Journal of Microbiological Methods 200 (2022) 106546

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Development of PCR-based markers for the identification and detection of Lophodermella needle cast pathogens on *Pinus contorta* var. *latifolia* and *P. flexilis*

Jessa P. Ata [a,b,*], Kelly S. Burns [c], Suzanne B. Marchetti [c], James J. Worrall [c], Stephen J. Mondo [a,d], Jane E. Stewart [a,*]

[a] Department of Agricultural Biology, Colorado State University, CO, USA
[b] Department of Forest Biological Sciences, University of the Philippines, Los Baños, Philippines
[c] USDA Forest Health Protection, CO, USA
[d] Department of Energy Joint Genome Institute, Lawrence Berkeley National Laboratory, California, USA

ARTICLE INFO

Keywords:
Needle disease
Emerging pathogens
Disease diagnosis
Pine

ABSTRACT

Morphological similarities and fastidious development of increasingly emerging fungal needle pathogens impede accurate disease diagnosis and early detection. This study analyzed the specificity and sensitivity of polymerase chain reaction (PCR)-based markers developed for emerging needle cast pathogens *Lophodermella concolor* and *L. montivaga* co-occurring on *Pinus contorta* var. *latifolia*, and *Bifusella linearis* and *L. arcuata* on *P. flexilis*. To design primers, we utilized sequences of the internal transcribed spacer (ITS) region and single-copy gene (RH_2175) of the TCP-1/cpn60 chaperonin family searched through genomes of related species. In addition to the DNA of target and non-target fungal species that were used for primer assays, environmental samples with next generation sequencing data were used to evaluate primer sensitivity. Direct amplification using ITS primer pairs generated 248–260 bp amplicons and successfully differentiated the needle pathogens used in this study. Nested amplification of single-copy gene RH_2175 primer pairs which produced 409–527 bp amplicons detected Rhytismataceae species and discriminated both *Lophodermella* pathogens on *P. contorta* var. *latifolia*, respectively. While ITS-based primers had higher sensitivity than the 2175-based primers, both primer sets for *L. concolor* and *L. montivaga* detected their respective pathogens in asymptomatic and symptomatic needles. These molecular tools can help monitor and assess needle diseases for forest management and phytosanitary regimes.

1. Introduction

Recent increasing prevalence of needle diseases has been attributed to several factors such as the enhanced activity of needle pathogens brought about by regional warm rain events (Gray et al., 2013; Rodas et al., 2016; Welsh et al., 2014), the emergence of new needle pathogens that may be previously cryptic or latent (e.g., Dick et al., 2014; Durán et al., 2008), and the introduction and spread of needle pathogens in vulnerable ecosystems due to the expansion of international trade (EFSA Panel on Plant Health, 2013). While previous occurrences caused minor damage, the increasing severity of needle diseases leads to severe ecological and economic losses of ecosystem goods and services (EFSA Panel on Plant Health, 2013; Jansons et al., 2020), and to possible forest decline as they predispose stressed forest trees to other diseases (Wyka et al., 2018). However, efficient monitoring and control of diseases caused by these emerging forest pathogens are often undermined by our limited understanding of their disease mechanisms and lack of tools for accurate disease diagnosis.

Lophodermella needle cast is a common disease in natural lodgepole pine (*Pinus contorta* var. *latifolia*; hereon referred to as *P. contorta*) stands in the Rocky Mountain Region (RMR), USA (Rocky Mountain Region, Forest Health Protection, 2010). In 2008 to 2011, two Lophodermella needle cast epidemics in natural *P. contorta* stands in Colorado were recorded with small but heavily infected forest stands (Worrall et al., 2012). Symptoms include discoloration and defoliation of infected needles; severely affected trees were highly compromised (Darker, 1932; Worrall et al., 2012). The recent epidemics were caused by *Lophodermella concolor* (Dearn.) Darker and *L. montivaga* Petrak

* Corresponding authors at: Department of Agricultural Biology, Colorado State University, CO, USA.
E-mail addresses: jpata@up.edu.ph (J.P. Ata), Jane.Stewart@colostate.edu (J.E. Stewart).

https://doi.org/10.1016/j.mimet.2022.106546
Received 28 March 2022; Received in revised form 18 July 2022; Accepted 29 July 2022
Available online 2 August 2022

(Rhytismataceae), which are characterized by their tanned or colorless subhypodermal hysterothecia, clavate ascospores with mucilaginous sheath, and asci wider than *Lophodermium* species (Darker, 1932).

While both pathogens occur in *P. contorta* stands in RMR, they have been reported infecting other two-needle pines in western states and provenances of USA and Canada, respectively, including *P. banksiana* for *L. concolor* and *P. attenuata* for *L. montivaga* (Darker, 1932; Minter and Millar, 1993). Recently, metabarcoding analysis detected both pathogens on symptomatic and asymptomatic *P. contorta* needles, indicating that *Lophodermella* pathogens were either part of the 'normal' needle mycobiota or existed as persistent infections that have evaded plant immune response (Ata et al., 2022). However, despite their presence, there was no pathogenic activity of the fungal community in asymptomatic needles which further suggested a latent phase of *Lophodermella* infection, a trait common on other needle pathogens, that was not previously observed for *Lophodermella* species. This also indicated that *Lophodermella* spp. may be needle endophytes that could transition to pathogenic necrotrophs when environmental perturbations (e.g., biotic and abiotic stresses; Bass et al., 2019; Sieber, 2007) and/or host adaptation and stress response persist (Précigout et al., 2020; Shetty et al., 2007).

Needle cast in limber pine (*P. flexilis*) stands in RMR is commonly caused by *Lophodermella arcuata* (Darker) Darker and *Bifusella linearis* (Peck) Höhn. *Bifusella linearis* has been recorded in *P. strobus* and *P. monticola* (Broders et al., 2015; Darker, 1932) while L. *arcuata* occurs only on five-needle pines along the western Rockies (Darker, 1932; Minter and Millar, 1993). *Lophodermella arcuata* can be distinguished among other *Lophodermella* species through the size of ascomata and ascospores, and host occurrence (Darker, 1932). Unlike its *Lophodermella* relatives that caused epidemics in *P. contorta*, *L. arcuata* historically only caused insignificant damage in natural stands (Minter and Millar, 1993). However, its presence together with *B. linearis* on *P. flexilis*, although not quantified, has been increasing recently in RMR based on personal observation. In addition, *B. linearis* has been attributed to white pine needle damage together with other needle pathogens such as *Lecanosticta acicola* and *Lophophacidium dooksii* (Broders et al., 2015).

Pathogen identification and detection are vital for early and accurate disease diagnosis and monitoring. It enables access to necessary information (e.g., host specificity, mating systems and lifestyles) in addressing their potential threats (Crous et al., 2016). However, among *Lophodermella* species, rapid and accurate identification are impaired by similarities in symptomology and morphological characteristics, varied features across developmental stages, and lack of asexual structures (Darker, 1932; Worrall et al., 2012). Due to their cryptic and fastidious or potentially obligate lifestyle, rapid detection is also hampered as *Lophodermella* pathogens could remain asymptomatic in their hosts and cannot be isolated in pure cultures. With the challenges in fungal systematics and phenotypic identification, molecular information of forest fungal pathogens and gene-based technologies need to be integrated in quarantine and management systems for accurate identification and rapid detection of phytopathogens (Crous et al., 2016).

Modern approaches improve plant fungal disease diagnosis with genetic and genomic tools, which are regarded as reliable and precise methods for rapidly identifying plant pathogens. While a variety of assays are now used to detect pathogens in forestry and agriculture, assays for conifer needle diseases are limited to only a few pathogens such as *Dothistroma pini*, *D. septosporum* and *Lecanosticta acicola* (Aglietti et al., 2021; Barnes et al., 2008; Janoušek et al., 2014; Myrholm et al., 2021; Siziba et al., 2016). Thus, this study aimed to develop molecular assays to rapidly identify and detect *Lophodermella* needle pathogens including *B. linearis* on *P. contorta* and *P. flexilis*. We developed specific PCR-based primers from the internal transcribed spacer (ITS) region for *L. arcuata*, *L. concolor, L. montivaga* and *Bifusella linearis*. To enhance robust detection and discrimination of co-existing *L. concolor* and *L. montivaga* on *P. contorta*, we also searched and designed primers from single-copy gene regions with the aid of genome sequences of related rhytismataceous species.

