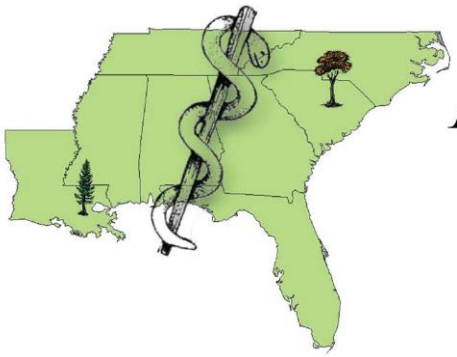

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ISOLATION AND IDENTIFICATION OF FUNGI ASSOCIATED WITH LOBLOLLY PINE DEFOLIATION AND MORTALITY IN THE SOUTHEASTERN UNITED STATES

by
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ABSTRACT

Loblolly pine is an economically important timber species in the southeastern United States. Over the past five years, loblolly pine stands have been observed with defoliation followed by stunted growth and tree mortality. Symptoms first appear as chlorosis following rapid necrosis of needles eventually resulting in complete tree defoliation and death. In an effort to determine the cause, this study investigated the potential causal agent(s) of loblolly pine defoliation and tree mortality in the southeastern United States. A total of 47 stands was sampled in an attempt to isolate and identify the causal agents of loblolly pine defoliation. Based on colony morphology and ITS-rDNA sequence data, a total of twenty-eight fungal species representing seventeen families were recovered from symptomatic loblolly pine needles. *Lecanosticta acicola* was repeatedly recovered (64%) from infected stands in Alabama. Other common needle cast, rust and tip blight fungi such as *Sydowia* spp. (60%), *Rhizosphaera* spp. (36%), *Coleosporium* spp. (9%), *Lophodermium* spp. (28%), and *Diplodia* spp. (6%) were recovered at one or few sites or recovered in a combination with *L. acicola* pathogen. *Lecanosticta acicola* was confirmed using morphological similarity and amplification of the internal transcribed spacer (ITS) region and the translation elongation factor (TEF) 1-alpha gene of interest. No sexual stage was observed for *L. acicola* and only the single mating type MAT-1- 1 was tested positive in Alabama. This suggests that there is a less genetic diversity of *L. acicola* in Alabama. Brown spot needle blight is an emerging disease in Alabama.

2.1. INTRODUCTION

Climatic factors such as increasing temperature and changing precipitation have potential influences on fungal disease emergence and spread in plant communities (Launay et al., 2014;

Wood at el., 2005). Warming and precipitation are expected to increase pathogen fitness and transmission by increasing growth rates and spore production, promoting mycelium growth, and extending the lengths of growth and reproduction times (Harvell et al., 2002). Changing interactions between the environmental factors and biotic agents can drive disease outbreaks (Wyka et al., 2017; Agrios, 2005) and may result in changing disease impacts. For example, water-stressed trees have been shown to be affected in the region where precipitation is reduced (Sturrock et al., 2011a).

Environmental conditions affect pathogen virulence, host susceptibility, and can modify interactions between the host and the pathogen (Agrios, 2005). For instance, the number of rainfall days between summer to winter and rainfall intensity have favored *Phytophthora* spp. in Central Europe and are expected to have bigger impacts on forest ecosystems that are dominated by susceptible *Fagus*, *Quercus*, *Alnus*, *Abies*, *Acer* and *Pinus* species (Jung, 2009). Yet, the relationship between climate change and its impact on disease intensity and severity depends on the pathosystem and the environment it happens (Sturrock et al., 2011b).

Fungi associated with needle diseases tend to be more sensitive to moisture as water promotes the rate of reproduction, fungal spread, and infection (Stone et al., 2007). Similarly, increasing temperatures alters fungal virulence by affecting growth and reproduction regardless of their host (Harvell, 2002). Since 1960s, large-scale mortality of trees reported in Chile, New Zealand, and Africa were found to be associated with *Dothistroma septosporum*, a fungus responsible for Dothistroma needle blight, with increasing temperatures and shifting precipitation patterns (Brown & Webber, 2008; Sturrock et al., 2011b; Woods et al., 2005).

Similarly, Swiss needle cast, caused by the fungus *Nothophaeocryptopus gaeumannii* (T. Rohde), occurrence in the Pacific Northwest was found to be positively correlated with increasing average winter temperatures and spring precipitation (Manter et al., 2005). Increasing *Phytophthora* foliar disease has been observed on water-stress forest trees (Jactel et al., 2012). Climatic factors favor needle pathogens, which in turn, limits water and nutrient availability of trees followed by less carbon stocks and forest productivity (Hicke et al., 2012).

In plantation forestry, threats are often imposed by the unintentional introduction of pests and pathogens (Wingfield et al., 2001). For example, *Phytophthora pluvialis* appeared suddenly in New Zealand in 2005 ((Dick et al., 2014) and *P. pinifolia* in Chile since February 2004 (Durán et al., 2008). Both fungi have caused large scale mortalities in exotic *Pinus radiata* (D Don) plantations in Chile and New Zealand and thought to be introduced from North America. Since 2016, loblolly pine has been experiencing repeated defoliation in Chatom, Alabama, Washington County. Symptoms first appear as chlorosis following rapid necrosis of needles eventually full crown defoliation and death. Second-and third-year needles are infected which resulted in tree crowns looking sparse after repeated defoliation. The objective of this study was to isolate and identify the causal agent(s) associated with loblolly pine defoliation and tree mortality (LPDM) in Alabama, Georgia, South Carolina, Mississippi and Louisiana.

2.2. MATERIALS AND METHODS

2.2.1. Study area and plant material collection

Seven permanent plots in Chatom, Alabama, Washington County were established in 2019 (Table 2.2). The climate of Chatom has an average air temperature from 61oF to 70oF and is rarely below 24oF or above 97oF. The average annual precipitation is 57 to 69 inches (Weatherspark.com). Poorly drained stands composed of Tibbie soils (40%), Pinebarren soils (35%) and 5% of other minor components. (Web Soil Survey). From 2019 to 2021, needle sampling was done once per month from March to November based on the sporulation period of five needle pathogens (Table 2.1). From seven experimental plots, five trees were sampled each time based on the expression of symptoms such as chlorosis, rapid necrosis and defoliation. GPS coordinates were also collected to locate the stands. Permanent plots were composed of 3 to 16 year old trees including 2004, 2009, 2010 and 2012 plantations. Destructive sampling was performed to collect needle samples from trees. To obtain upper and lower crown needles, a 0.22 mag caliber rifle was used to shoot foliage samples out of the tree. Among them, only 2004 plantations receive fertilization after 2-3 years of planting. Otherwise, no silvicultural treatments were applied to the rest of the sampled plots. Genetics of the seedlings were unknown.

2.2.2. Survey area and plant material collection

In addition to permanent sample plots, plots of various site conditions ranging from 3 to 32 year old plantations were assessed for defoliation and mortality and sampled. Private landowners and industry collaborators were sent a one-page information sheet about needle mortality and a collection guide (Appendix B) and instructed to send in infected needle samples (Munck & Burns, 2012). The study also scheduled a sampling visit to the infected stands based on needle disease incidence reports and collected needle samples and information about the stands. The sampling was conducted during the sporulation period of needle pathogens based on the disease similarity such as Dothistroma needle blight, Brown spot needle blight, Phytophthora needle blight, Lophodermium needle cast or Coleosporium needle rust (Table 2.1). Needle samples were collected from infected stands between March and November when landowners recognized symptoms and contacted the Forest Health Dynamics Laboratory. Information for each stand such as GPS coordinates (latitude and longitude of the stand), height (in meter), DBH (diameter at breast height in cm), stand type, site description, soil properties and visual crown rating was intended for later analyses.

2.2.3. Media and fungi isolation

To recover fungi from needle samples and induce sporulation from those samples, there were eight agar media were used in the study. Modified cornmeal agar (CMA-PARP) and V8 agar were used to recover *Phytophthora* species, dothistroma media was selected to recover *dothistroma* species, 2% malt extract and acid potato dextrose agar were used to recover *Lecanosticta* species in the study (Table 2.3).

