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CHARACTERIZATION AND DISTRIBUTION OF FUNGI ASSOCIATED WITH NEEDLE DEFOLIATION OF EASTERN WHITE PINE (*PINUS STROBUS*)

BY

STEPHEN A. WYKA

Marine and freshwater biology (BS), University of New Hampshire, 2012

THESIS

Submitted to the University of New Hampshire

In Partial Fulfillment of

The Requirements for the Degree of

Master of Science

In

Plant Biology

December, 2015

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ABSTRACT

CHARACTERIZATION AND DISTRIBUTION OF FUNGI ASSOCIATED WITH NEEDLE DEFOLIATION OF EASTERN WHITE PINE (*PINUS STROBUS*)

By

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University of New Hampshire, December, 2015

Over the past seven years eastern white pines (*Pinus strobus*) across the northeastern United States have been prematurely defoliating due to a disease complex known as White Pine Needle Damage (WPND). Previous research on WPND indicates that this outbreak, which started in 2010, is primarily attributed to the causal agent of brown spot needle blight, *Lecanosticta acicola* along with two other known *P. strobus* needle cast pathogens, *Bifusella linearis* and *Lophophacidium dooksii*. The current outbreak is thought to have arisen from several consecutive years of increased temperature and precipitation in the region. Current research, however, is lacking in both sampling distribution, the basic epidemiology of the primary causal agents, and the specific climatic factors that affect the development and spread of this outbreak. Furthermore, recent re-sampling of diseased *P. strobus* needles within this region has revealed a high isolation frequency of a putative new species closely related to *Septorioides pini-thunbergii*, a species associated with needle cast of *Pinus thunbergii*

in Japan, which was initially not known to be associated with WPND. This thesis intends to fill this lack of information, first by examining the putative new species of *Septorioides* through phylogenetic analysis of six gene loci (SSU, LSU, ITS, β-tubulin, EF1, and RPB2) to describe and classify its association with WPND. Secondly, by expanding field based sampling of WPND within Vermont, New Hampshire, Maine, and their neighboring states to construct a more detailed distribution map. Thirdly, by utilizing long-term WPND monitoring plots and data collected from land-based weather stations to create a climatic model to predict the severity of defoliation events in the next year. Lastly, by establishing a field-based spore trapping experiment to determine how climatic factors affect the development and dispersal of *L. acicola* spores. Results from this thesis demonstrate the widespread occurrence and establishment of a new family (Septorioideaceae), and a new species *Septorioides strobus*, as well as its association with the current WPND outbreak. Continued sampling provided further support for the disease complex theory that *L. acicoa, B. linearis, L. dooksii,* and *S. strobus* were ubiquitous across the region and neither an individual species nor a specific combination of species had a dominating presence in particular states or regions, but were generally found in forest stands that compromised >50% eastern white pine by basal area. Additionally, regional weather data confirmed the trend of increasing temperature and precipitation observed in this region with the previous year's May, June, and July rainfall being the best predictor of the following years defoliation event, while the current year's relative humidity was responsible for spore development and increased inoculum loads.

INTRODUCTION

The defoliation of the eastern white pine (*Pinus strobus* L.) in New England is an escalating concern threatening the ecological health of northern forests and economic vitality of the region's lumber industry. The white pine needle damage (WPND) epidemic, first documented in the spring of 2010 affecting 24,328 hectares in the state of Maine, has become of increasing concern to both the public and private landowners (Munck *et al.* 2012). *Pinus strobus* is not only an essential economic lumber resource but a crucial ecological component of the forests of northern USA and eastern Canada. Mature and regeneration *P. strobus* are similarly affected with symptoms including thin crowns, discoloration by tip necrosis or full needle chlorosis and pre-mature defoliation of 2nd year needles (known to some as one-year old needles) leaving behind only the newly emerged needles of the current growing season (Munck *et al.* 2012). The disease cycle of WPND is similar to that of typical needle cast fungi which includes; an infection period, spring through fall; a developmental period; and defoliation of infected needles usually one year after the infection period (Sinclair and Lyon 2005). It is during the defoliation event that infected needles develop fruiting structures that produce sexual or asexual spores which can then infect healthy needles (Sinclair and Lyon 2005). Symptoms of WPND typically manifest during the early spring and culminate by the end of July, leaving behind weakened trees during the summer that are more susceptible to other abiotic and biotic stresses. WPND does not appear to be diminishing since it was

first observed; rather it may actually be intensifying as a result of reported changes in the regional climate. These changes, namely increasing temperature, precipitation, and humidity are known to provide a favorable climate for infection by foliar pathogenic fungi associated with widespread outbreaks (Peterson 1973; Skilling and Nicholls 1974; Gadgil 1977; Hoff 1985; Coakley *et al*. 1999; Woods *et al.* 2005; Sturrock *et al.* 2011).

Previous needle blights of *P. strobus,* occurring at Acadia National Park, Maine in the 1980s, have been associated with several fungi including *Hendersonia pinicola*, *Truncatella truncata, Leptostroma* spp., a *Septoria* sp., a black yeast, a white nonsporulating hyphomycete, a *Bifusella*-like ascomycete, and *Lophodermium spp.* (Banfield 1960, 1962; Dreisbach 1989). Only the *Bifusella*-like ascomycete was considered to be pathogenic on *P. strobus*, while the others were classified as weak pathogens, saprophytes, and parasites (Dreisbach 1989). However, the *Bifusella*-like ascomycete could not be properly tested for pathogenicity, since it could not be cultured in artificial media and natural inoculum sources from needles also contained fruiting structures of these other weak pathogens; thus making it impossible to separate out the effects of each individual fungus (Dreisbach 1989). Other literature suggests that symptoms, caused by WPND, could be attributed to frost, ozone (Bennett *et al.* 1986), and "semimature-tissue needle blight" (Linzon 1960, 1964, 1967a, 1967b), further complicating diagnosis and analysis. Although winter injury caused by frost does discolor needles, these needles are not prematurely shed and can remain on the tree until normal needle-fall (Hauer 2008). While ozone-injured needles can often be shed prematurely, symptoms typically include tip necrosis and chlorotic mottling (Kohut *et al.* 1997); which do not resemble the total chlorosis observed on *P. strobus* needles.

Subsequent research conducted by Kohut *et al.* (1997) and Bennett *et al.* (1994) indicated that ozone treatments of up to 3x ambient ozone (103 ppb), was not responsible for foliar markings observed on eastern white pines in the field at Acadia National Park, Maine. Furthermore, semimature-tissue needle blight was first characterized by an orange-red discoloration that was caused by unfavorable conditions, including one or more days of wet weather followed by a continuous sunny period, inciting a condition that was inherent in certain individuals of a *P. strobus* population (Linzon 1960). This blight was considered to not be caused by any biotic factor as no micro-organisms were ever isolated from symptomatic tissues (Linzon 1960). However, this condition was successively studied by Wenner and Merrill (1998) who determined that semimature-tissue needle blight was caused by *Canavirgella banfieldii*, recently re-classified as *Lophophacidium dooksii* (Laflamme *et al.* 2015), a biotrophic fungal pathogen that could not be grown in culture.

Since the early 1900s reports of these previous needle blights of eastern white pines have occurred on mature trees from Maine to Pennsylvania, but have been generally found to affect random trees and in relatively low incidences at both good and poor sites (Merrill *et al.* 1996, 1997). These records also documented that only a few needles within each fascicle were affected at a time, symptomatic trees did not die, and outbreaks of symptoms did not generate much public concern (Dreisbach 1989; Merrill and Wenner 1996; Merrill *et al.* 1996, 1997). Unlike these previous reports, the current outbreak of WPND has documented that all needles in a fascicle can be affected, infected mature trees have succumb to mortality, and concern over the health of eastern white pines continues to rise in the northern U.S. and eastern Canada. (Munck *et al.*

2012; Broders *et al.* 2015). Current research is seeking to understand whether the current needle damage is caused by the factors previously reported, and if so, what change has caused the current outbreak, or if new fungal pathogens are involved in this current epidemic.

Munck *et al.* (2012) have found repeated incidences of *Lophophacidium dooksii*, the same species causing needle blight studied by Banfield (1960, 1962, 1963), Linzon (1960, 1964, 1967a, 1967b), Dreisbach (1989), Merrill *et al.* (1996), and Wenner and Merrill (1998). However, further study showed that three more fungal species were also frequently isolated from diseased needles: *Bifusella linearis* a known needle cast pathogen of eastern white pine, (Minter and Millar 1984; Munck *et al.* 2012); *Lecanostica acicola* (formerly known as *Mycosphaerella dearnessii*) the causal agent of brown spot needle blight, which was the most frequently observed and widely distributed pathogen (Jankovsky *et al.* 2009; Jurc and Jurc 2010; Munck *et al.* 2012); and a putative new *Septorioides*-like species which was the second most frequently isolated species (Broders *et al.* 2015).

Further research needs to be conducted on the eastern white pines of New England to better understand the primary causal agent(s) and environmental conditions conducive to the spread of the current WPND outbreak; whether it is the result of one fungal species or a combination of several fungal species, in conjunction with regional climatic factors.

My research into this epidemic is centered on three main objectives:

1. Characterize the fungi associated with WPND and their distribution across the northeastern United States, with emphasis on *Lecanosticta acicola,*

Bifusella linearis, Lophophacidium dooksii, and a putative new *Septorioides*-like species.

2. Develop a model to predict WPND severity and disease risk based on climatic data to assist foresters in developing management and control strategies.

3. Determine the effect of climate on the development and spore dispersal of *Lecanosticta acicola* both *in vitro* and *in situ*.

CHAPTER 1

Septorioides strobus sp. nov., a new species associated with needle defoliation of eastern white pine (*Pinus strobus* L.)

INTRODUCTION

The white pine needle defoliation (WPND) epidemic, suspected to be caused by multiple fungal pathogens was first documented in the spring of 2010 affecting 24,328 hectares in the state of Maine, and has since become an increasing concern to both the public and private landowners (Frament *et al.* 2011). In 2012 WPND caused damage to 4,897 and 1,496 hectares of white pine (*Pinus* strobus) National Forests in New Hampshire and Vermont, respectively (Frament per. comm.). *Pinus. strobus* is not only an essential economic lumber resource but a crucial ecological component to the forest of northern USA and eastern Canada. Mature and regeneration *P. strobus* are similarly affected with symptoms including thin crowns, discoloration and pre-mature defoliation of one-year old needles, leaving behind only the newly emerged needles of the current growing season (Munck *et al.* 2012). Symptoms along with current research point to three foliar pathogens: 1) *Lecanosticta acicola* (formerly known as *Mycosphaerella dearnessii*) the causal agent of brown spot needle blight; 2) *Lophophacidium dooksii* the causal agent of dooks needle cast (recently determined to be the same species as

Canavirgella banfieldii, Laflamme *et al.* 2015), and 3) *Bifusella linearis*, another needle cast pathogen (Minter and Millar 1984; Munck *et al.* 2012; Broders *et al.* 2015). However, continued sampling of diseased needles from 69 sites across New England revealed a putative new species, closely related to *Septorioides pini-thunbergii*, as the most frequently isolated species from symptomatic needles (Ch. 2, Fig. 7).

Septorioides pini-thunbergii was first documented to be associated with needle blight and the cause of sooty mold of *Pinus thunbergii* in Japan (Kaneko *et al.* 1989; Suto 2000), but has since been isolated as an endophyte from *P. densiflora* in Korea (Yoo and Eom 2012), confounding the true ecological role of this species. The genus *Septorioides* is characterized as resembling the genus *Septoria* by sharing hyaline filiform conidia with transverse eusepta along with a pycnidial conidiomata possessing conidophores reduced to conidiogenous cells. However, *Septorioides* is morphologically distinguished by having an acervulus, conidiomata that open by means of an irregular rupture, and paraphyses that are intermingled among conidiogenous cells (Quaedvlieg *et al.* 2013). Although recent complications in the *Septoria* complex brought up by Quaedvlieg *et al.* 2013, reveal that many *Septoria* morphological characters are conserved within many septoria-like genera which mostly reside in different phylogenetic clades rather than the tight cluster of *Septoria* within the family *Mycosphaerellaceae*. Their recent phylogenetic study further suggested that DNA sequence data was paramount when dealing with members of this complex. *Septorioides pini-thunbergii* is currently placed in the family *Botryosphaeriaceae* within the *Botryosphaeriales* (Quaedvlieg *et al.* 2013), an order that has undergone significant reconstruction over the past decade with the addition of several new genera (Crous *et*

al. 2004; Damm *et al.* 2007; Phillips *et al.* 2008; Hyde *et al.* 2011; Liu *et al.* 2012) as well as the creation of four new families; *Phyllostictaceae, Aplosporellaceae*, *Melanopsaceae*, and *Saccharataceae* (Slippers *et al.* 2013; Wikee *et al.* 2013).

The *Botryosphaeriales* is a highly varied order, containing species that range from endophytes to pathogens to opportunistic human pathogens (de Hoog *et al.* 2000; Slippers *et al.* 2013). This variety of lifestyles coupled with that fact that species are known to occur on a wide range of monocotyledonous, dicotyledonous, gymnosperm hosts, grasses, and lichens has allowed species of *Botryosphaeriales* to be globally distributed (Barr 1987; Denman et al. 2000; Mohali et al. 2007; Lazzizera et al. 2008; Marincowitz et al. 2008). While most of the taxa in the *Botryosphaeriales* exist as endophytes, living mostly in healthy tissue of woody plants for extended periods of time (Slippers and Wingfield 2007), many are characterized as pathogens that cause disease on a wide variety of economically and ecologically important plants (Slippers *et al.* 2013). However, these two ecological functions are not always exclusive. It has been documented that *Botryosphaeriales* cause disease after the onset of an initial stress factor, such as drought or infection by another weak pathogen (Schoeneweiss 1981; Swart and Wingfield 1991; Blodgett and Stanosz 1995). Once the disease is initiated, symptoms can develop rapidly and cause severe damage over large areas if the stress factor is widespread (Slippers and Wingfield 2007). The current WPND epidemic was speculated to be caused by *L. acicola*, *L. dooksii*, and *B. linearis* due to historical research and their ubiquitous presence across the Northeastern United States (Minter and Millar 1984; Merril *et al.*1996; Wenner and Merril 1998; Munck *et al.* 2012; Broders *et al.* 2014). Although these three pathogens are able to infect, they have been

previously documented to cause only limited disease on *P. strobus* (Skilling and Nicholls 1974; Merril *et al.*1996; Wenner and Merril 1998). It was hypothesized that this severe epidemic arose after several consecutive years of warmer and wetter weather during the spring, creating a more favorable environment for these foliar pathogens (Munck *et al.* 2012; Broders *et al.* 2014). While this might be true, these three foliar pathogens could instead be acting as a widespread stress factor, rather than the primary cause, to which the frequently isolated putative new species is exploiting. However, the putative new species might not be taking advantage of this stress factor, but may actually be working synergistically with *L. acicola*, *L. dooksii*, or *B. linearis,* or a combination of these three pathogens, resulting in a systematic downfall of eastern white pines.

It is therefore crucial to correctly categorize the ecological function and phylogenetic placement of this putative new species. Aside from morphological characteristics, the phylogenetic relationship of this unknown species to all known genera of the *Botryosphaeriales* is essential for understanding the evolution of this group of fungi. It is also important to try and answer critical questions pertaining to host association, patterns of disease outbreak, and origin as either a native or invasive species. Based on this information the objectives of this study were to: 1) complete a taxonomic description, including morphological features and optimal growth rates, of the putative new species and document its association with the WPND epidemic; 2) use DNA sequence data of five gene loci to accurately place this unknown species within the *Botryosphaeriales*. The taxonomic changes for the establishment of a new family and a new species are considered in this study.

MATERIALS AND METHODS

Isolates and DNA extraction

Pinus strobus needle samples were collected throughout Maine, New Hampshire, Vermont, and Massachusetts by Isabel Munck, Forest Health State Cooperators, and Forest Watch schools (www.forestwatch.sr.unh.edu), and the author from April to June 2011-2014. Diseased needles were surfaced sterilized in a 10% bleach solution for one minute, rinsed three times with de-ionized water, and allowed to dry in a fume hood. Dry needles were incubated in a humid chamber with 500µL of sterile H_2O for two days, to enhance sporulation. Single spore colonies were grown on 2% potato dextrose agar (PDA, Difco™) and incubated at room temperature with ambient light. Once pure cultures were established, isolates were transferred onto 2% PDA plates overlaid with a Cellophane membrane and grown for 1-2 weeks. Under aseptic conditions mycelium was scrapped off and placed inside 2mL microcentrifuge tubes (VWR), frozen for 1 hour at -80°C, and freeze dried for two days. Genomic DNA extraction from dried mycelium followed the modified CTAB method. (Kohler *et al.* 2011). Additional sequences for phylogenetic analyses were retrieved from GenBank (Table 1).

Table 1: Species subjected to phylogenetic analysis in this study.

Species	Isolate No.*	GenBank Accession No.					
		SSU	LSU	ITS	EF1	β-tubulin	RPB-2[†]
Aplosporella javeedii	CMW 38166	N/A	KC769980	KC769939	KC769847	KC769908	N/A
Aplosporella prunicola	CBS 121167	KF766229	JX681071	KF766147	N/A	N/A	N/A
Aplosporella yalgorensis	MUCC 511	N/A	EF591944	EF591927	EF591978	EF591961	N/A
Bagnisiella examinans	CBS 551.66	GU296139	KF766316	KF766148	GU349056	KF766126	GU371746
Barriopsis fusca	CBS 174.26	KF766230	DQ377857	KF766149	KF766395	EU673109	N/A
Botryosphaeria dothidea	CMW 8000, CBS 115476	KF766233	KF766319	KF766151	AY236898	AY236927	N/A
Cophinforma eucalypti	MFLUCC 110425	JX646833	JX646817	JX646800	JX646865	JX646848	N/A
Diplodia rosulata	CBS 116470	EU673211	DQ377896	EU430265	EU430267	EU673132	N/A
Dothiorella longicollis	CMW 26166, CBS 122068	KF766246	KF766328	KF766162	EU144069	KF766130	N/A
Endomelanconiopsis endophytica	CBS 120397	KF766249	EU683629	EU683656	EU683637	KF766131	N/A
Guignardia bidwellii (= *Phyllosticta parthenocissi*)	CBS 111645	EU673223	DQ377876	FJ824766	EU683653	FJ824777	N/A
Guignardia citricarpa (= *Phyllosticta citricarpa*)	CBS 102374	GU296151	DQ377877	FJ824767	FJ538376	FJ824778	N/A
Kellermania crassispora	CBS 131714	KF766261	KF766345	KF766175	KF766406	KF766135	N/A
Kellermania dasylirionis	CBS 131715	KF766263	KF766347	KF766177	KF766408	KF766137	N/A
Kellermania macrospora	CBS 131716	KF766264	KF766348	KF766178	KF766409	KF766138	N/A
Kellermania nolinae	CBS 131717	KF766266	KF766350	KF766180	KF766411	KF766140	N/A
Kellermania plurilocularis	CBS 131719	KF766267	KF766351	KF766181	KF766412	KF766141	N/A
Kellermania yuccifoliorum	CBS 131726	KF766271	KF766355	KF766185	KF766416	KF766144	N/A
Lasiodiplodia theobromae (Botryosphaeria rhodina in CBS)	CBS 164.96	EU673196	EU673253	AY640255	AY640258	EU673110	N/A
Lecanosticta acicola	LNPV 252	N/A	JX901844	JX901755	JX901639	JX902213	JX901968
Macrophomina phaseolina	CBS 227.33	KF766281	DQ377906	KF531825	KF952000	KF531806	N/A
Melanops sp. (*Botryosphaeria quercuum* in CBS)	CBS 118.39	FJ824763	DQ377856	FJ824771	FJ824776	FJ824782	N/A
Melanops tulasnei	CBS 116805	KF766282	FJ824764	FJ824769	FJ824774	FJ824780	N/A
Neodeightonia phoenicum	CBS 169.34	EU673203	EU673259	EU673338	EU673307	EU673138	N/A
Neofusicoccum umdonicola	CMW 14058	KF766293	KF766373	EU821904	EU821874	EU821844	EU821934
Neoscytalidium dimidiatum	CBS 499.66	KF531818	DQ377925	AY819727	EU144063	FM211167	N/A
Phaeobotryon mamane	CPC 12264	EU673183	DQ377898	EU673331	EU673297	EU673125	N/A
Phaeobotryosphaeria citrigena (*Botryosphaeria fusca* in CBS)	ICMP 16818	EU673181	EU673247	EU673329	EU673295	EU673141	N/A

Table 1: (Continued)

Species	Isolate No.*	GenBank Accession No.					
		SSU	LSU	ITS	EF1	β-tubulin	RPB-2†
Phyllosticta minima (= *Phyllosticta rubrum*)	CBS 111635	EU754095	EU754194	KF766215	KF766433	N/A	N/A
Phyllosticta philoprina	CBS 616.72	KF766257	KF766341	KF766171	KF766402	N/A	N/A
Phyllosticta podocarpi	CBS 111647	KF766304	KF766383	KF766217	KF766434	N/A	N/A
Pseudofusicoccum stromaticum	CBS 117448	EU673146	DQ377931	KF766223	N/A	EU673094	N/A
Saccharata capensis	CBS 122693	N/A	KF766390	KF766224	EU552095	N/A	N/A
Saccharata kirstenboschensis	CBS 123537	KF766310	FJ372409	KF766225	N/A	N/A	N/A
Saccharata proteae	CBS 115206	KF766311	DQ377882	KF766226	KF766438	KF531790	GU371729
Septorioides pini-thunbergii	CBS 473.91	N/A	KF251746	KF251243	N/A	KF252727	KF252248
Spencermartinsia viticola	CBS 117009	KF766313	DQ377873	AY905554	AY905559	EU673104	GU357795
Sphaeropsis visci	CMW 39386	N/A	KF575065	KF575004	KF575036	KF575100	N/A
Tiarosporella graminis	CBS 118718	KF531827	DQ377939	KF531828	KF531807	KF531808	N/A
Septorioides strobus	SW13-25	KT884663	KT884677	KT884691	KT884705	KT884719	KT884733
Septorioides strobus	SW14-36	KT884664	KT884678	KT884692	KT884706	KT884720	N/A
Septorioides strobus	SW14-52	KT884665	KT884679	KT884693	KT884707	KT884721	KT884734
Septorioides strobus	SW14-80	KT884666	KT884680	KT884694	KT884708	KT884722	KT884735
Septorioides strobus	SW14-90	KT884667	KT884681	KT884695	KT884709	KT884723	KT884736
Septorioides strobus	SW14-100	KT884668	KT884682	KT884696	KT884710	KT884724	KT884737
Septorioides strobus	SW14-102	KT884669	KT884683	KT884697	KT884711	KT884725	KT884738
Septorioides strobus	SW14-105	KT884670	KT884684	KT884698	KT884712	KT884726	KT884739
Septorioides strobus	WPF 25‡	KT884671	KT884685	KT884699	KT884713	KT884727	KT884740
Septorioides strobus	WPF 106‡	KT884672	KT884686	KT884700	KT884714	KT884728	KT884741
Septorioides strobus	WPF 147	KT884673	KT884687	KT884701	KT884715	KT884729	KT884742
Septorioides strobus	WPF 165‡	KT884674	KT884688	KT884702	KT884716	KT884730	KT884743
Septorioides strobus	V-3-11-1	KT884675	KT884689	KT884703	KT884717	KT884731	N/A
Septorioides strobus	V-5-2-3	KT884676	KT884690	KT884704	KT884718	KT884732	N/A

* CMW: Tree Pathology Co-operative Program, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; CBS: CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands; MUCC: Culture Collection, Laboratory of Plant Pathology, Mie University, Tsu, Mie prefecture, Japan; MFLUCC: Mae Fah Luang University Culture Collection, Chiang Mai, Thailand; LNPV: Laboratoire National de la Protection des Végétaux Mycologie, Malzéville, France; ICMP: International Collection of Microorganisms from Plants, Plant Diseases Division, DSIR, Auckland, New Zealand.

