THE BIOLOGY OF CALICIOPSIS CANKER DISEASES IN NORTH AMERICA

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Plant Pathology – Doctor of Philosophy

2025

ABSTRACT

Caliciopsis pinea is the causal agent of caliciopsis canker disease. The fungus is an emerging pathogen affecting *Pinus strobus* (eastern white pine) across its native range in North America. Infections result in canker formation, reduced tree vigor, and economic losses due to downgraded lumber quality. Despite increasing reports of severe disease outbreaks, many aspects of *C. pinea*'s life cycle, pathogenicity, and mechanisms of spread remain poorly understood. Current diagnostic challenges stem from the pathogen's morphological similarity to closely related species and its ability to infect hosts without production of visible fruiting structures, necessitating more precise molecular detection methods. This dissertation addresses gaps in the existing literature of this pathosystem by first presenting a comprehensive guide on the diagnostic options available to study the conifer-infecting members of the *Caliciopsis* genus. Microscopic and molecular tools for diagnosis are reviewed and methods for studying *Caliciopsis* spp. in the laboratory setting are discussed (Chapter 1).

This dissertation also investigates species diversity in the *Caliciopsis* genus through multigene phylogenetic analyses and the description of new species in Michigan. Microscopic and molecular techniques were combined to create a three-locus concatenated phylogenetic tree using sequences generated in the study and culled from GenBank for an analysis that showed with strong statistical support, the presence of three, previously undescribed *Caliciopsis* species present in Michigan. These findings are supported by microscopy which demonstrated that morphological differences were present between novel species and other known species of *Caliciopsis* including length and width of mazaedia, placement of the locule, and ascospore size. Specific epithets are proposed for species described in this study, and a review of the taxonomic history and documentation on the existence of type specimens for all known species of *Caliciopsis* is offered (Chapter 2).

Phylogenetic studies are followed by the development of a highly sensitive and specific quantitative polymerase chain reaction (qPCR) assay targeting the internal transcribed spacer (ITS) region of *C. pinea*. Several members of the *Caliciopsis* genus are known pathogens, but *C. pinea* is the most aggressive on its preferred host. Diagnostic options for this species are limited and are expanded on here with the development of new molecular techniques for detection, quantification, and diagnosis. The assay was validated against 51 target and non-target isolates from across the *P. strobus* native range, achieving a detection limit of 10 fg of *C. pinea* DNA. To

ensure reproducibility, the assay was tested in multiple laboratories and across different thermocycling platforms. In all cases the reaction detected only *C. pinea* when screened against non-target species and the target was picked up in the background of plant DNA from infected plant material (Chapter 3).

The diagnostic assay developed in these studies was then utilized to investigate the environmental factors influencing *C. pinea* spore dispersal. To discover optimal conditions for inoculum detection, a novel approach combining qPCR with rotating-arm air sampling was implemented over a six-month sampling period in 2021 and 2022. These air sampling devices were deployed in natural ecosystems to monitor airborne inoculum levels, providing critical insight into spore release patterns. By integrating advanced molecular diagnostics with field-based spore monitoring, this study enhances our ability to detect, diagnose, and understand the epidemiology of *C. pinea.* The findings have significant implications for forest management, disease mitigation, and the long-term health of eastern white pine (Chapter 4).

Cumulatively, this dissertation provides the most comprehensive examination of *C. pinea* to date, addressing key gaps in its diagnosis, taxonomy, and epidemiology. By integrating phylogenetic analyses, molecular diagnostics, and environmental monitoring, this research enhances our ability to detect, classify, and understand *C. pinea* and its impact on *P. strobus*. Understanding spore dispersal dynamics and optimal conditions for inoculum detection provides critical insights for predicting and managing disease outbreaks. Future research should build upon these findings by exploring host-pathogen interactions at the molecular level, assessing environmental factors driving disease progression, and expanding monitoring efforts across different regions and climate conditions. As forest ecosystems face increasing stress from climate change, invasive species, and emerging pathogens, proactive disease management is essential for maintaining forest populations and overall resilience. This dissertation contributes valuable tools and foundational knowledge toward that goal, providing a framework for future studies and informing strategies to mitigate the impact of caliciopsis canker disease on North American forests.

This thesis is dedicated to my dad.
1954-2018

ACKNOWLEDGEMENTS

I thank the present and past members of the MSU Forest Pathology and Small Fruit and Hop Pathology laboratories who provided invaluable friendship and research support during my PhD. Of these many amazing individuals, I am especially grateful to Lexi Heger and Jillian Check not only for their research guidance but for the frequent and wonderful ways they supported me outside of our program. They are both incredible scientists and even better friends, and I don’t know how I could have reached the end of my degree without them. They impress me every day and I look forward to seeing how our scientific careers will continue to connect us.

Thanks to my advisor, Dr. Timothy Miles, for his guidance through the many ups and downs of my program. I admire his enthusiasm for research and commitment to the scientific community at MSU. Tim, more than any boss I’ve ever had, took my (often over the top) attitude in stride. My other committee members, Dr. Gregory Bonito, Dr. Martin Chilvers, and Dr. James Jacobs provided critical support during my time at MSU, helping me to solve problems big and small. Thanks especially to Marty for his spore trapping guidance, to Greg for his expertise in mycology, and to James for his forest pathology knowledge and Lions vs. Packers heckling. My graduate experience was unique, and each committee member took it in stride, and I appreciate them for it.

I am especially grateful for the many wonderful people from state and federal forest service entities who assisted with my projects and helped me to manage my field sites. These include Linda Williams (WI-DNR), Chelsea Murawski (USFS), Keith Konen (USFS), Dr. Isabel Munck (USFS), Kyle Lombard (NH-DNCR), Stephen Eisenhauer (UNH), and Aaron Bergdahl (Maine Forest Service), among other technicians and support staff who kept my two-year field experiment going. I appreciate all of your hard work and investment in my projects and me as a student.

Of course, my academic career would not have reached this point without my loving parents, Jean and James Harkness. Their consistent and unwavering support of my continued education brought me to graduate school. I also thank my younger brother, Joseph, for always helping me remember that my family will have my back when life seems bleak. I thank my older brother, Dominic, and his wife and son, Carrie and Satoshi, for giving me reasons to celebrate when the stress of graduate school felt out of control. We had to endure a lot as a family during

my time in this program, but I’m grateful for the people I had to endure it with. Finally, I thank my cat, Curie, for all the snuggles.

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Chapter 1: A Diagnostic Guide for Caliciopsis Canker Disease on North American Conifers

1.1 Disease

Caliciopsis canker disease (CCD) of conifers.

1.2 Hosts

Known hosts of CCD include several coniferous tree species native to North America. *Caliciopsis pinea* is the most well studied causal organism and infects *Pinus strobus* naturally and *P. monticola* in pathogenicity trials (eastern white pine and western white pine, respectively) (Ray 1936; Funk 1963). There are many observations of *C. pinea* on other *Pinus* species, but most are not substantiated with species-level diagnostics of the ascocarps recovered on these hosts (see Table S.2.2 from chapter 2 of this dissertation). Koch's postulates have been completed for *C. pinea* only on the two North American *Pinus* reported here. Other hosts to different, disease-causing *Caliciopsis* species include *P. radiata, P. nigra, P. resinosa*, *P. banksiana*, *Tsuga canadensis, T. heterophylla, Pseudotsuga menziesii,* and *Abies grandis* (see Chapter 2; Migliorini et al. 2020; Funk 1963).

1.3 Pathogen

There are several pathogenic members of the *Caliciopsis* genus that can cause CCD on different hosts. *C. pinea* on *P. strobus* is the most well characterized host/pathogen pairing among the *Caliciopsis* species known to infect conifers (Munck et al. 2015, 2016). *C. pinea* is also known to infect *P. monticola* (Funk 1963). *C. moriondi* was recently observed causing cankers on multiple *Pinus* species including *P. resinosa* and *P. banksiana* in North America (see Chapter 2; Migliorini et al. 2020). *C. orientalis* was originally found on the margin of a canker on *Tsuga canadensis,* and *C. pseudotsuga* was demonstrated through *in plantae* pathogenicity trials to cause cankers to form on *Pseudotsuga menziesii, T. heterophylla,* and *Abies grandis* (Funk 1963; Fitzpatrick 1942). Recently, *C. pseudoorientalis* was discovered and described, co-occurring with *C. pinea* on cankered *P. strobus* (see Chapter 2).

1.4 Taxonomy

Kingdom: Fungi, phylum: Ascomycota, subphylum: Pezizomycotina, class: Eurotiomycetes, order: Coryneliales, family: Coryneliaceae, genus: *Caliciopsis*.

1.5 Symptoms and Signs

Caliciopsis canker disease has characteristic symptoms on all known coniferous hosts that range considerably in severity. Seedling and adult trees may become infected, and symptom development occurs most often in the lowest branches, progressing upward (Cram and Fraedrich 2022). In mature *P. strobus*, infections from *C. pinea* will not result in mortality. However, when preceded by drought, infections can be fatal in seedling trees (Munck et al. 2015; see Chapter 3). Other stand factors such as stocking density and soil composition that may lead to reduced retention of water also influence disease severity (Munck et al. 2016). These observations have not been made for other *Caliciopsis* species on their respective hosts but are suspected to be consistent with *C. pinea* given the similarity of disease presentation on other hosts.

A mature *P. strobus* infected with *C. pinea* will typically have a combination of three symptoms: flagging of lower branches (the needles on individual branches brown and the whole branch dies; Fig1.1A), coupled with red, sunken cankers that form on the trunk and branches of the host (Fig1.1B and C) that eventually dry and crack the bark, leading to pitching and profuse resin bleeding (Fig1.1D) (Cram and Fraedrich 2022; Costanza et al. 2019; Asaro et al. 2018; Munck et al. 2016 and 2015). These symptoms are consistent with disease development of other *Pinus* infecting *Caliciopsis* species (Fig1.2). When an infection is not severe, these symptoms may be observed in isolation but it is most common for them to occur together (Cram and Fraedrich 2022; Costanza et al. 2019; Munck et al. 2016 and 2015). Cankers may form as individuals with irregular margins but can coalesce, which sometimes results in girdling in seedlings (Cram and Fraedrich 2022; Asaro et al. 2018). Importantly, flagging and branch death not only reduce the overall vigor of the host, but pose a safety hazard for forest management professionals as dead limbs are more susceptible to wind-felling (Way et al. 2022).

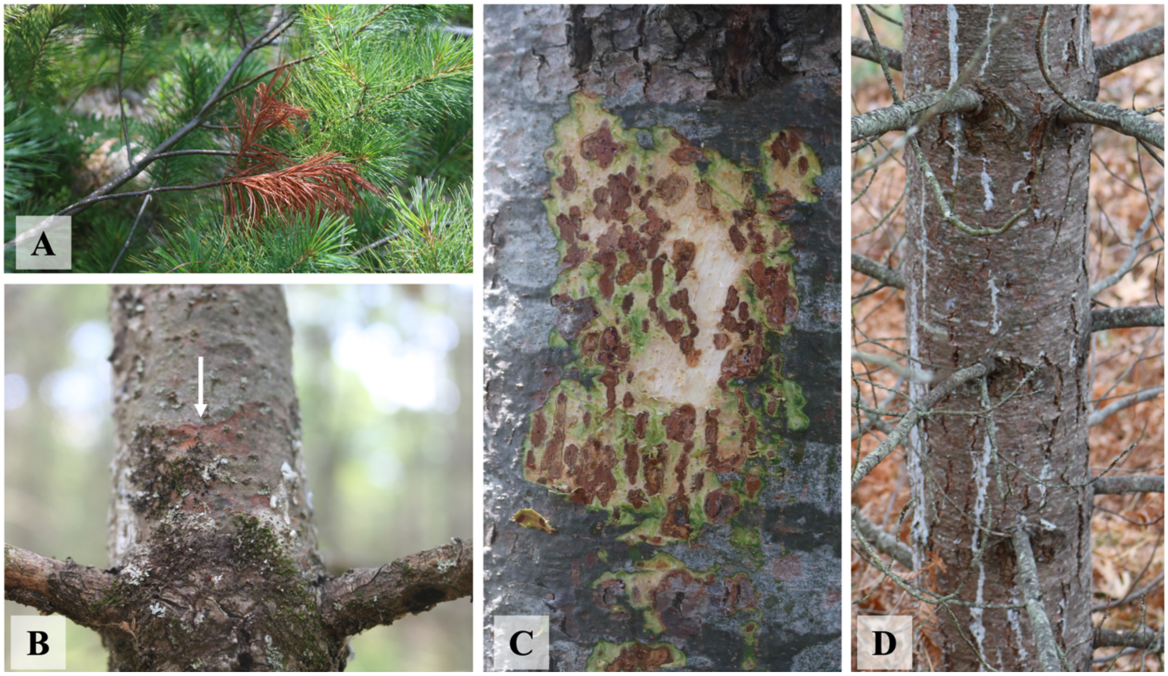

Figure 1.1. Symptoms on *Pinus strobus* infected with *Caliciopsis pinea*. **A)** Flagging, the needles of individual branches brown and the whole branch or branchlet dies, **B)** shallow, reddish-brown canker formation on the trunk and branches, canker margin indicated by white arrow, **C)** canker damage to the cambium apparent when bark is removed and **D)** profuse resin pitching from cracks caused by canker formation.

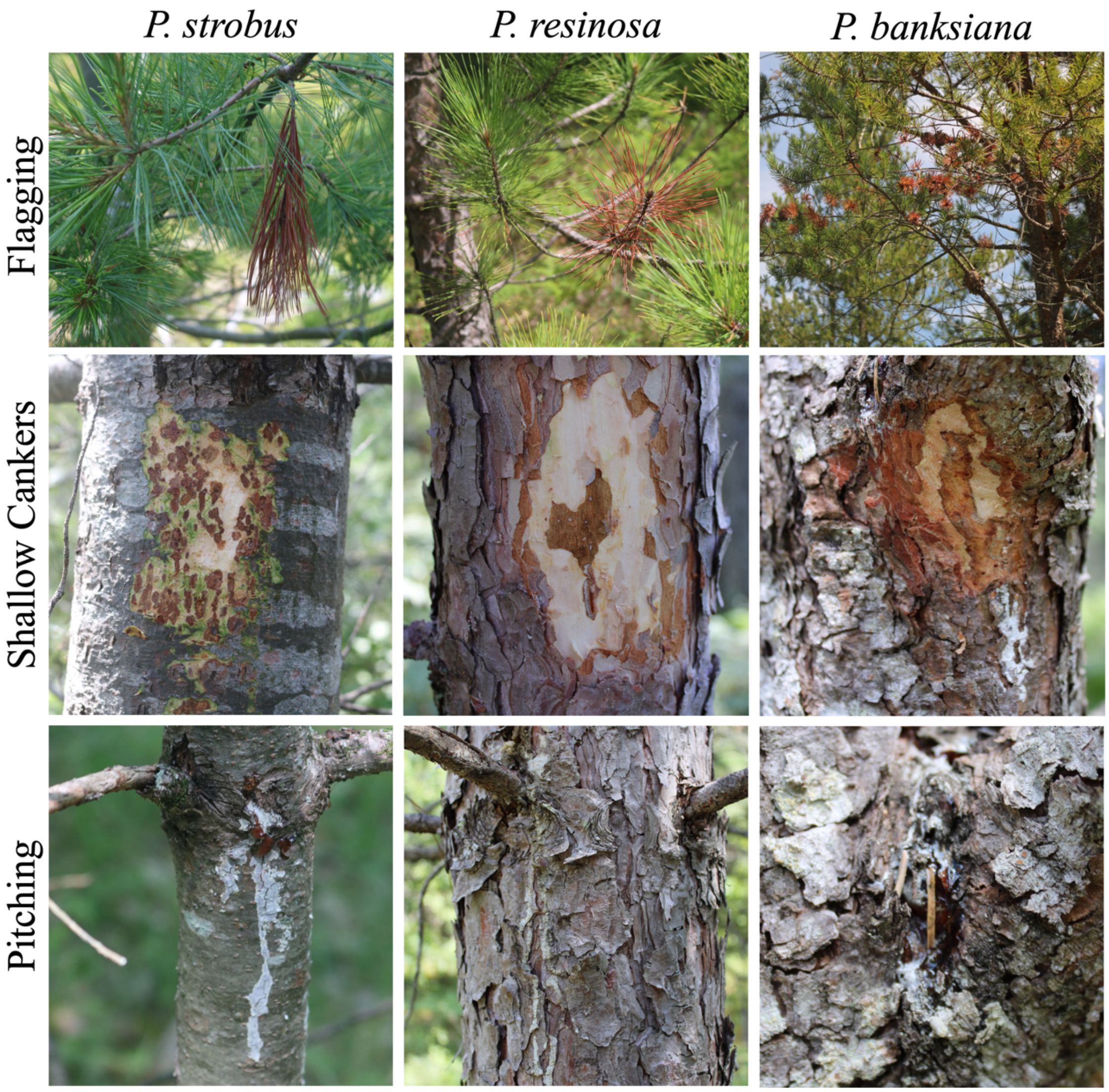

Figure 1.2. Common symptom development of caliciopsis canker disease observed across the *Pinus* genus. Photos of *P. strobus* show the impact of infection by *Caliciopsis pinea,* photos of *P. resinosa* and *P. banksiana* show the impact of infection by *C. moriondi*.

For *C. pinea,* infections will only occur at sites of prior natural or artificial wounding on the host (Cram and Fraedrich 2022). Once an infection is initiated, the sexual structures (mazaedia) of most *Caliciopsis* species are reported to emerge in the spring and have a perennial growth style, persisting for multiple years after emergence (Whitney et al. 2021; Schulz et al. 2018). Mazaedia are structures wherein ascospores accumulate in loose clumps for passive dissemination (Prieto et al. 2012). Emergence of new fruiting structures is presumed to occur in the spring (see Chapter 4). They most often form at the margins of cankers during the growing season and at branch nodes (Cram and Fraedrich 2022; Costanza et al. 2019; Asaro et al. 2018;

Munck et al. 2016 and 2015). Fruiting bodies of conifer-infecting *Caliciopsis* species are described as resembling eyelashes to the naked eye. *C. pinea* has the longest documented mazaedia, averaging 1.3 mm long and 0.07 mm wide at the widest point on the stalk (the locule) (Fig1.3A) followed by *C. moriondi* (average 0.9mm long and 0.04mm wide) (Fig1.3C) and *C. pseudoorientalis* (averaging 0.9mm long and 0.06mm) (Fig1.3E) (see Chapter 2). The spores of *C. pinea* are ellipsoidal and average 4.9 µm by 3.4 µm (Fig1.3B) and are nearly indistinguishable from *C. moriondi* (averaging 4.3 µm by 3.3 µm) (Fig1.3D) and *C. pseudoorientalis* (averaging 4.8 µm by 0.3 µm) (Fig1.3F) (see Chapter 2). All known species have dark brown to black stalks, with brown ascospores that are circular to ellipsoidal that do not vary dramatically in size (Fig1.3). Sexual structures are the only visible sign of disease, but symptoms can occur without their emergence (Schulz et al. 2018). Hand lenses are recommended to facilitate locating mazaedia. Molecular or microscopic observation is required to determine species identity, as multiple species can infect the same hosts (see Chapter 2).

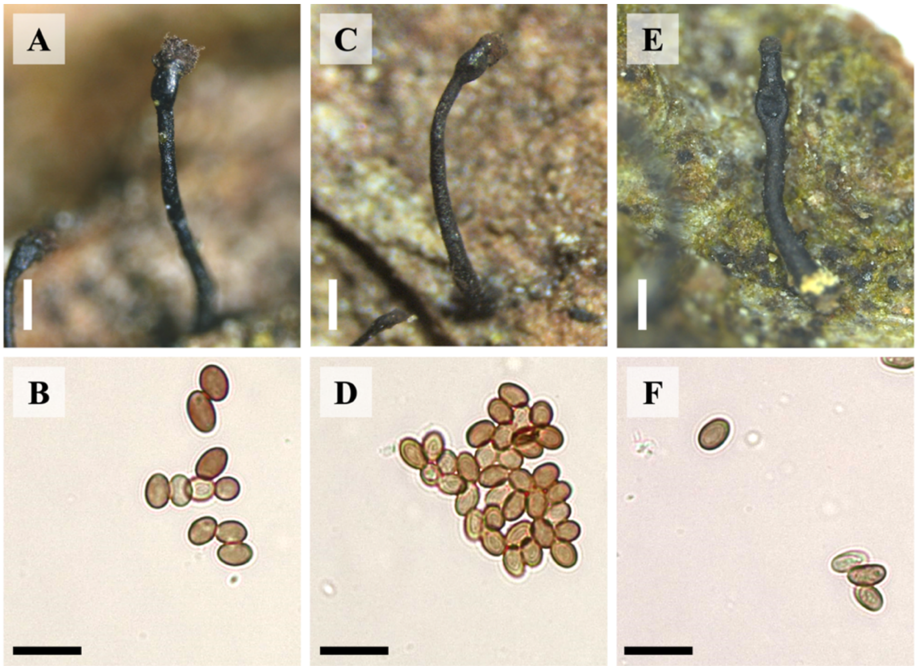

Figure 1.3. Sexual structures of conifer-infecting *Caliciopsis* species. **A)** Mazaedium and **B)** ascospores of *C. pinea* on *Pinus strobus*. **C)** Mazaedium and **D)** ascospores of *C. moriondi* on *P. resinosa.* **E)** Mazaedium and **F)** ascospores of *C. pseudoorientalis* on *P. strobus.* Scale bars on A, C, E indicate 250 µm and scale bars on B, D, F indicate 10 µm.

1.6 Host Range

There are 33 accepted species in the *Caliciopsis* genus and 29 of them were originally described from tree hosts with many reported to be pathogens (Table S.1.1). However, records of any substantial disease are limited primarily to conifers (Bergdahl et al. 2022; Costanza et al. 2019; Asaro et al. 2018; Schulz et al. 2018; Munck et al. 2016 and 2015). *Caliciopsis pinea* is considered an emerging pest of *P. strobus*, and this is the pathosystem on which there is the most published research (Cram and Fraedrich 2022; Whitney et al. 2021; Costanza et al. 2019; Asaro et al. 2018; Munck et al. 2016 and 2015). Though reported from numerous coniferous hosts, Koch's postulates were completed for *C. pinea* only on *Pinus strobus* and *P. monticola* in North America (Cram and Fraedrich 2022; Ray 1936; Funk 1963).

C. pinea has also been confirmed to infect the European pine *P. pinaster* by artificial inoculation (Capretti 1978; Delatour 1967 and 1969). There are reports of *C. pinea* on *Abies* (Spaulding 1961) but Funk's inoculation trials in 1963 with *C. pinea* on grand fir (*A. grandis*) resulted in no signs or symptoms of infection (Funk 1963). It is very likely that the *Caliciopsis* genus is far richer in species than researchers were aware of at the time of many of the original species descriptions, which may have led to mis-diagnoses of *C. pinea* on myriad hosts including *P. insignis, P. halepensis, P. echinate, P. pungens, P. rigida, P. mugo* var. *pumilio*, *Abies* spp., *Pseudotsuga menziesii,* and *Tsuga canadensis* (Capretti 1978; Delatour 1967; U.S. Department of Agriculture 1960; U.S. Department of Agriculture, Forest Service 1964; Ginns 1986). This hypothesis is supported by the recent discovery of *C. pseudoorientalis*, which was found causing disease alongside *C. pinea* on eastern white pine with no other known hosts (Harkness et al. 2025).

Caliciopsis moriondi was described in 2020 from symptomatic *P. radiata* in Italy (Migliorini et al 2020). Koch's postulates were completed on hosts *P. pinea, P. pinaster*, and *P. halepensis* (Migliorini et al 2020). Pathogenicity assays were not performed on *P. radiata, P. nigra*, and *P. resinosa*, though the fungal isolates used in formal pathogenicity trials came from those hosts (Migliorini et al 2020). *C. moriondi* is thus considered pathogenic on them. This species was experimentally proven to be a pathogen on three of its six reported hosts, though it is likely a pathogen of all six. In North America, *C. moriondi* has been recovered from symptomatic *P. resinosa* and *P. banksiana* (Harkness et al 2025). Symptom development on all

Pinus hosts is consistent in development with *C. pinea* on *P. strobus* with varying degrees of severity (Fig1.2) (Migliorini et al 2020).

C. orientalis was described from *Tsuga canadensis* (eastern hemlock) but no formal pathogenicity trials were conducted for that host (Funk 1963). Pathogenicity was assessed on *P. monticola* (western white pine) and data indicates an inability to cause disease (Funk 1963). Reports of *C. orientalis* on *T. canadensis* indicated that symptom development involved canker formation and eventual splitting of the periderm to expose the inner bark, resulting in pitching (Funk 1963). Symptom development is assumed to mirror the symptoms previously described for *C. pinea* on *P. strobus*, but this has not been formally tested since Funk's assessments (Funk 1963).

Funk also assessed pathology through artificial inoculation of *C. pseudotsuga* on 3 hosts (*Pseudotsuga menziesii, Tsuga heterophylla,* and *Abies grandis*), completing Koch's postulates on each of the 3 hosts and describing symptom development comparable to *C. pinea* on *P. strobus.* However, he noted that symptom development in *C. pseudotsuga*-infected *P. menziesii* was associated with phloem-feeding insects like weevils, and cankers would develop in a long series on lower branch surfaces, following patterns of feeding punctures by weevils. Funk offers more detailed descriptions of disease progression in *C. orientalis* and *C. pseudotsuga* infected trees, but largely these species have gone unstudied since these initial reports (Funk 1963).

1.7 Geographic Distribution

Caliciopsis species have been identified in North and South America, Europe, Asia, and Australia. Caliciopsis canker disease, however, is primarily observed on North American and European conifers (Bergdahl et al. 2022; Migliorini et al. 2020; Costanza et al. 2019; Asaro et al. 2018; Schulz et al. 2018; Munck et al. 2016 and 2015). Species in the genus can live as saprophytes on many different, primarily tree, hosts, acting as opportunistic pathogens (Migliorini et al. 2020; Mech et al. 2013; Funk et al. 1963; see Chapter 2, Table S.2.1). *C. pinea* is the most common species found in North America and is known from *P. strobus* naturally in North America and demonstrated to infect *P. monticola* in inoculation experiments (Funk 1963). *C. moriondi* is the next most-commonly found species in North America, reported on *P. resinosa* in New Hampshire and Michigan and on *P. banksiana* in Michigan and Wisconsin (Migliorini et al. 2020; see Chapter 2). Other than these two species, only isolated reports of disease can be found for *C. orientalis* and *C. pseudotsuga* in Canada (Funk 1963; Funk 1985). *C.*

pseudoorientalis has only been reported in Michigan (see Chapter 2). There are many reports of *C. pinea* on various hosts at several locations in Europe and North America that were never verified to species level (Capretti 1978; Delatour 1967; Spalding 1961; U.S. Department of Agriculture 1960; U.S. Department of Agriculture, Forest Service 1964; Ginns 1986). Because many species of *Caliciopsis* resemble each other (Fig1.23) it is possible that these are misdiagnoses, and the fungus would need to be reisolated from the reported hosts and identified to species using molecular testing to confirm.

1.8 Pathogen Isolation

Fungal mazaedia emerge in the spring and reach maturity by late spring, when they start producing ascospores (Fig1.4) (Funk 1963). They will be easiest to collect in early summer on dry days (Fig1.4); high humidity or rain will result in water droplets collecting in bark crevices and running down the trunk of the tree, obscuring mazaedia or introducing potential contaminants to the sample. Mazaedia can be nondestructively sampled by using forceps to pull them from the tree bark and placed in a microcentrifuge tube. Clusters of mazaedia can be removed from the tree by using a knife to scrape the top layer of bark and placing it in a paper bag (Fig1.4C). Bark shavings are recommended for samples that need to be shipped to a different location. Mazaedia that have been removed and placed in microcentrifuge tubes can be stored at room temperature for short periods of time but should be moved to -20°C or lower if long-term storage is desired.

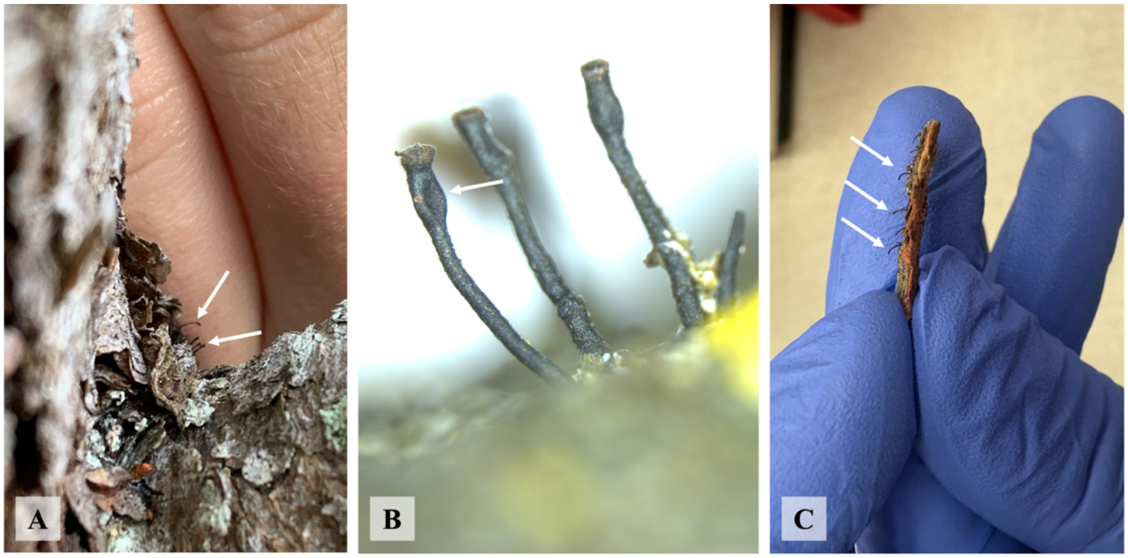

Figure 1.4. Images of locating and sampling *Caliciopsis* species in the field. **A)** Identify *Caliciopsis* species on their respective hosts using a hand lens. A hand was used as the

background of the image for a relative size comparison, and mazaedia are indicated with white arrows. **B)** Specimens can be further examined using a dissecting microscope to determine identity at the genus level, a white arrow indicates the placement of the locule. **C)** An example of a sample scraped from bark to preserve early growth stages, white arrows indicate mazaedia.

For samples brought to the laboratory on bark, the dissecting microscope may be used to magnify individual mazaedial stalks for removal with forceps and placed in a microcentrifuge tube. Using a micropestle, macerate individual stalks or multiple from a single tree in 50 µl of sterile water. Briefly spin the microcentrifuge tube to precipitate debris and remove as much of the supernatant as possible without disturbing the pellet. Create a serial dilution of this suspension down to 1:10^5 and pipette the three most dilute suspensions onto individual plates of 1.5% potato dextrose agar amended with antibiotics or lactic acid (PDA+). Spread each suspension onto its own plate using a sterile L-spreader. Incubate plates at room temperature for up to a week, checking daily for mycelial colony formation. Once colonies have started to form, subculture them onto a fresh plate of PDA+. Note that antibiotic or lactic acid amendments to the media are necessary to prevent bacterial growth. *Caliciopsis* spp. typically have slow growth rates in culture and are consequently outcompeted by contaminants. Alternatively, mazaedial stalks can be surface sterilized with ethanol and directly placed onto PDA+, but single spore isolation is recommended.