2. Methodology

2.1. Sample collection and DNA extraction

We used DNA from hysterothecia of *L. concolor, L. montivaga* and *L. arcuata* samples obtained by Ata et al. (2021), including the non-target species *Lophophacidium dooksii* (Table 1). In addition, DNA of *B. linearis* and non-target species (Table 2) were extracted from hysterothecia or potato dextrose agar-grown mycelia out of *P. contorta* needles using similar methods described by Ata et al. (2021). Briefly, hysterothecia on symptomatic needles or mycelia were excised or scraped, respectively. These were then ground into powder by submerging in liquid nitrogen and grinding using FastPrep-24™ (MP Biomedicals, Santa Ana, CA, USA) prior to the CTAB method (Cubero et al., 1999). Quality and quantity of all DNA samples were determined using NanoDrop™ spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Amplification of the ITS region of all the target and non-target species was performed in a 25-μL PCR reaction mixture of 1× standard Taq reaction buffer (New England BioLabs, Ipswich, MA, USA), 0.2 mM of each dNTP (Gold Biotechnology, St. Louis, MO, USA), 0.4 μM of each universal primer ITS1 and ITS4 (White et al., 1990), 0.625 units Taq polymerase (New England BioLabs), and ca. 40 ng template DNA. Cycle parameters included initial denaturation at 94 °C for 2 mins, followed by 30 cycles of denaturation at 94 °C for 40 s, annealing at 55–57 °C for 40 s, extension at 72 °C for 1 min, and final extension at 72 °C for 5 mins. Sterile molecular grade H_2O was used as negative control throughout the assays. All amplifications were carried out in Eppendorf Mastercycler ProS (Eppendorf, Hamburg, Germany) thermal cycler. PCR products were analyzed on 1.5% agarose gels in 0.5 × TBE and photographed using Azure™ gel imaging system (Azure Biosystems, Dublin, CA, USA). Enzymatic cleanup was performed among PCR amplicons using Applied Biosystems ExoSAP-IT™ (Thermo Fisher Scientific). All amplicons were sequenced by Eurofins Genomics (Louisville, KY, USA). The ITS sequences of *B. linearis* and non-target species were then matched to the National Center for Biotechnology Information (NCBI) database (NCBI Resource Coordinators et al., 2018) using NCBI BLAST to search for highly similar fungal sequences. Cloning of PCR products was performed using pGEM® T-Easy Vector Systems (Promega, Madison, WI, USA) among randomly selected samples to validate that the sequenced amplicon for each sample was that of a single species.

2.2. Primer development and assay from ITS region

Specific primers for *L. concolor* (LC_ITS), *L. montivaga* (LM_ITS), *L. arcuata* (LA_ITS) and *B. linearis* (BL_ITS) were developed based on the ITS sequences of the target fungal samples (Table 3). Primers were designed and initially screened using the Primer 3 module (v2.3.4; Untergasser et al., 2012) in Geneious (v R9.05). Primer sets were tested across *Lophodermella* spp. and among non-target species. Direct PCR amplifications were performed with similar 25 μL-PCR reaction mixtures as described above but using only ca. 5–10 ng template DNA. Cycle conditions were also similar as described above except for the annealing temperatures, as shown in Table 3.

2.3. Primer development and assay from genomic data

Genomic data of five Rhytismataceae species and three outgroup species from the Joint Genome Institute MycoCosm (Grigoriev et al., 2014) were used to explore primers from other genomic regions (Table 2). Multigene clusters among these genomes were identified from the Joint Genome Institute (JGI) MycoCosm (https://mycocosm.jgi.doe.gov/clm/run/Elyde1-comparative.2723;gwnsxm?organism=Elyde1).

Table 1
PCR amplification using specific primers designed from the internal transcribed spacer (ITS) and gene cluster 2175 on *Lophodermella* spp. and *Bifusella linearis* (Rhytismataceae) samples collected from *Pinus contorta* and *P. flexilis* stands in Colorado (CO) and in Maine (ME), USA. Plus (+) and minus (−) signs represent presence and absence of a single band with target amplicon size, respectively.

Sample ID	Host	Region	GenBank Accession	ITS				2175		
				LC_ITS	LM_ITS	LA_ITS	BL_ITS	RH_2175	LC_2175	LM_2175
***Lophodermella concolor* (Dearn.) Darker**										
CS9C	*P. contorta*	CO	MN937612	+	−	−	−	+	+	−
FS8C	*P. contorta*	CO	MN937610	+	−	−	−	+	+	−
OBJ9C	*P. contorta*	CO	NA	+	−	NA	NA	+	+	−
SR3C	*P. contorta*	CO	MN937617	+	−	NA	NA	+	+	−
FS6C	*P. contorta*	CO	MN937618	+	NA	−	−	+	+	−
***Lophodermella montivaga* Petrak**										
CU1M	*P. contorta*	CO	MN937633	−	+	−	−	+	−	+
NC2M	*P. contorta*	CO	MN937625	−	+	−	−	+	−	+
NC10M	*P. contorta*	CO	MN937637	−	+	−	−	+	−	+
NC6M	*P. contorta*	CO	MN937626	−	+	NA	NA	+	−	+
PT11M	*P. contorta*	CO	MN937630	−	+	NA	NA	+	−	+
***Lophodermella arcuata* (Darker) Darker**										
RMNP_LU1	*P. flexilis*	CO	MN937644	NA	−	+	NA	+	−	+
RMNP_LU16	*P. flexilis*	CO	MT906333	−	−	+	−	+	−	+
RMNP_LU12	*P. flexilis*	CO	NA	−	−	+	−	−	NA	NA
***Bifusella linearis* (Peck) Höhn.**										
BiTR	*P. flexilis*	CO	NA	−	−	−	+	+	−	−
BP20	*P. flexilis*	CO	NA	−	−	−	+	+	−	−
MB02	*P. strobus*	ME	NA	−	−	−	+	+	−	−

Primers are designed for *L. concolor* (LC), *L. montivaga* (LM), *L. arcuata* (LA), *Bifusella linearis* (BL) and species within Rhytismataceae (RH).

Table 2
Non-target species used in the study to test specificity of primers for *Lophodermella concolor* and *L. montivaga* on *Pinus contorta*, and *Bifusella linearis* and *L. arcuata* on *P. flexilis*.

Sample ID	ITS BLAST ID	GenBank	% Identity (e-value)	Family	Host	Material
DNA samples						
InfNSA1	*Alternaria alternata* YZU	MN615420.1	100 (0.0)	Pleosporaceae	*P. contorta*	Mycelium
EDSR19	*Davisomycella medusa* BPI842078	AY465525.1	94.86 (6e-105)	Rhytismataceae	*P. contorta*	Hysterothecia
PTKN9AP	*Hendersonia pinicola* EBJul30–4	KT000192.1	100 (0.0)	Phaeosphaeriaceae	*P. contorta*	Mycelium
T2-WY	*Mycosphaerella* sp. sd3cN2b	AY465456.1	100 (0.0)	Mycosphaerellaceae	*P. contorta*	Hysterothecia
GLRC	*Lophodermium nitens* NB-283-2D	KY485136.1	100 (4e-173)	Rhytismataceae	*P. flexilis*	Mycelium
SD_B	*Lophodermium resinosum* LPiPres2_12_3	MW466468.1	95.60 (0.0)	Rhytismataceae	*P. contorta*	Mycelium
MB05	*Lophophacidium dooksii*	KF889693.1	98.94 (0.0)	Rhytismataceae	*P. strobus*	Hysterothecia
InfNSAP2	*Thielavia* sp. SR-6	MK246011.1	99.78 (0.0)	Chaetomiaceae	*P. contorta*	Mycelium
WWFB_B.1	*Sydowia polyspora* ENDO-PINE669-BOTTOMA	MK762617.1	100 (0.0)	Dothioraceae	*P. ponderosa*	Mycelium
ED-AZ2	*Epicoccum layuense* isolate 17	MT573479.1	100 (0.0)	Didymellaceae	*P. ponderosa*	Mycelium

