SPRUCE DECLINE AND *DIAPORTHE*: INCIDENCE, TAXONOMY, VIRULENCE, AND TREE SUSCEPTIBILITY

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ABSTRACT

SPRUCE DECLINE AND *DIAPORTHE*: INCIDENCE, TAXONOMY, VIRULENCE, AND TREE SUSCEPTIBILITY

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In the early 2000s, spruce trees in Michigan began displaying basal needle drop and branch death that slowly progressed upwards, symptoms of what we call spruce decline. A survey in 2013 revealed that spruce decline was common throughout Michigan's Lower Peninsula, and *Diaporthe* was the most likely pathogen causing the cankers associated with these symptoms. Greenhouse inoculation studies completed Koch's postulates, confirming that *Diaporthe* could cause cankers that cause needle loss and branch death. The five different *Diaporthe* haplotypes isolated from symptomatic branches during the survey differed in virulence. Haplotypes 2, 4, and 5 were more virulent, and differed from each other by only one or two base pairs using the internal transcribed spacer (ITS) region and did not differ using the β-tubulin (TUB) gene. These haplotypes were unresolved phylogenetically. Haplotypes 1 and 3 were weakly virulent to avirulent on multiple spruce taxa, and fell into a resolved *Diaporthe eres* clade. Spruce taxa varied in susceptibility, with Colorado blue spruce (*Picea pungens*) the most susceptible, followed by Norway (*P. abies*), then white spruce (*P. glauca*). Spruce taxa that were much less susceptible were Black Hills (*P. glauca* var. *densata*), Serbian (*P. omorika*), and Meyer spruce (*P. meyeri*). We demonstrate that one or more *Diaporthe* species is causing cankers on declining spruce in Michigan, and these cankers elicit symptoms similar to the branch death expressed by declining spruce in the landscape. Future work will focus on further characterizing *Diaporthe* to species, and determining biotic and abiotic stressors that may predispose spruce trees to express decline symptoms.

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CHAPTER 1

Spruce trees are common throughout Michigan. In fact, Michigan conifer production encompassed nearly 70% of acreage in tree nurseries (MDA & USDA NASS, 2005). Frequently planted non-native species include Colorado blue spruce (*Picea pungens* Engelm.), Norway spruce (*Picea abies* (L.) H. Karst.), and white spruce (*Picea glauca* (Moench) Voss). White spruce and black spruce (*P. mariana* (Mill.) Britton, Sterns and Poggenb.) are the only spruce native to Michigan. Spruce are used for privacy screens, wind rows, and specimen trees due to their conical shape and dense branches, and in the case of Colorado blue spruce, the color. Spruce are also well suited for Christmas trees due to their shape, pleasant smell, and strength of branches. Ranked third in the country for acres of Christmas trees in production, Michigan cut 1,739,538 Christmas trees in 2012 alone (USDA 2012). According to the most recent estimate, 23% of Michigan Christmas tree acreage was spruce, with Colorado blue spruce nearly half of spruce Christmas tree production (USDA NASS 2014). Unfortunately, there are many diseases that affect these important trees. Some affect the needles, while others affect branches of the plant.

Beginning with needle diseases of conifers, the most well-known and common needle cast disease on spruce trees is *Rhizosphaera kalkhoffii* Bubák. *Rhizosphaera* kalkhoffii, an ascomycete, causes partial defoliation, or casting, of 1 year and older needles, with early symptoms of yellow mottling eventually turning to brown or, in Colorado blue spruce, purple-brown (Sinclair & Lyon, 2005). Given correct conditions, these symptoms will spread up and around the tree. If the disease persists for 3-4 years, branch death is likely to occur on trees less than 7 meters tall. Pycnidia are found in rows on all sides of the needles that plug the stomata. When mature, pycnidia are black spheres that produce single celled conidia. The life cycle of *R.*

kalkhoffii lasts for 12-15 months. The pathogen overwinters as mycelia or pycnidia in infected or dropped needles. In spring, conidia are produced that are water splashed to other needles. Germination occurs after approximately 48 hours with temperatures near 25°C; however, spores can survive for weeks in unsuitable conditions (Sinclair & Lyon, 2005). Pycnidia development occurs in late winter to early spring after the needles brown, and needles cast during summer and autumn. After multiple years of infection, the tree will resemble a skeleton, with just the current year's new flush of tips appearing green. These new tips will eventually become infected if conditions are moist enough for spore dispersal and germination. *Rhizosphaera kalkhoffii* can act as a primary pathogen or as an opportunistic invader, attacking needles stressed from other pathogens or environmental factors. This pathogen causes more intense damage in Christmas tree plantations, where trees are closely spaced with dense branches (Hansen & Lewis, 1997; Sinclair & Lyon, 2005). Conidia are easily moved in these plantations through water splash or mechanical transfer, and conditions become even more suitable when the branches are sheared, as they retain more moisture for spore germination.

Another fungus very similar to *R. kalkhoffii* was observed in 1999 in North Carolina, and found to be *Stigmina lautii* B. Sutton (Hodges, 2002). In 2006 it was confirmed in North Dakota (Walla & Kinzer, 2006) and in Michigan in 2008 (Fulbright, 2008a, 2010). *Stigmina lautii*, an ascomycete, is easily confused with *R. kalkhoffi*, as both emerge from stomata, but can be differentiated by the fruiting bodies (Hodges, 2002; Walla & Kinzer, 2006). *Rhizosphaera kalkhoffii* has smooth, rounded pycnidia, whereas *S. lautii* sporodochia have hair-like projections. However, Koch's postulates have not been completed to test *S. lautii* pathogenicity, and so it is unclear whether it truly is a pathogen of spruce.

Other needle cast diseases that affect spruce trees include ascomycetes *Lirula macrospora* (R. Hartig) Darker, *Isthmiella crepidiformis* (Darker) Darker, and *Lophodermium piceae* (Fuckel) Höhn. *Lirula macrospora* is distinguishable from other needle cast diseases by its long and narrow, brown/black ascomata in the midrib of needles. It infects current season needles which drop after 2-3 years (Hansen & Lewis, 1997). *Isthmiella crepidiformis* has similar symptoms, but the ascomata are smaller than those of *L. macrospora*. *Lophodermium piceae* have apothecia (hysterothecia) that are football-shaped and open and close in response to wetness to release ascospores (Sinclair & Lyon, 2005). These diseases are rarely serious, but can be unsightly on the tree.

Slightly different than needle cast diseases, spruce needle rust discolors current year needles (Sinclair & Lyon, 2005). Many different species of *Chrysomyxa* are found on spruce, and most spend half of their life cycle on alternate hosts in the Ericaceae family. Symptoms of these basidiomycetes include yellowing of current year needles, powdery orange spores in July or August, with needles falling off in September. This is an important disease of Christmas trees due to the loss of new needles. Heteroecious species will produce spermagonia and aecia on needles and cones of spruce. However, *C. weirii* H.S. Jacks, or Weir's cushion rust, is autoecious, completing its entire life cycle on spruce. Orange, cushion-like telia form in spring from yellow bands on one year old needles. These telia produce teliospores and eventually basidiospores that infect new needles. When compared to other rusts, *C. weirii* can build into epidemics more quickly due to its simpler life cycle (Hansen & Lewis, 1997).

Other diseases affect the needles and branches, for example, *Sirococcus strobilinus* G. Preuss. This ascomycete causes shoot blight of newer tissue and seedling death (Funk, 1985; Sinclair & Lyon, 2005). Symptoms start with drooping of new, succulent tissue on seedlings as

well as young shoots and year-old twigs due to lesion formation. These portions wilt and turn brown anywhere from a few days after infection to a few weeks. During June through August, shoot death and needle droop occur. Often the fungus overwinters in these dead tissues. Pycnidia typically form on these dead shoots and bases of needles in spring, but could form in as little as 2 weeks under optimum conditions. Conidia are water dispersed and germinate in moist, cool conditions. *Sirococcus strobilinus* can also infect seed, where 0.1-3% infested seed can lead to 30% of infected seedlings (Sinclair & Lyon, 2005).

Setomelanomma holmii M. Morelet was first found in North America in 1998 (Rossman et al., 2002). Observed on living twigs of Colorado blue and white spruce, *S. holmii*, an ascomycete, was present in 21 counties in Wisconsin. The small black perithecia developed in May and June on twigs have been associated with chlorosis and rapid needle loss, termed sudden needle drop, or SNEED. However, like *S. lautii*, Koch's postulates have not been completed with *S. holmii*, so it is not clear if it is a true pathogen of spruce, or may just associated with sudden needle drop.

Diplodia pinea (Desmaz.) J. Kickx. is a recognized pathogen on conifers that causes tip blight, cankers, and dieback (Hansen & Lewis, 1997; Sinclair & Lyon, 2005). It is a notorious opportunistic pathogen, causing severe damage on trees planted outside their native range that are stressed from drought, heat, frost, insect damage, and soil compaction. *Diplodia pinea*, an ascomycete, can also infect other conifers like spruces, when environmental stress occurs and there is a large supply of spores available from nearby pines. There are many symptoms in trees with Diplodia blight, including resinous cankers, blighted tips the color of straw, and cone death. *Diplodia pinea* typically affects newer tissues unless the tree is stressed, in which case it invades older woody tissue. The disease cycle starts with conidia dispersal from dead needles, cones and

sometimes twigs throughout the year. Black pepper-like pycnidia can be found on cones, branches, dead needles, and fascicle sheaths. The fungus enters through stomatal openings, where it can begin killing tissue, or become latent until stress triggers pathogenic activity. Second-year cones become infected during their rapid growth period in spring, and are important sources of inoculum for the disease cycle to continue. Diplodia blight epidemics are more likely to occur during wet springs, and nitrogen fertilization may further predispose trees to infection due to its promotion of young new succulent tissue for infection (Sinclair & Lyon, 2005). Currently, Michigan has an epidemic of Diplodia blight (O'Donnell, 2014)

Finally, the most prominent branch-killing disease of spruce trees is Cytospora canker, caused by *Cytospora kunzei* Sacc., an ascomycete. Cankers with profuse amounts of amber-colored resin that crystallizes to a white color found on branches or trunks, along with dead branches are indicators of this disease (Sinclair & Lyon, 2005). Stressed trees from drought, frost, or wrong site conditions are more susceptible to this disease, particularly Colorado blue spruce when it is planted outside of its Rocky mountain range. *Cytospora kunzei* typically attacks trees 10 years or older, with older branches more likely to be infected. Once a branch is infected, needles turn brown and drop in spring and summer, while the branch will stay attached to the tree. Cytospora canker typically moves from the bottom to the top of the tree, but occasionally will begin at a middle branch or kill the top of the tree. Cankers are inconspicuous at first because the bark is cemented onto the branch by resin, but eventually appear sunken a few years after infection, with resin flow around the perimeter (Hansen & Lewis, 1997; Sinclair & Lyon, 2005). Both conidia and ascospores are released in moist conditions, and are dispersed by air and water splash, though it is unknown if conidia become airborne. After infection,

usually from a wound, and girdling of the branch, areas beyond the canker are filled with perithecia and pycnidia to continue the life cycle.

Within the last 20 years, spruce trees across Michigan began dropping needles and having dead or dying branches, indicative of a needle cast disease or canker disease. The symptoms started with loss of year-old or older needles, along with entire branches dead starting from the base of the tree and moving upward. Mature trees throughout the state seemed to slowly deteriorate in health and vigor, telltale signs of tree decline (Manion, 1991). While plant pathology is traditionally viewed using the disease triangle as a model, taking into account a pathogen, a susceptible host, and a conducive environment, decline models are not as clear.

Tree declines involve a multifaceted etiology with numerous biotic and abiotic aspects, with the importance of each individual factor not always apparent, making for complex disease models (Manion, 1991). Sinclair and Lyon (2005) attempted to categorize tree declines, stating they are likely to fall into one of four models: continual stress by one factor, drastic injury then secondary stress, cohort senescence, and interchangeable predisposing, inciting, and contributing factors. To give examples of these models, many insects and viruses cause continual stress to trees that may lead to tree decline, and removing secondary stress and contributing factors would not reverse the process. Drought stress creates wounds, in the form of bark cracks, for opportunistic organisms or abiotic factors to affect otherwise healthy trees that would normally defend against those situations, with other factors adding to and intensifying the decline. Cohort senescence can occur if the environment is not able to sustain the biomass of a certain size or age of trees, but can be considered normal if trees are already considered old for a particular species. Finally, the model of predisposing, inciting, and contributing factors supports slow declines with pathogens playing an important role. Predisposing factors could include age, soil compaction,

poor fertility, low soil moisture, and other unsuitable site conditions. Inciting factors include insect defoliation, drought, freezing temperature, and pollution, and contributing factors like canker fungi, wood and bark boring insects, root rot fungi, and viruses should also be taken into account (Manion, 1991). If these stressing factors are removed or equalized with favorable conditions, the tree may be able to recover.