1.9 Pathogen Identification

Morphological identification

Caliciopsis species produce macroscopic fruiting structures, mazaedia, that can be found and identified with the naked eye (Figs1.3 and 1.4). However, all *Pinus*-infecting *Caliciopsis* species (*C. pinea, C. pseudoorientalis,* and *C. moriondi*) have marked similarity in mazaedial morphology that make rapid differentiation between them impossible, given their largely unstudied true host ranges (Fig1.3). Mazaedia of *C. pinea* are longer, on average, than those of *C. pseudoorientalis, C. pseudotsuga,* and *C. moriondi*. However, all three species are nearly indistinguishable, even at maturity (Fig1.3) and most species will produce mazaedia throughout their growing season resulting in various stages of mazaedial development at any given time of collection (Funk 1963). Consequently, mazaedial morphology alone is not a sufficient determinant of species identity in most circumstances. Similarly, colony morphology, coloration, and pigment production on PDA can be indicators of species identity but are not considered a diagnostic feature (Fig1.5). PDA is the most common media type for initial identification in culture because it is readily available in most laboratories and several *Caliciopsis* species have unique growth characteristics on it (Fig1.5). Cultures of the three *Pinus*-infecting *Caliciopsis* have several shared characteristics including pleomorphic growth that is initially white and may darken with age and pigment production, irregular colony margins, and penetrative growth into the media, rather than radial (Fig1.5). Due to the unreliability of morphology in differentiating *Caliciopsis* species, visual identification techniques should be coupled with molecular identification to make positive identifications at the species level.

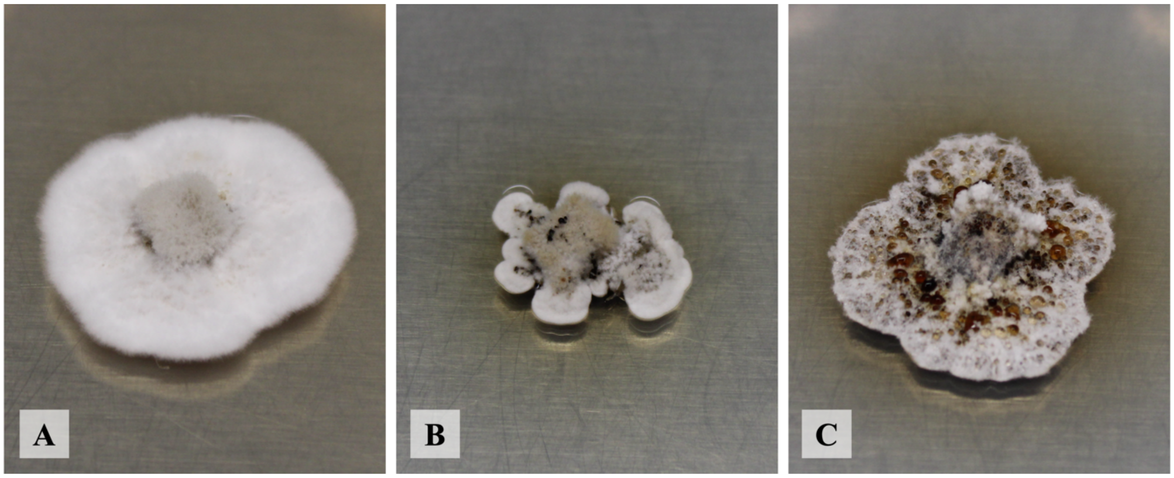

Figure 1.5. Culture morphologies of *Caliciopsis* species on full-strength potato dextrose agar (PDA) in ambient light and temperature conditions for three weeks. **A)** *C. pinea*, **B)** *C. pseudoorientalis,* and **C)** *C. moriondi.*

Molecular and genetic identification

Molecular diagnostic protocols for *Caliciopsis* species require the extraction of isolate DNA. Extracting DNA directly from mazaedia or infected plant tissue is not recommended unless a targeted downstream reaction will be used for diagnostics (this is discussed further below). However, if sequencing technologies are the chosen diagnostic technique, DNA extraction should be performed on single-spore isolated cultures. Cultures can be grown on PDA overlaid with sterile cellophane for 2 weeks, after which hyphae can be scraped into microcentrifuge tubes for use in DNA extraction protocols. Most *Caliciopsis* species produce a pigmented exudate on PDA that can interfere with downstream amplification of target genetic loci. To mitigate the impact of the exudate on downstream reactions, fungal hyphae can be rinsed with sterile water or lyophilized prior to DNA extraction, reducing the amount of exudate in the DNA extraction. Additionally, extraction kits that are optimized for inhibitor-rich sample types are ideal for *Caliciopsis* cultures, such as the Qiagen QIAmp® Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany). To maximize retention of DNA from the sample, combine extraction with an aggressive lysis technique; a shearing lysing matrix on a tissue homogenizer is recommended by the author.

The internal transcribed spacer region (ITS) is the most frequently used locus for identification of *Caliciopsis* at the species level (see Chapter 2). Amplification of the complete ITS region can be accomplished using the primers ITS1F and ITS4 (Gardes and Bruns 1993; White et al 1990). Depending on the chosen DNA polymerase the reaction mixture and thermocycling conditions may change, but how to achieve amplification using the Q5® High-Fidelity DNA Polymerase (New England Biolabs) is outlined here. Combine 5X Q5 reaction buffer to a final concentration of 1X, dNTPs to a final concentration of 200 µM, forward and reverse primer each to a final concentration of 0.5 µM, Q5 High-Fidelity DNA Polymerase to a final concentration of 0.02 U/µl, and less than 1,000 ng of template DNA. If amplification is inhibited or conservation of sample is desired, template DNA can be diluted to as low as 1 ng. Thermocycler conditions are as follows: a 30 second initial denaturation at 98°C, 35 cycles of 98°C for 10 seconds, 56°C for 30 seconds, and 72°C for 30 seconds, with a final extension of 72°C for two minutes. The resulting amplicon can be visualized on a 2% agarose gel, sequenced, and compared with voucher sequences on GenBank using their Basic Local Alignment Tool (BLAST). ITS, elongation factor 1-α (tEF1a), and β-tubulin (TUB) are the most populated genetic loci in GenBank for all *Caliciopsis* species**,** making tEF1a and TUB suitable alternatives for sequence-based identification. Primers for each gene can be found in Table 1.1.

Locus	Primer	Primer sequence (5'-3')	Size (bp)	Annealing Temperature (°C)	Reference
ITS	ITS1F	CTT GGT CAT TTA GAG GAA GTA A	500	56	Gardes and Bruns 1993
	ITS4	TCC GCT TAT TGA TAT GC		56	White et al. 1990
EF1-α	EF1-983F	GCY CCY GGH CAY CGT GAY TTY AT	760	54	Rehner and Buckley 2005
	EF-gr	GCA ATG TGG GCR GTR TGR CAR TC		54	Rehner and Buckley 2005
TUB	BT2a	GGT AAC CAA ATC GGT GCT TTC	370	54	Glass and Donaldson 1995
	BT2b	ACC CTC AGT GTA GTG ACC CTT GGC		54	Glass and Donaldson 1995

Table 1.1. Recommended primer sequences and annealing temperatures for amplification and sequencing of the internal transcribed spacer (ITS), translation elongation factor 1-alpha (EF1-α), and beta-tubulin (TUB) genetic loci for *Caliciopsis* species identification.

Diagnosis from infected plant material requires more targeted protocols than sequencing. Infected plant material will be home to other fungi in addition to *Caliciopsis.* PCR would consequently result in the production of multiple amplicons, making sequencing of the product impossible. For infected plant material, qPCR is recommended. However, only one qPCR-based diagnostic and detection assay is available, and it is for *C. pinea* (Harkness and Miles 2025). Given that *C. pinea* is assumed to be the most aggressive pathogen in the genus, this assay is still a useful tool, especially when diagnosing hosts with symptom development but no visible fruitification. Protocols for using this qPCR assay can be found in Chapter 3 of this document.

1.10 Pathogen Storage

Fungal mazaedia store well on bark or in microcentrifuge tubes at 4°C (Harkness et al. *unpublished data*). Previous studies have determined that ascospores stored at 2-5°C for two years had about 5% viability (Funk 1963). Cultural storage methods (serial subculturing and slants) were variably successful depending on the species and isolate of *Caliciopsis,* but most isolates did not maintain viability for longer than a year (Harkness et al. *unpublished data*). Cryogenic storage in microbanks with 40% glycerol resulted in 6 months of viability after which time the author observed no revival of the tested isolates (Harkness et al. *unpublished data*). Additional investigation on best practices for storage of *Caliciopsis* is needed.

1.11 Pathogenicity Tests

Caliciopsis pinea and *C. moriondi* signs and symptoms will develop the most reliably in *Pinus*-rich forests that are experiencing prolonged water stress (Costanza et al. 2020; Munck et al. 2016). Overstocking, soil profiles that do not retain water well, topography that promotes runoff, reduced precipitation, and increased temperatures for prolonged periods of time favor more severe disease development (Costanza et al. 2020; Munck et al. 2016). On their respective hosts, *C. pinea* and *C. moriondi* symptoms develop with similar symptoms, but varying degrees of severity (Fig2). The relationship between water stress and disease severity has only been investigated for *C. pinea* infections on *P. strobus,* but this phenomenon is assumed to be true for other species of *Caliciopsis* species given how similar disease development occurs on all conifer hosts, but further study on this subject is required (Costanza et al. 2020; Munck et al. 2016).

Greenhouse and growth chamber inoculations can be carried out according to protocols outlined in Cram and Fraedrich 2022. Migliorini et al. 2020 describe protocols for field inoculations using *C. moriondi* culture mycelia on seedlings planted in nursery rows. Both studies describe inoculum acquisition (both ascospores and culture mycelia), tree wounding and inoculum applications, as well as tree growth conditions to successfully complete Koch's postulates.

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Chapter 2: A description of three new species of *Caliciopsis* in Michigan forests

2.1 Abstract

Ascocarps of *Caliciopsis* spp. previously undescribed in the Midwest were observed on *Acer rubrum* (red maple), *Quercus velutina* (black oak), *Pinus resinosa* (red pine), *P. banksiana* (jack pine), and *P. strobus* in Michigan. Preliminary sequencing of the internal transcribed spacer region (ITS) revealed three putative new species of *Caliciopsis* on these hosts. Notably, this genus consists of species found primarily on trees and classified as pathogenic. The undescribed taxa were discovered on several tree species that are important economically and ecologically throughout North America. The aim of this study was to resolve where these species belong among existing phylogenetic data by describing the ecological niche they are known to occupy, their morphologies, and sequencing of additional genetic markers. After specimens were collected, morphological observations, measurements, and multi-locus phylogenetic analyses were completed. All species described in this manuscript had unique morphology (sexually and asexually) and genetics. The identification of these *Caliciopsis* spp. highlights the diversity present in native forests, where microbial ecology is understudied.

2.2 Introduction

The Coryneliaceae is a family of fungi made up primarily of pathogenic taxa (Balocchi et al 2022). This family is globally distributed and encompasses seven genera that have characteristic mazaediate ascomata often described as resembling eyelashes (Prieto et al. 2012; Migliorini et al 2020). Among these genera, *Caliciopsis* has been the subject of several recent investigations (Munck et al 2015; Munck et al 2016; Schulz et al 2018; Costanza et al 2020; Migliorini et al 2020; Cram and Fraedrich 2022; and others). The genus *Caliciopsis* contains many species pathogenic to trees. The most well characterized tree pathogens are *C. pinea* on eastern white pine (*Pinus strobus*) (Fitzpatrick 1920), *C. moriondi* on red pine (*P. resinosa*) (Migliorini et al 2020), *C. orientalis* on eastern hemlock (*Tsuga canadensis*) (Funk1963), *C. pseudotsuga* on Douglas fir (*Pseudotsuga taxifolia*) (Fitzpatrick 1942), *C. nigra* on Mediterranean cypress and juniper (*Cupressus sempervirens* and *Juniperus communis*, respectively) (Intini 1980), and *C. indica* on kokum (*Garcinia indica*) (Pratibha et al 2010). There are 33 accepted species in the genus, and 29 are confirmed or suspected pathogens to a tree host (Table S.2.1).

Caliciopsis pinea, the type species of the genus, was described from observations of fruiting bodies in New York state in 1880 (Peck 1880) after which, over the next forty years, more than 25 specimens of *C. pinea* were collected and deposited in the New York Botanical Gardens Steere Herbarium alongside the type specimen. However, the first published disease observations associated with *C. pinea* were not made until 1920, when Fitzpatrick reported "minor" canker disease associated with the fungus (Fitzpatrick 1920). In the last 15 years, reports of caliciopsis canker disease of North American *P. strobus* have increased in both frequency and severity (Munck et al 2015; Munck et al 2016; Whitney et al 2018; Costanza et al 2019; Costanza et al 2020). The known geographic distribution of both *C. pinea* and disease observations has expanded to include nearly every state where *P. strobus* is native and some Canadian territories (Migliorini et al 2020; Whitney et al 2021; Schulz et al 2018; Vermont DFPR 2021; Myren 1994; and others). There is evidence that environmental factors that lead to drought injury or water stress (i.e. poor soil quality, overstocking, reduced annual rainfall, climate change) increase the severity of *C. pinea* infections (Munck et al. 2015, 2016; Costanza et al. 2018, 2019). This has been observed in other pathosystems such as sooty bark disease of maple (*Acer* spp.) (Kespohl et al. 2022).

In the Midwest, climate modeling predicts that the frequency of drought is expected to increase, and overall water availability will be reduced which is anticipated to result in more frequent or dramatic wildfire events, and insect and pathogen outbreaks (Melillo et al. 2013). *C. pinea* and symptoms of canker disease were identified in Michigan in 2016, Wisconsin in 2018, and Minnesota in 2020 (Burgess et al. 2022, Wisconsin DNR 2021; Minnesota DNR 2020). Michigan forests are a critical part of the state's economic, social, and environmental health. Over half of Michigan's land area is forested and nearly all that land can be used for merchantable timber production (Poudel 2022). The forest products industry of Michigan directly employs an estimated 42,000 people and generates $13.4 billion in annual economic output (Poudel 2022). Changes to Michigan's climate that result in water stressed forests may lead to observations of more frequent and more severe *C. pinea* infections and negatively impact the successful regeneration of the state's *P. strobus* resource, like recent observations in the northeastern and southeastern USA (Munck et al 2016; Whitney et al 2021).

Surveys of Michigan's *P. strobus*-dominant forests revealed the presences of 3 putative new species of *Caliciopsis* identified on symptomatic *P. strobus* and asymptomatic *Acer rubrum* (red maple) and *Quercus velutina* (black oak). Furthermore, *C. moriondi* was identified on symptomatic *P. resinosa* and *P. banksiana* (red and jack pine, respectively). Given that most described *Caliciopsis* species are confirmed or suspected tree pathogens, assessing the diversity within the genus is critical for understanding the threats these species might pose to forest health. Therefore, this study aims to characterize these undescribed species and establish a more robust *Caliciopsis* phylogeny.

Morphological, phenotypic, and molecular characterization of species associated with tree hosts is key to identifying causal agents of disease and monitoring their presence within the geographic distribution of susceptible hosts. Previous species descriptions for members of the *Caliciopsis* genus have depended on standard fungal DNA barcoding loci such as the internal transcribed spacer (ITS) regions, beta-tubulin (TUB), and elongation factor-1α (EF1a) (Migliorini et al. 2020; Garrido-Benavent and Pérez-Ortega 2015; and Crous et al 2019). To characterize these putative new species found in Michigan, sequencing of these three loci for all isolates was performed. Further, morphological descriptions of mazaedia and cultures were made to assess both macro- and microscopic distinctions between species. Genetic data and descriptions from this paper will facilitate the improvement of both visual and molecular identification tools for this genus of fungi.

2.3 Methods

Origin and Identity of Isolates

Fifty-six single spored isolates were collected from various geographic locations from 2021 to 2023. *P. strobus*-dominant forests with poorly draining soils were targeted for isolate collection. Isolate identity was initially confirmed by grouping cultures with similar morphologies, then subsequent sequencing of the internal transcribed spacer (ITS) regions as described below. Host species and geographic locations are listed in Table 2.1. Cultures of all isolates were stored in 40% glycerol and maintained at room temperature (or about 20°C) in the Michigan State University Forest Pathology Laboratory.

Table 2.1. List of *Caliciopsis* spp. and other related taxa used for morphological and phylogenetic analyses conducted in this study. Isolates whose sequencing data was obtained from GenBank are indicated with an *.

				GenBank Accession Numbers		
Species	**Isoalte ID**	**Substrate**	**Location**	**ITS**	**tEF1-α**	**TUB**
C. pinea	CpMI3	*P. strobus*	Grayling, MI, USA	PQ778892	PV679966	PV714763
	CpMI4	*P. strobus*	Manistee, MI, USA	PQ778893	PV679967	PV714764
	CpMI5	*P. strobus*	Newberry, MI, USA	PQ778894	PV679968	PV714765
	CpMI6	*P. strobus*	Newberry, MI, USA	PQ778895	PV679969	PV714768
	CpMI7	*P. strobus*	Manistee, MI, USA	PQ778896	PV679970	PV714766
	CpMI8	*P. strobus*	Manistee, MI, USA	PQ778897	PV679971	PV714767
	CpUS42_NH*	*P. strobus*	Farmington, NH, USA	MK785367	MK913566	MN150096
	CpUS67_NH*	*P. strobus*	Greenfield, NH, USA	MK785365	MK913564	MN150098
	CpUS124_ME*	*P. strobus*	Albany, ME, USA	MK785359	MK913558	MN150090
	CpUS225a_MA*	*P. strobus*	Douglas, MA, USA	MK785346	MK913545	MN150078
	CpUS237_NC*	*P. strobus*	Macon, NC, USA	MK785342	MK913541	MN150074
	CpUS54_GA	*P. strobus*	Bogg's Creek, GA, USA	PQ778890	PV679964	PV714762
	CpUS55_GA	*P. strobus*	Bogg's Creek, GA, USA	PQ778891	PV679965	PV714761
	CpUS27_NH*	*P. strobus*	Blackwater, NH, USA	KY099598	MK913567	MN150097
	CpUS220_ME*	*P. strobus*	New Castle, ME, USA	MK785348	MK913547	MN150080
	CpUS230d_ME*	*P. strobus*	Peru, ME, USA	KY099602	MK913544	MN150077
	CpUS257_GA*	*P. strobus*	Unicio State Park, GA, USA	MK785336	MK913535	MN150079
	CpUS255_GA*	*P. strobus*	Unicio State Park, GA, USA	MK785338	MK913537	MN150070
	CpUS240_NH*	*P. strobus*	Lyme, NH, USA	MK785340	MK913539	MN150072
	CpUS238_VA*	*P. strobus*	Neola, VA, USA	MK785341	MK913540	MN150073
	CpUS234a_NH*	*P. strobus*	Hollis, NH, USA	MK785343	MK913542	MN150075
	CpUS232b_MA*	*P. strobus*	Barre, MA, USA	MK785344	MK913543	MN150076
	CpUS222b_MA*	*P. strobus*	Palmer, MA, USA	MK785347	MK913546	MN150099
	CpUS220_ME*	*P. strobus*	New Castle, ME, USA	MK785348	MK913547	MN150080
	CpUS206_ME*	*P. strobus*	Androscoggin River Park, ME, USA	MK785349	MK913548	MN150081
	CpUS199_ME*	*P. strobus*	Sauford, ME, USA	MK785350	MK913549	MN150082
	CpUS172_ME*	*P. strobus*	Naples, ME, USA	MK785351	MK913550	MN150083
	CpUS167_ME*	*P. strobus*	Bowdoinham, ME, USA	MK785352	MK913551	MN150084
	CpUS163_ME*	*P. strobus*	Androscoggin River Park, ME, USA	MK785353	MK913552	MN150085
	CpUS161_ME*	*P. strobus*	Androscoggin River Park, ME, USA	MK785354	MK913553	MN150086
	CpUS149_ME*	*P. strobus*	Brownfield, ME, USA	MK785356	MK913555	MN150088
	CpUS139_ME*	*P. strobus*	Sebago Lake, ME, USA	MK785357	MK913556	MN150100
	CpUS137_ME*	*P. strobus*	Alternate Brownfield, ME, USA	MK785358	MK913557	MN150089
	CpUS110_NH*	*P. strobus*	Burns Farm, Milford, NH, USA	MK785360	MK913559	MN150091
	CpUS100_NH*	*P. strobus*	Merrimack, NH, USA	MK785361	MK913560	MN150092
	CpUS76_NH*	*P. strobus*	Bear Brook, NH, USA	MK785363	MK913562	MN150102
	CpUS71_ME*	*P. strobus*	Parsonfield, ME, USA	MK785364	MK913563	MN150101
	CpUS52_ME*	*P. strobus*	Bethel, ME, USA	MK785366	MK913565	MN150095
	CBS 138.64	*P. strobus*	Chalk River, Ontario, Canada	KP881691	DQ677937	MN150093
C. moriondi	CmMI18	*P. banksiana*	Grayling, MI, USA	PQ778880	PV679954	PV714775
	CmMI19	*P. banksiana*	Grayling, MI, USA	PQ778881	PV679955	PV714776
	CmMI20	*P. banksiana*	Raco, MI, USA	PQ778882	PV679956	PV714778
	CmMI22	*P. banksiana*	Raco, MI, USA	PQ778883	PV679957	PV714782
	CmMI23	*P. resinosa*	Raco, MI, USA	PQ778884	PV679958	PV714781
	CmMI24	*P. resinosa*	Grayling, MI, USA	PQ778885	PV679959	PV714780
	CmMI25	*P. resinosa*	Grayling, MI, USA	PQ778886	PV679960	PV714774
	CmMI26	*P. resinosa*	Raco, MI, USA	PQ778887	PV679961	PV714779
	CmUS58_WI	*P. baksiana*	Newbold, WI, USA	PQ778888	PV679962	PV714777
	CmUS59_WI	*P. baksiana*	Newbold, WI, USA	PQ778889	PV679963	PV714783
	CmUS64_NH*	*P. resinosa*	Bear Brook State Park, NH, USA	MK785371	MK913570	MN150105
	CmUS65_NH*	*P. resinosa*	Bear Brook State Park, NH, USA	MK785370	MK913569	MN150104
	CmUS66_NH*	*P. resinosa*	Bear Brook State Park, NH, USA	MK785369	MK913568	MN150103
	CmSP1_Spain*	*P. radiata*	San Sebastian d Garabandal, Spain	MK785372	MK913571	MN150106
	CmIT22_Italy*	*P. radiata*	Fucecchio, Tuscany, Italy	MK785373	MK913573	MN150107
	CmIT20_Italy*	*P. radiata*	Carcheri, Tuscany, Italy	MK785374	MK913574	MN150108
	CmIT17_Italy*	*P. nigra*	Antella, Tuscany, Italy	MK785375	MK913575	MN150109
	CmIT15_Italy*	*P. radiata*	Carcheri, Tuscany, Italy	MK785376	MK913576	MN150110
	CmIT14_Italy*	*P. radiata*	Carcheri, Tuscany, Italy	MK785377	MK913577	MN150111
	CmIT13_Italy*	*P. radiata*	Carcheri, Tuscany, Italy	MK785378	MK913578	MN150112
	CmIT11_Italy*	*P. radiata*	Carcheri, Tuscany, Italy	MK785379	MK913579	MN150113
	CmIT9_Italy*	*P. radiata*	Carcheri, Tuscany, Italy	MK785380	MK913580	MN150114
	CmIT7_Italy*	*P. radiata*	Carcheri, Tuscany, Italy	MK785381	MK913581	MN150115
	CmIT6_Italy*	*P. radiata*	Carcheri, Tuscany, Italy	MK785382	MK913582	MN150116
	CmIT5_Italy*	*P. radiata*	Carcheri, Tuscany, Italy	MK785383	MK913583	MN150117
	CmIT4_Italy*	*P. radiata*	Carcheri, Tuscany, Italy	MK785384	MK913584	MN150118
	CmIT2_Italy*	*P. radiata*	Carcheri, Tuscany, Italy	MK785385	MK913585	MN150119
	Cm1233_France*	*P. radiata*	Pyrénées Atlantiques, France	MK785386	MK913572	MN150121
	CBS 146717*	*P. radiata*	Carcheri, Tuscany, Italy	MN156540	MK913586	MN150120
C. pseuoorientalis	CpsMI11	*P. strobus*	Grayling, MI, USA	PQ778898	PV679972	PV714786
	CpsMI12	*P. strobus*	Raco, MI, USA	PQ778899	PV679973	PV714787
	CpsMI13	*P. strobus*	Raco, MI, USA	PQ778900	PV679974	PV714788
	CpsMI15	*P. strobus*	Grayling, MI, USA	PQ778901	PV679975	PV714789
	CpsMI16	*P. strobus*	Grayling, MI, USA	PQ778902	PV679976	
	CpsMI17	*P. strobus*	Grayling, MI, USA	PQ778903	PV679977	PV714790
C. aceriae	CaMI27	*A. rubrum*	Grayling, MI, USA	PQ778867	PV679947	PV714752
	CaMI28	*A. rubrum*	Grayling, MI, USA	PQ778868	PV679939	PV714753
	CaMI29	*A. rubrum*	Grayling, MI, USA	PQ778869	PV679940	PV714754
	CaMI30	*A. rubrum*	Manistee, MI, USA	PQ778870	PV679941	PV714755
	CaMI31	*A. rubrum*	Manistee, MI, USA	PQ778871	PV679942	PV714756
	CaMI32	*A. rubrum*	Manistee, MI, USA	PQ778872	PV679943	PV714757
	CaMI33	*A. rubrum*	Raco, MI, USA	PQ778873	PV679944	PV714758
	CaMI34	*A. rubrum*	Raco, MI, USA	PQ778874	PV679945	PV714759
	CaMI35	*A. rubrum*	Raco, MI, USA	PQ778875	PV679946	PV714760
C. cilium	CcMI41	*Q. velutina*	Grayling, MI, USA	PQ778876	PV679948	PV714769
	CcMI42	*Q. velutina*	Grayling, MI, USA	PQ778877	PV679949	PV714770
	CcMI43	*Q. velutina*	Grayling, MI, USA	PQ778878	PV679950	PV714771
	CcMI44	*Q. velutina*	Grayling, MI, USA	PQ778879	PV679951	PV714772
	CcMI45	*Q. velutina*	Grayling, MI, USA	PQ778866	PV679952	PV714773
C. pseudotsuga	CBS 14064*	*Pseudotsuga menziesii*	Canada	MK785387	MK913587	MN150123
C. orientalis	CBS 13864*	*Canada*	Canada	KP881690	MK91358	MN150122
C. eucalypti	CBS 142066	*Eucalyptus marginata*	Australia	KY173391	PV679953	PV714784
Hypsotheca pleomorpha	CBS 144636	*E. piperita*	Australia	MK442588	PV679978	PV714785
C. indica	GFCC 4947*	*Garcinia indica*	India	NR119752		
C. valentina	MA 18176*	*Q. ilex* subsp. *rotundifolia*	Spain	NR132091		
C. beckhausii	MA 18186*	*Q. ilex* subsp. *rotundifolia*	Spain	NR132090		

DNA Extraction, PCR Amplification, and Sequencing

All isolates were grown for 14 days on potato dextrose agar (39 g of potato dextrose agar (PDA) in 1 liter of distilled water; Difco PDA, Sparks, MD) overlaid with sterile cellophane at ambient temperature (approximately 20C). Then, 100 mg wet weight mycelial mass was scraped from the cellophane into 2 mL tubes containing Lysing Matrix A (MP Biomedicals, Santa Ana, California). Next, 400 µL of the InhibitEX Buffer from the Qiagen QIAmp® Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) was added to each sample. Samples were then homogenized on the MP Biomedicals Fast-Prep®-24 for 1 minute at 6 m/s. Another 600 µL of InhibitEX Buffer was added to each tube, after which the remainder of the DNA extraction was performed according to manufacturer's instructions.

To assign isolates to species, three loci were amplified and sequenced. The internal transcribed spacer (ITS) regions, translation elongation factor 1-α (tEF1-α), and β-tubulin (TUB) were all targeted for amplification (Table 2.2). PCR amplification of each loci was performed on an ABI 2720 thermocycler (Applied Biosystems, Foster City, CA). The PCR mixture contained 5 µL of 5X Q5 reaction buffer (New England Biolabs Inc., Ipswich, MA), 0.5 µL of dNTP (100mM) (Invitrogen, Carlsbad, CA), 1.25 µL of each primer (10µM), 0.25 µL Q5 high-fidelity DNA polymerase (2,000U/mL) (New England Biolabs Inc.), 15.75 µL of PCR-grade water, and 1uL of gDNA template (5 ng/µL). All PCRs were run using the following thermocycler conditions: initial denaturation at 98°C for 30 seconds, then 35 cycles of 98°C for 10 seconds, annealing at the temperatures listed for each loci in Table 2.2 for 30 seconds, and 72°C for 40 seconds, and a final extension of 72°C for 2 min with a holding temperature of 4°C.

Table 2.2. List of primers used to amplify genetic loci for phylogenetic analyses.

Locus	**Primer**	**Primer sequence (5'-3')**	**Size (bp)**	**Annealing Temperature (°C)**	**Reference**
ITS	ITS1F	CTT GGT CAT TTA GAG GAA GTA A	500	56	Gardes and Bruns 1993
	ITS4	TCC GCT TAT TGA TAT GC		56	White et al. 1990
EF1-α	EF1-983F	GCY CCY GGH CAY CGT GAY TTY AT	760	54	Rehner and Buckley 2005
	EF-gr	GCA ATG TGG GCR GTR TGR CAR TC		54	Rehner and Buckley 2005
TUB	BT2a	GGT AAC CAA ATC GGT GCT TTC	370	54	Glass and Donaldson 1995
	BT2b	ACC CTC AGT GTA GTG ACC CTT GGC		54	Glass and Donaldson 1995

PCR products were purified using the Qiagen PCR purification kit (Qiagen, Valencia, CA) according to manufacturer's instructions. After PCR products were visualized on a 1% agarose gel, they were sequenced by the Michigan State University Research Technology Support Facility on an ABI 3730xl platform (Applied Biosystems, Foster City, CA) using Sanger sequencing technology. Forward and reverse sequences of each loci were manually trimmed and assembled using Geneious Prime (v2024.0.5, Dotmatics, Boston, MA, USA) (https://www.geneious.com) and aligned using the online alignment program M-Coffee (Notredame et al. 2000; available at https://tcoffee.crg.eu/).

Phylogenetic Analysis

For each isolate, phylogenetic relationships were determined for three individual loci (ITS, tEF1-α, and TUB) and for a concatenated analysis of all loci. Reconstructions of phylogenetic relationships for individual and concatenated trees were performed using maximum likelihood (ML) and Bayesian Markov chain Monte Carlo (MCMC). Maximum Parsimony analyses were not performed because several species included in our analyses were represented by only one isolate. Maximum likelihood was performed using RAxML (v4.0) and Bayesian analysis was done using MrBayes (v2.2.4); Both analyses were run as plug-ins in Geneious Prime.

The GTR+GAMMA evolutionary model was used to estimate the best-scoring trees in ML analyses with 1,000 bootstraps generated using the rapid bootstrapping algorithm in a single run. No correction for ascertainment bias was used and support values were added to the best-scoring tree (Sakalidis et al 2021). Bayesian analyses were performed using the default HKY85 substitution model with all other model parameters estimated by MrBayes. Markov chain Monte Carlo analysis of four chains started from random tree topology and lasted 1,100,000 generations. Trees were saved every 200 generations. Burn-in was set at a length of 110,000 and posterior probabilities were calculated from the remaining trees. For all analyses sequences from *Hypsotheca pleomorpha* (CBS144636) were used as the outgroup. Trees were visualized in R using the ggtree package (Yu et al. 2022). GenBack accession number for all sequences generated during this study are available in Table 2.1. There were no prominent differences in the topography of species clades between the two analyses, so the ML tree was selected to represent the final tree with Bayesian posterior probabilities (BPPs) annotated onto the ML tree.

