complex have been reported as pathogens; however, *N. abietis* and *N. microsporum* have not been as extensively researched as *N. germanicum* (Quaedvlieg et al. 2014; Markovskaja et al. 2016). *Neocatenulostroma germanicum* has been found

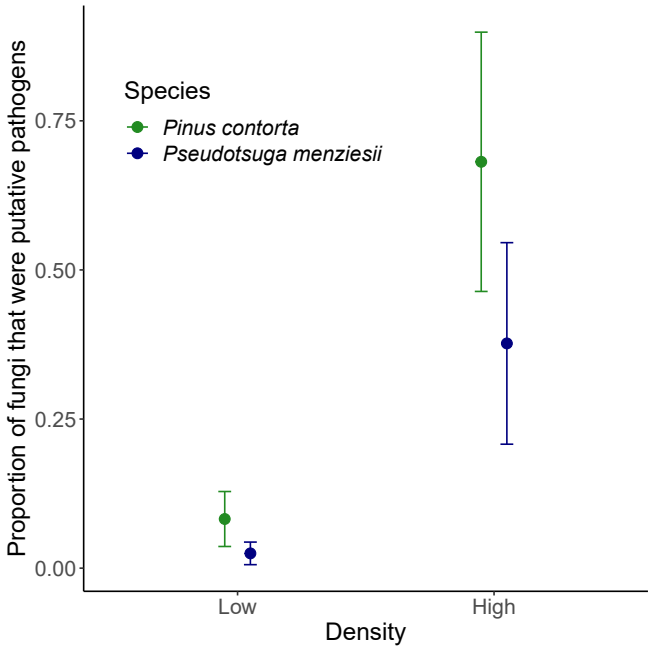

Figure 6. Mean (± standard error) proportion of fungi that were putative plant pathogens according to FUNGuild from *Pinus contorta* (green) and *Pseudotsuga menziesii* (blue) at low and high density, based on six needles sampled per tree. Means are fitted values from the best fit model.

co-occurring with the pathogens *Dothistroma* spp. and/or *Lecanosticta acicola* on *P. mugo*, *P. nigra*, and *P. sylvestris* (Markovskaja et al. 2016), in the knotwood of *P. sylvestris* (Szewczyk et al, 2017) and in association with symptoms of Cyclaneusma needle cast on *P. sylvestris* (Behnke-Borowczyk et al. 2019). Through inoculation experiments, *N. germanicum* was confirmed to be a weak opportunistic pathogen with a long latent phase (Markovskaja et al. 2016). Markovskaja et al. (2016) also suggested that the co-occurrence of *N. germanicum* with *Dothistroma* spp. and/or *Lecanosticta acicola* may be a result of a mutualistic interaction. Therefore, it is possible that *N. germanicum* may form synergistic pathogen-pathogen interactions in the future, thereby enhancing disease severity (Markovskaja et al. 2016).

Alternaria alternata is another potential pathogen of pines we detected. It is considered a saprobic fungus with pathogenic capabilities most frequently observed causing black spot in vegetable and fruit crops, although infections in pines have been reported (Troncoso-Rojas & Tiznado-Hernández 2014; Dobreva et al. 2016; Tozlu et al. 2018). *Alternaria alternata* has been identified as one of the causal agents of damping-off disease, a type of root rot, in pine nurseries around the world as well as in older trees (Lilja et al. 1995). However, once pines leave the nursery for planting, this disease becomes of little importance to plantation productivity as susceptibility decreases outside of the nursery environment due to plant age (Baranov et al. 2010). All the pathogens mentioned above cause damage to foliage and/or enhance the effects of other pathogens to cause reduced growth, reduced timber yield, and ultimately economic profit (Prihatini et al. 2015; Ridout & Newcombe 2015).

We obtained a close match to *Ramularia eucalypti* which is a pathogen known to infect a number of different plant hosts including *Pinus* spp. (Videira et al. 2015). There is also a collection of this species on *Coprosma robusta* (ICMP 20450) from mid-Canterbury (http://nzfungi2.landcareresearch.co.nz; accessed 22 July 2021). It has been suggested that some *Ramularia* spp., including *R. eucalypti*, may colonise different plant species as a means of dispersing onto their primary host (Videira et al. 2015), as many species in the *Mycospharaella* have been shown to do (Crous et al. 2008). Therefore, the *Ramularia* sp. culture we obtained may have been saprotrophic or pathogenic on pines and could potentially spread from invasive pines into *Eucalyptus* plantations (Videira et al. 2015).

Lophodermium pinastri has been in New Zealand since at least 1989 when it was first reported and is widely distributed across the country (Johnston et al. 2003). While historical records attribute Lophodermium needle cast to *L. pinastri* (Martinsson 1979), subsequent taxonomic treatment divided the pathogenic species *L. seditiosum* from *L. pinastri* which is considered a non-pathogenic endophyte. However, the abundance of *L. pinastri* is correlated with needle loss of *P. radiata* in Tasmania, complicating the functional assessment (Prihatini et al. 2015). Our sequenced isolate is a 98.99% BLAST match to the Tasmania *L. pinastri* and highly distinct from *L. seditosum* isolates (c. 91% matches). We suggest *L. pinastri* may be considered as a possible contributor to disease on the basis of Prihatini et al. (2015).