2.2.4. Isolation of fungi from loblolly pine needles

Needle samples collected from the plots and submitted to the lab were split into 3 subsamples: one for plating on growth media, one for moisture chamber sporulation and one for DNA extraction. These samples were processed and analyzed in the Forest Health Dynamics Laboratory following above mentioned fungal recovery methods for fungal identification and confirmation.

Plating: Needles were cut into 1-2 cm pieces and surface sterilized with 10% NaOCl (sodium hypochlorite) solution for 1 minute followed by 70% ethanol for another 1 minute and 2 subsequent washes for 1 minute each with distilled water. Surface-sterilized needles were 28 transferred to a sterile filter paper and blotted dried under a laminar flow hood for 20 minutes (Aboshosha et al., 2007). Needles (four needle pieces per Petri plates) were then placed onto the range of growth media (Table 2.3) and 5 replications per media per tree sample were followed. Petri plates were incubated at ambient temperature and light and regularly checked for fungal growth. Once growth was observed, hyphal tips and conidial heads were transferred to fresh 2% MEA media plates (Guo et al., 1998; Drenkhan et al., 2016). For long-term storage, pure cultures were maintained on MEA slants and stored at -4oC in the Forest Health Dynamics Laboratory collection in the School of Forestry and Wildlife Sciences at Auburn University.

Moist chamber: Symptomatic needles were placed in a moist chamber made up of a glass Petri plate, filter paper (Whatman) and ~500 uL of distilled H2O. In every moist chamber, 2-3 fascicles were incubated and total five moist chamber were incubated for each sample tree. Spores recovered on the needles were examined under a compound microscope and transferred onto a 2% MEA media for DNA extraction (Broders et al., 2015).

Direct DNA Extraction: Third subsample was cut into 2-3 mm pieces and kept in a refrigerator at 4oC until freeze drying. DNA extraction process from freeze dried needles is described in section 2.2.5.

2.2.5. DNA extraction

Hyphal tips from each of the pure cultures recovered from symptomatic needles were transferred into liquid MEA media and incubated at 25oC for 2 to 3 weeks. Liquid media were centrifuged (Thermo Scientific Sorvall BIOS 16 centrifuge) at maximum speed (≤16,000 rpm) for 10 minutes. The liquid suspension was discarded and fungal pellets were transferred to a lyophilizer and kept overnight at -550C. The genomic DNA was extracted from dried mycelium using a Phenol-chloroform method (Comey et al., 1994). 29 Fungal tissues were homogenized using liquid nitrogen followed by lysis buffer.

Suspension was transferred into a new 1.5 mL microcentrifuge tube, proteinase K (2-3 µL, 100 ng/µL) added, and kept at 55oC in a water bath for 15 minutes. Ammonium acetate (NH4AC, 250 µL) was added to the microtube which was then placed into ice to precipitate histone proteins for DNA denaturation. Next 750 µL phenol: chloroform: isoamyl alcohol (25:24:1) was

added to the suspension and vortexed for 5 minutes. Suspensions were added to 2-3 uL (100 ng/ μL) RNase

A. Two subsequent additions of 750 μL chloroform: isoamyl alcohol (24:1) and separation of suspensions followed. Finally, sodium acetate NaAc (120 mL) and 100% Ethanol (880 mL) were added to the suspension and kept in the refrigerator at 4oC for 30 minutes to precipitate the fungal DNA. The supernatant was discarded and 50 μL of molecular water was added to suspend DNA (Comey et al., 1994).

Total DNA was also extracted directly from symptomatic needle samples collected from loblolly pine. Needles cut at 2-3 mm were ground with liquid nitrogen. Each sample consisted of 5-100 mg of ground needles to extract DNA. DNA extraction was followed by DNeasy Plant Pro Mini Kit instructions. As per DNeasy Plant Pro Mini Kit, CD1 buffer was decreased from 500 uL to 400 uL and 50-100 uL PS buffer was added for highly symptomatic samples (Barnes et al., 2008). The rest of the procedures followed as described by DNeasy Mini Kit's instructions without any modification.

2.2.6. Polymerase Chain Reaction (PCR)

Internal transcribed spacer (ITS) regions were amplified using universal primers ITS1 and ITS4 (White et al., 1990). The thermal cycling reaction was carried out in an MJ Research PTC-100. PCR amplification was run at 25 uL reaction volume made up of 1uL of template DNA, 1uL of each primer pair, 12.5 uL Green Master Mix (GMM) and 9.5 uL nuclease-free water. The 30
reactions were as follows; initial denaturation of 95^{o}C for 2 minutes, annealing 56^{o}C for the 30S of primer pairs, 72^{o}C for 2 minutes, and 39 cycles were performed each time for maximum amplification. PCR products were kept in a molecular refrigerator for further analysis. However, PCR cycling conditions were varied at least for annealing temperature for other sets of primers used in the study (Table 2.4). PCR purification was conducted using E.Z.N.A purification kit. DNA concentration was maintained each time between 18 ng/uL to 100 ng/mL (Drenkhan et al., 2016).

2.2.7. DNA sequencing and phylogenetic analyses

Purified PCR products were sent to Laragen Inc. Biotechnology Company (Virginia Ave, Culver City, California) to sequence fungi. Raw sequence editing was done manually in BioEdit software where forward and reverse sequences were adjusted. Fungal sequences were identified based on their sequence similarity with fungi stored in NCBI GenBank. Edited sequences were deposited at Gen Bank and BankIt for future reference. Other closely related species and outgroups of fungi were imported from NCBI GenBank to serve as known reference sequences for comparison to the distance and character-based relation of fungi recovered in the study.

MEGA version 4.0 software was used to develop phylogenetic trees and subsequent molecular analyses. Sequence alignments were created using the ClustalW tool in MEGA where ambiguous sites were ignored, and gaps were counted as missing data. Both neighbor-joining (NJ) and maximum likelihood (ML) methods were performed to construct phylogenetic trees. Following that, 1000 bootstrap applications were performed for NJ analysis to increase statistical confidence and the relative support for the branches (Broders et al., 2015).

A total of 7 permanent plots and 40 private stands were sampled to identify pathogens associated with loblolly pine defoliation and tree mortality in Alabama, Georgia, South Carolina, Louisiana and Mississippi. Most (43 of the stands) were privately owned plantation forests. Soil 32 conditions of the sites were sandy to sandy loam and sandy clay loam to silty loam (Table 2.2). Stands were located at wet areas or near the edges of water bodies (within 1/2 mile) and at deep steep slopes. Needle samples were taken from a total of 282 trees and overall 2820 incubated needles and 1410 branch tips were examined from 2019 to 2021.

The fungal pathogens *L. acicola, S. polyspora*, *R. kalkhoffii*, *Lophodermium* spp., *D. sapinea*, and *Coleosporium* spp. were recovered from stands at 64%, 60%, 36%, 28%, 6%, and 9% respectively (Figure 2.2). *Lecanosticta acicola* was recovered at eleven sites in Alabama (Washington, Wilcox, Elmore, Butler, Crenshaw, Pickens, Lamar, Cullman, Colbert, Madison, and Greene) and one site in Mississippi (Greene). *Sydowia Polyspora* and *R. kalkhoffii* were recovered in Alabama in combination with *L. acicola*. *Lophodermium* spp. were recovered in Alabama (Bibb, Walker) and Georgia (Ware and Camden). *Diplodia sapinea* was recovered in South Carolina (Hampton) and Georgia (Upson). *Coleosporium* spp. was recovered in Louisiana (Natchitoches perishes) and Alabama (Colbert and Macon) (Figure 2.5).

Signs and symptoms for *L. acicola* were most frequently observed in a combination with *S. polyspora, R. kalkhoffii, Lophodermium* spp., and *Coleosporium* spp. in Alabama. *Lecanosticta acicola* produced hard small black fruiting bodies consisting of brown and banana-shaped spores. *Lophodermium* fungi were identified by their football-shaped black conidiomata that developed on the upper and lower portions of the infected needles. *Diplodia sapinea* formed brown to black circular spots and irregular sizes fruiting bodies which were densely recovered at the tip and base of the needles. Only *Coleosporium* spp. produced white aecia in the diseased needles (Figure 2.3).