† Sequences only used for alignment to determine p-distance of RPB-2 gene between *Septorioides pini-thunbergii* and *Septorioides strobus*.

‡ Isolates used in temperature growth trials; WPF 25, Dunbarton, NH; WPF 106, Lyman, ME; WPF 165, Springfield, VT.

PCR and sequencing

Six partial gene regions were amplified from 14 isolates of the unknown species across Maine, New Hampshire, and Vermont. The genes evaluated in this study included the 18s nuclear ribosomal small subunit (SSU), the 28s nuclear ribosomal large subunit (LSU), the internal transcribed spacer (ITS), the translation elongation factor 1-α (EF1), the β-tubulin, and the RNA polymerase II second largest subunit (RPB2), using primers sets listed in Table 2. PCR amplifications were performed with a modified version of that specified by Quaedvlieg *et al.* (2013); total volume of 12.5μL solution containing 30ng of template DNA, 1 X PCR buffer, 2mM $MgCl_2$, 0.4μM of each primer, 25μM of each dNTP and 1.0 U Taq DNA polymerase (GoTaq, Promega). Amplification conditions followed those provided by Quaedvlieg *et al.* (2013), with revised primer annealing temperatures (Table 2). PCR products were purified using the E.Z.N.A Cycle Pure Kit (Omega Bio-Tek, Norcross, GA) and sent to Genewiz (South Plainfield, NJ) for sequencing. Sequences were manually edited using BioEdit v7.2.5. All successful sequences were deposited into GenBank.

Phylogenetic analysis

A total of 39 species, covering all 25 genera known from culture and considered to reside in the *Botryosphaeriales*, were included in the ingroup with *Lecanosticta acicola* representing the outgroup. Relevant gene loci of these 39 species were imported from GenBank (Table 1). However, due to lack of representation of the RPB2 from species considered in this study this locus was excluded from the phylogenetic

Table 2: Primer combinations used in this study for genetic amplification and sequencing.

Locus	Primer	Primer sequence 5' to 3'	Annealing temperature (°C)	Orientation	Reference
18s Small subunit (SSU)	NS1	GTAGTCATATGCTTGTCTC	45	Forward	White *et al.* 1990
	NS4	CTTCCGTCAATTCCTTTAAG	45	Reverse	White *et al.* 1990
28s Large subunit (LSU)	LSU1 Fd	GRATCAGGTAGGRATACCCG	52	Forward	Crous *et al.* 2009
	LR5	TCCTGAGGGAAACTTCG	52	Reverse	Vilgalys & Hester 1990
Internal transcribed spacer (ITS)	ITS1 f	TCCGTAGGTGAACCTGCGG	54	Forward	Gardes & Bruns 1993
	ITS4	TCCTCCGCTTATTGATATGC	54	Reverse	White *et al.* 1990
Translation elongation factor-1α	EF1-983	GCYCCYGGHCAYCGTGAYTTYAT	55	Forward	Carbone & Kohn 1999
	EFgr	GCAATGTGGGCRGTRTGRCARTC	55	Reverse	
β-tubulin	Bt2a	GGTAACCAAATCGGTGCTGCTTTC	52	Forward	Glass & Donaldson 1995
	Bt2b	ACCCTCAGTGTAGTGACCCTTGGC	52	Reverse	Glass & Donaldson 1995
RNA polymerase II second largest subunit*	fRPB2-5F	GAYGAYMGWGATCAYTTYGG	49	Forward	Liu *et al.* 1999
	fRPB2-414R	ACMANNCCCCARTGNGWRTTRTG	49	Reverse	Quaedvlieg *et al.* 2011

* Was not used in phylogenetic tree development due to lack of sequences of other genera within *Botryosphaeriales* available on GenBank.

tree. All loci available for each species were concatenated and aligned using MAFFT v7.245 (Katoh and Standley 2013). Alignments were then exported into MEGA v6.0 to construct phylogenetic trees using both the neighbor joining (NJ) (maximum composite likelihood) and maximum likelihood (ML) algorithms (Hall 2013). A complete deletion treatment was used to handle gaps and missing data, while node reliability was determined with 1000 bootstrap replications (Felsenstein 1985). A Bayesian analysis was also performed using MrBayes v3.2.5 (Huelsenbeck and Ronquist 2001) to gather posterior probabilities to support bootstrap values. The final tree was edited in FigTree v1.4.2. Concatenated sequences of *S. pini-thunbergii* and the putative new species were also used to test for nucleotide similarity (P-distance). Since the RPB2 gene of *S. pini-thunbergii* and five other study species was available on GenBank (Table 1) this gene locus alone was used to run a separate nucleotide similarity analysis. Alignment of the RPB2 gene region was performed in the same manner described above.

Morphological evaluation

Morphological characteristics were described from both sporulating diseased needles and 2 week old isolates on PDA. Together, a stereomicroscope and light microscope with an attachable camera were used to capture morphological structures. Only macro and micro – conidia were measured with 50 replicates at 1000x magnification. Growth rates of three isolates from three states (Table 1 & 4) were conducted at four different temperatures. Four millimeter agar plugs from two week old colonies were placed mycelial side down on fresh PDA plates. Plates were incubated at 15, 20, 25, and 30°C with a 12hr light cycle. Colony diameters of ten replicates were

measured along two perpendicular lines weekly for three weeks. Statistical analysis of growth rates by isolate location and temperature were performed in JMP Pro 11.2.1 (SAS Institute Inc.).

RESULTS

Sequencing and phylogenetic analysis

Five of the partial gene regions were gathered for all 14 of the unknown isolates. Only 11 isolates were successful in the amplification of the RPB2 region. The phylogenetic reconstructions of the neighbor-joining (NJ), maximum likelihood (ML), and Bayesian inference (BI) were similar, with the ML tree being shown in Figure 1. As expected, our analysis confirmed the distinct lineages of *Planistromellaceae, Phyllostictaceae, Aplosporellaceae*, *Melanopsaceae*, and *Saccharataceae* within the *Botryosphaeriales* (Slippers *et al.* 2013). However, *S. pini-thunbergii* along with our closely related unknown species was not placed within the family *Botryosphaeriaceae*. Both species diverged from the *Saccharataceae* and clustered together with a bootstrap value of 82% and a posterior probability of 100%, providing evidence that these species form a distinct family hereafter called *Septorioideaceae.* Furthermore, our analysis showed that all isolates of our unknown species clustered together, forming a distinct phylogentic group from *S. pini-thunbergii*, with a bootstrap value of 99% and a posterior probability of 100%, revealing all 14 isolates as the same species hereby referred to as *Septorioides strobus* (Fig. 1). Concatenated sequences (LSU, ITS, and β-tubulin) of *S. pini-thunbergii* and *S. strobus* were analyzed by 1659 base pair positions resulting in a p-distance of 0.13±0.008 (Table 3), referring to 87% nucleotide similarity. Similarly, analysis of the RPB2 gene region alone resulted in a p-distance of 0.098±0.016, 90.2%

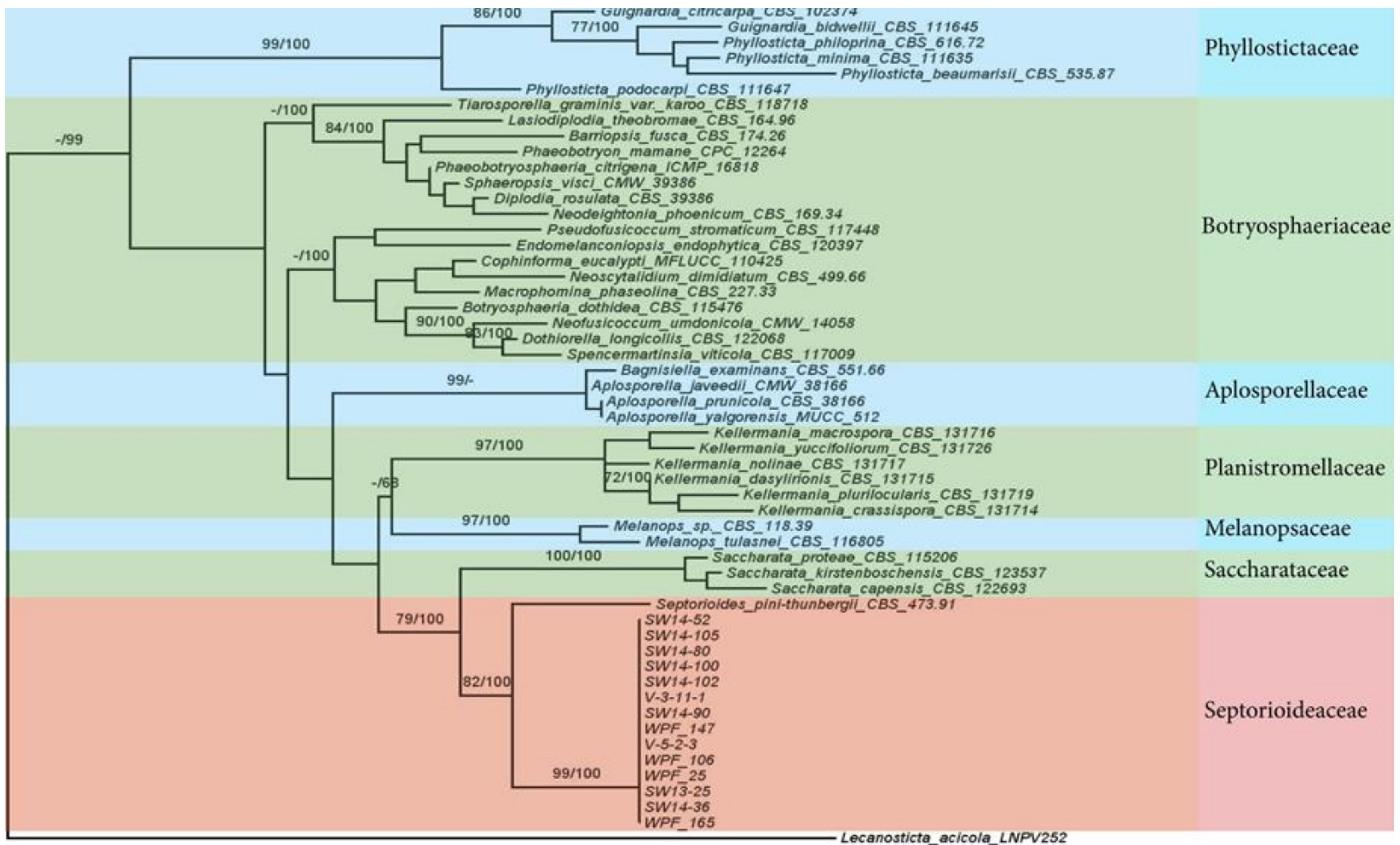

Figure 1: Phylogenetic reconstruction of representative members of each family within the *Botryosphariales* using maximum likelihood and five gene loci (SSU, LSU, ITS, β-tubulin, and EF-1α). Numbers above the branch indicate bootstrap values/posterior probabilities. Bootstrap values were determined with 1000 replicates.

Table 3: Estimate of evolutionary divergence between *Septorioides pini-thunbergii* and the type specimen of *S. strobus*. Two tests were performed, one with the concatenated sequence used in the phylogenetic study and the other using the RNA polymerase II second largest subunit (RPB2). All positions containing gaps and missing data were eliminated; standard error was estimated with 100 bootstrap replications.

Species	Strain	Gene region	Positions analyzed	p-distance (±SE)
Septorioides strobus	WPF 25	Concatenated sequence	1659	0.13 ± 0.008
		RPB2	337	0.098 ± 0.016

nucleotide similarity. Collective analysis of both the phylogenetic tree, nucleotide similarity, and morphological analysis indicates that *S. strobus* is a member of the genus *Septorioides*.

Taxonomy and growth

Septorioides strobus isolates were recovered from infected needles as early as mid-April to the end of June from 41 sites out of the total 69 sites sampled (Ch. 2 Table 5). It was found occurring: alone at 6 sites; together with *L. acicola* at 6 sites, *B. linearis* at 7 sites, *L. dooksii* at 5 sites; together with *L. acicola* and *B. linearis* at 8 sites and *L. acicola* and *L. dooksii* at 7 sites; all species were found together at 2 sites (Ch. 2 Table 5). Isolates were recovered throughout Maine, New Hampshire, Vermont and Massachusetts with a latitudinal range of 5.3° from Massasoit State Park in East Taunton, MA to Wallagrass, ME (Ch. 2 Table 5). Representative strains from both Maine and New Hampshire (WPF 106 and WPF 25, respectively) had the fastest growth rate at 20°C significantly higher than the growth rate of a Vermont strain (WPF 165) ($p < 0.05$), which had its highest growth rate at 15°C (Table 4, Fig. 2). However, the growth rate of WPF 165 at 15°C was not significant from the growth rate observed at 20°C ($p = 0.12$). Both WPF 106 and WPF 25 significantly decreased their growth rate at 25°C and 30°C; while strain WPF 165 did not have a significant decrease in growth rate from 20°C to 25°C ($p = 0.01$), but did show a significant decrease at 30°C. Infected *P. strobus* needles showed symptoms of yellowing while needles occasionally turned grey-whitish in severely infected regions of the needle. Black pycnidia, exuding a mucoid

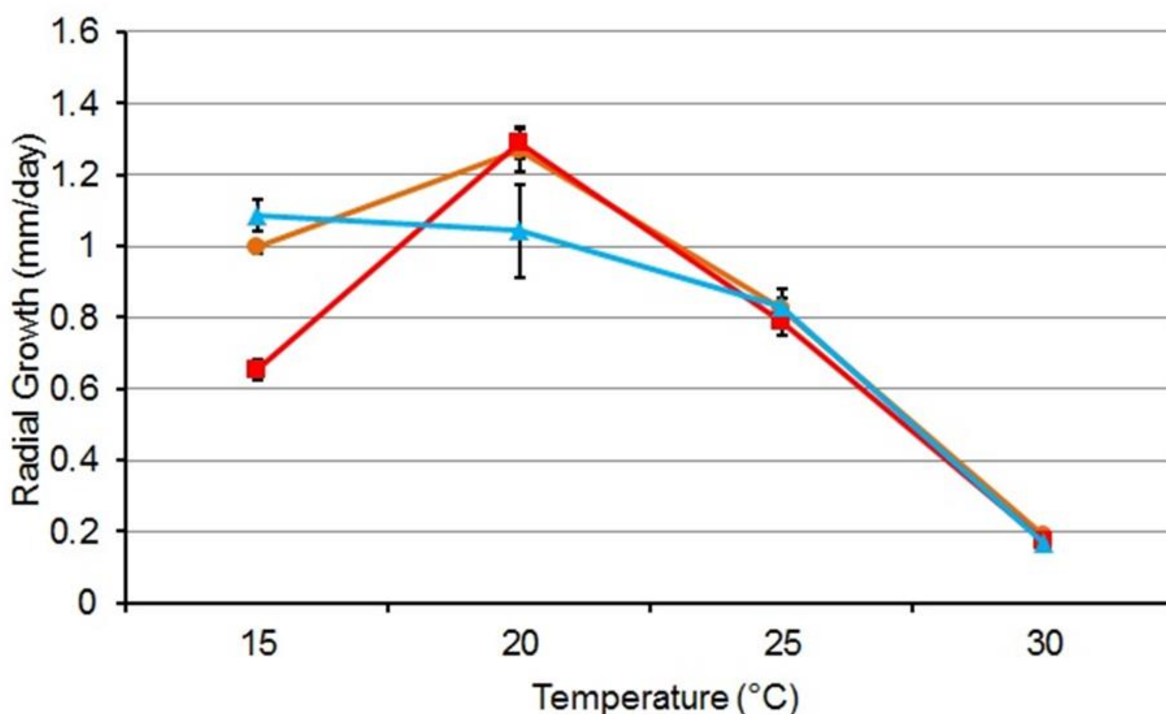

Figure 2: Radial daily growth rates at four different temperatures for three isolates of *Septorioides strobus*: red squares, WPF 25 (NH); orange circles, WPF 106 (ME); and blue triangles, WPF 165 (VT). Bars indicate standard error, n=10. WPF stands for (White Pine Fungus).

Table 4: Mean growth rate (mm/day) of three *Septorioides strobus* strains at four different temperatures over three weeks (±SE), n=10. Student t-test analysis was performed to find significant growth differences between strains and temperatures; capital letters correspond to growth differences between strains at a certain temperature, lower case letters correspond to growth differences between temperatures of a given strain. Values followed by the same letters are not significantly different ($p < 0.05$).

Strain	State	Temperature (°C)							
		15		20		25		30	
WPF 25	NH	0.65±0.027	A / c	1.29±0.061	A / a	0.79±0.039	A / b	0.17±0.01	A / d
WPF 106	ME	0.99±0.017	B / b	1.27±0.044	A / a	0.83±0.028	A / c	0.19±0.014	A / d
WPF 165	VT	1.09±0.044	B / a	0.97±0.086	B / ab	0.83±0.051	A / b	0.17±0.01	A / c

crystal conidial mass, were obvious signs of infections (Fig. 3). Colony morphology of *S. strobus* was nearly identical to *S. pini-thunbergii*. However, the distinctive feature between these two species was their macroconidia. Conidia lengths for *S. pini-thunbergii* were around 70-80µm while *S. strobus* conidia were nearly half that averaging 36µm. Secondly, macroconidia of *S. pini-thunbergii* were also found to contain 3-6 septate with a maximum of 10, while typical *S. strobus* macroconidia contained 1 septate, rarely possessing 2 or 3. Based on the morphological similarities and differences along with the results of our phylogenetic analysis and that *S. pini-thunbergii* has only been found in Japan and Korea on two needle pines, *Pinus thunbergii* and *P. densiflora,* we consider *Septorioides strobus* to be a member of the genus *Septorioides* as well as a new species for which we provide the following species description.

Septorioideaceae Wyka & Broders **fam. nov.**

Type genus: Septorioides (Quaedvlieg, Verkley & Crous) In: Quaedvlieg *et al.* Stud. Mycol. 75: 383. 2013.

Type species: S. pini-thunbergii (S. Kaneko) In: Quaedvlieg *et al.* Stud. Mycol. 75: 383. 2013.

See Quaedvlieg *et al.*2013 for description of genus.

Septorioides strobus Wyka & Broders **sp. nov.** (Fig. 3)

Type specimen: Strain: WPF 25

Sexual morph unknown. *Conidiomata* pycnidial, black, unilocular, globose, irregular, exuding a mucoid crystal conidial mass by means of an irregular rupture. *Paraphyses* intermingles among conidiogenous cells, hyaline, cylindrical, aseptate. *Conidiophores* reduced to conidiogenous cells, lining the inner cavity in basal layer, hyaline, subcylindrical to navicular. *Spermatia* hyaline, smooth, cylindrical, straight to curved. *Microconidia* hyaline, smooth, cylindrical to obovate, straight to slightly curved, base truncate, (5 –) 9.9 ± 2.7 (– 15) x (2.5 –) 2.6 ± 0.5 (– 5) (l/w ratio: 3.8). *Macroconidia* hyaline, smooth, granular content, fusiform-elliptical, straight to irregularly curved, 1-septate or rarely 2-3 septate or aseptate, apex rounded, base truncate, (22.5 –) 36 ± 6.8 (– 62.5) x (3.75 –) 5.6 ± 0.9 (– 7.5) (l/w ratio: 6.6).

Culture characteristics: On PDA, mycelium white to greyish, darkening with age, effuse. Can produce few to numerous, sparse or clustered conidiomata readily sporulating, becoming downy with age. Reverse white edges becoming dark olive to black in center, occasional presence of conidiomata in agar. Optimal growth rate of 1.18 ± 0.06 mm/day at 20°C.

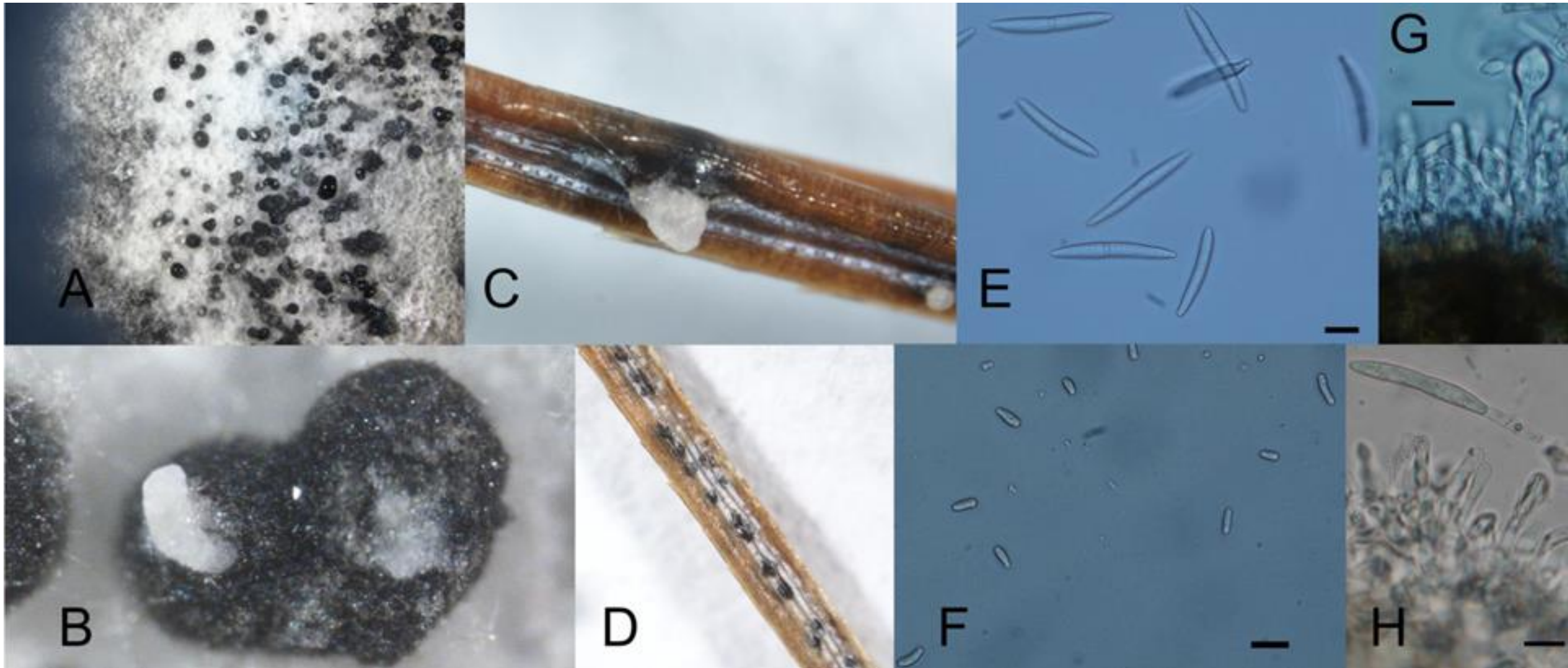

Figure 3: *Septorioides strobus*. A) 1 week colony sporulating on PDA. B-C) Crystal conidial mass protruding from conidiomata. D) Fruiting bodies on *P. strobus* needles. E) Macroconidia (400x). F) Microconidia (400x). G-H) Conidiogenous cells producing conidia (1000x). Scale bars = 10μm.

DISCUSSION

This study offers the establishment of a new family and species within the order *Botryosphaeriales,* an order than has undergone substantial reconstruction over the past decade with the addition of new species, genera, and families (Crous *et al.* 2004; Damm *et al.* 2007; Phillips *et al.* 2008; Hyde *et al.* 2011; Liu *et al.* 2012; Slippers *et al.* 2013; Wikee *et al.* 2013). While most of these additions were reclassifications of old species, brought to light through the use of molecular analysis, new species within the *Botryosphaeriales* have been and will continue to be discovered especially when sampling new hosts and new environments (Taylor *et al.* 2009; Perez *et al.* 2010; Mehl *et al.* 2011; Sakalidis *et al.* 2011; Jami *et al.* 2012, 2013, 2014).

