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Article

Characterization of Fungal Pathogens Associated with White Pine Needle Damage (WPND) in Northeastern North America

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Abstract: Eastern white pine is a crucial ecological and economic component of forests in the northern USA and eastern Canada, and is now facing an emerging problem in white pine needle damage (WPND). It is still unclear whether WPND results from one, or the combination of several fungal pathogens. Therefore, the first objective of this study was to characterize the fungi associated with WPND in the northeastern United States and document the damage being done to mature eastern white pine as a result of repeated defoliation. To date, 22 species of fungi, either cultured from diseased pine needles or formed fruiting bodies on pine needles were identified based on morphology and sequence data. *Lecanosticta acicola* and a putative new species of *Septorioides* were the species most frequently recovered from diseased needles, in addition to needle cast fungi *Lophophacidium dooksii* and *Bifusella linearis*, two obligate fungal pathogens that were frequently observed on pine needles in the northeast, but have not been known to cause excessive defoliation of eastern white pine. A second objective was to monitor yearly the health of 63 pairs of healthy and unhealthy trees in eight affected locations throughout New England. Since 2012, affected trees are increasingly and repeatedly chlorotic and defoliated every year. Trees that were initially healthy are now exhibiting symptoms. While *L. acicola* appears to be the primary pathogen causing WPND, several other common needle pathogens are being more frequently observed and the role of climate change may be important in the disease ecology of WPND. These defoliation events, while once a sporadic occurrence, have now become

more frequent as observed in continued crown deterioration of eastern white pine in long-term monitoring plots followed during the course of this three-year study.

Keywords: brown spot needle blight; needle cast; emerging disease; eastern white pine; defoliation

1. Introduction

In recent years, greater frequency and severity of biotic attacks and climate extremes—as well as interactions between biotic and climate stressors—have contributed to increased tree mortality rates worldwide [1,2] At the whole tree level, defoliation by pathogens and environmental stress can limit a tree's ability to take up water and nutrients, which can affect forest productivity and carbon stocks [3,4]. Eastern white pine (*Pinus strobus*) is a crucial ecological and economic component of forests in the northern USA and eastern Canada, and is now facing an emerging problem in white pine needle damage (WPND) a new disorder of eastern white pine that is likely due to one or more fungal pathogens.

During the summer of 2010, WPND was observed frequently throughout New England generating much public concern. Symptoms consisted of yellow and brown discoloration of one-year old needles, and affected needles dropped causing tree crowns to look thin a year after initial infection. Needles of both mature trees and regeneration were damaged. Preliminary diagnosis of this foliar damage has been attributed to frost and two foliar diseases, brown spot needle blight caused by the fungus *Lecanosticta acicola* (formerly known as *Mycosphaerella dearnessii*) [5] and *Canavirgella* needle cast caused by *C. banfieldii*, recently determined to be the same species as *Lophophacidium dooksii* [6] and herein referred to as *L. dooksii* as that name has precedence. Diagnosing the damage agent is difficult because both fungi cause similar symptoms, although they can be differentiated by their sexual and asexual fruiting bodies produced at different times in the growing season. The sexual fruiting structures of *L. dooksii* are produced through the winter and are visible earlier in the spring, whereas *L. acicola* pycnidia fruits in June [7]. Consequently, *L. dooksii* hysterothecia could be present in infected needles by April and fruiting bodies of both *L. dooksii* and *L. acicola* could be present by June. In addition, fruiting bodies of other fungi including *Bifusella linearis* and several undetermined species have been observed on diseased needles.

Both *L. dooksi* and *B. linearis* are known to cause needle cast diseases on eastern white pine in the northeast, but neither pathogen has been reported to cause the periodic large scale defoliation that has occurred over the last decade. While, brown spot caused by *L. acicola* has been frequently associated with WPND damage, this fungus has not historically been associated with disease on mature white pines in the northeast. Brown spot has been a persistent problem in long leaf pine plantations in the southeastern U.S. and scotch pine in Minnesota and Wisconsin [8,9], but severe defoliation of eastern white pine by *L. acicola* in the northeast was not reported prior to 2005 [10]. It is possible that a change in temperature and precipitation patterns over the past decade in the northeast have contributed to a potential range shift as well as contributed to repeated defoliation events by *L. acicola* and other pathogens in the northern forest. However, to date there is little data to substantiate this claim, but a

more thorough investigation of the fungi contributing to the defoliation event would be a positive first step in developing future management strategies.

The importance of this new disease has been observed in the substantial increase in the number of reports of WPND in the last four years as evidenced by the Forest Health Monitoring reports from Maine, New Hampshire, Massachusetts, and Vermont [11]. In addition, a recent forest service pest alert, "Eastern White Pine Needle Damage", outlined the importance of this emerging problem on white pine in the northeast [10] and its potential to be a problem in all regions of the United States and Canada within the range of eastern white pine. White pine foliar damage was mapped during 2010 aerial forest health detection surveys in New England. In Maine alone 24,328 hectares were reported damaged (Figure 1). However, several fungi and frost were associated with the foliar damage, thus coding the damage consistently during the aerial surveys was challenging. There was a need to understand the extent of the damage that could be directly related to foliar pathogens. Consequently, the first objective of this study was to identify the fungi commonly associated with the symptoms of white pine needle damage in the northeastern U.S., and the second objective, was to monitor the health of white pines affected by WPND over time.