Morphology

Caliciopsis fruiting bodies were removed from their respective hosts and mounted on glass slides in sterile distilled water. The length and width of 50 released ascospores and 50 mazaedia were measured and imaged using the Leica Application Suite X (LAS X) on a Leica dissecting microscope with integrated camera Leica ICC50W. Average dimensions were recorded and compared with those reported from the literature (Table 2.3). Asci and ascospores were characterized and imaged using the cellSens Entry software (v2.3) on an Olympus differential interference contrast (DIC) microscope. Culture morphologies were determined for cultures grown on full strength malt extract agar (MEA, DIFCO, Detroit, MI, USA) and full strength PDA after growth for 14 days with ambient light and temperature (about 20°C).

Table 2.4. Comparison of morphological characteristics of various species described in the literature compared to the species proposed in this study. Measurements are given as length x width, (minimum value) mean ± standard deviation (maximum value).

	C. pseudoorientalis	*C. aceriae*	*C. valentina*	*C. beckhausii*	*C. cilium*	*C. pinea*	*C. pinea*	*C. moriondi*	*C. moriondi*
Host	*Pinus strobus*	*Acer rubrum*	*Quercus ilex subsp. rotundifolia*	*Quercus ilex subsp. rotundifolia*	*Quercus velutina*	*P. strobus*	*P. strobus*	*Pinus radiata*	*Pinus resinosa*
Reference	This study	This study	Garrido-Benavent & Perez-Ortega 2015	Garrido-Benavent & Perez-Ortega 2015	This study	Funk 1963	This study	Migliorini et al. 2020	This study
Sampling location	Michigan	Michigan	Spain	Spain	Michigan	Canada	Michigan	France, Italy, Spain, NH	Michigan
Ascomata height (µm)	(382) 889±214 (1537)	(377) 608±154 (1042)	(600) 900 ± 200 (5000)	(500) 900 ± 200 (1500)	(381) 778±262 (1728)	1000-3000	(824.4) 1281±242.9 (1835.9)	(450) 845±24 (1240)	(599) 894±152 (1265)
Stalk width (µm)	(29) 56±16 (99)	(35) 57±11 (82)	(60) 80 ± 10 (110)	(50) 75 ± 10 (100)	(38) 70±20 (107)	100-125	(45.6) 66.3±13.5 (92.7)	(51) 79±2 (135)	(26) 38±7 (53)
Ascigerous swelling	Subterminal	Subterminal	Subterminal	Median to subterminal	Median to subterminal	Terminal	Terminal	Terminal	Terminal
Swelling size (µm)	(73) 170±42 (242) x (37) 98±31 (172)	(77) 148±42 (273) x (42) 83±23 (134)	(150) 190 ± 20 (240) × (100) 130 ± 15 (160)	(125) 190 ± 30 (260) × (75) 110 ± 20 (160)	(93) 147±33 (213) x (48) 98±23 (150)	Unreported length x 175	(96.3) 172.0±30.0 (230.6) x (56.7) 109.0±24.2 (155.4)	(106)281±8(406) × (81)142±5(268)	(80) 132±17 (172) x (53) 82±11 (104)
Ascospore shape	Subglobose to ellipsoidal	Globose, subglobose to ellipsoidal	Subglobose to ellipsoidal	Globose, subglobose to ellipsoidal	Globose to subglobose	Ellipsoidal	Ellipsoidal	Subglobose to ellipsoidal	Ellipsoidal
Ascospore color	Brown	Brown	Brown	Brown	Brown	Brown	Brown	Brown	Brown
Ascospore size	(3.4) 4.8±0.7 (8) x (2.9) 3.6±0.3 (4.5)	(4) 5±0.6 (6) x (3) 4±0.6 (6)	(5.3) 7 ± 0.8 (9.6) × (4) 5.7 ± 0.7 (8)	(5.4) 7.4 ± 1.1 (11) × (5) 6.6 ± 0.9 (10)	(3) 4.7±0.7 (6) x (3) 4.1±0.5 (5.9)	5-6 x 3	(3.5) 4.9±0.5 (6.1) x (2) 3.4±0.4 (4.3)	(3)4.4±0.07(6.2) × (1.8)2.5±0.04(3.5)	(2.7) 4.3±0.7 (6.5) x (2.5) 3.3±0.3 (4.5)
Conidia Shape	Capsular to allantoid	Capsular to allantoid	Subcylindrical to allantoid	Subcylindrical to allantoid	Allantoid, infrequently capsular	Rod-like to allantoid	Allantoid	Fusiform	
Conidia Color	Hyaline	Hyaline	Hyaline	Hyaline	Hyaline	Hyaline to pale yellow	Hyaline	Hyaline	Hyaline
Conidia Size (µm)	(4.4) 5.8±0.7 (7.8) x (1.2) 1.7±0.2 (2.2)	(4.7) 5.8±0.5 (7.0) x (1.3) 1.6±0.2 (2.2)	(4) 4.5±0.3 (5) x (1) 1.4±0.2 (1.6)	(3.5) 4.2±0.3 (4.7) x (1.2) 1.5±0.2 (1.7)	(4.4) 5.6±0.6 (7.0) x (1.1) 1.7±0.3 (2.5)	2.5-3.5 x 1.0	(3) 3.9±0.4 (5.2) x (1.0) 1.5±0.2 (2.1)	Not reported	
Colony description	Early growth is indistinguishable from *C. pinea,* but will brown more profusely with age (Supplemental Fig 2). Usually smaller growth radius than *C. pinea.*	Dark brown, sometimes white to beige at the margin. Growth is near-circular. Appressed with profuse staining of the media.	Not reported	Not reported	Beige to brown, often darker at culture margin, appressed to media, irregular borders.	From MEA: at first whitish, becoming fawn-colored with broad white margin [...] reverse brownish	White, fluffy when young but will become appressed to the media with age.	White-hyaline appressed to the agar. Turns brown with age.	White-hyaline appressed to the agar. Turns brown with age

2.4 Results

Phylogenetic analyses

A total of 48 ITS, 50 TUB, and 50 tEF1-α sequences were generated in this study and combined with previously published sequences for phylogenetic analyses (Table 2.1). Trimmed sequences of 478bp for ITS, 757 bp for tEF1-α, and 417 bp for TUB were used for both individual and combined alignments. The concatenated alignment of three loci (ITS— tEF1-α—TUB) included 91 ingroup sequences and one outgroup sequence all consisting of 1,652 characters. Bootstrap and BPP values strongly supported 11 clades corresponding to described or proposed species (Fig2.1A). Proposed species from this study include *C. pseudoorientalis,* sp. nov*., C. aceriae,* sp. nov., and *C. cilium,* sp. nov. All were supported as distinct species with ≥70% bootstrap support for ML analyses as well as ≥0.95 BPP in the concatenated analysis (formally described below). All species described in this study were represented by a minimum of six isolates. There was varying degrees of access to specimens of other described species, thus some were represented by a single specimen (Fig2.1 and Table 2.1).

Individual loci analyses varied in their ability to delineate all species; the concatenated tree resolved the relationships among species with a higher degree of support than the individual trees (Fig2.1). *C. aceriae* and *C. cilium* were supported as new species in both the tEF1-α and TUB analyses with > 90% bootstrap support and >0.95 BPP and were well differentiated in the Bayesian analysis for ITS (BPP = 0.98) but not supported in maximum likelihood analyses (FigS.2.1). Similarly, *C. pseudoorientalis* was more clearly resolved as a new species in the individual trees for tEF1-α and TUB (both with >80% bootstrap support and >0.70 BPP) (FigS.2.1). The concatenated analyses and the ITS individual loci analyses were run twice, once including isolates where only a single ITS sequence was available, and once without isolates which had missing data (Fig2.1 and S.2.1). Doing so did not appreciably change the topography or confidence of either the concatenated analyses or the ITS individual loci analyses.

Additionally, two clades separating *Caliciopsis* species derived from angiosperm or gymnosperm hosts were resolved within the genus (Fig2.1A). The later diverging clade consists of species found from gymnosperm hosts, while the early diverging clade consists of species found from angiosperm hosts. Both clades consist of isolates from Europe and North America. Each species that was resolved in this study also showed host specificity to the tree species from which they were originally collected. *C. moriondi* and *C. pseudotsuga* are the only species of *Caliciopsis* known to infect multiple hosts (this study; Miliorini et al. 2020; Funk 1963).

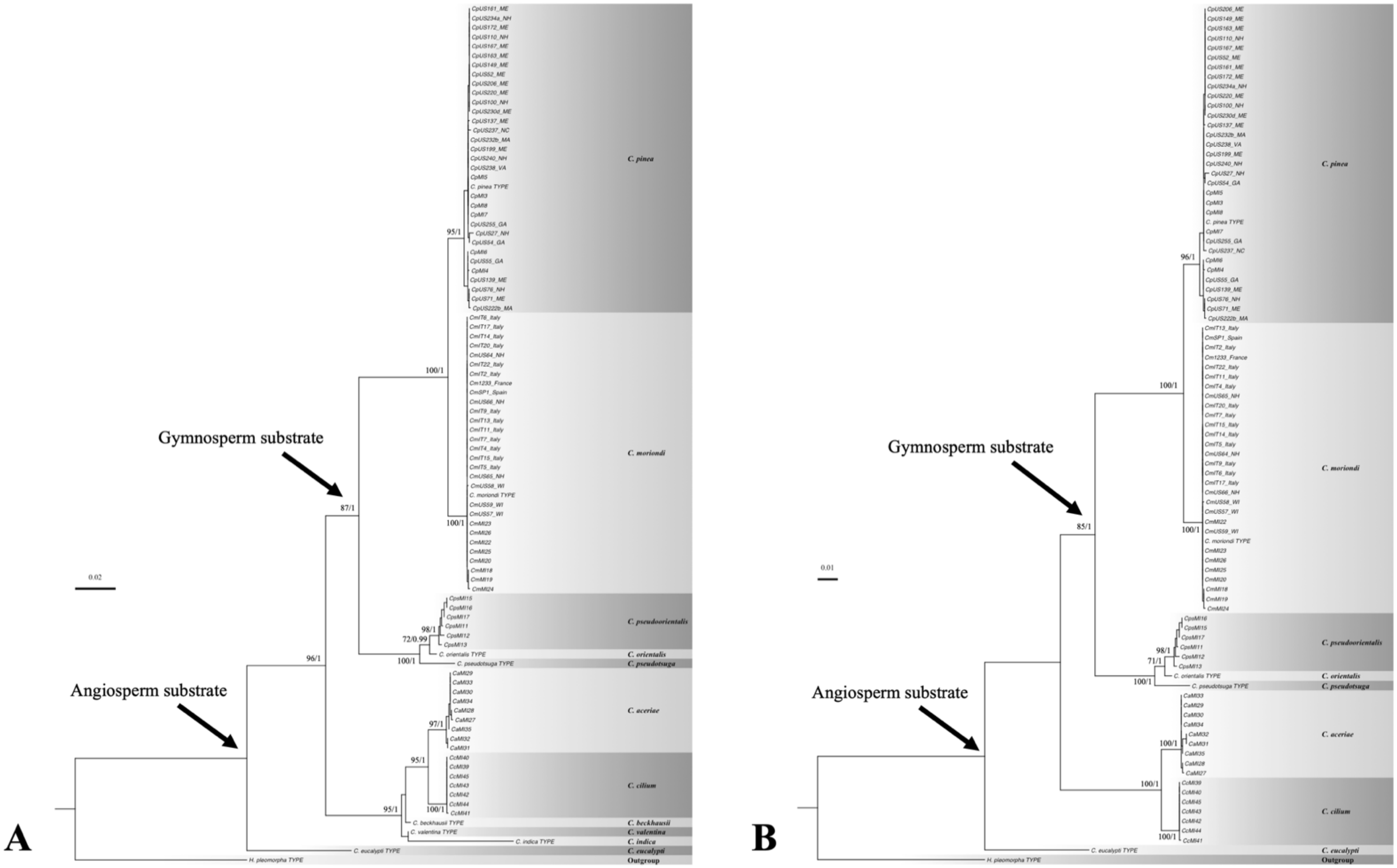

Figure 2.1. Three-locus (ITS— tEF1-α— TUB), maximum likelihood phylogeny of *Caliciopsis*. Bootstrap support values from maximum likelihood analyses that were ≥70% and Bayesian posterior probabilities ≥0.95 are reported as the first and second numbers, respectively, associated with internodes. **A)** Includes isolates listed in table 2.1 that are represented in GenBank by only a single gene, and **B)** excludes these isolates.

Morphology

Micromorphological characterization, hosts, and culture morphology were the most reliable means of differentiating species other than using molecular techniques which were more revealing of lineages (Table 2.3). *C. pseudoorientalis* was only identified on *P. strobus* over the duration of this study and has unique morphology differences in the mazaedium that allow it to be differentiated from the only other known species of *Caliciopsis* that grows on *P. strobus*, *C. pinea*. *C. pseudoorientalis* was considerably smaller than *C. pinea* with an average stalk length of 889µm compared to *C. pinea*'s 1,281µm (Table 2.3). Also, the ascigerous swelling of *C. pseudoorientalis* was subterminal compared to *C. pinea* which was terminal (Table 2.3). On full strength MEA their culture morphologies are indistinguishable; both start white and become beige to grey with age. The underside of the culture will brown with age but descension into the agar was not observed on MEA, as it was with PDA. On PDA, when young *C. pinea* and *C. pseudoorientalis* have very similar morphologies: both start white and appressed to the agar with irregular borders. With age, however, *C. pseudoorientalis* may become a light brown color. This can occur in *C. pinea* but was observed far less frequently in this study. Both species will usually produce a brown, hydrophobic exudate which may lead to browning of the PDA, but this is not known to occur on other media types.

It is more challenging to differentiate *C. aceriae* and *C. cilium* from mazaedia characteristics alone as they do not differ substantially using the characteristics measured in this study (Table 2.3). However, each was identified only on their respective hosts (*Acer rubrum* and *Quercus velutina*) indicating host specificity, but further study is needed to determine true host range for each species. On PDA *C. aceriae* is brown with white margins, fluffy when young but becomes flattened with age, and produces the brown exudate observed in *C. pseudoorientalis* (Fig2.5A). In *C. aceriae,* production of the exudate was only observed on the underside of the culture, never on top. This species also frequently grew with elastic morphology, changing between isolates, and displaying irregular zoning of the culture with irregular margins. *C. cilium* grew with a similar but distinct morphology on PDA: this species will grow down into the media and become appressed, as observed in *C. pseudoorientalis*. This species also produces a brown exudate on the underside of the culture, but it is reduced compared to *C. aceriae*, appearing more diluted in color and not typically reaching the plate margin (Fig2.7A). The growth of *C. cilium* is also usually a lighter beige-brown and does not darken dramatically with age. On PDA, *C. cilium* grew with an elastic morphology with zoning and irregular margins (Fig2.7A).

Taxonomy

Caliciopsis pseudoorientalis Harkness, Miles, and Sakalidis, sp. nov. (Figs2.2 and 2.3).

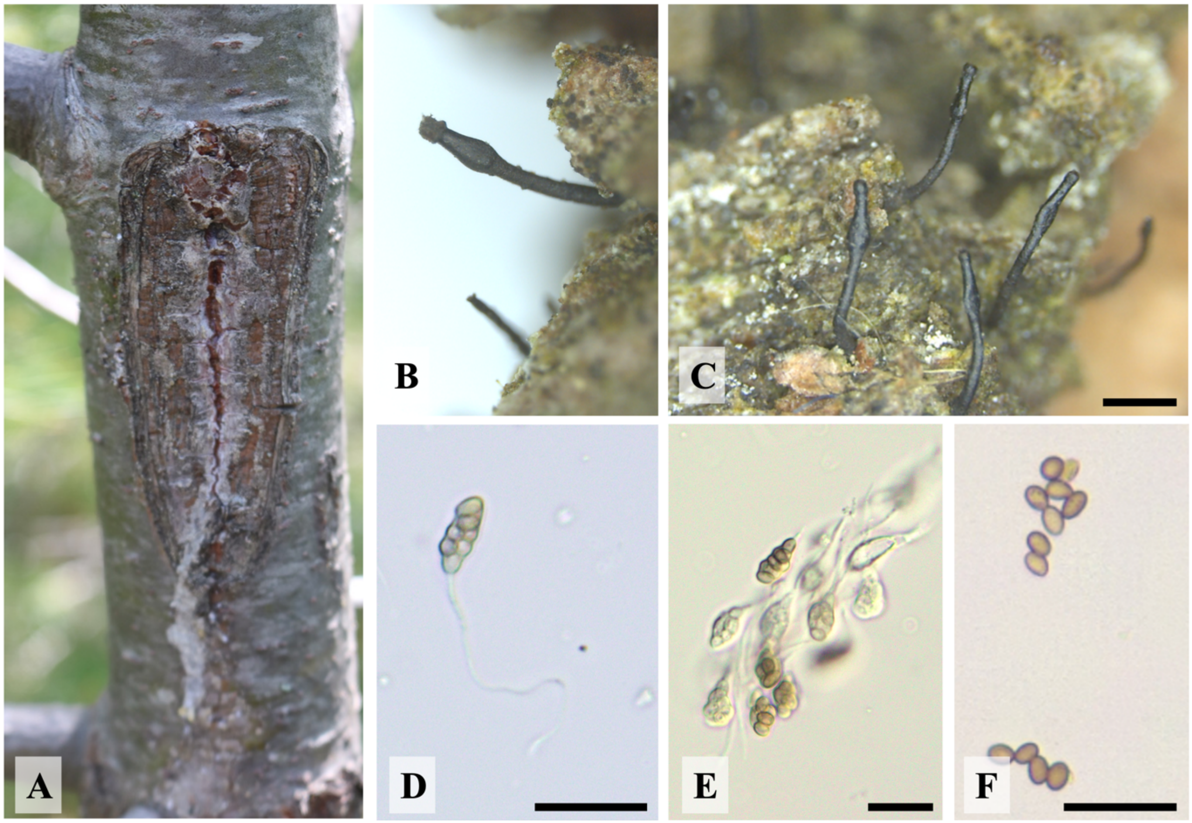

Figure 2.2. *Caliciopsis pseudoorientalis* sexual structures. **A)** Pitching canker on a eastern white pine (*Pinus strobus*) with *C. pseudoorientalis* mazaedia at the margin, **B-C)** mazaedia, scale bars: 500 µm, **D-E)** asci, scale bars: 20 µm, **F)** ascospores, scale bar: 25 µm.

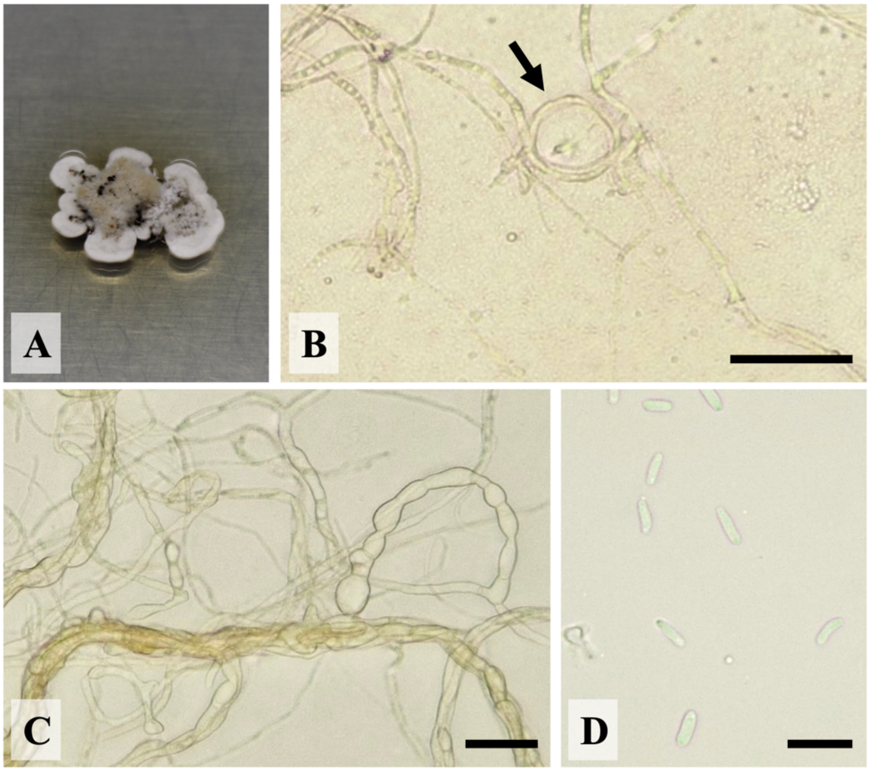

Figure 2.3. *Caliciopsis pseudoorientalis* asexual structures. **A)** A three-week-old colony grown on full strength PDA at room temperature, or approximately 20°C, **B)** hyphal coils observed from cultures grown on PDA, scale bar: 25 µm, **C)** hyphal swelling observed in cultures grown on PDA, bar: 25µm, and **D)** conidia, scale bar: 10 µm.

Etymology: From the Greek word *pseudo* (false) and the Latin *orientalis* (eastern), referring to the close phylogenetic placement and physical resemblance of this species with *C. orientalis.*

Diagnosis: This species can be distinguished by its affinity for *Pinus strobus* (no other known hosts) but exhibits a subterminal ascigerous swelling compared to the terminal ascigerous swelling of other *P. strobus*-infecting species, *C. pinea.* Furthermore, mazaedial stalks of *C. pinea* can grow to be much longer than was observed for *C. pseudoorientalis* at maturity: up to 3,000µm compared to 1,537µm, respectively.

Typification: USA, Grayling, Michigan, (coordinates: 44.662797, -84.767528), on the trunk of *Pinus strobus*, on the margin of a pitching canker. Collection made by Rebecca J. Harkness, 11/17/2021.

Description: Erumpent stromata black, gregarious but can be scattered, rounded spots which most frequently develop into a singular mazaedial stalk. Mazaedium black, straight to curved, never branched, gregarious to scattered, averaging 889µm tall and 56µm wide beneath the ascigerous swelling. Often individual mazaedial stalks are isolated but multiple emerging from the same stroma was observed. Ascigerous swelling subterminal, averaging 170µm long and 98µm wide, tapers to a red-brown mazaedium. Asci clavate, with eight ascospores, developing within a fasciculate hymenium, appearing bitunicate. Ascospores brown when mature, subglobose to ellipsoidal, averaging 4.8µm long and 3.6µm wide, with no visible ornamentation. Conidiogenous cells not observed. Conidia allantoid, hyaline, non-septate, averaging 5.8µm long by 1.7µm wide.

Habitat and known distribution: observations of this species were made at two distinct geographic locations in Michigan, one in the upper peninsula and one in the lower peninsula. In both localities mazaedia were found on the trunks of cankerous and pitching, pole-sized, *P. strobus* in mixed age hardwood and conifer forests with poorly draining soils.

Other specimens examined: USA, Raco, Michigan, (coordinates: 46.37708986, -84.70553482), on the trunk of *Pinus strobus* at a pitching branch node. Collection made by Rebecca J. Harkness, 11/18/21.

Caliciopsis aceriae Harkness, Miles, and Sakalidis, sp. nov. Figs2.4 and 2.5.

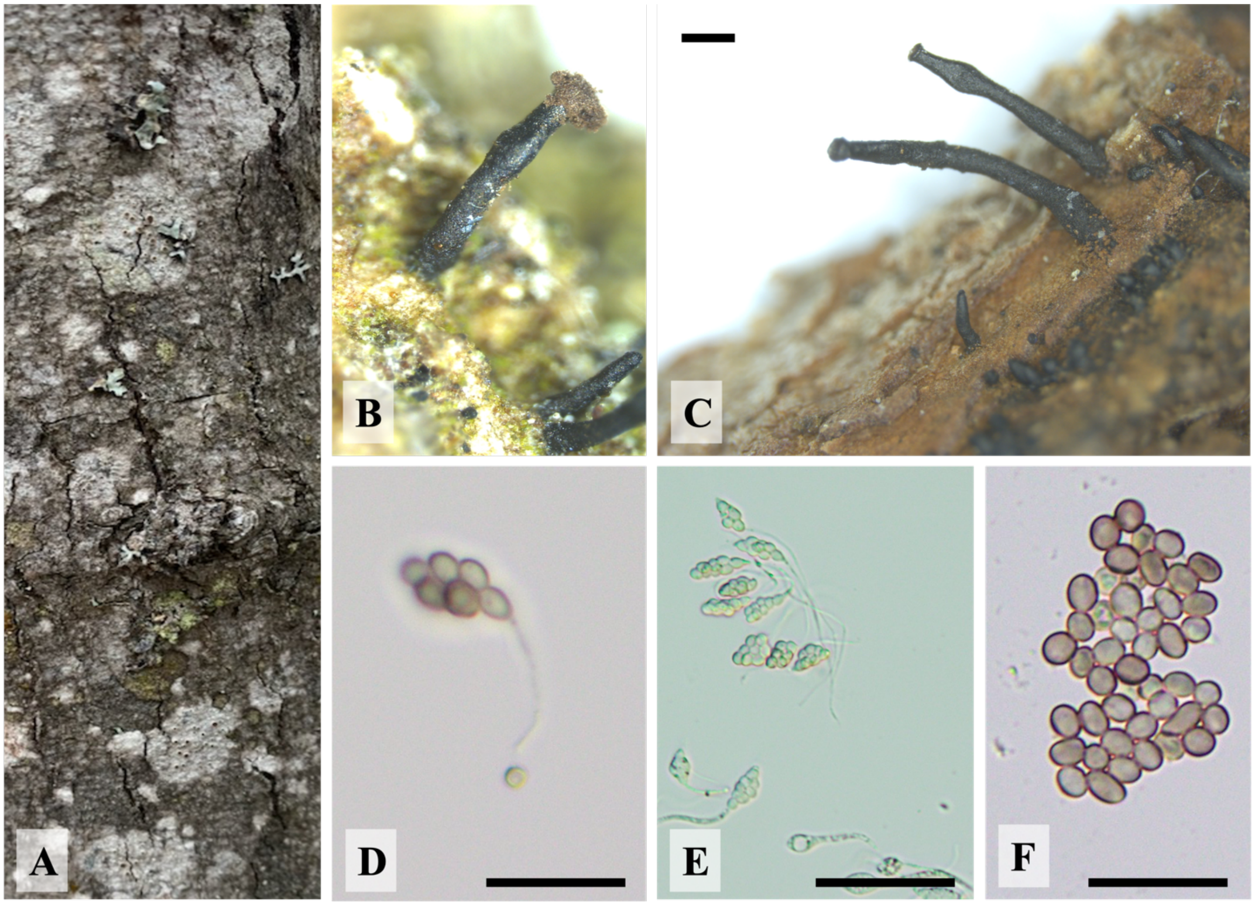

Figure 2.4. *Caliciopsis aceriae* sexual structures. **A)** Bark fissures from which mazaedia emerge, **B-C)** mazaedia, scale bars: 250 µm, **D)** ascus, scale bar: 20 µm, **E)** asci, scale bar: 50 µm, and **F)** ascospores, bar: 20 µm.

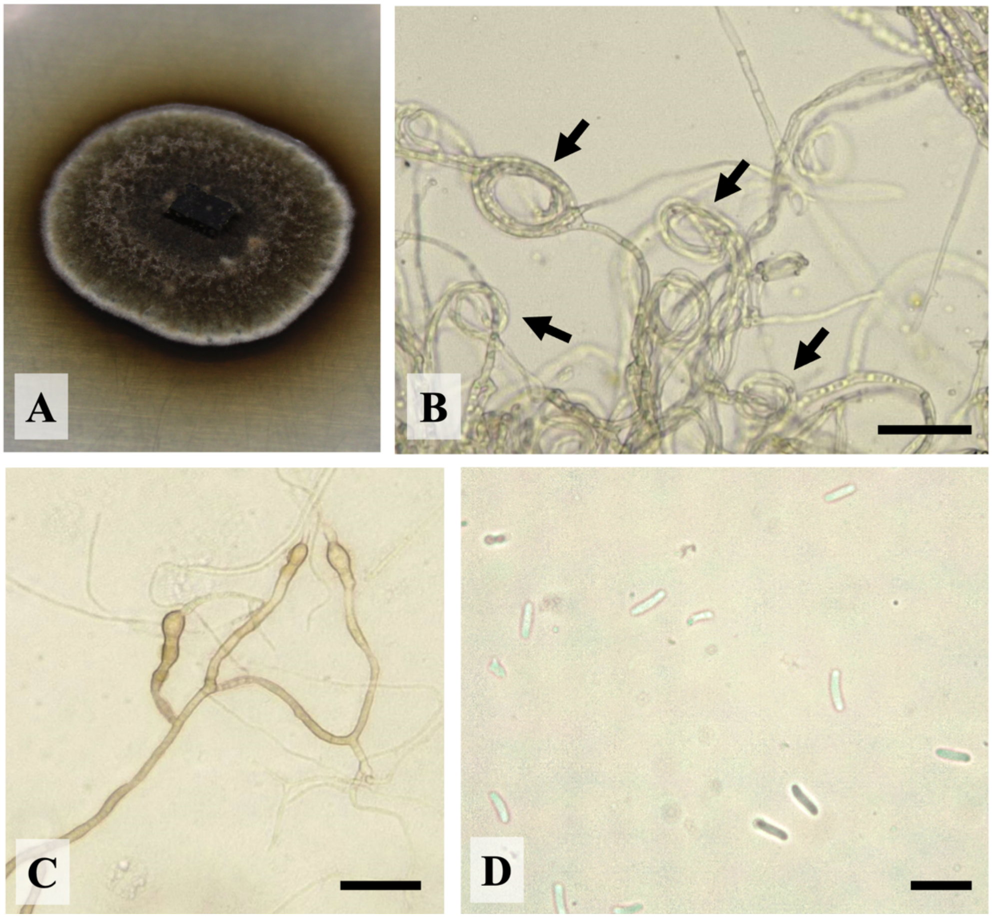

Figure 2.5. *Caliciopsis aceriae* asexual structures. **A)** A three-week-old colony grown on full strength PDA at room temperature, or about 20°C, **B)** hyphal coils observed from cultures grown on PDA, scale bar: 10 µm, **C)** hyphal swelling observed from cultures grown on PDA, bar: 25 µm, and **D)** conidia, bar: 10 µm.

Etymology: From the Latin word *acer* (sharp) referring to the identification of this species from *Acer rubrum,* the red maple.

Diagnosis: This species is differentiated from other related taxa primary by its size. *C. aceriae* is, on average, shorter and thinner than its sister species *C. cilium* (averaging 608x57µm and 778x70µm respectively). Furthermore, the ascigerous swelling of *C. aceria* is subterminal, compared to *C. cilium* which has a median to subterminal ascigerous swelling. Both *C. cilium* and *C. aceriae* are on average 100-300µm shorter than species described from a similar host in Europe (*C. valentina* and *C. beckhaussi*) (see Table 2.2). Additionally, *C. aceriae* has only been isolated from *Acer rubrum* and has no other known hosts.

Typification: USA, Manistee, Michigan, (coordinates: 44.197713, -86.188963), on the trunk of *Acer rubrum*, emerging from cracks in the bark, rarely observed on the surface. Collection made by Rebecca J. Harkness, 11/17/2021.