Our new species *Septorioides strobus* was the most common fungal species isolated from *P. strobus* needles, occurring at 41 locations, marking the first reported sighting of a *Septorioides* species found within the United States. Its presence was ubiquitous across the northeastern U.S., being found throughout Massachusetts, Vermont, New Hampshire and Maine (Ch. 2, Fig. 6). This species readily sporulated on diseased needles on both mature and regeneration trees. It was found to be slightly cold tolerant with maximum growth rates occurring at 20°C, and 15°C for a Vermont strain (WPF 165). While there was no significant difference between the growth rates of the Vermont and Maine strain at 15°C, the New Hampshire strain had a significantly reduced growth rate; suggesting that there may be genetically different lineages of this

species separated geographically. Our initial examination of *S. strobus* included molecular analysis of the ITS gene sequence alone, resulting in a 99% similarity to *S. pini-thunbergii*, a species associated with needle cast and the cause of sooty mold of *Pinus thunbergii* in Japan (Kaneko *et al.* 1989; Suto 2000), and isolated as an endophyte from *P. densiflora* in Korea (Yoo and Eom 2012). However, *S. pini-thunbergii* was the only species representing its genus, making it difficult to determine whether our species was *S. pini-thunbergii* or an undiscovered species. Morphologically the two species were very similar, only differentiating in the size, shape, and septum of the macroconidia. Morphological features alone have been shown to be insufficient when comparing species in the *Botryosphariales*, especially when comparing species occurring in different environments and on different hosts (Slippers *et al.* 2004, 2014; Crous *et al.* 2006; Pavlic *et al.* 2009). Additionally, molecular analysis of the ITS region alone has been found to be impractical when distinguishing species within the *Botryosphaeriaceae*, as it embodies species complexes (Slippers *et al.* 2014). Prior to this study *S. pini-thunbergii* was placed within the *Botryosphaeriaceae* (Quaedvlieg *et al.* 2013), and with only one species representing the genus it was not possible to determine whether *Septorioides* contained a species complex, therefore additional gene analysis was necessary. Slippers *et al.* (2014) recommended the use of the LSU, EF-1α, β-tubulin, and RPB2 gene to differentiate genera and resolve cryptic species. The authors also decided to sequence the SSU loci as previous research on the *Botryosphaeriales* stressed the importance of using reliable DNA sequence data when correctly placing species within this order (Liu *et al.* 2012; Slippers *et al.* 2014).

The increased resolution obtained from an additional five gene loci proved to be successful in correctly placing *S. strobus* in the *Botryosphaeriales*. Our analysis confirmed the presence and placement of the *Planistromellaceae, Phyllostictaceae, Aplosporellaceae*, *Melanopsaceae*, and *Saccharataceae* as separate families distinct from *Botryosphaeriaceae* (Minnis *et al.* 2012; Slippers *et al.* 2013; Wikee *et al*. 2013). However, our analysis revealed an unexpected outcome; both *S. strobus* and *S. pini-thunbergii* formed their own clade outside of the *Botryosphaeriaceae*, splitting most recently from the *Saccharataceae*. Initially, Kaneko *et al.* (1989) placed *S. pini-thunbergii* within the *Mycosphaerellaceae*, while a recent phylogenetic study of the genus *Septoria,* which included *Septoria*-like species, determined that *S. pini-thunbergii* was not a member of the *Mycosphaerellaceae* but resided in the *Botryosphaeriaceae* (Quaedvlieg *et al.* 2013). However, as this study focused primarily on species within the *Mycosphaerellaceae,* there was a lack of representation of species from the *Botryosphaeriales*, suggesting that *S. pini-thunbergii* could have easily been misplaced within the order. A more recent phylogenetic study of the *Botryosphaeriales* conducted by Slippers *et al.* (2013) undertook the task of re-evaluating this order through both morphological and molecular analysis. Their study resulted in the creation of four new families; however, *S. pini-thunbergii* was not included in their analysis. Based upon our resolution from five gene loci and the similarities between our phylogenetic tree and the Slippers *et al.* (2013) reconstruction, we conclude that the genus *Septorioides* should reside in its own family called *Septorioideaceae*.

There is still much to understand about this family as it only contains two species and neither of the sexual morphs for these species are known. Since the geographical

separation of these two species is so large (Japan to USA), it is the authors' opinion that *Septorioides* species are present in other parts of the world, particularly on other *Pinus* species. The true ecological role of these species, as either pathogens or endophytes, is still unknown, but it appears that they may be associated with needle blights of *Pinus* species, potentially as weak pathogens or latent endophytes (Kaneko *et al.* 1989; Suto 2000; Yoo and Eom 2012; Kihara *et al.* 2015). Similarly the *Saccharataceae* the closest related family to the *Septorioideaceae*, is also an understudied family consisting of only three species that have been considered pathogens by some researchers (Slippers *et al.* 2013) due to their associations with leaf spots and stem cankers, while separate studies have shown that species in the *Saccharataceae* were endophytes (Swart *et al.* 2000; Taylor *et al.* 2001). Further research should include pathogenicity tests of both species on their respective hosts and cross hosts as well as other *Pinus* species known to inhabit similar regions. A subsequent population study of both *S. strobus* and *S. pini-thunbergii* would provide valuable insight into the origin and divergence of these species.

CHAPTER 2

Emergence of White Pine Needle Damage (WPND) in the northeastern U.S. associated with changes in pathogen pressure in response to climate change

INTRODUCTION

Since 2010 eastern white pines (*Pinus strobus*) across the Northeastern United States have been under attack by a disease complex, likely caused by several fungal species, known as White Pine Needle Damage (WPND) (Munck *et al.* 2012). Mature and regeneration *P. strobus* are similarly affected with symptoms typically manifesting in the month of May with yellowing and browning of 2nd and 3rd year needles followed by defoliation of affected needles by the end of July. While these symptoms are more often observed on the lower portion of tree crowns, trees in long-term monitoring plots that have experienced defoliation for several consecutive years have shown advancement of disease symptoms to the upper portions of the crown. As the defoliation spreads upwards it leaves behind thinned and weakened branches that become increasingly susceptible to other biotic or abiotic stresses. While whole tree mortality associated with WPND was only first reported in 2014, death of lower branches was observed as early as 2012 (Munck per. comm.). The uncertainties regarding tree health have resulted in an increasing amount of concern from both public and private land owners. *Pinus strobus* is not only an essential economic lumber resource but a crucial ecological

component of the forest of northern U.S. and eastern Canada. This potential threat, to the habitat range of eastern white pines, has raised many questions related to methods on how to best manage and control the disease outbreak. However, management recommendations cannot be addressed satisfactorily due to a lack of knowledge regarding the cause of the outbreak and its distribution and spread across the northeastern U.S.

Previous research has focused primarily on monitoring and determining which fungal pathogens were causing WPND (Munck *et al*. 2012). Initial reports provided by the U.S. Forest Service highlighted the emerging concern and importance of this new disease complex on white pine growing regions of U.S. and eastern Canada (Munck *et al.* 2011, 2012). Since then the number of reports of WPND has increased, sparking more concern and stressing the need to understand the basic etiology and epidemiology of this disease (Munck per. comm.). A recent paper by Broders *et al.* (2015) documented and identified the majority of fungi associated with diseased needles of *P. strobus*, but in a limited geographic region. Their findings indicated that *Lecanosticta acicola* (previously known as *Mycosphaerella dearnessii*) (Quaedvlieg *et al.* 2012), the cause of brown spot needle blight, is the primary pathogen contributing to needle defoliation. Two needle cast fungi *Lophophacidium dooksii* (previously known as *Canavirgella banfieldii* (Laflamme *et al.* 2015)) and *Bifusella linearis* (Minter and Millar 1984), and a putative new species of *Septorioides*, described as *Septorioides strobus* (Wyka and Broders *Submitted*) were also associated with defoliation events of *P. strobus* throughout the northeastern U.S. (Broders *et al.* 2015).

Prior to the current WPND outbreak *L. acicola* was not historically associated with disease on mature white pine in the northeast, and was only first reported in Maine in 2006 (Munck *et al.* 2011). However, brown spot needle blight has been a continuous problem on both long leaf pine (*Pinus palustris*) and scotch pine (*Pinus sylvestris*) plantations in the southeastern and northcentral U.S., respectively (Skilling & Nicholls 1974;Huang *et al.* 1995; Heimann *et al.* 1997). While *L. acicola* is known to infect many species of pine, Skilling and Nicholls (1974) determined that, through natural inoculum from scotch pine, both long leaf pine and eastern white pine were moderately resistant to infection by *L. acicola*. However, since then the fungus has adapted or some other factor has allowed *L. acicola* to infect these hosts (Heimann *et al.* 1997; Munck *et al.* 2012; Broders *et al.* 2015). In addition, both *B. linearis* and *L. dooksii*, two known white pine needle cast fungi which have been present in the northeast since the early 1900s, were only reported on less than 0.1% of eastern white pines stands throughout this region (Minter and Millar 1984; Merril *et al.*1996; Wenner and Merril 1998). Furthermore, *Septorioides strobus*, a recent discovery within the United States on *P. strobus*, has shown to be an anomaly. The closest known related species, *Septorioides pini-thunbergii,* was found to be pathogenic, causing sooty mold on Japanese black pine (*P. thunbergii*) in Japan in 1989 (Kaneko *et al.* 1989; Suto 2000); but no fungi in this genus have been reported in North America. Therefore, it is essential to understand what regional shifts could have allowed an increase in the population of *L. dooksii* and *B. linearis*, as well as an establishment of both *L. acicola* and *S. strobus*.

A recent distribution map of *L. acicola*, *B. linearis*, and *L. dooksii* revealed that combinations of these three pathogens were present in only 14 of 22 locations; ranging

from northern to southern Vermont, southern New Hampshire, and central to southern Maine (Broders *et al.* 2015). Continued monitoring efforts aimed at tracking the extent of damage caused by WNPD have largely been conducted through the use of sketch mapping aerial surveys performed by the U.S. Forest Service. From 2010 to 2014, these surveys confirmed the wide distribution of WPND in Vermont and sparse distribution throughout both New Hampshire and Maine (USDA Forest Service, FHM. 2015.). Reports of some of the four fungal species have also come from southeastern Canada, Massachusetts, New York, and some European countries where *L. acicola* is causing a potential problem (Jankovsky *et al.* 2009; Jurc & Jurc 2010; Hintsteiner *et al.* 2012; Adams per. comm.). As the spread of WPND continues, it is essential to understand the exact range of these four fungal species in determining if *P. strobus* is in risk of infection throughout its native host range.

Although current data is inconclusive as to what factors have influenced this current WPND outbreak, it appears that WPND is not diminishing since it was first observed in 2010; and may actually be intensifying as a result of reported changes in the regional climate (Frumhoff *et al.* 2007; Campbell *et al.* 2010, 2011; Broders *et al.* 2015). While numerous papers have shown that increases in temperature, precipitation, and humidity provide a favorable environment for fungal foliar diseases, predictions of the impacts of climate change on foliar pathogen behavior are still challenging and constitute a highly uncertain field of study (Peterson 1973; Skilling and Nicholls 1974; Gadgil 1977; Hoff 1985; Woods *et al.* 2005, 2010; Sturrock *et al.* 2011). This task is especially more complex in natural forest settings due to the long-lived nature of trees and the relative short lifespan of pathogens, providing pathogens a greater ability to

adapt to new climatic conditions (Sturrock *et al.* 2011; Burdon *et al.* 2012; Gray *et al.* 2013). In addition to changes in disease impact through pathogen-host interactions, changes in environmental factors have been shown to affect the distribution and potential migration of pathogens into new locations where the regional climate is more suitable for their survival and reproduction (Sturrock *et al*. 2011). As research continues to discuss the complexity of the impacts of climate change on disease severity, it is still prudent to continue investigating potential disease risks in forest ecosystems that are experiencing increases in both temperature and precipitation. Environmental factors such as summer precipitation, frequency of warm-wet days, overnight minimum and winter temperatures have been shown to be strong drivers in the development, spread, and virulence of foliar diseases (Coakley *et al.* 1999; Woods *et al.* 2005). Although environmental factors often have varied effects on different species of fungi, it is reasonable to assume that the environmental conditions put forth by Woods *et al.* (2005) would favor the four foliar fungi associated with WPND, as their study also demonstrated the effects of climate change on the development and spread of a foliar fungal pathogen.

Based on the information presented above, we hypothesize that WPND represents a new disease complex of eastern white pine and that a combination of novel fungal pathogens in the region coupled with gradual changes in temperature and precipitation that are driving this current epidemic. This hypothesis will be tested by completed the following three objectives: 1) expand field based sampling of WPND within Vermont, New Hampshire, Maine, and their neighboring states to construct a more detailed distribution of *L. acicola*, *B. linearis*, *L. dooksii*, and *S. strobus* throughout

the region; 2) determine which environmental factors are driving the emergence and spread of WPND; and 3) develop a predictive model for disease severity, to assess whether this outbreak will continue to increase or decrease in the coming years. The results of these objectives will assist foresters and private landowners in establishing policy and management recommendations for the control of WPND.

MATERIALS AND METHODS

Fungal isolate collection and mapping

During the months of April through June in 2011-2014 *P. strobus* stands across the northeastern U.S. (43.10° to 45.17° N, 69.36° to 72.77°W) were sampled for diseased needles, by the author, Isabel Munck, Forest Health State Coordinators, and Forest Watch schools (www.forestwatch.sr.unh.edu) (Fig. 4). Forest Watch is an educational research program that has reached out to teachers and students (grades K – 12) across New England to help teach and conduct basic and applied research on the impact of air-quality on forest ecosystems. Over the past 24 years Forest Watch participants have studied the impact of ground-level ozone of eastern white pines by quantifying chlorosis on needles and measuring needle length and needle retention. However, they generally only looked at the current year's needles, disregarding the 2nd and 3rd year needles. Since our analysis pertained more to the 2nd and 3rd year needles, these needles from eight Forest Watch schools in Maine and New Hampshire were utilized to increase our distribution sample size.

At each location, branch tips, from three symptomatic trees were randomly sampled and shipped to the USFS Durham field office in quart-size (1L) paper bags. Branch samples were first visually inspected for symptomatic needles containing obvious fruiting structures, tip necrosis, and discoloration. All symptomatic needles were removed for further identification of fungal pathogens, with emphasis on *L. acicola, B.*

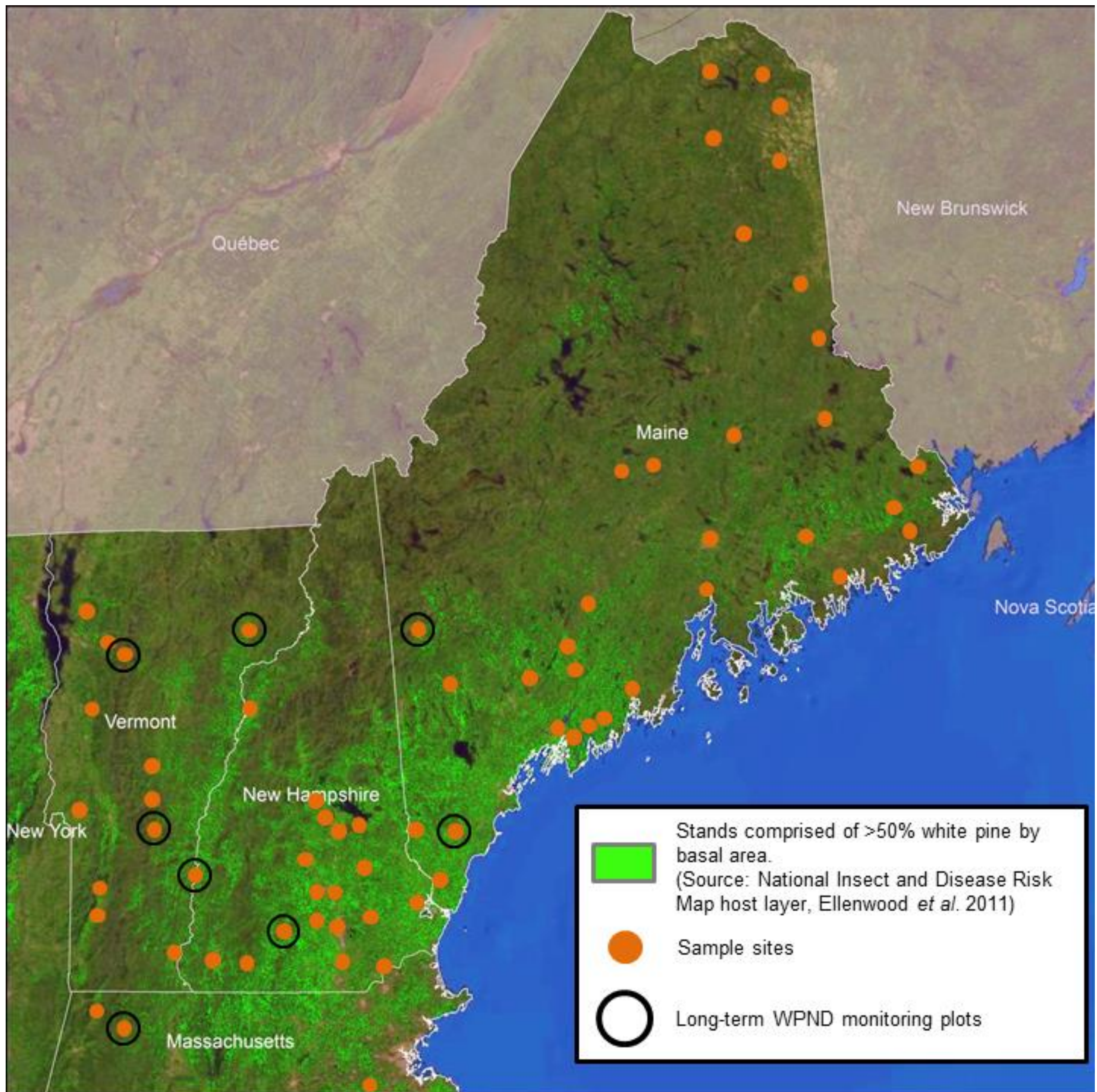

Figure 4: Site map of WPND sampled locations (orange circles) across the northeastern United States. Two separate sample sites are not depicted on this map; these sites were located in Lowville, NY and Scituate, RI. Black circles represent the locations of eight long-term WPND monitoring plots established in 2012. Background map was created by the USDA Forest Service using the National Insect and Disease Risk Map (NIDRM) eastern white pine host layer shows stands comprised of >50% white pine by basal area (bright green)

linearis, *L. dooksii*, and *S. strobus*. Twenty to thirty symptomatic needles per tree were placed in humid chambers and incubated at 25°C for no more than 48 hours. Humid chambers consisted of glass petri dishes with filter paper moistened with 500µL of sterile water sealed with Parafilm. Individual fruiting structures were then observed under a dissecting microscope, photographed, and subsequently transferred onto PDA plates. Fungal colonies were identified morphologically and were recorded as either being present or absent at all sampled locations. If morphological identification could not be made, fungi were identified based on their internal transcribed spacer (ITS) sequence, as described in Broders *et al.* (2015). Unidentified fungi were grown on PDA plates overlaid with cellophane (Flexel Sales Inc.). Under aseptic conditions mycelia was then gathered, frozen for 1 hour at -80°C, and freeze dried. DNA was extracted from the lyophilized mycelia using a modified CTAB extraction method (Kohler *et al.* 2011). PCR was performed in 28 µl reaction volume containing 6 µl of 5x Green GoTaq reaction buffer (Promega Corp.), 2.4 µl of 25 mM $MgCl_2$, 0.75 µl containing 10 mM each dNTP, 0.2 µl of GoTaq *Taq* DNA polymerase, 0.9 µl of each primer ITS1 and ITS4 (White *et al.* 1990) at 10 pmol $µl^{-1}$, 2 µl of 15 ng/µl DNA and 16.85 µl of sterile distilled water. Amplification conditions followed those provided by Broders *et al.* (2015). PCR products were purified using the E.Z.N.A Cycle Pure Kit (Omega Bio-Tek, Norcross, GA) and sent to Genewiz (South Plainfield, NJ) for sequencing. Raw sequences were manually adjusted and edited using BioEdit v7.2.5 and then blasted against NCBI's nucleotide GenBank database for closely related species.

The WPND distribution was mapped according to the presence or absence (pathogen prevalence) of *L. acicola, B. linearis*, *L. dooksii*, and *S. strobus*. The

frequency for each species was also recorded as disease incidence; whether species either occurred individually at sites or co-occurred with one or more of the other three species. A host layer risk map was created by the USDA Forest Service, with data from the National Insect and Disease Risk Map (NIDRM). The map displayed a host layer of stands compromised of >50% eastern white pine by basal area within the northeastern U.S. (Fig. 5) (Krist Jr. *et al.* 2014). Locations were plotted based on town centers of sampled sites or GPS coordinates, if available.

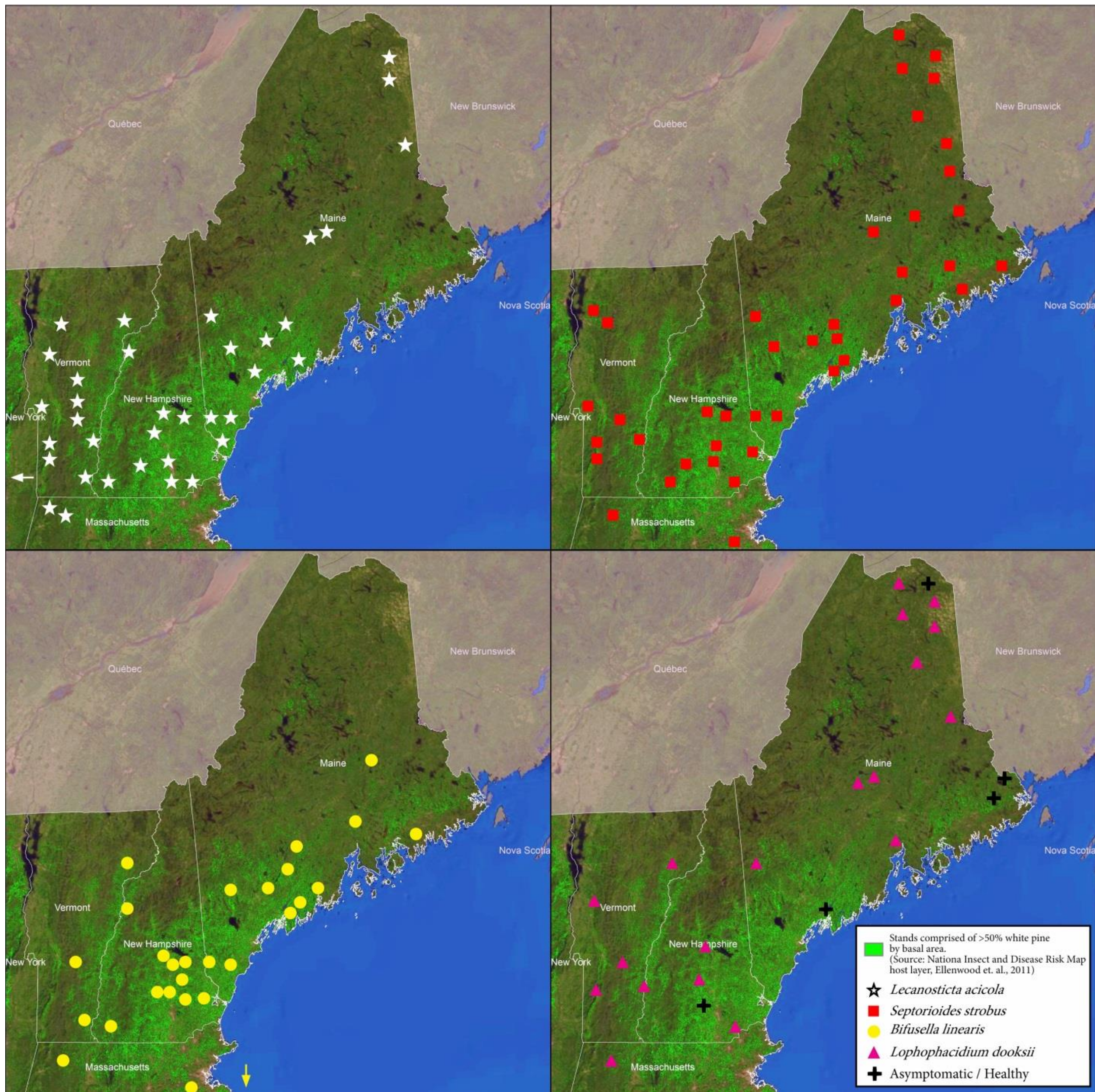

Figure 5: Distribution map of confirmed sightings (incidence) of *Lecanosticta acicola* (white stars), *Septorioides strobus* (red squares), *Bifusella linearis* (yellow circles), *Lophophacidium dooksii* (pink triangles), and asymptomatic/healthy sites (black crosses). Arrows indicate fungal presence outside the range of the map; white arrow corresponds to *L. acicola* in Lowville, NY and yellow arrow corresponds to *B. linearis* in Scituate, RI. Background map was created by the USDA Forest Service using of the National Insect and Disease Risk Map (NIDRM) eastern white pine host layer shows stands comprised of >50% white pine by basal area (bright green).