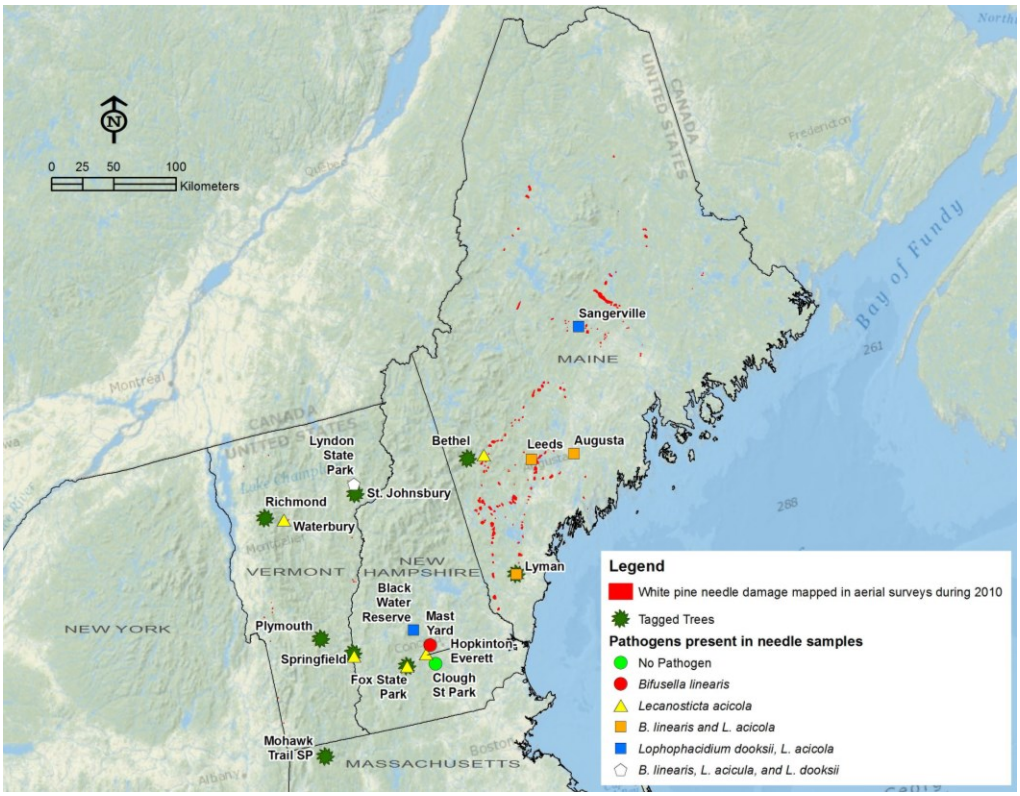

Figure 1. Map of white pine foliar damage detected during aerial detection surveys (**red**), long-term monitoring sites to evaluate repeated defoliation (**dark green**), and 13 sites sampled by State Forest Health Cooperators from Maine, New Hampshire, and Vermont during 2011 and the presence of *Bifusella linearis*, *Lophophacidium dooksii*, and *Lecanosticta acicola* at each of those locations.

2. Methods

Isolate collection. Forest Health State Cooperators from Maine, New Hampshire, and Vermont collected samples from at least three to five white pine stands per State that exhibited damage during 2011, along with stand information. These 13 forest units (stands) were located throughout New England (43.10° to 45.17° N, 69.36° to 72.77° W) (Figure 1). Most of these were natural stands located in wetland areas, but trees on dry steep slopes in one plantation in Vermont were also sampled. As multiple pathogens were associated with the damage, sexual and asexual reproductive structures would fruit at different times, stands were sampled between 25 April and 2 May and again during 13–22 June 2011. When available, samples were also collected from one healthy control tree. Each sample consisted of a quart-size (1 L) bag full of branch tips. Samples were taken from a total of 60 trees in all age classes. In May 2011, 729 branch tips and 1153 incubated needles were examined. Similarly, in June 2011, 384 branch tips and 901 incubated needles were examined. Additional samples from another eight sites were sent in by Forest Health State Cooperators during 2011 and 2012 in response to sporadic defoliation events in the region.

Samples were sent to the U.S. Forest Service Durham Field Office where they were processed for pathogen identification. All branch tips were visually examined for fungal fruiting structures. Disease incidence was recorded based on presence or absence of disease symptoms. *Bifusella linearis* was identified by Mary Inman; diagnostician for the Connecticut Agricultural Experiment Station. Twenty needles from one representative branch tip per tree were placed in a moist chamber; incubated at 25 °C for 24 to 72 h; and then examined with the aid of dissecting and light microscopes. To enable sporulation, moist chambers consisted of Petri plates with filter papers moistened with deionized water sealed with Parafilm. In order to observe the pathogens in culture as well as extract DNA from mycelia; pure cultures of each isolate were obtained. To do this; three to five symptomatic needles from each tree were surfaced sterilized in a 10% bleach solution for one minute; rinsed three times with de-ionized H_2O; and allowed to dry in a fume hood. Small segments (1 cm) of dry needles were either plated directly onto potato dextrose agar (PDA) or incubated in a humid chamber with 500 μL of sterile H_2O for two days; to enhance sporulation followed by transfer of spores to PDA and incubated at room temperature with ambient light (Difco™). Once pure cultures were established; isolates were transferred onto PDA plates overlaid with a Cellophane membrane (Flexel Sales Inc., Atlanta, GA, USA); and grown for one to two weeks. Under aseptic conditions mycelia were scrapped off and placed inside 2 mL microcentrifuge tubes (VWR); frozen for 1 h at −80 °C; and freeze dried for two days. Genomic DNA extraction from dried mycelium followed the modified cetyltrimethyl ammonium bromide (CTAB) method. DNA was then used for molecular identification described below.

In addition to morphological identification a subset of isolates were identified based on the sequence of their internal transcribed spacer (ITS) regions 1 and 2, including the 5.8s rDNA. Once isolates were in pure culture, they were organized based on morphology and growth characteristics in culture. Representative samples from each morphotype at each location were included for DNA extraction and molecular identification. DNA was extracted from mycelia as described above and stored at −20 °C. PCR was performed in 30 μL reaction volume containing 6 μL of 5× Green GoTaq reaction buffer (Promega Corp., San Luis Obisbo, CA, USA), 2.4 μL of 25 mM $MgCl_2$, 0.75 μL containing 10 mM each dNTP, 0.2 units of GoTaq *Taq* DNA polymerase, 0.9 μL of each primer ITS1 and ITS4 [12] at 10 pmol·μL^{-1},

2 μL of 15 ng/μL DNA and 16.85 μL of sterile distilled water. The PCR amplification conditions were initial denaturation at 94 °C for 2 min, followed by 35 cycles of 30 s at 94 °C, 30 s at 54 °C, and 30 s at 72 °C, with a final 5 min at 72 °C. Amplified products were purified using Qiaquick spin columns (Qiagen Inc., Gaithersburg, MD, USA). DNA was quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Amplified products were sequenced by including 14 ng of purified DNA with the BigDye version 3.1 Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) on an ABI 3730 DNA Analyzer (Applied BioSystems) at the University of New Hampshire Hubbard Center for Genome Studies.