Description: Erumpent stromata black, gregarious but can be scattered, rounded spots which most frequently develop into a singular mazaedium. Stalks black, straight to curved, never branched, gregarious to scattered, averaging 608µm tall and 57µm wide beneath the ascigerous swelling. Individual mazaedia are usually isolated but in some cases, multiple will emerge from the same stroma. Ascigerous swelling subterminal, averaging 148µm long and 83µm wide, tapers to a red-brown mazaedium. Asci clavate, with eight ascospores, developing within a fasciculate hymenium, appearing bitunicate. Ascospores brown when mature, globose to ellipsoidal, averaging 5µm long and 4µm wide, with no visible ornamentation. Conidiogenous cells not observed. Conidia allantoid, hyaline, non-septate, averaging 5.8µm long by 1.6µm wide.

Habitat and known distribution: observations of this species were made at three distinct geographic locations in Michigan, one in the northern lower peninsula and two in the central-lower peninsula. In all locations mazaedia were found in the cracks on the trunks of pole-sized to mature *Acer rubrum* in mixed age hardwood and conifer forests with poorly draining, sandy soils.

Other specimens examined: USA, Grayling, Michigan, (coordinates: 44.662797, -84.767528), on the trunk of *Acer rubrum*, in cracks on the trunk of the tree. Collection made by Rebecca J. Harkness, 11/17/2021. USA, Raco, Michigan (coordinates: 46.37708986, -84.70553482), on the trunk of *Acer rubrum*, in cracks on the trunk of the tree. Collection made by Rebecca J. Harkness, 11/18/2021.

Caliciopsis cilium Harkness, Miles, and Sakalidis, sp. nov. Figs2.6 and 2.7

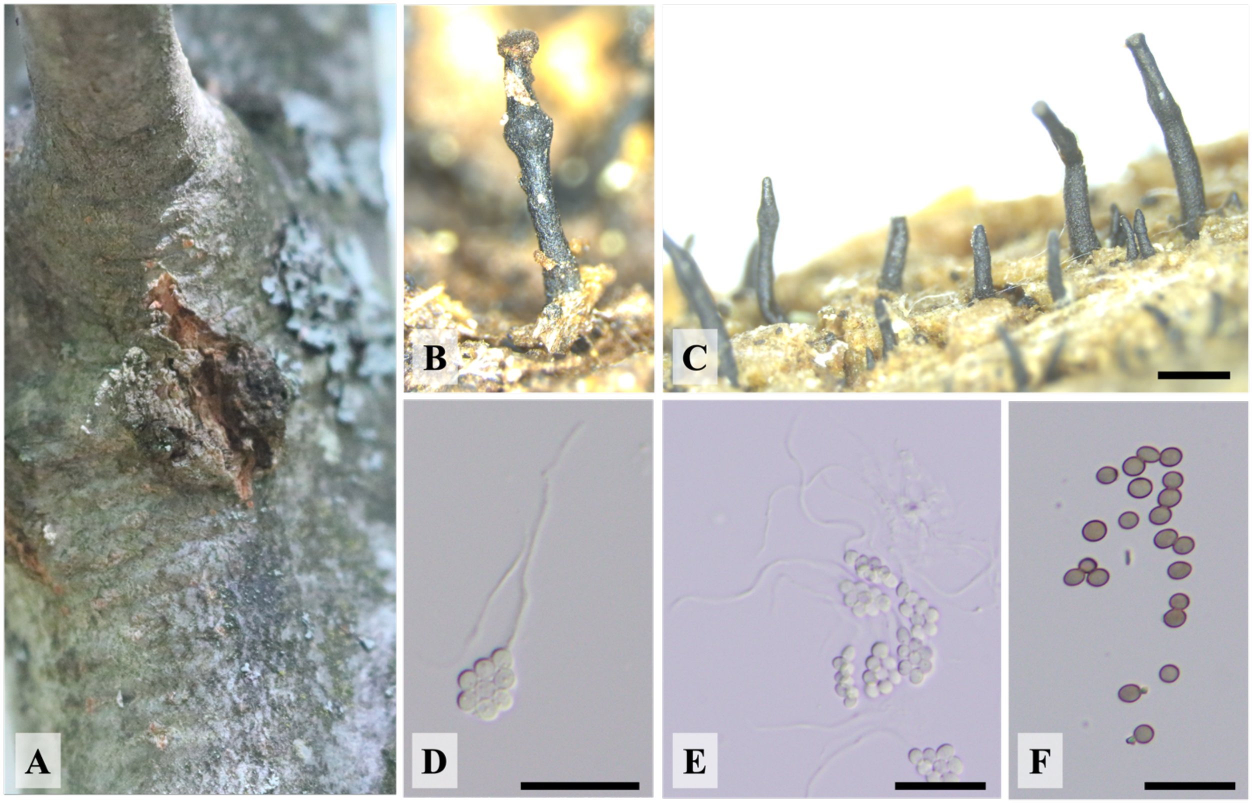

Figure 2.6. *Caliciopsis cilium* sexual structures. A) A site of prior wounding on *Quercus velutina*, where mazaedia were found along the interior margin, B-C) mazaedia, scale bar: 250 µm, E-F) asci, scale bars: 20 µm, and G) ascospores, scale bar: 20 µm.

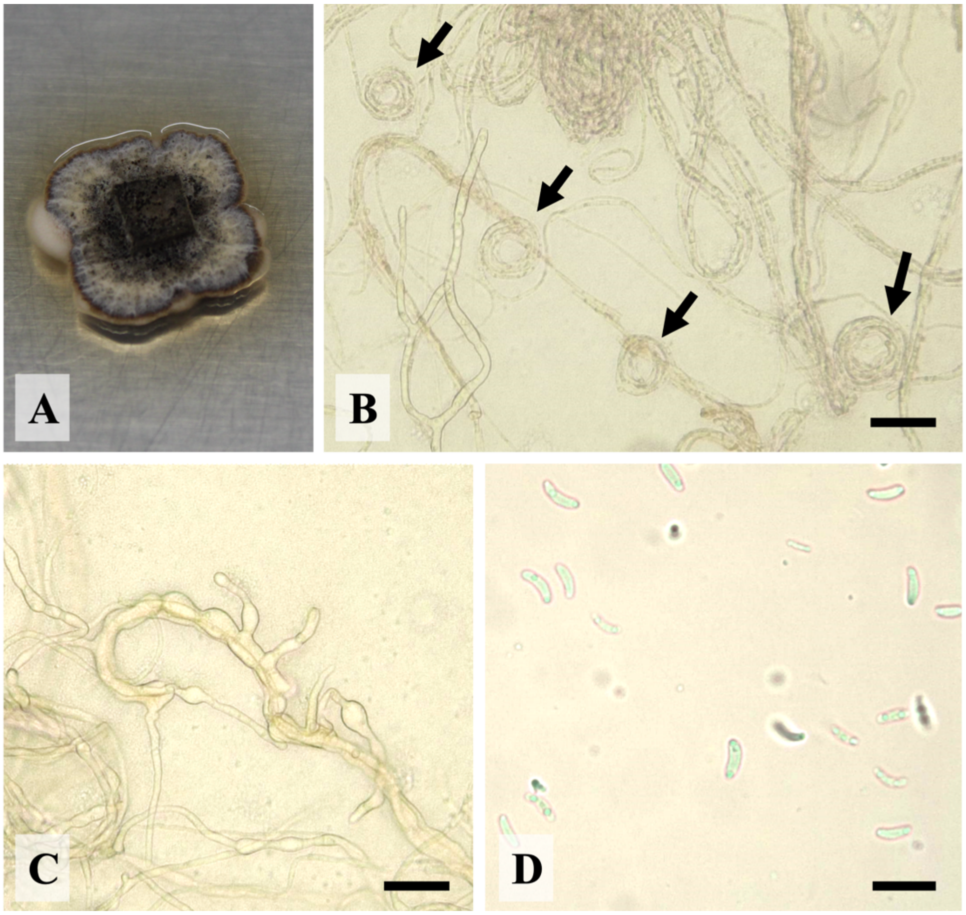

Figure 2.7. *Caliciopsis cilium* asexual structures. **A)** A three-week-old colony grown on full strength PDA at room temperature, or approximately 20°C, **B)** hyphal coils observed from cultures grown on PDA, scale bar: 25 µm, **C)** hyphal swellings observed in cultures grown on PDA, scale bar: 25 µm, and **D)** conidia, scale bar: 10 µm.

Etymology: From the Latin word *cilium* (eyelash) referring to the resemblance of many *Caliciopsis* species to eyelashes.

Diagnosis: This species is differentiated from other closely related species through the physical characteristics described above in the diagnosis of *C. aceriae*. Though it bares considerable morphological similarity to its sister species (*C. aceriae*) *C. cilium* was observed on a different host. *C. cilium* is only known from *Quercus velutina,* and morphological and molecular investigations only identified *C. cilium* on this host, never on the host of *C. aceriae* (*Acer rubrum*)*,* and vice versa. *C. valentina* and *C. beckhausii* are known from a European *Quercus* spp. and have considerable morphological similarity to *C. cilium*; However phylogenetic analyses offer a well-supported monophyletic group for *C. cilium* that diverges from these two closely related species (Fig2.1).

Typification: USA, Grayling, Michigan, (coordinates: 44.662797, -84.767528), on the trunk of *Quercus velutina*, on the margin of open wounds. Collection made by Max G. Helser, 9/7/23.

Description: Erumpent stromata black, usually scattered, rounded spots which most frequently develop into a singular mazaedium. Stalk black, straight to curved, sometimes branched, gregarious to scattered, averaging 778µm tall and 70µm wide beneath the ascigerous swelling. Individual mazaedia are usually isolated and were not observed developing from the same stroma. Ascigerous swelling subterminal to median, averaging 147µm long and 98µm wide, tapers to a red-brown mazaedium. Asci clavate, with eight ascospores, developing within a fasciculate hymenium, appearing bitunicate. Ascospores brown when mature, globose to ellipsoidal, averaging 4.7µm long and 4.1µm wide, with no visible ornamentation. Conidiogenous cells not observed. Conidia allantoid, hyaline, non-septate, averaging 5.6µm long by 1.7µm wide.

Habitat and known distribution: Observations of this species were made at one geographic location in Michigan's lower peninsula. Mazaedia were found in cracks on the trunks of pole-sized *Quercus velutina* in mixed-age hardwood and conifer forests with poorly draining, sandy soils. It is unclear if the cracks were caused by the emergence of mazaedia or if they were occupying a site of prior wounding (Fig2.6A).

Other specimens examined: Other specimens from the same geographic region as the type specimen, from the same host, collected in subsequent years of the original collection were observed. Collections made by Rebecca J. Harkness and Max G. Helser between 2023 and 2024.

2.5 Discussion

In this study three new species of *Caliciopsis* were described from ascocarps found on economically and ecologically important deciduous and coniferous hosts in *P. strobus* dominant forests. In is unclear if these species are all capable of behaving as pathogens, however, investigation of pathogenicity was not a priority in this study. The host range and distribution of *C. moriondi* were expanded to include *P. banksiana* and the midwestern USA, respectively. Three genetic loci were required to resolve all known species within *Caliciopsis* to a sufficient resolution and support. Newly described species had morphologies distinct from other closely related taxa, supporting the findings from phylogenetic analyses. The discovery of these previously undescribed *Caliciopsis* spp. and their genetic complexity highlights the diversity present in native forests, where microbial ecology is understudied.

The goal of this study was to characterize previously undescribed species of *Caliciopsis* using morphological and molecular analysis techniques. The availability of genetic data and macro- and micromorphological descriptions from this paper will improve visual and molecular identification tools for this genus of fungi. However, there were several distinct limitations to investigating *Caliciopsis* species: 1) species have been identified from a single, historic (pre-1970) collection; 2) no previously generated genetic data is available for them in public repositories, and 3) there is disagreement between studies on loci selected for phylogenetic investigation. Thus, some species are well characterized but sequences associated with those species do not exist, microscopes and methods for measuring microscopic structures can vary from lab-to-lab and are difficult to compare, and, finally, only ~33% of described species of *Caliciopsis* have a living type culture available for purchase from a nationally recognized culture collection (Table S.2.1). In this study we describe three new species of *Caliciopsis* and offer recommendations for continued investigation of the genus.

Morphological and molecular descriptions of all examined *Caliciopsis* species offer considerable evidence of diverging lineages. In this study no individual loci could resolve all species with high degrees of bootstrap support and BPP. TUB provided the highest resolution at the species rank except for a small subset of *C. pinea* isolates that did not group properly with other isolates of that species (FigS.2.1C). tEF1-α, similarly, resolved all species branches with sufficient support except *C. pseudoorientalis* which had a higher degree of intraspecies variation at this locus and thus was poorly resolved (FigS.2.1B). ITS was unable to resolve most species in this study with a high enough degree of confidence to determine independent lineage (FigS.2.1A). The individual trees for ITS and TUB had topographies in agreement with the concatenated analysis, while the topography of the tEF1-α individual analysis did not (Fig2.1 and S.2.1B). Concatenating three loci (ITS— tEF1-α— TUB) resulted in support at all nodes at the species rank with >90% bootstrap support and >0.95 BPP (Fig2.1).

Synapomorphies shared by all species observed over the course of this study include brown, circular to ellipsoidal ascospores that varied in size depending on species; the ascospores of *C. aceriae* and *C. cilium* were on average 2µm smaller in both length and width than their European sister species (*C. valentina* and *C. beckhausii*). Similarly, all species produced hyaline, allantoid conidia that were not as variable in size between species when compared to ascospores (Table 2.3 and Figs2.3D, 2.5D, and 2.7D). The three species described in this study produced hyphal coils when maintained in a vegetative growth state, as well as hyphal swellings which have been identified as ascogonial hyphae in previous studies (Figs2.3, 2.5 and 2.7) (Funk 1963). Funk speculated that, in nature, with such prolific production of receptive structures, "cells of the ascogonial hyphae may be fertilized by spermatia of a different genetic constitution, so that the ascospores which eventually arise in a single ascocarp are not necessarily of the same genotype" (Funk 1963). This may explain observations that were made in this study while trying to sequence genetic loci, which often resulted in multiple amplicons and a high degree of intraspecies single nucleotide polymorphisms (usually in the ITS regions but sometimes in tEF1-α).

C. pseudoorientalis was identified alongside *C. pinea* sometimes cohabitating on the same tree, but on average *C. pseudoorientalis* had shorter fruiting bodies and a subterminal ascigerous swelling compared to the longer mazaedia of *C. pinea* with terminal ascigerous swelling (Table 2.3). Both *C. aceriae* and *C. cilium* ascomata are very close in appearance to *C. beckhausii* and *C. valentina* (which also grow on a deciduous tree host) but are well supported in phylogenetic analyses as independent lineages (Fig2.1). Furthermore, they were identified from different hosts and on a different continent than *C. beckhausii* and *C. valentina* (Table 2.2). Additionally, over the course of this study we identified *C. moriondi* in the Great Lakes region on *P. banksiana* and *P. resinosa. C. moriondi* is a known pathogen of several *Pinus* species but has only been found in North America in New Hampshire on *P. resinosa* (Migliorini et al 2020). This study is the first report of *C. moriondi* on *P. banksiana* in Wisconsin and Michigan*.* The drivers of host specificity are poorly understood for this genus, but it does appear to play a role in the divergence of phylogenetic lineages.

Determining host preferences of individual *Caliciopsis* species requires additional investigation. Only three species of *Caliciopsis* (*C. pinea, C. moriondi,* and *C. pseudotsuga*) have ever been demonstrated through artificial inoculation to grow on multiple host genera (Ray 1936; Funk 1963; Delatour 1967 and 1969; Capretti 1978; and Migliorini et al 2020). All other known, pathogenic species are reported from a single host genus and in some cases a single host species (Table S.2.2). In the final, concatenated analysis, clades diverged based on host and not by geographic location (Fig2.1A). Future studies are necessary to determine more accurate host specificity, pathogenicity, and virulence for target species, such as *C. pinea* which in the last decade has come to be regarded as an emerging pathogen of *P. strobus* (Munck et al. 2015; Whitney et al. 2018).

There were several distinct limitations to investigating *Caliciopsis* species: species have been identified from a single, historic (pre-1970) collection and no previously generated genetic data is available for them in public repositories, there is disagreement between studies on loci selected for phylogenetic investigation and thus some species are well characterized but sequences associated with those species do not exist, microscopes and methods for measuring microscopic structures can vary from lab to lab and are difficult to compare, and, finally, only ~33% of described species of *Caliciopsis* have a living type culture available for purchase from a nationally recognized culture collection (Table S.2.1). In this study we describe three new species of *Caliciopsis* and offer recommendations for continued investigation of the genus to mitigate the aforementioned limitations.

Since the late 1800s, the genus *Caliciopsis* has periodically been a focus of scientific research. However, reports have been sporadic and isolated until 2015 (Munck et al 2015). Recent research has been driven by observations of *C. pinea* behaving as a more aggressive pathogen when water availability in forest stands is more limited, such as high-density planting, poor water-retentive soils, extreme heat, and drought conditions (Munck et al 2016; Costanza et al 2019 and 2020). Seedling mortality of *P. strobus* has also been reported in some regions (Munck et al 2015 and 2016). Due to the existence of other pathogenic species within *Caliciopsis,* further investigation is necessary to determine if the opportunistic nature of *C. pinea* infections could be true of other members of the genus. These findings highlight the need for continued research into the host specificity and ecological drivers of pathogenicity within the *Caliciopsis* genus, particularly as environmental changes may further elevate the risk of infection across diverse forest ecosystems.

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APPENDIX

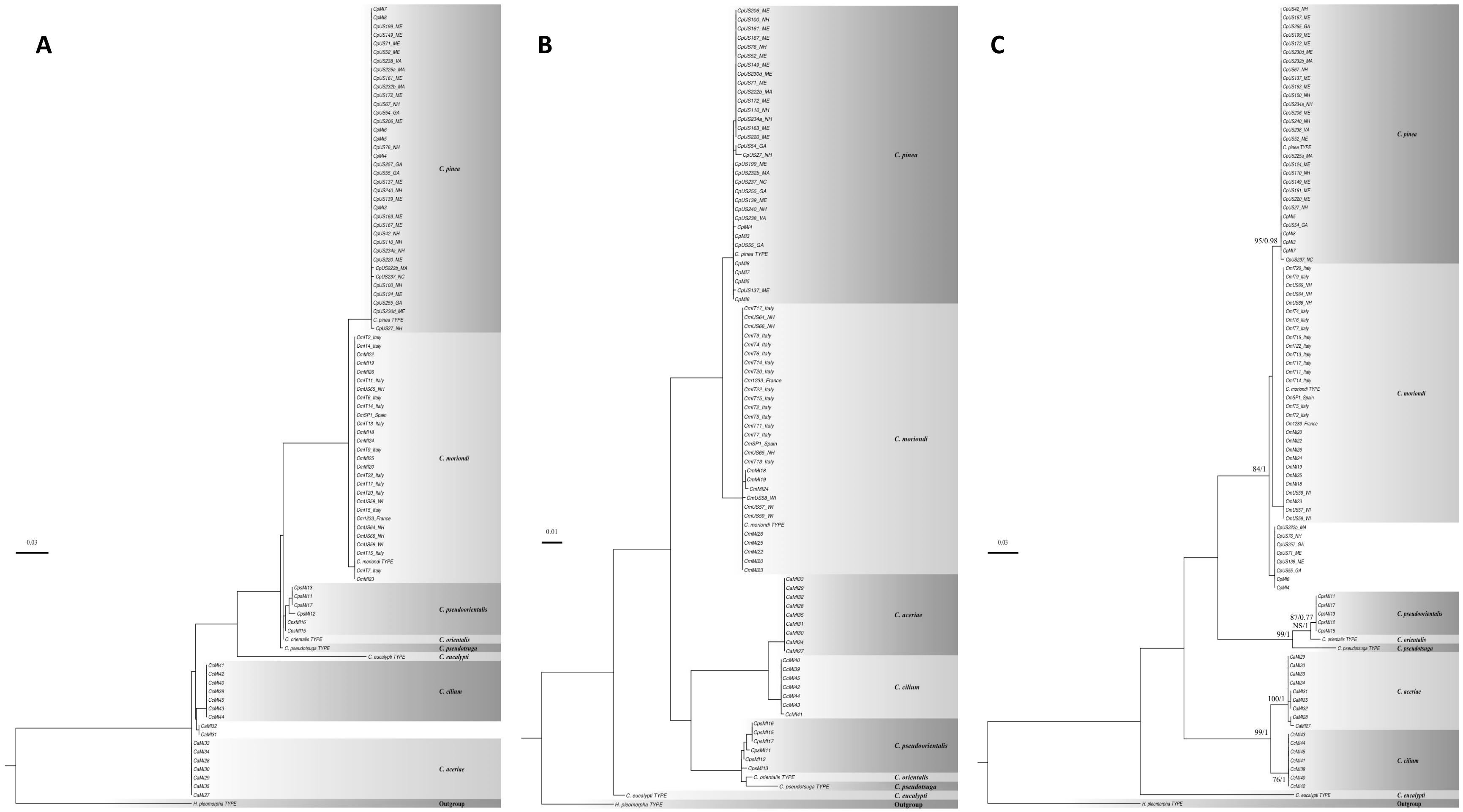

Figure S.2.1. Individual-locus maximum likelihood phylogenies of *Caliciopsis*. Bootstrap support values from maximum likelihood analyses that were ≥70% and Bayesian posterior probabilities ≥0.95 are reported as the first and second numbers, respectively, associated with internodes. Internodes with bootstrap support lower than 70% and posterior probability lower than 0.7 were labeled NS for not supported. A) nuclear internal transcribed spacer region (ITS), B) translation elongation factor 1-α (tEF1-α), and C) β-tubulin (TUB).

Table S.2.1. A table of all known *Caliciopsis* spp., the location of their discovery, location of type specimen, if available, synonyms, host(s), and notes/taxonomic history.

Caliciopsis Species	Location and Year of Description	Type	Synonyms	Host(s)	Notes
arceuthobii	New York, USA, 1873[1]	NYSf 312 New York State Museum Fungi Type Database Holotype-- unclear if culture is available http://www.nysm.nysed.gov/research-collections/biology/botany-mycology/collections/online-collection	1. *Sphaeria arceuthobii* 2. *Wallrothiella arceuthobii*	*Arceuthobium* (Dwarf Mistletoe species)	First discovered by Charles H. Peck in 1873 and described as *Sphaeria arceuthobii* , n.sp on *Arceuthobium pusillum* [1]. The specimen was not oberserved or mentioned again in the literature until 1882 when Pier A. Saccardo reclassified the specimen as *Wallrothiella arceuthobii* [2]. Therafter, this species was referred to as *W. arceuthobii* in all further literature. The second observation of this fungal species in nature was in 1900: C.F. Wheeler found *W. arceuthobii* on *Arceuthobium pusillum* in Michigan's Upper Peninsula[3,4]. At several points since 1900 there have been resurgences of interest in studying this organism as a potential bio-control for invasice Dwarf Mistletoe species (1968[5], 1979[6], 2009[7]). In 1969 Job Kuijt recognized that *W. arceuthobii* could be placed in the Coryneliaceae[8], but it wasn't until 1986 that Margaret E. Barr reclassified *W. arceuthobii* into the *Caliciopsis* genus[9]. Barr and Weir give detailed descriptions of the type specimen. Weir made his own collection in Coeur d'Alene, Idaho in 1916 that is in the NYBG Steere Herbarium, #2928717. Observations state this species is parasitic.
arrhiza	Venezuela, 1888[10]	Institute of Mycology of the University of Recife, IMUR 21562	1. *Capnodium arrhizum* 2. *Lagenula arrhiza*	Unidentified Rosaceous host[13]	First described by Patouillard and Gaillard as *Capnodium arrhizum* on "coriaceous, fallen leaves of an unidentified, probably dicotyledonous plant" in San Fernando de Atabaya, Venezuela and deposited in the herbarium of the National Museum of Natural History in Paris[10,11]. The species wasn't referenced again in literature until 1930 when Gabriel Arnaud renamed the original specimen as *Lagenula arrhiza* [12]. Fitzpatrick (1942) made observations of the Pat. and Gail. herbarium material and compared them to Arnaud's descriptions and drawings. Fitzpatrick noted that "Though the form of the ascocarp and the appearance of the ascospore initialy indicate that the species belongs in or near *Caliciopsis* its inclusion is only tentative." Fitzpatrick made observations of the Pat. and Gail. type material, but he describes how "broken ascocarps spoiled the specimen for photography" and stated that Arnaud's drawings were a sufficient substitute. There was no other reference to this species until 1964 when Batista and Bezzera made observations on a **mature** specimen, and asserted that their specimen and *Capnodiium arrhizum* were the same organism but the original *Capnodium arrhizum* specimen was immature and all descriptions of it were of an immature fruiting structure. The **mature** specimen was reclassified as *Caliciopsis* (Type found in Institute of Mycology of the University of Recife, IMUR 21562).
beckhausii	San Juan del Monte, Spain, 2015[14]	MA- 18186	1. *Calicium ephemerum* 2. *Caliciopsis ephemera* 3. *Coniocybe beckhausii* 4. *Sphaeronema subcorticalis* 5. *Hypsotheca subcoricalis* 6. *Hypsotheca ephemera*	*Quercus ilex* subsp. *rotundifolia*	The taxonomy of this species is complex, and described in great detail by Garrido-Benavent and Pérez-Ortega (2015). A North American specimen (originally collected by Cooke and Ellis and described as *Sphaeronema subcorticale* in 1878 on an oak[15]) was examined by Ellis and Everhart in 1885 and renamed *Hypsotheca subcorticalis* [16]. A European specimen was also collected by Zwackh-Holzhausen in 1864 and described as *Calicium ephemrum,* but Garrido-Benavent et al. note that this specimen was first labeled as *Coniocybe beckhausii* (a species described by Körber in 1865[18]), and when *Calicium ephemerum* was transferred to *Caliciopsis ephemera* by Rehm in 1896, *Coniocybe beckhausii* was listed as a synonym [17, 19]. In 1920 Fitzpatrick made observations **of both specimens** and agreed with Rehm's (1896) observation that both specimens were the same despite being from different continents. Rikkinen (2000) and Garrido-Benavent (2015) both assert that the two specimens are not the same, thus, according to nomenclature rules the North American specimen can be considered *Calicipsis subcorticalis* and the European isolate was combined under the name *Caliciopsis beckhausii* [14].
brevipes	Cordillera de Nahuelbuta (Mountain Range), Chile, 1970[21]	ETH Zürich ZT Myc 58038[26] Type specimen only, no culture	None	*Araucariae araucanae*	Discovered in 1970 by Heinz Butin and validated by Benny et al. 1985 who examined Butin's original collection. Butin cultured his specimen but it does not seem that he deposited that culture in an herbarium. His descriptions of the culture can be found in his original publication of the species, though.
calicioides	Sweden, 1832 (most likely from home parish of Elias Fries, Femsjö)	Suksdorf: NYBG Steere Herbarium #2928720 Ravenel: NYBG Steere Herbarium # 2928719 Specimens only, no cultures	1. *Sporocybe calicioides* 2. *Hypsotheca calicioides* 3. *Caliciopsis ellisii* 4. *Hypsotheca caliciodes* var. *caespitosa*	Original specimen: *Fagus* (beech) Fitzpartick "type": *Populus* (poplar)	This species was originally found by Elias Fries, described as *Sporocybe caliciodes* and marked "v.v." for "vivo vidi" or "seen alive" in 1832 and thus has no known herbarium specimen[23]. Fries found fruiting bodies "ad ligna putrida Fagi rarius," or on rotting beech trees. In 1885, J.B. Ellis and B.M. Everhart examined a fungal specimen sent to them by W.N. Suksdorf from Washington Territory (on Poplar bark). They considered this specimen to be the same organism as *Sporocybe calicioides* based on Elias Fries' original description, but renamed it *Hypsotheca calicioides* [24]. The Suksdorf material was rexamined by Saccardo in 1889 and determined to be a member of the *Caliciopsis* genus (observations were made on specimens from "Carolina" and Washington, both found on "*Populino*" bark) and thus he renamed it *C. ellisii* [25]. In this description the author noted "an species europaea, a Friesio descripta, indetica?" or "Is the European species described by Fries identical?". In Fitzpatrick's 1920 Monograph of the Coryneliaceae he would confirm Saccardo's suspicion that Fries' descriptions did not match those of *H. calicioides* given by Ellis and Everhart[11]: Fitzpatrick states, "Ellis obtained the specific name for this species from the older binomial, *Sporocybe calicioides* Fries which he cites as a synonym. The description published by Fries is, however, clearly based on a hyphomycetous fungus, and there seems to be no reason for assuming the two fungi to be the same." Fitzpatrick also noted that another specimen from Ellis' collection was labeled as *Hypsotheca calicioides* (Fr.) var. *caespitosa,* but Fitzpatrick notes that the Suksdorf specimen was most likely immature and that this second collection by Ellis may be mature fruiting structures of the same organism and because specimens were limited, he combined the two under the same name, *Caliciopsis calicioides.* The type specimen was considered by Fitzpatrick to be the Suksdorf collection which can be found in the herbarium of the NY Botanical Garden.