With the initial symptoms of spruce decline including needle loss and branch death, many diseases described above could be causal agents, or contribute to the decline. *Rhizosphaera kalkhoffii* and *S. strobilinus* have always been present, but have not seemed to increase in pressure with increasing spruce decline symptoms, while symptoms from rust diseases have not been present in declining spruce in Michigan. Cankers are a likely culprit, as they can incite both needle drop and branch death. There has been an increase in Diplodia blight in Michigan, which does cause cankers (O'Donnell, 2014). It is possible that *C. kunzei* could have increased, but curiously, there have been reports of *Diaporthe* on spruce in nurseries and tree farms causing tip blight and branch death with cankers, similar to spruce decline symptoms (Igoe et al., 1995; Sanderson & Worf, 1986).

In 1986, Sanderson and Worf reported needles and expanding tips of Colorado blue spruce curling and dying, with occasional entire young shoot dying in nursery and landscape trees in Wisconsin. Stem cankers with resin were sometimes found to occur in dead branches. While symptoms were similar to *S. strobilinus*, isolates collected from diseased plants were more often *Diaporthe occulta* (formerly *Phomopsis*) than *S. strobilinus*. Greenhouse studies demonstrated increasing disease severity with increasing temperature and relative humidity, with Colorado blue spruce the most susceptible species tested. In 1995, *D. occulta* was also confirmed in Michigan (Igoe et al., 1995). Initial symptoms included needle loss and branch

death on the lower part of the trees, associated with inconspicuous cankers. Through multiple experiments, they found that simulating transplant stress by pruning the roots increased canker lengths, current season growth resulted in longer cankers than older tissue, and covering the wound with Parafilm resulted in shorter cankers. Both mycelia and conidia were able to cause branch dieback. Interestingly, conidial infection was lessened by pruning the tips due to less succulent tissue, but wounding was necessary for mycelial infection. Thus, latent *D. occulta* mycelia on the branch could invade the wound if conidia were unsuccessful due to less succulent tissue. They also mentioned that while the symptoms were comparable to *C. kunzei*, they never isolated this pathogen while sampling.

These studies were not the first time *Diaporthe* was associated with spruce. In 1929, improperly cultivated spruce were reported to be infected with *Diaporthe occulta* in New Jersey (White, 1929). In 1989, *Diaporthe occulta* was isolated regularly from both healthy and diseased twigs in an endophyte community study, and was more associated statistically with diseased Norway spruce compared to healthy ones at one study site. In contrast, *Alternaria alternata, Epiccocum nigrum,* and *Cytospora* were only hardly ever isolated (Sieber). The genus *Diaporthe* encompasses many different species that act as plant pathogens, endophytes, and saprobes, often with wide host ranges (Gomes et al., 2013; Santos & Phillips, 2009; Udayanga et al., 2011). Species from this genus have traditionally been named according to host from which they were isolated (Uecker, 1988), however, this has caused many problems, since many *Diaporthe* species are not host-specific (Crous & Groenewald, 2005; Rehner & Uecker, 1994; Uddin et al., 1997), making positive confirmation of specific epithet complicated.

Spruce decline has been increasing in recent years, leaving us with many questions. Is spruce decline found throughout the Lower Peninsula of Michigan? What is/are the fungal

species involved in the decline? How virulent is the pathogen on different spruce species? What is predisposing trees to spruce decline? Are there any inciting factors? To answer some of these questions, research was planned, and is reported in this document. A survey of the Lower Peninsula was performed, sampling symptomatic trees for cankers, and discovering the likely fungal pathogens associated with spruce decline. Next, sequencing of two gene regions was carried out to determine the variability of *Diaporthe* haplotypes commonly isolated from trees exhibiting spruce decline. Finally, greenhouse inoculations were performed to a) determine the virulence of the five Diaporthe haplotypes on spruce host taxa, and b) assess the patterns of resistance and susceptibility across six spruce taxa. Finally, Koch's postulates were performed to demonstrate that *Diaporthe* recovered from trees displaying spruce decline is in fact pathogenic, while being mindful of the potential for other biotic and abiotic factors playing a role in the spruce decline complex.

CHAPTER 2

Reprinted from McTavish, CK, M. Catal, D.W Fulbright and A.M Jarosz, 2018, Spruce Decline and *Diaporthe*: Incidence, Taxonomy, Virulence, and Tree Susceptibility in Michigan, Plant Disease 102(11):2330-2340

INTRODUCTION

Narrow-leaved evergreens are an important commodity in Michigan, encompassing 65% of Michigan's woody plant acres in production (MDA & USDA NASS, 2005). Michigan ranks third in the country for acres of Christmas trees, and cut 1,739,538 Christmas trees in 2012 alone (USDA 2012), According to a recent estimate, 23% of Christmas tree acreage in Michigan was spruce, with Colorado blue spruce making up almost half of spruce Christmas tree production (USDA NASS 2014). Spruce trees are also important landscape trees, ranking second in abundance and comprising 12% of all urban and community trees in Midwestern cities (Wade 2010).

Unfortunately, in the early 2000s, spruce trees began displaying symptoms of what we term spruce decline (Jill O'Donnell and DW Fulbright, pers comm). Initial symptoms include the loss of needles more than one-year-old on the lowest branches of primarily mature trees. Over time, current year needles begin to die followed by the death of lateral branches closest to the trunk. Needle loss and lateral branch death continues outward from the trunk until the whole branch succumbs to disease. Whole branch death usually begins at the bottom of the tree and progresses slowly upward over multiple years, ruining the aesthetic appeal (Fig. 1A).

This symptomology is associated with many tree declines, which are multifaceted diseases that primarily affect mature trees. Characterized by slow deterioration in health and vigor, tree declines have a complex etiology influenced by multiple abiotic and biotic factors

Figure 1. A. Colorado blue spruce displaying spruce decline symptoms of needle drop and branch death beginning at the bottom of the tree and progressing upwards. B. A segment of the top layer of bark on the branch was removed to reveal a brown canker, with uninfected plant tissue appearing white.

(Manion, 1991). Spruce decline has caused havoc in the nursery and landscape industry, with sales decreasing and costs increasing. According to a recent grower poll, spruce decline is causing losses of $16,250 per grower (Jill O'Donnell, Survey at the Michigan Christmas Tree Association 2014 winter meeting; unpublished), which is a significant impact on growers because 70% of Michigan operations have less than 25 acres (USDA NASS 2014).

While partial needle loss could be due to diseases like *Rhizosphaera kalkhoffii*, or the unconfirmed pathogens *Stigmina lautii* and *Setomelanomma holmii* (SNEED), the ability to cause both needle loss and progressive mortality of entire branches suggested that a canker-causing pathogen was contributing to decline symptoms (Sinclair and Lyon 2005; Hodges 2002; Walla and Kinzer 2006; Rossman et al. 2002). Preliminary work found that cankers were indeed associated with branch death and needle loss. However, *Cytospora kunzei* Sacc, a well-characterized canker-causing pathogen of spruce, was only rarely isolated from these cankers (Sinclair and Lyon 2005; Hansen and Lewis 1997). Unlike Cytospora cankers, which commonly display resinous streaks on the branch above the canker, cankers causing spruce decline were not evident until the bark was scraped back to expose the phloem and vascular cambium layers (DW Fulbright and S Stadt, unpublished data). Cankers were brown with occasional resinous streaking found within the phloem and cambium layers, and did not spread into the xylem (Fig. 1B). Multiple cankers were found on most dying branches.

This study was undertaken to discover the canker causing pathogen associated with spruce decline in Michigan. Four fundamental questions were addressed concerning spruce decline: Four fundamental questions were addressed concerning spruce decline: i) where is spruce decline located in the Lower Peninsula of Michigan? ii) what fungi are associated with cankers, and what genus is the most likely pathogen causing cankers on declining trees? iii) how variable is the pathogen? and, iv) how susceptible are commonly planted spruce taxa to the decline pathogen?

MATERIALS AND METHODS

Spruce decline survey in the Lower Peninsula of Michigan

In the summer of 2013, spruce trees were surveyed to estimate the spatial distribution of spruce decline and to obtain canker samples for laboratory isolation. The Lower Peninsula was divided into four regions and within each region, six sites (five in the central region) were chosen so all areas of the Lower Peninsula were represented (Fig. 2, App Table 1). At each site, eight

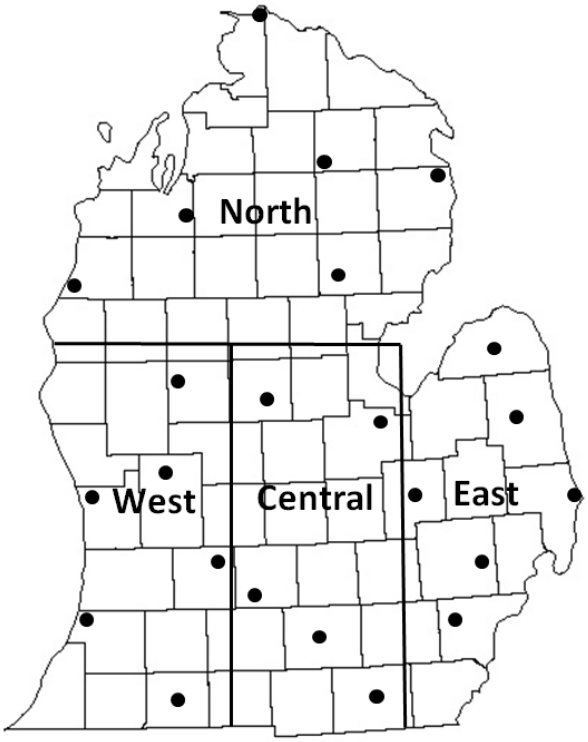


Figure 2. Regions and sites of the 2013 spruce decline canker survey. Dots represent sites (see App. Table 1).

mature trees from predominantly landscape settings were selected that were separated by at least 160 meters and exhibited spruce decline symptoms, with the exception of East 3, where 7 trees were selected. A single dying branch that displayed spruce decline symptoms but had not yet senesced completely was collected from each tree. Partially dying branches were chosen to maximize the likelihood of isolating true pathogens, because completely senesced branches

would have had weakened defense systems, allowing opportunistic microorganisms to invade. Conversely, a symptomless branch would have had a lower likelihood of locating the smaller, developing cankers on those branches as disease developed. To expose cankers on dying branches, the top layer of bark no deeper than the vascular cambium was scraped away (Fig. 1B). The first two cankers uncovered on each branch were sampled. In total, isolates were obtained from 393 cankers on the 159 branches collected across the Lower Peninsula of Michigan.

Fungal Isolation and Sequencing

Canker tissue samples were cut into approximately 3mm x 3mm segments and surface-sterilized in a 10% bleach solution for one minute, followed by two one-minute rinses in sterile deionized water. Three segments per canker were plated on petri plates containing full strength potato dextrose agar (PDA, Difco-Franklin Lakes, New Jersey). Four to six days later, a representative of each fungal morphology on the plate was sub-cultured to fresh PDA plates by hyphal tipping. Data from three sites in the west region were not included in summary statistics because slightly different protocols were followed. Once sub-cultured, isolates were grouped initially by morphotype, and taxonomic identification of the five most-common morphological groups was confirmed by sequencing the internal transcribed spacer (ITS) region of the nuclear ribosomal RNA gene operon using ITS1 and ITS4 primers (White et al. 1990). In addition to the five most common morphotypes, three known conifer pathogen genera, *Cytospora, Fusarium* and *Pestalotiopsis* were also confirmed by sequencing the ITS region. *Diaporthe* isolates were stored on filter paper and agar plugs in double distilled autoclaved water for future species identification.

ITS sequencing was executed using protocols revised from Catal et al. (2010). Single spores isolates were grown in potato dextrose broth (PDB, Difco Laboratories) for 14 days at

which time the liquid was vacuumed away using Büchner funnel with grade 1 Whatman cellulose filter paper (GE Healthcare). Between 50-100 mg of hyphae were lyophilized and ground in 1 ml of 2% hexadecyltrimethyl-ammonium bromide buffer until viscous and incubated at 65°C for 60 minutes. Following centrifugation at 13,000 rpm for 5 minutes, 0.7 ml of supernatant was pipetted to QIA shredder mini-column (Qiagen Inc.) and centrifuged at 13,000 rpm for 2 minutes. Approximately 0.5 ml of supernatant was precipitated with 0.3 ml cold 95% ethanol, and was transferred to DNeasy mini-columns. DNA was washed and eluted following Qiagen DNeasy Plant Mini Kit protocol. Amplification reactions had a total volume of 25 µl consisting of 12.5µl of GoTaq (Promega) Green Master Mix 2.5 µl of each ITS1 and ITS4, 2 µl DNA, and 5.5 µl of nuclease-free water. PCR conditions consisted of initial denaturation at 95°C for 2 minutes, followed by 30 cycles of 55°C for 30 seconds, 72°C for 60 seconds, and 95°C for 30 seconds, with a final extension at 72°C for 10 minutes. Samples were purified using ExoSAP-IT (Affymetrix, Santa Clara, CA), and were sequenced in the Genomics Core of the Research Technology Support Facility at Michigan State University using the Applied Biosystems BigDye v3.1 chemistry following manufacturer's instructions and then analyzed on an Applied Biosystems 3730xl sequencer.