Needles containing the fruiting structures of potential obligate fungi were washed in a 10% bleach solution for 1 min, triple rinsed with deionized water, placed in a sterile moist chamber, and incubated at 25 °C for 48 h. Fruiting structures were scrapped off the needle and directly placed into a sterile 2 mL tube with silica and two or three metal beads. Tubes were vortexed at max speed for 5 min and DNA extraction followed the Power Plant Pro DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA, USA). PCR was performed in the same manner as above.

2.1. Phylogenetic Analysis

Raw sequences were manually adjusted and edited using BioEdit and were deposited in GenBank as listed in Table 1. To infer species and outgroups, closely related species were imported from GenBank. When possible sequences were used only from vouchered cultures from either the Centraalbureau voor Schimmelcultures (CBS) fungal biodiversity center, or other published source. The phylogenetic and molecular analyses were completed using *MEGA* version 4.0 [13]. Alignments were completed using ClustalW and trees were inferred using the neighbor joining (NJ) method (Kimura two-parameter distance calculation) and maximum likelihood (ML) method. Gaps were treated as missing data with missing or ambiguous sites ignored for the affected pairwise comparison. All positions were included in the analyses and relative support for the branches was estimated with 1000 bootstrap replications [14] for NJ analysis. Analysis of polymorphic sites was completed using DnaSP [15].

Table 1. Cultured isolates or fruiting bodies of fungi recovered from symptomatic white pine needles in the northeastern United States.

Sample I.D.	Location	State	DNA [a] Source	Species	GenBank [b] Accession
EBJul30-10	Tolland	CT	Fruit body	*Hemiphacidium longisporum*	KT000188
EBJul30-11	Tolland	CT	Fruit body	*Hemiphacidium longisporum*	KT000189
WPF 111-12G	Augusta	ME	Mycelia	*Hendersonia pinicola*	KT000174
WPF 97-12G	Augusta	ME	Mycelia	*Lophodermium macci*	KT000171
WPF 180-12G	Augusta	ME	Mycelia	*Pyrenochaeta* spp.	KT000185
WPF 21-12A	Bethel	ME	Mycelia	*Allantophomopsiella pseudotsugae*	KT000147
WPF 19-12A	Bethel	ME	Mycelia	*Cladosporium*	KT000145
WPF 157-12G	Bethel	ME	Mycelia	*Hendersonia pinicola*	N/A
WPF 2-12A	Bethel	ME	Mycelia	*Lecanosticta acicola*	KT000153
WPF 32-12A	Bethel	ME	Mycelia	*Lecanosticta acicola*	N/A

Table 1. *Cont.*

Sample I.D.	Location	State	DNA [a] Source	Species	GenBank [b] Accession
WPF 33-12A	Bethel	ME	Mycelia	*Lecanosticta acicola*	N/A
WPF 4-12A	Bethel	ME	Mycelia	*Lecanosticta acicola*	KT000154
WPF 5-12A	Bethel	ME	Mycelia	*Lecanosticta acicola*	KT000155
WPF 9-12A	Bethel	ME	Mycelia	*Lecanosticta acicola*	KT000134
WPF 28-12A	Bethel	ME	Mycelia	*Phaeosphaeria pontiformis*	KT000144
WPF 26-12A	Bethel	ME	Mycelia	*Septorioides* sp. nov.	KT000142
WPF 15-12A	Bethel	ME	Mycelia	*Sydowia polyspora*	KT000149
WPF 17-12A	Bethel	ME	Mycelia	*Sydowia polyspora*	KT000156
WPF 23-12A	Bethel	ME	Mycelia	*Sydowia polyspora*	KT000151
WPF 69-12G	Blackwater	ME	Mycelia	*Septorioides* sp. nov.	N/A
1528	Leeds	ME	Mycelia	*Lecanosticta acicola*	KT007127
1666	Leeds	ME	Mycelia	*Lecanosticta acicola*	KT007128
WPF 37-12G	Leeds	ME	Mycelia	*Lecanosticta acicola*	N/A
WPF 56-12A	Leeds	ME	Mycelia	*Lecanosticta acicola*	KT000166
WPF 44-12G	Leeds	ME	Mycelia	*Lecanosticta acicola*	N/A
WPF 51-12G	Leeds	ME	Mycelia	*Lophodermium nitens*	KT000163
WPF 54-12G	Leeds	ME	Mycelia	*Pestalotiopsis jesteri*	KT000164
WPF 55-12G	Leeds	ME	Mycelia	*Pestalotiopsis jesteri*	KT000165
WPF 120-12G	Leeds	ME	Mycelia	*Phaeomoniella* sp.	KT000175
WPF 48-12G	Leeds	ME	Mycelia	*Phialocephala fluminis*	KT000162
WPF 42-12G	Leeds	ME	Mycelia	*Septorioides* sp. nov.	KT000161
WPF 46-12G	Leeds	ME	Mycelia	*Septorioides* sp. nov.	KT000138
WPF 103-12G	Lyman	ME	Mycelia	*Fungal endophyte*	KT000172
WPF 93-12G	Lyman	ME	Mycelia	*Hendersonia pinicola*	N/A
1671	Lyman	ME	Mycelia	*Lecanosticta acicola*	KT007129
WPF 102-12G	Lyman	ME	Mycelia	*Septorioides* sp. nov.	N/A
WPF 106-12G	Lyman	ME	Mycelia	*Septorioides* sp. nov.	KT000173
WPF 158-12G	Lyman	ME	Mycelia	*Septorioides* sp. nov.	N/A
WPF 90-12G	Lyman	ME	Mycelia	*Septorioides* sp. nov.	KT000170
WPF 151-12G	Sangerville	ME	Mycelia	*Fungal endophyte*	KT000179
WPF 152-12G	Sangerville	ME	Mycelia	*Hendersonia pinicola*	N/A
WPF 85-12G	Sangerville	ME	Mycelia	*Hendersonia pinicola*	KT000169
WPF 86-12G	Sangerville	ME	Mycelia	*Hendersonia pinicola*	N/A
WPF 87-12G	Sangerville	ME	Mycelia	*Hendersonia pinicola*	N/A
1675	Sangerville	ME	Mycelia	*Lecanosticta acicola*	KT007130
WPF 14-12A	Sangerville	ME	Mycelia	*Paraconiothyrium* spp.	KT000176
WPF 150-12G	Sangerville	ME	Mycelia	*Rhizosphaera kalkhoffii*	KT000178
WPF 121-12G	Sangerville	ME	Mycelia	*Septorioides* sp. nov.	KT000140
WPF 147-12G	Sangerville	ME	Mycelia	*Septorioides* sp. nov.	KT000139
WPF 149-12G	Sangerville	ME	Mycelia	*Septorioides* sp. nov.	KT000135
WPF 18-12A	Sangerville	ME	Mycelia	*Septorioides* sp. nov.	KT000157
WPF 22-12A	Sangerville	ME	Mycelia	*Sydowia polyspora*	KT000148
WPF 73-12G	Blackwater	NH	Mycelia	*Lecanosticta acicola*	KT000168