A culture with a near perfect match to *Austroafricana parva* (= *Teratosphaeria parva*, = *Mycosphaerella parva*), a pathogen of *Eucalyptus* spp., was also found (506 out of 507 base pairs, or 99.8% identical). *Mycosphaerella* spp. are known to be present in New Zealand (https://nzfungi2.landcareresearch.co.nz; accessed 22 July 2021). *Austroafricana parva* is part of the *Mycosphaerella* species complex which includes thousands of taxa, and causes disease symptoms such as leaf spot, leaf blotch, and shoot blight in *Eucalyptus* spp. (Crous et al. 2008; Chungu et al. 2010; Hunter et al. 2011). These symptoms can significantly reduce wood growth or result in tree death, which can lead to poor economic outcomes for growers (Hunter et al. 2011). In New Zealand, *Eucalyptus* spp. make up c. 1% of commercial forestry plantations, therefore this pathogen is unlikely to pose a major threat to the New Zealand forestry industry (Ministry for Primary Industries 2020). However, some species of *Austroafricana* do have a wide host range within the Myrtaceae and Proteaceae (Crous et al. 2008) and may threaten native species within this family if they were to spread as pine density increases due to invasion, and infect native plants (Bufford et al. 2019). We note that it is also possible our *Austroafricana* sp. was present as a spore on the surface of needles, rather than actively growing.

Tree density as a predictor of foliar fungi

Our results suggest that low density and high density wilding pines may play somewhat different roles in pathogen spill-over. Contrary to our hypothesis, low density pines supported a higher diversity of foliar fungi overall than high density trees. High density invasive pines, conversely, had a lower diversity of fungi overall, but equal pathogen richness compared to low density trees. However, as hypothesised, trees at high density had a greater prevalence (i.e. proportion) of potential plant pathogens than trees at low density. Equal richness of pathogens suggests that both low and high density wilding pine invasions may contribute to increasing pathogen abundance, thereby increasing inoculum pressure on surrounding landscapes (Burdon & Chilvers 1982; Plantegenest et al. 2007). The higher diversity of fungi on low density trees may be due to exposure

to environmental factors, such as high winds, which could damage foliage and provide entry points for fungi (Savatin et al. 2014). Due to the nature of our sampling, low density trees covered a somewhat larger geographic area than high density trees, which would be expected to increase beta and gamma diversity but is unlikely to contribute to the observed higher alpha diversity of fungi within individual trees. Having a higher diversity of plant-fungal interactions creates an opportunity for novel host-pathogen interactions to evolve (Navaud et al. 2018). For example, *Coleosporium ipomoeae* is able to infect more *Ipomoea* species outside of its native community than within, indicating that co-evolution may restrict the host range of *C. ipomoeae* (Chappell & Rauscher 2016). Therefore, invasive plants hosting pathogens may promote host jumps simply by supporting pathogen populations in proximity to novel potential hosts (Navaud et al. 2018).

Tree species as a predictor of foliar fungi

Our results show that *P. menziesii* hosts a significantly greater proportion of foliar fungi (i.e. a greater proportion of needles yielded culturable fungi), as well as a richer community of foliar fungi than *P. contorta*. This result is consistent with previous findings with other groups of fungi. For example, *P. menziesii* is a generalist and hosts a more diverse and rich community of mycorrhizal fungi than *Pinus* spp., which form fewer more specialised associations (Dickie et al. 2010; Moeller et al. 2015). As a generalist, *P. menziesii* is likely to accumulate a broader range of fungi over time, and therefore may become an important reservoir for pathogens and source of inoculum for spill-over into plantations in the future (Flory et al. 2013).

In this study, we focused solely on culturable fungi which limits our understanding of certain groups of fungi such as biotrophic pathogens (McTaggart et al. 2016) and non-fungal pathogens (e.g. viruses, oomycetes including *Phytophthora*, and bacteria). Our results are likely not representative of the complete foliar fungal communities in invasive pines, as we sampled from just one site, used a general growth media and did not target symptomatic needles. Sampling symptomatic needles would have increased our chances of isolating economically important pine pathogens, such as *Dothistroma* spp. (Mullet et al. 2014). Our sampling was also insufficient to saturate the species area curve, suggesting that many species remain to be detected, especially at high density where our sampling was less than at low density. Notwithstanding these limitations, this is the first study looking at foliar fungi in two of New Zealand's most important invasive weeds (Ministry for Primary Industries 2014). Thus, despite being limited in scope, our results demonstrate that further investigation of foliar pathogens on wilding pines, from a wider range of environments and with greater replication, is needed.

Invasive plants are recognised as a major threat to ecosystems, but their indirect effects through increasing pathogen loads are not as well understood. Even within our limited sampling, we detected several pathogens in invasive pine foliage, with the potential to spill-over onto plantation forests. As invasive pines continue to spread and increase in density across the landscape, this threat will increase. Further, invasive plant populations can function as pathogen reservoirs, limiting the ability to manage pathogens within planted ecosystems.

Acknowledgements

We thank Craig Galilee, Norma Merrick, Joanna Green, Vanita Thakur, Pieter Pelser, and Warwick Allen for assistance. This project was part of the Winning against Wildings research programme (MBIE contract C09X1611). We thank two anonymous reviewers whose comments greatly improved the paper.

Author Contributions

IAD and SJS developed the project concept; GS, IAD and SJS collected foliar samples. GS cultured fungi and extracted DNA. SJS and GS led analyses. GS wrote the first draft of the manuscript, and all authors contributed substantially to revisions.

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Received: 1 August 2021; accepted: 22 November 2021
Editorial board member: Peter Bellingham

Supplementary material

Additional supporting information may be found in the supplementary material file for this article:

Appendix S1. Meta-data for *Pinus* individuals sampled.

Appendix S2. Operational taxonomic unit data.

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