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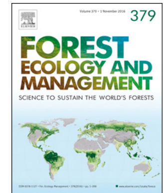

Association of *Caliciopsis pinea* Peck and *Matsucoccus macrocicatrices* Richards with eastern white pine (*Pinus strobus* L.) seedling dieback☆

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ABSTRACT

Matsucoccus macrocicatrices Richards (Hemiptera: Matsucoccidae) is the only species within this genus that feeds and reproduces on eastern white pine (*Pinus strobus* L.), and at the time of its description, was not observed or known to cause serious damage. With eastern white pine dieback occurring extensively throughout the Appalachian Mountains, researchers are now in search of the contributors to this dieback phenomenon. Since its recent discovery (2007) far outside its historical range, *M. macrocicatrices*, and cankers associated with *Caliciopsis pinea* Peck, are regularly present on symptomatic trees throughout the range of eastern white pine. Little is known about the relationship between *M. macrocicatrices* and the fungal cankers commonly found on eastern white pines expressing dieback symptoms. For this study, we evaluated the relationships between both focal organisms and the extent of dieback symptoms on tree seedlings to identify contributing factors affecting symptomatic trees. We assessed the insect-pathogen complex on 270 eastern white pine seedlings from nine states that include the Appalachian Mountain range. There were positive correlations between *M. macrocicatrices* and seedling dieback, cankers and seedling dieback, and *M. macrocicatrices* and cankers in both the southern and northern portions of the Appalachians. About 95% of the observed *M. macrocicatrices* cysts and shells were associated with cankers, especially *C. pinea*-dominated cankers, which were exceptionally abundant on severely affected seedlings. The most prevalent fungi isolated from cankers without apparent fruiting bodies of *C. pinea* were in the genus *Phaeomoniella*. Trials were conducted to test the pathogenicity of *C. pinea* and other fungal isolates. Of the 15 fungal species tested, *C. pinea* was the only pathogenic species that formed girdling cankers on eastern white pine seedlings. We postulate that there is a facultative relationship between *M. macrocicatrices* and *C. pinea*, forming an insect-pathogen complex that is contributing to eastern white pine dieback and significantly impacting its regeneration dynamics in North America.

1. Introduction

The genus *Matsucoccus* (Hemiptera: Matsucoccidae), also known as the pine bast scales, has some of the most serious sap-sucking pests of pine forests and plantations in Asia, Europe, and North America (Foldi, 2004; Liu et al., 2014). There are 19 species of *Matsucoccus* in North America, all of which feed exclusively on trees in the family Pinaceae (Foldi, 2004; Liu et al., 2014). Among these is *Matsucoccus macrocicatrices* Richards that feeds and reproduces on eastern white pine (*Pinus strobus* L.) (Foldi, 2004; Kosztarab, 1996; Richards, 1960; Watson et al., 1960). Richards (1960) described the different stages of the *M. macrocicatrices* life-cycle, including the egg, crawler (first instar and mobile nymphs), cyst (heavily sclerotized and legless stage between legged crawler and adult), shell (shed exoskeleton from adult emergence), and adult (winged male and wingless female) stages, but did not document the duration that *M. macrocicatrices* remains in each life stage. Watson et al. (1960) documented the life history of *M. macrocicatrices* in association with a potentially symbiotic fungus, *Septobasidium pinicola* Snell, suggesting that the scale insect may have a two-year life-cycle in Canada, and it may be parthenogenetic due to a lack of

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males. Like all hemipterans, *M. macrocicatrices* possesses modified mandibles and maxillae which form a stylet that they pierce into the tissues of eastern white pine to feed. It is unknown whether they feed on xylem or phloem cells, but are placed within the bast scale group that feeds on bast cells in the phloem. Since these foundational studies conducted in the early- to mid-20th century, little biological or phenological research has been conducted on *M. macrocicatrices*. Until its discovery in the southern Appalachians in 2007, *M. macrocicatrices* was only known to exist on eastern white pine trees in Canada (New Brunswick, Nova Scotia, Ontario, and Quebec) and the northeastern United States (Massachusetts, New Hampshire, and Vermont) (Kosztarab, 1996; Martineau, 1964; Mech et al., 2013; Richards, 1960). However, in the last decade, foresters and researchers have observed *M. macrocicatrices* on eastern white pine in the southern Appalachian Mountains, specifically in Georgia, North Carolina, South Carolina, Tennessee, Virginia, and West Virginia (Asaro, 2011; Asaro et al., 2018; Mech et al., 2013).

With eastern white pine dieback being reported extensively throughout the Appalachian Mountains (Asaro, 2011; Rose, 2011; Rosenholm, 2012), it is essential to evaluate the influential abiotic and biotic factors that may be contributing to this phenomenon. Symptoms observed on eastern white pine include cankerous growths, significant resinosis, crown thinning, branch flagging, and decreases in crown density (Asaro, 2011; Rose, 2011; Rosenholm, 2012). A common characteristic observed among the dying pines includes the presence of *M. macrocicatrices* (Fig. 1), usually embedded under lichen or in branch crotches or cankers, especially those developed by the fungus, *Caliciopsis pinea* Peck (Fig. 2A and B) (Mech et al., 2013; Whitney et al., 2018). Damage to eastern white pine by *M. macrocicatrices* was historically considered to be negligible (Watson et al., 1960), but recent observations have led researchers to consider the contribution of the insect to eastern white pine dieback.

Another common and potentially contributing biotic agent is *C. pinea*. This canker-forming, ascomycetous fungus is native to the eastern United States (Funk, 1963). *Caliciopsis pinea* is primarily found on eastern white pine, but has been documented on other *Pinus* species east of the Mississippi River, including pitch pine (*P. rigida* Mill.), shortleaf pine (*P. echinata* Mill.), table mountain pine (*P. pungens* Lamb.), and Virginia pine (*P. virginiana* Mill.) (Funk, 1963). These cankers have been described as "reddish brown depressions in the bark that have small, globose, clustered, black pycnidia and stalked perithecia that arise from a stromatic cushion" (Overholts, 1930; Ray, 1936) (Fig. 2A, B, and C). Development of fruiting structures begins with the aggregation of fungal hyphae under the bark of an infected tree. This aggregation creates a flattened stroma, which continually grows, and eventually erupts from the bark of the tree. Once the stroma erupts, it provides a foundation for the production of ascocarps (hair-like structures), which enlarge and elongate, and go on to bear ascospores (main disseminating agents). Any conical lobes that do not elongate and form ascocarps are referred to as spermagonia. These spermagonia produce spermatia, which can also disperse, germinate, and grow new colonies of *C. pinea* on the already infected tree, as well as spread to other adjacent trees (Funk, 1963). Once established, the perennial *C. pinea* cankers are capable of producing annual crops of ascocarps with ascospores, and spermagonia with spermatia (Funk, 1963; McCormack, 1936; Ray, 1936). It is unknown whether *C. pinea* spores require existing tree wounds to colonize the tissues of eastern white pine trees, though it has been noted that other species in the genus *Caliciopsis* take advantage of old lenticels and wounds from mechanical damage, insect feeding, oviposition, or boring (Funk, 1963). Past studies suggest that *C. pinea* can create sharply delimited cankers on the trunks and branches of eastern white pine (Cram et al., 2009; Ray, 1936). In recent years, reports from the field have indicated that *C. pinea* cankers cause damage that potentially leads to mortality in thousands of hectares of eastern white pine in both the northern (Maine, Massachusetts, New Hampshire, New York, and Vermont) and southern (Georgia, Virginia, and West Virginia) regions of the Appalachian Mountains (Asaro, 2011; Munck et al., 2015; Rose, 2011; Rosenholm, 2012). Some suggest the disease is most serious on suppressed seedlings and saplings (Overholts, 1930), but *C. pinea* has been found on mature trees as well (Rose, 2011).

Eastern white pine remains one of the most economically and ecologically important species in eastern North America, so there is a need to better assess the relationships among eastern white pine dieback, *M. macrocicatrices*, and canker-forming fungi for the purpose of identifying the contributing factors associated with the now extensive eastern white pine dieback observed throughout the range of the tree. Using eastern white pine seedlings, we aimed to: (1) determine the prevalence and distribution of *M. macrocicatrices* with *C. pinea*, other cankers, and the absence of cankers; (2) assess associations (*via* correlations) among seedling dieback, canker surface area, and *M. macrocicatrices*; (3) map the individual tree-level spatial patterns of *C. pinea* and *M. macrocicatrices*; (4) isolate and identify other fungi associated with cankers; and (5) conduct pathogenicity tests using *C. pinea* and other fungal species isolated from cankers.

For our first objective, we hypothesized that *M. macrocicatrices* and *C. pinea* would be present in most sites in the southern and northern regions of the Appalachian Mountains, since these organisms have been observed and documented by other studies in these regions (e.g. Asaro, 2011; Asaro et al., 2018; Mech et al., 2013; Munck et al., 2015; Rose, 2011; Rosenholm, 2012). For our second objective, we hypothesized that seedling dieback would be positively correlated with cankers and *M. macrocicatrices*, and *M. macrocicatrices* and cankers would be positively correlated. This prediction was based on the idea that *M. macrocicatrices* was creating feeding wounds that were then colonized by canker-forming fungi, similar to other *Matsucoccus* insect-fungus systems (e.g. Furniss and Carolin, 1977). These fungi then create cankers that slowly amalgamate, cut off the flow of water and nutrients, and ultimately lead to stress and mortality of the tree (Houston, 1994). For our third objective, we hypothesized that cankers and *M. macrocicatrices* would be more prevalent on the older tissue located at the base of the stem of the eastern white pine seedlings. For our fourth objective, we hypothesized that many of the isolates from the cankers without obvious fruiting structures of *C. pinea* would be ubiquitous, non-pathogenic fungi or weakly pathogenic fungi. For our final objective, we hypothesized that fungi of unknown pathogenicity on eastern white pine were unlikely to cause a significant canker, while the pathogen, *C. pinea*, would cause a significant canker (Munck et al., 2015).