Table 1. *Cont.*

Sample I.D.	Location	State	DNA [a] Source	Species	GenBank [b] Accession
EBJul30-4	Gilmanton	NH	Fruit body	*Hendersonia pinicola*	KT000192
1678	Hillsboro	NH	Mycelia	*Lecanosticta acicola*	KT007131
WPF 75-12G	Hopkinton	NH	Mycelia	*Hendersonia pinicola*	N/A
WPF 21-12A	Hopkinton	NH	Mycelia	*Septorioides* sp. nov.	N/A
WPF 25-12A	Hopkinton	NH	Mycelia	*Septorioides* sp. nov.	KT000160
WPF 27-12A	Hopkinton	NH	Mycelia	*Septorioides* sp. nov.	KT000143
WPF 71-12G	Mast Yard	NH	Mycelia	*Dothiodiomycete*	KT000167
1679	Merrimack	NH	Mycelia	*Lecanosticta acicola*	KT007132
EBJul30-16	Monadnock	NH	Fruit body	*Hendersonia pinicola*	KT000191
EBJul30-2	New Hampton	NH	Fruit body	*Hemiphacidium longisporum*	KT000190
EBJul30-9	New Hampton	NH	Fruit body	*Lophophacidium dooksii*	KT000187
EBJul30-15	Sanbornton	NH	Fruit body	*Bifusella linearis*	KT000195
EBJul30-5	Scituate	RI	Fruit body	*Bifusella linearis*	KT000193
WPF 16-12A	Bethel	VT	Mycelia	*Sydowia polyspora*	KT000150
1681	Brookfield	VT	Mycelia	*Lecanosticta acicola*	KT007133
EBJul30-6	Plymouth	VT	Mycelia	*Bifusella linearis*	KT000194
EBJul30-1	Plymouth	VT	Mycelia	*Lophophacidium dooksii*	KT000186
V-3-4	Richmond	VT	Mycelia	*Septorioides* sp. nov.	KT000137
WPF 172-12G	Springfield	VT	Mycelia	*Fungal endophyte*	KT000183
WPF 165-12G	Springfield	VT	Mycelia	*Septorioides* sp. nov.	KT000180
WPF 167-12G	Springfield	VT	Mycelia	*Septorioides* sp. nov.	KT000181
WPF 175-12G	Springfield	VT	Mycelia	*Septorioides* sp. nov.	KT000184
WPF 170-12G	Springfield	VT	Mycelia	*Sydowia polyspora*	KT000182
WPF 176-12G	Springfield	VT	Mycelia	*Sydowia polyspora*	N/A
WPF 174-12G	Springfield	VT	Mycelia	*Hendersonia pinicola*	N/A
V-5-2	Underhill	VT	Mycelia	*Septorioides* sp. nov.	KT000136
V-1-4	Waterbury	VT	Mycelia	*Hendersonia pinicola*	KT000152
1506	Waterbury	VT	Mycelia	*Lecanosticta acicola*	KT007125
1518	Waterbury	VT	Mycelia	*Lecanosticta acicola*	KT007126
WPF 144-12G	Waterbury	VT	Mycelia	*Pestalotiopsis uvicola*	KT000177
WPF 20-12A	Waterbury	VT	Mycelia	*Septorioides* sp. nov.	KT000146
WPF 24-12A	Waterbury	VT	Mycelia	*Septorioides* sp. nov.	KT000141
V-1-9	Waterbury	VT	Mycelia	*Discostroma fuscellum*	KT000158

[a] Indicates whether DNA was extracted from mycelia grown in culture or direction from a fruiting structure on an symptomatic white pine needle; [b] Those isolates identified by morphology, but for which the internal transcribed spacer (ITS) regions was not sequenced are labeled as N/A.

2.2. Long-Term White Pine Health Monitoring

Eight long-term monitoring plots have been established throughout New England in sites where pines were maintaining adequate growth and healthy crowns prior to the disease outbreak within four different frost hardiness zones to determine the long-term effects of repeated defoliations on affected trees (Figure 1). At each of the eight long-term monitoring plots, pairs of healthy and unhealthy trees were

tagged during 2012 and their crown condition has been monitored yearly by U.S. Forest Service and State Forest Health Cooperators. It was not possible to find completely unaffected trees in affected sites; thus trees with the greenest and fullest crowns were chosen as the healthy controls. At each site, pairs of healthy and unhealthy trees were tagged at the base of their stems. The following variables were collected for each tagged tree: latitude and longitude coordinates, diameter at breast height (1.3 m from the ground), overall health rating, and chlorosis and defoliation severity rating. Tree crowns were visually inspected to determine disease severity defined as the proportion of the crown with chlorosis or defoliation as follows: crown not affected, less than a third of the crown affected, one-third to two-thirds of the crown affected, or more than two-thirds of the crown affected. The location of the damage was also recorded: bottom, middle, or top of the crown. To establish the effect of "year" and "initial health status" on WPND severity, data were analyzed using a linear mixed model (PROC GLIMMIX) in SAS (SAS Institute Inc., version 9.4, 2012, Cary, NC, USA). "Year" and "initial health status" were fixed effects and "site" was a random factor, and the response variables were either "chlorosis rating per tree" or "defoliation rating per tree".