A diverse group of fungi were isolated from symptomatic needles and identified based on colony morphology and ITS-rDNA sequence data. A total of six pathogens were recovered from symptomatic needles (Figure 2.2). *Lophodermium* spp. and *Coleosporium* spp. were recovered at 6% and 2% stands respectively. Otherwise, pathogens were recovered in the study as a combination of two or three fungal species. For example, *L. acicola* and *S. polyspora* were 35 recovered at 19% of stands followed by *L. acicola* and *Lophodermium* spp. at 13% trees.

Lecanosticta acicola and *Coleosporium* spp. combinations were found at 4% of stands and *Lophodermium* spp. and *Coleosporium* spp. from 3% of stands. *Diplodia sapinea* and *Lophodermium* spp. were recovered at 9% of stands in the study. Based on stand prevalence i.e., disease severity, pathogens were occasionally recovered as a combination of three fungal species such as *L. acicola, S. polyspora* and *R. kalkhoffii* were recovered at 25% of stands followed by *L. acicola, S. polyspora* and *Lophodermium* spp. were recovered at 13% of stands (Figure 2.4). Additionally, other endophytic and saprophytic fungi were recovered in the study. The distribution map demonstrates the fungal pathogen distribution in Alabama, Georgia, Mississippi, South Carolina and Louisiana (Figure 2.5).

Fungi recovered in the media from plated needles and fungal spores recovered in the moist chambers from formed fruiting bodies in the symptomatic needles making it total 904 fungal cultures in the study. Pure cultures were divided into 58 groups based on colony color, shape, height, marginal growth, and surface characteristics. Each morphological group was examined and identified based on their distinct hyphal shape and spore structures (Webster & Weber, 2007). Twenty-eight species of fungi representing seventeen families were identified and confirmed based on morphological and molecular analysis. Twelve species of fungi appeared to be pathogens or weak parasites and they were *L. acicola, R. kalkhoffii, Lophodermium* spp., *D. sapinea, D. seriata, D. scrobiculata, Coleopsorium* spp., *S. polyspora, Ramularia weberiana, Meyerozyma caribbica, Meyerozyma carpophila* and *Hormonema macrosporum*. Eight species were likely to be associated as endophytes such as *Hendersonia pinicola, Epicoccum nigrum, Alternaria tenuissima, Alternaria alternata, Preussia isomera, Penicillium spp., Sardiniella celtidis,* and *Pestalotiopsis spp*. Eight additional species appeared to be saprophytes such as *Sordaria fimicola, Myrmaecium rubricosum, Talaromyces amestolkaie, Talaromyces purpureogenus, Cladosporium anthropophilum, Cladosporium cladosporioides, Trichoderma caerulescens,* and *Paraconiothyrium brasiliense* in the study (Figure 2.4). *Pestalotiospsis* spp. were the most common endophyte recovered (100%) from both asymptomatic and symptomatic loblolly pine needles at all sites in Alabama, Georgia, Mississippi, Louisiana and South Carolina.

2.3.2. Lecanosticta acicola associated with loblolly pine defoliation and mortality in Alabama

Lecanosticta acicola was recovered from needles collected from 32 diseased stands at 11 different sites in Alabama and one site in Mississippi including Washington (7 stands), Colbert (2 stands), Crenshaw (2 stands), Elmore (1 stand), Madison (2 stands), Pickens (1 stand), Greene (AL, 1 stand), Lamar (3 stands), Cullman (5 stands), Wilcox (1 stand), Butler (5 stands) and Greene (MS, 2 stands) counties. A foliar endophyte and pathogen, *Sydowia polyspora* was frequently recovered along with *L. acicola* in Alabama (Washington, Elmore, Butler, Madison, Greene, Lamar, Cullman, Wilcox and Pickens) where nearly 80% of trees were infected and tree mortality was observed due to defoliation.

The desired length of PCR products for fungal identification were confirmed by gel electrophoresis. For universal ITS1/ITS4 primers and species-specific LAtef-F/LAtef-R primers, successful amplification was about 500 (Figure 2.5) and 230 base pairs (Figure 2.6) respectively.

The sequences recovered in the study were identified to have three distinct lineages of *L. acicola* globally (Figure 2.6). The first lineage was related to a *L. acicola* population in Spain where it was reported to infect *Pinus radiata* stands (Janoušek et al. 2016). The second lineage was associated with fungus populations in France where *L. acicola* infects *P. radiata* plantations (Alvère et al. 2010). Finally, the third relationship was related to a *L. acicola* population in the northeastern United States where it infects *Pinus strobus* (eastern white pine) (Broders et al., 2015).

Mating type analyses of *L. acicola* were confirmed using mating-type primers Md MAT1-1F, Md MAT1-1R, and Md MAT1-2F, Md MAT1-2R (Janousik et al., 2016). Positive *L. acicola* isolates were amplified for a single mating type, MAT-1-1. However, amplification did not successfully detect *L. acicola* mating-type 2 loci, MAT-1-2. Microscopic examination of fruiting bodies recovered from needles in Alabama and Mississippi did not result in the formation of a sexual stage in those infected stands. Morphological examination of fungal isolates of *L. acicola* black to olive green mucilaginous conidiomata was recovered from 42

symptomatic loblolly pine needles. Conidia were light green to olivaceous to pale brown in color and rough verruculose thick walls. Spore shapes varied from fusiform to cylindrical with straight to curved conidia ranging from 2 to 4 septation with truncate base and rounded apex. Dimensions of the conidia varied between 22.15 - (33.34) - 38.38 × 3.59 - (4.84) - 7.32 μm (Figure 2.3A).

2.4. DISCUSSION

The loblolly pine defoliation and mortality (LPDM) were predominantly due to brown-spot needle blight fungus, *L. acicola* in Alabama. This pathogen was recovered from Central (1), Southern (3), Northern (6), and Southwestern (1) counties of Alabama and indicates that this fungal presence is ubiquitous in Alabama. The mating-type analysis of *L. acicola* confirmed that a single mating type MAT-1-1 was present in the samples in Alabama. No sexual state was observed during the study indicating that there is less possibility of sexual outcrossing (Janoušek et al. 2016). A similar study of mating-type loci analysis of *L. acicola* from *Pinus palustris* Mill. seedlings in Mississippi was negative also supporting that only one mating types was present in Alabama and Mississippi (Bartlett, 2015).

Lecanosticta acicola wasn't recovered at infected stands in South Carolina, Georgia or Louisiana where infected trees showed varied symptomology compared to Alabama. Trees sampled on those sites did not mimic *L. acicola* symptomology (chlorosis followed by rapid necrosis leading to premature defoliation) which supported the study results. In South Carolina (Hampton) and

Georgia (Upson), infected trees showed tip blight of the needles and were located at deep steep slopes. In Louisiana (Natchitoches Perishes), yellow to orange spots on the green needles, yellowing over time and defoliation was observed. Other needle casts were also associated with LPDM but their recovery was only at a site or in combination with *L. acicola* pathogen in Alabama. Several stands in Alabama and Mississippi were considered healthy

because no needle pathogens were recovered from those stands. They were located at Noxubee, Lauderdale, Clark counties in Mississippi, and Walker and Bullock counties in Alabama (Figure 2.2). Infected trees on those sites showed symptoms such as chlorotic needles, shortened internodes and sparse crown which is indicative of a root disease such as pine decline (Eckhardt et al., 2010; Hess et al., 1999; Lorio, 1966) or littleleaf disease (Crandall et al., 1945).
One of the more intensifying recovery of the fungus was *Sydowia polyspora*, a foliar endophyte (Ridout & Newcombe, 2018) and pathogen (Talgø et al., 2010). *Sydowia polyspora* was recovered at more than 80% of brown spot needle blight affected stands in Alabama. Nearly all loblolly trees were affected and one-third trees have started dying due to defoliation. Frequent recovery of this *S. polyspora* along with *L. acicola* indicates *S. polypora* may have a potential role associated with increasing disease severity. Other studies reported that *S. polypora* fungus changes its role from endophytic to pathogenic when exposed to warm climate and/or if the host tree is under stress (Muñoz-Adalia et al., 2017; Pan et al., 2018). Most of the positive stands were situated in the wet areas and/or close to a water body and in poor drainage conditions. Although loblolly pine grows and survives in poor soil conditions, it is also possible that trees are stressed due to site conditions and inoculum pressure. Spore survival might have increased in those sites (Gadgil, 1970). Edaphic factors such as soil types, slope, aspect, depth of soil to impermeable layer and nutrient availability are thought to be associated with disease incidence and occurrence. Average infection of dothistroma needle blight is positively correlated with sulfur-deficient basalt parent materials and infection levels are related to soil and topographic factors (Eldridge et al., 2013).