2. Materials and methods

2.1. Study sites

We established 40 sites in the major range of eastern white pine (Little, 1971) in the southern region of the Appalachian Mountains, specifically in Georgia, North Carolina, South Carolina, Tennessee, Virginia, and West Virginia (Schulz et al., 2018) (Fig. 3; Appendix A). One additional site, not used in Schulz et al., 2018, was established in South Carolina. Eight sites were also established in the northern portion of the Appalachian range, including the Atlantic Maritime Highlands of New Hampshire, Massachusetts and Maine (Fig. 3; Appendix A). These sites encompass the eastern temperate (Appalachian) and northern (mixed wood plain and Atlantic highland) forests of the eastern United States (CEC, 1997; Omernik and Griffith, 2014). The general soil orders (and dominant suborders) found at these sites include Inceptisols (Udepts), Ultisols (Udults), Spodosols (Orthods), and, to a lesser extent, Entisols (Orthents) (USDA NRCS, 1998; Wendel and Smith, 1990). Average annual precipitation was variable across the sites, with sites in the southern extent of the Appalachians (with a range of 1,095–1,316 mm) generally having higher annual precipitation than sites in the northern part of the Appalachian range (1,083–1,152 mm) (NOAA, 2014). Similar to annual precipitation, average annual

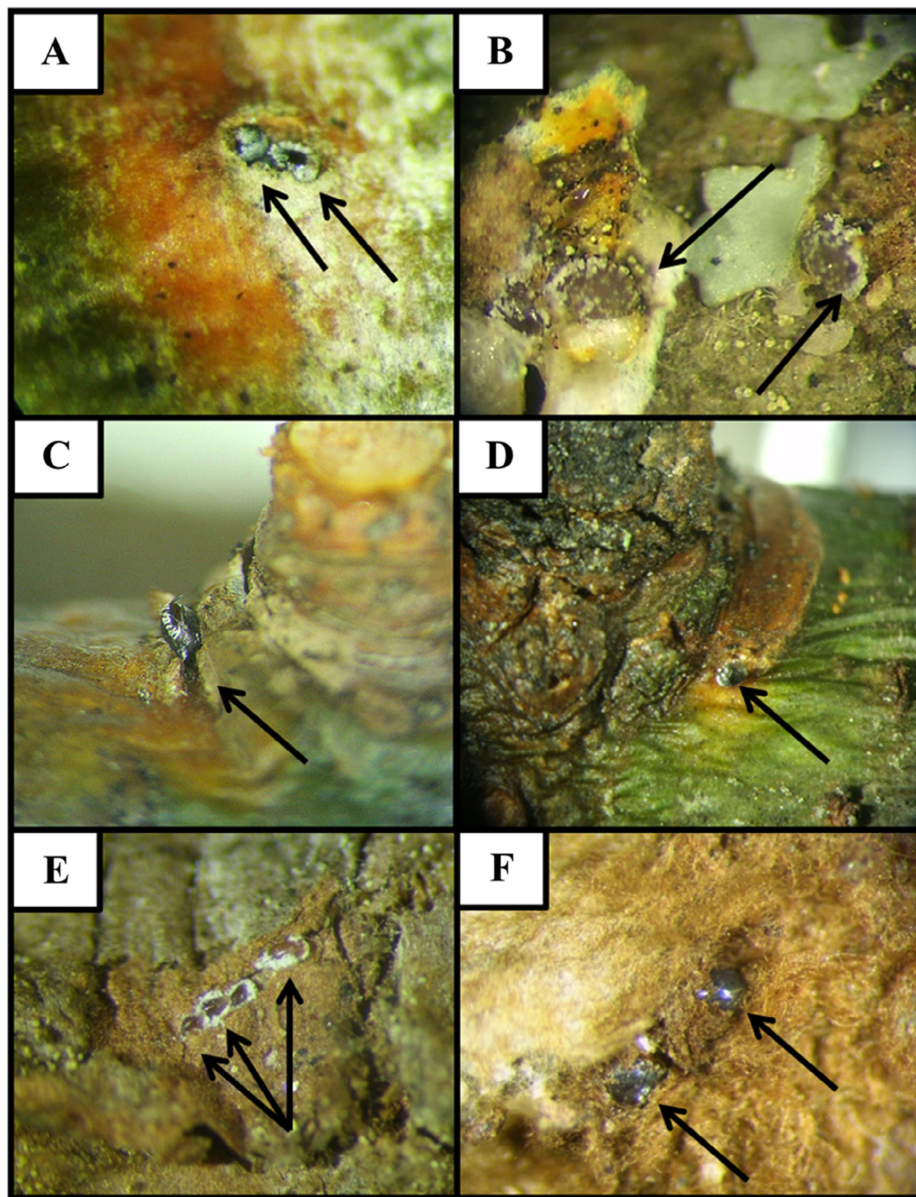

Fig. 1. Immature cyst stage of *Matsucoccus macrocicatrices* (A) in a leaf scar with apparent yellowing around their feeding site, (B) under a lichen, (C) wedged in a branch node, (D) settled and feeding on the edge of a canker, (E) in a canker, and (F) in a *Septobasidium pinicola* mat. Photos by A.N. Schulz.

temperature was higher in the southern Appalachians (10.9–17.4 °C) than the northern Appalachians (4.6–8.4 °C) (NOAA, 2014).

All sites were within one of five major Society of American Foresters (SAF) forest cover types for this region: red pine (Type 15), eastern white pine-northern red oak-red maple (Type 20), eastern white pine (Type 21), eastern white pine-eastern hemlock (Type 22), or eastern white pine-chestnut oak (Type 51). In the southern Appalachian region, eastern white pine occupied all crown classes, whereas, in the northern Appalachian region, eastern white pine was often a dominant or co-dominant tree. Other overstory vegetation within the southern and northern Appalachian sites included American beech (*Fagus grandifolia* Ehrh.), birch (*Betula* spp.), hemlock (*Tsuga canadensis* (L.) Carrière), maple (*Acer* spp.), pitch pine, red oak (*Quercus rubra* L.), red pine (*Pinus resinosa* Aiton), table mountain pine, tulip poplar (*Liriodendron tulipifera* L.), Virginia pine, and white oak (*Q. alba* L.) (Wendel and Smith 1990). Bracken fern (*Pteridium aquilinum* (L.) Kuhn), buckberry (*Vaccinium stamineum* L), dogwood (*Cornus* spp.), mountain laurel (*Kalmia latifolia* L.), and rhododendron (*Rhododendron maximum* L.) were all common understory species (Wendel and Smith 1990).

2.2. Collection of eastern white pine

Within each of the 41 sites in the southern Appalachian Mountains, we collected six eastern white pine seedlings (< 2.54 cm; n = 246) randomly and adjacent to permanent plots as established by Schulz et al. (2018). Three seedlings were collected from the eight sites in New Hampshire, Massachusetts, and Maine (n = 24), for a total of 270 seedlings. Seedlings in the southern region of the Appalachian Mountains were collected from January–August 2014, and seedlings in the northern region were collected in July and September 2014. Each of the 270 seedlings were assessed and given an overall dieback rating based on the proportion of live to dead nodes on the tree. Seedling length (cm) and small and large end diameters (mm) of the main stem were taken. These measurements were used to calculate the surface area (mm^2) of each seedling. Seedlings were stored in a refrigerator at 4.4 °C to prevent mold and temporarily preserve the material. Clippings from the seedling specimens from each site were vouchered by dry-pressing and deposited at The University of Georgia Herbarium [GA] in Athens, Georgia.

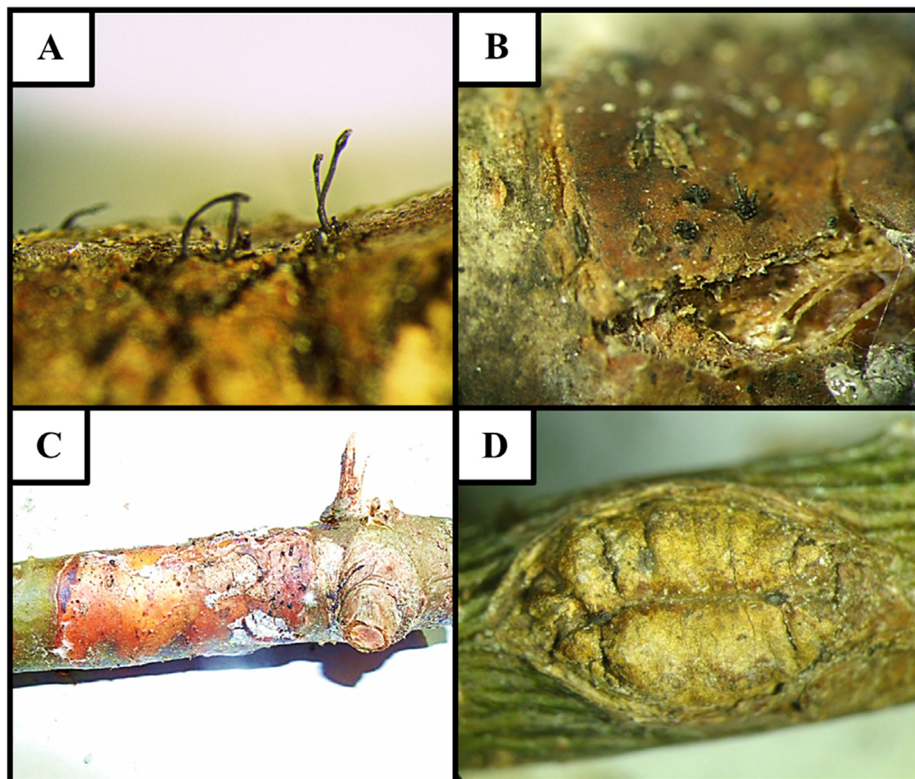

Fig. 2. Sexual (A) and asexual (B and C) structures of *Caliciopsis pinea* and cankers, and an example of other cankers (D) on the eastern white pine seedlings. Photos by A.N. Schulz.

2.3. Sampling of *Matsucoccus macrocicatrices*

We visually examined each seedling using a dissecting microscope and searched for all life stages of *M. macrocicatrices*, including eggs, crawlers, cysts, shells, and adults. Due to the time of sampling (January–September 2014) and the hypothesized phenology of the insect (i.e., they were not hatching or emerging at the time when we cut the seedlings), we only found cysts and shells on the seedlings (see Costanza et al., 2018 for the hypothesized life-cycle of the insect). A subset of cysts were preserved in ethanol and deposited at The University of Georgia, Museum of Natural History in Athens, Georgia.