3. Results

3.1. Morphologic Diversity of Fungi Associated with WPND

A total of 13 forest units (stands) were sampled throughout northern New England during 2011 in addition to receiving samples from another eight sites in 2011 and 2012, and samples were taken from a total of 60 trees in all age classes. In May 2011, 11 of the 60 trees were asymptomatic and considered to be controls. By June 2011, only nine of these trees were asymptomatic. Needles from two of these trees had *L. acicola* fruiting bodies. The needles of the remaining six apparently initially asymptomatic trees did not yield signs or symptoms of foliar pathogens. In May 2011, 729 branch tips and 1153 incubated needles were examined. Of the 1153 needles collected and incubated in May, 6% had *B. linearis* ascomata, 4% had *L. acicola* conidiomata, and 6% had *L. dooksii* ascomata. Similarly, in June 2011, 384 branch tips and 901 incubated needles were examined. Of the 901 needles collected and incubated in June, 2% had *B. linearis* ascomata, 22% had *L. acicola* conidiomata, and 1% had *L. dooksii* ascomata.

Signs and symptoms of *L. dooksii*, *B. linearis*, and *L. acicola* (Figure 2) were the most frequently observed pathogens. Both *B. linearis* and *L. dooksii* produce elongated (4–22 mm), ascomata (Figure 2A,B) [7,16]. The ascomata of *B. linearis* are shiny and black (Figure 2A), whereas *L. dooksi* ascomata are grey and embedded in the needle (Figure 2B) [16]. These two fungi can be distinguished by the shape of their hyaline ascospores. *Bifusella linearis* ascospores are constricted in the middle [17], whereas *L. dooksi* ascospores are oval [6,7]. *Lecanosticta acicola* produces acervular, erumpent, brown conidiomata (Figure 2C) and solitary, brown conidia that are straight to curved, and subcylindrical [18–20]. Several other fungi were found fruiting on needles, but these fungi were not associated with needle blight or needle cast symptoms and appeared to be secondary invaders.

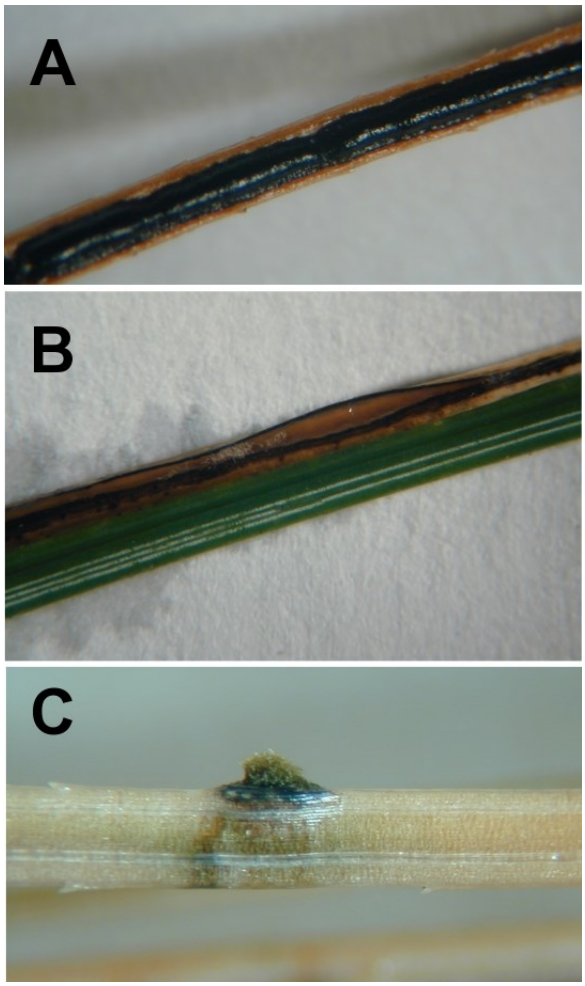

Figure 2. Disease symptoms and reproductive structures of (**A**) *Bifusella linearis*; (**B**) *Lophophacidium dooksii*; and (**C**) *Lecanosticta acicola* on eastern white pine.

Diagnostic *B. linearis* fruiting bodies were present in samples collected in April and June 2011 from the same five sites (Figure 1). Similarly, *L. dooksii* fruiting bodies were observed in samples collected in April and June from the same three sites. Both these needle cast fungi produce sexual fruiting structures that take a year to develop. In contrast, *L. acicola* was only found fruiting at four sites in April; however, by June, it was fruiting in samples from ten sites (Figure 1). Unlike the needle cast fungi, *L. acicola* produces asexual fruiting structures that result in more than one disease cycle through the growing season.

Samples were disease free from only one site, Clough State Park, which is in New Hampshire. Although needles from trees at Clough State Park were symptomatic, their needles did not yield fungal fruiting bodies when incubated suggesting that the symptoms observed at this site were potentially caused by abiotic agents such as frost. *Lecanosticta acicola* was the most widely distributed pathogen occurring in 14 of 21 sites, and was found alone or co-occurring with the needle cast fungi. All three pathogens were present in one tree at one site, Lyndon State Park in Vermont (Figure 1).

In April, fruiting structures of all three fungi were found in less than 20% of the trees sampled. By June, 48% of the trees yielded samples with signs of *L. acicola*. Between April and June, the proportion of trees with symptoms of chlorosis and defoliation increased from 25% to 50% and 0% to 45%, respectively (Figure 3). In April, 68% of the trees yielded samples with necrotic needles, although the necrosis was

limited to less than one-third of the needle. In contrast, the chlorosis of samples collected in June exceeded more than two-thirds of the needle. It is possible that the proportion of necrotic needles decreased due to the needle drop in June.