JGI Mycocosm genome sequences				
Species	Label/ID	Family	Assembly Length (bp)	Genes Count
Elytroderma deformans	CBS 183.68 v1.0	Rhytismataceae	50,483,512	12,886
Lophodermium nitens	PLMe3–1-3 v1.0 (Salas-Lizana and Oono, 2018)	Rhytismataceae	74,665,558	19,985
Pseudographis elatina	Pseel1	Rhytismataceae	36,124,988	11,338
Coccomyces strobi	CBS 202.91 v1.0	Rhytismataceae	32,666,196	11,537
Spathularia flavida	Spafl1	Rhytismataceae	35,536,079	9941
Bulgaria inquinans	CBS 118.31 v1.0	Bulgariaceae	26,108,822	9864
Rutstroemia firma	CBS 116.86 v1.0	Rutstroemiaceae	44,266,318	13,359
Thelebolus microsporus	ATCC 90970 v1.0	Thelebolaceae	27,344,100	10,290

Amino acid sequences were extracted and aligned using MUSCLE. Codon alignments per cluster were generated using PAL2NAL with -nomismatch parameter (Suyama et al., 2006). Single-copy orthologs were selected to limit sequence variations within a species. To further reduce the number of orthologs to be analyzed, only clusters with a gene length < 2000 bp were considered. Sequence alignments of candidate clusters were then randomly selected and manually inspected for variations between Rhytismataceae species and outgroups. Primer sets were then designed from eight candidate gene clusters using Primer 3 and screened for specificity to rhytismataceaous target and non-target species. Of these, the primer set (exterior primer) of only one gene cluster (RH_2175) was selected due to its clear amplification among rhytismataceous species. This region was then used for designing nested PCR primers specifically for *L. concolor* and *L. montivaga*. PCR amplification of RH_2175 was carried out using a 25 μL-PCR reaction mixture described above with 10 ng of template DNA with cycle parameters shown in Table 3.

The amplified RH_2175 region of *L. concolor* and *L. montivaga* were

Table 3
Primers developed for *Lophodermella concolor, L. montivaga* and *Bifusella linearis* and their parameters for direct and nested PCR amplification.

Gene Region/ Cluster	Target Pathogen	Primer Name	Direction	Sequence	Annealing Temperature (°C)	Number of Cycles	Product Length (bp)
Direct PCR							
ITS	*L. concolor*	LC_ITS	Forward	TGAGCTTCTCACCCCCTGTA	66	35	260
			Reverse	GAGCTTGAGGGCTGGTTGAT			
	L. montivaga	LM_ITS	Forward	CCTGGTAAAACTCGCACCCT	70	30	259
			Reverse	GCTTGAGGGTTGTAATGACGC			
	B. linearis	BL_ITS	Forward	TTGCAGTCTGAGTACCACAC	65	30	248
			Reverse	TACTGCGCTGGAGCTTAGAT			
	L. arcuata	LA_ITS	Forward	GCCTGGTAACTCACACCTC	58	30	338
			Reverse	GTACTACGCTTAGGGGGCA			
Nested PCR							
2175	Rhytismataceae	RH_2175	Forward	CTGCTATCGGAGAAGAAGAT	49	35	525
			Reverse	TTGATGTTTCCAAGAGCTTG			
	L. concolor	LC_2175	Forward	TCTCTGACGAGCGTGATATT	68	35	215
			Reverse	ATGAACCTCCAACCCTAATC			
	L. montivaga	LM_2175	Forward	CTGACCAGCTCGACATCAAA	65	30	212
			Reverse	ATGAGCCTCCGACCTTGATA			

then sequenced to identify nucleotide polymorphisms in both *Lophodermella* species. Species-specific primers (interior primers) LC_2175 and LM_2175 were then designed from polymorphic sites using Primer 3 (Table 3). PCR amplification using these specific primers was carried out using the previously described 25 μL-PCR reaction mixture with 4 μL of diluted PCR product (1:100) generated from the RH_2175 amplification and cycle parameters presented in Table 3. Cycle conditions, except for annealing temperature, for the nested PCR amplification assay were similar to those described above.

2.4. In silico and in vitro primer testing

The designed ITS and 2175 primers were tested for specificity in silico using NCBI Primer BLAST with nr and Refseq representative genomes databases, respectively, prior to in vitro assays. We also tested the *Lophodermella* specific 2175 gene primers against the genome sequences of related species available at JGI Mycocosm using Primer 3. In vitro primer assays were performed on available non-target species (Table 4) following the PCR conditions for each specific primer set. Available ITS sequences of other *Lophodermella* spp. and closely related species, including RH_2175 amplicon sequences were aligned using the MUSCLE module in Geneious (v R9.05).

2.4.1. Sensitivity of primers

To verify DNA concentration, each DNA sample extracted from fruiting bodies of target species was measured using Qubit 3.0 (Invitrogen, Thermo Fisher Scientific). Sensitivity of primers was tested using ten-fold serial dilutions of each sample, ranging from 1 ng – 1 pg.

2.5. Detection of Lophodermella pathogens on environmental samples

Given the endophytic lifestyle of some needle pathogens, we used whole symptomatic and asymptomatic needles of *P. contorta* and *P. flexilis* to assess the sensitivity of primers. Specifically, two sets of environmental samples were tested. The first set of samples (SET 1) were obtained from Ata et al. (2022). These were DNA samples from asymptomatic ($n = 12$) and symptomatic ($n = 8$) *P. contorta* needles infected with either *L. concolor* or *L. montivaga* (Fig. 1, Table 5) with estimates of *L. concolor* and/or *L. montivaga* contigs which were obtained through sequencing of the internal transcribed spacer (ITS) region [ITS3: (GCATCGATGAAGAACGCAGC) and ITS4: (TCCTCCGCTTATTGATATGC)] via Illumina MiSeq (NCBI SRA Bioproject PRJNA7531). The second set (SET 2) were asymptomatic needles of *P. contorta* and *P. flexilis* collected from Colorado and Wyoming, USA without associated metabarcoding data.

Needle cleaning and DNA extraction were performed prior to DNA extraction. Briefly, two to three needles per tree were pooled for each sample. Needles were washed by vortexing in a 0.2% Tween 20 solution and cleaned in 70% ethanol. To evaluate the removal of contamination, we followed Rajala et al. (2013) with modifications: randomly selected clean symptomatic and asymptomatic needles were washed with distilled water and vortexed for approximately 10 mins. The resulting rinse solution was used as template for ITS amplification with primers ITS1 and ITS4 (White et al., 1990). Amplification was only observed in symptomatic needles as spores can easily disperse from mature hysterothecia. Approximately 1-mm segment from the sheath-covered base was removed. Whole needle DNA was extracted using the CTAB method with similar modifications presented above. DNA samples were stored in

Table 4
Amplification using primers designed for *Lophodermella concolor* (LC) and *L. montivaga* (LM) on *Pinus contorta*, and *Bifusella linearis* (BL) and L. *arcuata* (LA) on *P. flexilis* on non-target species. RH represents primers designed for species within Rhytismataceae. Single asterisk (*) represents single band but not the target size, double asterisk (**) represents multiple bands. Plus (+) and minus (−) signs represent presence and absence of a single band with target amplicon size, respectively.