Phylogenetic analyses:

The genus *Diaporthe* encompasses many different species that act as plant pathogens, endophytes, and saprobes, often with wide host ranges (Udayanga et al. 2011; Santos & Phillips 2009; Gomes et al. 2013). Species from this genus have traditionally been named according to host from which they were isolated (Uecker 1988), however, this has caused many problems, since many *Diaporthe* species are not host-specific (Rehner & Uecker 1994; Uddin et al. 1997; Crous & Groenewald 2005), making positive confirmation of specific epithet complicated. Due

to this, it was decided to focus on genus-level variability in order to identify the potential intensity of disease and population dynamics and determine species-level information at a later date. To assess the genus level variability among the *Diaporthe* isolates, 23 samples sequenced using ITS as described above were also sequenced for the β-tubulin (TUB) gene using primers Bt2a and Bt2b (Glass and Donaldson, 1995). PCR conditions were the same as described for ITS amplification. Sequences were edited using BioEdit (Hall 1999), aligned separately by gene using ClustalW, visually checked and minimally adjusted. A partition homogeneity test using the branch and bound method was performed in PAUPv4.0a159 (Swofford, 2002), and resulted in a p-value of 0.001, thus the genes were analyzed separately. Sequences were visualized as minimum spanning trees in PopART (Bandelt, Forster & Röhl 1999, http://popart.otago.ac.nz).

After comparing these haplotypes to each other, we then compared them to other known species. To do this, a representative of each *Diaporthe* haplotype above was chosen for analysis using both ITS and TUB sequences. To choose comparative sequences, a BLAST search was performed using the ITS sequence from each haplotype. Species that were closely related were then compared to the phylogenetic tree from Gomes et al. (2013), and 15 species were chosen, downloading the ITS and TUB genes from the NCBI database using the accession numbers provided by Gomes et al. (Table 1). Neighbor-joining (Saitou and Nei, 1987), maximum-likelihood, and maximum parsimony phylogenetic analyses were performed using MEGA6 (Tamura et al., 2013) and Bayesian phylogenetic analyses were performed using MrBayes v3.2.6 (Ronquist and Huelsenbeck, 2003). All trees were rooted with *Diaporthella corylina*. The Kimura 2-parameter nucleotide substitution model was used for evolutionary distance analysis with an estimated gamma distribution for all ITS analyses (Kimura, 1980), and the Tamura 3-parameter model with invariant sites was used for TUB analyses (Tamura, 1992). Bootstrap

analysis (Felsenstein, 1985) was performed for statistical support of branches in neighbor-joining (2000 replicates), maximum-likelihood, and maximum parsimony analyses, bootstrap analysis (all 1,000 replicates), while Bayesian analyses used posterior probabilities.

Maximum likelihood analysis used nearest-neighbor-interchange (NNI) as the optimal tree search. To search the tree space in the parsimony analysis, Tree-Bisection-Reconnection (TBR) was employed using all characters (Nei and Kumar, 2000), and the consensus tree was formed using the most parsimonious trees. Bayesian phylogenetic trees were generated under default priors and likelihood settings, as not much was known prior to the start of analysis. The Markov Chain Monte Carlo (MCMC) analysis of four chains was run for 250,000 generations, sampling every 100 generations using a burn-in of 5,000 generations (20%), with the average standard deviation of split frequencies resulting in less than 0.01. The phylogenetic tree was formatted in FigTree v1.4.2 (Rambaut, 2012).

Pathogen virulence and spruce susceptibility inoculations

Three inoculation experiments were carried out in different Michigan State University greenhouses from 2013 to 2014. For all experiments, temperature and sunlight varied with natural day/night cycle, and internal temperatures were controlled by venting. In the 2013 experiment and the seed source experiment, swamp coolers were used to control conditions, while the greenhouse for the 2014 experiment involving 6 spruce taxa was not properly equipped to use them. Greenhouses were whitewashed in early summer to reduce light intensity and reduce day-time temperatures. At the conclusion of each experiment, cankers were resampled for *Diaporthe* and Koch's postulates were confirmed by sequencing the ITS region following above-mentioned protocols to confirm that the canker was caused by the haplotype originally inoculated onto the tree.

Table 1. Strain, isolation source, country of origin, and GenBank accession numbers used in the study

Species	**Strain**	**Isolation source**	**Country of Origin**	**GenBank accession number**	
				ITS	**TUB**
Diaporthe alleghaniensis	CBS 495.72	*Betula alleghaniensis*	Canada	KC343007	KC343975
Phomopsis amygdali	CBS 126679	*Prunus dulcis*	Portugal	KC343022	KC343990
Diaporthe celastrina	CBS 139.27	*Celastrus scandens*	-	KC343047	KC344015
Diaporthe eres	CBS 102.81	*Juglans regia*	Italy	KC343074	KC344042
Diaporthe eres	CBS 186.37	*Picea ab*ies	UK	KC343079	KC344047
Diaporthe eres	CBS 287.74	*Sorbus aucuparia*	Netherlands	KC343084	KC344052
Diaporthe eres	CBS 375.61	*Malus sylvestris*	-	KC343088	KC344056
Diaporthe eres	CBS 439.82	*Cotoneaster* sp.	UK: Scotland	KC343090	KC344058
Diaporthe helianthi	CBS 592.81	*Helianthus annuus*	Serbia	KC343115	KC344083
Diaporthe vaccinii	CBS 122112	*Vaccinium macrocarpon*	USA: New Jersey	KC343224	KC344192
Diaporthe vaccinii	CBS 122114	*Vaccinium corymbosum*	USA: Michigan	KC343225	KC344193
Diaporthella corylina	CBS 121124	*Corylus* sp.	China	KC343004	KC343972
Diaporthe bicincta	CBS 121004	*Juglans* sp.	USA: Tennessee	KC343134	KC344102
Diaporthe nobilis	CBS 587.79	*Pinus pentaphlla*	Japan	KC343153	KC344121
Diaporthe decedens	CBS 109772	*Corylus avellana*	Austria	KC343059	KC344027
Diaporthe haplotype 1	-	*Thuja occidentalis*	USA: Michigan	MG452402	MG735768
Diaporthe haplotype 2	-	*Picea pungens*	USA: Michigan	MG452403	MG735771
Diaporthe haplotype 3	-	*Picea pungens*	USA: Michigan	MG452404	MG735769
Diaporthe haplotype 4	-	*Picea pungens*	USA: Michigan	MG452405	MG735772
Diaporthe haplotype 5	-	*Picea pungens*	USA: Michigan	MG452406	MG735770

Experiment 1: Evaluating virulence across *Diaporthe* isolates:

Six *Diaporthe* isolates collected in Michigan were inoculated onto 3 common spruce taxa grown in the state: Colorado blue (*Picea pungens* Engelm.), Norway (*P. abies* (L.) H. Karst.), and white spruce (*P. glauca* (Moench) Voss). Collectively, the six isolates encompassed the range of variability for ITS sequence, colony color, geographic region and host of origin (Table 2). The three-year-old trees (two years in a seed bed, one year in a transplant bed, or 2-1) were

Table 2. *Diaporthe* isolates used to assess virulence patterns. Collectively, the isolates encompass the known variability for ITS haplotype, colony color, geographic region, habitat and host of origin

Isolate code	*Diaporthe* haplotype[a]	Colony color[b]	Region within Michigan[c]	Habitat	Host
90	1	white	West	Forest	*P. pungens*
5	2	grey	Central	Nursery	*P. glauca*
15	3a	white	Central	Landscape	*Thuja occidentalis*
68	3b	grey	West	Nursery	*P. pungens*
53	4	white	North	Landscape	*P. pungens*
82	5	grey	Central	Nursery	*P. pungens*

a. Haplotypes were determined using sequences from the internal transcribed spacer region (ITS). The five haplotypes differed based on substitutions and insertions at 11 variable positions along the 600 base pair sequences. Isolates 15 and 68 have identical ITS sequences corresponding to haplotype 3.
b. Cultures were grown on full strength PDA agar for 10 days in ambient lighting
c. Regions shown in Figure 2.

purchased from a nursery on the west side of the Lower Peninsula of Michigan. One hundred trees from each spruce taxon were planted from May 5-8, 2013 into pots measuring 10.2cm by 10.2cm by 35.6cm long pots filled with Suremix perlite soil (Michigan Grower Products Inc., Galesburg, MI). Tree roots were cut to mimic transplant damage as well as to fit in pots properly. During May 28-30 of 2013, approximately 20 days after planting during which

budbreak had occurred, 15 trees from each spruce taxon were inoculated per *Diaporthe* haplotype, and an additional 10 trees served as negative controls that were inoculated with a sterile plug of PDA. Prior to inoculations, all *Diaporthe* isolates were grown on PDA under ambient lighting for approximately 10 days to allow full expansion of the mycelia across the 90mm diameter petri plate. Inoculations were carried out by making a small 2x2x3mm hole in the stem approximately 4cm above the soil line, and placing a 2x2mm mycelial disc in the hole. The inoculation site was sealed by wrapping Parafilm around the stem and was not removed. Wounding was necessary for mycelial infection (Igoe, Peterson, and Roberts 1995).

Trees were watered every other day to saturation. Approximately 56 days post-inoculation, canker area was determined by destructively sampling the inoculated stems. Length and width of the canker formed at the inoculation site were measured after scraping off the top layer of bark.

Experiment 2: Evaluating spruce taxa for susceptibility to *Diaporthe*:

Six commonly planted spruce taxa were inoculated with *Diaporthe* to determine differences in susceptibility: Colorado blue (*Picea pungens*), Norway (*P. abies*), white (*P. glauca*), Black Hills (*Picea glauca* var. *densata* Bailey), Serbian (*Picea omorika* (Pancic) Purk.), and Meyer (*Picea meyeri* (Rehder) E.H.Wilson) spruce. The trees were purchased from a nursery on the west side of the Lower Peninsula of Michigan. Most trees were three years old (2-1), with the exception of *P. glauca* var. *densata* and *P. meyeri*, which were four years old (2-2) to ensure proper diameter for inoculations. Stem diameter of the trees averaged 0.85cm and ranged from 0.40cm to 1.96cm. Three *Diaporthe* isolates were used for inoculations: two from virulent haplotypes (4 & 5) and a third from the largely avirulent haplotype (3b) based on the results of the previous experiment.

Trees were planted between April 10 and April 21, 2014. For each spruce taxon, 330 trees were planted as described in the first experiment. Approximately 19 days after planting (May 7-14), during which time the trees had broken bud, trees were inoculated as described above with one of the three *Diaporthe* isolates. Fungal isolates were grown on PDA plates for approximately 10 days to allow mycelia to cover the plate. A sterile PDA plug served as a negative control. Sixty trees from each spruce taxon were inoculated per treatment (i.e., three separate *Diaporthe* isolates and the negative control), and sixty trees were not inoculated to serve as environmental controls to confirm symptoms were not caused by environmental conditions in the greenhouse.

Trees were watered every other day to saturation, and were treated for aphids on June 23, 2014. Approximately 78 days post-inoculation (July 23-31), stems were sampled destructively, as described above, to measure the canker area at the inoculation site.

Experiment 3: Evaluating susceptibility in nine different seed sources of *P. pungens*:

Nine different seed sources of Colorado blue spruce, *P. pungens*, were inoculated to test for differences in susceptibility to *Diaporthe* (Table 3). Trees were planted on May 20, 2014. For each seed source, 25 trees were planted as described in the first experiment (50 for Baby Blue and Majestic). Tree roots were cut to mimic transplant damage. Fifty one days after planting (July 10, 2014), during which time bud break had occurred, trees were inoculated as described above using an isolate from *Diaporthe* haplotype 4 grown on PDA for approximately 10 days in ambient light to allow full expansion across the plate. Twenty trees from each seed source (40 for Baby Blue and Majestic). The remaining trees from each seed source were inoculated with a fungus-free disc of PDA.

Table 3. Seed sources selected for susceptibility trial. Seed sources were chosen to represent the widest variability available in Michigan and the surrounding area.

Common name	Seed source type	Age
Apache	Ecotype	3
Blue Wonder	Variety	3
Dolores	Ecotype within San Juan	3
Baby Blue	Cultivar	1+ (plug start)
Majestic	Variety	3
San Juan	Ecotype	4
Kaibab	Ecotype	4
Misty Blue	Variety	3
Wolf Creek (Pagosa)	Ecotype within San Juan	3

Trees were watered every other day to saturation. Approximately 76 days post-inoculation (September 23-24, 2014), stems were destructively sampled as described above to measure the canker area at the inoculation site.

Statistical Analyses

Data analyses were performed in SAS Institute (2013). Generalized linear model (GLM) analysis of variance was carried out on each inoculation experiment using the PROC GLIMMIX function. Fixed effects included species, inoculum haplotype, and species by inoculum interaction. Post-hoc analyses were performed on least-squares means using the Tukey-Kramer method. To reduce heteroscedasticity, canker area data were $\log_{10}$ transformed after adding one to each datum. Results have been back-transformed for presentation.