Based on the study, needle pathogens are increasing in appearance in Alabama. Most notably, brown spot needle blight, *L. acicola* has resulted in tree mortality of loblolly pine trees

in Alabama. This pathogen has been a major problem for grass-stage longleaf pine (*P. palustris* Mill.) in the southeastern United States (Siggers, 1944) and Scotch pine Christmas tree plantations in Minnesota and Wisconsin (Skilling & Nicholls, 1974). However, this is the first report of loblolly pine defoliation and mortality due to this pathogen in Alabama. In the context of climate change, this disease is likely to continue if the pathogen is favored by changes in temperature and moisture conditions. Since loblolly pine is one of the most productive timber species native to the southern United States, it is crucial to manage this disease for the sustainability of the species. Current research is underway to attempt to predict loblolly pine defoliation severity in the coming years (see Chapter V).

2.5. CONCLUSION

A total of twenty-eight different fungi were recovered from symptomatic loblolly pine needles in Alabama, Mississippi, South Carolina, Georgia, and Louisiana. The brown-spot needle blight fungus, *L. acicola*, was the predominant needle pathogen associated with loblolly pine needle defoliation and tree mortality in Alabama. Only mating type 1, MAT-1-1 was present in Alabama indicating a less genetically diverse population and reduced recombination. Other needle cast, rust, and tip blight pathogens were recovered at one or few sites or in combination with *L. acicola*. Their role in LPDM was secondary and might be associated with increasing disease severity in the *L. acicola* infected stands. A foliar endophyte and pathogen *S. polyspora* was also recovered with *L. acicola* in Alabama where the disease was most severe. Based on the study, *L. acicola* has become a problem to forest managers in Alabama. Due to the nature of the disease, stand characteristics, and other factors, an understanding of LPDM is critical to guiding management and policy recommendations.

Table 2.1. Sporulation period of five needle pathogens

Needle Disease	**Causal Agent**	**Host**	**Sporulation Period**
Phytophthora needle blight	*Phytophthora* spp.	Pines, Oak, Douglas fir,	May to November
Brown spot needle blight	*L. acicola*	Over 53 different pine species	March to October
Dothistroma needle blight	*D. septosporum* *D. Pini*	82 *Pinus* taxa	Late summer to fall (August-October)
Lophodermium needle cast	*L. seditiosum* *L.* spp.	Scots, Austrian and Red pine	Late summer (August-September)
Coleosporium needle rust	*Coleosporium* spp.	2 or 3-needled Pines	Spring (March-May)

Table 2.2. Descriptive data for sampled stands surveyed from 2019 to 2021 in the southeasternUnited States.

State	County	Stand type	Site description	Soil Properties	Latitude	Longitude
*Alabama	Washington	Plantation	Wet area	Sandy loam	31.2729	-88.3094
*Alabama	Washington	Plantation	Edge of water	Sandy loam	31.2725	-88.3094
*Alabama	Washington	Plantation	Wet area	Sandy loam	31.2719	-88.3098
*Alabama	Washington	Plantation	Wet area	Sandy clay loam	31.2649	-88.3169
*Alabama	Washington	Plantation	Wet area	Sandy clay loam	31.2607	-88.2937
*Alabama	Washington	Plantation	Wet area	Sandy clay loam	31.2395	-88.2807
*Alabama	Washington	Plantation	Wet area	Sandy clay loam	31.2422	-88.2769
South Carolina	Hampton	Natural	N/A	Silty clay	33.8361	-81.1637
Alabama	Bibb	Plantation	N/A	Sandy	33.1388	-87.1820
Alabama	Butler	Plantation	Flat Plain land	Sandy clay loam	34.8526	-82.3941
Alabama	Colbert	Plantation	Edge of water	Sandy	34.8267	-87.6128
Alabama	Crenshaw	Plantation	Edge of water	Sandy	31.6671	-86.2641
Alabama	Macon	Plantation	Flat plain land	Sandy	32.3731	-85.6846
Alabama	Walker	Plantation	N/A	Sandy	33.0154	-87.0099
Alabama	Bullock	Plantation	N/A	Sandy	32.0574	-85.7256
Alabama	Colbert	Natural	Edge of water	Clay loam	34.8192	-87.6119
Alabama	Elmore	Plantation	Steep slope	Sandy	32.6153	-86.0498
Alabama	Madison	Plantation	Flat plain land	Sandy	34.5672	-86.3605
Alabama	Crenshaw	Plantation	Wet area	Clay loam	31.6671	-86.2641
Alabama	Lamar	Plantation	Wet area	Sandy loam	34.0469	-88.1839

Alabama	Lamar	Natural	Flat plain land	Sandy loam	33.8179	-88.1429
Alabama	Lamar	Natural	Dry steep slope	Sandy loam	33.8737	-88.1808
Alabama	Cullman	Plantation	Flat plain land	Sandy loam	34.1301	-87.0793
Alabama	Cullman	Plantation	Edge of water	Sandy loam	34.1292	-87.0782
Alabama	Cullman	Plantation	Edge of water	Sandy loam	34.1139	-87.0783
Alabama	Cullman	Plantation	Steep slope	Sandy loam	34.1263	-87.0816
Alabama	Cullman	Plantation	Flat plain land	Sandy loam	34.1279	-87.0774
Alabama	Pickens	Plantation	Edge of water	Sandy	33.3341	-88.0901
Alabama	Wilcox	Plantation	Wet area	Sandy loam	32.1282	-87.4203
Alabama	St. Clair	Plantation	N/A	Silty loamy	33.8338	-86.2124
Alabama	Butler	Plantation	Wet area	Sandy Clay Loamy	31.7115	-86.4233
Alabama	Butler	Plantation	Wet area	Sandy Clay Loamy	31.7118	-86.4441
Alabama	Butler	Plantation	Wet area	Sandy Clay Loamy	31.7116	-86.4444
Alabama	Greene	Plantation	Wet area	Sandy clay loamy	31.7114	-86.4446
Georgia	Upson	Plantation	Steep slope	Sandy	32.8029	-84.3105
Georgia	Upson	Plantation	Steep slope	Sandy	32.8001	-84.1547
Georgia	Ware	Plantation	Steep slope	Sandy	31.1344	-82.4753
Georgia	Camden	Plantation	Flat plain land	Sandy	30.8983	-81.6035
Mississippi	Noxubee	Plantation	Flat plain land	Sandy loam	33.2451	-88.5642
Mississippi	Noxubee	Plantation	Wet area	Clay loam	33.1331	-88.1931
Mississippi	Kemper	Plantation	Flat plain land	Clay loam	33.2481	-88.3346
Mississippi	Kemper	Plantation	Flat plain land	Silty loam	33.2481	-88.3346
Mississippi	Lauderdale	Plantation	Steep slope	N/A	32.5738	-88.8298

Mississippi	Clarke	Plantation	Steep slope	Sandy	31.9654	-88.6579
Mississippi	Clarke	Plantation	Flat plain land	Sandy loam	32.0042	-88.6979
Mississippi	Greene	Plantation	Edge of water	Sandy	31.1979	-88.4847
Mississippi	Greene	Plantation	Edge of water	Sandy	31.3153	-88.4441
Louisiana	Natchitoches Perishes	Plantation	Dry steep slope	N/A	31.6801	-93.1780

N.B. Asterisk (*) sites are permanent sample plots and others are survey plots based on the random reports from private landowners and industry collaborators.