Sample (NCBI BLAST ID)	Location	ITS				2175		
		LC_ITS	LM_ITS	LA_ITS	BL_ITS	RH_2175	LC_2175	LM_2175
InfNSA1 (*Alternaria alternata*)	Wyoming	−	−	−	−	*	−	−
PTKN9AP (*Hendersonia pinicola*)	Colorado	−	−	−	−	−	−	−
GLRC (*Lophodermium nitens*)	Colorado	−	−	−	−	+	−	−
SD_B (*Lophodermium resinosum*)	South Dakota	−	−	−	−	+	−	−
InfNSAP2 (*Thielavia* sp.)	Wyoming	−	−	−	−	*	−	−
WWFB_B.1 (*Sydowia polyspora*)	Colorado	−	−	−	−	**	−	−
ED-AZ2 (*Epicoccum layuense*)	Arizona	−	−	−	−	**	−	−
MB05 (*Lophophacidium dooksii*)	New Hampshire	−	−	−	+	+	−	+
ED-19 (*Davisomycella* sp.)	Colorado	−	−	−	−	+	−	−
T2-WY (*Mycosphaerella* sp.)	Wyoming	−	−	−	−	+	−	−

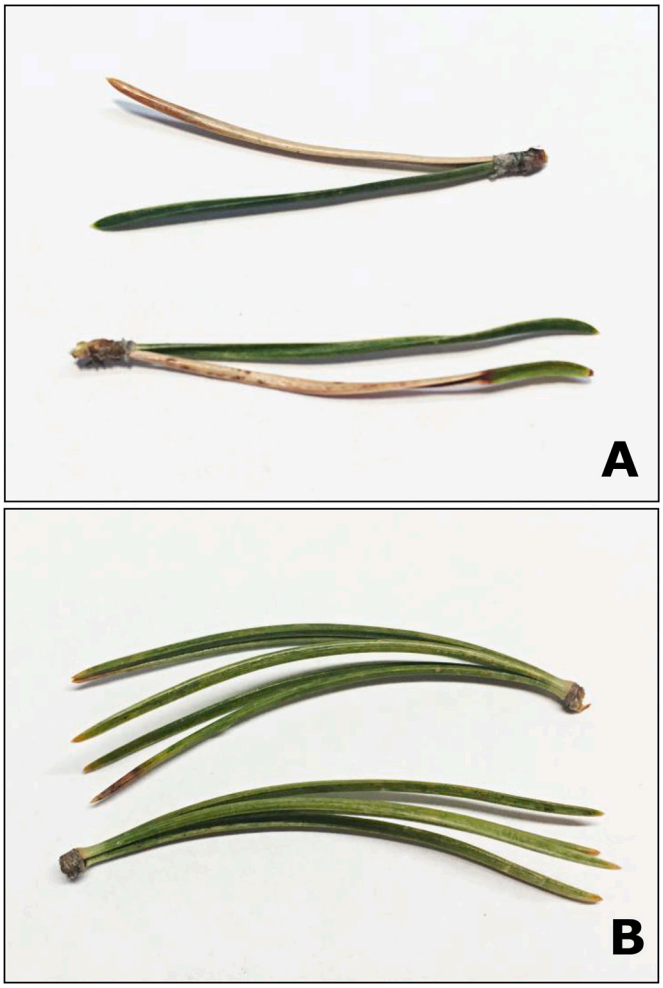

Fig. 1. Needles of *P. contorta* that were asymptomatic and symptomatic of either *L. concolor* and *L. montivaga* (A), and asymptomatic needles of *P. flexilis* (B).

−20 °C prior to primer assays.

Direct and nested PCR amplifications were performed following the described assays for primers designed from regions ITS and cluster 2175, respectively. To determine the optimal DNA concentration, amplification was evaluated using the presence of *L. concolor* and/or *L. montivaga* on samples with associated metabarcoding data (Table 5). A prior test was performed that showed that 10 ng and undiluted DNA with >400 ng amount did not yield any amplification. Thus, DNA concentration of asymptomatic needles was adjusted to be within the 110 to 150 ng range. A total of 15 ng of DNA was used for symptomatic needle samples. Since no contigs of *L. arcuata* nor *B. linearis* were detected from the previous metabarcoding analysis, the SET 1 samples were only tested for the sensitivity of *L. concolor* and *L. montivaga* primers. All designed primers were tested among the SET 2 samples. To check the accuracy of amplification in only target species in asymptomatic needles, cleaned amplicons from direct and nested PCR assays were randomly selected for sequencing.

3. Results

3.1. Identification of needle pathogens and non-target species

Amplification of the ITS region using the universal fungal primers ITS1 and ITS4 for *B. linearis* and non-target fungal species yielded quality sequences. All *B. linearis* samples matched to *B. linearis* sequence in NCBI (KT000195.1) with 99.02–99.55% identity (e-value 4e-152 to 0.0). Four out of the 10 non-target samples were identified as species within Rhytismataceae belonging to genera *Davisomycella* and *Lophodermium* with >90% similarity to the NCBI sequences (Table 2). The other non-target samples belonged to six fungal families which include Pleosporaceae, Phaeosphaeraiaceae, Mycosphaerellaceae, Chaetomiaceae, Dothioraceae and Didymellaceae, with percent identities ranging from 99.78 to 100%.

3.2. Primer specificity

In silico analyses showed no match of either the forward or reverse LC_ITS and the forward LM_ITS to the NCBI fungal database. However, the BL_ITS and LA_ITS matched the ITS sequences of *B. linearis* (GenBank accession numbers KT000195.1, KT000194.1, AY465527.1 and KT000193.1) and *L. arcuata* (AY465518.1) in the database, respectively. While exterior primer 2175 (RH_2175) had BLAST hits to other non-rhytismataceous genomes, none were predicted to produce the target amplicon length. Using JGI genome sequences of closely related species and three outgroups, RH_2175 only matched to *Elytroderma deformans, Lophodermium nitens, Pseudographis elatina, Coccomyces strobi* and *Spathularia flavida*, all within Rhytismataceae. The interior primers LC_2175 and LM_2175, however, had no match to any of the genome sequences.

For the in vitro assays, the PCR conditions were optimized for each primer to enhance specificity. The LM_ITS primer set had the highest annealing temperature at 70 °C. Primers designed from the ITS region amplified the expected sequence of the target species with amplicon lengths ranging from 248 to 338 bp (Tables 1 and 3), with a clear singular band in the electrophoresis gel (Supplementary Figs. 1–4). There were no amplifications among the non-target samples using LC_ITS, LM_ITS and LA_ITS primer pairs based on the absence of a single band. While most of the non-target species did not yield any amplifications using the BL_ITS primer set, a single band was observed in *Lophophacidium dooksii* hysterothecia on *P. strobus* (MB05; Supplementary Fig. 4), which may be due to *B. linearis* co-existing on the same *Pinus* host. After amplicon sequencing, the sequence matched to the *B. linearis* ITS sequences rather than to that of *L. dooksii*.

Out of the eight gene clusters screened for specificity, only the singleton gene region 2175 amplified the target size (409–527 bp) in most Rhytismataceae species. This gene was annotated as domain TCP-1/cpn60 chaperonin family for all the five rhytismataceous species and three outgroups. The tail end of this gene cluster in *Pseudographis elatina* and *Spathularia flavida* genomes was further annotated by JGI as cofilin/tropomyosin-type actin-binding protein domain. The low annealing temperature (49 °C) for the exterior primer RH_2175 assay allowed the amplification of most target and non-target rhytismataceaous species used in this study with an amplicon size of 525 bp, except for one sample of *L. arcuata* (RMNP_LU1) which contained only 1.2 ng DNA.

Most non-target species had either no amplification or had differently sized band/s using RH_2175 (Table 4, Supplementary Fig. 5). A target amplicon size was produced in *Mycosphaerella* sp. (T2-WY) using RH_2175, but not with the species-specific primers LC_2175 and LM_2175. Assays for interior primers LC_2175 and LM_2175 showed clear amplification among *L. concolor* and *L. montivaga* samples, respectively, and discriminated these two coexisting pathogens (Table 1, Supplementary Figs. 6 and 7). Except for *L. arcuata* and *Lophophacidium dooksii*, other non-target species did not amplify using LC_2175 and LM_2175.