WPND monitoring and climatic records

In 2012 eight long-term monitoring plots, within WPND infected *P. strobus* stands, were established throughout the northeastern U.S. to determine the effect of repeated defoliation (Fig. 4). At these plots, pairs of mature trees were selected and tagged to monitor yearly crown yellowing and defoliation severity. Pairs were intended to include a healthy control and an infected tree. However, it was not always possible to find completely asymptomatic trees at these sites; therefore trees with the fullest and greenest crowns were treated as healthy controls. Yellowing and defoliation severity were ranked on an ordinal scale: 0: crown was asymptomatic; 1: if the lower one-third of the crown; 2: if two-thirds of the crown; and 3: if greater than two-thirds of the entire crown was symptomatic.

Weather data for the eight monitoring plots was obtained from land-based stations maintained by the National Oceanic and Atmospheric Administration (NOAA/National Climatic Data Center 2015). An interactive mapping tool provided by the National Climatic Data Center was utilized to locate land-based stations within a 15 mile radius of each site. Stations meeting the criteria were selected, and rainfall and minimum and maximum temperatures dating back to 1950 were used to create a climatic timeline for each site. Minimum and maximum temperatures were also averaged to estimate the daily average temperature. Data from multiple stations were combined and averaged to produce a more precise and complete timeline.

WPND climatic prediction model

To best quantify the influence of climatic factors on the defoliation severity caused by WPND, four year annual defoliation severity data (2012-2015) collected from the eight long-term monitoring plots was used as the basis for our disease prediction model. Broders *et al.* (2015) recently determined that pairs of both healthy controls and infected trees have experienced increased defoliation severity since establishment of the monitoring plots in 2012. For this reason healthy controls were no longer considered as such and were deemed infected, increasing the sample size, and allowing for a better evaluation of site defoliation severity. Preliminary research has also suggested that climatic conditions of the previous year (one-year lag time) were the best predictors of the following year's defoliation event (Broders *et al.* 2014).

For model preparation three primary climatic variables: precipitation, temperature, and relative humidity, were considered to be the most important factors affecting disease spread (Manter *et al.* 2005; Gray *et al.* 2013; Welsh *et al.* 2014). However, due to the lack of measurements at monitoring sites for years 2011 and 2012, relative humidity was excluded. Approximately 34 climatic variables were included for model parameterization. Cumulative precipitation (mm) variables included: spring (March-May), summer (June-August), modified spring (May-July), and for individual months within these seasons. Similarly, temperature (°C) variables included: overall mean as well as average minimum and maximum values for spring, summer, modified spring, winter (December-February) and for individual months within these seasons. Modified spring was chosen to be used as a separate variable because it more closely

correlated with the life cycle and sporulation peak of *L. acicola* (Siggers 1944; Skilling and Nicholls 1974; Munck *et al.* 2012; Broders *et al.* 2014).

Variable selection and model development was done using JMP Pro 11.2.1 (SAS Institute Inc.). A linear stepwise multiple regression analysis with a forward direction was performed in order to select for variables that most accurately predicted defoliation severity (Card *et al.* 1988; Liu *et al.* 2007). Several models were examined for collinearity between variables using the variance inflation factor (VIF) with a threshold of VIF<4, a more stringent threshold than the rule-of-thumb of 5-10 (Montgomery and Peck 1992). If collinearity was present the variable that contained the larger VIF factor was removed from future analysis. All remaining variables were used to fit all possible models. Several criteria were examined to identify the best model. The Akaike information criterion with small sample correlation (AICc) (Hurvich and Tsai 1989; Burnham and Anderson 2002), adjusted R^2, VIF factor of variables (VIF<4), and root mean square error (RMSE) were used to assess the models support of the data. The amount of factors present in the model was also considered when analyzing all possible models; preferred models had ≤5 factors. This was taken into account to provide a more simplified prediction model that could be used by foresters and private landowners to plan and manage for future defoliation events. The best models containing five, four, three, two and one factors were reported to permit flexibility so that landowners could use their discretion in choosing which model to follow based upon resources available to them.

RESULTS

Distribution of fungi associated with WPND

From 2011 to 2014 a total of 69 *P. strobus* stands were sampled across the northeastern U.S., encompassing Maine, New Hampshire, Vermont, Massachusetts, New York, and Rhode Island (Fig. 4, Table 5). Samples were taken from a total of 207 trees of all age classes in sites that ranged from natural wetlands to dry steep slopes to urban and rural areas.

The main focus of this sample collection was to determine the incidence and distribution of *L. acicola*, *B. linearis*, *L. dooksii*, and *S. strobus*, the primary fungal pathogens associated with WPND. However, in the process of isolating these fungi from diseased needles, a diverse range of other fungal species was also discovered. A total of 34 fungal species were identified from *P. strobus* needles using morphological and ITS sequence data, of which twenty two corresponded to those found by Broders *et al.* (2015). Based on megablast search results of closely related species from NCBI's GenBank nucleotide database 12 different species, other than those found by Broders *et al.* (2015), were observed. These species consisted of known pathogens, weak parasites, and endophytes. Known pathogens include *Diplodia pinea*, *Teratosphaeria microspore*, *Cytospora schulzeri*, *Schizothyrium pomi*, and *Pseudocercosporella fraxini*. The remaining species incorporate both weak parasites and endophytes; *Meloderma*

Table 5: Detailed locations of sampled sites showing presence or absence of *Lecanosticta acicola, Bifusella linearis*, *Lophophacidium dooksii*, and *Septorioides strobus*.

State	Location	County	WPND Fungal Species			
			L. acicola	***B. linearis***	***L. dooksii***	***S. strobus***
Massachusetts	East Taunton (Massasoit S.P.*)	Bristol		**X**		**X**
Massachusetts	Charlemont (Mohawk S.P.)	Franklin	**X**	**X**	**X**	**X**
Massachusetts	Williamstown	Berkshire	**X**			
Maine	Acton	York	**X**	**X**		**X**
Maine	Augusta	Kennebec	**X**	**X**		**X**
Maine	Bangor	Penobscot		**X**		**X**
Maine	Baring	Washington	Asymptomatic†			
Maine	Bath	Sagadahoc		**X**		**X**
Maine	Bethel	Oxford	**X**		**X**	**X**
Maine	Brunswick	Cumberland	Asymptomatic†			
Maine	Caribou	Aroostock	**X**		**X**	**X**
Maine	Cherryfield	Washington		**X**		**X**
Maine	East Machas	Washington				**X**
Maine	Guilford	Piscataquis	**X**		**X**	
Maine	Kossuth	Washington				**X**
Maine	Leeds	Androscoggin	**X**	**X**		**X**
Maine	Lincoln	Penobscot		**X**		**X**
Maine	Littleton	Aroostock	**X**			**X**
Maine	Lyman	York	**X**	**X**		**X**
Maine	No. 14 Cathance Township	Washington	Asymptomatic†			
Maine	North Yarmouth	Cumberland	**X**			
Maine	Norway	Oxford	**X**	**X**		**X**
Maine	Orient	Aroostock			**X**	**X**
Maine	Pittston	Kennebec				**X**
Maine	Portage Lake	Aroostock			**X**	**X**
Maine	Presque Isle	Aroostock	**X**		**X**	**X**
Maine	Prospect	Waldo			**X**	**X**
Maine	Sangerville	Piscataquis	**X**		**X**	**X**
Maine	South Berwick	York	**X**			
Maine	T8 R5 W.E.L.S	Aroostock			**X**	**X**
Maine	Township 22	Hancock				**X**
Maine	Waldoboro	Lincoln		**X**		
Maine	Wallagrass	Aroostock			**X**	**X**
Maine	Waterville	Kennebec		**X**		
Maine	Woolwich	Sagadahoc	**X**	**X**		**X**
Maine	T16 R4 W.E.L.S	Aroostock	Asymptomatic†			

Table 5: (continued)

State	Location	County	WPND Fungal Species Fungal Species			
			L. acicola	*B. linearis*	*L. dooksii*	*S. strobus*
New Hampshire	Alton	Belknap	X	X		X
New Hampshire	Allenstown (Bear Brook S.P)	Merrimack		X		
New Hampshire	Concord	Merrimack		X		X
New Hampshire	Dublin	Cheshire				X
New Hampshire	Dunbarton	Merrimack	X			X
New Hampshire	Durham (USDA)	Strafford		X		X
New Hampshire	Gilmanton	Belknap		X		
New Hampshire	Hillsboro (Fox Forest S.P.)	Hillsborough	X			X
New Hampshire	Hopkinton (Mast yard S.P.)	Merrimack		X		
New Hampshire	Keene	Cheshire	X	X		
New Hampshire	Loudon	Merrimack		X		
New Hampshire	Merrimack	Hillsborough	X			
New Hampshire	New Hampton	Belknap			X	
New Hampshire	Pike	Grafton		X		
New Hampshire	Salem	Rockingham	X		X	X
New Hampshire	Sanbornton	Belknap	X	X		X
New Hampshire	Weare (Clough S.P.)	Hillsborough	Asymptomatic†			
New Hampshire	Webster (Black Water Reserve)	Merrimack	X		X	
New Hampshire	Woodsville	Grafton	X			
New York	Lowville	Lewis	X			
Rhode Island	Scituate	Providence		X		
Vermont	Bethel	Windsor	X			
Vermont	Brookfield	Orange	X			
Vermont	Castleton	Rutland	X			X
Vermont	Dorset	Bennington	X		X	X
Vermont	Dummerston	Windham	X	X		
Vermont	Lincoln	Addison	X		X	
Vermont	St. Johnsbury (Lyndon S.P.)	Caledonia	X	X	X	

Table 5: (continued)

State	Location	County	WPND Fungal Species			
			L. acicola	*B. linearis*	*L. dooksii*	*S. strobus*
Vermont	Manchester	Bennington	**X**			**X**
Vermont	Plymouth	Windsor	**X**	**X**	**X**	**X**
Vermont	Springfield	Windsor	**X**		**X**	**X**
Vermont	Underhill	Chittenden				**X**
Vermont	Waterbury	Washington	**X**			**X**
Total sightings			**37**	**28**	**19**	**41**

* S.P. = State Park

† Asymptomatic signifies locations where none of these four species were present

desmazieresii, *Scleroramularia asiminae*, *Epicoccum nigrum*, *Tumularia aquatic*, *Geastrumia polystigmatis*, *Lachnum virgineum*, and *Trichomerium spp.* Further phylogenetic analysis of these species were not carried out in this study as these species were isolated infrequently and generally occurred at no more than two sites. In respect to the abundance of fungal species *S. strobus*, *L. acicola*, *B. linearis*, and *L. dooksii* were the most common species identified, with confirmed detections at 41 (59%), 37 (54%), 28 (41%), and 19 (28%) of the sites, respectively (Table 5, Fig. 5 & 6). These species were followed in abundance by several *Lophodermium spp.*, *an Allantophomopsis sp.*, and a Massarinaceae species which was morphologically determined to be *Hendersonia pinicola*, with confirmed detections at 26 (38%), 17 (25%), and 6 (9%) of the sites, respectively (data not shown). However, since these species are more commonly regarded as secondary invaders or weak pathogens they were not considered to be causing the needle defoliation that is associated with the current WPND outbreak and their distributions were not mapped (Banfield 1960, 1962; Darker 1967; Dreisbach 1989; Carris 1990; Putnam 2004).

Results indicated that not one of the four main fungal species associated with WPND was found at all sample sites. Five of the sites were found to be disease-free (no presence of any of the four primary fungal species) while *S. strobus, L. acicola, B. linearis,* and *L. dooksii* or combinations of two three or all four species were detected in the other 64 sites (Table 5, Fig. 7). None of these species had a dominating presence (>50%) at sites containing only one species; with sole sightings of *L. acicola* and *B. linearis* at eight locations, *S. strobus* at six locations, and *L. dooksii* at one location (Table 5, Fig. 7). These individual occurrences totaled 23 sites, 36% of diseased sites,

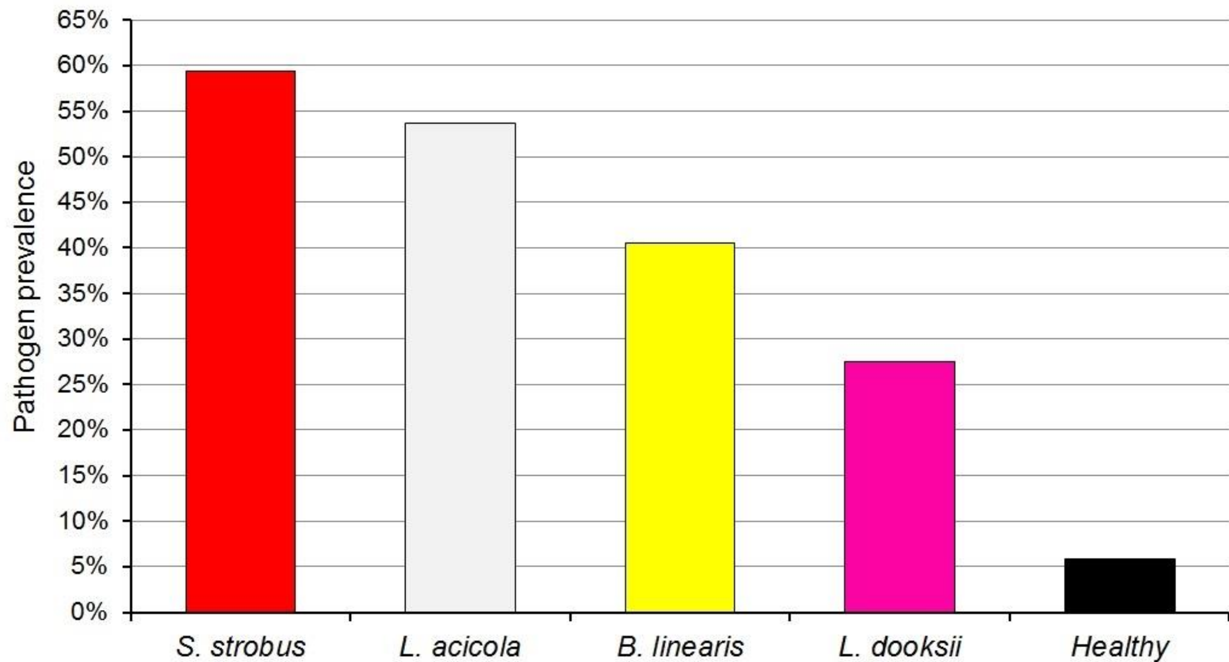

Figure 6: Pathogen prevalence of *Septorioides strobus*, *Lecanosticta acicola*, *Bifusella linearis*, *Lophophacidium dooksii*, and healthy sites as percent of total of 69 sampled locations.

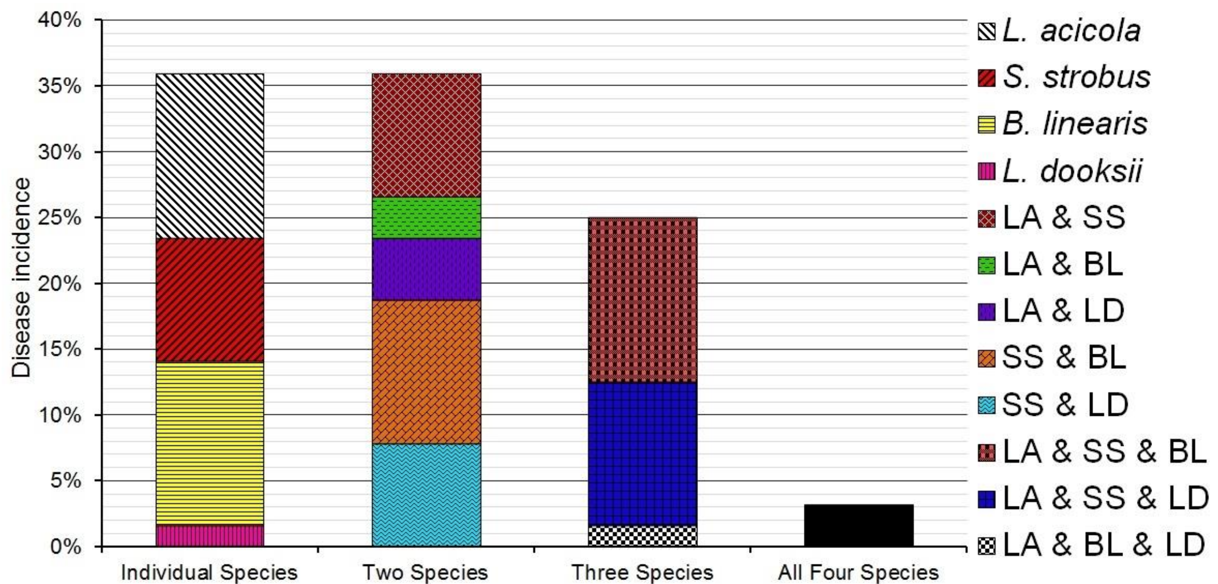

Figure 7: Pathogen prevalence of each species and all species combinations (one to all four species present) of *Septorioides strobus* (SS), *Lecanosticta acicola* (LA), *Bifusella linearis* (BL), *Lophophacidium dooksii* (LD) as percent of total of 64 infected sites.

while incidences where more than one species was present totaled 41 sites, 64% of diseased sites (Table 5, Fig. 7). Evidently, none of these sites with co-occurring fungi contained a combination of species that had a dominating presence. At best, both *L. acicola* and *S. strobus* were the most frequently co-occurring species; being found together at six sites and at seventeen sites with either *B. linearis*, *L. dooksii,* or both fungi present (Table 5, Fig. 7).

Geographically, *S. strobus*, *L. acicola*, *B. linearis*, and *L. dooksii* were found throughout Maine, New Hampshire, Vermont and Massachusetts. *Lecanosticta acicola* was found at the New York site and *B. linearis* was found at the Rhode Island site (Fig. 5). Clustered sightings of *L. acicola*, *S. strobus*, and *B. linearis* occurred in central to southern Vermont and New Hampshire and in southcentral to southwestern Maine, corresponding to a vast forested area with stands comprising >50% eastern white pine by basal area. *Bifusella linearis* occurred more frequently in coastal areas with fewer sightings in Vermont and no sightings in northern Maine. *Septorioides strobus* was more frequently found in eastern and northern Maine, more than any of the other species, and had the largest latitudinal range of 5.3° from Massasoit State Park in East Taunton, MA to Wallagrass, ME.

Effects of climate on the spread on WPND

The northeastern U.S. has been experiencing an increase in average temperature (~1°C) and precipitation (~10mm) during the growing season (April-September) since 1950 (Fig. 8). More recently, this region has experienced above average precipitation and temperature from 2003-2014, with six years being ranked in

the top 10% for precipitation, with 2011 being the wettest year on record, and four years ranked in the top 10% for temperature (NOAA/National Climatic Data Center 2015). These climatic shifts provide the region with favorable weather conditions that promote the development and spread of foliar fungal pathogens.

Our data indicates that cumulative precipitation during the modified spring season (May-July) was positively correlated with, and determined to be the best individual climatic predictor for the following year's WPND defoliation event ($R^2 = 0.223$, $p = 0.0037$) (Table 6). This factor was also represented in the 4-, 3- and 2-factor models, but was replaced by cumulative summer (June-August) and spring (March-May) precipitation in the 5-factor model. All models and variable coefficients were found to be significant in predicting defoliation severity caused by WPND ($p < 0.01$). All model residuals were normally distributed showing no bias with either predicted or independent variables (data not shown). While some models contained individual month variables instead of full season variables, all models exhibited a trend that cumulative precipitation during the months of May, June, and July as well as warmer winter and cooler spring temperatures were the most important factors affecting disease outbreak in the following year. The 5-factor model was the best-fit model, meeting all criteria considered by the authors (Adj. $R^2 = 0.565$, $p = <0.0001$) (Table 6, Fig. 9).

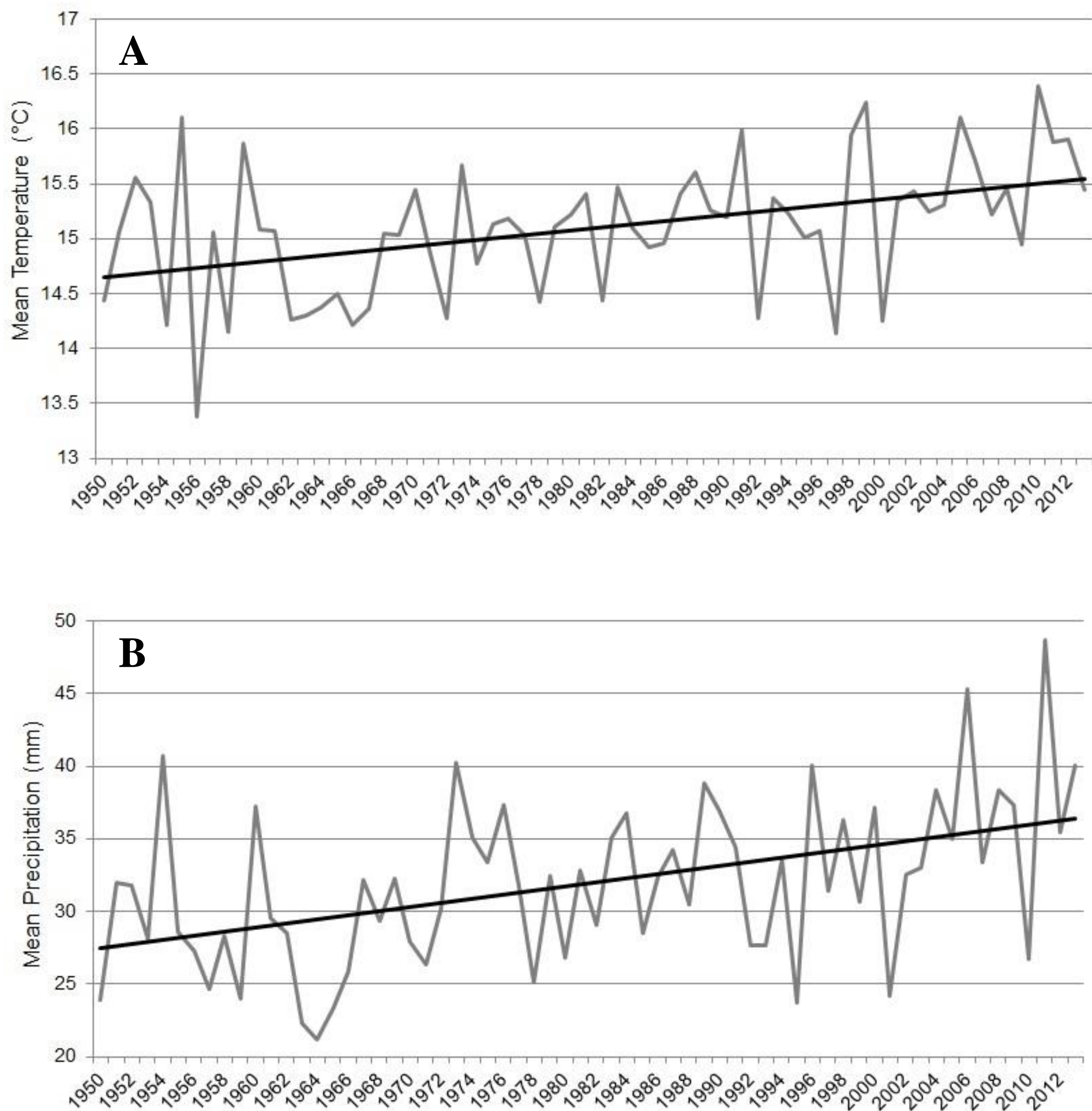

Figure 8: Northeastern regional mean temperature (A) and precipitation (B) during eastern white pine growing season (April – September) from weather station within a 15 mile radius around eight WPND long-term monitoring sites across the northeastern United States from 1950-2014.