RESULTS

Spruce decline survey

Spruce decline was found throughout the Lower Peninsula of Michigan, and declining trees were never difficult to locate at any location. The majority of sampled trees were blue

spruce trees (77%), as this species is a popular landscape tree in Michigan and our results indicated it was highly susceptible to spruce decline (App. Table 2). Other trees sampled included Norway and white spruce. Cankers were not visible on collected branches until the bark was scraped back, where brown cankers were observed in the bark, phloem and vascular cambium, but not penetrating further into the branch (Fig. 1B). Cankers were found on all but one of the 159 branches collected in the survey.

Isolations were attempted from 393 cankers: 109 cankers in the central region (7 of which did not yield fungi): 48 cankers in the west (5 of which did not yield fungi), 134 cankers in the east, and 102 cankers in the north (10 of which did not yield any fungi). The overall average for all regions was 1.8 isolates per canker with slightly different numbers across the four regions. An average of 2.1 isolates per canker were obtained from the central region, 1.4 isolates in the west, 2.2 isolates in the east, and 1.6 isolates in the north. The five most common fungal genera isolated from cankered tissue accounted for 58% (439 out of 759) of the total isolates. No other morphological group had an occurrence greater than 3% of the total isolates, and thus was not identified, as they were not common enough to be causing widespread spruce decline. Three exceptions were made for *Cytospora, Fusarium* and *Pestalotiopsis*, genera which contain known conifer pathogens.

The two most common genera isolated were *Paraconiothyrium* and *Diaporthe*. While their prevalence differed slightly among regions, these two taxa were either the first or second most common genus isolated within each region, (Fig. 3). *Paraconiothyrium* was more common than *Diaporthe* in both the north and east regions of Michigan, while *Diaporthe* was the most common in the west and central regions. The widespread occurrence of both fungal genera presents the possibility that either could be involved in causing spruce decline.

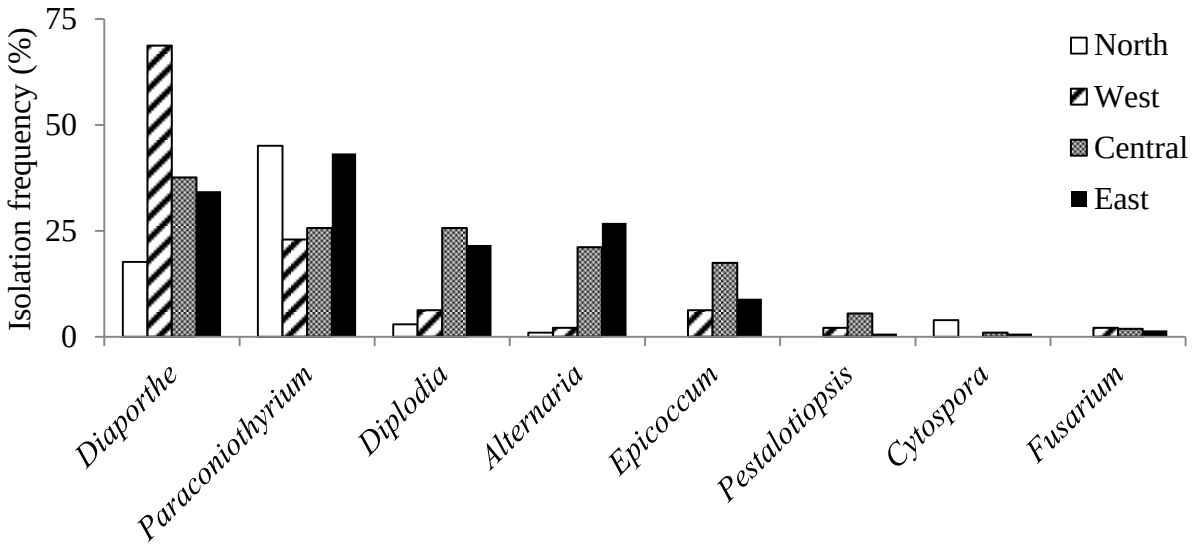


Figure 3. Frequency of isolation of fungal taxa from cankers collected as part of the survey. Regional total percentages are based upon cankers that contained the taxon.

Diplodia and *Alternaria* were the only other genera isolated from spruce cankers in all areas of Michigan (Fig. 3). However, both genera were rarely sampled in both the north and west regions of the state, being found on less than 7% of sampled cankers in both regions (Fig. 3). The fifth most common genus, *Epicoccum*, was not detected in the north region of the Lower Peninsula. The three known canker pathogens of conifers, *Cytospora*, *Pestalotiopsis*, and *Fusarium* never exceeded 6% of the isolates recovered in any one region and their cumulative frequencies across the whole of the Lower Peninsula were 1%, 2% and 1%, respectively. *Pestalotiopsis* and *Fusarium* were not detected in the north region, while *Cytospora* was not found in the west region of the state. The rarity of these three potential pathogen taxa across Michigan argues against them being major components of the current spruce decline epidemic.

Phylogenetic results

A total of 452 base pairs (hereafter: bp) from the ITS and 351 bp TUB sequences from 23 *Diaporthe* isolates collected from declining spruce in Michigan were aligned for comparison.

Analyses confirmed the presence of the same five haplotype sequences detected during preliminary studies. Haplotypes 2, 4, and 5 were closely related; differing by only one or two bp using ITS and identical using TUB. Using ITS, haplotype 1 differed from haplotype 5 by three bp changes, while haplotype 3 was the most distinct, differing from haplotype 1 by five additional bp changes. Using TUB, haplotypes 1 and 3 were also identical to each other, and differed from the grouping of haplotypes 2, 4, and 5 by five bp using TUB. There was no obvious geographical localization or host specialization for any haplotype, although the small sample size for sequenced isolates precluded drawing any definitive conclusions.

The phylogenetic datasets consisted of 19 ingroup and one outgroup (*Diaporthella corylina*) taxa. The ITS and TUB alignments contained 487 and 379 characters, respectively, including alignment gaps. Phylogenetic trees generated by neighbor-joining, maximum-likelihood, maximum-parsimony, and Bayesian analyses were topographically identical within gene, while there were slight differences between genes (Figs. 4, 5, & 6). For ITS maximum-parsimony, 362 characters were constant, 78 characters were parsimony-uninformative, and 47 were parsimony-informative. Six most parsimonious trees of tree length 173 were found, and for all sites and parsimony-informative sites in parentheses, the consistency index was 0.850 (0.701), the retention index was 0.814 (0.814), and the composite index was 0.692 (0.571). For TUB maximum-parsimony, 241 characters were constant, 81 characters were parsimony-uninformative, and 57 characters were parsimony-informative. Ten most parsimonious trees of tree length 211 were found, and the consistency index was 0.863 (0.726), the retention index was 0.743 (0.743), and the composite index was 0.641 (0.540) for all sites and parsimony-informative sites in parentheses.

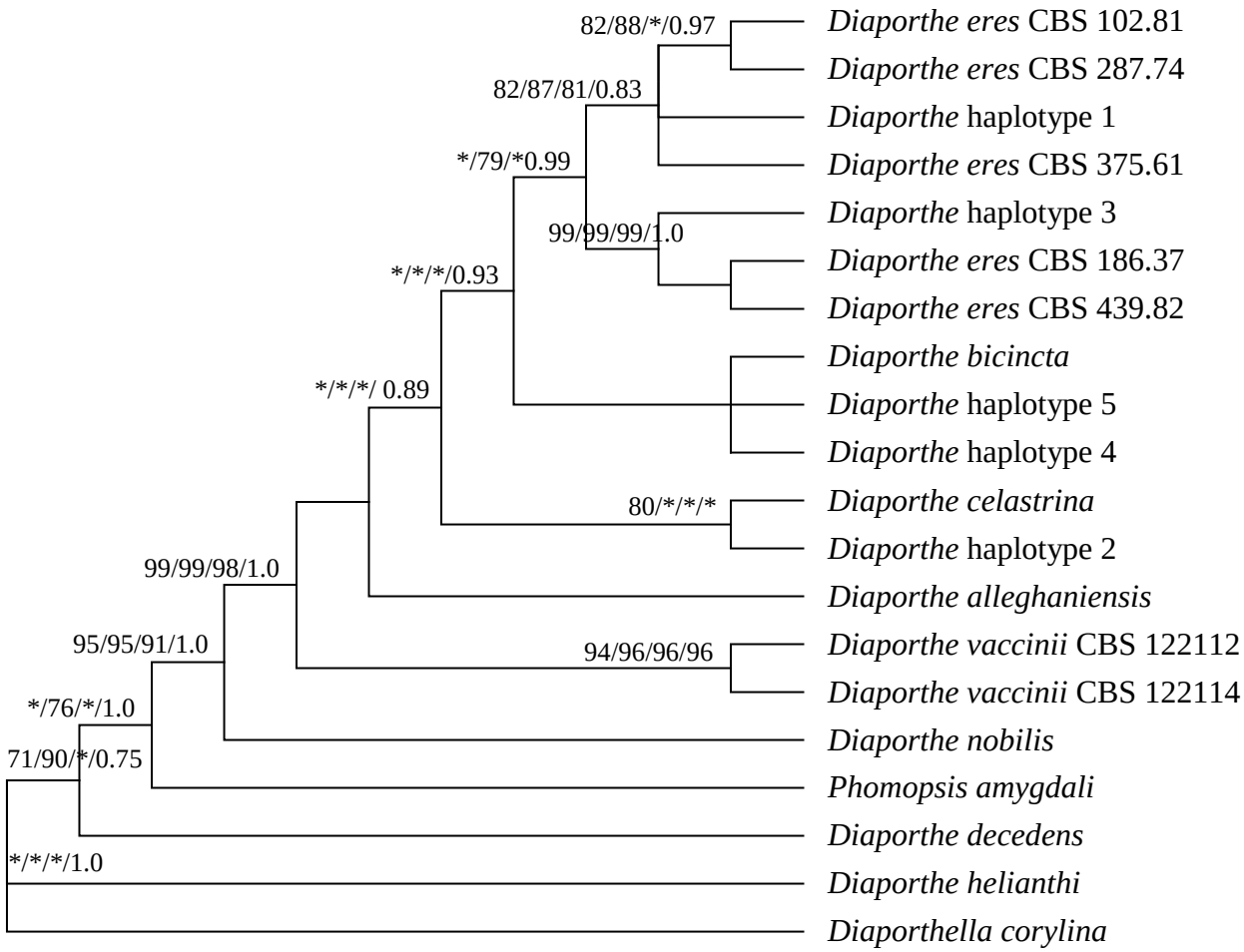


Figure 4. Cladogram of the consensus tree (tree length 173) from parsimony analysis of ITS sequence data. Tree-bisection-reconnection was used to search the tree space, using all sites. Bootstrap values are shown at the nodes, from left to right: neighbor-joining, first most-parsimonious tree, maximum-likelihood, and Bayesian (posterior probabilities) analyses with a cutoff value of 70% or 0.7. A * indicates a below-threshold support value. The cladogram was rooted with *Diaporthella corylina*. Analyses were performed in MEGA6 and MrBayes.