2.4. Sampling of fungal species

For each seedling, we assessed the size (using a mm^2 grid) and location (B1: first branch whorl and below, B2: second branch whorl to just above first branch whorl, etc.) of cankers, and the presence/absence of *S. pinicola*, which is identifiable by the presence of dark brown hyphae surrounded by light brown to cream colored hyphae (Fig. 1F) (Snell, 1922). Cankers were identified as having either *C. pinea* or other unknown fungi. *Caliciopsis pinea* cankers were identifiable by their characteristic asexual spermagonia and sexual ascocarp fruiting structures (Fig. 2A and B). For the southern Appalachian region, any cankers > 10 mm^2 that were not identified as *C. pinea* and any tissue that did not have visible canker, but had at least one settled *M. macrocicatrices* were extracted and preserved for further analyses (Fig. 2D). All fungi samples were stored in individual sterile sampling bags in a 4.4 °C refrigerator until they were delivered to the USDA Forest Service in Athens, Georgia for isolation. Samples were maintained at 4.4 °C at the USDA facility during the one to two days of processing for fungal isolation.

Cankers processed for fungal isolation were categorized as either: (1) *M. macrocicatrices* with no canker; (2) *M. macrocicatrices* absent but canker present; or (3) both canker and *M. macrocicatrices* present. The outer bark surface of each canker was shaved off, and cankers that were > 3 mm were divided into three parts and sliced off the branch at a depth of 2–5 mm. Pieces of canker were surface sterilized in 95% ethanol for 10 seconds, then soaked in 1.05% NaOCl solution for four minutes, washed in sterile water for one minute, and blotted dry with sterile paper towels (Blodgett and Stanosz, 1997). Three pieces from each canker were placed on three types of media: modified Nash-Snyder media (Nelson et al., 1983), pine needle agar (PNA) (Blodgett et al., 2003), and potato dextrose agar (PDA) with streptomycin and tergitol (PDA + S + T) (Steiner and Watson, 1965). Stem tissue that was associated with *M. macrocicatrices* and could not be divided was placed on PNA media. Plated samples were incubated at 20 °C for 4–8 weeks, with weekly observation for identification or transfer of isolates to other media. Sporulating cultures were single-spored and placed on PDA. Samples with unidentifiable mycelium isolates were transferred to carnation-leaf water agar (Nelson et al., 1983) or PNA in an attempt to induce the isolate to produce spores for identification. Secondary transfers were observed weekly for another four weeks. Fungi were identified morphologically to genus or labeled unknown if a morphological identification was not possible. Two *C. pinea* isolates were also obtained by collecting single ascospores from asci collected in Unicoi State Park, Georgia (34°43′20.14″N, 83°43′15.77″W) and Lone Mountain State Park, Tennessee (36°4′14.91″N, 84°32′44.80″W). These *C. pinea* isolates were used for comparison purposes during pathogenicity testing. Isolates from branches collected in Georgia, Tennessee, and South Carolina were initially placed in 10% glycerin at 20 °C for temporary storage, and all isolates were subsequently placed on or moved to PDA slants for up to six months prior to inoculation studies.

2.5. Identification of unknown canker samples

Several representative isolates of each genus or unknown morphological types were sent to Iowa State University for DNA sequencing either as living cultures on PDA or, in some cases, as dead frozen cultures. DNA was extracted from hyphae and spores using PrepMan™ Ultra (Applied Biosystems, Foster City, California). Amplification of the

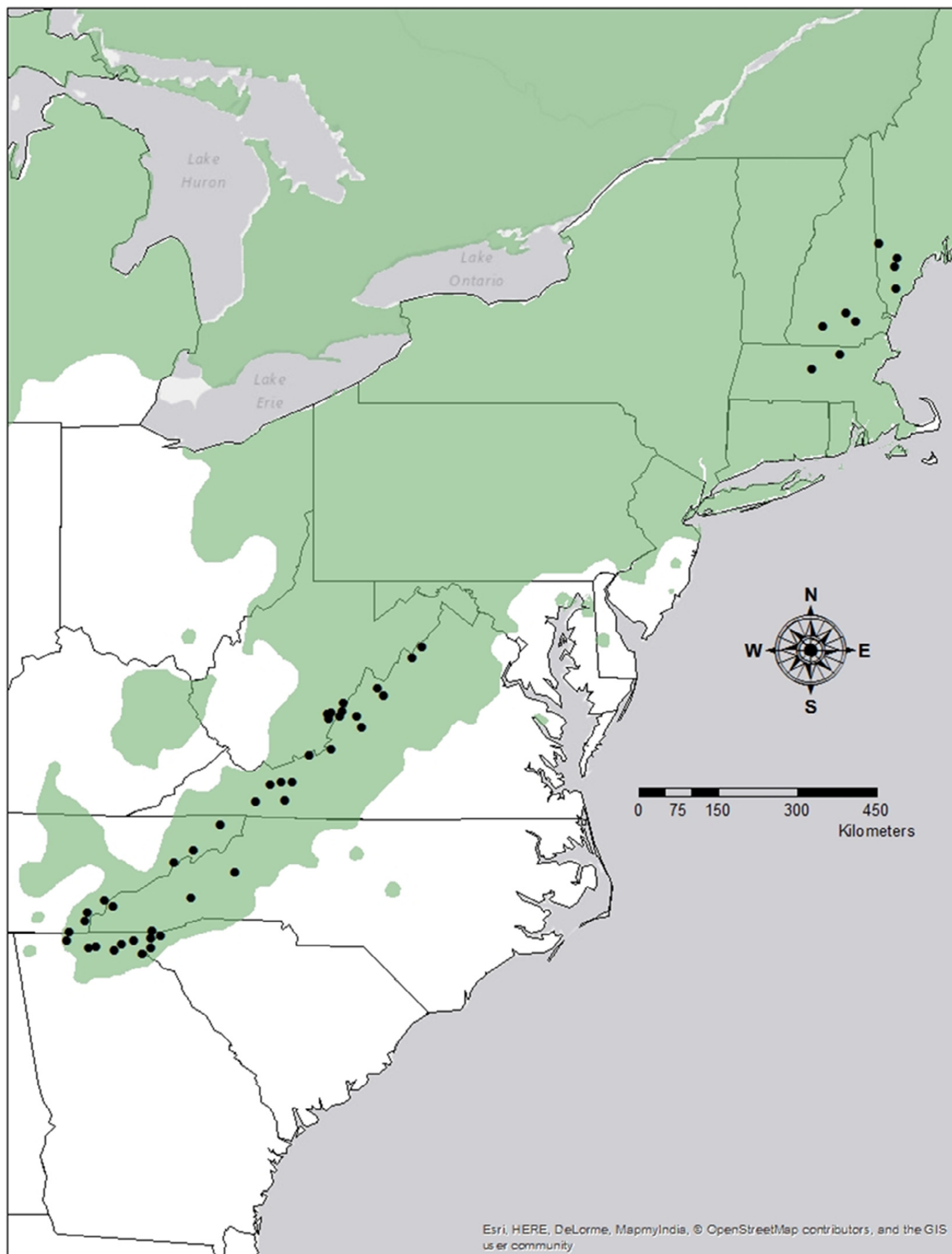

Fig. 3. Distribution of eastern white pine seedling collection sites in the eastern United States. Black dots indicate site locations, and the green area indicates the native distribution of eastern white pine. Map created using ArcGIS 10.2 (ESRI 2013).

ITS rDNA region utilized primers ITS1F (5′-CTTGGTCATTTAGAGGAA GTAA-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3) (Schoch et al., 2012), as well as the following cycling conditions: 85 °C for 2 min, 95 °C for 95 s, and then 36 cycles of 58 °C for 1 min, 72 °C for 80 s, and 95 °C for 70 s, followed by 52 °C for 1 min, and 72 °C for 15 min. The fragments were purified using Ilustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare Life Sciences, Buckinghamshire, United Kingdom) and sequenced at the Iowa State University DNA Facility. In most cases, quality sequences were obtained with the ITS1F primer only. If a better sequence was needed, then the PCR product was also sequenced with ITS4, and a consensus sequence between the forward and reverse-complement sequence was developed. The sequences were compared to GenBank and other accessions using BLAST searches (NCBI). If a clear ITS sequence could not be obtained or there was no close match, then the D1/D2 region of the large subunit (LSU, 28S rDNA) was amplified using primers LROR and LR5, the region was sequenced with the primer LR3, and a BLAST search was performed as for the ITS sequences (Xu et al., 2010). Taxa that had the most similar ITS sequences, at ≥99%, were considered the most closely related species (Gazis et al., 2011). In cases where an isolate did not have a close match, the isolate morphology and percent similarity of sequences to other referenced fungi were used to determine the likely genus, family, or order. Canker samples that yielded mold contaminants, such as *Penicillium* spp., or lichenized fungi, such as *Sarea* spp., were removed

from the data analysis. A total of 278 cankers were analyzed for this study.

2.6. Pathogenicity testing of fungal isolates

Containerized eastern white pine seedlings used for the pathogenicity testing were obtained from Linville River Nursery (Crossnore, North Carolina) from one seed source (seedlot WP-USFS-PU-O-03-6-M-N: USDA Forest Service, Cleveland, Tennessee). We transplanted 250 dormant, two-year old seedlings on 23 February 2015 into D40 cells (656 mL) and placed them in the greenhouse of the USDA Forest Service Resistance Screening Center, Asheville, North Carolina. Seedlings were maintained at a temperature range of 17 °C to 26 °C and watered by hand three days a week until saturated. Seedlings were fertilized during the 2015 and 2016 growing seasons with a water-soluble fertilizer at 15-30-15 (Plantex®) at a concentrated rate of 1.25 lbs per gallon of water, dispensed at a rate of 1:100 with a Dosatron chemical injector. The first pathogenicity trial of fungi isolates was established in the greenhouse on 19 October 2015, and the second trial was established 1 March 2016. The first trial consisted of 24 treatments with five replications each for a total of 120 seedlings, and the second trial consisted of 20 treatments with five replications each for a total of 100 seedlings. The containerized seedlings were slightly larger in diameter in the second trial. The average diameter of seedlings just below the inoculation site was 7.1 mm for the first trial and 8.1 mm for the second trial. Fungal isolates for pathogenicity testing were selected based on their frequency of isolation and high similarity to the nearest ITS match (Appendix B). Fungal isolates used in each trial were transferred to PDA plates four weeks prior to inoculation (Table 1).