Table S.2.1. (cont'd)

clavata	Chile, 1846[27]	National Museum of Natural History, Paris In the Mountain Herbarium Under *Corynelia clavata* (Mont.) MNHN-FC-FUSION94850 Specimens only, no cultures https://science.mnhn.fr/institution/mnhn/collection/pc/item/fusion94850?listIndex=1&listCount=2	1. *Sphaeronema clavatum* 2. *Aposphaeria clavata* 3. *Corynelia clavata* 4. *Sorica clavata*	1. *Drimys winteri var. chilensis*	The first description of this species was as *Sphaeronema clavatum* in 1846[27]. The original specimen was collected by Claude Gay in Chili "on the branches, leaves, and galls" of *Drymis chilensis* , a tree native to Chili. The species was formally described by Joseph-Henri Léveillé[27], and is only known by the original specimen, according to Fitzpartick 1942. Jean Pierre Montagne was the next to make observations of the original specimen, and he renamed it *Corynelia clavata,* but never published this name change and the same name was later used by Saccardo to identify an entirely different organism[11]. The original specimen would later be examined by A.L. Jaczewski, he noted that the author of the description did not notice "two very distinct mushrooms on leaves and galls of *Drymis* which lie side by side." He noted that one was clearly an asexual structure (pycnidium), while the other could not be Sphaeropsidea, as Léveillé claimed, because it looked like an elongated club with asci and paraphyses (i.e. a sexual structure). He concluded that the proximity of the "two" fungi on its hosts was not coincidental and that the asexual state should be reassigned to *Aposphaeria clavata,* and that the sexual state of *Aposphaeria* was *Caliciopsis*[28]. Finally, Gabriel Arnaud (who was aware of all previous names and mentions Fitzpatrick's 1920 publication) asserted that, while Jaczewski felt the sexual fruiting bodies belonged in the *Caliciopsis* genus, he felt that *Sorica* was a more appropriate assignment and placed the specimen there[12]. Fitzpatrick later returned it to *Caliciopsis* in 1942 after making his own observations of the type specimen.
cochlearis	Cordillera pelada (Mountain Range), Chile, 1970[21]	ETH Zürich ZT Myc 58041[26] Type specimen only, no culture	None	1. *Pilgerodendri uviferi* 2. *Araucariae araucanae* 3. *Podocarpus nubigenus* 4. Several Cupressaceae	Discovered in 1970 by Heinz Butin and validated by Benny et al. 1985 who examined Butin's original collection. Described originally as a parasite to it's host but also capable of growing as a saprophyte.
confusa	Nepal, 1821[29] Sumatra, 1927[29]	New York Botanical Garden Nepal Specimen: 02928722 Sumatra Specimen: 02928723	1. *Capnodium fructicolum*	1. *Myrsine africana* 2. *Embelia microphylla*	This species was discovered in Nepal and Sumatra in 1821 and 1927 (respectively). The first specimen has an unnamed collector, but the second was discovered by a person who only identified themselves as "Barlett". This may refer to Harley Harris Bartlett who has many other (primarily plant) specimens deposited in the NYBG Steere Herbarium and who was in Indonesia during the time the specimen was discovered. Both the original specimen and the second collection were labeled "*Capnodium fructicolum"* , a taxon described by Patouillard in 1889. A.C. Batista attempted to revise the *Capnodiales* fungi and found the original specimen. In their revision of the order, they determined that the original *Capnodium fructicolum* collection was wrongly grouped in *Capnodiaceae* and should instead be grouped into the *Coryneliaceae.* Batista then reassigned the original specimen to *Caliciopsis.* This species was described as parasitic in the species description.
elytranthicola	North Borneo, 1957[30]	Herbarium Bogoriense BO 13537 Dried specimen only	None	1. *Elytranthis albidae*	This species was discovered in North Borneo in 1957 by C.G. Hansford. He made this discovery on the edges and tips of sepals of *Elytranthis albidae. T*here was no mention of disease on the host and the species has not been investigated outside of the original collection. Descriptions (without images or illustrations) are available in Hansford, 1957.
eucalypti	Western Australia, 2016[31]	CBS H-22863, culture available	None	1. *Eucalyptus marginata*	This species was described from Western Australia on *Eucalyptus marginata* in 2016. It was considered, upon its discovery, to be a "minor" pathogen of *Eucalyptus*[32].
indica	India, 2010[33]	Herb. GUBH No. P166 Holotype only--no culture	None	1. *Garcinia indica*	This species was described in Western Ghats, India in 2010 on kokum. It was discovered on leaf lesions of kokum and is therefor considered to be pathogenic.
maxima	Cuba, 1869[11]	Herbarium of Brown University Dried specimen only--no culture https://www.gbif.org/occurrence/2013961216	1. *Capnodium maximum* 2. *Sorica dusenii* 3. *Sorica maxima* 4. *Capnodiella maxima* 5. *Corynelia pteridicola*	1. *Polypodium* spp.	The original collection of this fungal species was by Charles Wright in Cuba between 1856-1867. The original specimen can be found in the Herbarium of Brown University (CBRU00008244). The specimen was formally described as *Capnodium maximum* by M.J. Berkley in the Journal of the Linnean Society, Botany[34]. In 1904 a second collection of the species was made "by Dussen in Brazil" and described as *Sorica dusenii,* the type species of a new proposed genus, formally described by K.F. Giesenhagen. Giesenhagen placed this collection in the *Sphaeriaceae* but could not determine a genus that the specimen fit into. He concluded "there is nothing left but to regard the fungus as a representative of a new genus, for which I propose the name *Sorica"*[35]. After this new genus and species description was published, a colleague of Geisenhagen brought his attention to the original collection named *Capnodium maximum.* After making observations that the original, type specimen and the Dussen collection were the same, Giesenhagen asserted that both were NOT *Capnodium* and comprise their own genus, *Sorica.* He acknolwedge that, by latin nomenclature rules, the type specimen and Dussen collection should be recombined under the name *Sorica maxima.* In Saccardo's 17th volume of *Sylloge fungorum* (between 1904-1906) Saccardo made observations of the Wright and Dussen specimens and agreed with Geisenhagen that they did not belong in the genus *Capnodium* . Saccardo went on to regroup both specimens under the name *Capnodiella maxima* and listed *S. dusenii* and *S. maxima* as synonyms. Then in 1917 another collection was made of the species from a different genus of Polypodiaceae (*Campyloneurum*) and called it *Corynelia pteridicola*[37]. In 1919, Hohnel determined that all of these specimens should be grouped as one in *Caliciopsis maxima*[38]. In Fitzpatrick's 1942 *Caliciopsis* genus revision, he made observations of all of these independent collections (plus some additional ones) and outlined their differences (likely related to specimen age, both when collected and at the time of his own observation), and determined that they were all the same species, and reassigned them to *Caliciopsis maxima.*

Table S.2.1. (cont'd)

moriondi	Italy, 2020[39]	CBS146717, culture available	None	1. *P. radiata* 2. *P. nigra* 3. *P. resinosa*	Described in 2020 from Italian *P. radiata,* Koch's postulates were completed to confirm pathogenicity[39].
myrticola	New Caledonia, 1969[40]		None	1. Uromy*rtus emarginata*	Discovered by B. Hugeunin in 1966[40] at the Faux Bon Secours, New Caledonia. Hugeunin considered this species pathogenic, and observed it parasitizing the leaves of its host. Hugeunin gives detailed descriptions and drawings.
nigra	Switzerland, 1805[11]		1. *Stilbum nigrum* 2. *Ceratostoma juniperinum* 3. *Ceratostoma stromaticum* 4. *Corynelia juniperina* 5. *Lagenula nigra*	1. *Juniperus communis*	Fitzpatrick (1942) compared various collections of this species and determined them all to be the same organism. He gives a detailed nomenclature history as well as detailed descriptions in his 1942 Revisionary Studies in the Coryneliaceae. It was unclear from the various specimens he examined if the *Caliciopsis* spp. was the cause of the galls on which the specimens were recovered.
orientalis	Ontario, Canada, 1963[41]	CBS 138.64, culture available	None	1. *Tsuga canadensis*	*C. orientalis* was described by A. Funk in 1963 on *Tsuga canadensis* (though pathogenicity was not experimentally demonstrated Funk did observe canker formation on the host tree).
pinea	New York, USA, 1880[42]	CBS 139.64, culture available	1. *Calicium stenocyboides* 2. *Cyphelium stenocyboides* 3. *Caliciopsis stenocyboides* 4. *Caliciopsis ventricosa*[14]	1. *Pinus strobus*[43] 2. *P. pinaster*[44, 45, 46] 3. *P. monticola*[41]	This species was first described from a specimen collected by C.H. Peck in New York state in 1880 from *Pinus strobus* . It is the first use of "*Caliciopsis"* as a genus name and *C. pinea* is regarded as the type species for the genus (other taxa were discovered before pinea and later re-grouped into this genus after its description). Various collections were made of this species after its original description that revised its name, but Fitzpatrick (1942) firmly places it as *C. pinea.* It has many suspected hosts but few confirmed through pathogenicity trials, however Fitzpatrick noted its definite pathogenicity to *Pinu strobus* and many oberservations have been made since on pathogenicity to *P. strobus*.
pleomorpha	Australia, 2018[47]	VPRI 17721 (Victorian Plant Pathology Herbarium) Holotype deposit unsure if culture exists	None	1. *Eucalyptus cladocalyx* 'Nana' 2. Several other *Eucalyptus* spp.	A recently described species causing disease on Australian eucalypts. Koch's postulates were completed on all hosts listed in the species description[47].
podocarpi	New Caledonia, 1969[40]		None	1. *Podocarpus minor* 2. *Podocarpus comptonii*	Discovered by B. Hugeunin in 1966 at the Plaine des Lacs, New Caledonia. Hugeunin considered this species pathogenic, claiming it was the cause of cankers observed on its hosts' leaves. Hugeunin offered detailed descriptions and drawings of his specimen and another, thought to be the same *Caliciopsis* species, discovered on *Podocarpus comptonii*[40].
pseudotsugae	British Columbia, Canada, 1942[11]	CBS 140.64, culture available	None	1. *Pseudotsuga taxifolia*	Collected by J.R. Hansbrough in 1930 but formally described by Fitzpatrick in 1942. Early specimens were mis-identified as *C. pinea* by Fitzpatrick and there were subsequent references to this species as *C. pinea* until it was described as a separate species in 1942. It is considered parasitic.
quercina	Mexico		None	1. *Quercus spp.*	A recently described species recovered from galls of *Quercus canbyi*[50]. The author of the original description was unsure if the *Caliciopsis* was the cause of the galls it was recovered from and no further testing has been done.
rapaneae	New Caledonia, 1969[40]		None	1. *Myrsine lanceolata*	Discovered by B. Hugeunin in 1966 at Mont Mou, New Caledonia[40]. Hugeunin considered this species pathogenic, claiming it was the cause of cankers observed on its hosts' twigs. Hugeunin offered detailed descriptions and drawings of his specimen.
rhoina	Hunan Province, China, 2000[20]	Herbarium of Hunana Forest Botanical Garden (990296) Unclear if culture is available	None	1. *Rhus chinensis*	A recently described species that is described in more detail by the original descriptor[20]. It is unclear if a culture was deposited in an herbaria or just original specimen. It was assumed to be parasitic by species descriptor.
sambaibae	Brazil, 2020[48]		None	1. *Davilla elliptica*	A recently described species causing disease on Brazillian *Davilla elliptica*[48]. Detailed descriptions can be found in the original description.
struthanthi	Belize, 1974[49]	Deposited in an herbarium that is no longer active	None	1. *Struthanthus belizensis*	This species was originally escribed as a pathogen of a mistletoe in Belize[49]. Detailed descriptions are given by Funk but no pictures[49]. The holotype was deposited in an herbarium which is no longer active and it is unclear if the original specimen has been lost.

Table S.2.1. (cont'd)

subcorticalis	New Jersey, 1885[14, 16]		1. *Hypsotheca subcorticalis* 2. *Sphaeronema subcorticale*	1. Unknown *Quercus* sp.	Detailed descriptions of the nomenclature history for this organism are given by Garrido-Benevant; they assert the North American specimen of *Hypsotheca subcorticalis* made by Ellis and Everhart should truly be name *Caliciopsis subcorticalis* . The original specimen was found on dead oak branches that had fallen from a tree in New Jersey in 1877 and was described as *Sphaeronema subcorticale* . Ellis and Everhart reexamined the collection and regrouped it into *Hypsotheca.* Described as a saprophyte.
symploci	Sri Lanka[11]		None	1. Unsecified dichotyledenous tree	Detailed descriptions are given by Fitzpatrick (1942).
thujina	New Jersey[11]		1. *Hypsotheca thujina*	1. *Chamaecyparis thyoides*	Detailed descriptions are given by Fitzpatrick (1942). This species was revised from Everhart and Ellis & Ev. Jour
tiliae	France		1. *Caliciopsis ellissii*	1. *Tilia sylvestris*	Described from the margin of a canker, and found by Arnaud (1912), but revised by Fitzpatrick (1942).
toonae	Hunan Province, China, 2000[20]	Herbarium of Hunana Forest Botanical Garden (991084) Unclear if culture is available	None	*Toona sinensis*	A newly described species with details given by the author in the original description; It is unclear from the description if a culture was also deposited in herbaria or just the original specimen.
valentina	Valencia Province, Spain, 2015[14]	MA- 18176	None	*Quercus ilex* subsp. *rotundifolia*	A newly described species that is detailed in its original description[14]. The type was deposited in Real Jardín Botánico (MA18176), both a culture and specimen.
veillonii	New Caledonia, 1969[40]		None	Unknown	A species detailed by Hugeunin (1969). There have been no references to this species beyond its original description an dit is unclear if a type specimen was ever depositied into any herbarium.
xanthostemonis	New Caledonia, 1969[40]		None	1. Unidentified *Myrtaceae*	A species detailed by Hugeunin (1969). There have been no references to this species beyond its original description an dit is unclear if a type specimen was ever depositied into any herbarium.

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Table S.2.2. A table detailing the report vs. confirmed host of conifer-infecting *Caliciopsis* spp.

Caliciopsis spp.	**Hosts Confirmed with Experimental Evidence**	**Hosts the pathogen species has been found/reported on**	**Notes**
pinea	1. *Pinus strobus*[2] 2. *P. pinaster*[3, 4, 5] 3. *P. monticola*[6]	1. *P. insignis*[3] 2. *P. halepensis*[5] 3. *P. echinata*[7] 4. *P. pungens*[7] 5. *P. mugo* var. *pumilio*[8] 6. *Abies spp.*[8] 7. *P. rigida*[9] 8. *P. virginiana*[7, 9] 9. *Pseudotsuga menziesii*[10] 10. *Tsuga canadensis*[9]	Artificial inoculations and Koch's postulates were completed for this species on *Pinus strobus, P. pinaster,* and *P. monticola*[2, 3, 4, 5, 6]. The *C. pinea* used for artifical inoculations onto *P. pinaster* was, in some cases, sourced from *P. halepensis*[5], indicating that this tree is also a susceptible host though it was not experimnetally demonstrated. Spaulding reported *C. pinea* on *Abies* in 1961 but Funk did inoculation trials in 1963 with *C. pinea* on grand fir (*Abies)* and observed no sign of infection. It is very likely that the *Caliciopsis* genus is far more rich in species than researchers were aware of at the time of its description, which led to many mis-diagnoses of *C. pinea* on many hosts[7].
moriondi	1. *P. pinea*[11] 2. *P. pinaster*[11] 3. *P. halepensis*[11]	1. *P. radiata*[11] 2. *P. nigra*[11] 3. *P. resinosa*[11]	Described in 2020 from Italian *P. radiata.* Koch's postulates were completed but only on hosts *P. pinea, P. pinaster,* and *P. halepensis*. Pathogenicity assays were not performed on *P. radiata, P. nigra,* and *P. resinosa*, though the fungal isolates used in formal pathogenicity assays came from those hosts. This species was therefor experimentally proven to be a pathogen on 3 of its 6 reported hosts, though it is likely a pathogen of all 6[11].
orientalis	None	1. *Tsuga canadensis*	*C. orientalis* was described from *Tsuga canadensis* in 1960 but no formal pathogenicity trials were ever conducted for this host. Pathogenicity was assessed on western white pine[6] and it was determined that *C. orientalis* could not infect this host. Outside of observation of disease on eastern hemlock, pathogenicty through Koch's postulates has never been demonstrated on this host or any others.
pseudotsuga	1. *Pseudotsuga menziesii*[6] 2. *Tsuga heterophylla*[6] 3. *Abies grandis*[6]		Artificial inoculations demonstrated pathogenicity of this species on the 3 listed hosts[6].

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Chapter 3: A quantitative PCR-based assay for the species-specific detection of *Caliciopsis pinea*, causal agent of Caliciopsis Canker Disease of *Pinus strobus*

3.1 Abstract

Caliciopsis pinea is the ascomycete plant pathogen that causes Caliciopsis Canker Disease on North American *Pinus strobus* (eastern white pine). Infections result in downgrading of lumber due to canker formation and overall loss of vigor in *P. strobus*, which is a critical cover species throughout its native range. *C. pinea* is challenging to distinguish from closely related species (some of which can coinfect *P. strobus*) based on macroscopic and microscopic observations alone. *C. pinea* and other *Caliciopsis* species that infect conifers all have sign development that is indistinguishable to the naked eye and often indistinguishable using microscopy and exhaustive measurement. Additionally, symptoms may develop on a host without fruitification, making molecular techniques necessary for detection and diagnosis. Improved sensitivity and specificity of diagnostic and detection tools are critical for the early identification of *C. pinea* infections. Therefore, this study aimed to develop a quantitative polymerase chain reaction (qPCR) assay for the detection, diagnosis, and quantification of *C. pinea* from infected plant material. This assay targets the internal transcribed spacer (ITS) region of *C. pinea*, and specificity was confirmed on a panel of 51 target and nontarget isolates from throughout the *P. strobus* native range. The limit of detection for the assay was determined to be 10 fg of *C. pinea* DNA. The technology was tested in additional facilities and other thermocycling platforms to ensure reproducibility of results. This study reports the most sensitive and specific assay for *C. pinea* detection from our current understanding of species diversity within the genus.

3.2 Introduction

Pinus strobus (eastern white pine) has a rich history in North America and is one of the most culturally significant tree species alive today. Before European colonization, *P. strobus* was important to indigenous people throughout the tree's native range including using the pitch as a sealant for canoes, various tree parts as treatments for illness and injury, and seeds as a source of food during times of scarcity (Arnason et al 1981). To many indigenous people the white pine is a symbol of peace (Schroeder 1992). Prior to the arrival of Europeans, forested stands in North America supported approximately 3.4 billion m^3 of *P. strobus* lumber (Wendel and Smith 1990). However, most of the abundant white pine forest cover was cut for a variety of uses by 1850 (Wendel and Smith 1990). To reforest the heavily cut stands, white pine seedlings were imported to the eastern US from Europe by the millions in the early 1900s (Van Arsdel 2011). These seedlings carried with them *Cronartium ribicola* (the causal agent of white pine blister rust), which further devastated North American populations of white pine (Van Arsdel 2011).

Logging, disease, and destructive fires reduced available seed source and have since inhibited white pine regeneration efforts in the US (Ostry et al 2010). Today, the remaining *P. strobus* is a valuable source of lumber and is an important component of forest stands throughout its native range, providing food and wildlife habitat to local ecosystems (Wendel and Smith 1990; Woudenberg et al 2010). *P. strobus* is a rapidly growing conifer that can thrive in a wide range of elevations and nutrient availabilities. These characteristics have led to the species being the most widely planted tree in eastern North America (Wendel and Smith 1990; Whitney et al 2019). However, the last 20 years have seen reports of a complex dieback of white pine, attributed to many coinciding biotic and abiotic factors, which has provoked new concerns for the future of *P. strobus* (Costanza et al. 2018). White pine dieback has been attributed, in part, to several fungal pathogens.

Caliciopsis pinea (Ascomycota) is the causal agent of caliciopsis canker disease on *P. strobus*. *Caliciopsis* infections are characterized by flagging of lower branches coupled with pitching and profuse resin bleeding from sunken cankers that form on the trunk and branches of the tree (Munck et al. 2015, 2016; Costanza et al. 2018; Migliorini et al. 2020; This study, Fig 1). *C. pinea* has also been demonstrated through *in planta* trials as a pathogen to *P. pinaster* (maritime pine) and *P. monticola* (western white pine) (Funk 1963, Delatour 1967, Delatour 1969, and Capretti 1978). Both *C. pinea* and caliciopsis canker disease have been reported on *P. strobus* in the United States, Canada, and Europe (Munck et al. 2015, 2016; Luchi et al. 2018). Observations of frequent and severe *Caliciopsis*-associated disease on *P. strobus* have increased in the last twenty years (Munck et al. 2015, 2016; Whitney et al. 2017; Schulz et al. 2018; Asaro et al. 2018). These observations have been attributed to environmental conditions that result in drought injury (poor soil quality, overstocking, changing climates, etc.) (Munck et al. 2015, 2016; Costanza et al. 2018, 2019). Since 2015 there have been reports of *C. pinea* behaving as a more aggressive pathogen than previously documented; mortality of seedlings was even observed in severely infected sites (Munck et al. 2015; this study, Fig3.2).

Figure 3.1. Typical symptom development of a *Caliciopsis pinea* infection on *Pinus strobus.* **A)** Sunken, red cankers and the cambium, **B)** "flagging", or when the needles on individual branches brown and the whole branch dies, and **C)** pitching and profuse resin bleeding from cankers that form on the trunk and branches of the tree.

Figure 3.2. Observations of mortality in drought-stressed *Pinus strobus* seedlings in Michigan. **A)** A dead standing seedling with **B)** a canker that girdled its stem with *Caliciopsis pinea* mazaedia emerging at the margin.

Existing methods for diagnosis and detection of *C. pinea* are limited. *Caliciopsis* species produce macroscopic ascocarps (mazaedia) that can be visually identified (Voigt and Williams 2021). However, ascocarps of *C. pinea* are between one and five millimeters long and are easily overlooked, especially on trees with profuse lichen growth on their bark. Symptoms may develop on a host without the production of sexual structures, and *C. pinea* is not known to reproduce asexually in nature (Schulz et al. 2018; Funk 1963). A quantitative polymerase chain reaction (qPCR) assay was previously developed for species-specific detection of *C. pinea,* however new species of *Caliciopsis* have since been described that are indistinguishable using this assay (Luchi et al. 2018; Migliorini et al. 2020). *Caliciopsis* species are slow-growing and consequently difficult to isolate in culture from diseased plant tissue (Migliorini et al. 2020; Schulz et al. 2018). Species-specific diagnosis is further inhibited by the presence of eight other *Caliciopsis* species endemic to North America, commonly found in forests with *C. pinea* (see

Chapter 2; this study, Table 3.2). Despite some reports of host specificity within the genus, the true host range of most *Caliciopsis* species is still unknown. Furthermore, there is little existing research about the biology of *C. pinea* and its disease progression once an infection is initiated. A sensitive and specific detection and diagnostic assay is necessary for continued study of *C. pinea* and the *Caliciopsis* genus.

Here we describe a specific and highly sensitive qPCR-based assay for the diagnosis, detection, and quantification of *C. pinea* which can be applied to infected plant tissue. We also report primers and probes for detection of other members of the *Caliciopsis* genus which have been designed *in silco*. We assessed the limit of detection for the *C. pinea* assay, tested it against a range of other *Caliciopsis* species, closely related genera, and other common white pine pathogens, and finally we demonstrated utility of the assay by detecting and quantifying *C. pinea* DNA in infected plant samples. This research will facilitate future lifecycle studies within the genus *Caliciopsis,* inform management strategies for *C. pinea* on *P. strobus*, and serve as a resource for future diagnostic assay development for other forest pathogens.

3.3 Materials and Methods

Identification of Isolates

Caliciopsis mazaedia from North America and Europe were collected from infected plant tissue by the author or cultures/tissue samples were sent to the Michigan State University Forest Pathology lab by cooperating scientists. Single spore isolations were made from ascocarps, and all cultures were maintained on full strength potato dextrose agar (PDA) at room temperature (about 20°C) then plated onto full strength PDA overlaid with sterile cellophane for DNA extraction (39 g of PDA in 1 liter of distilled water; Difco PDA, Sparks, MD). Sequencing of the nuclear internal transcribed spacer region (ITS) was used to confirm species identities according to methods described in Chapter 2 of this dissertation.

DNA Extraction and Sequencing

Genomic DNA was collected from single-spore isolates of *Caliciopsis* spp. 100mg of mycelium was scraped from 2-week-old cultures grown on PDA with cellophane overlay and transferred to 2mL reinforced bead beating tubes containing Lysing Matrix A (MP Biomedicals, Sana Ana, CA, USA). 400µL of the InhibitEX Buffer from the Qiagen QIAmp® Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) was added to each sample. Samples were then homogenized on the MP Biomedicals Fast-Prep®-24 for 1 minute at 6 m/s. Another 600µL of

InhibitEX Buffer was added to each tube, after which the remainder of the DNA extraction was performed according to manufacturer's instructions. DNA was further cleaned using a Monarch® PCR and DNA Cleanup Kit (New England Biolabs, Ipswitch, MA, USA) due to considerable production of pigmentation by most isolate cultures. Final DNA concentrations were quantified using an Invitrogen ™ Qubit™ 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). To identify suitable candidate loci for a species-specific detection assay, sequences generated during a previously conducted phylogenetic study were aligned in Geneious Pro (version 2024.0.2) (Dotmatics, Boston, MA, USA) and assembled into individual gene trees (see Chapter 2). Loci which could effectively distinguish between species were targeted for *in silico* assay design.

Development of C. pinea-specific qPCR assay

Multigene phylogenetic analyses conducted previously identified the ITS region of *Caliciopsis* as the most phylogenetically informative, multi-copy region at the species-level (see Chapter 2). Multi-copy loci were preferred to increase reaction sensitivity. The ITS region was thus targeted for primer and probe development. Primers Cali4FP and Cali7RP and the dual-labeled probe Cali2Pr were designed *in silico* using Geneious Pro (Table 3.1), which produce a 157-bp amplicon. Additionally, a previously developed exogenous internal control (EIC) was included in the reaction to distinguish between truly negative samples and samples which did not amplifying due to inhibition (Table 3.1) (Haudenshield and Hartman 2011). Primers for the internal control were elongated from their original sequences to increase their melting temperature such that the reaction could operate at a higher annealing temperature to increase the specificity.

Table 3.1. Primers and probes utilized in this study.

Primer	Primer Sequence (5’-3’)	Tm (°C)	Reference
Cali4FP	CGTGGGCGGTCGTCG	60.2	This study
Cali7RP	ACTTTCAACAACGGATCTCTTGGTTC	61.9	This study
Cali2Pr	[FAM] CGCCGAAGACCCCTCTAAACTCTGACTGT [BHQ1]	69.1	This study
HHIC-F	CTAGGACGAGAACTCCCACAT	58.6	Haudenshield and Hartman, 2011
HHIC-R	CAATCAGCGGGTGTTTCA	55.6	Haudenshield and Hartman, 2011
HHIC-Pr	[CalRed610] TCGGTGTTGATGTTTGCCATGGT [BHQ2]	63.4	Haudenshield and Hartman, 2011
HHIC-DNA	CACGCCTAGGACGAGAACTCCCACATCGAGCTTGACGCAAACGACCACGCCAGGACCATGGCAAACATCAACACCGAGCGCAACGCCTTGTGCTGAAACACCCGCTGATTG	73.7	Haudenshield and Hartman, 2011

These primers, probes, and exogenous DNA were labeled HHIC-F and HHIC-R, HHIC-Pr, and HHIC-DNA, respectively (Table 3.1). Primers and probes were supplied by Biosearch Technologies (LGC, Hoddesdon, United Kingdom). The reaction mix and cycler protocol were optimized initially for the *C. pinea* target individually, then the EIC was added. The reaction consisted of 8uL of Perfecta qPCR Toughmix (Quanta Biosciences, Gaithersburg, MD, USA), 400nM of Cali4FP and Cali7RP primers, 200nM of HHIC-F primer, 160nM of HHIC-R primer, 200nM of Cali2Pr probe, 160nM of HHIC-Pr probe, 5fg HHIC-DNA as the target for the internal control, 2mM of $MgCl_2$, and water all to a final reaction volume of 25uL. The cycling conditions were set at 95°C for a three-minute initial denaturation, followed by 49 cycles of 95°C for 15 seconds and annealing at 66°C for 30 seconds with a plate reading taken after each cycle.

A standard curve was then generating using DNA from the type isolate of *C. pinea* (CBS 139.64) purchased from the Westerdijk Fungal Biodiversity Institute (Utrecht, Netherlands). The culture was grown on full strength PDA overlayed with sterile cellophane, then DNA was extracted as described above. Eight 10-fold dilutions from 10ng to 0.1fg of the extracted DNA were used for standard curve generation and replicated in triplicate on the optical plate. The reaction was tested both with and without the internal control to determine if the EIC would impact reaction efficiency. Reactions were performed using a BioRad CFX96 Real-Time System and outputs from the qPCR instrument were visualized using the BioRad CFX Maestro Software (v2.3).

Primer Specificity

To verify that primers and probes were specific only for *C. pinea*, 51 isolates were screened including 13 isolates of *C. pinea* from 5 states and the type isolate of *C. pinea* from Italy described above (Table 3.2). Isolates used for this study also included 8 species of *Caliciopsis* sourced from multiple hosts and geographic locations, and one isolate of an unrelated common white pine pathogen, *Cronartium ribicola* (Table 3.2). All were grown in culture and DNA-extracted as described above, with the exception of *C. ribicola* due to its obligate-parasite nature. For this species DNA was sourced from a *C. ribicola* infected *Ribes* sample that was brought to the Michigan State University Plant Diagnostic Clinic (MSU-PDC). *C. ribicola* DNA extraction was performed using the Qiagen DNeasy Plant Mini Kit with modifications to the manufacturer protocol; volumes of buffer AP1 and RNase A were adjusted to 600 µL and 6 µL, respectively, plant tissue was homogenized on a Fisherbrand Bead Mill 24 for 40 seconds at 6.0 m/s and heated for 15 minutes at 65°C, and for the precipitation step 195 µL of buffer P3 was added and samples were placed on ice for 5 minutes. DNA was further purified using a QIAcube instrument and eluted into 100 µL of elution buffer. Sequencing of the 28S rRNA gene confirmed the presence of *C. ribicola* in the sample prior to its use in this study.

Table 3.2. Panel of all target and non-target isolates screened using the *Caliciopsis pinea* qPCR detection assay.

CCC#	Location	Coordinates	Substrate	Date Received/ Collected	Species	Average Ct
139.64	Canada	Unknown	*P. strobus*	7/29/60	*Caliciopsis pinea*	15.20
3	MI	44.662797, -84.767528	*P. strobus*	11/16/21	*C. pinea*	12.97
4	MI	44.197713, -86.188963	*P. strobus*	11/17/21	*C. pinea*	12.97
5	MI	46.256035, -85.473712	*P. strobus*	8/21/19	*C. pinea*	12.81
6	MI	46.256035, -85.473712	*P. strobus*	8/21/19	*C. pinea*	12.96
7	MI	44.197713, -86.188963	*P. strobus*	11/17/21	*C. pinea*	14.45
8	MI	44.197713, -86.188963	*P. strobus*	11/17/21	*C. pinea*	15.65
48	NH	Unknown	*P. strobus*	Unknown	*C. pinea*	15.33
49	NH	Unknown	*P. strobus*	Unknown	*C. pinea*	18.12
50	ME	Unknown	*P. strobus*	Unknown	*C. pinea*	20.91
53	NC	Unknown	*P. strobus*	Unknown	*C. pinea*	21.81
54	GA	34.70265, -83.88583	*P. strobus*	2/8/18	*C. pinea*	18.78
55	GA	34.70265, -83.88583	*P. strobus*	2/8/18	*C. pinea*	17.35
11	MI	44.662, -84.775	*P. strobus*	10/27/20	*C. pseudoorientalis*	0.00
12	MI	46.37709, -84.70553	*P. strobus*	10/27/20	*C. pseudoorientalis*	0.00
13	MI	46.37709, -84.70553	*P. strobus*	11/17/21	*C. pseudoorientalis*	0.00
15	MI	44.662797, -84.767528	*P. strobus*	11/16/21	*C. pseudoorientalis*	0.00
16	MI	44.662797, -84.767528	*P. strobus*	11/16/21	*C. pseudoorientalis*	0.00
17	MI	44.662797, -84.767528	*P. strobus*	11/16/21	*C. pseudoorientalis*	0.00
18	MI	44.662797, -84.767528	*P. banksiana*	11/16/21	*C. moriondi*	0.00
19	MI	44.662797, -84.767528	*P. banksiana*	11/16/21	*C. moriondi*	0.00
20	MI	46.37709, -84.70553	*P. banksiana*	11/16/21	*C. moriondi*	0.00
22	MI	46.37709, -84.70553	*P. banksiana*	11/16/21	*C. moriondi*	0.00
23	MI	44.662797, -84.767528	*P. resinosa*	11/16/21	*C. moriondi*	0.00
24	MI	44.662797, -84.767528	*P. resinosa*	11/16/21	*C. moriondi*	0.00
25	MI	46.37709, -84.70553	*P. resinosa*	10/27/20	*C. moriondi*	0.00
26	MI	46.37709, -84.70553	*P. resinosa*	10/27/20	*C. moriondi*	0.00
27	MI	44.662797, -84.767528	*A. rubrum*	11/16/21	*C. aceriae*	0.00
28	MI	44.662797, -84.767528	*A. rubrum*	11/16/21	*C. aceriae*	0.00
29	MI	44.662797, -84.767528	*A. rubrum*	11/16/21	*C. aceriae*	0.00
30	MI	44.197713, -86.188963	*A. rubrum*	11/17/21	*C. aceriae*	0.00
31	MI	44.197713, -86.188963	*A. rubrum*	11/17/21	*C. aceriae*	0.00
32	MI	44.197713, -86.188963	*A. rubrum*	11/17/21	*C. aceriae*	0.00
33	MI	44.197713, -86.188963	*A. rubrum*	11/17/21	*C. aceriae*	0.00
34	MI	44.197713, -86.188963	*A. rubrum*	11/17/21	*C. aceriae*	0.00
35	MI	44.197713, -86.188963	*A. rubrum*	11/17/21	*C. aceriae*	0.00
41	MI	44.662797, -84.767528	*Q. velutina*	9/7/23	*C. cilium*	0.00
42	MI	44.662797, -84.767528	*Q. velutina*	9/7/23	*C. cilium*	0.00
43	MI	44.662797, -84.767528	*Q. velutina*	9/7/23	*C. cilium*	0.00
44	MI	44.662797, -84.767528	*Q. velutina*	9/7/23	*C. cilium*	0.00
45	MI	44.662797, -84.767528	*Q. velutina*	9/7/23	*C. cilium*	0.00
46	NH	Unknown	*P. resinosa*	Unknown	*C. moriondi*	0.00
47	NH	Unknown	*P. resinosa*	Unknown	*C. moriondi*	0.00
57	WI	45.882115, -89.435017	*P. banksiana*	3/13/24	*C. moriondi*	0.00
58	WI	45.882129, -89.435289	*P. banksiana*	3/13/24	*C. moriondi*	0.00
59	WI	45.882494, -89.434466	*P. banksiana*	3/13/24	*C. moriondi*	0.00
146717	Italy	Unknown	*P. radiata*	5/27/20	*C. moriondi*	0.00
140.64	Canada	Unknown	*Pseudotsuga menziesii*	4/23/59	*C. pseudotsuga*	0.00
138.64	Canada	Unknown	*Tsuga canadensis*	11/26/60	*C. orientalis*	0.00
142066	Australia	Unknown	*Eucalyptus marginata*	11/11/16	*C. eucalypti*	0.00
144636	Australia	Unknown	*E. piperita*	7/6/18	*Hypsotheca pleomorpha*	0.00
NA	MI	Unknown	*Ribes* spp.	Unknown	*Cronartium ribicola*	0.00

Technology Transfer

To ensure reproducibility of the assay, the MSU-PDC blind-tested the reaction. Reagents and template DNA were provided to the clinic to recreate the standard curve and test the reaction on unknown samples. The MSU-PDC utilizes the same qPCR platform as MSU Forest Pathology

(BioRad CFX96 Real-Time System), so the assay was further validated by the author on a BioRad CFX Opus system in the MSU Small Fruit and Hop Pathology lab where results were visualized using the CFX Maestro Software as described above.