Table 6: Five best-fit climatic regression models to predict defoliation severity of eastern white pine (*Pinus strobus*) by WPND for the following year.

Model, variables*	Parameter estimate	SE†	Prob. > t	VIF‡	Model Prob. > F	Adj. R^2	RMSE	AICc††
5-Factor								
Intercept	0.08							
Sum summer P	0.0029	0.00064	0.0001	1.3				
Sum spring P	-0.0048	0.00083	<.0001	2.12				
Sum May P	0.0081	0.0018	<.0001	2.38				
Avg. max winter T	0.19	0.048	0.0006	3.18				
Avg. min March T	-0.086	0.019	0.0001	3.00				
Total model					<0.0001	0.565	0.253	14.89
4-Factor								
Intercept	0.0053							
Sum mod. spring P	0.0033	0.0008	0.0003	1.59				
Sum April P	-0.0032	0.0013	0.0198	1.13				
Avg. max winter T	0.18	0.049	0.0010	2.95				
Avg. min March T	-0.062	0.018	0.0014	2.31				
Total model					<0.0001	0.507	0.27	16.79
3-Factor								
Intercept	-0.0083							
Sum mod. spring P	0.0036	0.0008	0.0001	1.44				
Avg. max winter T	0.195	0.054	0.0011	3.13				
Avg. min spring T	-0.13	0.039	0.0027	2.51				
Total model					0.0002	0.445	0.286	18.63
2-Factor								
Intercept	-0.82							
Sum mod. spring P	0.0028	0.00077	0.0010	1.045				
Avg. max April T	0.089	0.044	0.0533	1.045				
Total model					0.0024	0.295	0.322	24.63
1-Factor								
Intercept	0.41							
Sum mod. spring P	0.0025	0.00079	0.0037*	-				
Total model					0.0037	0.223	0.339	26.19

* Season measurements were averaged over three months in the year preceding defoliation ratings: winter (December, January, February); spring (March, April, May); mod. Spring (May, June, July); summer (June, July, August). P = cumulative precipitation, T = mean temperature

† Standard error.

‡ VIF = variance inflation factor, variables with values <4 were considered not to be collinear.

†† AICc = Akaike information criterion with small sample correlation.

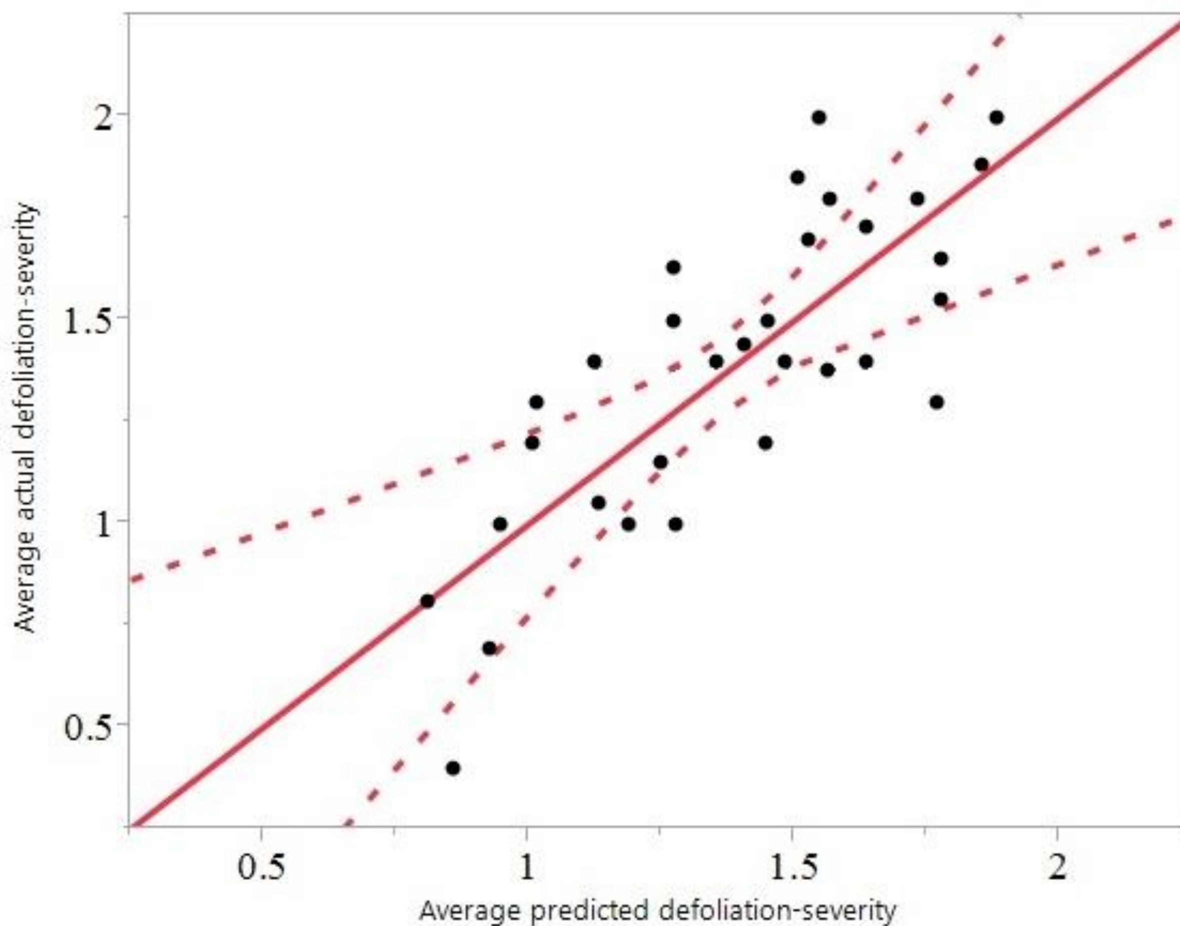

Figure 9: Best fit 5-factor climatic model for data from eight long-term WPND monitoring plots across the northeastern U.S. from 2012-2015. Comparison is between actual and predicted defoliation severity (1-3 ordinal rating) of eastern white pine (*Pinus strobus*) by WPND. Model equation [Y = 0.08 + (0.0029*sum summer P) + (-0.0048*sum spring P) + (0.0081*sum May P) + (0.19*avg. max winter T) + (-0.086*avg. min March T) Adj. $R^2 = 0.565$, $p = <0.0001$]. Dotted lines signify a 95% confidence interval.

DISCUSSION

Our results confirmed the hypothesis that WPND is a disease complex consisting of several fungal species, most notably *Lecanosticta acicola*, *Bifusella linearis*, *Lophophacidium dooksii*, and *Septorioides strobus* as all four species were found throughout the northeastern U.S., occurring at a variety of sites and on a variety of tree age classes. Neither individual nor co-occurring combinations of these species were found to exhibit a dominating presence in any particular state or region; therefore, diagnosticians and foresters should examine trees for the presence or absence of all four of these fungal species, to better determine and evaluate management strategies for a particular site. Furthermore, our expanded sampling has revealed a greater distribution of WPND than what has been previously reported (Broder *et al.* 2015). Ground based sampling has confirmed occurrences of WPND in New York, Massachusetts, and Rhode Island. Although these reports comprise only 5 sites and thus do not give a sufficient representation of disease severity in these states, they do provide evidence of the presence of WPND in these states, and emphasize the need for further sampling throughout these states. Similarly, sampling efforts throughout Maine confirmed presence of WPND that was suggested through aerial surveys conducted from 2010 – 2011 (USDA Forest Service, FHM. 2015.), and also confirmed incidences of WPND in northern and eastern Maine that were not observed in the aerial surveys. Since 2010, aerial surveys showed a decrease in mapped damage, while personal

reports of WPND from foresters and private land owners have only continued to increase (Adams per.comm.; Munck per. comm.; Ostrofsky per. comm.), suggesting that sketch mapping aerial surveys could be underestimating damage caused by WPND. This could be due to surveys being conducted either before or after the manifestation of symptoms attributed to WPND or by a lack of visible evidence as infections are more likely to be found occurring on the lower portion of tree crowns making it difficult to detect from the air (Munck *et al.* 2012; Broders *et al.* 2015). While aerial surveys can be useful when mapping certain forest health risks, it is the authors' opinion that damage caused by WPND is particularly difficult to map from the air and should be examined primarily through ground based sampling.

While ground based sampling alone can have its discrepancies, previous research has shown that it can be improved through the use of host layer mapping (Guo *et al*. 2005; Kelly *et al.* 2007). Together these can provide a better representation of potentially infected areas by determining high risk zones, locations where there are an abundance of susceptible host species. This application has been demonstrated by Guo *et al*. (2005) and Kelly *et al.* (2007) who showed that similar methods were useful in determining high risk locations, predicting the distribution, and future spread of sudden oak death (*Phytophthora ramorum*) in California. Our host layer map (Fig. 5) has revealed that the majority of New Hampshire, eastern Vermont, northern Massachusetts, and southwestern Maine are heavily forested with eastern white pine. Although this amount of white pine was anticipated as Vermont, New Hampshire, and Maine are all top producers of eastern white pine lumber, it was surprising to see a contiguous span of eastern white pine that connected all four states. Needle samples

from within this area, as well as other heavily forested white pine regions in eastern Maine and western Vermont, have revealed numerous incidences of WPND, suggesting that stands comprising >50% eastern white pine by basal area should be considered high risk locations. However, sample locations from northern Maine indicate that WPND is present in stands that contain less than 50% eastern white pine. Therefore, further sampling of varied stand densities and subsequent mapping on different basal area host layers would help determine exactly which stand densities should be considered high risk. Nonetheless, it would be reasonable to conclude that this large span of eastern white pine may act as a conduit facilitating the spread of these fungal species throughout the northeastern U.S., particularly *L. acicola* and *S. strobus* which have recently become established in this region (Munck *et al.* 2011; Broders *et al.* 2015).

The establishment of this high density belt of eastern white pine within the northeastern U.S. was not a recent establishment as both New Hampshire and Maine were the top two producers of white pine lumber as far back as the 1940s (Betts 1954). Therefore, the current WPND outbreak was not incited by a recent increase in host abundance, but, an abundance of host species is necessary for a disease epidemic to occur (van Maanean and Xu 2003). When sufficient host species are present, several other factors are of primary concern when determining the driving force behind a recent emergence of disease outbreak, such as inoculum loads (van Maanean and Xu 2003), dispersion patterns of spores (Ingold 1971, 1978), and most importantly environmental changes which directly affects both spore development and dispersal (Ingold 1971, 1978; Fitt *et al.* 1989; Rabbinge and Bastiaans 1989; Lacey 1996; Hardwick 1998; de Vallavieille-Pope *et al.* 2000; Woods *et al.* 2005). While inoculum loads are perhaps the

most basic requirement for an epidemic and are correlated to the abundance of host species available (van Maanean and Xu 2003), we can safely assume that the high density of eastern white pine in the region has allowed for an increasing amount of inoculum. This high density of eastern white pine also favors the potential distribution of these foliar fungi (Ingold 1978) as all four species are dispersed through rain-splash (Skilling and Nicholls 1974; Merrill *et al.*1996; Sinclair and Lyon 2005; Wyka and Broders *Submitted*).

While the effect of climatic variables on the dispersal patterns and development of *L. dooksii*, *B. linearis* and *S. strobus* are unknown, current research is underway in determining what climatic variables precisely affect the dispersal pattern and development of *L. acicola* on eastern white pine (Chapter 3). Despite this fact, certain climatic parameters, specifically increases in summer precipitation, frequency of warm-wet days, overnight minimum and winter temperatures have been shown to favor the development, spread, and virulence of foliar diseases (Coakley *et al.* 1999; Woods *et al.* 2005). Our results largely agree with these findings as our 5-factor model was significantly able to predict the following years WPND defoliation severity based upon the previous years' maximum winter temperatures, minimum spring temperatures, and spring and summer precipitation (Table 6).

Based upon these results, we conclude that the current outbreak of WPND in the northeastern U.S. is due to the singular and combined effects of four fungal species, *Lecanosticta acicola*, *Bifusella linearis*, *Lophophacidium dooksii*, and *Septorioides strobus.* A naturally high density of eastern white pine within this region, coupled with increases in annual temperatures and precipitation has provided a favorable

environment for these foliar species to become emergent within this region and to cause disease on eastern white pine. We believe these factors specifically allowed for increased inoculum loads of both *B. linearis* and *L. dooksii* which have been present on *P. strobus* throughout the region since the early 1900s (Wenner and Merrill 1998), as well as providing the necessary ecological requirements for the rapid development and dispersal of *L. acicola* and *S. strobus* throughout the region, as both of these species have only recently been reported causing widespread damage in the northeastern U.S. (Munck *et al.* 2011; Broders *et al.* 2015). The current WPND epidemic will likely increase in coming years as current climate scenarios predict increases in annual spring and summer precipitation and warmer winter temperatures (Frumhoff *et al.* 2007; Campbell *et al.* 2010, 2011) in the northeastern U.S., as these factors directly affect inoculum loads and spore dispersal (Ingold 1971; Fitt *et al*. 1989; Lacey 1996; de Vallavieille-Pope *et al.* 2000). In addition, we suggest that future surveys of WPND should be conducted primarily via ground-based sampling coupled with host layer mapping of eastern white pine stand densities. Currently stands that comprise >50% eastern white pine by basal area should be considered high risk for potential WPND infections. Continued sampling throughout this region, especially in western Maine, northern New Hampshire, northern Massachusetts, New York, Connecticut, and Rhode Island with subsequent plotting on varied host layer stand density maps would provide a better resolution, allowing foresters to determine whether stands comprising <50% eastern white pine should also be considered high risk. Lastly, we believe that the mutual use of both host layer mapping and our climatic predictive

models will provide the necessary requirements to guide foresters and private landowners in establishing management and policy recommendations for the control of WPND.

CHAPTER 3

Epidemiology of *Lecanosticta acicola* from eastern white pine (*Pinus strobus* L.) in Southern Maine.

INTRODUCTION

Previous research on the white pine needle damage (WPND) that has been affecting eastern white pines across the northeastern United States has revealed that four fungal species *Lecanosticta acicola*, *Bifusella linearis*, *Lophophacidium dooksii*, and *Septorioides strobus* are primarily responsible (Munck *et al.* 2012; Broders *et al.* 2015; Wyka and Broders *Submitted*). While previous studies have mainly focused on monitoring and determining which fungal pathogens were causing WPND, it is now crucial to begin to understand the epidemiology, or specifically, the effects of environmental factors on the development and dispersal patterns of the four fungal species associated with WPND. Of these species *Lecanosticta acicola* is considered to be the primary fungal pathogen attributing to disease symptoms on eastern white pine (*Pinus strobus*) (Broders *et al.* 2015), and is thus the focal species of this study.

Lecanosticta acicola, a foliar pathogen causing brown spot needle blight, is known to infect up to 28 species of pine across a large geographic range including

North America, Central America, Europe, Asia, and South Africa (Skilling and Nicholls 1974; Phelps *et al.* 1978; OEPP/EPPO 2005; Jankovsky *et al.* 2009, Jurc & Jurc 2010; Hintsteiner *et al.* 2012; EPPO/CABI 2013). However, brown spot needle blight is most notably known for its outbreaks in the Southeast and northern Midwest United States where it has caused devastating economic loses to both longleaf pine (*Pinus palustris* Mill.) and scotch pine (*Pinus sylvestris*) plantations (Siggers 1944; Skilling and Nicholls 1974; Phelps *et al.* 1978; Huang *et al.* 1995; Heimann *et al.* 1997) These outbreaks have thus led to the majority of research on the etiology and epidemiology of *L. acicola* to be conducted on scotch pine and longleaf pine (Kais 1971, 1975; Skilling and Nicholls 1974; Phelps *et al.* 1978; Heimann *et al.* 1997). Skilling and Nicholls (1974) demonstrated that mature acervuli of *L. acicola* on scotch pine release conidia (asexual spores) upon rainfall from June-September, with major infections occurring during June and July, with no ascospores (sexual spores) being reported throughout their study. *Lecanosticta acicola* conidia penetrate the needle through the stomata (Appendix C). Rainfall, moisture, and relative humidity were found to be necessary for spore dissemination, production, and germination (Kais 1975; EPPO/CABI 2013). Infected scotch and longleaf pine needles begin to show symptoms consisting of yellow bands fading to light brown with a dark brown or orange border within 4-7 months on older foliage and 1-2 months on younger needles (Phelps *et al.* 1978; EPPO/CABI 2013). Needles of all ages begin to die back from the tips until the entire needles turns brown and are cast during normal needle drop in October and November (Skilling and Nicholls 1974; Phelps *et al.* 1978; EPPO/CABI 2013). Skilling and Nicholls (1974) further discovered that *L. acicola* varied in virulence on different pine species; scotch pine was

found to be moderately resistant to susceptible, while longleaf and eastern white pine were found to be highly to moderately resistant.

However, these reports are not consistent with the observations documented for WPND-infested eastern white pines of this region, as symptomatic needles turn yellow (chlorosis) and are cast in mid-June to early July (Munck *et al.* 2012). Kais (1971) documented that northern races of *L. acicola* were not as pathogenic on longleaf pine as southern races, suggesting that eastern white pine could be more susceptible to another race, than the northern race Skilling and Nicholls (1974) used to test virulence. In scotch pine and longleaf pine, *L. acicola* infects needles in the spring, causing symptoms to develop in early to late August, and needles to drop in the autumn (Kais 1971, 1975; Skilling and Nicholls 1974; Phelps *et al.* 1978; Heimann *et al.* 1997). On eastern white pine, *L. acicola* symptoms have only been observed on 2nd and 3rd year needles (one- and two-year old needles) after a full year incubation period, with defoliation occurring the following spring and not during the autumn immediately following infection (Munck *et al.* 2012). Additionally, infection is generally localized to the lower portion of the tree crown with disease slowly progressing upwards over several consecutive years of defoliation (Munck *et al.* 2012). This disease leaves eastern white pines with thin crowns, with infected branches that contain only the new emergent needles of the current growing season, which were infected during late May and June by the 2nd year needles prior to their defoliation (Munck *et al.* 2012). Similarly, La Porta and Capretti (2000) observed that *L. acicola* affected only the 2nd and 3rd year needles on mountain pine (*Pinus mugo*) with symptoms arising in the spring and initially occurring on the bottom portion of the crown. Other variations in the onset of brown spot

needle blight between tree species and in the degree of virulence between *L. acicola* isolates, even within a specific geographic area, have been indicated by numerous studies and reports (Skilling and Nicholls 1974; Phelps *et al.* 1978; Holdenrieder and Sieber 1995; Huang *et al.* 1995; La Porta and Capretti 2000; Jankovsky *et al.* 2009; Jurc & Jurc 2010). While eastern white pine was initially found to be highly to moderately resistant to *L. acicola* infection, studies indicate that a change in regional climate producing a more favorable environment for foliar fungal pathogens provided the necessary ecological requirements for *L. acicola* to develop more readily and become established in the region (Munck *et al* 2012; Broders *et al*. 2015). Therefore, we hypothesize that *L. acicola* will exhibit a difference in the onset of disease, development, and dispersal patterns on mature eastern white pine than what has been previously documented for scotch and longleaf pine. To test this hypothesis we will study *L. acicola* both: *in situ*, by establishing a spore trapping field site to determine how climatic variables affect the abundance and distance of spores dispersal; and *in vitro*, by examining how temperature affects growth rates of *L. acicola* isolates from across the northeastern United States.

MATERIALS AND METHODS

Spore sampling and weather data

This study was conducted at the Massabesic Experimental Forest (MEF), a research forest owned and operated by the Northern Research Station of the USDA Forest Service, in Lyman, Maine (Fig. 10). The land is relatively flat with elevation ranging from 200ft (61m) to 450ft (137m), with stony to sandy loam soils of glacial origin over granite bedrock (Stone 2006). After a fire in 1947 that burned much of the land, the forest regenerated to hardwoods and eastern white pine (Stone 2006). A recent thinning of eastern white pine within the past decade left three clear cuts approximately 400ft (122m) in diameter. Regeneration within these clear cuts consisted mainly of: common woody shrubs such as winterberry, witch-hazel, and sheep-laurel; common herbaceous plants such as mayflower, bracken fern, and raspberry; hardwoods; and eastern white pine. During our study period (spring and summer of 2014 and 2015) however, white pine regeneration never exceeded 8ft (2.4m). In 2012, the MEF was designated as a representative forest affected by the current WPND outbreak (Munck *et al*. 2012). Sixteen trees were selected for annual monitoring of yellowing and defoliation severity caused by WPND, GPS coordinates were recorded with a handheld GPS unit (Garmin, GPSmap 62s) for each tree. Three trees infected with *Lecanosticta acicola* (#670, #676,

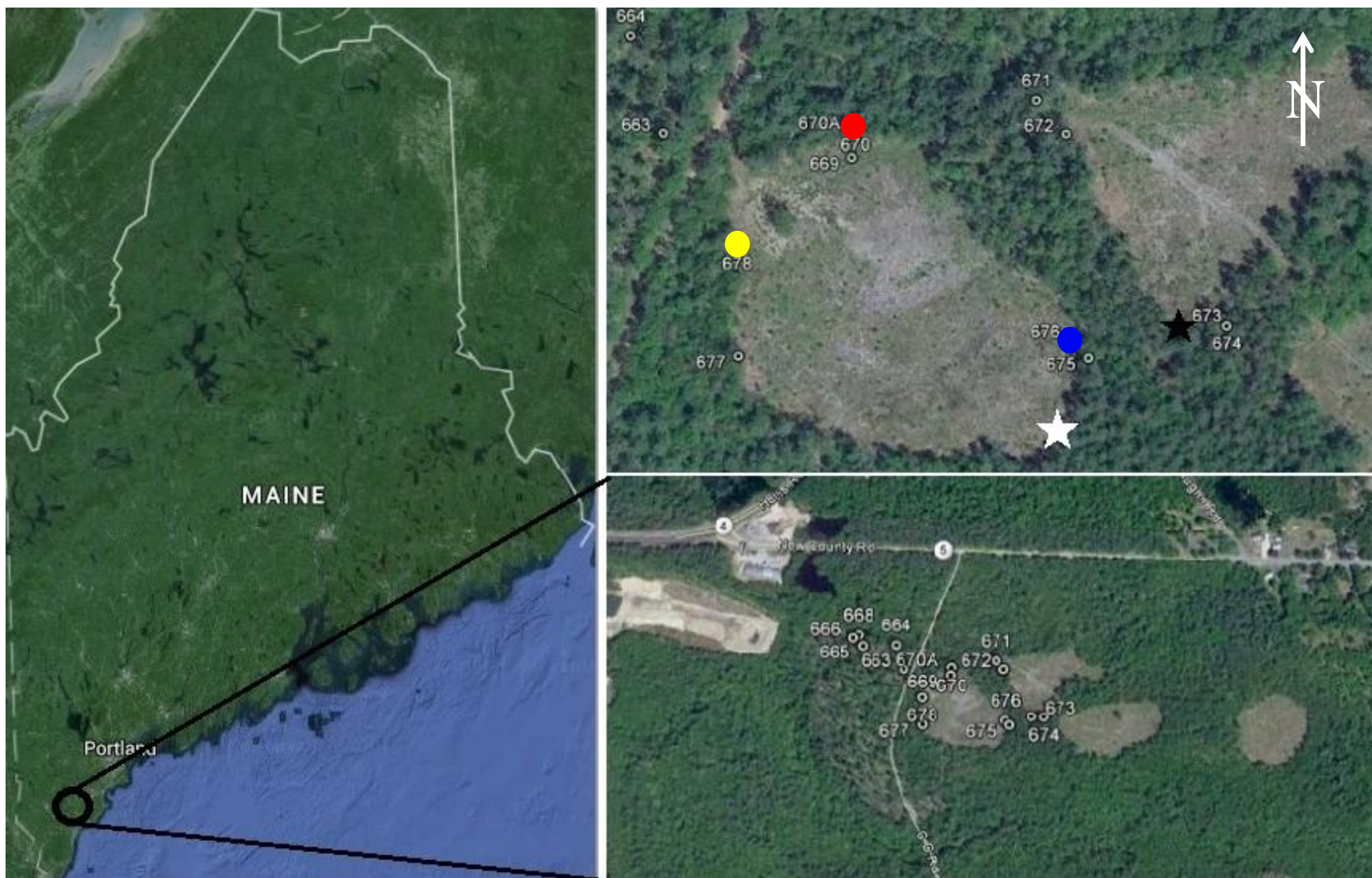

Figure 10: Map of Maine (left) with the location of the Massabesic Experimental Forest (MEF). MEF showing the clear cuts and tagged trees used by the USDA Forest Service for annual defoliation ratings (bottom right). Clear cut and trees used for spore sampling study site (top right) and the position of infected source trees (#670, red circle; #676, blue circle; and #678, yellow circle). Stars represent HOBO data loggers; white represents HOBO placed in the canopy, black represents HOBO place at ground level which was used for 2015 relative humidity measurements.

and #678) were bordering the larger clear cut and were thus chosen as source trees for a spore dispersal study (Fig. 10).