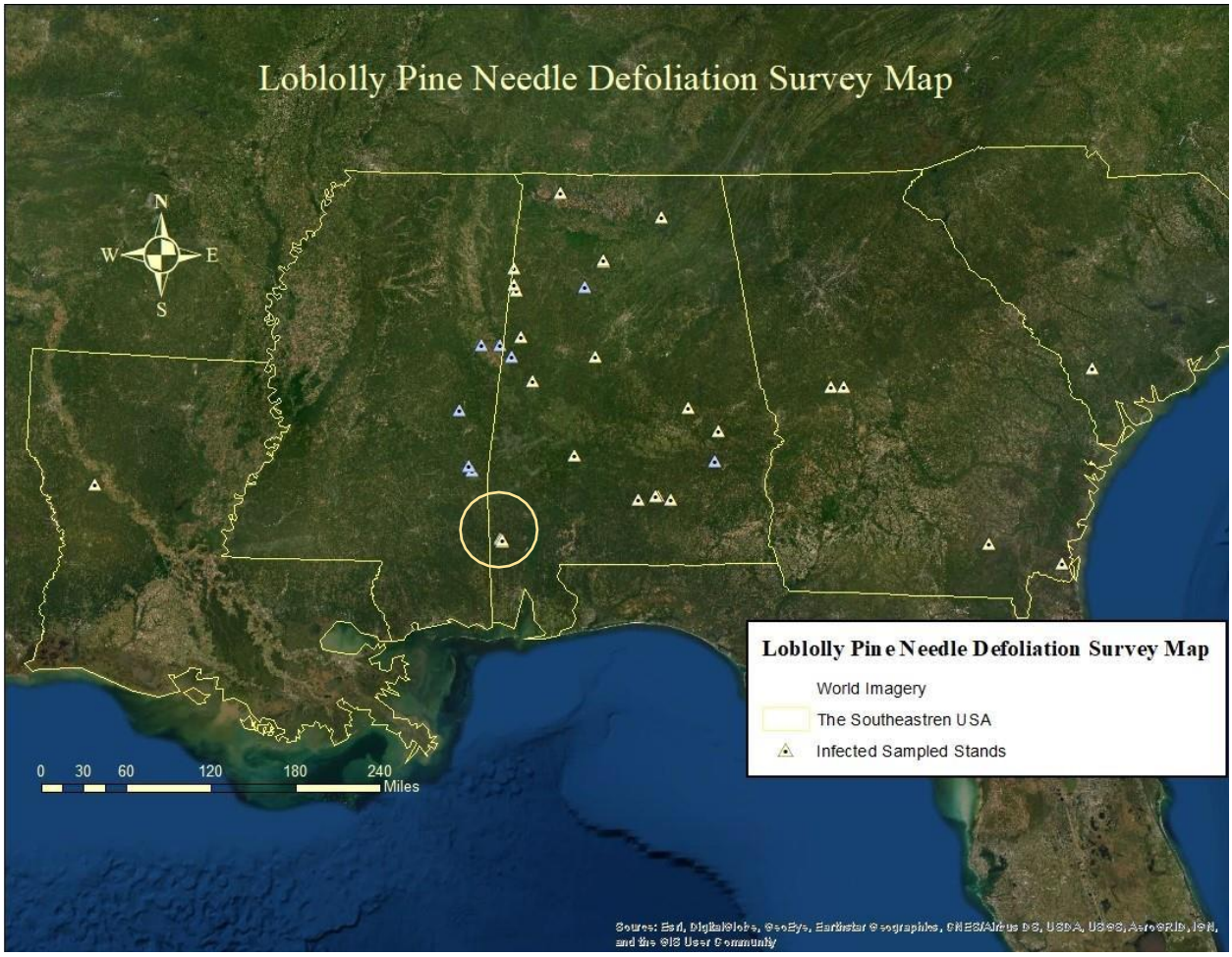

Figure 2.1. Map showing sampled stands in South Carolina, Georgia, Alabama, Mississippi, and Louisiana from 2019 to 2021 from surveys and the permanent study area. Circled stands indicate permanent sampled plots in Chatom, Washington County, Alabama.

Table 2.3. List of agar growth media used in the study

Growth media	Basal Ingredients	Amount	Amendments	Amount	References
CMA-PARP	Difco Cornmeal Agar	17 g	Pimaricin (50%)	10 mg	Jeffers and Martin, 1986 Ferguson and Jeffers, 1999)
	Distilled Water	1000 ml	Rifamycin-Sodium Salt	66.7 mg	
			PCNB (75%)	50 mg	
			Hymexazol (70%)	71.4 mg	
V8 Agar	Clarified V8 Concentrate	50 ml	β-sitosterol	0.03 mg/ml	Jeffers, 2006
	Difco Bacto Agar	15 g			
	Distilled Water	950 ml			
2% Malt Extract Agar	Malt Extract	20 g			Barnes et al., 2004
	Agar	15 g			
	Distilled Water	1000 ml	Streptomycin	100 g/L	
Dothistroma Medium	Malt Extract	50 g			Bradshaw et al., 2000
	Nutrient Agar	23 g			
	Distilled Water	1000 ml			

2% Dothistroma Sporulating Medium	Malt Extract	20 g	Streptomycin	100 g/L	Bradshaw et al., 2000 Nest et al., 2019
	Yeast Extract	5 g			
	Agar	15 g			
	Distilled Water	1000 ml			
Fresh Pine Needle Agar	Pine Needle Extract	500 ml			Luchi et al., 2007
	Agar	30 g			
	Distilled Water	1000 ml			
Modified Ground Pine Needle Agar	Ground pine needle	100 g			Luchi et al., 2007
	Agar	30 g			
	Distilled Water	1000 ml			
Acid Potato Dextrose Agar	Potato Dextrose Agar	39 g			Wyka, et al., 2015
	Concentrated Lactic Acid (85%)	1 ml			
	Distilled Water	1000 ml			

Table 2.4. List of primers used in this study

Marker name	Forward or Reverse	Sequence (5' to 3')	References
ITS1	Forward	TCCGTAGGTGAACCTGCGG	White et al., 1990
ITS4	Reverse	TCCTCCGCTTATTGATATGC	White et al., 1990
LAtef-F	Forward	GCAAATTTTCGCCGTTTATC	Ioos et al., 2009
LAtef-R	Reverse	TGTGTTCCAAGAGTGCTTGC	Ioos et al., 2009
Rust ITS1-F	Forward	GAAGTAAAAGACGTAACAAGGT	McTaggart & Aime, 2018
Rust ITS2-R	Reverse	CACCTGATTTGAGGTCTTAAAA	McTaggart & Aime, 2018
LRust1R	Forward	TAAGACCTCAAATCAGGT	Beenken et al., 2017

LR6	Reverse	CGCCAGTTCTGCTTACC	Beenken et al., 2017
FM35	Forward	CAGAACCTTGGCAATTAGG	Martin, 2000; Durán et al., 2008
FM58	Reverse	CCACAAATTTCACTACATTGA	Martin, 2000; Durán et al., 2008
DStub2	Forward	CGAACATGGACTGAGCAAAAC	Ioos et al., 2009
DStub2	Reverse	GCACGGCTCTTTCAAATGAC	Ioos et al., 2009
DPtef	Forward	ATTTTTCGCTGCTCGTCACT	Ioos et al., 2009
Dtef	Reverse	CAATGTGAGATGTTCGTCGTG	Ioos et al., 2009
MdMAT1-1F	Forward	CGCATTCGCACATCCCTTTGT	Janoušek et al. 2014
MdMAT1-1R	Reverse	ATGACGCCGATGAGTGGTGCG	Janoušek et al. 2014
MdMAT1-2F	Forward	GCATTCCTGATCTACCGTCT	Janoušek et al. 2014
MdMAT1-2R	Reverse	TTCTTCTCGGATGGCTTGCG	Janoušek et al. 2014