Detection from Infected Plant Material

To demonstrate reaction feasibility, the assay developed in this study was tested on samples taken from five separate trees in each of the following categories: symptomatic white pine that had *C. pinea* mazaedia present, symptomatic white pine without mazaedia present, and asymptomatic white pine. Samples were taken from the cambium at the edge of sunken cankers characteristic of infection on symptomatic hosts, and from the cambium at breast-height (about 1.35 m) of asymptomatic trees. About 100 mg of plant tissue was scraped from the bark and phloem of the tree and DNA was extracted using the same protocols described above for fungal mycelium extractions.

3.4 Results

Development of C. pinea-specific qPCR assay

We identified conserved single nucleotide polymorphisms (SNPs) in the ITS region of *C. pinea* not present in any other closely related species that were the target of the fluorescent probe developed over the duration of this study (Fig3.3, Table 3.1). For each isolate included in this study the ITS region was sequenced previously and the resulting alignment was used to ensure specificity of the probe for *C. pinea, in silco* (Harkness et al. 2025 submitted). To ensure reproducibility, the assay was performed by the MSU-PDC with DNA from cultures of various *Caliciopsis* species as well as infected plant material (Table 3.3). Both the MSU-PDC and the Opus qPCR system achieved the same results for both sensitivity and specificity that were achieved in this study (Table 3.3).

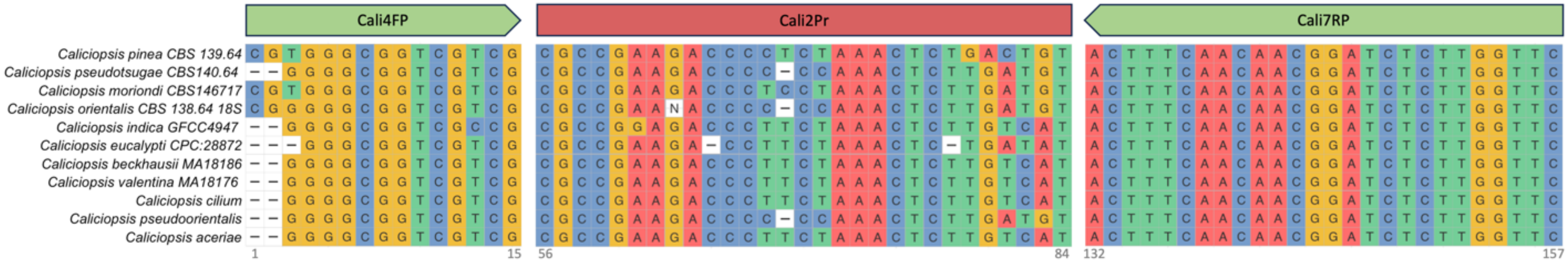

Figure 3.3. A limited-isolate sequence alignment of the region targeted for qPCR assay development showing SNPs in the *Caliciopsis pinea* forward primer and probe sequences that enable species-specificity of the reaction.

Table 3.3. Technology transfer and cross-platform validation of *C. pinea* qPCR detection assay. "STwM" indicates infected plant samples taken from symptomatic trees with mazaedia, "STwoM" indicates infected plant samples taken from symptomatic trees without mazaedia, and "Asymp" indicates samples taken from asymptomatic trees. NA's indicate samples not tested by the given laboratory.

	Laboratory		
Sample	**Forest Pathology**	**Plant Diagnostic Clinic**	**Small Fruit and Hop Pathology**
Caliciopsis pinea CBS 139.64	13.74	12.40	13.79
C. pinea - NH	18.12	11.94	16.34
C. pinea - ME	20.91	14.10	15.35
C. pinea - GA	17.35	NA	NA
C. pinea- MI3	12.97	25.10	12.56
C. pinea- MI4	12.97	NA	NA
C. pinea- MI5	12.81	NA	NA
STwM	25.87	25.10	27.14
STwM	24.89	23.29	26.26
STwM	26.83	NA	NA
STwM	30.57	NA	NA
STwoM	35.70	31.09	34.47
STwoM	36.7	36.59	38.3
STwoM	0	NA	NA
STwoM	0	NA	NA
Asymp	0	0	0
Asymp	0	0	0
Asymp	0	NA	NA
Asymp	0	NA	NA
Caliciopsis moriondi CBS 146717	0	0	0
Caliciopsis pseudotsuga CBS 140.64	0	0	0
Caliciopsis orientalis CBS 138.64	0	0	0
Cronartium ribicola	0	0	0

Sensitivity and Specificity

Initially, the reaction was optimized using 10-fold dilutions from 10 ng to 1 fg of *C. pinea* DNA sourced from the type-culture, CBS139.64. We set the limit of detection (LOD) at the minimum amount of template material that amplified in all technical replicates which was determined in this study to be 10 fg of *C. pinea* DNA (Fig3.4). The LOD did not vary in the presence of the EIC (Fig 4B). Reaction efficiency was also determined using the same 10-fold serial dilutions which was 90%, both with and without the internal control, with an R^2 of 0.9997 and slope of -3.6 after linear regression was performed (Fig3.4). The cycle cutoff was set at 35 using the 10 fg LOD average Ct (Fig3.4). There was no reduction in reaction sensitivity, specificity, or efficiency after the inclusion of the internal control (Fig3.4, Tables 3.2 and 3.3).

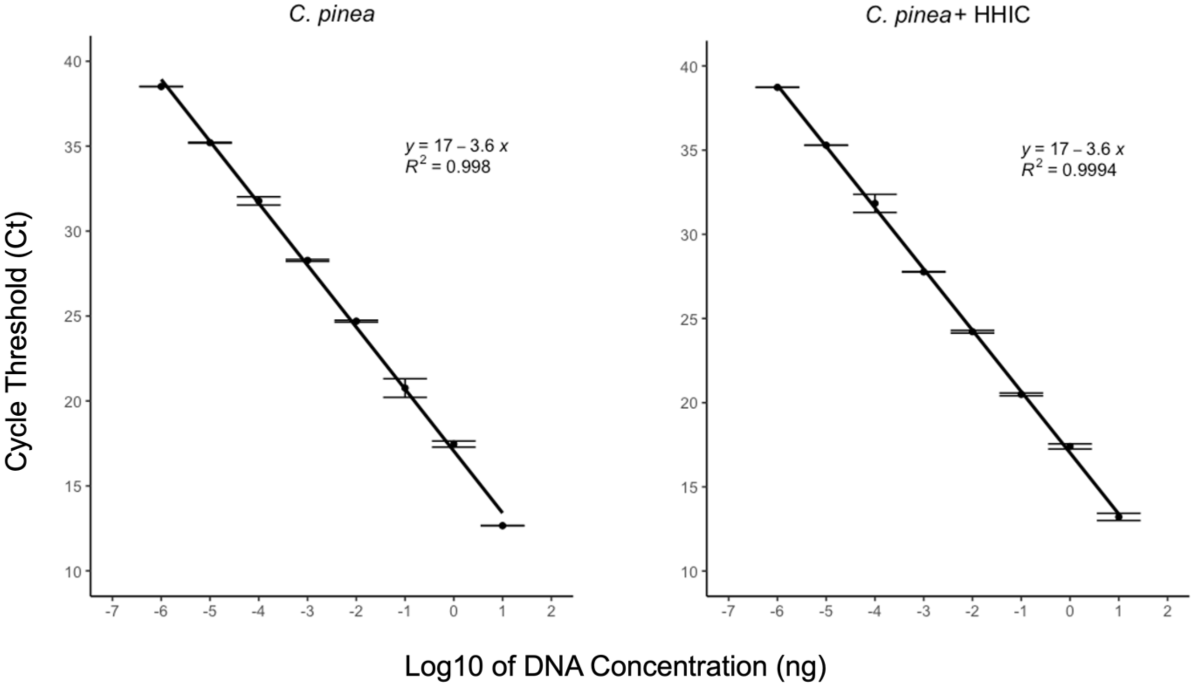

Figure 3.4. Standard curves generated **(A)** without and **(B)** with the exogenous internal control. Linear regression equations and R^2 values are included for each in the top right corner of their respective plots.

The target reaction was screened against a limited panel of closely related *Caliciopsis* species to determine an annealing temperature that resulted in species-specificity of the reaction for *C. pinea*. Once the appropriate annealing temperature to achieve species-specific detection was determined, a previously published EIC (Haudenshield and Hartman 2011) was incorporated into the reaction to distinguish between truly negative samples and samples that were inhibited. The originally developed primers for this reaction had insufficient relative fluorescence at the target annealing temperature and were modified by adding three nucleotide bases for both the forward and reverse primer such that the EIC could still amplify at the higher annealing temperature required by the primary target (Table 3.1). After the EIC was incorporated into the reaction, a complete panel of 51 isolates were screened using the assay, of which only the 13 isolates identified through sequencing as *C. pinea* amplified (Table 3.2).

Transferability testing

Technology transferability was assessed by using a subset of target and non-target samples for testing in other laboratories (MSU-PDC and Small Fruit and Hop Pathology) as well as on a qPCR platform different from that used by MSU Forest Pathology and PDC (the BioRad CFX Opus system in the Small Fruit and Hop Pathology Laboratory). No false identifications of *C. pinea* occurred when the reaction was tested by participating labs (Table 3.3). There was agreement in the cycle of amplification at all participating laboratories for all *C. pinea* culture DNA and infected plant material tested (Table 3.3). Furthermore, standards curves generated by each lab were nearly identical: both Forest Pathology and MSU-PDC had regression lines with equation y=17-3.6x, R^2 values of >0.99, and efficiency values of 90% (Fig3.5, Table 3.3). The Small Fruit and Hop Pathology Laboratory generated a regression line with equation y=17-3.4x, R^2 value of >0.99, and an efficiency value of 97% (Fig3.5, Table 3.3). The only instrument on which all replicates of the 0.1 fg concentration of the standard amplified was the Opus; the limit of detection was thus determined to be 10 fg, the next-lowest concentration tested in the standards (Fig3.5). At 10 fg of target DNA the cycle threshold was determined to be 35 cycles, or the average from all three labs (Fig3.5, Table 3.3).

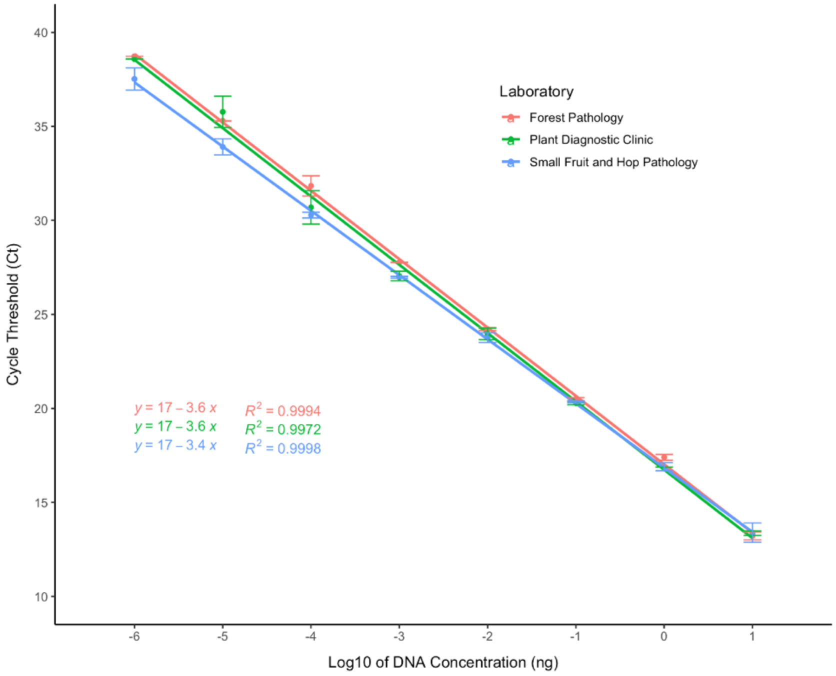

Figure 3.5. Cross laboratory comparison of standard curves generated in different labs and on different platforms. Regression equations and R^2 values for each standard are reported in the bottom right corner of the plot.

Detection of C. pinea DNA in plant tissue

As a demonstration of reaction feasibility, the assay developed in this study was tested on both infected and uninfected plant host tissue. At a single *P. strobus*-rich stand in Michigan's lower peninsula, bark scrapings from trees with the following disease presentations were collected: five symptomatic trees with mazaedia, five symptomatic trees without mazaedia, and five asymptomatic trees (n=15). *C. pinea* DNA was only detected using the detection assay described in this study from symptomatic hosts, although less target DNA was present in symptomatic samples taken from trees with no visible signs of disease (Table 3.3). A subset of these samples was tested by the MSU-PDC and Small Fruit and Hop Pathology, neither showed disagreement with number of detections made nor the cycle at which amplification occurred from infected plant material.

3.5 Discussion

Over the course of this study a species-specific qPCR assay was developed to address cross-reactivity in existing detection protocols for *C. pinea*. The assay was specific to *C. pinea* when screened against a panel of 51 isolates of related species and common *P. strobus* pathogens. The limit of detection for the assay was determined to be 10fg of *C. pinea* DNA and *C. pinea* was successfully detected against the background of plant DNA in symptomatic *P. strobus* samples. Reproducibility was ensured by additional independent lab validation of the assay, which resulted in consistent amplification of only *C. pinea* DNA against nontarget isolates and infected plant material.

ITS was the preferred target for this reaction given its multiple copies in the genomic DNA of *C. pinea.* Selection of a multicopy locus has been consistently demonstrated to improve the sensitivity of qPCR detection assays (Roggenkamp et al. 2024). Furthermore, the ITS region was the most populated with sequence data in the National Center for Biotechnology Information (NCBI) GenBank sequence repository making *in silico* assay design possible, ensuring consistency across wide geographic locations.. When ITS sequences were aligned, the DNA fragment targeted for probe design had identical sequence between geographically distant isolates indicating that the reaction will be specific across the distribution of *C. pinea* (Harkness et al. 2025 submitted). Additionally, the high annealing temperature of the reaction improved reaction specificity when tested against closely related species.

For qPCR-based detection assays the limit of detection has been previously defined asthe lowest amount of DNA detected by the reaction in ≥95% of replicates (Roggenkamp et al. 2024; Groth-Helms et al. 2022; Bustin et al. 2009). Here the limit of detection for the developed assay was determined to be 10 fg of genomic *C. pinea* DNA, which was the lowest tested concentration that amplified in all replicates, across all laboratories. PCR efficiency, depending on the qPCR platform used, ranged from 90-97%, above the recommended minimum value of 90% (Taylor et al. 2010).

C. pinea specimens from across North America and some from Europe were detected 100% of the time, across laboratories and geographic origin further demonstrating reaction reproducibility. Nearly identical standard curve generation and detection results were achieved by all three laboratories and both qPCR platforms (BioRad CFX96 Real-Time System and the BioRad CFX Opus system). Efficiency from all three test labs was >90% and R^2 were all >0.99. Furthermore, pathogen DNA was only detected in infected plant material and from *C. pinea* isolates . The reaction also successfully detected *C. pinea* in the background of plant DNA, which had no impact on amplification of the internal control. The reaction developed in this study was sensitive, specific, and reproducible between labs and personnel indicating high robustness for our current understanding of *Caliciopsis* phylogenetics.

Caliciopsis species are not well studied, and sequence availability for any genetic locus besides ITS is poor (Harkness et al. 2025; Migliorini et al. 2020; Garrido-Benavent and Pérez-Ortega 2015; Pratibha et al. 2010). *Caliciopsis* as a genus is likely more diverse than currently recognized and will require additional detection resources as our understanding of the phylogenetic relationships between species continue to be investigated. As sequencing data continues to grow, more refined DNA-targeted assays for detection of *C. pinea* and other members of the genus will continue to get more accurate, efficient, and accessible for diagnostic purposes and ecological investigations. In this study, a highly sensitive and species-specific *C. pinea* detection assay was developed for diagnostic and early detection purposes, but it has the potential to be used for continued investigation of the biology and epidemiology of *C. pinea* and CCD.

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Chapter 4: Quantification of *Caliciopsis pinea* aerial dispersal in diseased white pine (*Pinus strobus*) forests throughout the host native range

4.1 Abstract

The causal agent of caliciopsis canker disease of *Pinus strobus, Caliciopsis pinea,* is considered an emerging pest in forests throughout the native range of *P. strobus*. Due to the recency of observations of severe disease associated with *C. pinea,* there is little existing research on many aspects of its life cycle, pathogenicity, and mechanisms of spread. Notably, the environmental factors affecting spore release in natural ecosystems are largely unknown. To elucidate optimal environmental conditions for increased inoculum detection in the air and study the reproductive cycle of *C. pinea,* quantitative PCR was coupled with rotating-arm air sampling to investigate this critical gap in knowledge. Rotating-arm air sampling devices were constructed and deployed in nine field sites from June to November of 2021 and 2022. Samples were collected weekly, DNA was extracted, and spores were quantified using a novel, species-specific qPCR assay. The timing of peak spore collection was assessed over both years of the experiment and optimal environmental conditions for spore collection were determined by graphing spore quantity against different, measured environmental variables. This is the first study to investigate dissemination of *C. pinea* in North America and demonstrates the feasibility of a qPCR detection assay for spore quantification in forested settings.

4.2 Introduction

Pinus strobus, the eastern white pine, is an economically and ecologically important cover species in forests throughout eastern North America. *P. strobus* was once widespread in the eastern United States and Canada until the arrival of colonizers and by the early 1800's nearly all the *P. strobus* in the US was logged (Wendel and Smith 1990). To revitalize populations, *P. strobus* was shipped in mass back to the US from Great Britain resulting in the introduction of *Cronartium ribicola* (white pine blister rust) (Geils and Vogler 2010). Disease coupled with destructive fires led to the devastation of *P. strobus* populations in North America by the mid 20th century (Geils and Vogler 2010). Federal harvest and exportation laws were enacted that promoted sustainable forest harvesting and offered protection to the remaining *P. strobus* resource (Huffman 1978). Today, *P. strobus* is favored for reforestation projects due to its rapid growth rate and is the most widely planted tree in many parts of its native range (Whitney et al. 2019; Costanza et al. 2018).

However, efforts to restore *P. strobus* populations for sustainable harvesting are inhibited by the presence of a complex dieback throughout the native range of the tree (Costanza et al. 2018 and 2020; Whitney et al. 2019). Several biotic and abiotic stressors contribute to observed *P. strobus* dieback, but it is most frequently associated with the presence of *Caliciopsis pinea* (Costanza et al. 2018 and 2020; Whitney et al. 2019). *C. pinea* is the causal agent of caliciopsis canker disease (CCD) of *P. strobus*. Early publications report *C. pinea* as a minor pathogen of *P. strobus,* however observations of widespread and severe disease have been increasing for the last decade (Munck et al. 2023, 2016, and 2015; Costanza et al. 2020, 2019, and 2018; Migliorini et al. 2020; Whitney et al. 2019). The relatively recent increase in reports of large-scale and severe CCD in forests has been attributed to environmental factors that result in water-stress (Costanza et al. 2020; Munck et al. 2016). These factors include overstocking, poorly draining soils, prolonged high temperatures, reduced or absent precipitation, and regional droughts that are exacerbated by anthropogenic climate change (Costanza et al. 2020; Munck et al. 2016). Given the disease was originally classified as "minor" and the relative recency of the latest reports of increased incidence and severity of CCD, there is little existing research on most aspects of the *C. pinea* biology and lifecycle.

C. pinea is present throughout the native range of *P. strobus* and is assumed to be native to North America (Migliorini et al. 2020; Harrison 2009). It is thought to emerge in late winter to early spring, and reaches maturity by the end of spring, but this aspect of its lifestyle has only been documented once (Funk 1963). When mature, spores exude through the locule they are borne in and are presumed to passively disseminate by wind or rain splash (Fig4.1A-B) (Cram and Fraedrich 2022; Botella et al. 2020; Funk 1963). After being blown or washed away from its origin, a spore must land on an existing wound on a host for infection to occur as it cannot directly penetrate host cells (Cram and Fraedrich 2022). Once infection is initiated, *C. pinea* remains in the living tissue of the cambium, where it will cause characteristic symptoms of infection (Cram and Fraedrich 2022; Migliorini et al. 2020; Munck et al. 2016 and 2015). These include the formation of shallow, reddish-brown cankers on the trunk and branches of the host which eventually leads to bark cracking (Fig4.1C) (Cram and Fraedrich 2022; Costanza et al. 2020; Munck et al. 2016 and 2015). Once the bark has cracked open, resin will bleed down the trunk of the tree (Fig4.1C) (Cram and Fraedrich 2022; Costanza et al. 2020; Munck et al. 2016 and 2015). These characteristic symptoms are usually accompanied by flagging, or when the needles on individual branches brown, and the whole branch eventually dies (Fig4.1D) (Cram and Fraedrich 2022; Costanza et al. 2020).

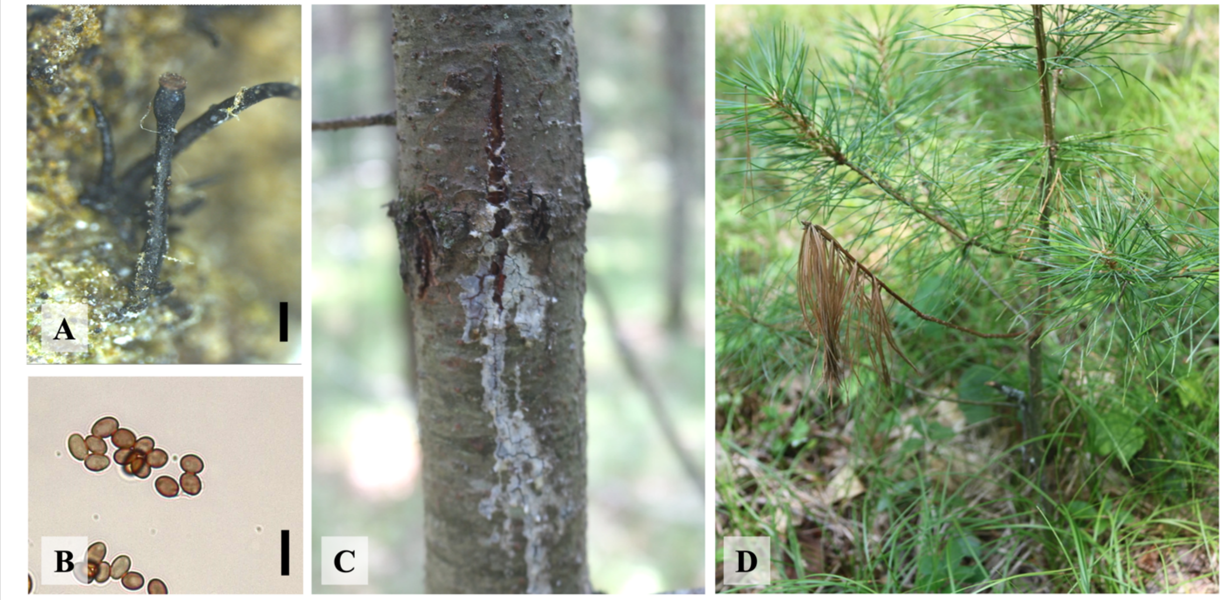

Figure 4.1. Typical signs and symptoms of *Caliciopsis pinea* infection on *Pinus strobus*. **A)** *C. pinea* mazaedia, scale bar indicates 250 µm, **B)** *C. pinea* ascospores, scale bar indicates 10 µm, **C)** pitching and resin bleeding from a canker that has cracked the bark of the host *P. strobus*, and **D)** flagging in an understory seedling infected with *C. pinea.*

There have only been two attempts to characterize *C. pinea* ontogeny, both occurred before molecular tools were developed which hindered the authors' ability to distinguish individual specimens (Funk 1963; McCormack 1936). However, the work of Funk (1963) is the most recent and detailed account of maturation in *C. pinea* and begins with ascospore germination on a susceptible host. Following contact with a wounded host, *C. pinea* ascospores germinate and vegetative growth spreads throughout the cambium (Funk 1963). As hyphae aggregate, a stroma will form and emerge from beneath the periderm of the tree, leading to the characteristic bark cracking and pitching symptoms of a *C. pinea* infection (Funk 1963). Mazaedia and spermogonia can both emerge from the same stroma in *C. pinea,* and often clusters of fruitifications are borne together (Funk 1963). Importantly, it is still unclear from Funk's original study if spermatia contribute to disease development and spread in nature, but they are assumed to contribute to the genetic constitutions of the ascospores that are born from the process of spermatia fertilizing ascogonial cells, causing the formation of asci (Funk 1963). Asci production is believed to lead to the formation of the ascogenous swelling, or the terminal cavity of the mazaedium from which *C. pinea* ascospores will extrude at maturity (Funk 1963). Dehiscence of the cavity is thought to contribute to the release of spores by forcing them to the mazaedial surface where their asci deliquesce, and spores are liberated by wind and rain (Funk 1963). Spores are released repeatedly during the growing season, ensuring a continuous supply of inoculum for ongoing infections. However, it's uncertain if disease is polycyclic, as it is unknown if new mazaedia form after initial infection, potentially causing further infections within the same season.

Much remains to be learned about the reproductive strategies of *C. pinea.* The timing of reproductive events and optimal environmental conditions for inoculum dissemination and disease development are still poorly understood, especially in modern climates. Disease is reported to be most severe in the years following prolonged drought, but the immediate impact of the environment on pathogen development and spore release has never been investigated in the US (Costanza et al. 2020; Munck et al. 2016). Most existing research on CCD has focused on *in vitro* study of specimens, silvicultural perspectives on disease which primarily investigate economic consequences of disease on timber, and pathogenicity trials that are useful for assessing pathogen host ranges but do not adequately imitate natural infections or pathogen and host genetic diversity in larger landscapes (Cram et al. 2022; Migliorini et al. 2020; Costanza et al. 2020).

Various air samplers have historically been used by plant pathologists to study fungal biology, mechanisms of spore release and environmental condition requirements for spore dispersal (Check et al. 2024a and 2024b). Of the various sampler types, rotating-arm samplers (commonly referred to as spore traps), an active impaction-based sampling instrument, are particularly popular for studying fungal biology due to their relatively low cost per unit, flexibility in sampler design as determined by study needs, and high degree of accessibility for a wide range of researchers and study designs (Check et al. 2024a; Boutry et al. 2023; Botella et al. 2019). This type of sampler has been used to investigate forest fungal pathogens, but most of these study designs did not include a mechanism by which spore trap rotational speed could be regulated over the duration of the sampling period (Boutry et al. 2023; Botella et al. 2019; Quesada et al. 2018). Without a speed regulating component, the rotational speed of the trap will slow as the battery depletes over the course of the sampling period, making sampling rate and the active sampling period between time points inconsistent (Check et al. 2024a). Additionally, existing studies of spore release in *C. pinea* were performed in Europe and before the description of a sister *Caliciopsis* species that was later found to cross-react in the detection assay utilized in the study (Migliorini et al. 2020; Botella et al. 2019).

A better understanding of *C. pinea* biology is critical for making informed recommendations to state and federal forest management entities. Using rotating-arm samplers coupled with weather data acquisition is a cost-effective way to determine the environmental factors that influence inoculum dispersal, make timely detections of pathogens and their movement using molecular tools, quantify inoculum loads over the duration of a growing season, and facilitate rapid response to disease incursions (Check et al. 2024a and b; Mahaffee et al. 2023). A quantitative polymerase chain reaction (qPCR)-based assay for detection of *C. pinea* has been developed and screened against a 52-isolate panel of other closely related *Caliciopsis* species and common *P. strobus* pathogens and was determined to be specific to *C. pinea* alone and could detect down to 10 fg of target DNA (see Chapter 3). This study couples rotating-arm samplers with this molecular detection technique to monitor spore release in *C. pinea* and relate it to environmental factors that may lead to inoculum spread and reinfection. Similar studies have been conducted in high-value cropping systems but rarely for investigations in forests (Check et al. 2024a and b; Boutry et al. 2023; Mahaffee et al. 2023; Botella et al. 2019; Quesada et al. 2018). With the expansion of this type of study into forested ecosystems, more specific, sensitive, and widespread disease monitoring can elaborate on the biology of target forest pathogens, lead to more accurate pathogen identification, and facilitate response to disease across wider forested land areas.