Containerized seedlings were inoculated by removing the epidermis and phloem on the stem at approximately 12 cm above the soil line with a 2 mm cork borer. Mycelium from isolates and blank PDA media were cut with a 3 mm cork borer to form plugs, which were placed into the wound. Cork borers and probes were flame-sterilized in between samples to reduce any potential cross contamination. Plugs were secured with Parafilm® M film wrapped around the stem. Inoculation treatments were replicated five times, and completely randomized on the greenhouse bench. Inoculated stems were collected after 19 weeks for both trials. Cankers were measured by affected surface area of the stem using a mm^2 grid. Affected surface area was defined as having discolored tissue (brown to yellow brown), and measurements included the inoculation area where the epidermis was removed. Healthy stem tissue of seedlings was green in color.

All inoculated stems were processed for reisolation for fungi. The stem was cut above and below the inoculation area and then cut in half length-wise. The inoculated stem sections were surface sterilized with 95% ethanol for 10 s, then soaked in 1.05% NaOCl solution for four minutes, washed in sterile water for one minute, and blotted dry with sterile paper towels (Blodgett and Stanosz, 1997). The stem pieces were then placed on PNA with a sterilized section of an eastern white pine branch, or on PDA + S + T media. Identification of fungi from inoculated stems was performed after four to six weeks. Identification was based on spore production and morphology matching inoculant cultures (Table 1).

2.7. Statistical analyses

For each of the 270 eastern white pine seedlings, we counted the total number of *M. macrocicatrices* (cysts and shells) and assessed seedling dieback based on the proportion of dead nodes. Since surface area of each seedling varied, we standardized both the *M. macrocicatrices* and canker data by dividing the total number of *M. macrocicatrices* and the total canker coverage (mm^2) by the respective surface area of each seedling, thus creating proportions. After standardization, all data were analyzed using R v. 3.4.0 (R Core Team, 2017). The data were first checked for normality using a Shapiro-Wilk normality test.

Table 1
Lesion area (mm^2) development and re-isolation success (%) of fungi from three-year-old eastern white pine seedlings 19 weeks after inoculation in trials one (October - March) and two (March - July).

			Trial 1		Trial 2	
Code	Taxon	Nearest ITS	Lesion area mm^2 (± SE)	Re-isolation	Lesion area mm^2 (± SE)	Re- isolation
G3	*Caliciopsis pinea* Peck	KP881691	**157.8 (± 31)***	80	**640.4 (± 60.9)***	100
CPtn	*Caliciopsis pinea* Peck	KP881691	**292.6 (± 77.1)***	60	**712.2 (± 42.8)***	100
CPga	*Caliciopsis pinea* Peck	KP881691	–	–	**612.2 (± 155.1)***	100
M10	*Cytospora* sp.	KC464341	37.0 (± 3.1)	100	58.2 (± 5.9)	60
N6	*Cytospora* sp.	AB470827	39.2 (± 4.2)	60	105.6 (± 9.1)	40
N7	*Coniochaeta velutina* (Fuckel) Cooke	JQ346221	37.4 (± 1.1)	60	94 (± 17.6)	80
N8	*Coniochaeta velutina* (Fuckel) Cooke	JQ346221	53.8 (± 3.3)	40	57.8 (± 10.5)	60
C12	*Diaporthe phaseolorum* (Cooke & Ellis) Sacc.	AF001018	61.0 (± 2.8)	100	–	–
L10	*Diaporthe eres* Nitschke	KJ210516	61.2 (± 1.7)	60	–	–
L15	*Diaporthe eres* Nitschke	KJ210520	33.6 (± 3.6)	80	59.2 (± 5.8)	100
L7	*Diplodia scrobiculata* J. de Wet, Slippers & M.J. Wingf.	KF766160	37.8 (± 0.6)	80	151 (± 19.9)	80
M16	*Diplodia scrobiculata* J. de Wet, Slippers & M.J. Wingf.	KF766160	48.2 (± 3.7)	100	215.8 (± 120.1)	60
G7	*Paraconiothyrium brasiliense* Verkley	KP050565	60.4 (± 1.7)	60	109 (± 9.5)	100
L1	*Pestalotiopsis* sp.	KP900734	40.4 (± 4.3)	80	–	–
L8	*Pestalotiopsis* sp.	KP900734	68.8 (± 5.5)	80	91.8 (± 5.6)	100
L9	*Pestalotiopsis uvicola* (Speg.) Bissett	KF374685	48.6 (± 4.8)	80	102.8 (± 8.2)	100
L12	*Pezicula cinnamomea* (DC.) Sacc.	KF376148	43.2 (± 3.7)	100	102.8 (± 8.0)	80
F9	*Pezicula cinnamomea* (DC.) Sacc.	KF376148	66.2 (± 11.1)	100	56.8 (± 3.2)	100
B6	*Phaeomoniella* sp.	JX421733	57.2 (± 3.4)	100	63 (± 5.2)	100
D6	*Phaeomoniella* sp.	JF440607	39.6 (± 2.6)	80	–	–
G2	*Phaeomoniella* sp.	JX421733	–	–	118.4 (± 6.1)	100
A8	*Phomopsis quercella* (Sacc. & Roum.) Died.	AY853216	37.8 (± 2.6)	100	128.6 (± 5.3)	100
C13	*Phomopsis quercella* (Sacc. & Roum.) Died.	AY853216	58.8 (± 5.3)	100	67.6 (± 6.9)	100
D4	*Xylaria* sp.	HQ608148	34.6 (± 3.5)	60	–	–
F10	*Xylaria acuta* Peck	DQ491493	49.0 (± 4.3)	0	–	–
Blank	Blank PDA		39.4 (± 2.6)		84.8 (± 6.8)	

-Isolate not used in trial.
* Lesion area (± SE) was significantly different than the control (Blank PDA). Data were analyzed with Welch's ANOVA and Dunnett's test for comparisons of lesion area vs. the control.

Since data were not normal, we used a non-parametric Spearman's Rank Correlation Coefficient (function: *cor.test,* method = "spearman") to analyze the associations among *M. macrocicatrices*, cankers, and eastern white pine seedling dieback within the southern and northern regions of the Appalachian Mountains. For each region, we used these correlations to specifically assess the associations between: (1) the proportion of *M. macrocicatrices* and eastern white pine seedling dieback; (2) the proportion of total canker and dieback; and (3) the proportions of *M. macrocicatrices* and total canker area. Since many types of cankers were present on the seedlings, we calculated the percent of *M. macrocicatrices* per seedling found either: (1) in cankers with fruiting bodies of *C. pinea*; (2) in other cankers with no obvious fruiting bodies of *C. pinea*; and (3) without a canker (usually under lichen, moss, or in a node). Kruskal-Wallis Rank Sum tests (function: *kruskal.test*) were used to compare these three groups within each of the two regions.

To assess the qualitative spatial distribution of the total canker surface area (cm^2) within eastern white pine seedlings, we summed the total canker area for each portion (e.g., B1, B2, B3, B4, etc.) of the 270 seedlings. For instance, B1 includes all of the cankers from the first eastern white pine branch whorl to the base; B2 includes all of the cankers from the second branch whorl to just above the first branch whorl, etc. Similarly, we assessed the distribution of *M. macrocicatrices* on the seedlings, where we summed the total number of *M. macrocicatrices* for each portion of the seedlings.

Data from the pathogenicity trials were analyzed using SAS software (SAS 9.4, SAS Institute, Inc., Cary, North Carolina). All data were evaluated for normality using a Shapiro-Wilk normality test, and residuals were used to test homogeneity of variances. Some treatments were not normally distributed, and a comparison of residuals indicated non-homogeneity of variance. As a result, data were analyzed with Welch's ANOVA ($P < 0.05$) and Dunnett's test ($\alpha = 0.05$) for comparisons of canker area of fungi versus the blank PDA control inoculations.

3. Results

Matsucoccus macrocicatrices scale insects were found on 116 of the 246 eastern white pine seedlings from the southern Appalachian region (Georgia, North Carolina, South Carolina, Tennessee, Virginia, and West Virginia) in the United States. We found a total of 2,402 shells and cysts with a mean ($\pm$ SE) of 9.8 $\pm$ 1.9, and range of 0 (absence) to 265 individual *M. macrocicatrices* per seedling. Approximately 53% of the seedlings had no *M. macrocicatrices*, 37% had between 1 and 20 individuals of *M. macrocicatrices*, and 10% had $\geq$21 individuals of *M. macrocicatrices* (Fig. 4A). Cankers or lesions were found on 235 of the 246 seedlings. Seedlings had cankers with a mean ($\pm$ SE) surface area (cm^2) on each seedling of 87.9 $\pm$ 9.7, and ranged from 0.6 to 1,054.3. Mean ($\pm$ SE) *C. pinea* canker size (cm^2) was 114.9 $\pm$ 16.2 (N = 651), while the mean for other types of cankers was 31.2 $\pm$ 2.9. *Septobasidium pinicola* was collected from three seedlings from three different study sites: two in Virginia and one in West Virginia.

Matsucoccus macrocicatrices scale insects were very abundant on all 24 seedlings from the northern Appalachian region (Maine, Massachusetts, and New Hampshire). We found a total of 929 shells and cysts with a mean ($\pm$ SE) of 38.7 $\pm$ 7.1, a nearly fourfold increase, and a range of 1 to 110 individual *M. macrocicatrices* per seedling. Altogether, none of the seedlings had no *M. macrocicatrices*, 46% had between 1 and 20 individual *M. macrocicatrices,* and 54% had $\geq$21 individual *M. macrocicatrices* (Fig. 4B). Cankers were also found on all 24 seedlings. Mean ($\pm$ SE) canker surface area (cm^2) on each seedling was 277.9 $\pm$ 43.9, and ranged from 25.6 to 812.6 cm^2. Mean ($\pm$ SE) *C. pinea* canker size (cm^2) was 270.7 $\pm$ 51.5 (N = 75), while the mean size of other cankers was 97.5 $\pm$ 10.7. *Septobasidium pinicola* was collected from two seedlings originating from two separate study sites in Maine and New Hampshire.