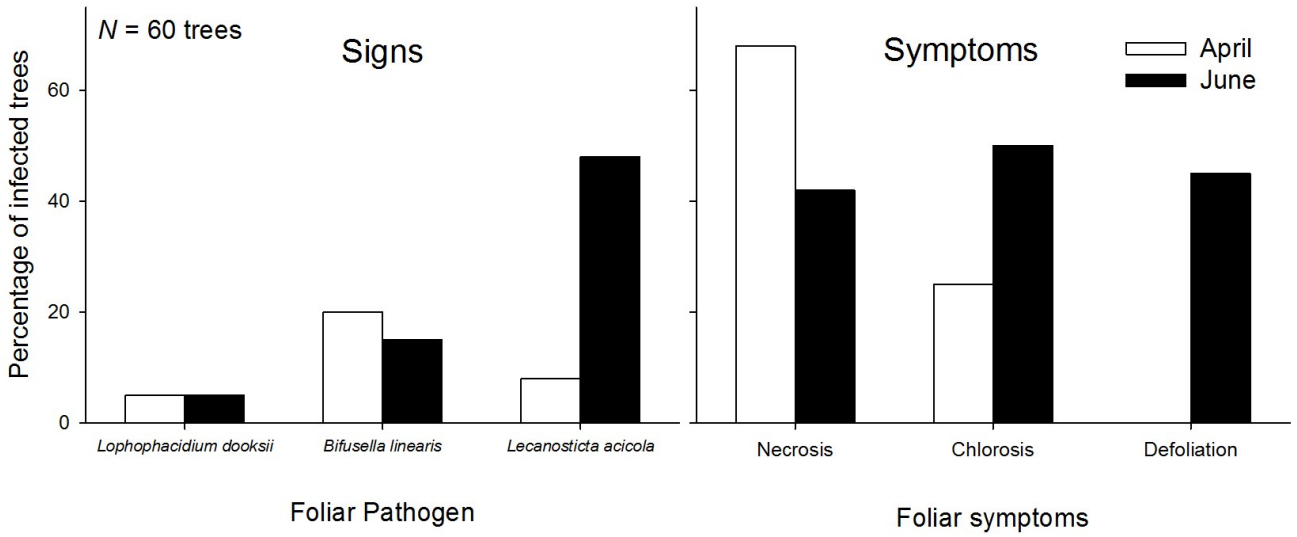

Figure 3. Frequency of foliar disease signs and symptoms on infected trees.

3.2. Genetic Diversity of Fungi Associated with WPND

A total of 186 isolates were recovered from infected needles and grown in pure cultures. Representative individuals from each of the morphological groups identified above were also identified to species based on the ITS sequence. In total 87 isolates from 22 locations across New England were sequenced. The primary focus of this study was to identify pathogenic fungi that are associated with diseased needles on trees suffering from WPND. However, in the process of isolating fungi from the needles several putative fungal endophytes were also identified. In addition to the expected pathogens *B. linearis*, *L. acicola*, and *L. dooksii*, several other known pine pathogens were identified in the course of this study, and for many of these pathogens this represents the first time the ITS region has been sequenced. As many of the species are obligate pathogens and have not been successfully cultured, DNA needed to be extracted directly from the fruiting bodies.

In total, 22 species representing 13 families were identified, of which 12 species represent either pathogens or weak parasites and 10 species represent saprophytes or endophytes based on phylogenetic similarity to known pathogens or saprophytes (Figure 4). The pathogens included the expected species of *B. linearis*, *L. acicola*, and *L. dooksii*, but also included several other known pathogens including *Rhizosphaeria kalkhoffii*, *Hemiphacidium longisporum*, and *Allantophomopsis cytisporea*. Other weak parasites and endophytes included *Discostroma fuscellum*, *Lophodermium macci*, *L. conigenum*, *L. nitens*, *Neostagonospora caricis*, *Pyrenochaeta cava*, *Paraconiothyrium* spp., *Phialocephala* sp., *Phaeomoniella* spp., *Sydowia polyspora*, and three species of *Pestiolopsis*.