3.3. Primer sensitivity

To test and assess the sensitivity of primers, we utilized serial dilutions of DNA samples of target species and DNA samples extracted from whole *P. contorta* and *P. flexilis* needles. Except for the LA_ITS assay which obtained a clear band at 10 pg, singular bands remained clear and visible in all other primer assays using DNA of target species diluted at 1

Table 5
Amplification using primers designed for *Lophodermella concolor* (LC) and *L. montivaga* (LM) on *Pinus contorta*, and *Bifusella linearis* (BL) and *L. arcuata* (LA) on *P. flexilis* on *P. contorta* needles asymptomatic and symptomatic of *L. concolor* and *L. montivaga* and asymptomatic *P. flexilis* needles. RH represents primers designed for species within Rhytismataceae. Asterisk (*) represents faint single band. Plus (+) and minus (−) signs represent presence and absence of a single band with target amplicon size, respectively. Pound sign (#) represents primer assay requiring high concentration of primer set. Number of contigs obtained from NCBI Bioproject PRJNA753461.

Sample ID	Host	DNA Amount (ng)	Number of Contigs		ITS				2175		
			LC	LM	LC_ITS	LM_ITS	BL_ITS	LA_ITS	RH_2175	LC_2175	LM_2175
SET 1: Asymptomatic needles											
CS02-19CN	*P. contorta*	150	271	0	−	−	NA	NA	−	−	−
LV01-19CN	*P. contorta*	148	34,489	0	+	−	NA	NA	+	+	−
LV02-18MN	*P. contorta*	146	22,078	18,744	+	+	NA	NA	−	+	+
LP02-19CN	*P. contorta*	150	4260	336	+	−	NA	NA	−	−	−
MP02-19CN	*P. contorta*	150	18,629	33	+	−	NA	NA	−	+	−
NC02-19CN	*P. contorta*	150	859	6300	−	−	NA	NA	−	−	−
NC11-19MN	*P. contorta*	150	14,933	2360	+*	+*	NA	NA	−	+*	−
NC13–19MN	*P. contorta*	150	23,931	152	+	−	NA	NA	−	+	−
PT11-19CN	*P. contorta*	150	6363	7066	−	−	NA	NA	−	−	−
TC03–19MN	*P. contorta*	150	19,183	121	+	+	NA	NA	−	+	−
TC07-19CN	*P. contorta*	150	3033	28	−	−	NA	NA	−	−	−
TC09-19CN	*P. contorta*	110	10,857	0	+#	−	NA	NA	−	+	−
SET 1: Symptomatic needles											
NC04-18MP	*P. contorta*	15	3	63,789	−	+	NA	NA	+	−	+
OBJ10-19CP	*P. contorta*	15	42,459	0	+	−	NA	NA	+	+	−
TC03–19CP	*P. contorta*	15	53,718	9	+	−	NA	NA	+	+	−
TC05-19CP	*P. contorta*	15	52,241	0	+	−	NA	NA	+	+	−
LP06-19CP	*P. contorta*	15	38,751	0	+	−	NA	NA	+	+	−
NC11-19CP	*P. contorta*	15	48,366	0	+	−	NA	NA	+	+	−
PT01-19CP	*P. contorta*	15	34,845	0	+	−	NA	NA	+	+	−
NC11-19MP	*P. contorta*	15	17	53,102	−	+	NA	NA	+	−	+
SET 2											
T1U	*P. contorta*	150	NA	NA	−	−	−	−	−	+	−
SA1U	*P. contorta*	150	NA	NA	−	−	−	−	−	−	−
SEED2U	*P. contorta*	150	NA	NA	−	−	−	−	−	−	−
PN22U	*P. flexilis*	50	NA	NA	−	−	+	−	−	−	−
BP20U	*P. flexilis*	50	NA	NA	−	−	−	−	−	−	−
BP17U	*P. flexilis*	50	NA	NA	−	−	+	−	−	+	−

pg (Supplementary Table 1). Using the designed ITS and 2175 primers, amplifications were observed in most SET 1 DNA samples from *P. contorta* needles which were symptomatic and asymptomatic of *L. concolor* and *L. montivaga* (Table 5). Among symptomatic needles, the primers were able to detect the dominant pathogen despite the marginal presence of the other *Lophodermella* species. For example, LC_ITS and LC_2175 detected L. *concolor* in six symptomatic needle samples that contained more *L. concolor* contigs (<35,000) than *L. montivaga* contigs. Conversely, LM_ITS and LM_2175 detected L. *montivaga* in two L. *montivaga* dominated symptomatic needles.

Among SET 1 asymptomatic needles, LC_ITS and LM_ITS detected 58% of the 12 samples and 33% of the 9 samples that contained *L. concolor* and *L. montivaga*, respectively. Two samples (LV02-18MN and TC03–19MN) had an amplification for both LC_ITS and LM_ITS regions. Further, in sample TC03–19MN, LM_ITS detected *L. montivaga* despite low pathogen concentration (121 contigs). In contrast, despite a relatively heavy presence of *L. concolor* in TC09-19CN (10,857 contigs), a target size band was produced only after increasing the concentration of LC_ITS primer (100 μM) although some faint bands were also observed.

Amplification of RH_2175 produced multiple faint bands among most *P. contorta* asymptomatic needles with no visible target size band (Supplementary Fig. 5), which could suggest the presence of multiple non-target species as was observed in the previous metabarcoding study (Ata et al., 2022). However, a clear target-sized band was observed in LV01-19CN and all symptomatic needle samples, which may be due to the large number of rhytismataceous species (e.g., *L. concolor* and *L. montivaga*) relative to other asymptomatic needle samples. Interestingly, from the diluted amplicons produced in the first round of amplification, LC_2175 and LM_2175 detected both *L. concolor* and *L. montivaga,* respectively, in asymptomatic samples that had a relatively heavy pathogen presence (>11,000 and > 19,000 *L. concolor* and *L. montivaga* contigs; Table 5 and Supplementary Figs. 6 and 7). While most of the amplifications were consistent between the specific ITS and 2175 primers, three asymptomatic samples (NC11-19MN, TC03–19MN and LP02-19CN) with relatively low *Lophodermella* contigs did not have a visible band using either LC_2175 or LM_2175 despite amplification using LC_ITS or LM_ITS. All sequenced amplicons from both specific ITS and 2175 primer assays matched to either *L. concolor* and/or *L. montivaga*.

Among SET 2 samples, BL_ITS amplification produced a band in two out of three asymptomatic *P. flexilis* samples, but none were produced with RH_2175 (Table 5, Supplementary Figs. 4 and 5). *Lophodermella arcuata*, on the other hand, was not detected among *P. flexilis* asymptomatic needles which may be attributed to the low sensitivity of LA_ITS primer set (Supplementary Table 1). Interestingly, the DNA concentration of *P. flexilis* asymptomatic needles needed to be reduced from 150 ng to 50 ng to yield a band in the BL_ITS reaction. Sequencing of BL_ITS amplicons that showed a faint band across the two samples yielded only poor-quality sequences which is likely due to the low number of amplicons. Further, an LC_2175 band was observed in a 50-ng asymptomatic sample of *P. flexilis* (BP17U). For SET 2 *P. contorta* asymptomatic needles, *L. concolor* was the only pathogen detected using LC_2175 in one sample (TIU). The amplicon sequence matched correspondingly to that of *L. concolor*.

4. Discussion

Here, we explored the use of multi-copy and single-copy gene regions to develop primers that would accurately identify and rapidly detect

emerging needle cast pathogens, *Lophodermella* spp. and *Bifusella linearis*, on *P. contorta* and *P. flexilis*, respectively. In silico and in vitro primer assays revealed the specificity and sensitivity of markers developed from ITS and 2175 gene regions, which will be useful in the early detection of the target pathogens for efficient forest disease monitoring. Further, through amplification of target regions using the designed primers, this study determined for the first time the latent lifestyle of obligate *Bifusella linearis* in *P. strobus* and *P. flexilis*.

4.1. Pathogen identification and detection using specific ITS and RH_2175 primers

The developed primers based on single nucleotide polymorphisms in the ITS and 2175 gene regions enabled delimitation of species and pathogen detection of asymptomatic needles, indicating the efficiency of multi- and single-copy gene regions in pathogen identification and disease diagnosis. The primers distinguished the target needle pathogens from the non-target fungal species, including foliar endophytes that are common members of the needle mycobiota (Deckert et al., 2002; Del Frari et al., 2019; Guo et al., 2004; Ridout and Newcombe, 2018; Soltani and Hosseyni Moghaddam, 2015; Tanney and Seifert, 2017) and needle pathogens that occur on *P. contorta* in the RMR (Rocky Mountain Region Forest Health Protection, 2010).