Over 95% of *M. macrocicatrices* were found to be associated with

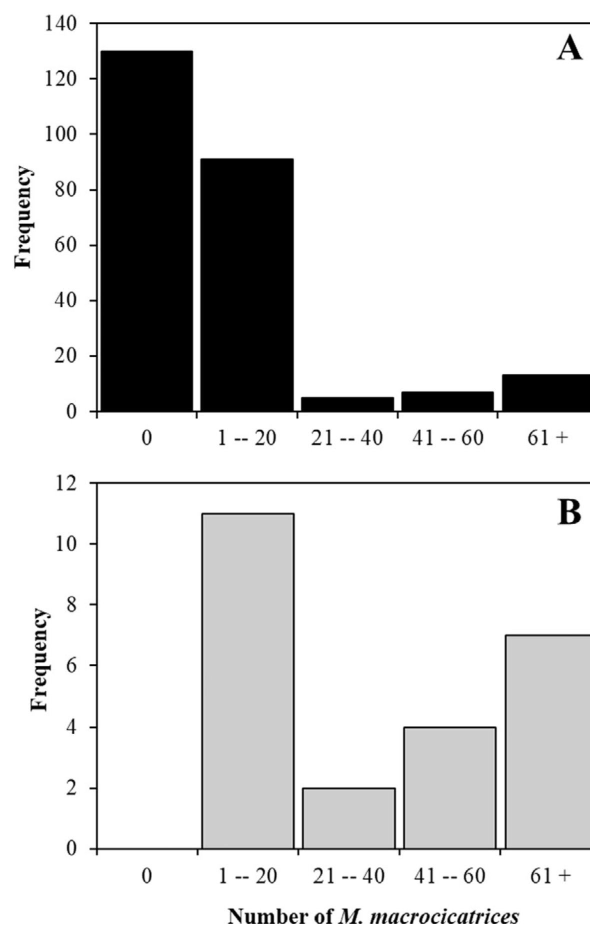

Fig. 4. Frequency of observation of 0, 1–20, 21–40, 41–60, and ≥61 *Matsucoccus macrocicatrices* insects per seedling in the southern (A) and northern (B) Appalachian regions of the United States.

cankers on seedlings in the southern and northern Appalachians. The 246 seedlings from the southern Appalachian region had significantly different numbers of *M. macrocicatrices* associated with *C. pinea* cankers and no canker ($\chi^2 = 28.93$, df = 1, $P < 0.001$), as well as other cankers and no canker ($\chi^2 = 39.79$, df = 1, $P < 0.001$), but there were no differences between *C. pinea* and other cankers ($\chi^2 = 0.30$, df = 1, $P > 0.1$) (Fig. 5). Similarly, for the 24 seedlings from the northern Appalachian region, there were differences in the number of *M. macrocicatrices* found between *C. pinea* cankers and no canker ($\chi^2 = 9.63$, df = 1, $P = 0.002$), as well as other cankers and no canker ($\chi^2 = 21.42$,

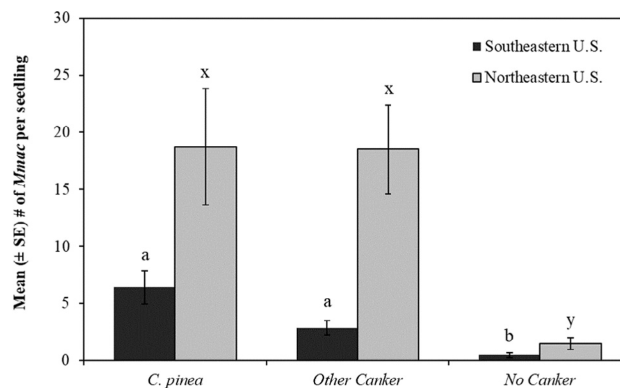

Fig. 5. Mean ($\pm$ SE) number of *Matsucoccus macrocicatrices* (*Mmac*) associated with *Caliciopsis pinea* cankers, other cankers (non-*Caliciopsis pinea* cankers), and no cankers in the southern Appalachian (black) and northern Appalachian (gray) seedlings. Different letters above bars indicate statistically significant differences.

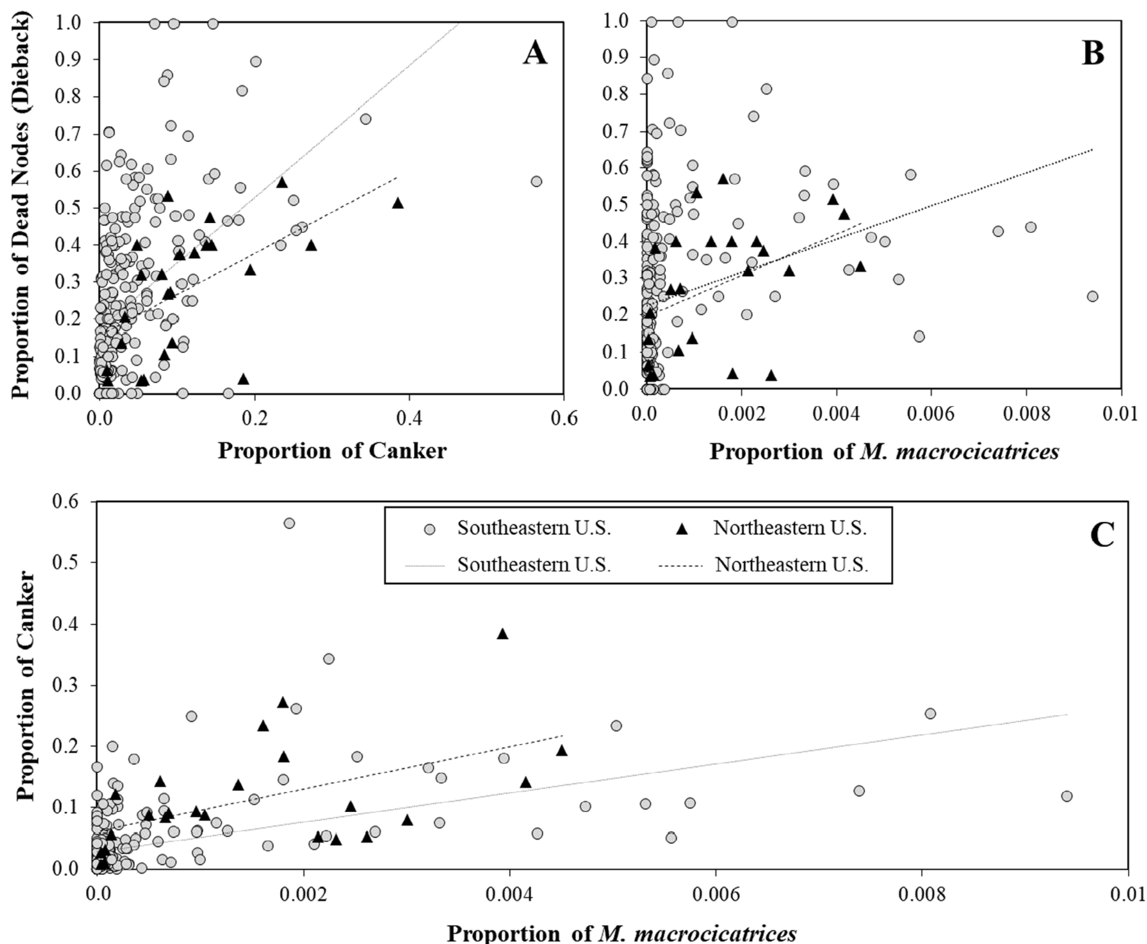

Fig. 6. Positive correlations between eastern white pine seedling dieback and proportion of total canker (A), seedling dieback and proportion of *Matsucoccus macrocicatrices* (B), and proportion of total canker and proportion of *Matsucoccus macrocicatrices* (C) in the southern (○) and northern (▲) portions of the Appalachian Mountains.

df = 1, $P < 0.001$), but there were no differences between *C. pinea* and other cankers ($\chi^2 = 0.45$, df = 1, $P > 0.1$) (Fig. 5). Overall, more *M. macrocicatrices* were associated with *C. pinea* cankers than other cankers or no canker, though there was no significant difference between the number of scale insects found in *C. pinea* cankers and other cankers.

In the 246 seedlings from the southern Appalachian region, we found positive correlations between the proportion of canker and seedling dieback ($r_s = 0.62$, $P < 0.001$, Fig. 6A), *M. macrocicatrices* and dieback ($r_s = 0.46$, $P < 0.001$, Fig. 6B), and *M. macrocicatrices* and total cankers ($r_s = 0.55$, $P < 0.001$, Fig. 6C). We found similar results for the 24 seedlings from the northern Appalachian region, in which there were positive correlations between the proportion of canker and dieback ($r_s = 0.62$, $P < 0.01$, Fig. 6A), *M. macrocicatrices* and dieback ($r_s = 0.45$, $P < 0.05$, Fig. 6B), and *M. macrocicatrices* and total cankers ($r_s = 0.52$, $P < 0.01$, Fig. 6C).

Approximately 36% of the total canker area (cm^2) observed on the 270 seedlings were found below the first branch whorl. Similarly, 29% of *M. macrocicatrices* were found below the first branch whorl, often in cankers or under moss or lichen growing at the base of the trees. Overall, the total canker area (cm^2) and number of *M. macrocicatrices* both decreased when going from the older, base tissue to the younger, meristem tissue (Fig. 7).

Fungal isolations from 278 cankers and lesions without the characteristic *C. pinea* fruiting structures resulted in only 1.4% being identified as *C. pinea* (Fig. 8). The most prevalent fungi isolated were in the genus *Phaeomoniella* (33.1%), the second most prevalent fungi were in the genera *Phomopsis-Diaporthe* (7.6%), and the rest of the genera were found to be ≤4% (Fig. 8). Fungi in the "other" category (10.8%) included approximately 27 different genera (listed in Appendix B). A further 9.7% of isolates were classified as "unknown," and 11.2% of the 278 cankers did not result in any fungal isolation (Fig. 8).