4.3 Materials and Methods

Air sample and field data collection

Nine forest sites heavily infected with *C. pinea* were selected for this study in Maine, New Hampshire, Michigan, Wisconsin, and Minnesota in collaboration with state and federal Forest Service and Department of Natural Resource entities (Fig4.2). For some parts of the analysis sites were grouped into regions: the three sites in Maine and New Hampshire (grouped as “New England”), 3 sites in Michigan, and 3 sites in Wisconsin and Minnesota. Site selection prioritized presence of *C. pinea* mazaedia or disease and accessibility to participating researchers. Rotating-arm spore samplers (constructed according to Check et al. 2024) were built by the Michigan State University Forest Pathology laboratory, shipped to collaborators, and deployed from June to November of 2021 and 2022 to account for spring emergence of *C. pinea* and the growth season of the host (Funk 1963) (Figs4.2 and 4.3). Samplers utilized, 1.6 mm diameter steel tig welding rods rated ER308/308L (Griainger, Lake Forest, IL, U.S.), cut to 31.75 mm in length as the sampling surface. All samplers were operated at 2,400 ±100 rotations per minute (RPM). The theoretical sampling rate was calculated according to Check et al. 2024b and Check et al. 2025 and determined to be 66 L/min. Sampling speed of 2,400 RPM is recommended for most general-use purposes (Check et al. 2024). Vacuum grease (Dow Corning, Midland, MI, U.S.) was applied to sampling rods in a thin layer using hand gloves to act as the sampling surface (Check et al. 2024a and b; Check et al. 2025; Hirst 1952; Lacey and West 2006). Individual spore traps were attached to a T-post at each site at a sampling height of 1.5 m and remained in place for the duration of each study year (Fig4.3). Three traps were placed at each site in an equilateral triangle formation, each about 100 m apart (Botella et al. 2019) (Figs4.2 and 4.3). Sampling rods were collected once a week for the duration of the study and placed in 2 mL microcentrifuge tubes, transported to the respective participating laboratories for each site and stored at -20 °C until DNA extraction. At the end of each season, participating labs shipped all samples to MSU Forest Pathology for processing.

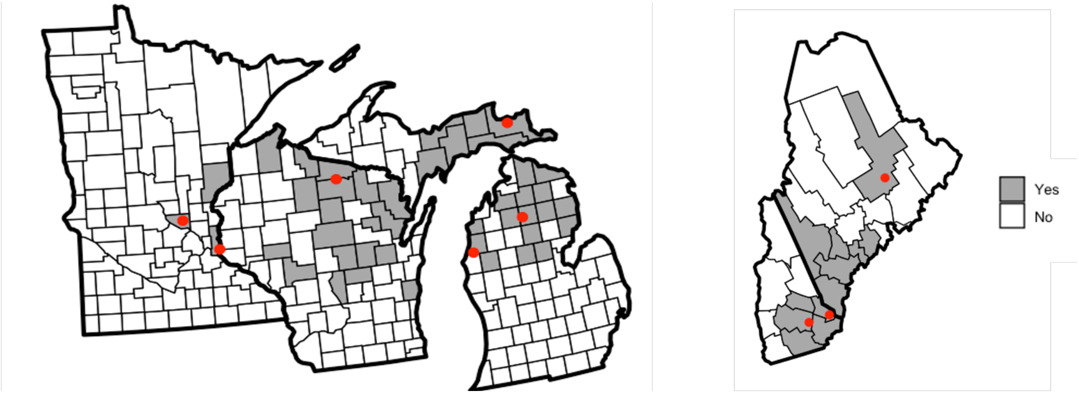

Figure 4.2. A map of the geographic locations of field sites utilized in this study, overlayed with *Caliciopsis pinea* incidence data at the county level in each state. Counties shaded in grey indicate the presence of *C. pinea* and red dots indicate field sites.

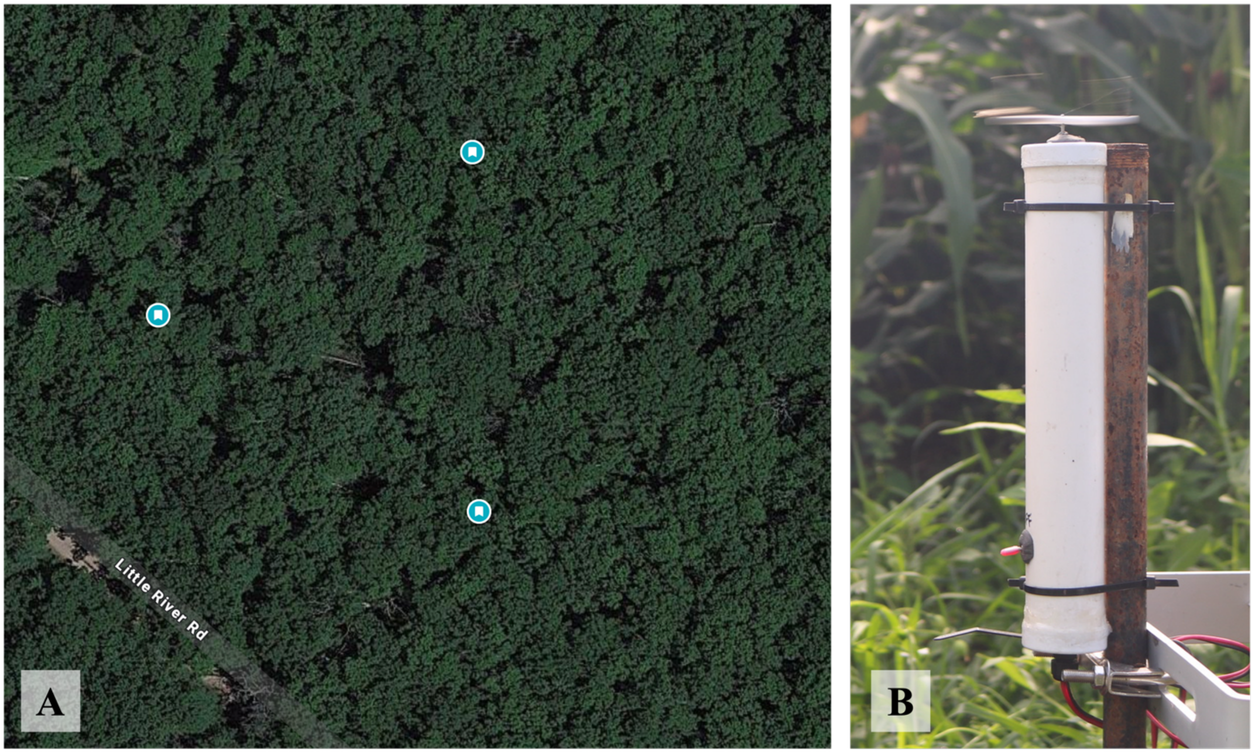

Figure 4.3. Representative photos of field site set-up. **A)** Topographical map of the equilateral triangle formation of spore traps at each site. **B)** An example rotating-arm spore trap.

DNA extraction

Eight different kits were tested on three positive control samples each, mock inoculated with 10,000 *C. pinea* spores. Positive control samples were screened using the quantitative PCR (qPCR) assay described below. The PowerSoil Pro Kit yielded the lowest Cq from positive control samples, indicating the highest retention of target DNA. This was done to determine an optimum extraction protocol for *C. pinea* DNA from this sample type. Sampling rods were removed from their collection tubes using sterile forceps and placed into lysis tubes from the Qiagen DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany). DNA extraction was performed according to manufacturer instructions, with the extension of the lysis step for 20 minutes.

Quantitative PCR

A standard curve was generated for quantification of spores from environmental samples (Fig4.4). *C. pinea* spores were suspended in water and quantified on a hemacytometer, then serially diluted from 10,000 spores/µL to 1. One µL of each concentration was pipetted onto two sampling rods thinly coated in vacuum grease inside a lysis tube from the PowerSoil Pro kit. This was performed in triplicate at each concentration. DNA was extracted from these samples as described above and tested using qPCR. A previously developed qPCR assay for *C. pinea* species-specific detection (described in Chapter 3) was utilized on both the mock inoculated and environmental samples on the BioRad CFX96 Touch Real-Time System (BioRad Laboratories, Hercules, California, U.S.). Importantly, qPCR protocols included the addition of an exogenous internal control (EIC) to differentiate truly negative samples and samples that appeared negative because of PCR inhibition (Haudenshield and Hartman 2011). Primers, probes, and exogenous DNA names and sequences can be found in Table 4.1. Primers and probes were supplied by Biosearch Technologies (LGC, Hoddesdon, United Kingdom). The reaction consisted of 8uL of Perfecta qPCR Toughmix (Quanta Biosciences, Gaithersburg, MD, USA), 400nM of Cali4FP and Cali7RP primers, 200nM of HHIC-F primer, 160nM of HHIC-R primer, 200nM of Cali2Pr probe, 160nM of HHIC-Pr probe, 5fg HHIC-DNA as the target for the internal control, 2mM of $MgCl_2$, and molecular-grade water for a final reaction volume of 25uL. The cycling conditions were set at 95°C for a three-minute initial denaturation, followed by 49 cycles of 95°C for 15 seconds and annealing at 66°C for 30 seconds with a plate reading taken after each cycle. At each spore concentration all three mock samples were tested, each in triplicate, for a total of nine reactions at each concentration. The average and standard deviation of all nine reactions was used to generate the spore standard curve. Linear regression was performed on these values to determine reaction efficiency, slope, and coefficient of determination (R^2). This standard was used to determine the limit of detection and the limit of quantification for this assay on *C. pinea* spores.

Table 4.1. All primers and probes utilized in this study.

Primer	Primer Sequence (5’-3’)	Tm (°C)	Reference
Cali4FP	CGTGGGCGGTCGTCG	60.2	This study
Cali7RP	ACTTTCAACAACGGATCTCTTGGTTC	61.9	This study
Cali2Pr	[FAM] CGCCGAAGACCCCTCTAAACTCTGACTGT [BHQ1]	69.1	This study
HHIC-F	CTAGGACGAGAACTCCCACAT	58.6	Haudenshield and Hartman, 2011
HHIC-R	CAATCAGCGGGTGTTTCA	55.6	Haudenshield and Hartman, 2011
HHIC-Pr	[CalRed610] TCGGTGTTGATGTTTGCCATGGT [BHQ2]	63.4	Haudenshield and Hartman, 2011
HHIC-DNA	CACGCCTAGGACGAGAACTCCCACATCGAGCTTGACGCAAACGACCACGCCAGGACCATGGCAAACATCAACACCGAGCGCAACGCCTTGTGCTGAAACACCCGCTGATTG	73.7	Haudenshield and Hartman, 2011

Weather data acquisition

Daily weather data including precipitation (mm), and the maximum, minimum, and average relative humidity (%), temperature (°C), dew point (°C), and wind speed (m/s) were sourced using historical weather data services with a 2.5-km resolution (The Weather Company, IBM). This was done for each site from 2021 to 2022. The daily weather data for each site at each year was transformed into a moving average over a 7-day window to keep analysis consistent with weekly sample collections. This was done using the rollmean() function from the R package “zoo” v.1.8-13 (Zeileis and Grothendieck 2005) according to methods developed by (Check et al. 2025, https://github.com/checkjill/Phyllachora-maydis-spore-trapping). Spore quantities and weather data were then plotted by various methods in R to test relationships between collection efficiency and the measured environmental variables.

Data analysis

Spore numbers in environmental samples were estimated from their average Ct value across 2 replicates. The below equation was used for this process by taking the slope and intercept from the spore quantity standard curve linear regression according to Check et al. 2025:

$$Quantity = 10^{\left[\frac{(Ct-Intercept)}{Slope}\right]}$$

For each region and year included in the study (New England, Michigan, and Wisconsin and Minnesota) data was plotted using the square root of spore quantity as a function of time; square roots were chosen over raw values for data visualization. For each year, data from all sites were pooled, transformed into logarithmic values, and used to perform linear regression analysis of spore quantity at all sites over the duration of the study. Spearman correlation analyses between spore quantities and 7-day moving averages (MAs) were performed using the cor() function for the R package stats (R Core Team 2024) for each year at each site to determine positive, negative, or neutral correlation between spore quantity and the various weather variable averages (FigsS.4.1, S.4.2, and S.4.3) (Check et al. 2025). Finally, all data points were pooled across both years and plotted as the square root of spore quantity as a function of the average weekly precipitation (mm), average temperature (C°), dew point (°C), and wind speed (m/s) all individually. Patterns were assessed by fitting Gaussian curves by non-linear least squares analysis and generating density plots for each data set.

4.4 Results

Standard curve generation

The chosen qPCR assay had a limit of detection of one spore, but a limit of quantification of 10 spores (Fig4.4). A single spore amplified in all three qPCR replicates across all three extraction attempts (n=9) at an average Ct of 36.3. However, 10 spores amplified at an average Ct of 36.2. The closeness of the cycle thresholds makes it impossible to differentiate between one spore and 10, so for quantification purposes, spore number was only estimated for samples that cycled before 36.2 (Fig4.4). Reaction efficiency from the spore-rod sample type was determined to be 110.2% with an R^2 value of 0.9406, demonstrating that the relationship between spore number and amplification timing were highly correlated.

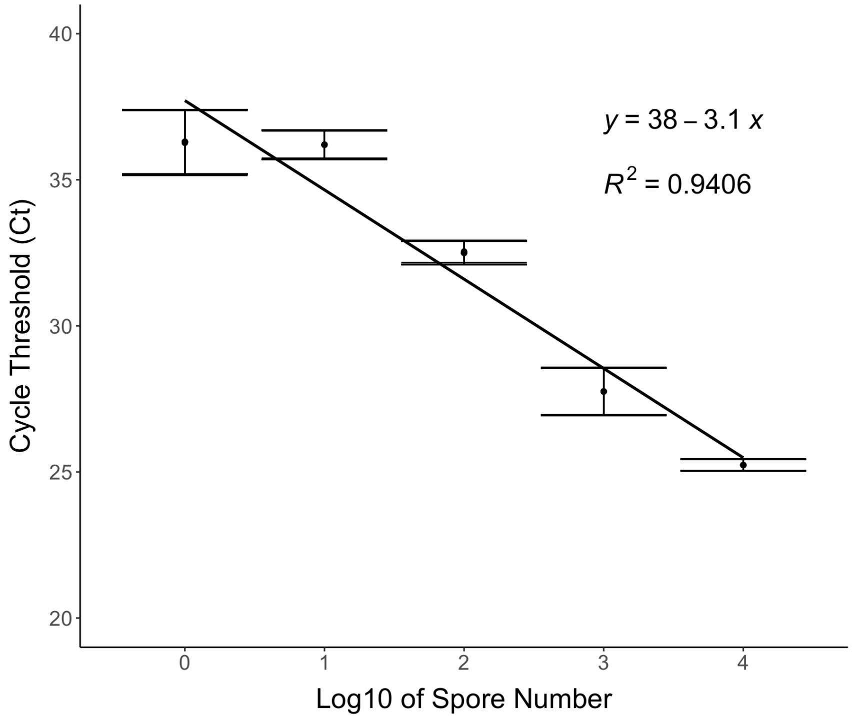

Figure 4.4. Linear regression analysis of the relationship between the Log10 of spore number and the Cycle Threshold (Ct) value after qPCR analysis. Each point represents the mean Ct value (± standard deviation) for a given spore concentration.

Time series and linear regression analysis

A total of 926 samples were collected over the duration of the study. For each region and each trap, the square root of the spore quantity detected from each sample was plotted as a function of time (Fig4.5). The EIC ensured that any samples which failed to amplify were truly negative. At all sites and years, inoculum was consistently recovered from samples over the duration of the study, in varying amounts depending on time point and trap (Fig4.5). All the highest spore quantities detected over the duration of the study were found at New England sites in 2022 (Fig4.5). Conversely, Wisconsin and Minnesota sites showed spore quantities consistently lower than both New England and Michigan sites during both years of sampling (Fig4.5). There were considerable differences in spore quantity collected even from traps stationed at the same site (Fig4.5), but when data were pooled across sites by year and plotted as a function of time, linear regression revealed a highly significant ($p<2.2e-16$) reduction of inoculum present in the samples over the course of the growing season, both years (Fig4.6).

However, this reduction did not translate to total absence of inoculum at any point during the study, consistent with observations made in several early reports of disease that *C. pinea* is a perennial fungus and fruiting structures will remain on the host through the winter (Figs4.5 and 4.6) (Funk 1963). A February visit to one of the Michigan sites utilized for this study revealed fruiting structures with viable spores, further validating these findings (Harkness et al., *unpublished data*).

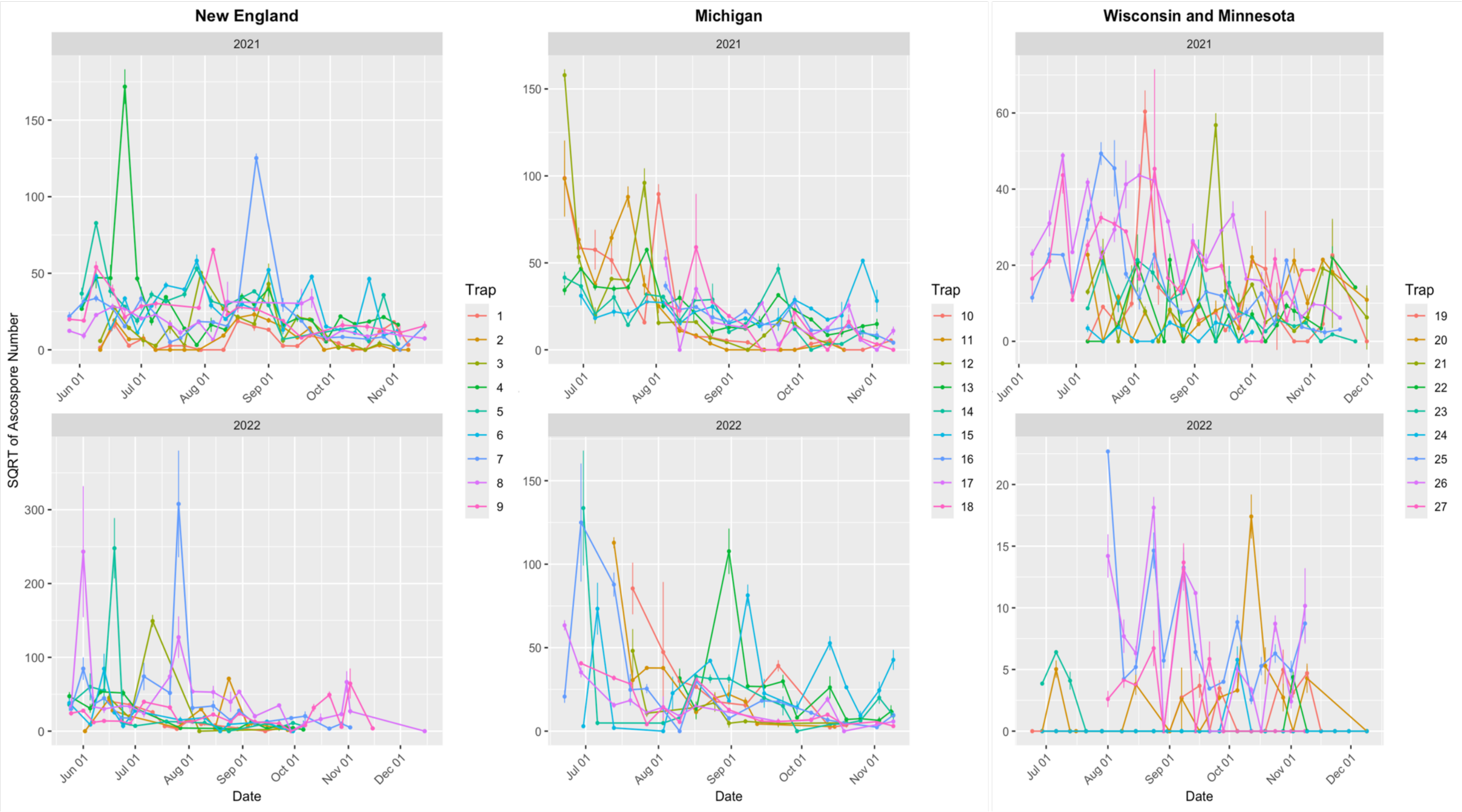

Figure 4.5. The square root of spore quantity plotted as a function of time for each trap, in each region, for each year. The spore quantity for each trap, recovered at each collection time is reported (± standard deviation between qPCR replicates).

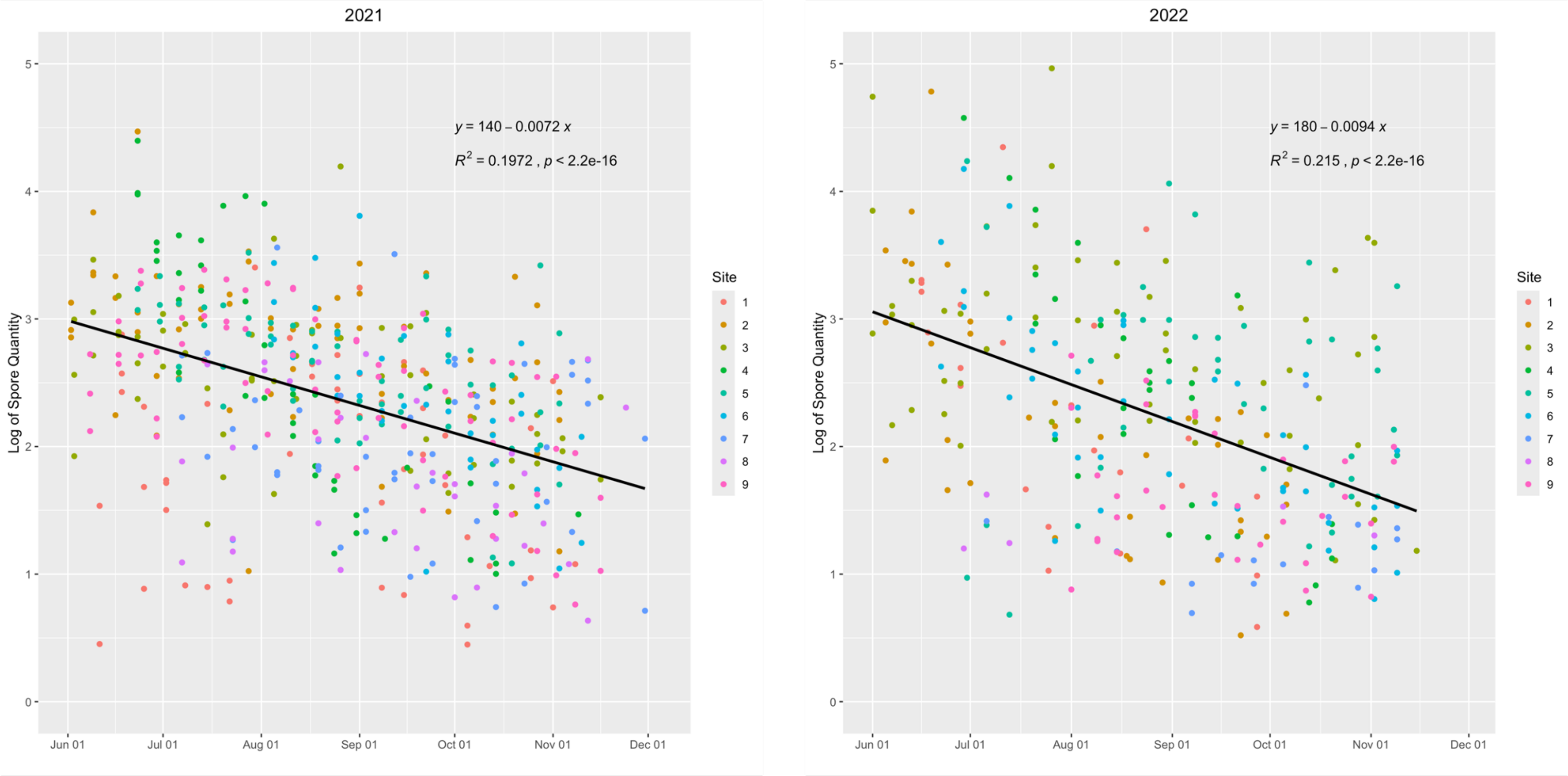

Figure 4.6. Linear regression analysis of the log of spore quantity as a function of time. The equation of the regression line, R^2 value, and the p-value are reported in each plot.

Collection efficiency was particularly high at the Maine site, especially during the second year of collection, when the highest quantity of spores (~95,000 in an individual sample from Trap 7) was detected out of the entire study (Fig4.5 and S.4.4). Michigan sites did not achieve the same peaks in spore quantity (maximum recorded capture was ~23,000 spores), but trends remained very similar to New England during both years, all showing highly significant ($p<0.05$) reduction in inoculum loads over the course of the study (Fig4.5 and S.4.4). The Wisconsin and Minnesota region showed the lowest recovery of *C. pinea* spores from both years the study was conducted (Fig4.5 and S.4.4). The maximum number of spores recovered from any of the sites in this region over the duration of the study was ~3,600 (Fig4.5 and S.4.4). The recovered spore quantities dropped between the first collection year and the second, with almost no recovery of spores from both Minnesota sites in 2022.

***Epidemiology of* Caliciopsis pinea**

Correlation analysis revealed that at each site each year, different environmental variables showed significant correlation with spore collection efficiency (FigsS.4.1, S.4.2, and S.4.3). In many cases there was no correlation to any measured variable (FigsS.4.1, S.4.2, and S.4.3). This was the case when data were pooled, and correlations were assessed across both years and all sites (Fig4.7). No measured variable had a significant positive or negative correlation with collection efficiency (Fig4.7). However, non-monotonic trends were still observed in the data, when spore quantity was plotted against 7-day MAs for each measured environmental variable. Lower average weekly precipitation led to greater collection efficiency. Density plotting revealed that most samples were collected at an average weekly precipitation of 0.57 mm (Fig4.8A). *C. pinea* spore capture peaked at an average weekly precipitation of 2.97 mm, as determined by fitting a Gaussian curve using non-linear least squares analysis (Fig4.8A). Mild temperatures facilitated more frequent detection of spores, and greater quantities of spores collected. Most samples were collected at 18.99°C, and spore capture peaked at an average weekly temperature of 23.55°C (Fig4.8B). Breezy, but not windy, conditions resulted in higher collection efficiency and more frequent collections (Fig4.8C). Most samples were collected at an average weekly wind speed of 7.71 m/s, and the collection efficiency peaked at average wind speeds of 10.35 m/s (Fig4.8C). The final weather parameter assessed in this study was dew point, from which it was found that average weekly dew points of 13.9°C resulted in the most detections of spores, and dew points of 14.9°C were where collection efficiency peaked.

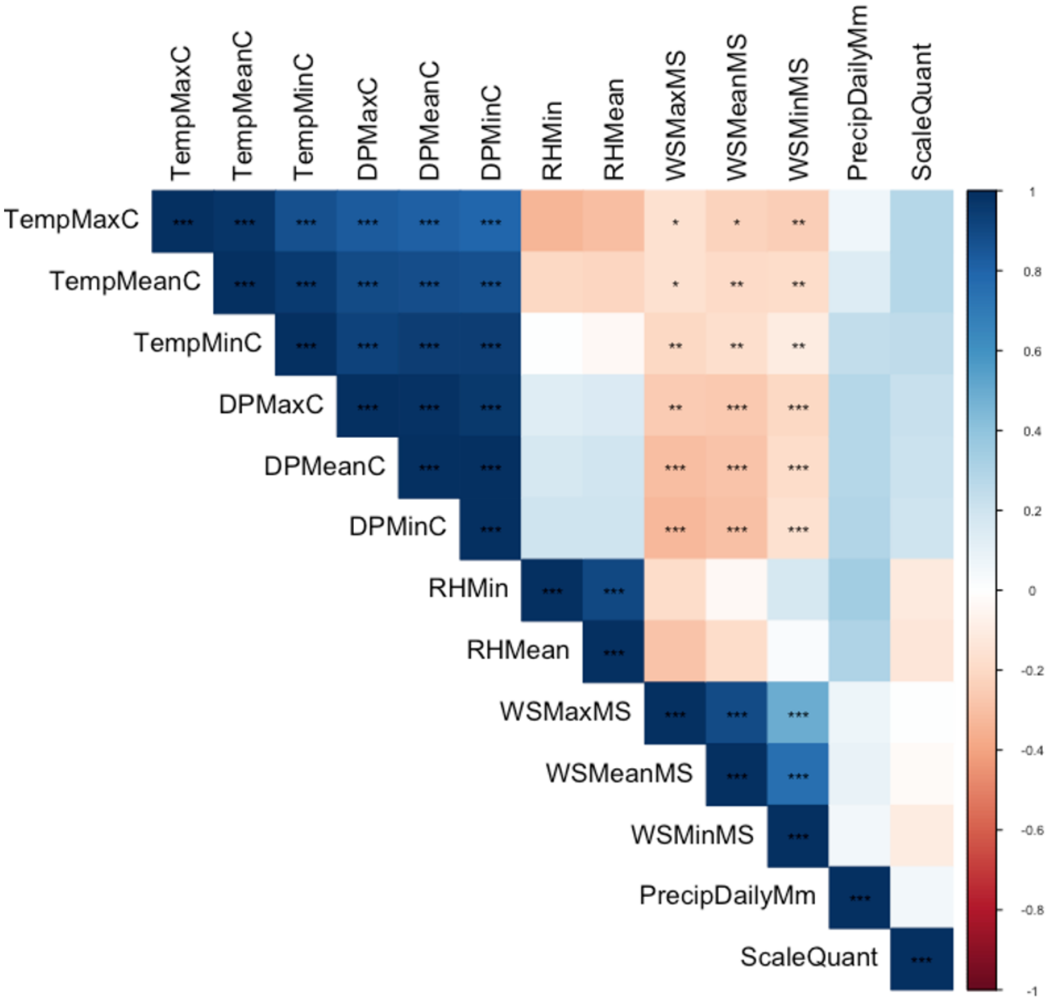

Figure 4.7. Spearman correlation analyses between spore quantities and 7-day moving averages (MAs) across all sites and study years. Maximums, means, and minimums of temperature (Temp), dew point (DP), relative humidity (RH), wind speed (WS), and daily precipitation (Precip) were averaged over one-week intervals. These variables were assessed for correlation to spore number (ScaleQuant). Spearmans's rho is represented by color and shade. The significance value is visualized using the following codes: * represents a p<0.05, ** represents a p<0.01, and *** represents a p<0.001.