However, one drawback was the amplification using LM_2175 in two non-target species that were closely related to *L. montivaga*, which could suggest insufficient polymorphic sites to allow further discrimination between close relatives. Phylogenetic analysis found that *L. arcuata*, *L. sulcigena* and *Lophophacidium dooksii* clustered together with *L. montivaga* in the *Lophodermella* (LOD) subclade, with 99.9 Bayesian posterior probability and 80.1 bootstrap support (Ata et al., 2021). Using the tools developed herein, while not specific to *L. montivaga*, LM_2175 could be used to discriminate *L. montivaga* from *L. concolor* coexisting within *P. contorta* and to identify species within the LOD subclade, and then the presence of *L. montivaga* could be further confirmed through ITS-specific primer LM_ITS.

We detected both *Lophodermella* pathogens in some asymptomatic *P. contorta* needles using the designed primers (i.e., LC_ITS and LC_2175 for *L. concolor*, and LM_ITS and LM_2175 for *L. montivaga*), although a higher concentration of template DNA was needed. This further suggests primer efficiency using environmental DNA samples without potential interference from the fungal endophytes and *Pinus* hosts DNA. Interestingly, the ITS-based primers LC_ITS and LM_ITS had about 9% more positive detections than the 2175 primers, indicating a higher sensitivity of the ITS-based primers likely due to the multiple copies within the ITS region (Jurado et al., 2006; Salvioli et al., 2008; Tekpinar and Kalmer, 2019). Despite primer specificity, with a low amount of target DNA, the detection efficacy of single copy gene-based primers is typically low (Kulik et al., 2020). This is likely why no band was observed in an *L. arcuata* sample with low DNA amount with RH_2175 despite this sample amplifying the target region using the LA_ITS, and why no amplification occurred with LC_2175 and LM_2175 in some asymptomatic needles despite low pathogen contig counts observed from previous metabarcoding data.

We further observed no amplification using LC_ITS in some asymptomatic needles despite pathogen presence or detection with LC_2175, which could be attributed to competition from mixed DNA templates for reaction reagents resulting in inefficient amplification of relatively low abundant target DNA templates (Kalle et al., 2014). Thus, the increase in primer concentration that resulted in visible bands in the TC09-19CN asymptomatic sample could have likely allocated primers for the relatively less abundant DNA templates including that of *L. concolor*. For these reasons, we recommend the use of both the ITS- and 2175-based primers to determine pathogen presence in asymptomatic needles.

With the aid of metabarcoding data, this study showed that the lack of amplification could suggest a low colonization of one or both pathogens. In contrast, the amplification/s of *L. concolor* and/or *L. montivaga* specific ITS and/or 2175 regions among asymptomatic samples could indicate the increasing presence and biomass changes of the *Lophodermella* pathogen/s which may influence disease occurrence. This enhanced abundance of latent needle pathogens, such as *Lophodermella* spp. in the microbiome, due to abiotic and biotic stresses is a common characteristic during pathogenic transitions from normal to a diseased state of the host (Bass et al., 2019; Sieber, 2007).

4.2. Genome sequences of related species to search for markers

To search for single-copy gene regions among species with unavailable genome sequences (i.e., *L. concolor* and *L. montivaga)*, this study demonstrated the use of genome sequences of non-target but closely related species to explore candidate gene regions with distinct polymorphisms to develop markers for non-model target species. Among the primers designed from eight candidate gene regions, only RH_2175 from gene cluster 2175 amplified samples that represented at least five rhytismataceous genera at a low optimal annealing temperature. Gene 2175 was annotated as a TCP1/cpn60 chaperonin family member (Grigoriev et al., 2014), a ubiquitous protein found in prokaryotes and eukaryotes. While essential in the assembly of actin and tubulin among fungi (Stoldt et al., 1996), the role of cytosolic chaperonin among eukaryotes in disease remains unknown. Interestingly, chaperonin members among pathogenic bacteria are major antigenic proteins important in infection and immunity (Gupta, 1995; Ranford, 2002). However, in this present study, the relationship of amplifications using RH_2175 across target and non-target samples with the gene function was not further explored.

4.3. Detection of Lophodermella concolor on P. flexilis

Our assays detected *L. concolor* in one of the three asymptomatic *P. flexilis* samples using LC_2175. This is a surprising finding since, on the basis of hysterothecia development, *L. concolor* has only been reported to occur on two-needle pines *P. banksiana* and *P. contorta* of subsection Contortae and *P. sylvestris* of subsection Pinus (Darker, 1932; Millar, 1984; Minter and Millar, 1993). As *P. flexilis* and *P. contorta* commonly co-occur in the southern parts of Wyoming and northern Colorado (Steele, 1990), it is possible that *L. concolor* inoculum from infected *P. contorta* stands could infect, albeit rarely, nearby *P. flexilis* trees asymptomatically like other needle pathogens that thrive as endophytes (Magan and Smith, 1996; Sieber et al., 1999; Stone et al., 2004). Currently, there is no evidence to suggest pathogenicity of *L. concolor* in *P. flexilis*. Since there was no associated metabarcoding data for the SET2 asymptomatic needle samples, we cannot confirm the presence nor the amount of *L. concolor* within the samples. However, given the amplification observed in asymptomatic *P. contorta* needles, we surmise that a relatively heavy *L. concolor* load may have been present.

4.4. Detection of B. linearis on environmental samples

Using BL_ITS, we detected *B. linearis* present in the *P. strobus* needle tissue surrounding *Lophophacidium dooksii* hysterothecia and in asymptomatic *P. flexilis* needles, which likely indicates an endophytic or latent pathogen lifestyle of *B. linearis* and coinfection of both pathogens on *Pinus* hosts. Similar to *Lophodermella* spp., the inability of *B. linearis* to grow or have only ephemeral growth in culture (Broders et al., 2015; Merrill et al., 1996) may have contributed to the lack of evidence on its latency in *Pinus* needles. Ganley et al. (2004) further reported that none of the media-grown endophytes of *P. monticola* were synonymous or closely related to the host's known foliar parasites such as *B. linearis* and *L. arcuata*. As *B. linearis* and *L. dooksii* were both attributed to *P. strobus* needle damage (Broders et al., 2015), the amplification using BL_ITS in *L. dooksii* from *P. strobus* could further indicate the ability of *B. linearis* to coexist asymptomatically with other needle pathogens in an individual host. As *B. linearis* causes damage to multiple white pine hosts (Darker,

1932), a cryptic lifestyle of this species could pose a greater threat to pine forests.

With climate change favoring the growth of needle pathogens, molecular tools for needle pathogen identification and diagnosis are promising for efficient monitoring of needle disease outbreaks. While more sophisticated tools are available for a few needle pathogens, the PCR-based markers from single and multi-copy gene regions developed from this study can help diagnose needle diseases caused by emerging non-model fungal pathogens such as *Lophodermella* spp. and *Bifusella linearis*. Further, the ability of the primers to detect these pathogens on asymptomatic needles at a given pathogen abundance can help predict the onset and enable early detection of needle cast. With the taxon-specific primers, our study also demonstrates the use of environmental DNA for the early detection and surveillance of latent needle pathogens. However, re-evaluation of primer assay performance from this study is necessary to include more geographically diverse specimens of target and closely related non-target species as these become available. Additionally, the cryptic lifestyles of some conifer needle pathogens in tree species, which may or may not be their known hosts, observed in this study warrant further investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Acknowledgements

The authors thank Dr. Jorge Ibarra Caballero of Colorado State University for his valuable insights in experimental and primer designs, and Drs. James Blodgett and Isabel Munck and Brent Oblinger of USDA Forest Service for providing samples for the primer assays. This project is funded through the USDA Forest Service Forest Health Protection and Special Technology Development Program (STDP-R2-2017-3). The authors are also grateful to the 1000 Fungal Genomes project, especially to the following principal investigators, for access to unpublished genome data: Dr. Richard Hamelin (*Elytroderma deformans*), Dr. Ryoko Oono (*Lophodermium nitens*), and Drs. Joseph Spatafora and Jon Magnuson (*Spathularia flavida, Pseudgraphis elatina*, *Coccomyces strobi, Bulgaria inquinans, Rutstroemia firma* and *Thelebolus microsporus*). Genome data was produced by the Joint Genome Institute, a Department of Energy (DOE) Office of Science User Facility supported by the Office of Science of the US DOE under Contract no. DE-AC02-5CH11231.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2022.106546.