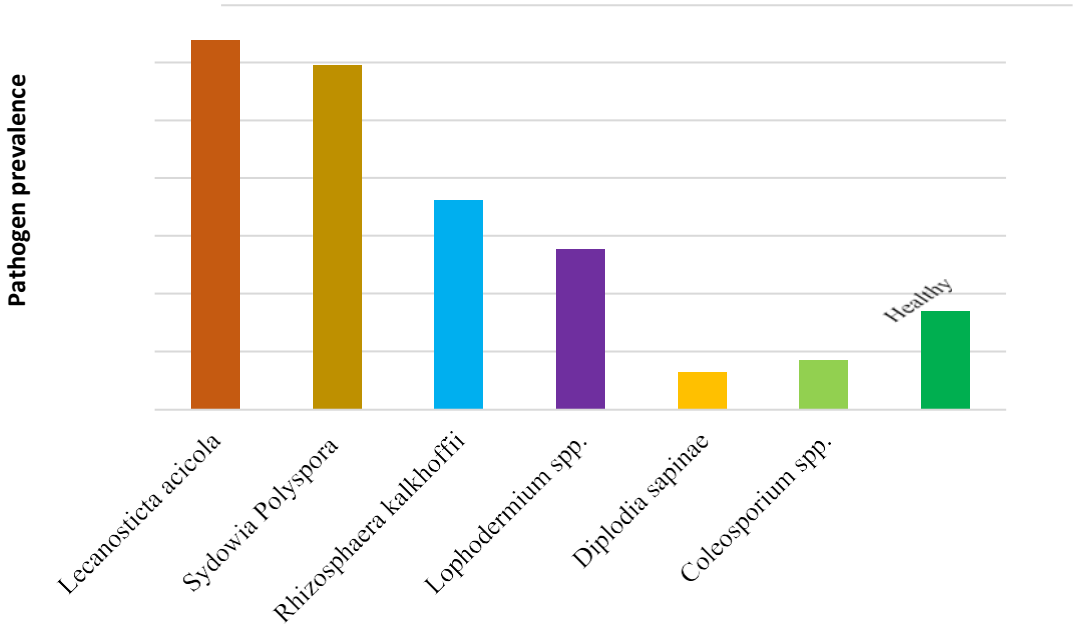

Figure 2.2. Pathogen prevalence by stands of *Lecanosticta acicola*,*Sydowia polyspora*, *Rhizosphaera kalkhoffii*, *Lophodermium* spp., *D. sapinae*,*Coleosporium* spp. and healthy sites.

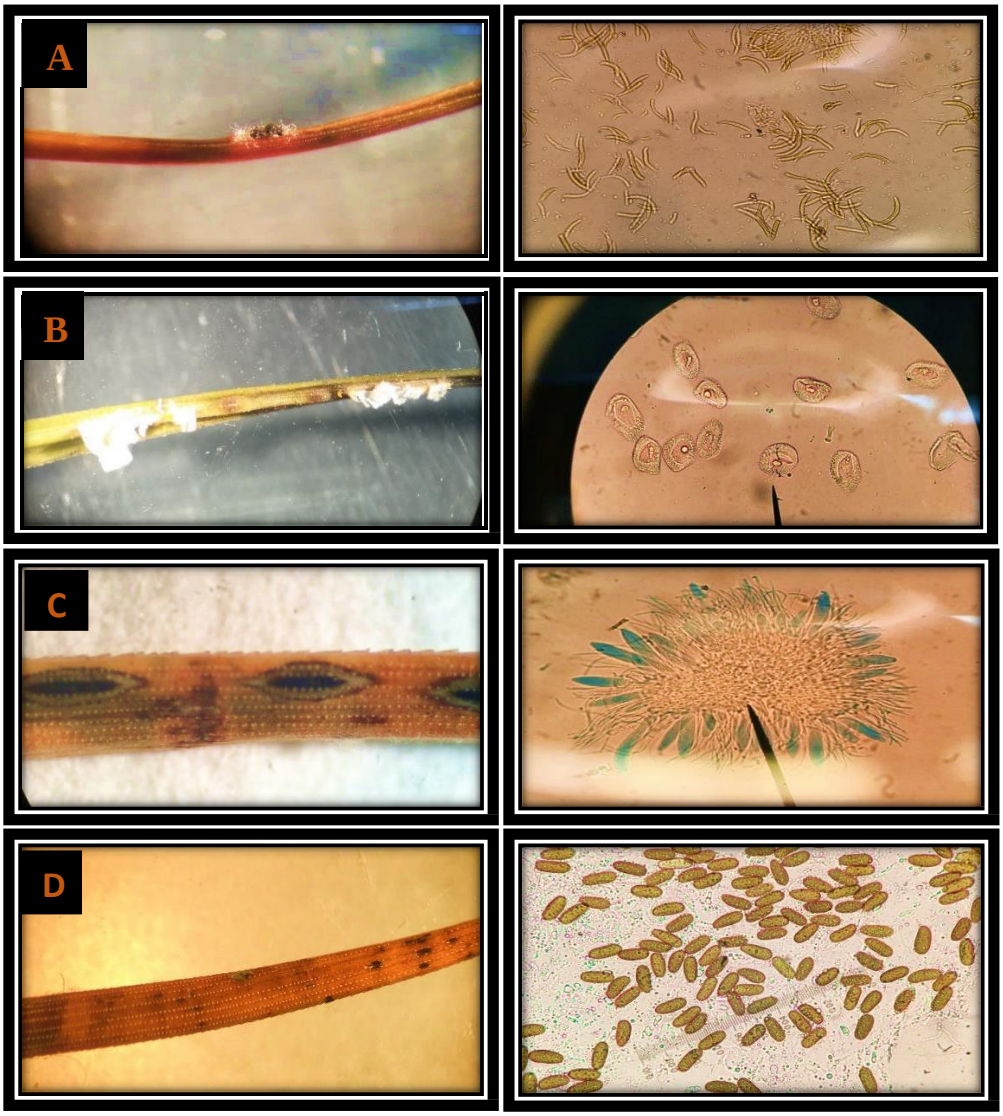

Figure 2.3 Expression of disease symptoms and reproductive structures of dominant fungal pathogens such as (A) *Lecanosticta acicula* (B) *Coleosporium* sp. (C) *Lophodermium* sp. and (D) *Diplodia sapinea* in the infected loblolly pine needles.