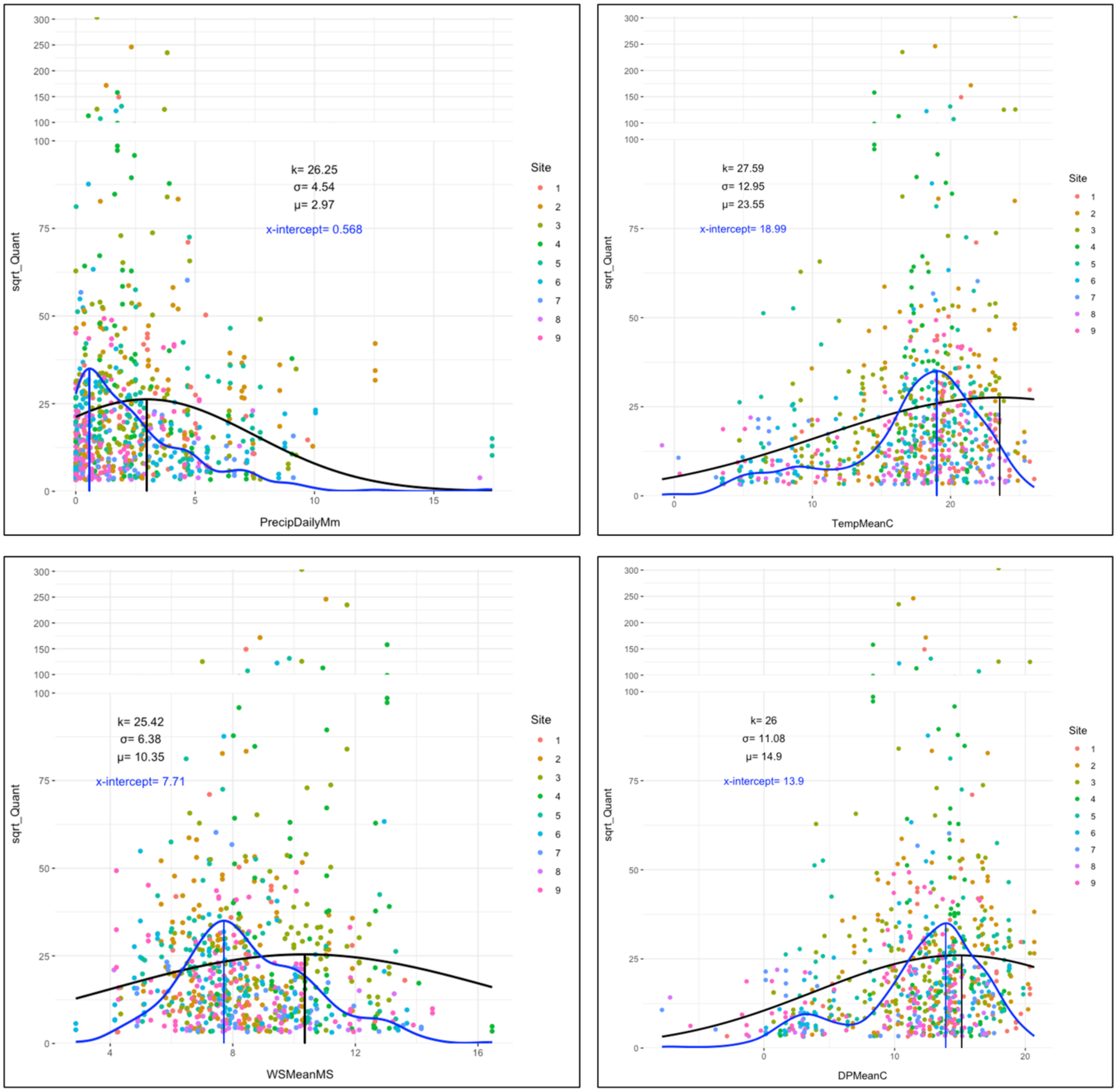

Figure 4.8. Square root of spore quantity, pooled over both study years graphed against the average weekly value of different environmental variables including temperature (Temp), dew point (DP), wind speed (WS), and daily precipitation (Precip). Blue lines represent the density of the data, with the peak occurring at the provided x-intercept. Gaussian curves were fitted to the data in black and the mean (μ), standard deviation (σ), and amplitude (k) are listed for each.

4.5 Discussion

This study is the first investigation of the factors influencing spore release in *Caliciopsis pinea* in North America. There has only been one other investigation of *C. pinea* spore release, conducted in Europe (Botella et al. 2019). Though the body of existing research is limited, most previous studies of North American *C. pinea* have focused on economic impacts of disease (Costanza et al. 2019) or the silvicultural and land management consequences of disease (Costanza et al. 2019 and 2020; Munck et al. 2023, 2016, and 2015). Very few studies have been conducted to evaluate pathogen ontogenesis, dispersal, and most aspects of its life cycle (Migliorini et al. 2020; Botella et al. 2019; Funk 1963; McCormack 1936). Even fewer of these studies were published recently (Migliorini et al. 2020; Botella et al. 2019). There is a distinct lack of research on pathogen biology, and such research could lead to critical insights on minimizing the impact of *C. pinea* as an emerging native disease.

Previous studies (Migliorini et al. 2020; Botella et al. 2019; Munck et al. 2015) have utilized various extraction methods for *C. pinea* DNA recovery, but this study identified the DNeasy PowerSoil Pro Kit as ideal for the spore rod sample type and had optimal DNA recovery compared to other tested extraction methods (Harkness et al. *unpublished data*). The methods used for determining the best kit for extraction from this sample type were not exhaustive, but it is recommended that as many available protocols as possible are tested for recovery of DNA from the target organisms tissue type (spores, hyphae from pure culture, etc.) for better retention of target DNA and more accurate quantification when conducting studies similar to this one (Balduque-Gil et al. 2024). Furthermore, this study validated a new sample type for use with the qPCR assay adopted for *C. pinea* detection. The assay has been previously demonstrated to detect *C. pinea* in the background of plant DNA, but this study demonstrates that the assay is also functional in the background of environmental DNA recovered from air samples (see Chapter 3). Additionally, consistent amplification of the EIC confirmed that the reaction was not inhibited by anything present in air samples collected over the duration of this study (*data not shown*). There are many sources of contamination in air samples that may lead to inhibition including pollen, non-target spores, insects deposited onto the sample, or any number of other organic or inorganic particles that have become aerosolized (Check et al. 2025). The chosen reaction for detection and quantification of spores was not impacted by inhibition from contaminants. The qPCR assay efficiency and sensitivity when used on the spore rod sample type were also ideal: efficiency was determined to be 110%, and the R^2 value was determined to be 0.9406 after linear regression, indicating that the model was well fit to the data and acceptable to infer spore quantity from cycle thresholds.

Trends in spore quantity as a function of time remained consistent between regions each year. Variation in the modern cultivation of *P. strobus* depending on region and stand-level differences (composition, age, etc.) may account for variation in inoculum levels observed between regions at any given point during the study. The five highest spore quantities observed in this study were all collected from New England sites. All New England sites were moderately dense to densely stocked and almost completely composed of *P. strobus.* Both New Hampshire sites were primarily mature trees (50-80 years old), while the Maine site was younger (25-30 years old). Collection efficiency was particularly high at the Maine site, (maximum spore quantity ~95,000) which could be related to the difference in stand age; younger trees will generally be shorter, and *C. pinea* infections typically progress from the bottom of the tree, growing upward with the host as it ages (Whitney et al. 2021). All traps at all sites were deployed at the same height, as described in the materials and methods. Sources of inoculum at the Maine site may have been closer to each trap, increasing the probability that spores moving through the air could interact with the sampling surface, leading to increased collection efficiency (Mahaffee et al. 2023). Michigan sites were compositionally very different from New England sites, and while they did not reach the same peaks in spore collection efficiency (the maximum from any Michigan site was ~23,000 spores), their trends in inoculum availability over the course of the study remained consistent with New England (Fig4.5 and S.4.4). Two of three Michigan study sites were mixed age and composition forests (*P. strobus*-rich but with the presence of other *Pinus* species, as well as *Acer* and *Quercus* spp). The third site was mostly uniform in composition and age: mature trees, primarily *P. strobus* with some *P. resinosa* and *P. banksiana. P. strobus* was naturally regenerating in the understory and most understory regeneration within the study area was observed to have *C. pinea* mazaedia during both years of the study. Despite differences in stand characteristics, all sites in New England and Michigan showed a 1 to 2-fold reduction in inoculum recovered from samples from June to November (Fig4.5 and S.4.4). Wisconsin and Minnesota sites had nearly half the amount of inoculum present at the start of each year of sample collection compared to both New England and Michigan (Fig4.5 and S.4.4). The maximum number of spores recovered from any of the sites in Wisconsin and Minnesota was ~3,600 spores (Fig4.5 and S.4.4). This may have to do with the age of the stands used as study sites: Minnesota sites were the oldest included in this study at 75 to 100 year-old plantations and the site in Wisconsin was a mixed-age *P. strobus* stand, but the trees ranged from 50-100 years old. As previously discussed, *C. pinea* will continue to grow with its host, consequently mazaedia are most likely to be found within the canopy. Besides the Wisconsin/Minnesota region, the oldest stands included as study sites were between 50 and 80 years old. This could result in a 6-to-12 m (20-to-40-foot) height difference in the trees at the Wisconsin/Minnesota sites compared to all others, which may explain the observed reduced collection efficiency (Curtis et al. 1990).

When all sites were pooled a statistically significant ($p<2.2e-16$) reduction in inoculum loads was observed during both years of data collection over the sampling period. These findings are consistent with the very few existing studies on *C. pinea* ontogenesis, which observe that mazaedia reach maturity in the spring at which time the spores are passively released after extruding through the locule of the mazaedium, and that mazaedia with viable spores can be found year-round providing a constant source of inoculum (Funk et al. 1963; McCormack 1936). This study supports these observations as spore quantities peaked at the start of the summer and became reduced by November and December but were still present in the samples to a quantifiable degree. Microclimate differences, host genetics, site characteristics, weather patterns, age and myriad other variables that differ from site to site make direct comparison impossible, even between sites in the same region. Pooling data from all sites allows for the separation of real trends from local anomalies and offers a more generalized perspective on the *C. pinea* life cycle.

Data were similarly pooled to investigate the relationship between the quantity of spores recovered from samples and different environmental variables, across site and year. In general, spore collection efficiency improved when there was low weekly rainfall, mild weekly temperatures, breezy conditions, and average dew points that would feel comfortable for a person. Density plotting revealed the precipitation, temperature, windspeed, and dewpoints at which the most spore detections were made, regardless of spore quantity. Gaussian curve fitting by non-linear least squares analysis considered spore quantity to show the average of each weather variable (μ) at which the most spores were collected. Additionally, it estimated the peak spore count (k) predicted by the non-linear regression model, which corresponds to the mode if the data were perfectly normally distributed. Though most environmental parameters measured in this study were unimodal in collected spore quantity, there was disagreement on “optimal” conditions for spore collection depending on analysis type. This likely resulted from tails in the data: when density plots were visualized it was clear that the spore quantities tapered in either a positive or negative direction in number of detections, depending on the measured weather variable (Fig4.8). In the case of precipitation and wind speed, the tail curve was in the positive direction (Fig4.8A and C). Conversely, in the case of temperature and dew point, density tailed in the negative direction (Fig4.8A and C). Both analysis types offer critical insights on the data set as a whole, across both year and site. While fitting Gaussian curves theoretically presents the optimum conditions for spore collection, it smooths skewness that would otherwise reflect important aspects of the data that are lost with forced Gaussian fit. In contrast, the peak of the density plots highlights the mode of the data set, or the value of the measured weather variable at which the most detections were made, but do not account for the magnitude of spores collected from each sample.

This study was unique among existing research in understanding the factors impacting aerial spore dispersal of *C. pinea* in North America. As the impact of *C. pinea* and CCD continue to increase studies such as this one will continue to address methodological gaps in the existing body of knowledge of emerging endemic pathogens. Results from this study contribute to the current understanding of *C. pinea* epidemiology in North America, and they serve as the first descriptor of the relationship between environmental variables and inoculum availability in CCD afflicted forests. Furthermore, an existing qPCR assay was validated for use on a novel sample type over the duration of this study, and inoculum availability was assessed over the growing season to determine that probability of spread (as determined by inoculum availability) decreases over the growing season but can persist throughout. Further study is required to determine how tree maturity impacts disease impact, progression, and spread. Future studies would ideally prioritize the impact of *C. pinea* infections as trees age, inoculum availability in older tree canopies, and mechanisms of spread between forest stands that have disease presentation and those that do not.

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APPENDIX

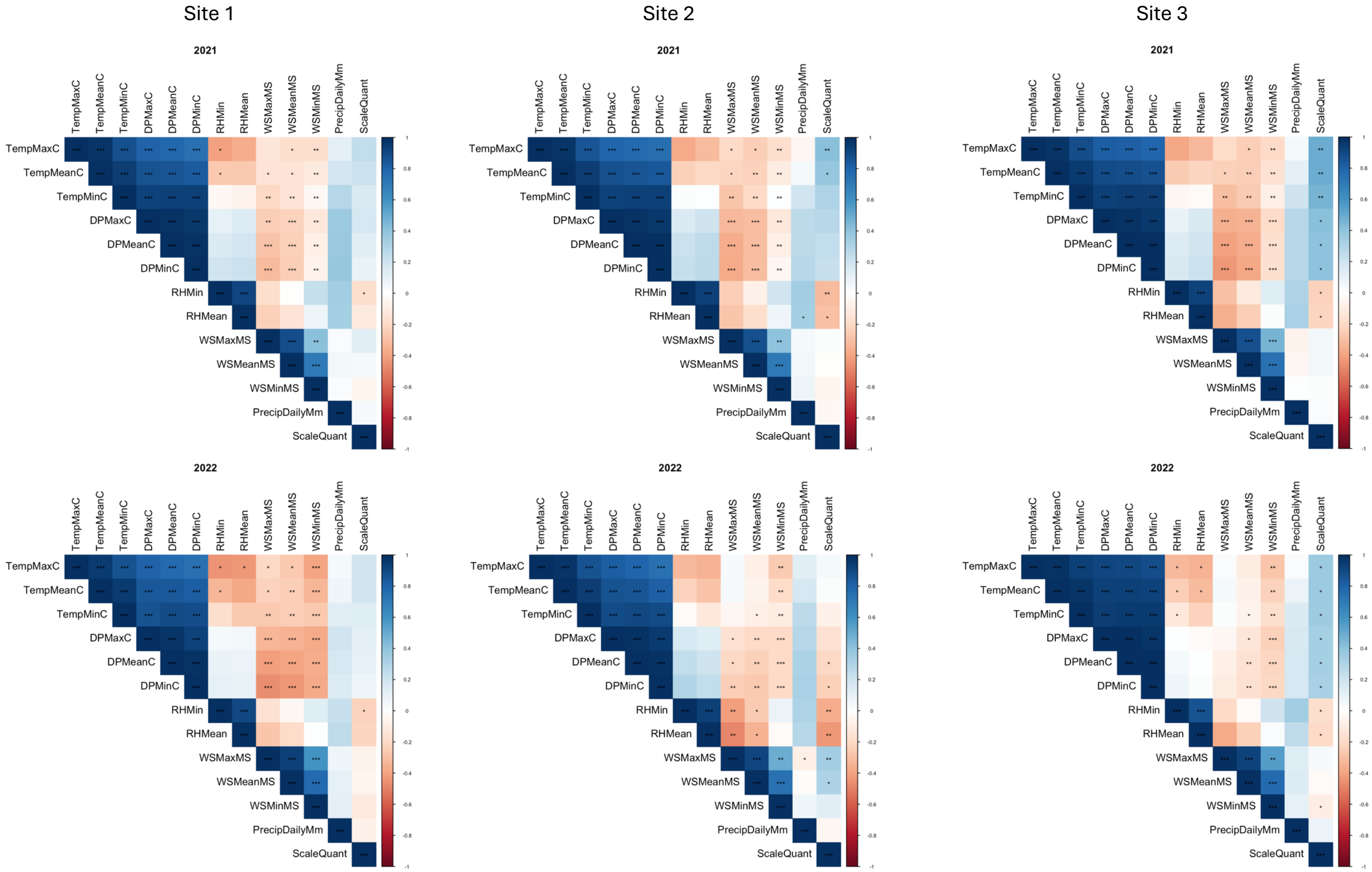

Figure S.4.1. Spearman correlation analyses between spore quantities and 7-day moving averages (MAs) at each New England site for each year. Maximums, means, and minimums of temperature (Temp), dew point (DP), relative humidity (RH), wind speed (WS), and daily precipitation (Precip) were averaged over one-week intervals. These variables were assessed for correlation to spore number (ScaleQuant). Spearmans's rho is represented by color and shade. The significance value is visualized using the following codes: * represents a $p<0.05$, ** represents a $p<0.01$, and *** represents a $p<0.001$.

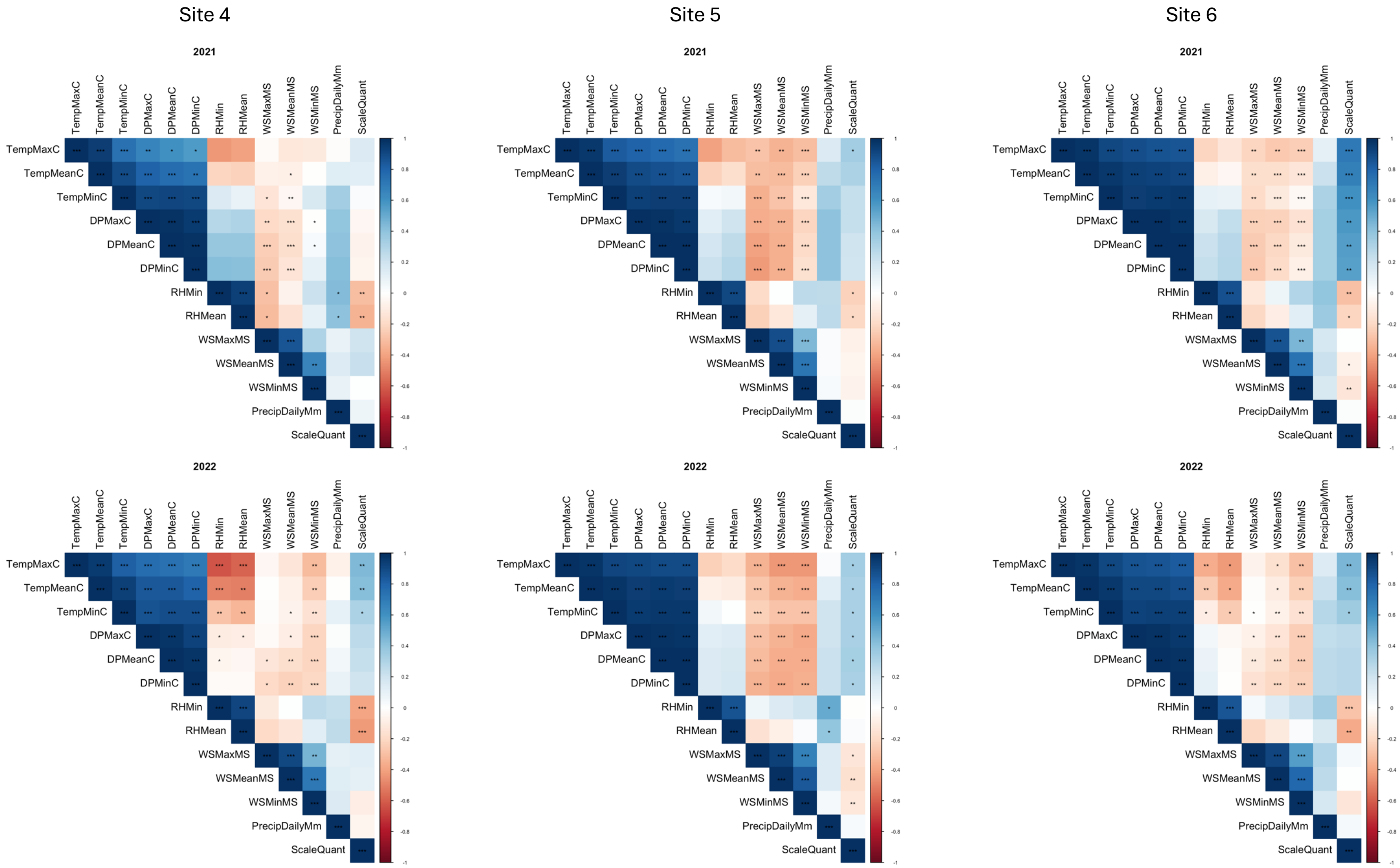

Figure S.4.2. Spearman correlation analyses between spore quantities and 7-day moving averages (MAs) at each Michigan site for each year. Maximums, means, and minimums of temperature (Temp), dew point (DP), relative humidity (RH), wind speed (WS), and daily precipitation (Precip) were averaged over one-week intervals. These variables were assessed for correlation to spore number (ScaleQuant). Spearmans's rho is represented by color and shade. The significance value is visualized using the following codes: * represents a $p<0.05$, ** represents a $p<0.01$, and *** represents a $p<0.001$.

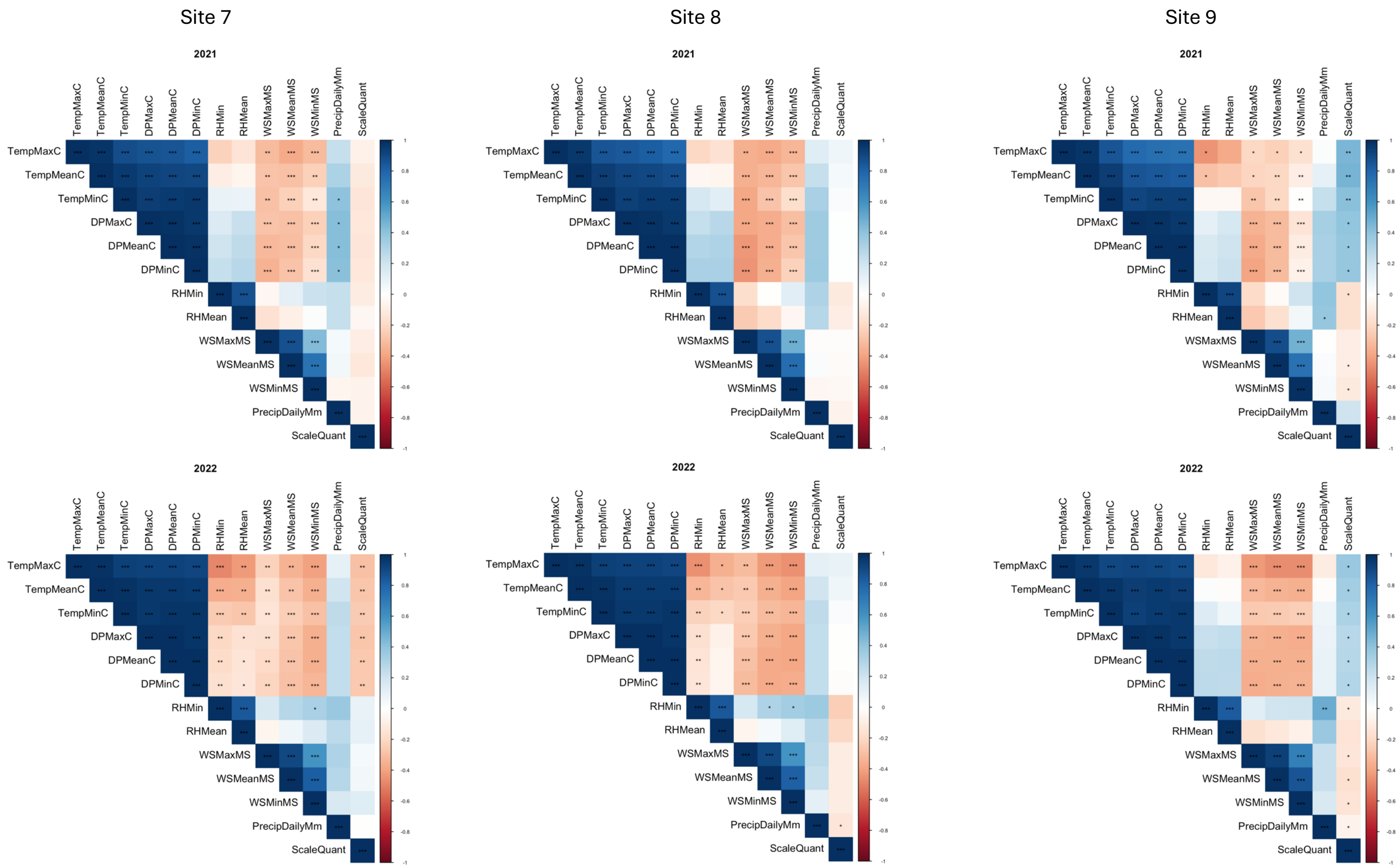

Figure S.4.3. Spearman correlation analyses between spore quantities and 7-day moving averages (MAs) at each Wisconsin and Minnesota site for each year. Maximums, means, and minimums of temperature (Temp), dew point (DP), relative humidity (RH), wind speed (WS), and daily precipitation (Precip) were averaged over one-week intervals. These variables were assessed for correlation to spore number (ScaleQuant). Spearmans's rho is represented by color and shade. The significance value is visualized using the following codes: * represents a $p<0.05$, ** represents a $p<0.01$, and *** represents a $p<0.001$.

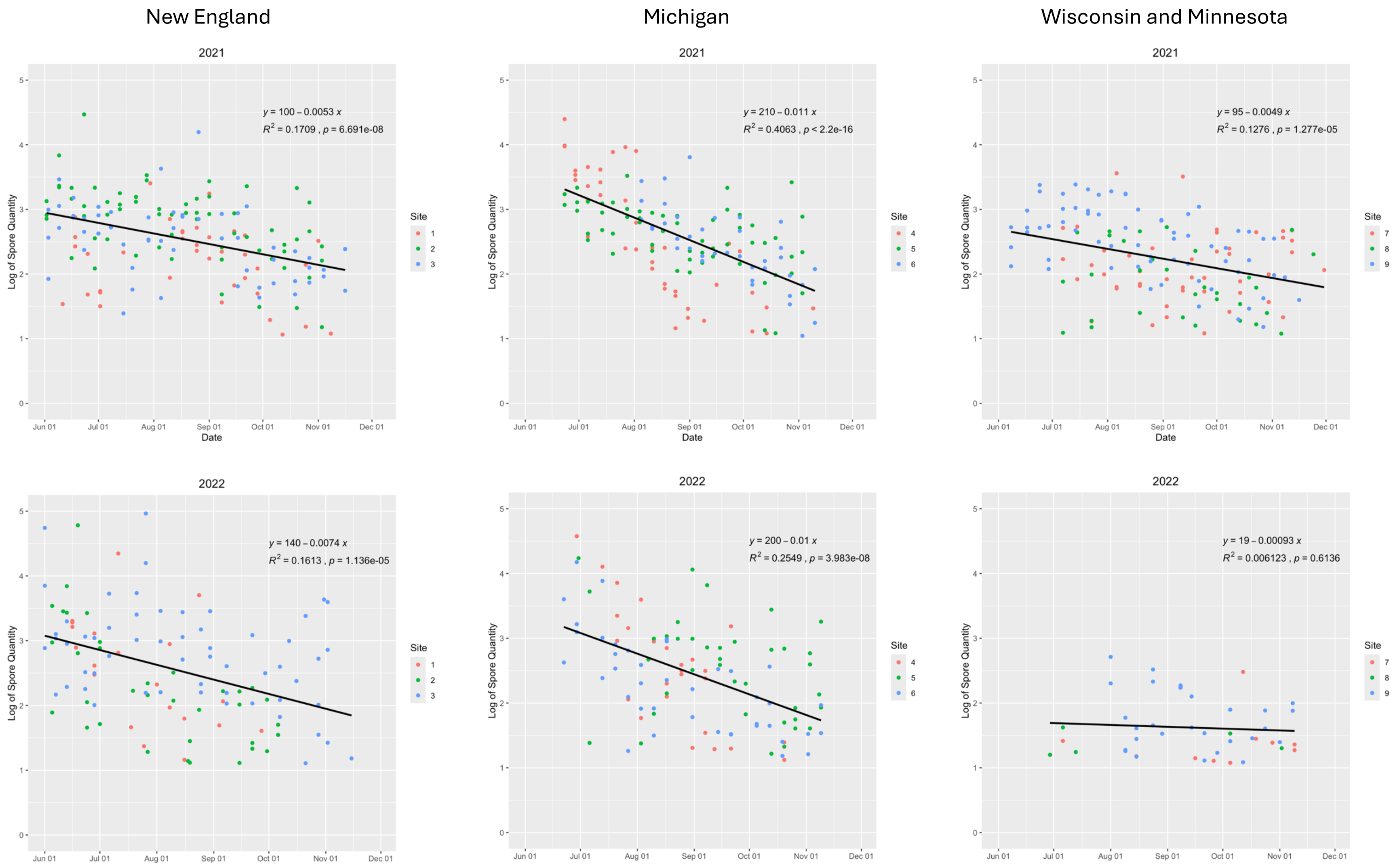

Figure S.4.4. The log of spore quantity plotted as a function of time with linear regression analysis to identify significant changes in spore quantity over the study duration. This was done for each region and year. The regression equation, R^2 value, and p-value is included in the top right corner for each plot.

Chapter 5: Conclusions

5.1 Conclusions

Pinus strobus is a critical cover species throughout its native range and has substantial ecological, economic, and social value. Investigations on factors impacting its health and successful regeneration are pivotal to sustainable management of this important timber species. *Caliciopsis pinea* threatens North American *P. strobus* populations and is nearly ubiquitous throughout the native range of its host. It is presumed by most researchers that *C. pinea* can transition back and forth between saprophytic and pathogenic lifestyles when the host is persistently stressed, which may account for the relatively recent observations of increasing frequency and severity of disease reports. As abiotic stress from changing global climates continues to shape forest ecosystems, understanding *C. pinea* biology has become a necessary part of maintaining healthy *P. strobus* populations.

The investigations herein demonstrate the *Caliciopsis* genus to be more diverse than previously known. Almost 90% of the known species of *Caliciopsis* are reported from tree hosts, most with some degree of pathology (see Chapter 2, Tables S.2.1 and S.2.2). It is unclear if they too will act as more aggressive pathogens in the face of climate change. If this phenomenon, observed in *C. pinea,* is true of other members of the *Caliciopsis* genus, then further phylogenetic investigations are critical to ensuring the health of other forest cover species. This dissertation broadens the growing body of sequences publicly available for such purposes and offers extensive documentation on the growth forms of three, previously unknown *Caliciopsis* species. Additionally, diagnostic strategies for *C. pinea*, the most aggressive pathogen within the genus, were expanded such that epidemiological investigations could be conducted. Ultimately, these studies can be used to advise state and federal forest service entities to prevent further disease spread and applied to other forest pathogens to improve our understanding of how diseases impact forests.

5.2 Broader impacts

These studies represent an important expansion point for future forest health investigations. Here, critical knowledge gaps concerning *Caliciopsis pinea*, the causal agent of caliciopsis canker disease, which has an increasing impact on eastern white pine (*Pinus strobus*) across its native range. By developing a comprehensive diagnostic guide and a highly sensitive qPCR assay, this study provides essential tools for accurate and rapid detection of the pathogen. This has significant implications for forest health management and conservation efforts, enabling early intervention and potentially mitigating economic and ecological losses. These kinds of technologies can empower forest pathologists, land managers, and regulatory agencies to monitor disease spread, conduct risk assessments, and implement effective and efficient management strategies. Furthermore, the expansion of diagnostic options for forest pathogens prioritizes investigations on pathogen biology where most studies approach disease in forests from the management or silvicultural perspectives.

The discovery and characterization of three new *Caliciopsis* species in Michigan contributes to a broader understanding of fungal biodiversity and their ecological roles. This taxonomic work is crucial for accurate identification and management of *Caliciopsis* pathogens, preventing misidentification and ensuring targeted control strategies. Furthermore, the phylogenetic analyses provide a framework for future studies on *Caliciopsis* evolution, which will facilitate improved understanding of how populations are and will change over time. The methodologies and findings presented in this dissertation can serve as a model for addressing similar challenges in other pathosystems, contributing to the broader field of plant pathology and disease management.

APPENDIX

* Denotes first authorship

† Denotes co-first authorship

Harkness, R.J.*, Suslow, K., Luecke, N., Sharma, S., Miles, T., Sakalidis, M.L., and Schweigkofler, W. 2024. Diversity and pathogen risk of *Phytophthora* spp. found in native plant nurseries and interstate shipping nurseries on plant stocks. First Look, *PhytoFrontiers.* https://doi.org/10.1094/PHYTOFR-06-24-0064-R

Check, J. †, **Harkness, R.J**. †, Heger, L. †, Chilvers, M.I., Mahaffee, W.F., Sakalidis, M.L., and Miles, T.D. 2024. It's a trap! Part II: An approachable guide to constructing and using rotating-arm air samplers for plant pathologists. *Plant Disease,* 108(7): 1923-1936. https://doi.org/10.1094/PDIS-01-24-0131-SR ***Editor's Pick***

Check, J. †, **Harkness, R.J**. †, Heger, L. †, Sakalidis, M.L., Chilvers, M.I., Mahaffee, W.F., and Miles, T.D. 2024. It's a trap! Part I: Exploring the applications of rotating-arm impaction samplers in plant pathology. *Plant Disease,* 108(7):1910-1922. https://doi.org/10.1094/PDIS-10-23-2096-FE ***Feature Article***