The Welch's ANOVA tests of the lesion area for both pathogenicity trials one ($F_{(23,34)} = 14.25$, $P < 0.001$) and two ($F_{(19,29)} = 22.50$, $P < 0.001$) were statistically significant. *Caliciopsis pinea* isolates caused significantly larger lesions than the PDA blanks in both trials 19 weeks after inoculations (Table 1). The *C. pinea* lesions formed girdling cankers on most of the seedlings, and often produced spermagonia fruiting structures. No other isolates were demonstrated to be statistically pathogenic, although, in trial two, the lesion development for *Diplodia scrobiculata* J. de Wet, Slippers & M.J. Wingf. (M16) was highly variable with one replicate forming a much greater (574 mm^2) lesion area (girdling canker) than the other replicates (70–130 mm^2). Occasionally, reisolation was affected by other secondary fungi, including: *Alternaria* spp., *Fusarium* spp., and *Pestalotiopsis* spp. These fungi were likely present as endophytes in the seedling stems prior to inoculation. *Xylaria* was the only genus not retested in the second trial due to the poor reisolation success in the first trial.

4. Discussion

Our findings demonstrate there were positive correlations between the dieback of eastern white pine seedlings, canker size, and *M. macrocicatrices* in the northern and southern Appalachian Mountain region in the United States. Furthermore, *M. macrocicatrices* was found on 66% and 48% of *C. pinea* cankers in southern and northern sites, respectively. The prevalence of *M. macrocicatrices* in *C. pinea* cankers indicates

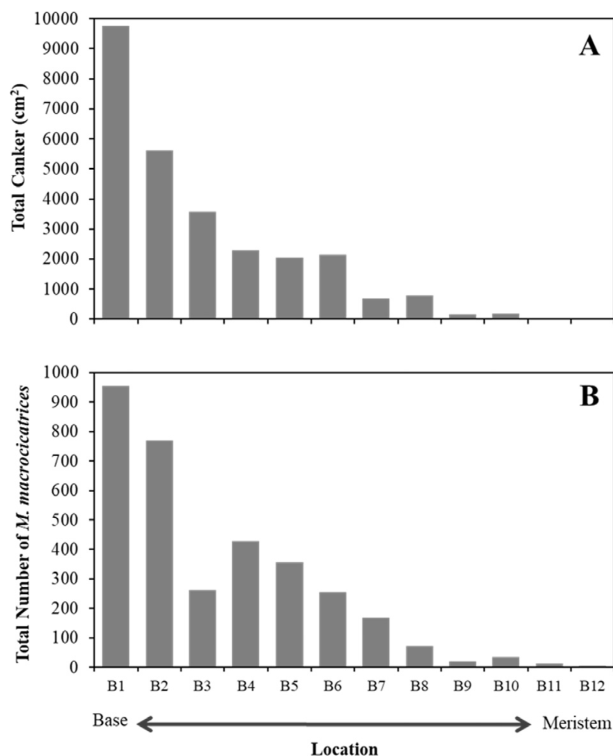

Fig. 7. Distribution of (A) the total canker surface area (cm^2), and (B) *Matsucoccus macrocicatrices* from the base to the meristem of the eastern white pine seedlings (N = 270).

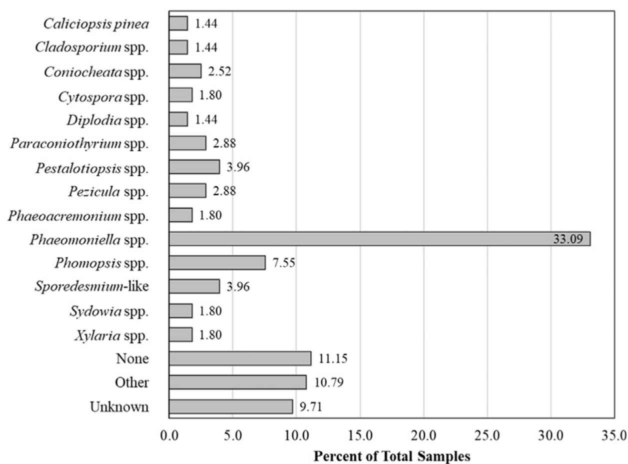

Fig. 8. Genera-level identification of fungi isolated from other cankers (N = 278) on the eastern white pine seedlings collected in the southern Appalachian Mountains. Other cankers did not have *C. pinea* fruiting bodies.

that the scale insect may assist in colonization of trees by the fungus, thereby enhancing spread. Similar to the beech scale (*Cryptococcus fagisuga* Lind.) on American beech, and Israeli pine bast scale (*Matsucoccus josephi* Bodenheimer and Harpaz) associated with *Sphaeropsis sapinea* (Fr.) on Aleppo pine (*Pinus halepensis* Mill.), *M. macrocicatrices* may be an important contributing factor since canker-forming fungi likely require entry wounds for the successful establishment of infection (Houston, 1994; Madar et al., 2005). We hypothesize that feeding wounds created by the stylet of *M. macrocicatrices* may allow the spores of canker-forming fungi entry into optimal tree tissues (Fig. 9A and B; Whitney et al., 2018). In the genus *Matsucoccus*, the Prescott scale (*Matsucoccus vexillorum* Morrison) has been documented to cause girdling lesions and necrosis on ponderosa pine (*Pinus ponderosa* Dougl. ex Laws), which then become infected with fungi that further girdle the tree, leading to mortality (Carter, 1952; Furniss and Carolin, 1977). Like other sapsucking insects, such as *M. josephi*, *M. macrocicatrices* may also be secreting defense-inhibiting saliva into the feeding wound, allowing the insect to feed freely in its cyst stage without having to combat tree defenses (Liphschitz and Mendel, 1989). We observed many cysts of *M. macrocicatrices* surrounded by yellowing tissue, which might be discolored due to feeding and/or salivary excretions (Fig. 1A and D). Defense-inhibiting salivary secretions could leave lasting damage in the tissues even after *M. macrocicatrices* emergence. This damage could predispose the tree to secondary invaders, such as opportunistic fungi, and prevent the tree from defending itself (Jactel et al., 2006). After successful establishment in the feeding wounds left by the scale insect, the canker-forming fungus may be able to colonize the xylem tissues (Fig. 9C). The amalgamation of cankers cuts off the flow of water and nutrients through the tracheids of the xylem, which results in reduced radial growth, crown dieback, girdled branches, and eventual mortality (Coulson and Witter, 1984; Houston, 1994).

There may also be an inverse relationship in which the callous tissue that develops as a defense around cankers may benefit *M. macrocicatrices*, as has been suggested for related systems (Houston, 1994). Our observations indicate that the living edge of a canker will curl up, which provides a crack or ridge in the tissue where the immature stages of *M. macrocicatrices* can settle (Fig. 1D and 9D). Since *M. macrocicatrices* spends most of its life-cycle in the cyst stage, nestling into branch crotches or the margins of a canker allows it to passively remain secured to the tree. If *M. macrocicatrices* settles on the edge of a canker, it can feed by probing its long stylet into the living tissue surrounding the cankerous tissue. *Matsucoccus macrocicatrices* can thus create additional wounds for canker-forming fungal spores to more easily establish. This provides a positive-feedback between the tree and insect, generating increased incidence of canker formation on trees. It is important to note that the exact mechanistic contribution(s) of *M. macrocicatrices* and fungal pathogens, like *C. pinea*, to the phenomenon of eastern white pine dieback remain unclear.

Septobasidium pinicola was found on seedlings from sites in Maine, New Hampshire, Virginia, and West Virginia. Early descriptions of *M. macrocicatrices* and *S. pinicola* suggest that *S. pinicola* lives as an epiphyte on eastern white pine, but it parasitizes *M. macrocicatrices* (Snell, 1922). Although the relationship is not fully understood, *S. pinicola* may protect *M. macrocicatrices* from adverse weather conditions, and possibly from other natural enemies while deriving nourishment from the insect (Watson et al., 1960). Other studies within the genus *Septobasidium* also concluded that this fungus is either symbiotic with (Couch, 1931) or parasitic on *M. macrocicatrices* (Burt, 1916; Coker, 1920; Petch, 1921). Although *S. pinicola* was described as a species commonly found on the bark of eastern white pine stems (Watson et al., 1960), observations from this study indicate that the fungus appears to be rare. Additional research on the relationship between *M. macrocicatrices* and *S. pinicola* warrants consideration, despite its relatively low frequency of occurrence.

About two-thirds of the total assessed canker surface area was identified as *C. pinea* in sites located in both the northern and southern portions of the Appalachians. The majority of these cankers were identified based on the presence of *C. pinea* ascocarps and spermagonia fruiting structures. *Caliciopsis pinea* fruiting structures can be found on cankers throughout the year, allowing for year-round identification (McCormack, 1936; Ray, 1936). Our inoculation tests, as well as tests conducted in other studies, indicate that stromata and spermagonia fruiting structures of *C. pinea* can develop within three months after inoculation (Hepting and Roth, 1946; Ray, 1936). The readiness of *C. pinea* to produce spermagonia and the low isolation frequency of *C. pinea* from cankers and lesions with no fruiting structures indicate that the use of *C. pinea* fruiting bodies to confirm the presence of the pathogen is adequate for field identification and surveys.