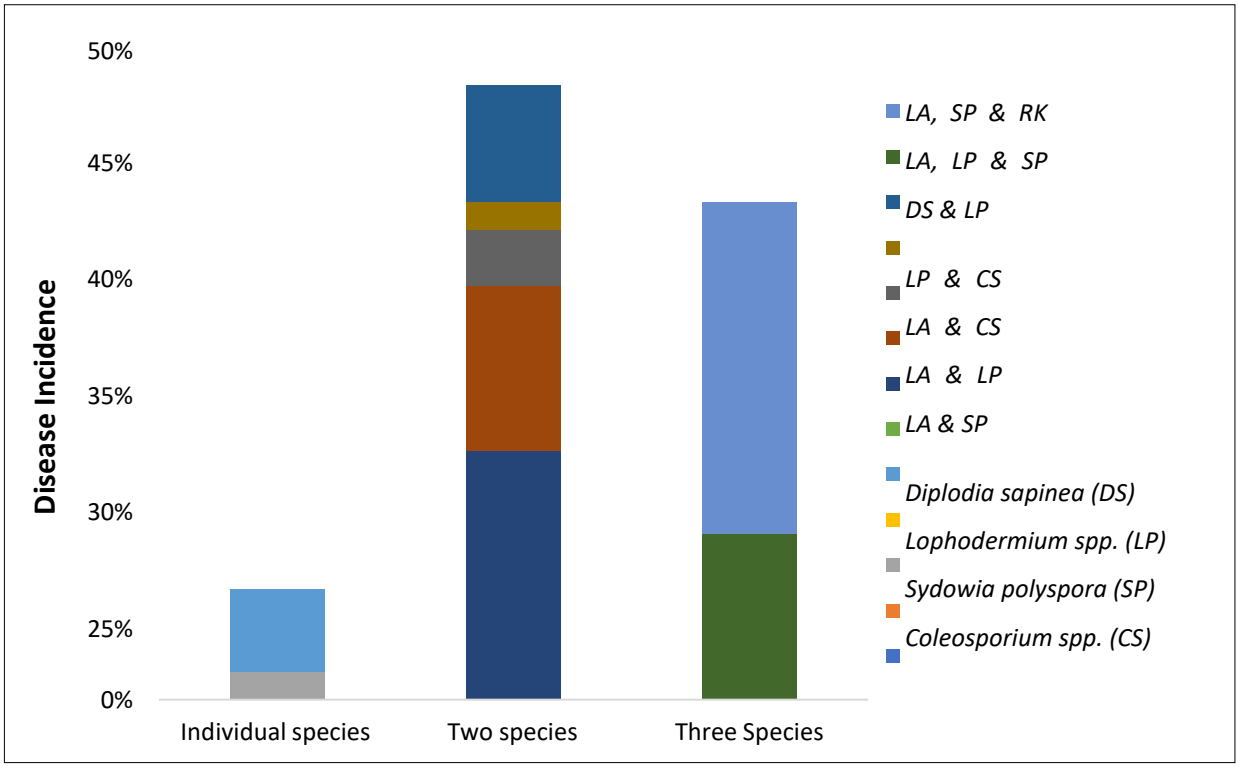

Figure 2.4. Fungal prevalence of individual species and species in combinations (one to three species present) of Lecanosticta acicola (LA), Sydowia Polyspora (SP) Lophodermium sp. (LP), Rhizosphaera kalkhoffii (RK), Coleosporium sp. (CS), and Diplodia sapinae (DS), as a percent of a total of 47 infected stands in the southeastern United States.

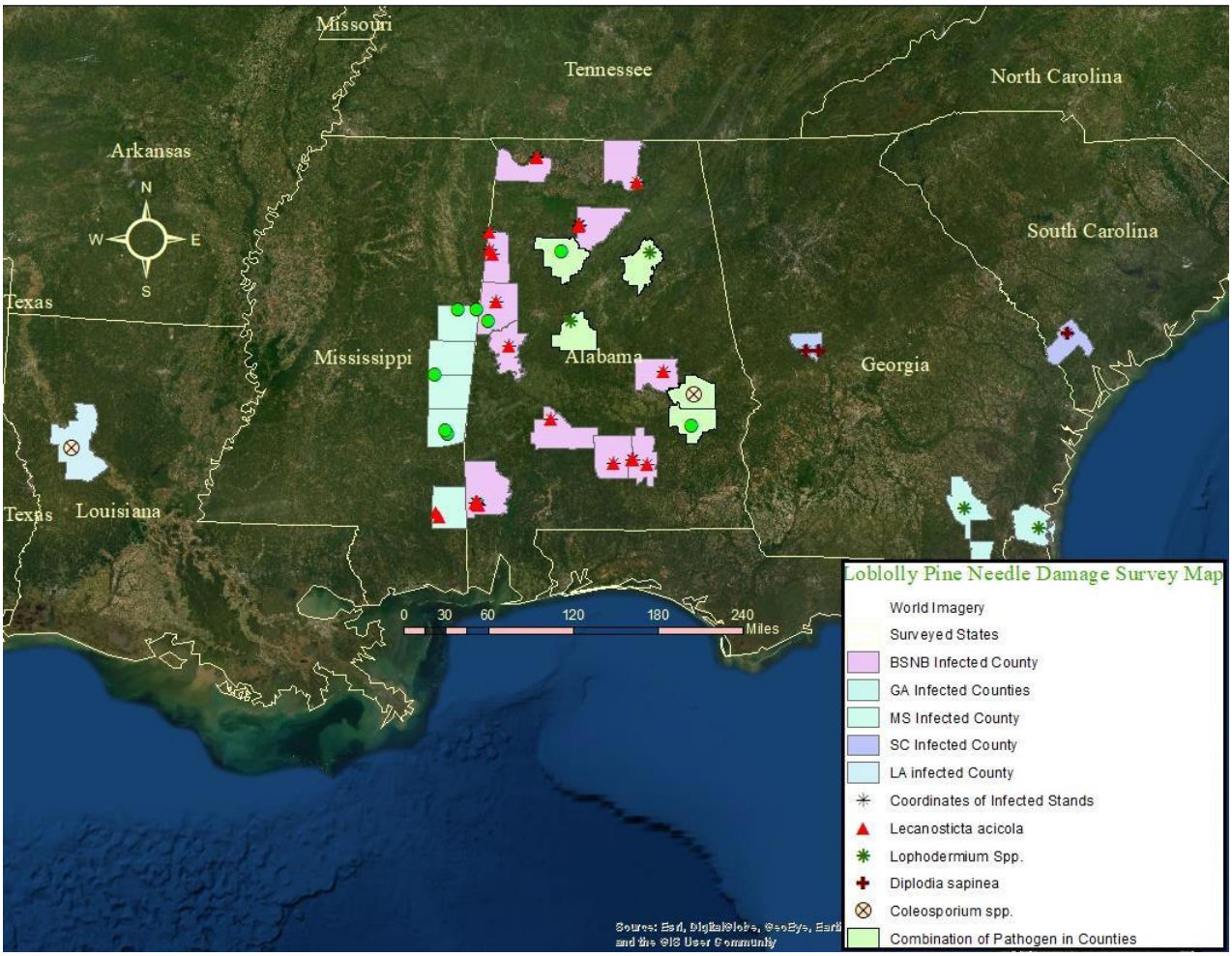

Figure 2.5. Map of pathogen distribution and loblolly pine foliar defoliation observed in stands inthe southeastern United States

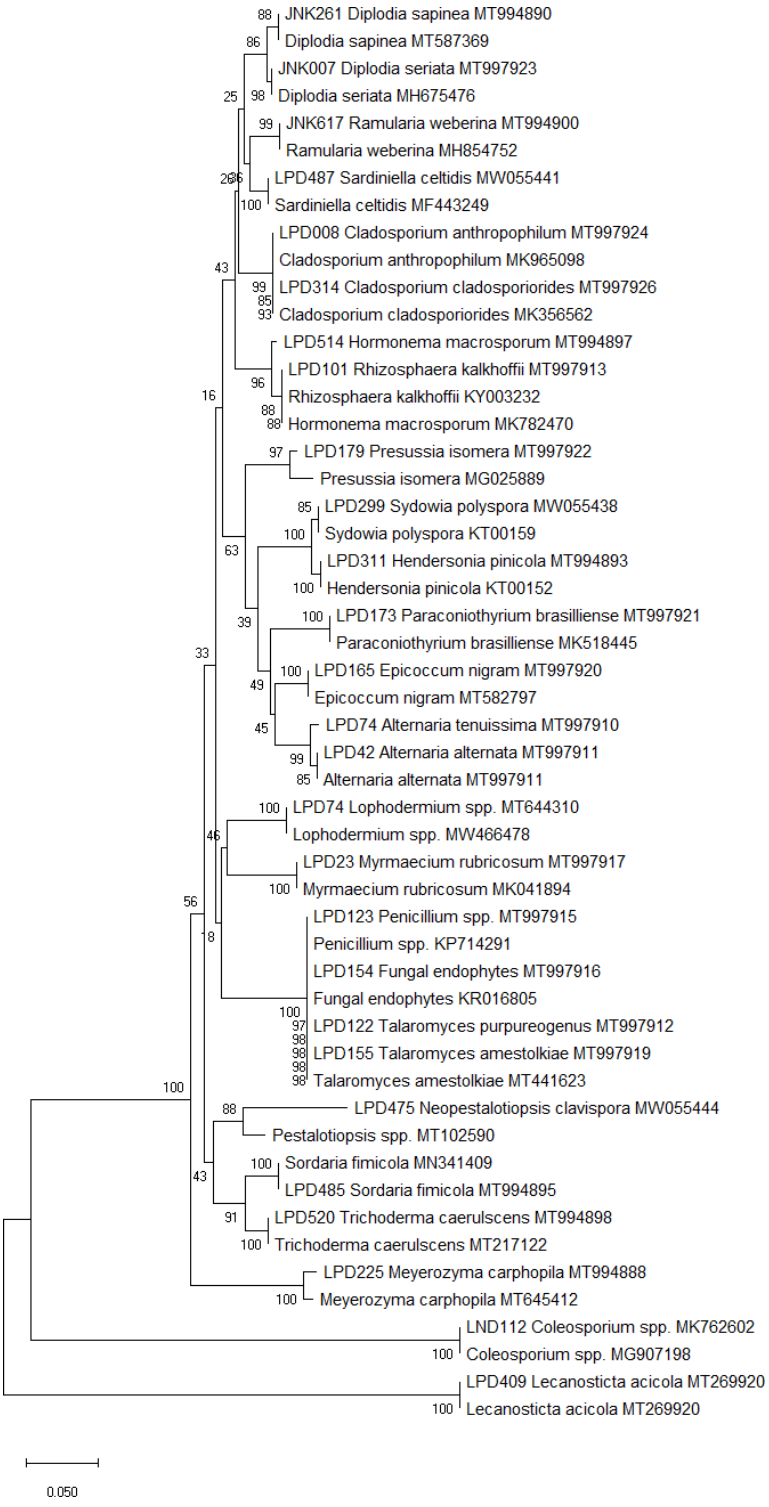

Figure 2.6. Neighbor-joining phylogenetic tree based on ITS1 and ITS4 sequences of fungi recovered from loblolly pine needles in the study.