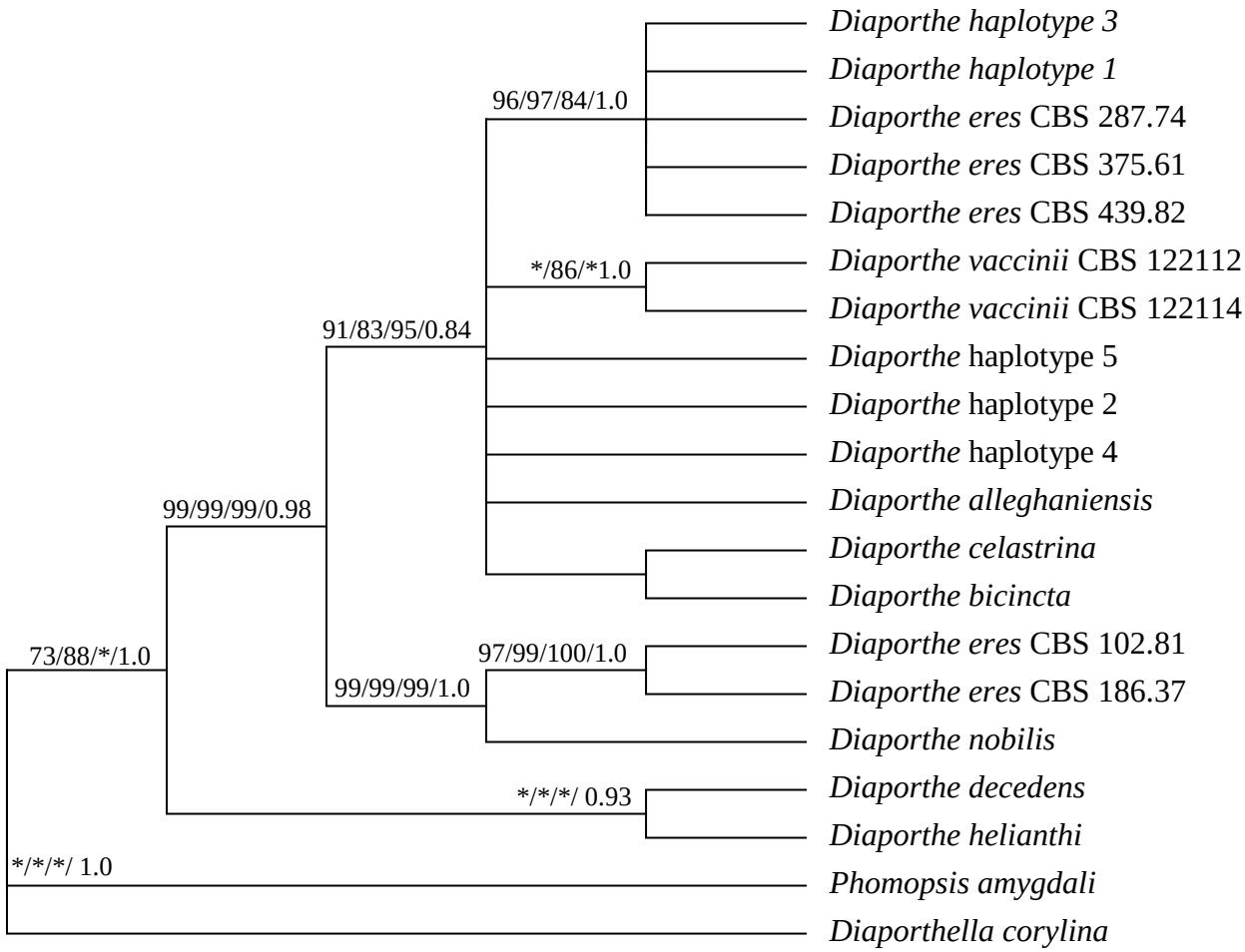


Figure 5. Cladogram of the consensus tree (tree length 211) from parsimony analysis of TUB sequence data. Tree-bisection-reconnection was used to search the tree space, using all sites. Bootstrap values are shown at the nodes, from left to right: neighbor-joining, first most-parsimonious tree, maximum-likelihood, and Bayesian (posterior probabilities) analyses, with a cutoff value of 70% or 0.7. A * indicates a below-threshold support value. The cladogram was rooted with *Diaporthella corylina*. Analyses were performed in MEGA6 and MrBayes.

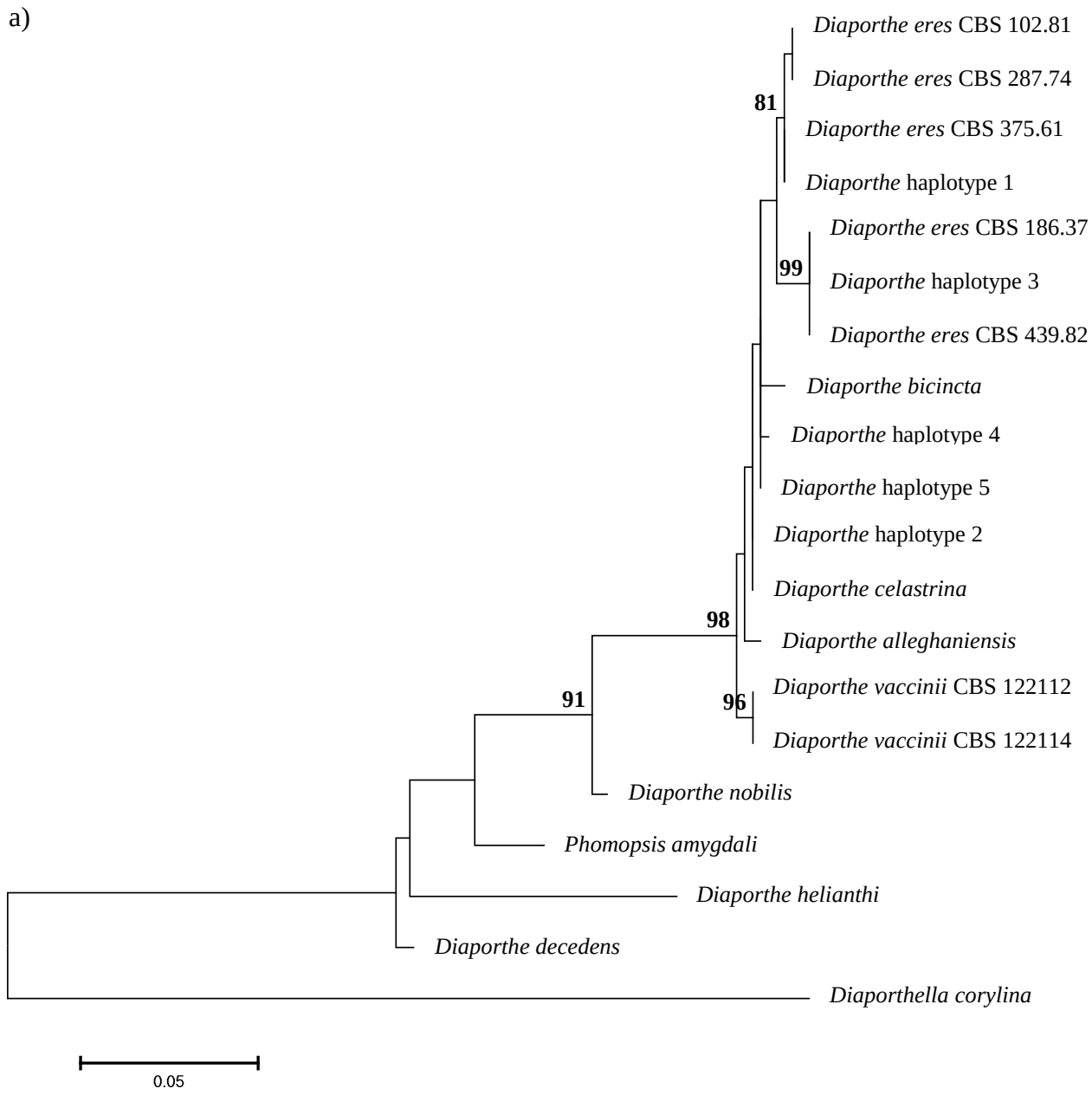
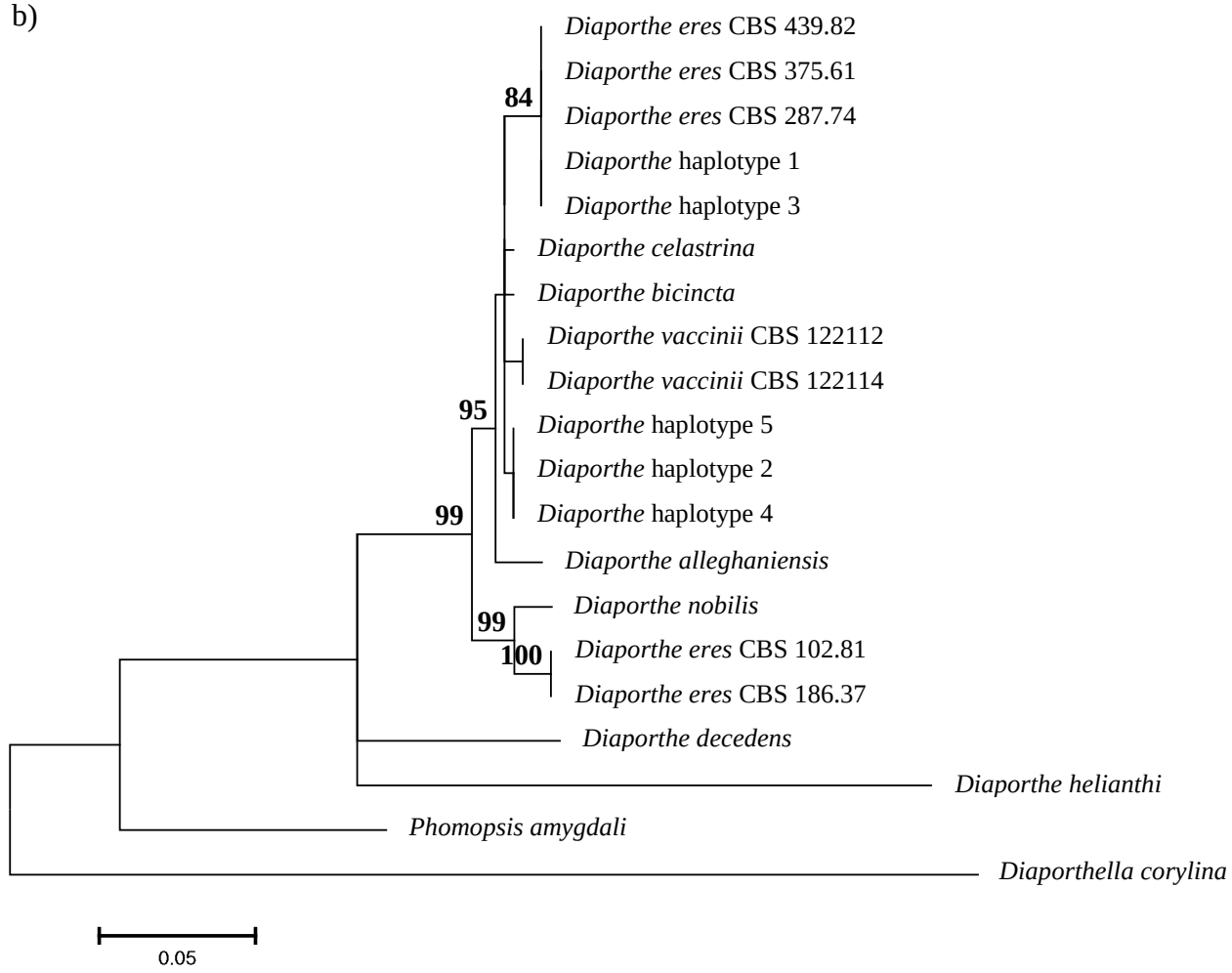


Figure 6. Phylograms of the maximum-likelihood analysis of a) ITS and b) TUB data using the best-fit models (Kimura 2-parameter with estimated gamma distribution and Tamura 3-parameter with invariant sites, respectively). Both used the nearest-neighbor-interchange as a heuristic search method using all sites. Trees with the highest log likelihoods are shown (-1497.7039, -1441.3485), with bootstrap values (1000 replications) displayed at nodes with a 70% cutoff value. Trees are rooted with *Diaporthella corylina*.

Figure 6 (cont'd)

b)



The five haplotypes detected in Michigan did not group into a single, well-supported clade in either gene analyzed (Figs. 4, 5, & 6). However, *Diaporthe* haplotypes 1 and 3 fell into well-supported *Diaporthe eres* clade(s) for both genes. The ITS sequence for *Diaporthe* haplotype 3 was identical to *Phomopsis occulta*, the same species identified in spruce canker diseases (Igoe et al., 1995; Sanderson & Worf, 1986) but no TUB data were available for *P. occulta*. Additionally, *P. occulta*, formerly *Phoma occulta*, has been proposed to be renamed *D. eres* in an effort to reduce competing names within the genus (Rossman et al., 2014).

Despite their high sequence similarity, haplotypes 2, 4, and 5 did not form a single clade, but instead grouped into poorly resolved portion of the cladogram that included representatives from *Diaporthe bicincta* and *D. celastrina*.

Pathogen virulence and spruce susceptibility inoculation

Experiment 1: Evaluating virulence across Diaporthe haplotypes

There were significant differences among the five *Diaporthe* haplotypes for virulence and among three spruce taxa for their level of resistance (p=0.0001 and p=0.0098 respectively, Table 4). There also was a weak *Diaporthe* haplotype by spruce taxa interaction (p=0.06). This

Table 4. ANOVA analysis for inoculation experiment to determine differences in virulence across five *Diaporthe* haplotypes. The degrees of freedom in the denominator was 214 for all comparisons.

Effect	Degrees of Freedom	F-value	Probability > F
Diaporthe haplotype (DH)	6	22.62	<0.0001
Spruce species (SS)	2	4.73	0.0098
DH by SS interaction	12	1.73	0.0626

interaction was caused by the two patterns of virulence across the three spruce taxa. Virulence for haplotypes 3 and 5, which exhibited low and high levels of virulence, respectively, did not differ across the three spruce taxa (Fig. 7). Haplotypes 1, 2 and 4 exhibited a second pattern where virulence on Colorado blue spruce was greater than on Norway and white spruce. Negative controls did not yield cankers. Overall, Colorado blue spruce was the most susceptible of the three spruce taxa tested. Cankers were significantly larger in Colorado blue spruce ($0.92cm^2$; 0.77; 1.10 for mean; lower confidence interval; upper confidence interval respectively) than either Norway ($0.38cm^2$; 0.29; 0.48) or white ($0.54\ cm^2$; 0.45; 0.63). Average canker sizes

across haplotypes fell into three statistical groupings, with some overlap between the two groups exhibiting higher virulence (Table 5).