Ten spore trapping supports were constructed to passively sample spores (Fig. 11). Supports were 6ft (1.8m) high and contained four branched supports on top that were angled at a 45° slant towards the tree, except for one central support which laid flat. Each of the four supports contained a microscope slide with a drawn sampling surface area of $10cm^2$ divided into four quadrants covered by a thin coat of petroleum jelly (Vaseline) (Podger 1978; Bingzhang *et al.* 1992; Boateng and Lewis 2014). Three supports were arranged in a line from the source tree towards the center of the clear cut, making sure to avoid tall white pine regeneration. The first support was placed at 0ft (0m) from the furthest reach of tree's branches; the subsequent two supports were placed at 10ft (3.03m) intervals behind the first. One support was placed in the center of the clear cut, approximately 200ft (60.6m) from the source trees, to determine if spores were capable of dispersing this distance. All microscope slides were collected and replaced with new slides at 4 day intervals from May 5th to August 29th 2014. The sampling period in 2015 was shortened from May 5th to August 1st based on results obtained in 2014. Slides were examined under a compound microscope at 100x magnification to count the number of spores present in two randomly selected quadrants of the $10cm^2$ sampling area (Boateng and Lewis 2014). Microscope slides that collected fallen infected needles, with sporulating *L. acicola* acervuli, were not included in analysis as these slides exhibited an excess amount of spores and were deemed outliers. Conidia were identified based on the morphological description of

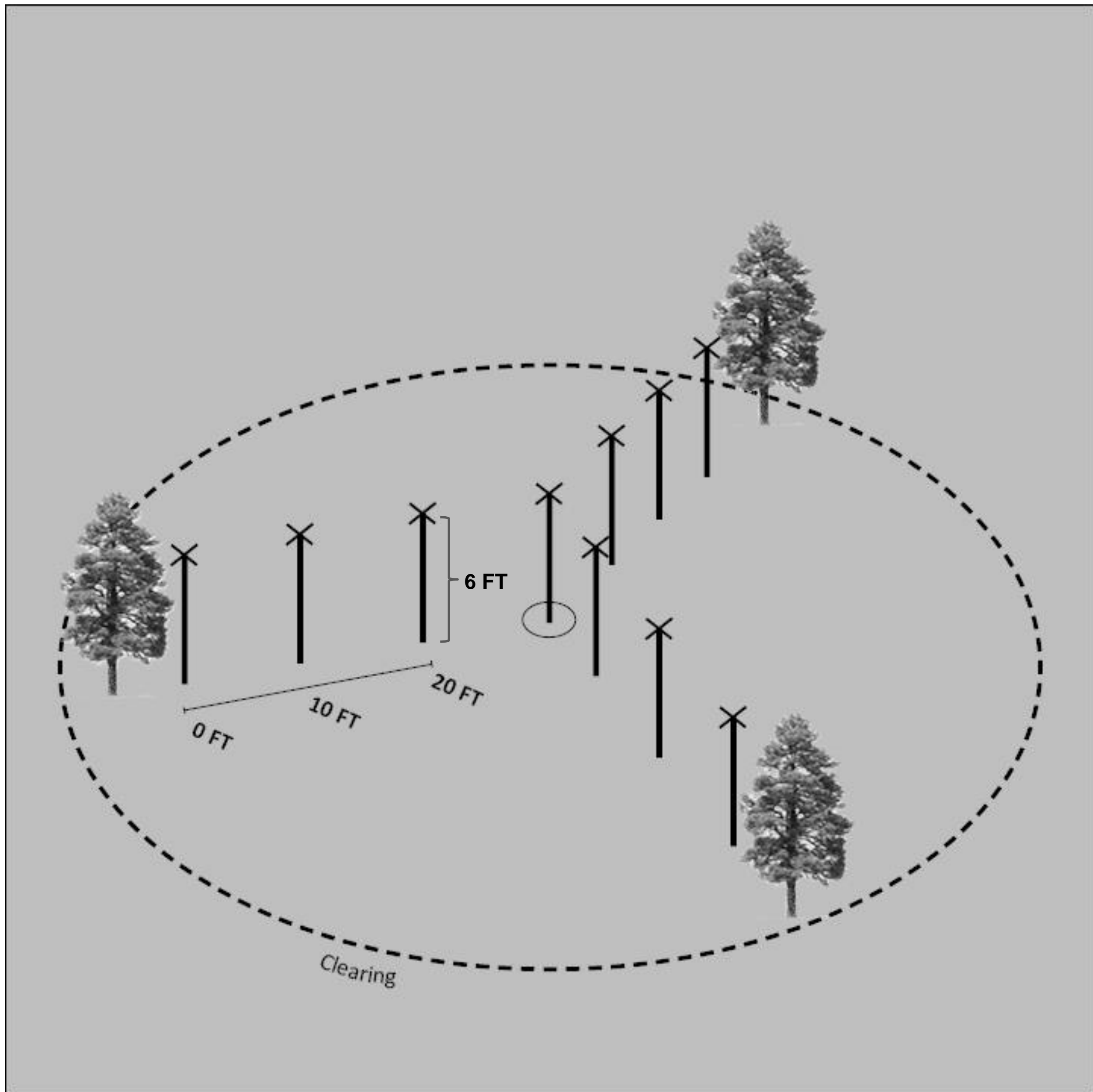

Figure 11: Pictorial diagram of spore trap support set-up based on three source trees bordering a clear cut used to determine the dispersal distance of *Lecanosticta acicola* spores at the Massabesic Experimental Forest in Lyman, ME in 2014 and 2015. Spore trap supports at 0ft, 10ft, and 20ft had mounting heads that were slanted at a 45° angle towards the source tree. The central spore trap support was approximately 200ft away from any source tree and contained a flat mounting head.

L. acicola described previously (OEPP/EPPO 2005; Jankovsky *et al.* 2009), and were easily distinguished from other spores due to their banana shape and olive color (Appendix A).

A HOBO data logger (HOBO Pro v2; Onset, Bourne, MA) was placed in the canopy of a nearby mature eastern white pine, also bordering the clear cut, to log daily temperate (°C) and relative humidity (%) at 1 hour intervals throughout both sampling seasons. Due to a malfunction of the HOBO in 2015 relative humidity was not recorded. However, the MEF had another HOBO data logger located on the trunk of an eastern white pine in the adjacent clear cut (Fig. 10), which tracked relative humidity for the 2015 sampling period. One rain gauge was placed in the center of the clear cut, alongside the central spore trapping support, to record cumulative rainfall (in millimeters) collected at 4 day intervals, daily rainfall was not recorded.

Growth rates

Growth rates studies of three isolates from three states (#1692 ME, #1676 NH, #1680 VT) were conducted at four different temperatures. Four millimeter agar plugs from two week old colonies were placed mycelial side down on fresh 2% potato dextrose agar (PDA) plates (Difco^{TM}). Plates were incubated at 15, 20, 25, and 30°C with a 12hr light cycle. Colony diameters of ten replicates were measured along two perpendicular lines weekly for four weeks.

Statistical analysis

All data collected from the three source trees during both sampling periods were used for statistical analysis performed using JMP Pro 11.2.1 (SAS Institute Inc.). The central spore trapping support was left out of analysis as conidia were rarely found on these slides. Spore counts from the four slides on each support were averaged together, as these were sub-samples for each distance, and each support interval (0ft, 10ft, 20ft) was then averaged together for the three source trees. To determine when peak spore release occurred, the average number of spores observed every 4 days, for each interval, were subsequently added to the previous 4 day spore count to create a cumulative spore release progress curve (Kranz 1974). A logistic 3P curve, a logistic curve that assumes the data does not have a lower asymptote, fit to the sigmoid nature of the data to determine slope for peak spore release, was used for graphical analysis. A 3P curve was used because the data was assumed to not contain a lower asymptote as initial spore release was zero prior to the first spore sighting. To determine overall spore release all support intervals were averaged and plotted separately by year for graphical analysis of the relative number of spores dispersed at 4 day intervals. Ascospores counts could not be determined as none were found on any slide during the study. For further statistical analysis spore counts for both years were combined and log transformed ($\log_{(x + 1)}$) as our data contained many zeros (Boateng and Lewis 2014). ANOVA and Student t-tests were used to determine differences in spore release by month, year, and distance. Statistical analysis of growth rates by isolate location and temperature were performed in JMP Pro 11.2.1 (SAS Institute Inc.).

Model Development

To determine the effects of environmental parameters on spore loads three climatic variables were considered for model parameterization, 4 day cumulative rainfall, temperature, and relative humidity (Skilling and Nicholls 1974; Fitt *et al.*1989; Boateng and Lewis 2014). Temperature and relative humidity variables were expanded for model parameterization to include average daily (24 hours), daytime (6am – 8pm), overnight (8pm – 6am) as well as average minimum and maximum values for these three time intervals. To better understand how climate variables affected spore release and abundance, variables were modeled against spore data collected from June, July, and both June and July together. A linear stepwise multiple regression analysis was utilized to select for variables that most accurately predicted spore abundance (Card *et al.* 1988; Liu *et al.* 2007). Models were manually examined for collinearity between variables using the variance inflation factor (VIF) with a threshold of VIF<4, a more stringent threshold than the rule-of-thumb of 5-10 (Montgomery and Peck 1992). Variables showing the larger VIF factors were removed for further analysis. Remaining variables were fit for all possible models. The Akaike information criterion with small sample correlation (AICc) (Hurvich and Tsai 1989; Burnham and Anderson 2002) and adjusted R^2 values were used to assess the models support of the data. We reported both individual and multiple variable models that best fit and explained field observations.

RESULTS

Patterns of spore dispersal

Spore trapping supports were successfully able to passively trap *L. acicola* spores released from *P. strobus* source trees. During both sampling periods only conidia (asexual spores) were trapped on microscope slides, no ascospores (sexual spores) were observed. Similarly, only a few spores were observed on the central support throughout both study periods, and thus was excluded from analysis. Both study years showed similar trends in spore dispersal, with differences only in the onset of spore dispersal and abundance of spores released (Fig. 12, 13, & 14). In the 2014 sampling period the first spores were observed on May 13th, but not again until May 29th despite the fact that rainfall occurred between these dates (Fig. 12 A). The initial spore release in the 2015 sampling period was not observed until June 2nd, immediately following substantial rainfall (Fig. 13 A). Aside from this difference in the initial dispersal event, both years showed identical trends of increased spore release during the month of June, peaking around June 14th – 18th and slowly decreasing throughout the remainder of the summer (Fig. 12, 13, & 14). The amount of spores observed in the month of June was significantly greater than all other months, overall and at each distance interval ($p < 0.001$). This was especially true in the 2015 study period when the highest amount of spores observed throughout both studies was

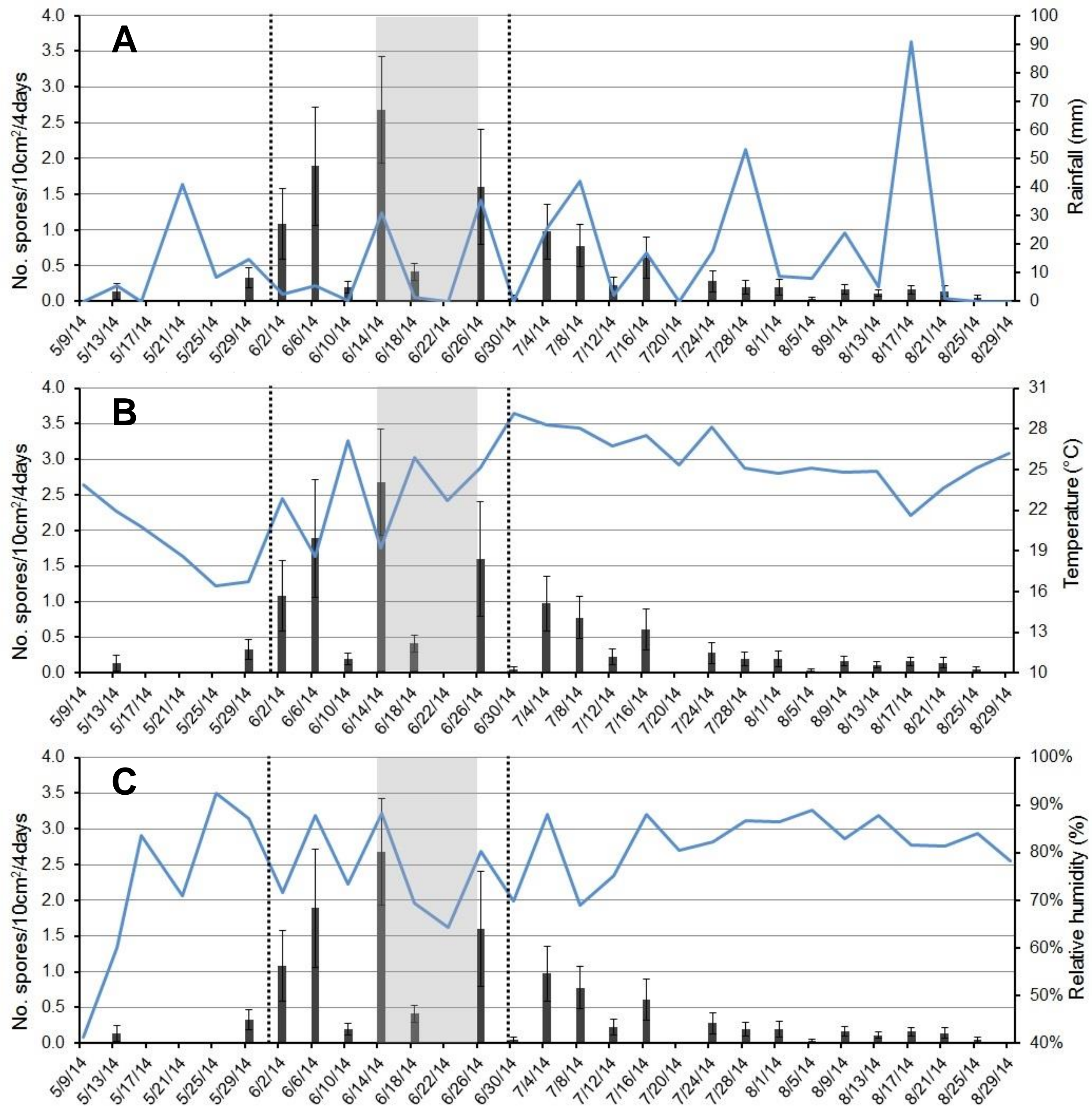

Figure 12: Mean count of *Lecanosticta acicola* conidia collected (black bars) from all intervals (0ft, 10ft, and 20ft) from source trees in 2014 with imposed climatic variables: A) 4 day cumulative rainfall; B) mean 4 day maximum daily temperature; and C) mean 4 day daily relative humidity. Errors bars indicate standard error, dotted vertical lines indicate the month of June, shaded area indicates field observed defoliation event of *Pinus strobus* needles at the Massabesic Experimental Forest in Lyman, ME.

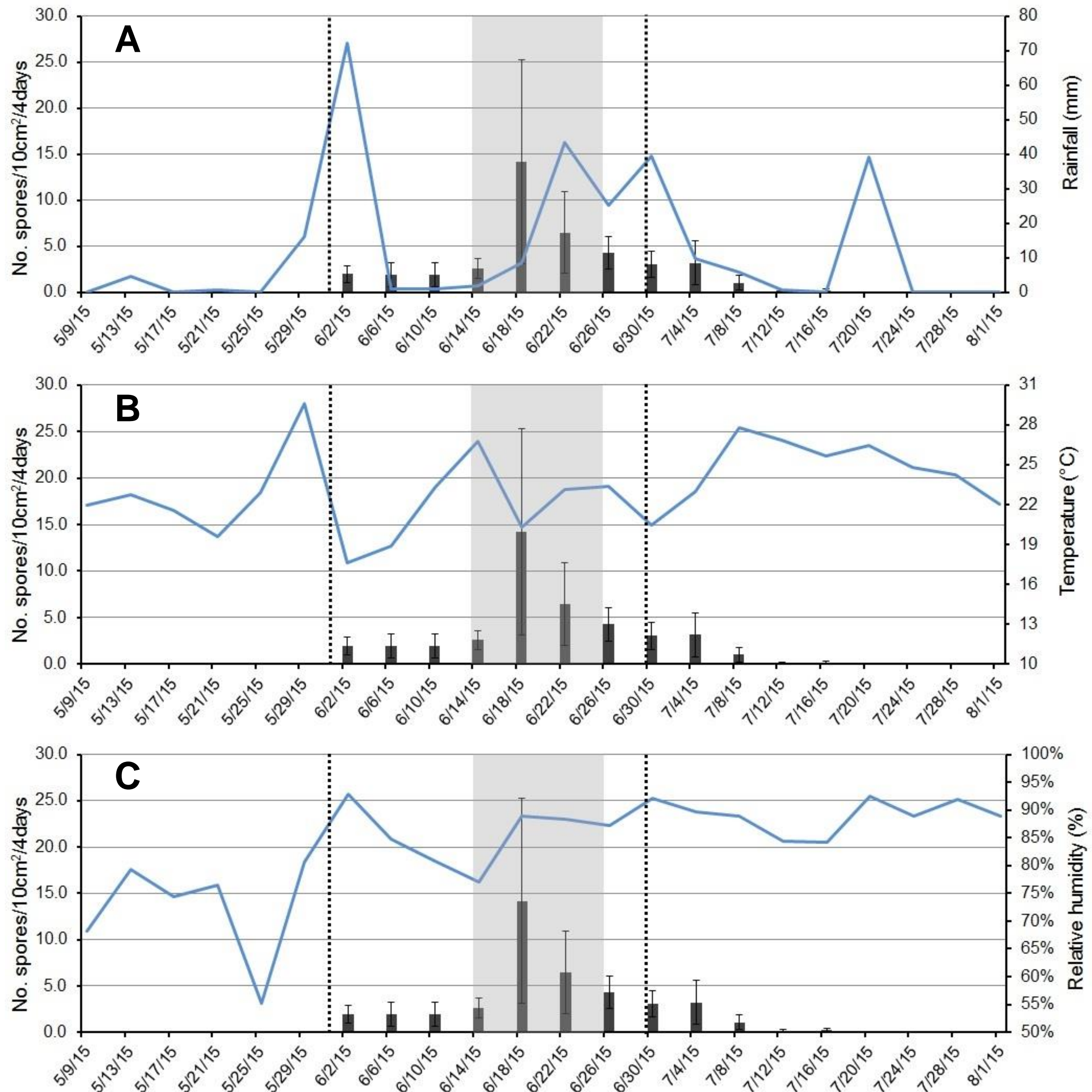

Figure 13: Mean count of *Lecanosticta acicola* conidia collected (black bars) from all intervals (0ft, 10ft, and 20ft) from source trees in 2015 with imposed climatic variables: A) 4 day cumulative rainfall; B) mean 4 day maximum daily temperature; and C) mean 4 day daily relative humidity. Errors bars indicate standard error, dotted vertical lines indicate the month of June, shaded area indicates field observed defoliation event of *Pinus strobus* needles at the Massabesic Experimental Forest in Lyman, ME.