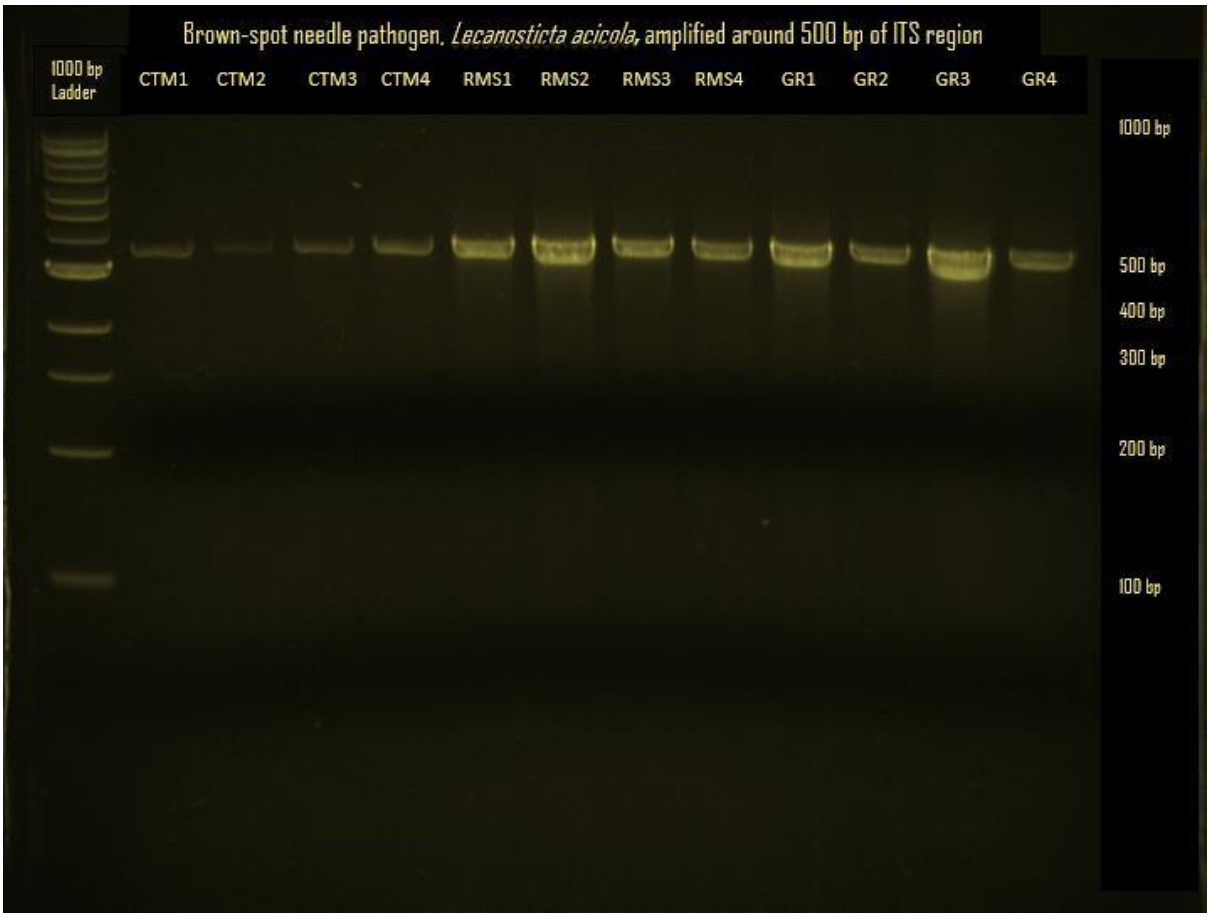

Figure 2.7. Gel electrophoresis results of PCR products showing amplification of internal transcribed spacer (ITS) region of *L. acicola* isolates recovered in the study. The top of the gelindicates *L. acicola* isolates recovered from Chatom, Butler, and Greene counties.

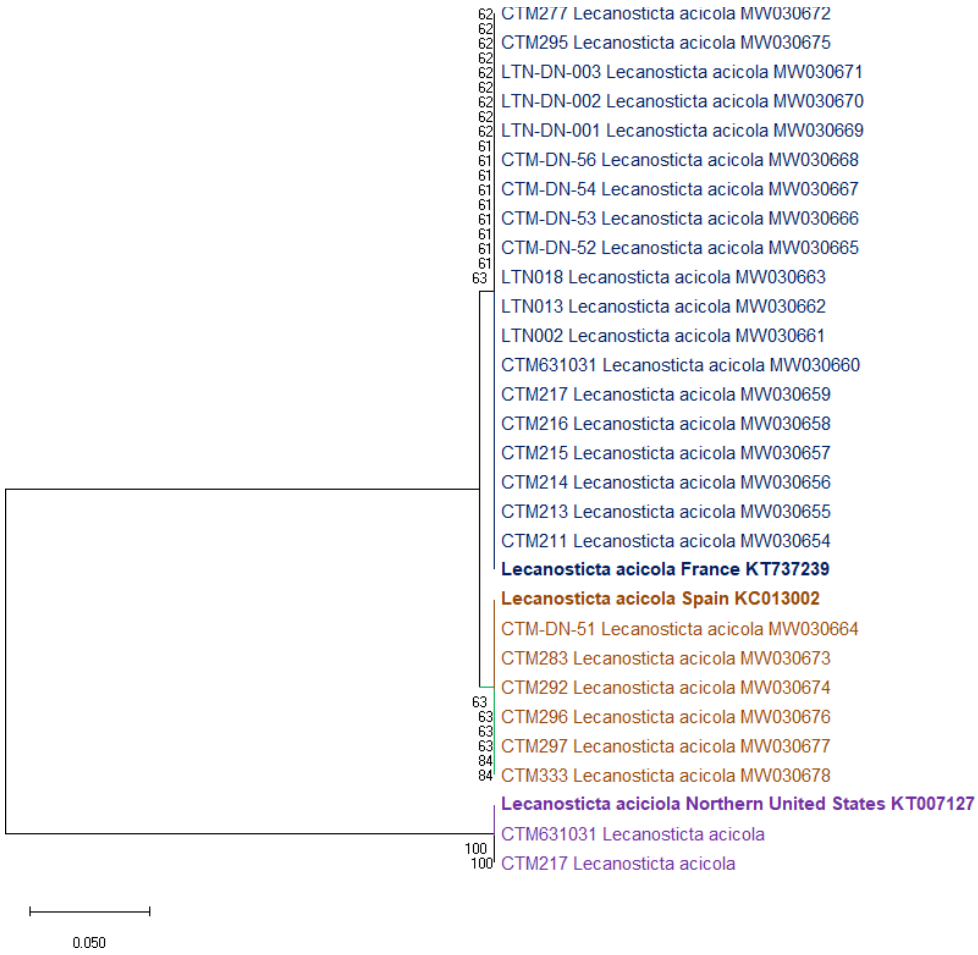

Figure 2.8. Maximum likelihood (ML) phylogenetic tree representing *L. acicola* and itsassociated lineages globally.