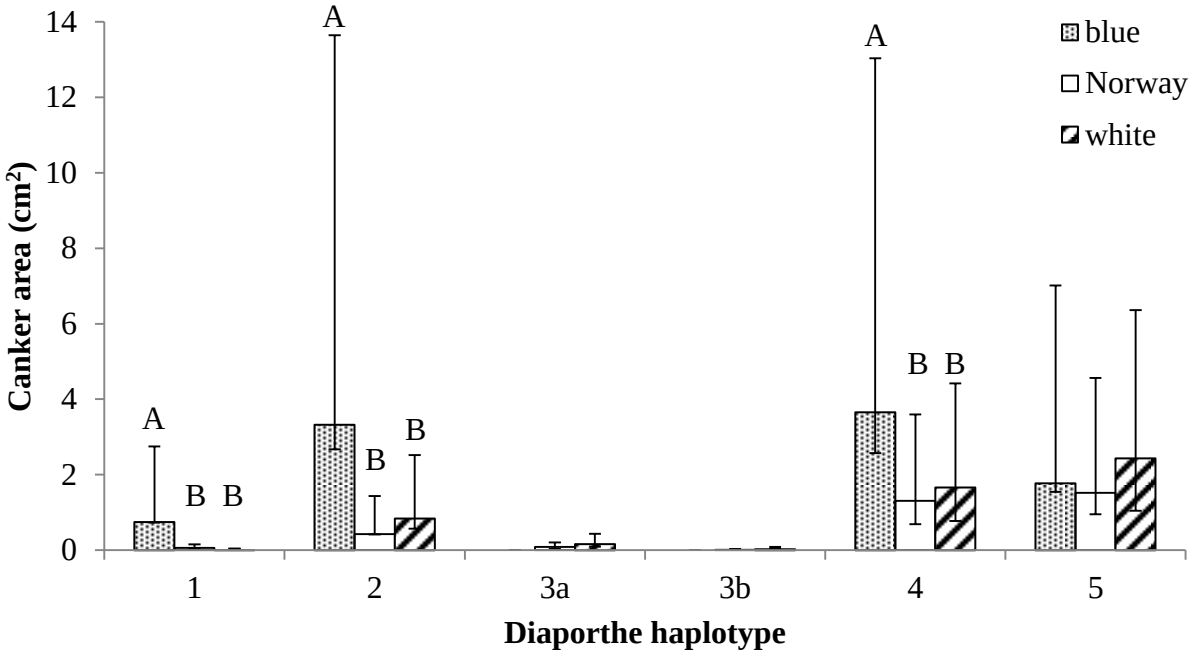


Figure 7. Back-transformed mean canker area (cm^2) caused by *Diaporthe* haplotypes on blue, Norway, and white spruce. Error bars represent back-transformed 95% confidence interval. Species designations are: blue = *P. pungens*, Norway = *P. abies*, and white = *P. glauca*. Capital letters above the bars indicate differences among tree species susceptibility to a particular *Diaporthe* haplotype. Undisplayed negative controls yielded no cankers.

Table 5. Back-transformed average canker sizes for *Diaporthe* isolates. Values followed by the same letter do not differ statistically.

Diaporthe Haplotype (Isolate #)	Average canker size	Tukey-Kramer comparisons
4 (53)	2.058	A
5 (82)	1.885	AB
2 (5)	1.249	B
1 (90)	0.231	C
3 (15)	0.080	C
3 (68)	0.011	C

Experiment 2: Evaluating spruce taxa for susceptibility to *Diaporthe*

Six spruce taxa were inoculated with isolates from three *Diaporthe* haplotypes; haplotype 4 was highly virulent, haplotype 5 was moderately virulent and haplotype 3 was avirulent on Colorado blue spruce. Spruce taxa were chosen because they are planted in Michigan as landscape trees. Analysis of variance indicated large differences among spruce taxa for their level of susceptibility to *Diaporthe* and significant difference among *Diaporthe* haplotypes to cause disease (Table 6). Additionally, the ANOVA indicated a highly significant interaction

Table 6. ANOVA analysis for inoculation experiment to determine differences in spruce taxa susceptibility using three *Diaporthe* isolates.

Effect	Num DF	F Value	Pr > F
Spruce Taxon	5	55.32	<.0001
Diaporthe haplotype	3	395.71	<.0001
Main stem diameter	101	1.15	0.1556
Taxon*Haplotype	15	23.86	<.0001

effect between spruce taxon and *Diaporthe* haplotype ($p<0.0001$). Two spruce taxon by haplotype interaction patterns were evident. Haplotype 3 displayed the first pattern, and was uniformly avirulent across all six spruce taxa (Fig. 8). The more virulent haplotypes 4 and 5 displayed the second pattern where Colorado blue spruce was the most susceptible spruce taxon, followed by Norway, then white spruce. There was a slight tendency among these susceptible spruce taxa for cankers to be larger when trees were inoculated with the haplotype 4 isolate, but the difference was not significant statistically. Black Hills, Serbian and Meyer spruce were largely resistant to *Diaporthe* haplotypes 4 and 5, but Meyer spruce was significantly more resistant to haplotype 4 than either Black Hills or Serbian spruce.

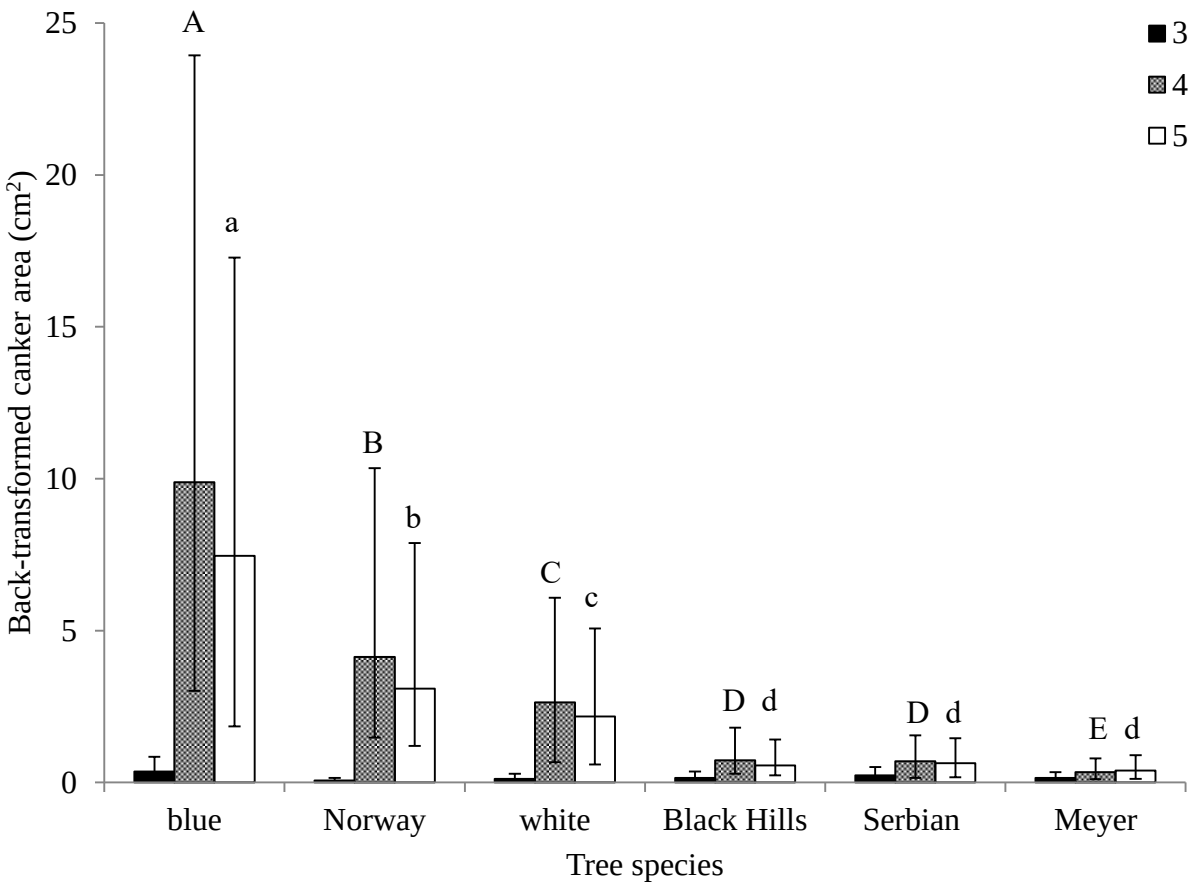


Figure 8. Back-transformed mean *Diaporthe* canker area (cm^2) of isolates on 3 year old blue (*P. pungens*), Norway (*P. abies*), white (*P. glauca*), Black Hills (*P. glauca* var. *densata*), Serbian (*P. omorika*), and Meyer (*P. meyeri*) nursery spruce trees. Error bars represent the 95% confidence interval, upper and lowercase letters represent post hoc analyses of haplotype 4 and 5 differences, respectively. Canker sizes did not differ among the six spruce taxa when inoculated with haplotype 3. Negative controls did not yield cankers (data not presented).

While sampling cankers for trees inoculated with *Diaporthe* haplotype 5, there was one *P. glauca,* nine *P. abies,* and three *P. pungens* trees with dark spore structures on the outer bark overlaying the canker on the main stem (Fig. 9). These spore structures also occurred on six *P. abies* and one *P. pungens* trees inoculated with *Diaporthe* haplotype 4. These structures were harvested and observed under a compound microscope, where alpha and beta spores typical of *Diaporthe* were observed (Fig. 10).

Figure 9. Norway spruce inoculated with haplotype 5 exhibiting dark spore structures and canker indentation. Note the inoculation hole on the right side of the image.

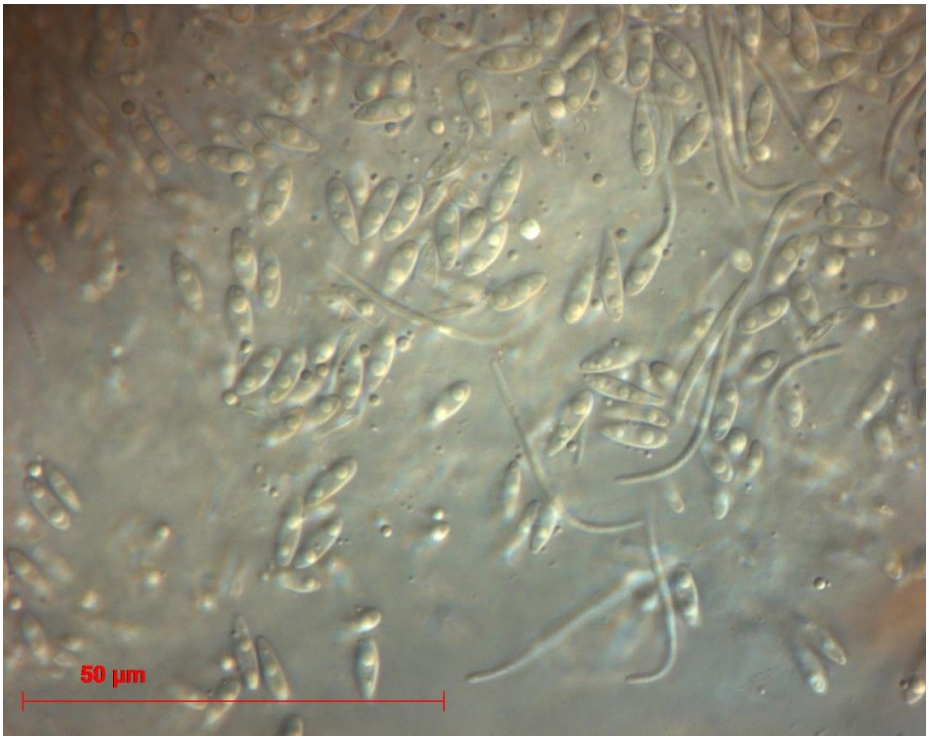


Figure 10. Spheroid-shaped alpha conidia and long, thin, and curved beta conidia isolated from spore structures found in Fig. 9

Experiment 3: Evaluating susceptibility in nine different seed sources of *P. pungens*

Nine different seed sources of Colorado blue spruce representing different varieties, cultivars, and ecotypes were inoculated with a virulent isolate from *Diaporthe* from haplotype 4 (Table 3). Seed sources differed statistically ($F_{(8,162)} = 4.17$; $p = 0.0001$). Average canker size ranged from 0.55 cm^2 for Apache to over 5.41 cm^2 for Misty Blue, but standard errors were relatively large (Fig. 11). The seed sources fell into three overlapping groups, in which Misty blue and Majestic were more susceptible than the other seven seed sources. However, overall canker sizes for Colorado blue spruce were smaller for this inoculation experiment (Fig. 11) compared to Experiment 2 (Fig. 8), with the average canker sizes from the haplotype 4 isolate in this experiment approximately one-quarter the size of experiment 2. There was a considerable amount of death in the experiment (Fig. 12). While more trees died following inoculation with *Diaporthe*, trees also died in the negative control treatment. In some cases death in the negative controls exceeded that seen in the comparable *Diaporthe* inoculation. Tree death was not correlated with the level of susceptibility to *Diaporthe*, and four of seed sources that displayed moderate susceptibility, Dolores, Kaibab, Baby Blue and Wolf Creek, had the highest death rates.