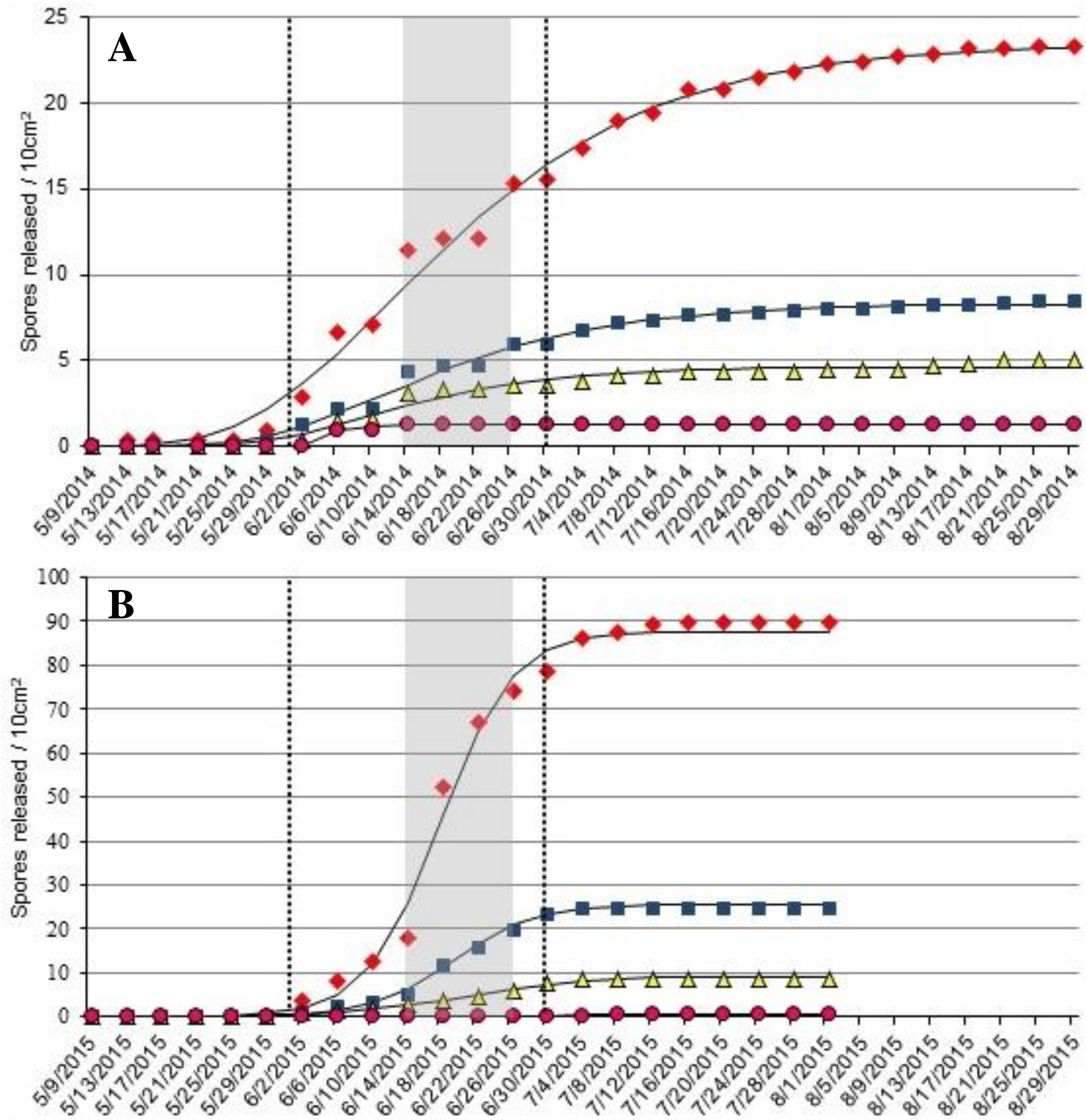

Figure 14: Cumulative spore release curve of *Lecanosticta acicola* conidia (per 10cm^2) for each interval: 0ft (red diamonds); 10ft (blue squares); 20ft (yellow triangle); and central (pink circles) throughout 2014 (A) and 2015 (B) at the Massabesic Experimental Forest in Lyman, ME. Points were fit with a 3P logistic curve to determine peak spore release. Dotted vertical lines indicate the month of June, shaded area indicates field observed defoliation event of *Pinus strobus* needles. Sampling did not continue throughout August in 2015 due to low spore loads collected in August in 2014

observed on June 18th and 22nd, directly in the middle of observed field defoliation event of *P. strobus* needles (Fig. 13). Overall data from both years revealed that there was also a significant increase in the amount of spores observed in the 2015 study period than in the 2014 study period ($p = 0.02$). Although differences between the years at each distance interval only showed significant increases in spore abundance in 2015 at 0ft ($p = 0.03$) and 10ft ($p = 0.04$); both years showing similar spore counts at 20ft ($p > 0.05$). While there was a greater amount of spores observed at the 10ft interval from 2014 to 2015, both years exhibited the greatest abundance of spores at 0ft ($p < 0.05$) (Fig. 14).

Effects of climate on dispersal

Weather data during noticeable spore release was used to correlate the effects of climate on spore abundance and dispersal. While the peak spore release was observed during the month of June, spores were also present through mid-July and were therefore included in model analysis. While more spores were generally seen when rainfall was recorded, spores were occasionally seen after periods of no rain, but in much lower abundance (Fig. 12 A, Fig. 13 A). Consequently, cumulative 4 day rainfall during the months of June ($R^2 = 0.14$, $p = 0.15$), July ($R^2 = 0.01$, $p = 0.72$), and both June and July ($R^2 = 0.07$, $p = 0.14$) were not correlated with the abundance of spores dispersed. Cumulative rainfall was only found to be significant when modeled with temperature for combined June and July weather data ($R^2 = 0.32$, $p = 0.002$) (Table 7). Consequently, temperature and relative humidity were found to be the climatic variables

Table 7: Regression and model statistics for effects of climatic variables on the average abundance of *Lecanosticta acicola* conidia found on spore traps placed at different distance intervals (0ft-20ft) from three mature eastern white pines (*Pinus strobus*) at the Massabesic Experimental Forest in Lyman, ME.

Regression/Model, variable*	Parameter estimate	SE†	Prob. > t	VIF‡	Model Prob. > F	R^2††	AICc‡‡
June							
Univariate							
Intercept	-1.8317	0.4244	0.0007				
Min overnight RH	0.0273	0.005	<0.0001	---			
Total model					<0.0001	0.678	-2.933
Intercept	1.4468	0.5055	0.0125				
Max daily Temp.	-0.0432	0.0219	0.0686	---			
Total model					0.0686	0.217	11.28
Multivariate							
Intercept	-1.3358	0.6344	0.0553				
Min overnight RH	0.0255	0.0053	0.0004	1.126			
Max overnight Temp.	-0.0214	0.0204	0.3135	1.126			
Total model					0.0004	0.658	-0.596
June & July							
Univariate							
Intercept	1.6006	0.3581	0.0001				
Max daily Temp.	-0.0526	0.0145	0.0012	---			
Total model					0.0012	0.318	6.837
Intercept	-0.4711	0.3893	0.2364				
Daytime RH	0.0103	0.0051	0.0509	---			
Total model					0.0509	0.129	14.18
Multivariate							
Intercept	0.7680	0.358	0.0411				
Min. daytime RH	0.0077	0.0036	0.0409	1.001			
Max overnight Temp.	-0.0526	0.0157	0.0024	1.001			
Total model					0.0018	0.328	6.94
Intercept	1.225	0.2798	0.0002				
Rainfall	0.0048	0.0023	0.0455	1.008			
Max overnight Temp.	-0.0564	0.0158	0.0014	1.008			
Total model					0.0019	0.324	7.141

* Climatic measurements were averaged together over a 4 day interval: daily (24 hours), daytime (6am-8pm), overnight (8pm-6am). RH = relative humidity, Temp. = temperature
† Standard error
‡ VIF = variance inflation factor, variables with values <4 were considered not to be collinear.
†† Adjusted R^2 values are shown for multivariate models.
‡‡ AICc = Akaike information criterion with small sample correlation.

driving spore abundance and dispersal during the months of June and July. However, analysis of the effect of individual month's climatic parameters revealed that, neither July temperature nor relative humidity were correlated with spore release ($p > 0.05$). Results showed a general negative correlation between temperature and spore abundance, particularly average maximum daily temperature. This trend was evident during the month of June ($R^2 = 0.16$, $p = 0.07$), but was only significantly correlated during the combined months of June and July ($R^2 = 0.32$, $p = 0.001$) (Table 7). Fewer conidia were being trapped as average maximum temperatures began to increase above 25°C, coinciding with the start of July (Fig. 12 C, Fig. 13 C). Results of relative humidity revealed a positive correlation to the amount of spores trapped, and was shown to be the most influential climatic variable. During the month of June all relative humidity parameters were found to be positively correlated with spore abundance, with average minimum overnight relative humidity being the best predictor ($R^2 = 0.66$ $p < 0.0001$) (Table 7). In the combined analysis of June and July only average daytime relative humidity was found to be significantly correlated ($R^2 = 0.13$, $p = 0.05$) (Table 7). It was generally rare to find spores during periods when relative humidity was <70%. These results are most evident during the period of field observed defoliation (June 14th – June 26th) where in 2014 this period exhibited the lowest relative humidity of the entire sampling period and average maximum daily temperatures were > 25°C resulting in very little spore release (Fig. 12 B & C). In 2015 this period showed relative humidity values > 80% and average maximum daily temperatures < 25°C resulting in spore abundances of up to 6x greater than in 2014 (Fig. 13 B & C). Although June weather appeared to be the best predictor of spore abundance, the addition of July weather data

combined with June's data showed significant correlations with some individual climatic variables and two factor models containing both temperature and relative humidity (Table 7); providing more understanding of the nature of *L. acicola* spore dispersal from mature eastern white pines in the field.

Growth rates

All isolates showed similar trends in growth rates at all different temperatures (Fig. 15). Due to excessive contamination in replicates of isolate #1680 at 15°C, this data set was excluded from analysis. Growth rates of all isolates were significantly slower at 15°C and faster at 25°C ($p < 0.05$) (Table 8). There were significant differences in growth rates at 20°C, and both #1676 and # 1692 exhibited significantly greater growth rates than #1680 at 30°C ($p < 0.05$) (Table 8). However, growth rates for all isolates were not significantly different at the optimal growth rate of 25°C ($p > 0.05$).

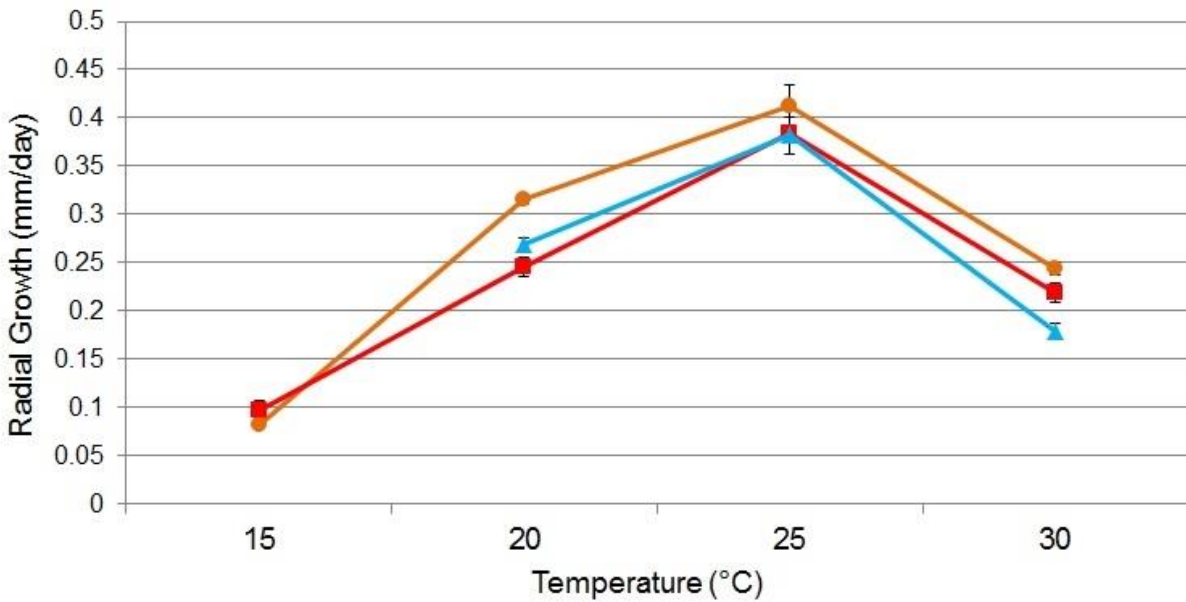

Figure 15: Radial daily growth rates at four different temperatures for three isolates of *Lecanosticta acicola*: red squares, #1676 (NH); orange circles, #1692 (ME); and blue triangles, #1680 (VT). Bars indicate standard error, n=10.

Table 8: Mean growth rate (mm/day) of three *Lecanosticta acicola* strains at four different temperatures over four weeks (±SE), n=10. Student t-test analysis was performed to find significant growth differences between strains and temperatures; capital letters correspond to growth differences between strains at a certain temperature, lower case letters correspond to growth differences between temperatures of a given strain. Values followed by the same letters are not significantly different ($p < 0.05$).

Strain	State	Temperature (°C)			
		15	20	25	30
#1676	NH	0.097±0.009 A / a	0.25±0.01 A / b	0.38±0.009 A / c	0.22±0.007 A / b
#1692	ME	0.082±0.01 A / a	0.32±0.005 B / b	0.41±0.02 A / c	0.24±0.01 A / d
#1680	VT	N/A	0.27±0.006 C / a	0.38±0.02 A / b	0.18±0.008 B / c

DISCUSSION

The objective of this study was to understand the effects climatic factors had on the development and dispersal of *Lecanosticta acicola*, a primary foliar pathogen associated with the defoliation of eastern white pine. Currently, only one study has examined the pathogenicity of *L. acicola* on eastern white pine with results indicating that it was highly to moderately resistant (Skilling and Nicholls 1974). Their findings also demonstrated that longleaf pine was highly to moderately resistant. This contrasts with numerous other reports however, that documented large economic losses in longleaf pine plantations due to *L. acicola* (Hedgcock 1929; Siggers 1944; Mann 1969; Kais 1971; Phelps *et al.* 1978; Heimann *et al.* 1997). The study by Skilling and Nicholls (1974) however, used natural inoculum from scotch pine to infect both eastern white pine and longleaf pine, suggesting possible variations in virulence of *L. acicola* isolates from different host species. Support for this variability was reported by Kais (1971) who indicated that northern strains of *L. acicola* were not as pathogenic on longleaf pine as southern strains. While we did not explicitly perform a pathogenicity trial of *L. acicola* on eastern white pine, *L. acicola* has been found occurring on mature and regeneration eastern white pine trees since 2006 and has since continued to spread across the northeastern United States (Munck *et al.* 2011, 2012; Broder *et al.* 2015) (Ch. 1 Table 1, Ch. 1 Fig 1). Based upon its ubiquitous presence throughout the region and evidence of

variability in virulence among strains (Kais 1971), we believe that *L. acicola* is able to infect eastern white pine, either through the introduction of a new race of *L. acicola* or a change in the regional climate that has allowed southern strains to have spread north or endemic strain to increase in abundance to epidemic proportions.

While prior epidemiological studies of *L. acicola* have primarily been performed on scotch and longleaf pine, our results generally agree with their findings (Skilling and Nicholls 1974). In a two-year spore dispersal study conducted on scotch pine plantations in Wisconsin and Minnesota, *L. acicola* ascospores were never observed on any of the 1,300 microscope slides used as passive spore traps (Skilling and Nicholls 1974). They further confirmed the absence of the sexual stage by examining numerous infected needles and finding no sexual fruiting bodies (pseudothecium). Other studies of *L. acicola* have primarily found the sexual stage present in the southern U.S. (Kais 1971; Phelps *et al.* 1978), with the most northern record, of the sexual stage, occurring near Ashland, Missouri on ponderosa pine (*Pinus ponderosa* L) (Luttrell 1949). Our results support those found by Skilling and Nicholls (1974), suggesting that northern climates are unfavorable for the production and development of the sexual life stage of *L. acicola*. While our sampling method could be considered ineffective in capturing wind-dispersed ascospores, sampling surveys of diseased eastern white pine needles throughout the northeastern U.S. have not revealed any sightings of sexual fruiting structures (Munck *et al.* 2011, 2012; Broders *et al.* 2015), indicating that the primary means of dispersal is through rain-splash dispersed conidia.

Lecanosticita acicola conidia were first observed in the beginning of May in 2014, but consistent dispersal did not occur until the start of June. Conidial dispersal peaked

during the middle of June (14th – 26th), after which the number of trapped conidia began to decrease steadily throughout July and August. This peak during the month of June corresponded to a significant *P. strobus* defoliation event at the MEF and throughout the northeastern U.S. (Munck per. comm.; Ostrofsky per. comm.). Skilling and Nicholls' (1974) results showed a more consistent onset time of conidia release occurring in early May, but exhibited a very similar conidia dispersal peak from June 5th to June 19th. Similarly, after the peak, conidia abundance began to drastically decrease throughout the rest of the summer (Skilling and Nicholls 1974). This inconsistency in onset time may be caused by drier conditions during the month of May, which may have slowed the development of conidia as high humidity is necessary for the development of conidia in infected needles (Kais 1975). It is not possible to compare these results to those reported by Skilling and Nicholls (1974) as they did not report average relative humidity along with their observed conidia counts. In addition, their study indicated a smaller second peak in conidia dispersal from August 21st – September 30th, which coincided with a defoliation event of infected current year needles. While our study did not extend into September, we believe that a second spore peak would not have occurred at the MEF for two reasons. Firstly, over the past decade as *L. acicola* has spread throughout the region, infection symptoms and signs have only been observed on 2nd and 3rd year needles, with no sightings on current year needles. Secondly, defoliation events caused by *L. acicola* have only been observed during the months of June and July. This has been confirmed through preliminary research of litter fall plots from WPND infected eastern white pine stands, which was successful in capturing the defoliation event in June and July, with normal abscission of un-infected 2nd and 3rd year needles occurring

in October; no shedding of current year needles was observed during August or September (McIntire per. comm.).

Along with slight differences in the timing of spore dispersal, our study also showed greater dispersal distances compared to the results of Skilling and Nicholls (1974). From scotch pine, conidia were rarely seen to be dispersed further than 5ft from a source tree, with significant reduction in spore abundance occurring at 1ft from the tree (Skilling and Nicholls 1974). In our study conidia were found in greater abundances at 0ft from the furthest reach of the branches (~12ft from the trunk), it was not uncommon to find conidia 10ft and even 20ft away. This difference in dispersal distance however, is most likely due to the difference in height between the two trees species, rather than a variation in isolates or climates. Since *L. acicola* produces mucilage conidial masses on both scotch pine and eastern white pine, rain splash is the primary method of dispersal as the mucilage prevents dispersal by wind (Fitt *et al.* 1989). During our study, spore trapping supports were placed well below the canopy, as it would have required a support of approximately 40ft (12m) to reach the bottom portion of the canopy. This large distance could explain the observed ability of conidia to disperse 20ft away from a tree; providing conidia caught in water droplets a greater chance of being further dispersed by strong wind. Although we tried to account for this factor by placing supports in three cardinal directions, conidia were still observed at 20ft for all three source trees, suggesting that strong wind could play a role in the dispersal of rain-splashed spores if host height is relatively large. Although we do not believe that the tree height and wind could have allowed for the few collected conidia to reach the central support, which was around 200ft away. Skilling and Nicholls (1974) suggested

that *L. acicola* mucilage conidia could be dispersed greater distances by insects, as they found incidences where 25 conidia were attached to scales of a lepidopterous insect. Similarly, we occasionally found insects stuck to microscope slides and believe that this could be the reason for the few observed conidia on the central support.

Despite the fact that wind direction or speed was not considered in this study, our results demonstrated the importance of other climatic variables such as rainfall, temperature, and relative humidity on the abundance and dispersal of *L. acicola* conidia. While, rainfall is one of the primary climatic factors affecting spore dispersal of foliar pathogens (Fitt *et al*. 1989, Skilling and Nicholls 1974, Boateng and Lewis 2014), this was not the case in our study. Unlike Skilling and Nicholls's (1974) where *L. acicola* spores were only released during rainy weather, we observed conidia on slides even after periods of no rain. In general, more conidia were observed on days when rainfall was recorded, and it appears that rainfall frequency was more important than the amount of rainfall. While rainfall was most likely the primary mechanism for spore dispersal, it was not a factor in affecting the number of spores trapped. Our data indicates that relative humidity and temperature were the primary climatic factors affecting spore abundance; more conidia were seen as relative humidity increased and temperature decreased. These results agree with previous research of *L. acicola*. It is known that needles containing mature acervuli can readily sporulate after one day in a moist chamber (OEPP/EPPO 2005), and that high summer temperatures are detrimental to germination, growth, and sporulation (Skilling and Nicholls 1974; Kais 1975). Kais (1975) determined that consecutive days of high humidity were essential for the development of infection, coinciding with development of mature acervuli. Similarly,

during the month of June spore abundance decreased when relative humidity dropped below 80% and spores were rarely observed when humidity was below 70%. Regression analysis specifically determined that high minimum overnight (8pm-6am) relative humidity was the most important factor. As relative humidity continued to increase throughout the summer there was, however, an observed decrease in spore abundance. This decrease in spore abundance was most likely caused by the parallel increase in temperature, which was found to negatively impact spore dispersal. There appears to be a shift in importance of climatic factors from relative humidity to temperature throughout the summer. Our observation of reduced spore abundance above daily maximum temperatures of 25°C corresponds to our *in vitro* growth rate experiments which indicate that temperatures above 25°C begin to negatively affect growth. While other studies have also shown that increased temperature negatively affects spore dispersal of foliar fungi (Skilling and Nicholls 1974) and development of *L. acicola* (Kais 1975; Huang *et al.* 1997), their findings report that temperatures between 25 – 30°C are more beneficial for growth (Kais 1975; Huang *et al.* 1997). Kais (1975) determined that northern isolates of *L. acicola* were more virulent on longleaf pine at daytime temperatures of 30°C, while Huang *et al.* (1997) documented that both northern and southern isolates grew best at temperatures of 28°C.

It appears that *L. acicola* strains found in the northeastern United States could represent a new race. Further population genetic studies would need to be conducted to determine whether the northeastern *L. acicola* isolates were adapted from northern - Midwest or southeastern isolates, sexual recombination of isolates from both regions, or if they were introduced from another region or country. *Lecanosticta acicola* isolates

from Japan were observed to have an optimal growth rate of 25°C, after which growth rate drastically decreased (Suto and Ougi 1998). Aside from the origin of *L. acicola*, our results showed that *L. acicola* strains within the region start to mature on infected needles during May with spore production beginning at the end of May, peaking in June, and slowly decreasing throughout July. During the peak spore production period in June, minimum overnight relative humidity was found to have the greatest influence on spore abundance. This was most notable during the defoliation event; if relative humidity was lower during this period spore abundance was significantly decreased. After the month of June, relative humidity was no longer the most influential climatic factor on spore abundance. During this time, spore abundance was observed to be negatively correlated with increases in maximum daily temperatures. As temperatures began to consistently increase throughout the rest of the summer, spore abundance began to rapidly decrease. In our study we were able to also demonstrate that while the relationship between rainfall and spore abundance was not significantly correlated, it was believed to be the primary mechanism for conidia dissemination from infected needles. This differed from our previous study in which we found cumulative spring precipitation to be the primary factor in predicting defoliation severity in the following year (Ch. 2, Table 6). However, this new finding does not dispute our previous finding. While the amount of rainfall did not directly affect the number of spores trapped, we generally saw more spores after rain events with only few to no spores being observed after periods of no rain. This suggests that while rainfall does not affect the abundance of spores produced from an infected needle, it is responsible for the dispersal of spores from infected needles. This dispersal allows for the spread and subsequent infection of

new needles which then have the potential to increase the observed defoliation of a tree, i.e. defoliation severity. Thus, during the peak spore production period in June, relative humidity is the driving factor in the development of spores (spore abundance). However, without rainfall during periods of high relative humidity few to no spores will disperse from infected needles, decreasing the potential of new infections of needles within 20ft.

DISCUSSION

Based upon previous studies and our results we conclude that the current outbreak of WPND in the northeastern United States is attributed to a fungal disease complex consisting of *Lecanosticta acicola*, *Septorioides strobus*, *Bifusella linearis*, and *Lophophacidium dooksii*. However, results from our spore trapping experiment revealed that the defoliation of eastern white pines throughout the region coincided with the peak spore release of *L. acicola*, suggesting that *L. acicola* is the primary pathogen responsible for the widespread defoliation that is seen in June. Our sampling survey however, revealed that *L. acicola* was not present everywhere, indicating that defoliation caused by *S. strobus*, *B. linearis*, and *L. dooksii* must be occurring, but their exact defoliation period is unknown.