References

Aglietti, C., Meinecke, C.D., Ghelardini, L., Barnes, I., van der Nest, A., Villari, C., 2021. Rapid detection of pine pathogens *Lecanosticta acicola*, *Dothistroma pini* and *D. septosporum* on needles by probe-based LAMP assays. Forests 12 (4), 479. https://doi.org/10.3390/f12040479.

Ata, J.P., Burns, K.S., Marchetti, S., Munck, I.A., Beenken, L., Worrall, J.J., Stewart, J.E., 2021. Molecular characterization and phylogenetic analyses of *Lophodermella* needle pathogens (Rhytismataceae) on *Pinus* species in the USA and Europe. PeerJ 9, e11435. https://doi.org/10.7717/peerj.11435.

Ata, J.P., Ibarra Caballero, J.R., Abdo, Z., Mondo, S.J., Stewart, J.E., 2022. Transitions of foliar mycobiota community and transcriptome in response to pathogenic conifer needle interactions. Sci. Rep. 12 (1), 7832. https://doi.org/10.1038/s41598-022-11907-0.

Barnes, I., Cortinas, M.N., Wingfield, M.J., Wingfield, B.D., 2008. Microsatellite markers for the red band needle blight pathogen, Dothistroma septosporum. Mol. Ecol. Res. 8 (5), 1026–1029. https://doi.org/10.1111/j.1755-0998.2008.02142.x.

Bass, D., Stentiford, G.D., Wang, H.-C., Koskella, B., Tyler, C.R., 2019. The Pathobiome in animal and plant diseases. Trends Ecol. Evol. 34 (11), 996–1008. https://doi.org/10.1016/j.tree.2019.07.012.

Broders, K., Munck, I., Wyka, S., Iriarte, G., Beaudoin, E., 2015. Characterization of fungal pathogens associated with white pine needle damage (WPND) in northeastern North America. Forests 6 (12), 4088–4104. https://doi.org/10.3390/f6114088.

Crous, P.W., Groenewald, J.Z., Slippers, B., Wingfield, M.J., 2016. Global food and fibre security threatened by current inefficiencies in fungal identification. Philos. Trans. Royal Soc. B: Biol. Sci. 371 (1709), 20160024. https://doi.org/10.1098/rstb.2016.0024.

Cubero, O.F., Crespo, A., Fatehi, J., Bridge, P.D., 1999. DNA extraction and PCR amplification method suitable for fresh, herbarium-stored, lichenized, and other fungi. Plant Syst. Evol. 216 (3–4), 243–249. https://doi.org/10.1007/BF01084401.

Darker, G., 1932. The Hypodermataceae of Conifers, Vol. 1. Contributions from the Arnold Arboretum of Harvard University.

Deckert, R.J., Hsiang, T., Peterson, R.L., 2002. Genetic relationships of endophytic *Lophodermium nitens* isolates from needles of *Pinus strobus*. Mycol. Res. 106 (3), 305–313. https://doi.org/10.1017/S0953756201005494.

Del Frari, G., Cabral, A., Nascimento, T., Boavida Ferreira, R., Oliveira, H., 2019. Epicoccum layuense a potential biological control agent of esca-associated fungi in grapevine. PLoS One 14 (3), e0213273. https://doi.org/10.1371/journal.pone.0213273.

Dick, M.A., Williams, N.M., Bader, M.K.-F., Gardner, J.F., Bulman, L.S., 2014. Pathogenicity of *Phytophthora pluvialis* to *Pinus radiata* and its relation with red needle cast disease in New Zealand. N. Z. J. For. Sci. 44 (1), 6. https://doi.org/10.1186/s40490-014-0006-7.

Durán, A., Gryzenhout, M., Slippers, B., Ahumada, R., Rotella, A., Flores, F., Wingfield, B. D., Wingfield, M.J., 2008. *Phytophthora pinifolia* sp. nov. associated with a serious needle disease of *Pinus radiata* in Chile. Plant Pathol. 57 (4), 715–727. https://doi.org/10.1111/j.1365-3059.2008.01893.x.

EFSA Panel on Plant Health, 2013. Scientific Opinion on the risk to plant health posed by *Dothistroma septosporum* (Dorog.) M. Morelet (*Mycosphaerella pini* E. Rostrup, syn. *Scirrhia pini*) and *Dothistroma pini* Hulbary to the EU territory with the identification and evaluation of risk reduction options. EFSA J. 11 (1), *3026*. https://doi.org/10.2903/j.efsa.2013.3026.

Ganley, R.J., Brunsfeld, S.J., Newcombe, G., 2004. A community of unknown, endophytic fungi in western white pine. Proc. Natl. Acad. Sci. 101 (27), 10107–10112. https://doi.org/10.1073/pnas.0401513101.

Gray, L.K., Russell, J.H., Yanchuk, A.D., Hawkins, B.J., 2013. Predicting the risk of cedar leaf blight (*Didymascella thujina*) in British Columbia under future climate change. Agric. For. Meteorol. 180, 152–163. https://doi.org/10.1016/j.agrformet.2013.04.023.

Grigoriev, I.V., Nikitin, R., Haridas, S., Kuo, A., Ohm, R., Otillar, R., Riley, R., Salamov, A., Zhao, X., Korzeniewski, F., Smirnova, T., Nordberg, H., Dubchak, I., Shabalov, I., 2014. MycoCosm portal: gearing up for 1000 fungal genomes. Nucleic Acids Res. 42 (D1), D699–D704. https://doi.org/10.1093/nar/gkt1183.

Guo, L.-D., Xu, L., Zheng, W.-H., Hyde, K.D., 2004. Genetic variation of Alternaria alternata, an endophytic fungus isolated from *Pinus tabulaeformis* as determined by random amplified microsatellites (RAMS). Fungal Divers. 16, 53–65.

Gupta, R., 1995. Evolution of chaperonin families (Hsp60, Hsp10 and Tcp-1) of proteins and the origin of eukaryotic cells. Mol. Microbiol. 15 (1), 1–11.

Janoušek, J., Krumböck, S., Kirisits, T., Bradshaw, R.E., Barnes, I., Jankovský, L., Stauffer, C., 2014. Development of microsatellite and mating type markers for the pine needle pathogen *Lecanosticta acicola*. Australas. Plant Pathol. 43 (2), 161–165. https://doi.org/10.1007/s13313-013-0256-5.

Jansons, Ā., Zeltiņš, P., Donis, J., Neimane, U., 2020. Long-term effect of lophodermium needle cast on the growth of scots pine and implications for financial outcomes. Forests 11 (7), 718. https://doi.org/10.3390/f11070718.

Jurado, M., Vázquez, C., Marín, S., Sanchis, V., Teresa González-Jaén, M., 2006. PCR-based strategy to detect contamination with mycotoxigenic *fusarium* species in maize. Syst. Appl. Microbiol. 29 (8), 681–689. https://doi.org/10.1016/j.syapm.2006.01.014.

Kalle, E., Kubista, M., Rensing, C., 2014. Multi-template polymerase chain reaction. Biomol. Detect. Quant. 2, 11–29. https://doi.org/10.1016/j.bdq.2014.11.002.

Kulik, T., Bilska, K., Żelechowski, M., 2020. Promising perspectives for detection, identification, and quantification of plant pathogenic fungi and oomycetes through targeting mitochondrial DNA. Int. J. Mol. Sci. 21 (7), 2645. https://doi.org/10.3390/ijms21072645.