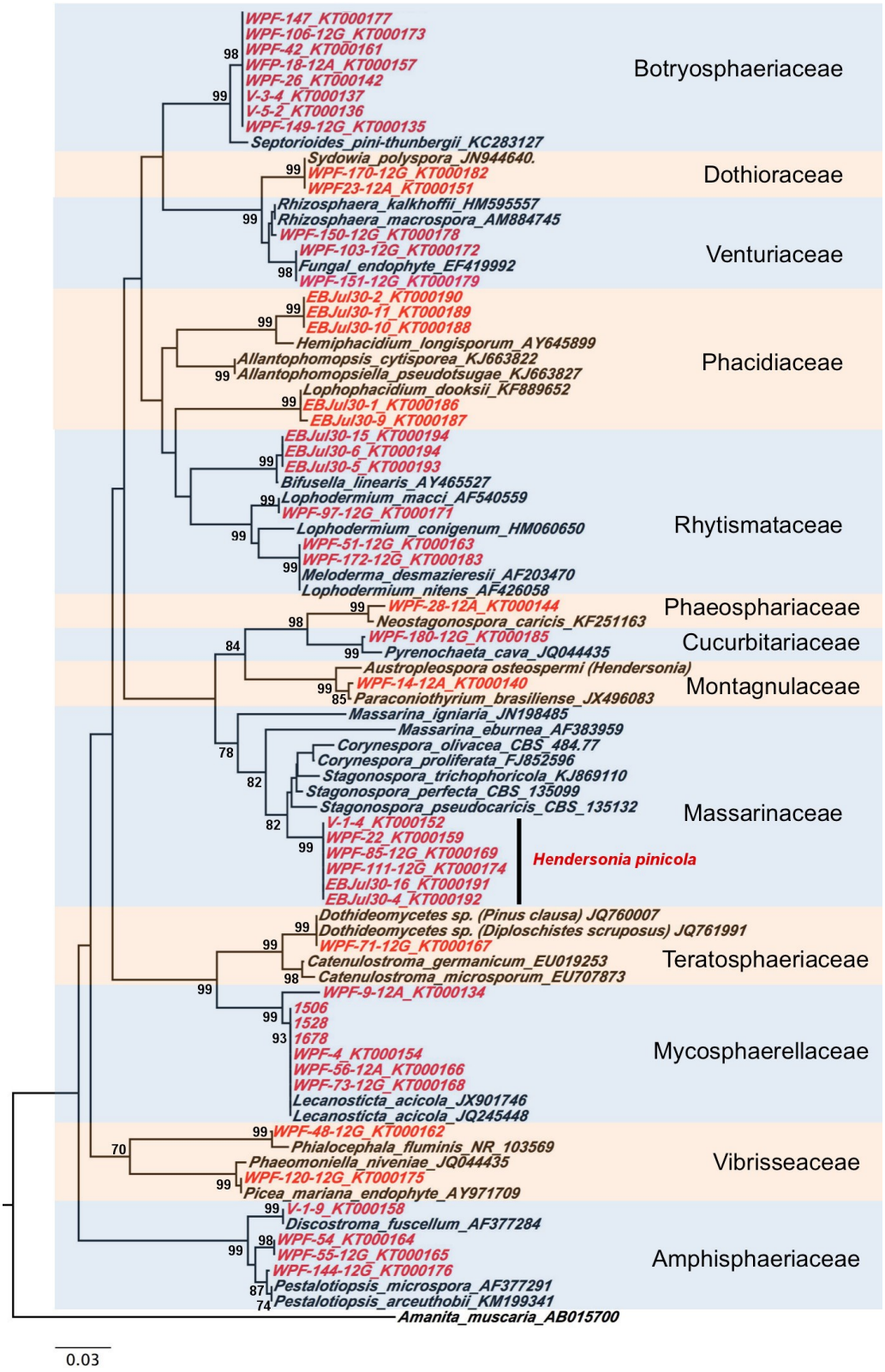

Figure 4. Neighbor-joining phylogenetic tree based on the sequence of the Internal Transcribed Spacer regions 1 and 2 of fungi isolated from symptomatic eastern white pine needles (red font) as well as closely related species imported from Genbank (black font). Isolates are followed by their respective Genbank accession numbers. Bootstrap values > 70% were included at the nodes.

Several of the isolates recovered in this study appear to be putative new species based on sequence similarity to known species (Figure 4). Of these the three that were recovered on more than one occasion include a species closely related to *Hemiphacidium longisporium*, a species closely related to *Septorioides pini-thunbergii*, and a species that is a member of the Massarinaceae and closely related to the genus *Stagonospora*. All of these appear to be either pathogens or weak parasites. In the case of the putative *Hemiphacidium* species, all three ITS sequences were generated from DNA that was extracted from the fruiting structure, and no cultures of this likely biotroph were recovered. The isolate belonging to the Massarinaceae, was morphologically determined to be *Hendersonia pinicola*, a well-known weak parasite and secondary colonizer of pine needles [20]. *Hendersonia* is an anamorphic genus that appears to by polyphyletic and difficult to categorize as it was recently listed as an asexual state of Phaeosphaeriaceae [21,22]. However, the genus was synonymized under *Stagonospora* and is currently placed in Massarinaceae [23], which agrees with the results of our analysis. Most recently, *Hendersonia osteospermi*, which is pathogenic on the invasive shrub Bitous bush (*Chrysanthemoides monilifera*) in Australia, was determined to be the anamorphic stage of the newly named genus and species *Austropleospora osteospermi* [24], which happens to be in a different family than *H. pinicola.* This would indicate that further taxonomic clarification is needed for this organism and the genus *Hendersonia*. Finally, the second most frequently isolated species from diseased white pine needles after *L. acicola* in this study appears to be a putative new species of *Septorioides*. Very little is known about this genus and to date only a single species, *S. pini-thunbergii*, which is pathogenic on *Pinus thunbergii* in Japan, has been described [25] This putative new species is a member of the *Botryosphaeriales* but current research indicates that members of the genus *Septorioides* form a new distinct family from the *Botryosphariaceae*, tentatively named *Septorioidiaceae* [26]. Further phylogenetic, morphological, and ecological analyses are currently in progress in order to understand this organism, its evolutionary history, and its role in WPND [3].

3.3. Long-Term White Pine Health Monitoring

Crown conditions for a total of 127 tagged trees are assessed every year by U.S. Forest Service and State Forest Health Cooperators from mid-June to early July when the affected pines exhibit most obvious symptoms of chlorosis. Crown deterioration progresses from bottom to top consistent with rain-splash dispersed foliar diseases. The effect of "year" ($p < 0.0001$), "initial health status" ($p < 0.0001$), and their interaction ($p \leq 0.05$) had a significant effect on the severity of both chlorosis and defoliation. Initially unhealthy trees continued to exhibit chlorosis and defoliation every year of the survey (Figure 5). In contrast to initially healthy trees, for which chlorosis and defoliation was limited to less than a third of the crown, chlorosis and defoliation was between a third and two-thirds of the crown for initially unhealthy trees (Figure 5). The proportion of the crown with chlorosis increased consistently every year for both initially healthy and unhealthy trees (Figure 5). Initially unhealthy trees did not recover from year to year. The proportion of the crown with defoliation increased for initially unhealthy trees from 2013 to 2015. Similarly, the proportion of the crown with defoliation increased for initially healthy trees from 2012 to 2013 and did not change after 2013 (Figure 5).