Prior to this outbreak, beginning in 2010, needle blights of eastern white pine were reported sporadically throughout the northeastern U.S., with some researchers attributing the damage to ozone (Bennett *et al.* 1986), semimature tissue needle blight (Linzon 1960, 1964, 1967a, 1967b), or several fungal species (Banfield 1960, 1962, 1963; Dreisbach 1989). Most of these claims have since been refuted. Ozone was determined not to be the causal agent responsible for foliar markings observed on eastern white pines in the field at Acadia National Park, Maine (Bennett *et al.* 1994; Kohut *et al.* 1997). Additionally, most of the fungal species found associating with needle blights discovered by Dreisbach (1989) were previously or later determined to be

weak pathogens or endophytes (Darker 1967; Deckert & Peterson 2000; Deckert *et al*. 2002; Sokolski *et al.* 2004), except a *Bifusella*-like species that was determined to be pathogenic on *P. strobus* (Dreisbach 1989). It wasn't until the late 1990's when Wenner and Merrill (1998) finally determined that the *Bifusella*-like species found by Dreisbach (1989), a *Lophodermium sp.* discovered by Banfield (1960), and the causal agent of semimature tissue needle blight were all *Lophophacidium dooksii*. It was around the same time that a separate species known as *Bifusella linearis* was found to be parasitizing *P. strobus* (Minter and Millar 1984; Ganley *et al.* 2004), and was thought to be present in the northeastern U.S. since the early 1900s (Darker 1932). This species is remarkably similar to *L. dooksii* as they express similar symptoms and signs, including an early onset of symptoms (April-May), chlorosis, partial tip die-back, needle casting during spring after infection, and long black sexual fruiting bodies (Minter and Millar 1984; Merrill *et al.* 1996; Munck *et al.* 2012). These similarities have led to several misdiagnosed cases of *L. dooksii,* as the elongate dark grey hysterothecium of *L. dooksii* are often confused with the shiny black elongate stroma of *B. linearis* (Merrill *et al.* 1996). Proper identification can be completed by microscopic observation of the sexual spores. Ascospores of *B. linearis* are constricted in the middle (Horst and Westcott 2008) whereas *L. dooksii* ascospores are not (Merrill *et al.* 1996). These features were not known to earlier researchers, so it is not possible to determine which species had a greater presence on *P. strobus* in the early 1900s. Currently, these morphological differences as well as the success in our DNA sequencing of excised fruiting structures has allowed us to correctly determine the geographic distribution of these species. Our findings agree with those of previous researchers, that both *B.*

linearis and *L. dooksii* were found to be present on *P. strobus* needles throughout the northeastern U.S (Merrill *et al.* 1996). Due to previous misdiagnoses and the lack of recorded sightings however, we cannot conclude whether the abundance of these two species has increased or decreased over the past century. While it is certain that the current WPND outbreak is larger than any previously reported needle blight, our findings indicate that this may be due in large part to the arrival of two new fungal pathogens and the increase of temperature and precipitation that has been experienced in this region.

Our results confirmed the presence of both *B. linearis* and *L. dooksii* across this region, and also revealed the occurrence of *L. acicola* and *S. strobus*, two species that are both new to this region and the eastern white pine fungal community. While *L. acicola* has been present in North America throughout the past century, documented cases have only occurred on other *Pinus* species, particularly scotch pine and longleaf pine (Skilling and Nicholls 1974), with the first report on *P. strobus* occurring in Maine in 2006 (Munck *et al.* 2011). These reported cases on scotch and longleaf pine have largely occurred in the northern Midwest and southeastern U.S., respectively (Skilling ad Nicholls 1974). Research has indicated that *L. acicola* isolates from these respective geographic regions constitute two different races that were found to be less virulent on the other host. Northern races were more virulent on scotch pine than longleaf pine, while southern races were more virulent on longleaf pine than scotch pine (Kais 1971; Skilling and Nicholls 1974). Additionally, Skilling and Nicholls (1974) examined virulence on other *Pinus* species and determined that eastern white pine was highly to moderately resistant to the northern races of *L. acicola*, suggesting that eastern white pine could be

more susceptible to these southern races. While pathogenicity trials were not conducted in this study, the scenario of fungal pathogens from southern latitudes moving northward due to increases in temperature and precipitation, has been recently documented with crop pathogens (Bebber *et al.* 2013). Our research and existing data has confirmed a regional increase in summer temperature and precipitation, and supports other studies regarding changing climate in this region (Frumhoff *et al.* 2007; Campbell *et al.* 2010, 2011). Thus, a northern migration of *L. acicola* from the southeastern U.S. due to changing climatic conditions could explain the recent arrival within the northeastern U.S.

This study marks the first reported case of a *Septorioides* species, *Septorioides strobus*, in North America with the only other species within the genus, *S. pini-thunbergii*, occuring only in Japan and Korea (Kaneko *et al.* 1989; Suto 2000; Yoo and Eom 2012; Kihara *et al.* 2015). It is unclear how *S. strobus* became established in North America. Several papers suggest that new species within the order *Botryosphaeriales* will continue to be discovered especially when sampling new hosts and new environments (Taylor *et al.* 2009; Perez *et al.* 2010; Mehl *et al.* 2011; Sakalidis *et al.* 2011; Jami *et al.* 2012, 2013, 2014), as this order comprises a large portion of the endophytic fungal community of monocotyledonous, dicotyledonous, and gymnosperm hosts with many species also being characterized as pathogens (Slippers and Wingfield 2007; Slippers *et al.* 2013). Based upon these findings and the geographical separation between *S. strobus* and *S. pini-thunbergii* (USA to Japan) we believe that *Septorioides* species are present in other parts of the world, particularly on other *Pinus* species, and

appear to be associated with needle blights of *Pinus* species (Kaneko *et al.* 1989; Suto 2000; Yoo and Eom 2012; Kihara *et al.* 2015).

Based upon studies of endophytic and pathogenic fungi on *Pinus thunbergii* it appears that both *S. pini-thunbergii* and *L. acicola* are often found co-occurring in the same needle and are believed to be weak pathogens or latent endophytes (Kihara *et al.* 2015). Additionally, both of these species have been reported on *P. thunbergii* prior to 2000 (Kaneko *et al.* 1989; Suto and Ougi 1998). With this knowledge one could suggest that both the *L. acicola* and *S. strobus* strains within the northeastern United States could have been introduced through an infected *P. thunbergii* event. However, it would be impossible to suggest that *S. pini-thunbergii* diverged into a new species, *S. strobus*, within that short a time span. This scenario of an introduction from Japan could be the case for *L. acicola* as isolates studied in Japan were found to have an optimal growth rate of 25°C (Suto and Ougi 1998), which matches the results of our *L. acicola* isolates from Vermont, New Hampshire, and Maine (Fig. 15). This contrasts however, with the growth rates observed for northern and southern races of *L. acicola* which were found to grow faster at 28°C versus 24°C (Kais 1975). While the temperatures tested were not identical, both Suto and Ougi (1998) and our results show a drastic decrease in growth rates from 25°C to 30°C, with growth rates at 30°C rates being slower than those found at 20°C (Suto and Ougi 1998). Therefore, it would be reasonable to suggest that both the northeastern and Japanese *L. acicola* isolates would grow slower at 28°C. While it appears that there could be an association between *L. acicola* and *Septorioides* species, shown by the co-occurrence in Japan (Kihara *et al.* 2015) and our distribution results showing occasional co-occurrence (Fig. 7). Further research examining the

parasitic or symbiotic relationship between these two species and their host, along with population studies of both *Septorioides* species and different *L. acicola* races would be needed to provide valuable insight into the origin and divergence of these species.

Our research has revealed that both *L. acicola* and *S. strobus*, along with *B. linearis* and *L. dooksii* are all established and are widespread throughout this region, and their occurrence and distribution may be related to increases in precipitation and temperature. While this region has experienced such increases since 1950 (Fig. 8), we believe that the specific increases observed from 2003-2014 (NOAA/National Climatic Data Center. 2015), were primarily responsible for the current WPND outbreak. Six of the eleven years were ranked in the top 10% for precipitation and four years ranked in the top 10% for temperature. These weather patterns created a favorable environment conducive to the development and spread of all four fungal species. With climate change scenarios predicting further increases for the northeastern U.S. (Frumhoff *et al.* 2007) and data shown by our defoliation prediction models (Table 6), particularly warmer winters and wetter springs and summers, we expect to see continued defoliation by WPND with the potential for increases in disease severity. Furthermore, we anticipate seeing increased distribution of disease incidences due to a naturally high density of eastern white pine within this region and neighboring states.

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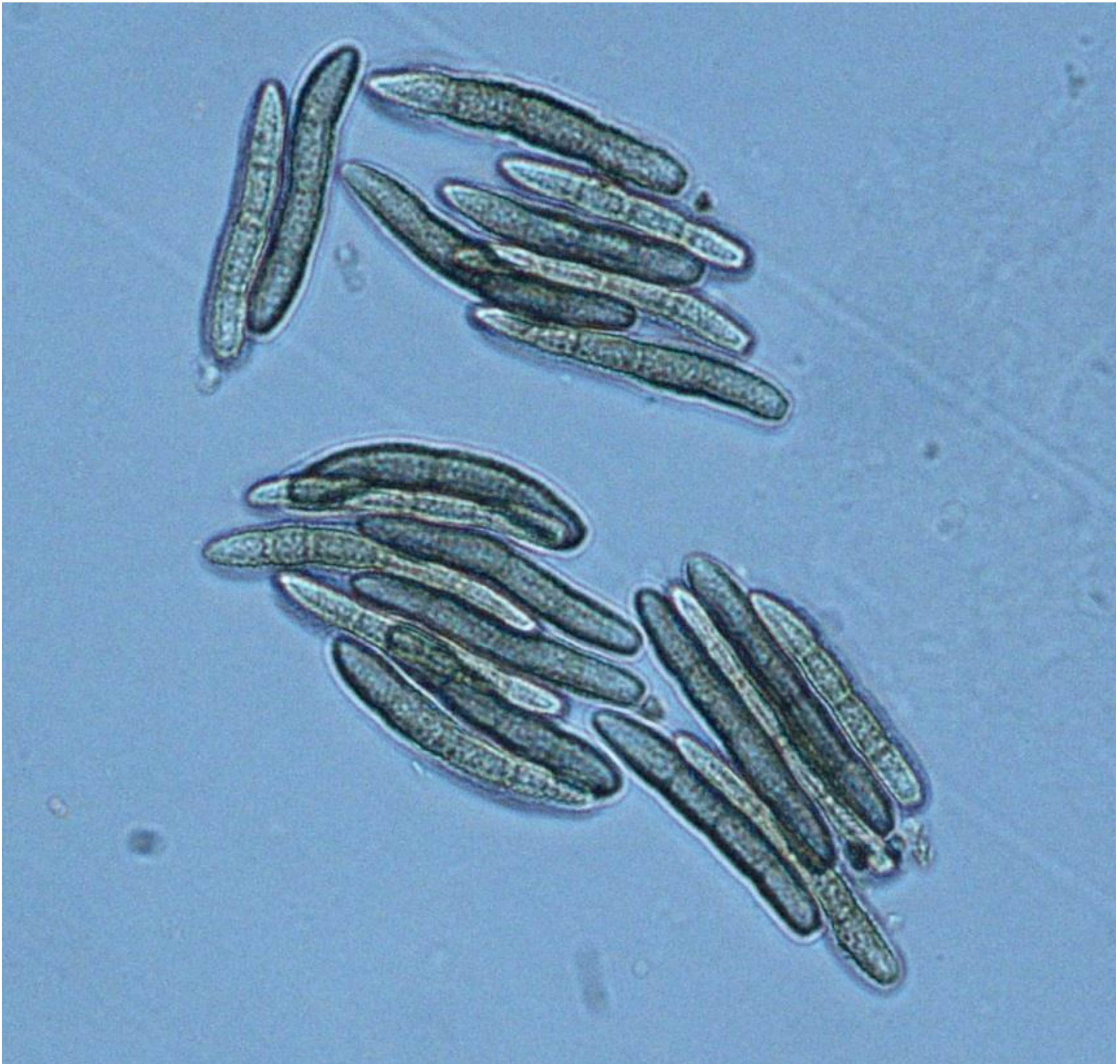
APPENDIX

APPENDIX A

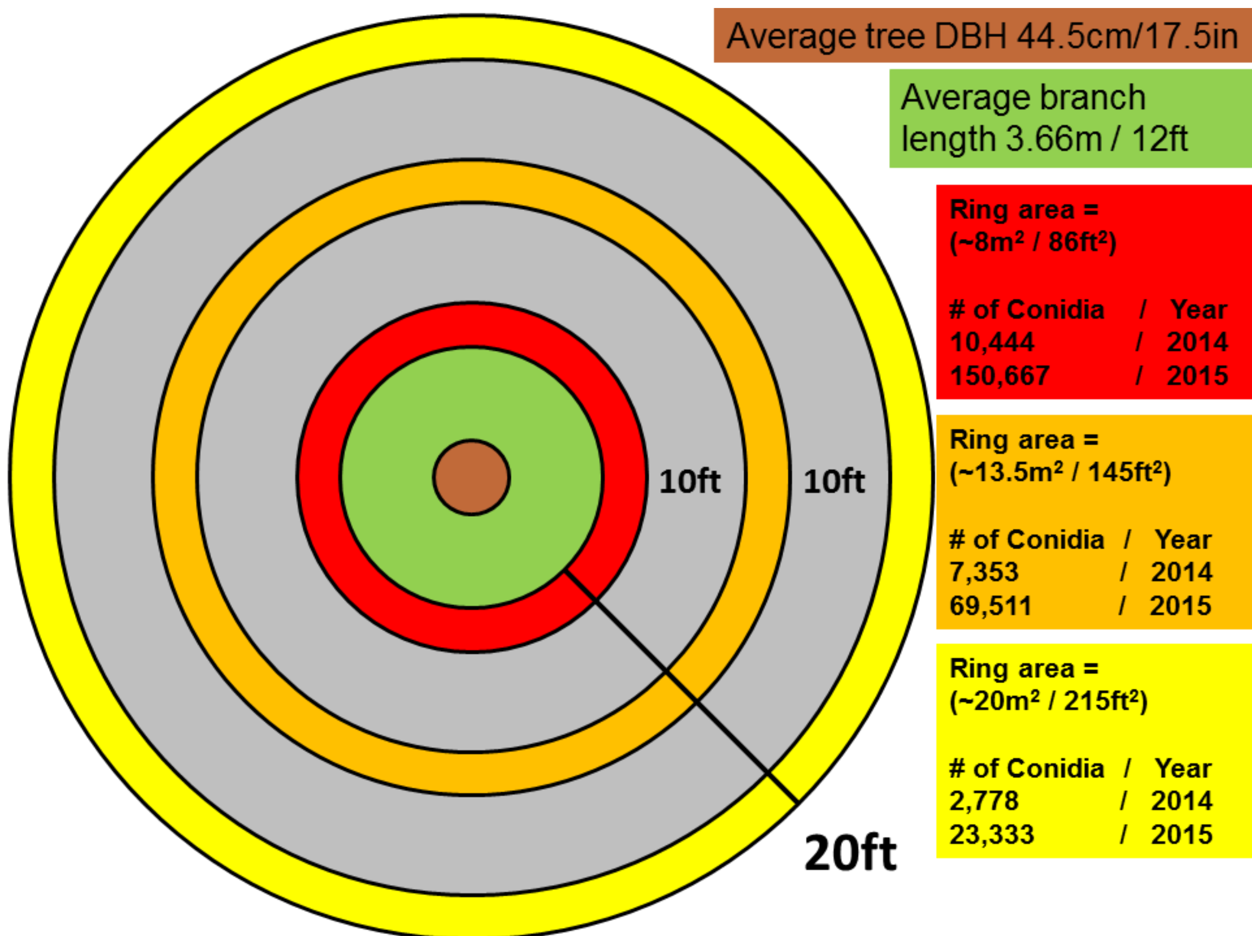
Lecanosticta acicola conidia



Appendix A: *Lecanosticta acicola* conidia (1000x), showing the general banana shape and olive color. These features made it easy to identify and differentiate *L. acicola* conidia from other spores trapped on petroleum jelly coated microscope slides. Scale bar not displayed.

APPENDIX B

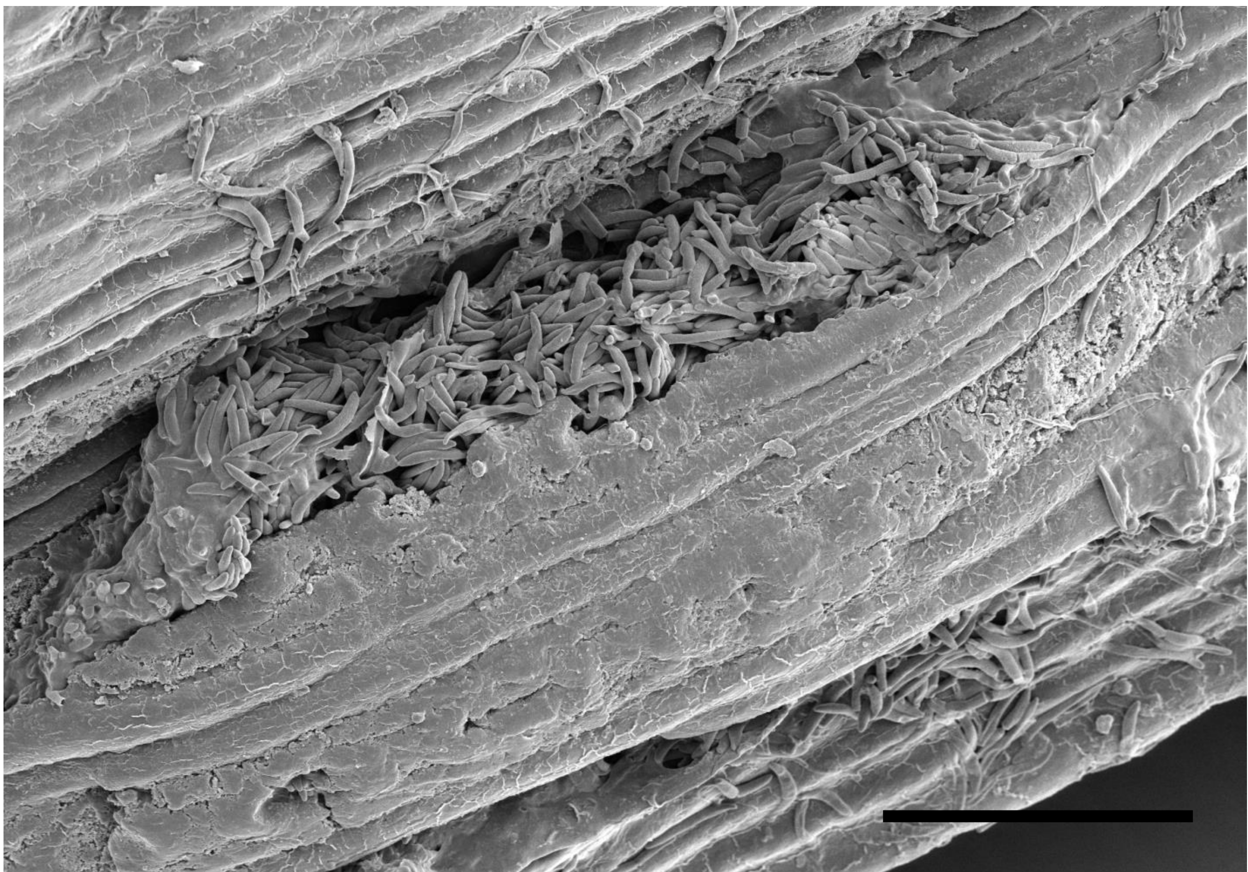
Estimates of *Lecanosticta acicola* conidia dispersal from single source tree



Appendix B: Pictorial diagram depicting an estimation of *Lecanosticta acicola* conidia dispersed around a single source tree at the Massabesic Experimental Forest in Lyman, ME. Brown indicates an average diameter at breast height (DBH) of the three source trees utilized in the experiment, while green represents the average furthest branch length. Each sampling interval (0ft, red; 10ft, orange; and 20ft, yellow) is represented by a one foot ring around the source tree. Conidia values were averaged from peak spore release (June 14th – June 26th) in both sampling years and indicate the estimated number of conidia trapped within each ring area per year.

APPENDIX C

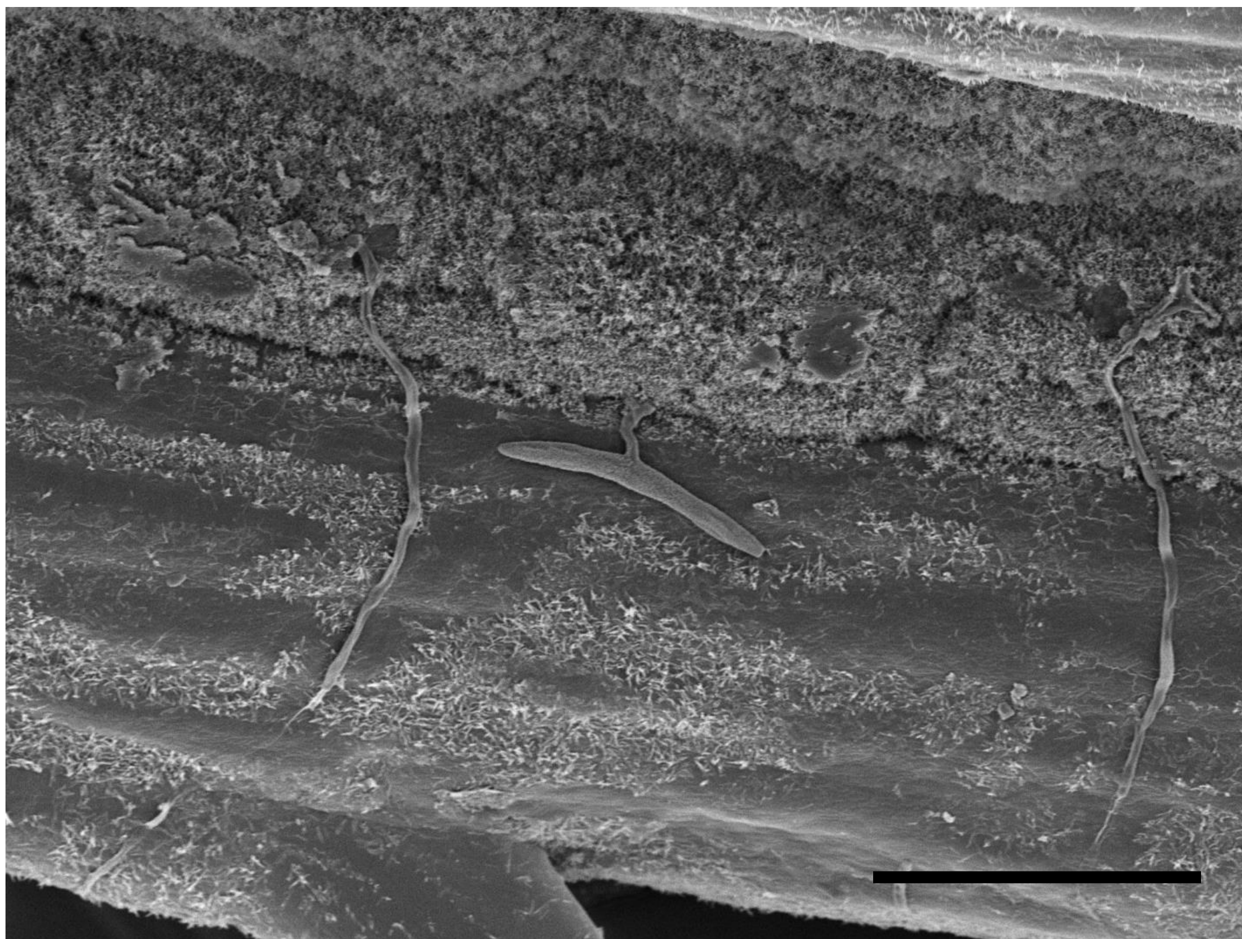
SEM image of *Lecanosticta acicola* acervuli



Appendix C: Scanning electron microscope (SEM) image, taken at the University of New Hampshire. Image shows a mature *Lecanosticta acicola* acervulus (asexual fruiting structure) producing conidia on a 2nd year *Pinus strobus* needle taken from Hubbard Brook, NH on June 11th 2015. Acervulus was observed at 1.24kx magnification, scale bar = 100µm.

APPENDIX D

SEM image of *Lecanosticta acicola* conidial germination



Appendix D: Scanning electron microscope (SEM) image, taken at the University of New Hampshire. Image shows a *Lecanosticta acicola* conidium germinating and infecting though the stomata of a current year *Pinus strobus* needle taken from Hubbard Brook, NH on June 11th 2015. Condium was observed at 2.91kx magnification, scale bar = 50µm.