Magan, N., Smith, M.K., 1996. Isolation of the endophytes *Lophodermium piceae* and Rhizosphaera kalkhoffii from Sitka spruce needles in poor and good growth sites and in vitro effects of environmental factors. Phyton 36 (3), 103–110.

Merrill, W., Wenner, N.G., Dreisbach, T.A., 1996. *Canavirgella banfieldii* gen. and sp. nov.: a needle-cast fungus on pine. Can. J. Bot. 74, 1476–1481.

Millar, C.S., 1984. *Lophodermella* species on pines. *In: Recent Research on Conifer Needle Diseases. Eds. Glenn W. Peterson.* USDA Forest Service, General Technical Report GTR-WO, 50, pp. 45–55.

Minter, D.W., Millar, C.S., 1993. IMI descriptions of fungi and bacteria. Lophodermella arcuata. Mycopathologia 121 (1), 49–50.

Myrholm, C.L., Tomm, B.D., Heinzelmann, R., Feau, N., Hamelin, R.C., McDougal, R., Winkworth, R.C., Ramsfield, T.D., 2021. Development of a rapid loop-mediated isothermal amplification assay for the detection of *Dothistroma septosporum*. Forests 12 (3), 362. https://doi.org/10.3390/f12030362.

Précigout, P.-A., Claessen, D., Makowski, D., Robert, C., 2020. Does the latent period of leaf fungal pathogens reflect their trophic type? A meta-analysis of biotrophs, hemibiotrophs, and necrotrophs. Phytopathology 110 (2), 345–361. https://doi.org/10.1094/PHYTO-04-19-0144-R.

Rajala, T., Velmala, S.M., Tuomivirta, T., Haapanen, M., Müller, M., Pennanen, T., 2013. Endophyte communities vary in the needles of Norway spruce clones. Fungal Biol. 117 (3), 182–190. https://doi.org/10.1016/j.funbio.2013.01.006.

Ranford, J.C., 2002. Chaperonins in disease: mechanisms, models, and treatments. Mol. Pathol. 55 (4), 209–213. https://doi.org/10.1136/mp.55.4.209.

Resource Coordinators, N.C.B.I., Agarwala, R., Barrett, T., Beck, J., Benson, D.A., Bollin, C., Bolton, E., Bourexis, D., Brister, J.R., Bryant, S.H., Canese, K., Cavanaugh, M., Charowhas, C., Clark, K., Dondoshansky, I., Feolo, M., Fitzpatrick, L., Funk, K., Geer, L.Y., Zbicz, K., 2018. Database resources of the National Center for biotechnology information. Nucleic Acids Res. 46 (D1), D8–D13. https://doi.org/10.1093/nar/gkx1095.

Ridout, M., Newcombe, G., 2018. *Sydowia polyspora* is both a foliar endophyte and a preemergent seed pathogen in *Pinus ponderosa*. Plant Dis. 102 (3), 640–644. https://doi.org/10.1094/PDIS-07-17-1074-RE.

Rocky Mountain Region, Forest Health Protection, 2010. Field Guide to Diseases & Insects of the Rocky Mountain Region (Gen. Tech. Rep. RMRS-GTR-241). Department of Agriculture, Forest Service, Rocky Mountain Research Station.

Rodas, C.A., Wingfield, M.J., Granados, G.M., Barnes, I., 2016. Dothistroma needle blight: an emerging epidemic caused by *Dothistroma septosporum* in Colombia. Plant Pathol. 65 (1), 53–63. https://doi.org/10.1111/ppa.12389.

Salas-Lizana, R., Oono, R., 2018. A comparative analysis of *Lophodermium fissuratum*, sp. nov., found in haploxylon pine needles in the Pacific Northwest, and other *Lophodermium* endophytes. Mycologia 110 (5), 797–810. https://doi.org/10.1080/00275514.2018.1499991.

Salvioli, A., Lumini, E., Anca, I.A., Bianciotto, V., Bonfante, P., 2008. Simultaneous detection and quantification of the unculturable microbe *Candidatus Glomeribacter gigasporarum* inside its fungal host *Gigaspora margarita*. New Phytol. 180 (1), 248–257. https://doi.org/10.1111/j.1469-8137.2008.02541.x.

Shetty, N.P., Mehrabi, R., Lütken, H., Haldrup, A., Kema, G.H.J., Collinge, D.B., Jørgensen, H.J.L., 2007. Role of hydrogen peroxide during the interaction between the hemibiotrophic fungal pathogen *Septoria tritici* and wheat. New Phytol. 174 (3), 637–647. https://doi.org/10.1111/j.1469-8137.2007.02026.x.

Sieber, T.N., 2007. Endophytic fungi in forest trees: are they mutualists? Fungal Biol. Rev. 21 (2–3), 75–89. https://doi.org/10.1016/j.fbr.2007.05.004.

Sieber, T.N., Rys, J., Holdenrieder, O., 1999. Mycobiota in symptomless needles of *Pinus mugo* ssp. *uncinata*. Mycol. Res. 103 (3), 306–310. https://doi.org/10.1017/S0953756298007229.

Siziba, V.I., Wingfield, M.J., Sadiković, D., Mullett, M.S., Piškur, B., Barnes, I., 2016. Development of microsatellite markers for the pine needle blight pathogen, Dothistroma pini. For. Pathol. 46 (5), 497–506. https://doi.org/10.1111/efp.12282.

Soltani, J., Hosseyni Moghaddam, M.S., 2015. Fungal endophyte diversity and bioactivity in the mediterranean cypress *Cupressus sempervirens*. Curr. Microbiol. 70 (4), 580–586. https://doi.org/10.1007/s00284-014-0753-y.

Steele, R., 1990. Pinus flexilis James. In: Silvics of North America, Vol. 1. U.S. Department of Agriculture, pp. 348–354.

Stoldt, V., Rademacher, F., Kehren, V., Ernst, J.M., Pearce, D.A., Sherman, F., 1996. Review: the Cct eukaryotic chaperonin subunits of *Saccharomyces cerevisiae* and other yeasts. Yeast 12, 523–529.

Stone, J.K., Polishook, J.D., White, J.F., 2004. Endophytic fungi. In: Biodiversity of Fungi. Elsevier, pp. 241–270. https://doi.org/10.1016/B978-012509551-8/50015-5.

Suyama, M., Torrents, D., Bork, P., 2006. PAL2NAL: Robust conversion of protein sequence alignments into the corresponding codon alignments. Nucleic Acids Res. 34, W609–W612. https://doi.org/10.1093/nar/gkl315.

Tanney, J.B., Seifert, K.A., 2017. *Lophodermium resinosum* sp. nov. from red pine (*Pinus resinosa*) in eastern Canada. Botany 95 (8), 773–784. https://doi.org/10.1139/cjb-2017-0012.

Tekpinar, A.D., Kalmer, A., 2019. Utility of various molecular markers in fungal identification and phylogeny. Nova Hedwigia 109 (1–2), 187–224.

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B.C., Remm, M., Rozen, S. G., 2012. Primer3—new capabilities and interfaces. Nucleic Acids Res. 40 (15), e115. https://doi.org/10.1093/nar/gks596.

Welsh, C., Lewis, K.J., Woods, A.J., 2014. Regional outbreak dynamics of Dothistroma needle blight linked to weather patterns in British Columbia, Canada. Can. J. For. Res. 44 (3), 212–219. https://doi.org/10.1139/cjfr-2013-0387.

White, T.J., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.), PCR Protocols: A Guide to METHODS and applications. Academic Press Inc, pp. 315–322.

Worrall, J.J., Marchetti, S.B., Mask, R.A., 2012. An Epidemic of Needle Cast on Lodgepole Pine in Colorado. In: In Biological Evaluation R2–12-01 (p. 16). USDA Forest Service, Rocky Mountain Region, Forest Health Protection.

Wyka, S.A., Munck, I.A., Brazee, N.J., Broders, K.D., 2018. Response of eastern white pine and associated foliar, blister rust, canker and root rot pathogens to climate change. For. Ecol. Manag. 423, 18–26. https://doi.org/10.1016/j.foreco.2018.03.011.