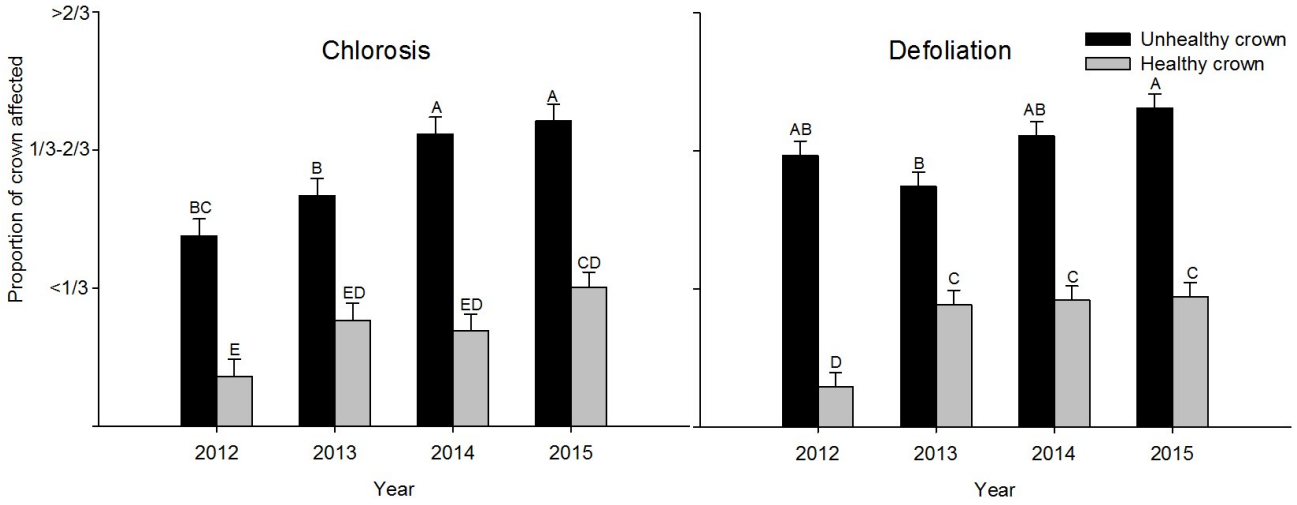

Figure 5. Frequency of chlorosis and defoliation of healthy and unhealthy eastern white pine at eight long-term monitoring sites from 2012 thru 2015. Bars represent means and standard errors (N = 127 trees). Values with the same letter are not statistically different ($\alpha = 0.05$).

4. Discussion

Based on morphological observations and genetic data, the recent white pine defoliation events in the northeastern U.S. and eastern Canada are due in large part to the brown spot pathogen *L. acicola*, in addition to several needle cast fungi including *L. dooksii*, *B. linearis*, and the putative new species of *Septorioides*. At one site all three pathogenic fungi were present and multiple pathogens were found on the same tree at another location. *Lecanosticta acicola* was the most frequently observed, widely distributed pathogen, and most consistently associated with chlorosis and defoliation in late June. It is likely that wet spring weather, favorable to disease development, during several consecutive years has led to an outbreak of foliar diseases. Trees in a variety of sites across northern New England were affected. Thus, the observed foliar damage is probably not site related.

Prior to this outbreak, damage caused by Dook's needle blight was reported on less than 0.1% of eastern white pines [7]. Similarly, although brown spot needle blight is common on two- and three-needle pines, it typically is not associated with white pine. In addition, Bifusella needle cast is rarely reported in northeastern North America; however, this disease may have been misdiagnosed or overlooked in the past.

Affected trees are repeatedly symptomatic and defoliated every year since the WPND outbreak was first observed in 2010. The disease is spreading from symptomatic trees to initially healthy trees. The consequence of repeated defoliations by these pathogens is unknown, but these fungi are expected to continue to cause damage in years following unusually wet springs.

The role of climate in these defoliation events is still not clear, however in many areas of the world, including the northeastern U.S., climate-change scenarios are predicting a wetter and warmer climate. This is indeed the case, as the northeastern U.S. has experienced above average precipitation and average to above average temperatures from 2003–2014 including six years that were in the top 10% for precipitation with 2011 being the wettest on record, and four years in the top 10% for temperature [27]. These represent optimum conditions to promote foliar disease caused by a number of foliar fungal

pathogens. This future outlook not only raises the likelihood of increased disease outbreaks caused by endemic fungal pathogens, but also increases the likelihood of pathogens from southern latitudes moving northward, as has been recently documented with crop pathogens [28]. This scenario is playing out in eastern white pine trees in the northeastern U.S. and eastern Canada, which have experienced a series of defoliating events in the last decade caused by fungi associated with WPND [10]. In 2010, these fungi were responsible for 24,369 hectares of damage to white pine forests in Maine [11] while in 2012 they caused additional damage to 4900 and 1500 hectares of white pine forests in New Hampshire and Vermont, respectively (*Frament* per. comm.). This study was able to document that these symptoms were primarily attributed to the fungal pathogen *Lecanosticta acicola* and on occasion *Lophophacidium dooksi* and *Bifusella linearis*. While WPND-infested white pine trees are generally capable of recovering from a single defoliation event, in 2014 the first cases of mortality in eastern white pine due to repeated WPND-induced defoliations were observed in our research sites (*Munck* per. comm.). Concern over WPND has expanded to an international level as important pine growing regions including Canada and Europe have been affected [18,29].

Based on our results, we believe that the brown spot pathogen *L. acicola* has recently become established in the northeast as several consecutive years with wetter and warmer than average weather during the spring created an environment conducive to an epidemic of this fungus. Brown spot has been a persistent problem in long leaf pine plantations in the southeastern U.S. and scotch pine in Minnesota and Wisconsin [8,9], but severe defoliation of eastern white pine by *L. acicola* in the northeast was not reported prior to 2005 [10]. Given current climate change scenarios for the region [30], the potential for WPND epidemics will likely increase, especially if the region continues to see greater than average rainfall in June, which in preliminary studies has been determined to drive epidemics of WPND [31]. Given the ecological and economic importance of eastern white pine in the northern U.S. and eastern Canada, reliably predicting the spread and impacts of WPND is critical to guiding management and policy recommendations. Current research is underway that will provide valuable information needed about the climatic factors that favor or inhibit epidemics of these fungi, the long-term effects of repeated defoliation on eastern white pine, and the impact on ecosystem processes, including carbon dynamics, water balance, and nutrient cycling, in white pine dominated stands.

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Author Contributions

Kirk Broders and Isabel Munck conceived and designed the project and experiments. Stephen Wyka, Gloria Iriarte, and Eric Beaudoin developed and executed the methods for fungal cultivation, DNA

extraction, and identification. Kirk Broders, Isabel Munck, and Stephen Wyka analyzed the data. Kirk Broders and Isabel Munck wrote the paper.

Conflicts of Interest

The authors declare no conflict of interest.

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