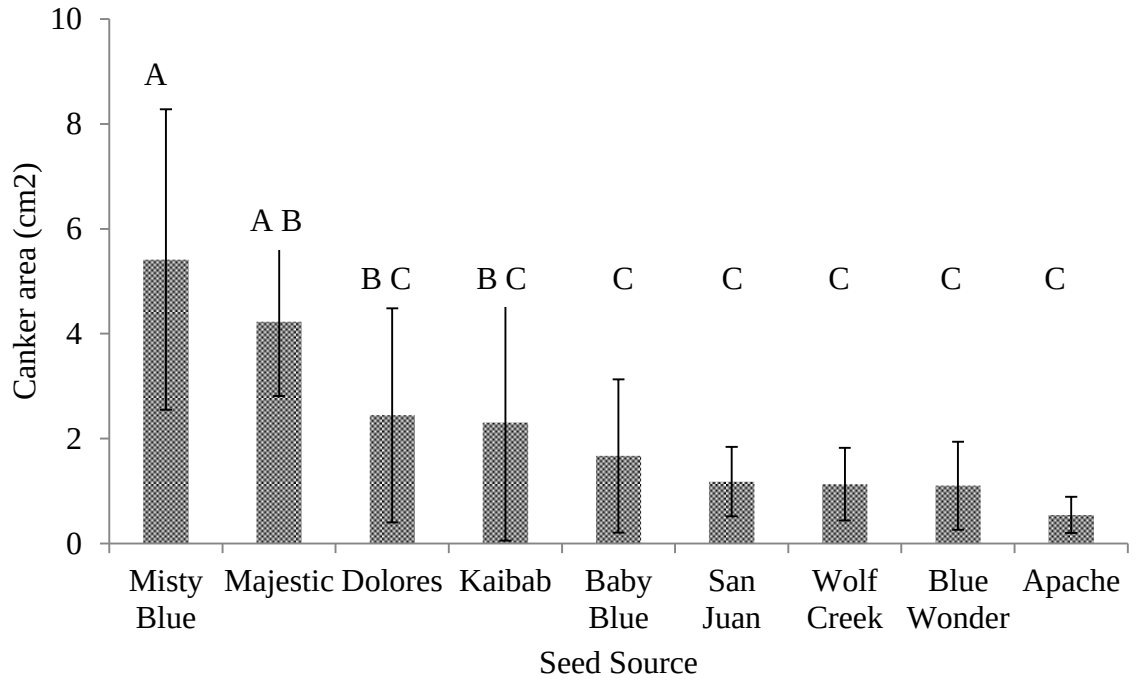


Figure 11. Mean canker area (cm^2) caused by *Diaporthe* haplotype 4 on 9 different seed sources of blue spruce. Error bars represent the 95% confidence interval.

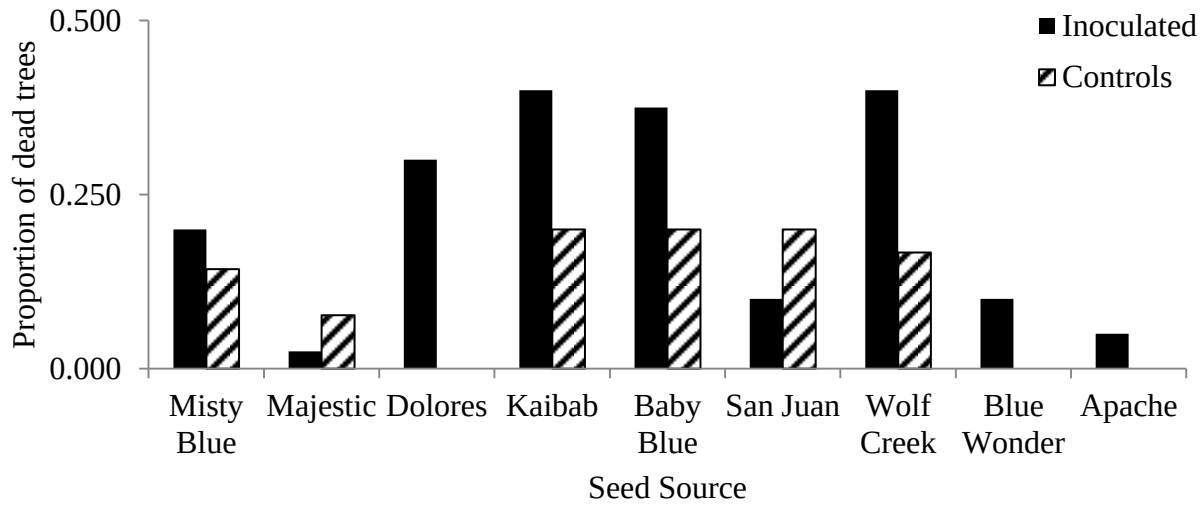


Figure 12. Proportion of dead trees per *Diaporthe* haplotype before the conclusion of the experiment. The legend refers to treatments, with inoculated referring to *Diaporthe* haplotype 4, and controls referring to the negative control (blank plug).

DISCUSSION

Spruce decline was found throughout the Lower Peninsula of Michigan with no obvious epicenter. While the disease was first noted on landscape trees starting in the early 2000s, it is surprisingly widespread. We also suspect spruce decline is not limited to Michigan, as descriptions from growers, extension agents, and tree companies in other states match spruce decline symptoms (Jill O'Donnell, D.W. Fulbright, and C.K. McTavish, *pers. comm.*). Cankers were found on every branch exhibiting spruce decline symptoms in our survey, with one exception, thus cankers play an important role in spruce decline. Interestingly, branches did not display any outward symptoms of cankers; they were only evident once the bark layer was removed (Fig. 1B). Cankers were brown with a distinct margin, restricted to the bark and vascular cambium, and with only occasional resinous streaking.

In order to identify the likely pathogen(s) causing cankers in the spruce decline system, the following criteria were considered: i) There was a wide distribution of the fungal genus throughout Michigan, since spruce decline was found throughout Michigan, ii) the frequency of isolation from cankers was consistently high in all regions, iii) there was evidence for pathogenicity and iv) the symptoms of infection following greenhouse inoculations matched spruce decline symptoms in the field.

Using these criteria, *Cytospora*, which is reported to be a common and damaging canker-causing pathogen of spruce in the central and eastern United States (Sinclair and Lyon 2005), was detected in only 1.4% of the cankers sampled from trees exhibiting spruce decline. *Cytospora* also was not widely distributed, as it was not recovered from all regions studied (Fig. 3). Similar to spruce decline, *Cytospora kunzei* attacks older trees and involves cankers that kill branches, often starting from the bottom of the tree, but *C. kunzei* will usually disfigure the tree

instead of kill it. Additionally, a tree infected with *C. kunzei* will exude copious amounts of resin that will form a white crust around the canker site. In contrast, we have not detected any similar outward symptoms on trees with spruce decline at the canker site. Thus we assert that *C. kunzei* is unlikely to be the main pathogen causing spruce decline in Michigan. The other two known canker pathogens of conifers, *Pestalotiopsis* and *Fusarium,* were isolated at very low rates within the survey, suggesting that they are also not likely to be a major cause of branch death in declining spruce.

Of the five most commonly isolated genera in the survey, *Diplodia*, *Alternaria* and *Epicoccum* had patchy distributions where the genera were rare or absent in northern and western regions of Michigan's Lower Peninsula. Thus, violating our criterion that the spruce decline pathogen should be common in all regions where spruce decline is common. However, *Diplodia* (isolated from14.75% of all sampled cankers) was detected in all regions, but less so in the West and North regions (Fig. 3). While sequencing is underway, we suspect many are *D. pinea,* the pathogen causing Diplodia tip blight of pines (Sinclair and Lyon 2005). Michigan is currently experiencing a severe Diplodia tip blight epidemic on pines, and it is likely these pines are serving as reservoirs for nearby stressed spruce (Sinclair and Lyon 2005; O'Donnell 2014). Our preliminary work also provides evidence that at least some of these *Diplodia* isolates can cause cankers on mature blue spruce (CK McTavish, unpublished data). Thus, *Diplodia* is associated with spruce decline in areas where Diplodia tip blight is a problem, but due to the patchiness of recovery, it cannot be a main pathogen contributing to spruce decline.

Paraconiothyrium (isolated from 33.5% of all sampled cankers) was one of the most common genera in this study, and was ubiquitous throughout Michigan (Fig. 3), which suggests it might be involved in the spruce decline epidemic. However, *Paraconiothyrium* was regularly

isolated from both healthy spruce tissue as well as canker tissue (CK McTavish, unpublished data). It was also reported as an endophyte with the ability to elicit taxol production in the tree that acts as a fungicide (Soliman et al. 2013). The chemical properties of this taxon could conceivably limit other fungi from invading, making it a beneficial fungus for the tree. Taking all this into account, while this group fit the distribution and frequency criteria, there was a lack of pathogenicity evidence and no previously described symptoms to our knowledge for *Paraconiothyrium* to be a pathogen causing spruce decline.

Diaporthe was also frequently isolated from cankers on symptomatic branches (isolated from 32.32% of all cankers) (Fig. 3). Found in every region, it was also the only fungus isolated from every site studied. The *Diaporthe* genus contains species that are plant pathogens, saprobes, and endophytes, many of which have wide host ranges (Udayanga et al. 2011; Santos and Phillips 2009; Gomes et al. 2013). Similar to *Diaporthe juniperova,* which causes juniper blight, *Diaporthe occulta* was reported to attack *P. pungens* and *P. engelmanni* when trees were stressed due to improper cultivation conditions (White 1929). Sieber (1989) studied twigs on healthy and diseased trees, and found that *Diaporthe occulta* was isolated frequently and, at one site, was statistically more linked with diseased Norway spruce than healthy ones, while *Alternaria alternata, Epiccocum nigrum,* and *Cytospora* were only rarely isolated. Furthermore, *Diaporthe occulta* has already been reported as disease-causing on younger spruce trees: it was reported to cause tip curling and death along with stem cankers on spruce nursery and tree farm stocks in Wisconsin (Sanderson and Worf 1986) and later causing cankers on spruce in nursery and tree farm settings in Michigan (Igoe, Peterson, and Roberts 1995). While other fungi may exacerbate the expression of spruce decline symptoms, and stress due to abiotic and biotic factors may play important roles in tree decline progression, *Diaporthe* was the only genus

encountered in this study that met all four criteria of frequency, distribution, symptomology, and evidence of pathogenicity, and therefore is the most important canker causing pathogens responsible for spruce decline symptoms.

Only five *Diaporthe* haplotypes were detected with ITS using 43 samples collected across the northern Lower Peninsula of Michigan, confirming preliminary work (DW Fulbright and S Stadt, unpublished data). The β-tubulin gene sequences only differentiated haplotypes 2, 4, and 5 further from haplotypes 1 and 3 by 5 additional bp. Phylogenetic results did not classify the haplotypes to species level, but there were notable findings. The low virulence haplotypes, 1 and 3, fell into well-supported *Diaporthe eres* clade(s) (Figs. 4, 5, & 6), which has a large host range including *Acer, Castanea, Corylus, Juglans, Picea, Prunus,* and *Viburnum*, and has been reported to be pathogenic or weakly pathogenic on many of these hosts (Gomes et al., 2013; Thomidis & Michailides, 2009; Udayanga et al., 2014). *Phomopsis occulta,* the pathogen identified for spruce canker and tip blight, has been proposed to be renamed *D. eres* (Igoe, Peterson, and Roberts, 1995; Rossman et al., 2014; Sanderson & Worf, 1986). In contrast, Haplotypes 2, 4, and 5 were grouped into unresolved portions of the cladograms, which included *D. bicincta* (host: dead *Juglans* spp.) and *D. celastrina* (host: *Celastrus scandens*). These findings hint at the possibility that more than one *Diaporthe* species is involved in spruce decline. The genetic variability of haplotypes further supported the theory of multiple *Diaporthe* species involved in spruce decline. Haplotypes 4, 5, and 2 were only different by 1 or 2 bp changes in ITS and identical in TUB, were more virulent than the less similar (up to 9 bp changes from haplotypes 4, 5, and 2 using ITS) but resolved haplotypes 1 and 3. Results were similar with TUB, with 5 bp differences between the groupings of haplotypes 2, 4 and 5 and

haplotypes 1 and 3. Taxonomical work is not complete: further sequencing with additional genes is already underway to identify the species names of *Diaporthe* causing spruce decline.

The five *Diaporthe* haplotypes differed in their virulence on Colorado blue, Norway and white spruce (Fig. 7). The genetically similar haplotypes 2, 4 and 5 were virulent on Colorado blue, Norway and white spruce (Fig. 7). Haplotype 1, which differed from the virulent group by seven or eight bp changes, was only slightly virulent on Colorado blue spruce and was essentially avirulent on Norway and white spruce. The most distinct haplotype 3 was essentially avirulent on spruce. Additionally, *Diaporthe* haplotypes inoculated onto spruce trees could be isolated readily from the resulting cankers, thus completing Koch's postulates. The grouping of haplotypes 2, 4, and 5 were therefore differentiated biologically (virulence) and genetically (increased bp changes) from haplotypes 1 and 3.