We found a distinct spatial pattern of cankers and *M. macrocicatrices*

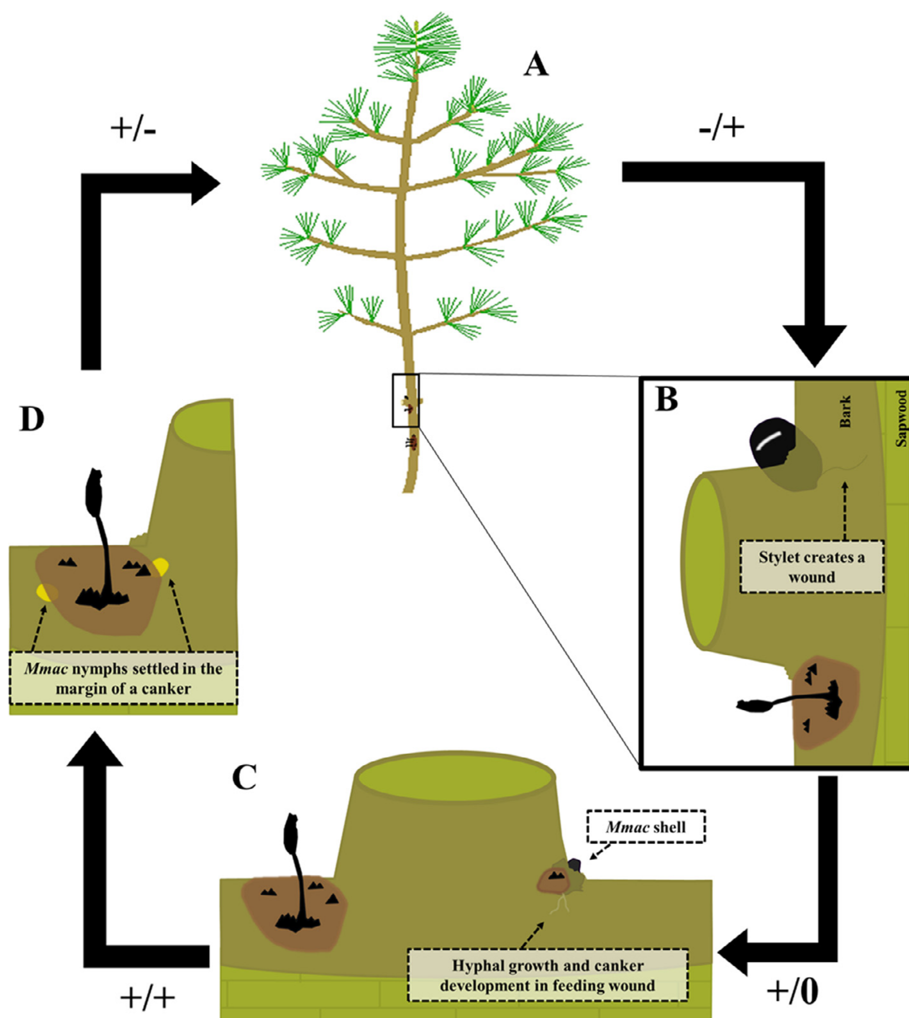

Fig. 9. Proposed complex, including (A) eastern white pine; (B) *Matsucoccus macrocicatrices (Mmac)*; and (C) cankers, which may (D) provide a crevice for more scale insects to colonize. Plus and minus signs represent the hypothesized feedback loop for this *M. macrocicatrices-C. pinea* complex. The seedling benefits (+) *M. macrocicatrices*, which then creates a feeding wound that benefits colonization of *C. pinea*. The cankers developed by *C. pinea* may in turn benefit the scale insects by providing a crevice in which to settle. Both *M. macrocicatrices* and *C. pinea* cankers are a detriment (–) to the eastern white pine seedling.

on individual seedlings, where most of the cankers and scale insects were found at the base. Often times, ovoid to elongate cankers appeared as large swellings centered at the base of the eastern white pine stems. These larger, older cankers at the base of the stem may have been present longer than the younger, smaller cankers on the younger tree tissue. Similar to bark beetles (Coulson, 1979), girdling of the stem at the base is more detrimental and damaging than girdling at the apical portion of the tree. As cankers continue to grow over time, there may be little chance for seedlings and small diameter saplings to recover from the cutoff of vital nutrients and water, thus reducing the overall fitness of the tree. Therefore, the most severe symptoms of dieback are expressed in the seedling (≤2.54 cm) and sapling (2.54–12.45 cm) size classes (Schulz et al., 2018). Similar tree-level colonization patterns by both focal organisms have been reported in sapling-, poletimber- (12.7–22.61 cm), and sawtimber-sized (≥22.86 cm) eastern white pine trees (Whitney et al., 2018). Specifically, the bottom-up pattern of presence and relative abundance of both *M. macrocicatrices* and cankers were greatest at lower canopy levels and most abundant on smaller trees (Whitney et al., 2018).

The isolation of fungi from the other cankers and tissue surrounding *M. macrocicatrices* were diverse and reflective of the types of genera that are associated with other tree species (Damm et al., 2010; Kowalski and Kehr, 1992; Sieber, 2007). The most commonly isolated genus, *Phaeomoniella*, was not found to be pathogenic in our study, and appears to be an endophyte on eastern white pine. Although *Phaeomoniella* species can be pathogenic (e.g., causing lesions on apricot), they are more typically endophytic on *Pinus* and *Prunus* (Damm et al., 2010; Sanz-Ros et al., 2015). The other endophytic fungi isolated from the seedlings are common on conifers and are not considered highly virulent (Kowalski and Kehr, 1992; Sieber, 2007). Fungi often classified as pathogenic, such as those in the *Phomopsis*, *Pezicula*, *Diplodia*, and *Cytospora* genera, are able to infect host tissue and enter into a quiescent state until conditions become unfavorable for the host (Ooki et al., 2003; Schulz and Boyle, 2005; Sieber, 2007). Many endophytic species are opportunists and could cause disease of hosts compromised by other factors (Schulz and Boyle, 2005; Sieber, 2007). Results from the pathogenicity tests indicate that *C. pinea* was the only proven pathogen that caused significant canker growth on healthy eastern white pine tissue. Based on past research, *C. pinea* would be expected to create sharply delimited cankers on the trunks and branches of otherwise healthy seedlings (Funk, 1963; Munck et al., 2015; Ray, 1936). This is supported by recent reports that have indicated *C. pinea* may be a major factor affecting eastern white pine health in the northeastern United States (Munck et al., 2015; Rose, 2011; Rosenholm, 2012). *Diplodia scrobiculata* has been found to be variable in producing lesions on eastern white pine saplings in Virginia (Cram et al., 2009), and is considered a latent-weak pathogen on other conifers (Blodgett and Stanosz, 1997; Santamaría et al., 2011). The isolates of *D. scrobiculata* used in our trials primarily

had no effect, with the exception of one replicate. These results indicate that the condition of the host tree appears to determine the ability of *D. scrobiculata* to produce disease, which is true of many endophytes with low virulence.

Most of the other taxa tested for pathogenicity in this study have been noted as pathogens of other tree species in other studies (Damm et al., 2010; Gramaje et al., 2012; Ooki et al., 2003; Thomidis and Michailides, 2009). However, in eastern white pine, these other taxa are likely endophytic fungi that were present prior to damage by the *M. macrocicatrices* scale insect or other damaging agents (e.g., falling branches, insect feeding, etc.). Endophytic fungi are ubiquitous on healthy as well as damaged and dying tree hosts (Sieber, 1989; Kowalski and Kehr, 1992). The endophytic fungi-host interaction is postulated to range from saprophytic to mutualistic to weakly pathogenic (Schulz and Boyle, 2005; Sieber, 2007). Ultimately, these types of fungi are beneficial in the natural pruning process and breakdown of dying branches (Kowalski and Kehr, 1992; Ooki et al., 2003), and are unlikely contributors to the current and emerging eastern white pine dieback phenomenon.

5. Conclusions

Overall, our results indicate that (1) *M. macrocicatrices*, cankers, and pathogenic fungi, such as *C. pinea*, are associated with eastern white pine seedling dieback in the mixed hardwood-conifer forests of the Appalachian Mountains; (2) more cankers and *M. macrocicatrices* occur at the base of seedlings; and (3) *C. pinea* was the only fungal species that produced large cankers, while the other isolated fungi were primarily endophytic. Although our results have demonstrated positive relationships between this novel insect-pathogen complex and eastern white pine dieback, there is still much to be learned about the mechanisms contributing to and the long-term consequences of eastern white pine dieback. It is imperative to gain a better understanding of what is driving the spread of this insect-pathogen complex and the rate of progression. Contemporary research of disease complexes such as beech bark disease have suggested that insect-pathogen interactions often involve pest-induced changes in host physiology or antagonisms among plant defense hormones (Cale et al., 2015a; Hatcher, 1995; Stout et al., 2006; Thaler et al., 2002). Factors such as soil and bark chemistry, temperature, precipitation, host population diversity and dynamics, and insect and pathogen dispersal syndromes may be involved in the success or failure of the insects and pathogens in the insect-pathogen complexes (Cale et al., 2015b; McClure, 1989). Future research may aim to determine: (1) feeding mechanisms, reproductive strategies, and phenology of *M. macrocicatrices*; (2) the relationship between *S. pinicola* and *M. macrocicatrices*; and (3) strategies to best manage pathogenic fungi and *M. macrocicatrices*. The results from this study and future research on eastern white pine dieback will help direct management decisions that will promote eastern white pine health, including seedling and sapling health, to ensure that regeneration is able to survive to replace senescing mature eastern white pine trees in eastern North America.

Acknowledgements

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Appendix A

Eastern white pine collection locations in the eastern United States.

State	County	National forest	Latitude and longitude	Collection date
Georgia	Habersham	Chattahoochee	N34.69767°, W83.41489°	1/31/2014
	Rabun	Chattahoochee	N34.92200°, W83.25822°	2/5/2014
	Rabun	Chattahoochee	N34.88304°, W83.56023°	2/21/2014
	Towns	Chattahoochee	N34.83934°, W83.76933°	2/25/2014
	Union	Chattahoochee	N34.75518°, W83.89426°	2/26/2014
	Murray	Chattahoochee	N34.87796°, W84.70926°	3/9/2014
	Gilmer	Chattahoochee	N34.77939°, W84.32744°	3/9/2014
	Fannin	Chattahoochee	N34.79816°, W84.18977°	3/11/2014
North Carolina	Buncombe	Pisgah	N35.48113°, W82.59150°	6/16/2014
	Burke	Pisgah	N35.82455°, W81.84663°	6/16/2014
	Graham	Nantahala	N35.35266°, W83.90728°	6/17/2014
	Macon	Nantahala	N35.00928°, W83.24178°	6/17/2014
South Carolina	Oconee	Sumter	N34.96559°, W83.09306°	3/19/2014
	Oconee	Sumter	N34.79610°, W83.20348°	4/15/2014
	Oconee	Sumter	N34.79885°, W83.31249°	5/21/2014
Tennessee	Monroe	Cherokee	N35.26812°, W84.33806°	3/30/2014
	Polk	Cherokee	N35.15207°, W84.37422°	3/30/2014
	Sullivan	Cherokee	N36.48812°, W82.08171°	4/22/2014
	Unicoi	Cherokee	N36.12572°, W82.53853°	4/22/2014
	Greene	Cherokee	N35.97279°, W82.85342°	4/23/2014
	Monroe	Cherokee	N35.43005°, W84.06290°	4/23/2014
	Polk	Cherokee	N34.99749°, W84.63921°	5/30/2014