Interestingly, spruce taxa differed in their resistance to the virulent haplotypes 4 and 5. Colorado blue spruce was the most susceptible host while Norway and white spruce were moderately susceptible (Fig. 8). Black Hills, Serbian and Meyer spruce were the least susceptible to these *Diaporthe* haplotypes. It was surprising that Black Hills spruce (*P. glauca* var. *densata*) differed from the closely-related white spruce (*P. glauca*). This suggests that resistance to spruce decline could be relatively easy to evolve. Our data suggests that more resistant spruce could be considered as substitutes for the relatively susceptible Colorado blue and Norway spruce in future landscape plantings. However, these potential replacements may have other drawbacks that reduce their appeal as plantings in the landscape. For example, Serbian spruce is more susceptible to the white pine weevil, and Meyer spruce grows more slowly than Colorado blue spruce (Bert Cregg, *pers.* comm).

The third inoculation experiment utilized nine different seed sources of Colorado blue (Table 3) to investigate variability in resistance to a haplotype 4 isolate of *Diaporthe*. Misty blue and Majestic seed sources were the most susceptible, with the least susceptible including Apache, Blue Wonder, Wolf Creek, and San Juan (Fig. 11). However, it should be noted that the San Juan seed source was utilized for experiment 2, and was the most susceptible taxon. A relatively low sample size for each seed source (50 trees for Baby Blue and Majestic and 25 for the remaining seven seed sources) and high variability for canker size among trees within a given seed source decreased the power to further discriminate among the seed sources. High variability for canker size among trees from the same seed source is to be expected since trees from a single seed source are not clones, but are outcrossed progeny and therefore are genetically variable. More in-depth experiments should be carried out to confirm these results.

When comparing inoculation experiments, there were differences between canker sizes, although the patterns remained the same. This could be due to improvement on inoculation technique, the impact of tree stress on canker size, as the second experiment was performed in a different greenhouse without swamp coolers and the third experiment came from multiple growers. Additionally, while the sample size of the 2014 experiment was larger (60 treatment replications versus 15), the confidence intervals were not improved upon, particularly the larger upper confidence intervals. Sometimes there would be more susceptible trees within a spruce taxon, particularly in Colorado blue spruce. This reiterates that trees are not clones of each other, thus one tree will not have exactly the same reaction as another in the same taxon, even with the same general stressors. This is further echoed by the Colorado blue spruce seed source experiment, where even though the tree is the same species, different sources of that species will have an impact on the ability to respond to stressors. Unfortunately, these inoculation

experiments were not designed to explicitly evaluate tree stress on spruce decline symptoms; therefore the role of stress will need to be determined in more detail with future experiments.

Based on past studies of *Diaporthe* and spruce trees, the etiology suggests that *Diaporthe* is in nurseries and tree farms (Sanderson and Worf 1986; Igoe, Peterson, and Roberts 1995). Indeed, growers have reported problems associated with *Diaporthe* in their nurseries and tree farms with similar spruce decline symptoms of needle loss and death of lower branches, but full spruce decline symptoms as seen on mature spruce do not usually develop due to aggressive disease management regimes. Currently, it is unclear whether *Diaporthe* causing symptoms in nurseries and tree farms is the same species recovered in this study from landscape spruce. Based on our haplotype findings, there could be more than one *Diaporthe* species causing disease.

It is also unclear how spruce decline incidence became so widespread in a relatively short amount of time. *Diaporthe* may normally be a latent endophyte in mature trees, or a weak pathogen (Udayanga et al. 2011; Gomes et al. 2013; Diogo, Santos, and Phillips 2010) until biotic or abiotic stressors trigger a more virulent phase that leads to spruce decline. Results reported here suggest that *Diaporthe* has a large role in the spruce decline epidemic, but it may not be the only factor responsible for spruce decline. Tree declines are complex and often include complicated etiologies that include environmental influences that play a key role in host decline (Manion 1991). Spruce trees are widely planted outside of their native range, perhaps predisposing these trees to stress. It could be site selection factors, changing seasonal rainfall patterns in Michigan (Andresen et al. 2014) or climate change generally that may be acting to stress spruce over wide areas. Sanderson and Worf (1986) reported that higher temperatures and humidity induce more symptom development in nursery settings, and those conditions may

predispose mature landscape trees to develop spruce decline symptoms when *Diaporthe* is present. Alternatively, trees may be weakened due to shallow roots and nutrient deficiencies which may further exacerbate spruce decline. Needlecast and insect diseases, while individually cannot cause all the spruce decline symptoms, are common throughout Michigan, and another fungus, *Stigmina lautii*¸ was reported as associated with needle cast symptoms at the same time spruce decline symptoms were first being identified (Fulbright 2008b; Fulbright 2010; Fulbright et al. 2011). What remains to be determined is which stressor or combination of stressors is responsible for the sudden increase in spruce decline. Further work also needs to explore spruce taxa, including Colorado blue spruce, for useful sources of resistance.

Though studies presented in this work were limited to Michigan, surrounding states as well as more distant states like New York have reported similar symptoms, particularly with Colorado blue spruce. Genetic work at a larger scale could shed light on where *Diaporthe* originated, if it is not an endophyte, or became latent after infection in a nursery. It is currently unknown how *Diaporthe* is spreading both within the tree and among trees, particularly because a sexual stage has not been observed. Due to these unsolved questions, methods to control spruce decline, other than reducing stress and using good site selection, remain uncertain. In the meantime, dead branch removal on trees should help to reduce inoculum. Avoiding damage during wet conditions will also assist the control of spruce decline. Using good arboricultural techniques to maintain tree vitality as well as reducing competition from other plants and pathogens will minimize tree stress, lowering susceptibility to disease.

CHAPTER 3

Within the last decade, spruce trees in Michigan began exhibiting symptoms of needle drop and branch dieback, starting basally and continuing upwards, symptoms of what we call spruce decline. Earlier reports of *Diaporthe* (formerly *Phomopsis*) causing these disease symptoms on nursery and tree farms (Igoe, Peterson, and Roberts, 1995; Sanderson & Worf, 1986) as well as isolations of *Diaporthe* from cankers on symptomatic branches of trees displaying spruce decline spurred us to complete a survey of the Lower Peninsula of Michigan to detect how widespread spruce decline was, and what fungal pathogen was likely causing these cankers associated with needle loss and branch dieback. The two most common genera isolated from cankers that were collected during the survey were *Diaporthe* and *Paraconiothyrium*. *Diaporthe* was found at every site, and had previous evidence of pathogenicity, unlike *Paraconiothyrium* which was not found everywhere and had no data indicating pathogenicity on spruce. *Paraconiothyrium* was probably an opportunistic, secondary invader on existing cankers.

Once *Diaporthe* was confirmed to be the likely pathogen, greenhouse inoculation studies were performed to complete Koch's postulates, and to test the: 1) virulence of the five *Diaporthe* haplotypes found from the Michigan survey, and 2) susceptibility of six spruce taxa as well as Colorado blue spruce seed sources. The haplotypes varied in virulence with haplotypes 2, 4, and 5 being more virulent, while haplotypes 1 and 3 were weakly virulent to avirulent. Spruce taxa differed in their susceptibility to *Diaporthe*, with Colorado blue spruce the most susceptible, followed by Norway, then white spruce. Black Hills, Serbian, and Meyer spruce were much less susceptible to *Diaporthe*, though small cankers still formed. Haplotypes 4 and 5 caused statistically similar cankers in all taxa except Meyer spruce. Seed sources of Colorado blue

spruce were also differing in susceptibility, with Misty blue and Majestic more susceptible than others. Thus, *Diaporthe* was confirmed to be pathogenic on spruce, haplotypes of *Diaporthe* differed in their virulence on spruce taxa, and spruce taxa and seed sources differed in their susceptibility to *Diaporthe*.

My next work determined the amount of genetic variability and taxonomic identity of *Diaporthe*. This step is particularly useful for disease management, as it can elucidate the evolutionary forces controlling the pathogen population. In turn, this information can be used to predict the progress of spruce decline epidemics, guide resistance breeding, and ascertain the effectiveness of certain control methods, such as fungicides. The Michigan population of *Diaporthe* displayed very limited variability, with only five haplotypes detected across the Lower Peninsula. The five haplotypes can be divided into two biologically important sub-groups. One sub-group contained three haplotypes, 2, 4 and 5, which were virulent on several spruce taxa with Colorado blue spruce being highly susceptible to all three haplotypes. Genetically, the haplotypes in this sub-group differed by only one or two bp substitutions for the ITS sequence and were identical for the TUB sequence. The second sub-group contained the largely avirulent haplotypes 1 and 3. This sub-group was well differentiated from the virulent sub-group by two bp differences in the ITS sequence and five bp differences in the TUB sequence. Haplotypes 1 and 3 fell into a well-supported *Diaporthe eres* clade, while the more virulent haplotypes were more basal to this clade, with poor resolution. Future studies should include sequencing additional gene regions for clearer phylogenetic resolution as well as more isolates for better pattern differentiation in regards to genetic variability. More inoculation studies should be completed using different isolates within haplotypes to confirm that virulence is uniform within a haplotype.

Future work must also begin investigating the predisposing and inciting biotic and abiotic factors that add to the stress of spruce trees and set the stage for *Diaporthe* infection. Climate change and other environmental influences, other diseases and pests, and site-specific factors could all have the potential individually or in concert to provoke spruce decline. Conversely, many of these factors have the potential to be controlled in order to lessen the risk of spruce decline. In order to find these predisposing and inciting factors, a survey detailing tree factors such as age and size of the tree, tree species, whether it is in an open area or in a row of trees, distance to road, soil type grade, along with noting other pests and pathogens present is recommended. Overlaying climate data with these factors would further illuminate important influences in the spruce decline system.

The role of other fungi found in cankers of declining spruce, particularly *Diplodia* and *Paraconiothyrium,* remains to be determined. Are these fungi endophytic, opportunistic, or competitors with *Diaporthe*? Is spruce decline accelerated or decelerated with these additional fungi? Studies involving competition and dependence among these fungi would further illuminate this disease complex.

As mentioned in chapter 2, this thesis work was limited to Michigan, but neighboring states to as far as New York reported comparable symptoms, especially in Colorado blue spruce. How widespread is the spruce decline epidemic in North America? Are there differences in the variability of *Diaporthe* in these areas? More genetic work on a larger geographic scale is necessary to begin answering these questions. Mechanisms of dispersal within and among trees are unknown, as is if there is a sexual stage present in spruce decline. Is there a fungicide that could be sprayed or injected to halt the spread within the tree? Would trimming dead branches near the bottom of the tree slow decline symptoms, or create new wounds and stress, further

accelerating decline? Much is yet to be discovered in this interesting disease complex; work presented in this thesis only scratches the surface.

APPENDIX

Table 1. Sites sampled in the 2013 spruce decline canker survey with region, population, town name, code, and latitude and longitude indicated

Code	Region	Population	Location (town name)	Latitude	Longitude
WE-4	West	4	Three Rivers	N 41° 94′	W 85° 63′
WE-5	West	5	Big Rapids	N 43° 70′	W 85° 48′
WE-6	West	6	Cedar Springs	N 43° 22′	W 85° 55′
CE-1	Central	1	Jackson	N 42° 25′	W 84° 40′
CE-3	Central	3	Adrian	N 41° 90′	W 84° 04′
CE-4	Central	4	Olivet	N 42° 44′	W 84° 92′
CE-5	Central	5	Saginaw	N 43° 42′	W 83° 95′
CE-6	Central	6	Mount Pleasant	N 43° 60′	W 84° 77′
EA-1	East	1	Rochester Hills	N 42° 66′	W 83° 15′
EA-2	East	2	Port Huron	N 42° 98′	W 82° 44′
EA-3	East	3	Flint	N 43° 01′	W 83° 69′
EA-4	East	4	Livonia	N 42° 40′	W 83° 37′
EA-5	East	5	Marlette	N 43° 33′	W 83° 08′
EA-6	East	6	Bad Axe	N 43° 80′	W 83° 00′
NO-1	North	1	Manistee	N 44° 24′	W 86° 32′
NO-2	North	2	Fife Lake	N 44° 58′	W 85° 35′
NO-3	North	3	West Branch	N 44° 28′	W 84° 24′
NO-4	North	4	Mackinaw City	N 45° 78′	W 84° 73′
NO-5	North	5	Spruce	N 44° 82′	W 83° 50′
NO-6	North	6	Lewiston	N 44° 88′	W 84° 31′

Table 2. Tree species sampled from the west, central, east, and north regions of the Lower Peninsula of Michigan in 2013

	West	Central	East	North	Total
Colorado	16	30	35	41	122
Norway	3	5	3	3	14
White	0	2	2	0	4
unknown	5	3	7	4	19

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