Virginia	Smyth	Jefferson	N36.79331°, W81.49586°	7/7/2014
	Carroll	Jefferson	N36.79961°, W80.98392°	7/7/2014
	Wythe	Jefferson	N37.01903°, W81.23375°	7/8/2014
	Bland	Jefferson	N37.05272°, W81.06964°	7/8/2014
	Pulaski	Jefferson	N37.05269°, W80.87303°	7/8/2014
	Giles	Jefferson	N37.41422°, W80.59153°	7/9/2014
	Craig	Jefferson	N37.49433°, W80.19567°	7/9/2014
	Alleghany	Jefferson	N37.78928°, W79.70103°	7/9/2014
	Bath	George Washington	N37.92225°, W79.79086°	7/10/2014
	Highland	George Washington	N38.30536°, W79.43025°	7/10/2014
	Augusta	George Washington	N38.22136°, W79.32392°	7/10/2014
	Rockingham	George Washington	N38.71194°, W78.84325°	7/11/2014
	Shenandoah	George Washington	N38.86669°, W78.68550°	7/11/2014
West Virginia	Greenbrier	Monongahela	N37.98781°, W80.21772°	8/14/2014
	Greenbrier	Monongahela	N37.97897°, W80.28106°	8/14/2014
	Greenbrier	Monongahela	N37.90417°, W80.25161°	8/14/2014
	Greenbrier	Monongahela	N38.00278°, W80.02261°	8/14/2014
	Pocahontas	Monongahela	N38.11914°, W80.01197°	8/15/2014
	Greenbrier	Monongahela	N37.94239°, W80.07422°	8/15/2014
Maine	York		N43.426772°, W70.649808°	9/19/2014
	York		N43.705993°, W70.674035°	9/19/2014
	Cumberland		N43.809592°, W70.638708°	9/19/2014
	Oxford		N43.98127°, W70.93385°	9/19/2014
Massachusetts	Middlesex		N42.618196°, W71.61146°	7/25/2014
New Hampshire	Hillsborough		N42.961983°, W71.884363°	7/20/2014
	Rockingham		N43.018726°, W71.326695°	9/8/2014
	Merrimack		N43.133605°, W71.493854°	9/8/2014

Appendix B

Taxon table of fungi identified from other cankers without the characteristic *Caliciopsis pinea* fruiting structures taken from seedlings collected in the Southern Appalachian Mountains, United States.

Code	Taxon	Closest blast match (GenBank accession No.)	Query/reference ITS length (Similarity %)
L2	*Alternaria sp.*	*Alternaria alternata* (LN808867) *Alternaria tenuissima* (KM979980)	482/482 (100) 482/482 (100)
G3	*Caliciopsis pinea*	*Caliciopsis pinea* (KP881691)	528/528 (100)
N5	*Capnodiales*	*Devriesia strelitziicola* (GU214635)	394/440 (89.5)
G1	*Capnodium* sp.	*Capnodium* sp. (HQ631045)	443/447 (99.1)
G12	*Capronia* sp.	*Capronia kleinmondensis* (EU552107)	501/530 (94.5)
M2	*Cladosporium cladosporioides*	*Cladosporium cladosporioides* (KM816685)	461/461 (100)
N3	*Cladosporium sphaerospermum*	*Cladosporium sphaerospermum* (JX966572)	463/463 100)
F3	*Collophora* sp.	*Collophora hispanica* (JN808839)	432/464 (93.1)
C1	*Coniochaeta velutina*	*Coniochaeta velutina* (JQ346221)	485/485 (100)
N8	*Coniochaeta velutina*	*Conichaeta velutina* (JQ346221)	485/485 (100)
F16	*Cytospora* sp.	*Cytospora* sp. (KC464341)	515/521 (98.8)
M10	*Cytospora* sp.	*Cytospora* sp. (KC464341)	533/540 (98.7)
N6	*Cytospora* sp.	*Cytospora mali* (AB470827) *Cytospora chrysosperma* (KJ739458)	525/529 (99.2) 525/529 (99.2)
M15	Dermateaceae	*Pezicula* sp. (JN225939) *Dermea viburni* (AF141163)	479/502 (95.4) 472/497 (94.5)
F2	Dermateaceae	*Dermea acerina* (AF141164) *Cryptosporiopsis diversispora* (JF340249)	449/492 (91.3) 429/473 (90.7)
L10	*Diaporthe eres*	*Diaporthe eres* (KJ210516) *Phomopsis quercina* (JX262803)	494/494 (100) 494/494 (100)
L15	*Diaporthe eres*	*Diaporthe eres* (KJ210520) ITS1-F; LR3*Phomopsis* sp. (HE774484)	496/496 (100) 496/496 (100)
C12	*Diaporthe phaseolorum*	*Diaporthe phaseolorum* (AF001018)	488/492 (99.1)
L14	*Diaporthe* sp.	*Diaporthe terebinthifolii* (KC343217)	484/491 (98.6)
L7	*Diplodia scrobiculata*	*Diplodia scrobiculata* (KF766160) *Sphaeropsis sapinea* (HM467659)	492/492 (100) 492/492 (100)
M16	*Diplodia scrobiculata*	*Diplodia scrobiculata* (KF766160) *Sphaeropsis sapinea* (HM467659)	492/492 (100) 492/492 (100)
F12	*Discostroma fuscellum*	Seimatosporium lichenicola (AB594806)	496/500 (99.2)
G13	*Ellisembia asterinum*	*Helminthosporium asterinum* (AF073918)	477/479 (99.1)
M1	*Ellisembia asterinum*	*Helminthosporium asterinum* (AF073917)	478/479 (99.7)

M14	*Epicoccum nigrum*	*Epicoccum nigrum* (KP271952)	456/456 (100)
C15	*Fusarium solani*	*Fusarium solani* (KP001165)	478/478 (100)
B9	*Helminthosporium velutinum*	*Helminthosporium velutinum* (AB551948)	476/477 (99.7)
G10	*Helotiales* sp.	*Helotiales* sp. (AB598098)	733/786 (93.3)
F4	*Hyalodendriella* sp.	*Hyalodendriella* sp. (HM992804)	494/495 (99.8)
A6	*Hyalodendriella* sp.	*Hyalodendriella* sp. (HM992804)	453/454 (99.7)
E14	Hypoxylon sp.	*Hypoxylon* sp. (JQ009308)	524/541 (97.4)
E15	*Kirschsteiniothelia* sp.	*Kirschsteiniothelia thujina* (KM982718)	502/517 (97.1)
F11	*Lachnum virgineum*	*Lachnum virgineum* (AB481268)	816/817 (99.8)
F13	*Lophiostoma* sp.	*Lophiostoma* sp. (HQ914838)	572/577 (99.1)
A3	Lophiostomataceae	*Lophiostoma chamaecyparidis* (EU552143)	406/471 (86.2)
C3	*Nectria* sp.	*Nectria cucurbitula* (JN995624) *Nectria balsamea* (JN995618)	503/524 (96.0) 503/525 (95.8)
N10	*Ophiostoma floccosum*	*Ophiostoma floccosum* (KF854000)	570/570 (100)
G7	*Paraconiothyrium brasiliense*	*Paraconiothyrium brasiliense* (KP050565)	517/517 (100)
A10	*Pestalotiopsis* sp.	*Pestalotiopsis* sp. (KP217182)	516/516 (100)
L1	*Pestalotiopsis* sp.	*Pestalotiopsis* sp. (KP900734)	515/515 (100)
L8	*Pestalotiopsis* sp.	*Pestalotiopsis* sp. (KP900734)	515/515 (100)
L9	*Pestalotiopsis uvicola*	*Pestalotiopsis uvicola* (KF374685)	486/487 (99.7)
F9	*Pezicula cinnamomea*	*Pezicula aff. cinnamomea* (KF376148)	472/472 (100)
L12	*Pezicula cinnamomea*	*Pezicula aff. cinnamomea* (KF376148)	472/472 (100)
M11	*Phaeoacremonium aleophilum*	*Phaeoacremonium aleophilum* (JF275866) *Togninia minima* (KP083231)	504/504 (100) 504/504 (100)
B6	*Phaeomoniella* sp.	*Phaeomoniella effusa* (JX421733)	727/731 (99.5)
B15	*Phaeomoniella* sp.	*Phaeomoniella effusa* (JF440607)	474/506 (93.7)
D6	*Phaeomoniella* sp.	*Phaeomoniella effusa* (JF440607)	474/507 (93.5)
D8	*Phaeomoniella* sp.	*Phaeomoniella prunicola* (GQ154595)	451/492 (91.7)
F15	*Phaeomoniella* sp.	*Phaeomoniella dura* (GQ154597)	431/469 (91.9)
G2	*Phaeomoniella* sp.	*Phaeomoniella effusa* (JX421733)	494/496 (99.5)
B5	*Phialophora* sp.	*Phialophora* sp. (FN386268)	465/472 (98.5)
A14	*Phomopsis* sp.	*Phomopsis* sp. (AB107890)	494/494 (100)
L16	*Phomopsis* sp.	*Phomopsis asparagi* (JQ613999)	482/491 (98.2)
A8	*Phomopsis quercella*	*Phomopsis quercella* (AY853216)	490/490 (100)
C13	*Phomopsis quercella*	*Phomopsis quercella* (AY853216)	490/490 (100)
F14	Pleomassariaceae	*Dendryphiopsis* sp. (KJ159066)	338/423 (79.9)
F1	Pleosporales	*Camarographium koreanum* (JQ044432)	384/435 (88.3)
G9	*Proliferodiscus* sp.	*Proliferodiscus alboviridis* (U57990)	464/471 (98.5)
B8	*Sarea difformis*	*Sarea difformis* (JF440614)	475/478 (99.3)
N1	*Sydowia polyspora*	*Sydowia polyspora* (KJ589589)	499/499 (100)
C11	*Therrya* sp.	*Therrya fuckelii* (JF793672)	412/450 (91.6)
E7	*Trametes versicolor*	*Trametes versicolor* (KC176325)	547/547 (100)
D4	*Xylaria* sp.	*Xylaria* sp. (HQ608148)	718/723 (99.3)
F10	*Xylaria acuta*	*Xylaria acuta* (DQ491493)	430/430 (100)

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