2020

Occurrence, mating strategy, and pathogenicity of members of Nectriaceae in Central Appalachia

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in Central Appalachia

Cameron Michael Stauder

Dissertation submitted to the Davis College of Agriculture, Natural
Resources and Design at West Virginia University in partial fulfillment of the
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in
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2020

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mating type

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ABSTRACT

Occurrence, mating strategy, and pathogenicity of members of Nectriaceae in Central Appalachia

Cameron M. Stauder

Members of the Nectriaceae occupy many ecological niches including dominant canker pathogens, such as *Neonectria ditissima* and *N. faginata*. These two pathogens contribute to the decline of American beech (*Fagus grandifolia*) forests across the Appalachian Mountains due to beech bark disease (BBD). Interestingly, *N. ditissima* represents a well-known canker pathogen many hardwood species, while *N. faginata* has not been observed outside of BBD. Additionally, *N. faginata* occurs at higher incidences than *N. ditissima* in BBD stands. Nectriaceae in Central Appalachia were surveyed as to further characterize the diversity and possibly identify a non-beech host of *N. faginata*. This resulted in the recovery of ten nectriaceous species from twelve tree species. *Neonectria faginata* only occurred on BBD trees. *Neonectria ditissima* was recovered eight tree species including *Acer spicatum*, *Ilex mucronata*, and *Sorbus americana*. *Fusarium babinda* was often recovered from BBD trees, but its role in BBD remains unclear. *Corinectria gaudineerii* sp. nov. was recovered from *Picea rubens* and *Neonectria magnoliae* comb. nov. from cankered *Liriodendron tulipifera* and *Magnolia fraseri*. The pathogenicity of *N. magnoliae* was confirmed, but the pathogenicity of *C. gaudineerii* was less apparent. Heterothallism for *N. ditissima*, *N. faginata*, and a number of other Nectriaceae was confirmed using molecular data and *in vitro* assays. This was important different mating strategies might explain differences in the ecology of *N. faginata* and *N. ditissima*. Together, these results demonstrate the diversity of

Nectriaceae in eastern North America and their mating strategies as to further our understanding of dominant diseases affecting Appalachian forests.

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INTRODUCTION

Members of the fungal family Nectriaceae occupy a number of diverse ecological niches, including mycoparasites, entomopathogens, and phytopathogens. Many species across multiple genera have been implicated in causing annual and perennial cankers on diverse woody plant hosts. Among these are generalist canker pathogens capable of infecting multiple host species, such as *Neonectria ditissima* ([Tul. & C. Tul.] Samuels & Rossman) (Lohman and Watson 1943; Spaulding et al. 1936; Booth 1967), and apparent specialists only known to infect a single host species, such as *N. faginata* ([Lohman, Watson, & Ayres] Castl. & Watson) (Castlebury et al., 2006). Interestingly, both of these are implicated in a disease known as beech bark disease (BBD).

BBD is a disease complex involving both insect and fungal agents of American beech (*Fagus grandifolia*) trees throughout eastern North America and European beech (*Fagus sylvatica*) in Europe (Hewitt, 1914; Erhlich, 1934; Cale et al., 2017). In both locations, the disease requires prior infestation by an exotic scale insect (*Cryptococcus fagisuga*) (Erhlich, 1934). Infestation by this scale insect predisposes the host tissues to invasion by one or more closely related canker fungi: *Neonectria ditissima*, *N. faginata*, and *N. coccinea* ([Pers.] Rossman and Samuels) (Houston 1994b; Thomsen et al. 1949). *Neonectria ditissima* (formerly *N. galligena* Bres.) infects beech in both North America and Europe, while *N. faginata* (formerly *N. coccinea* var. *faginata*) appears restricted to North America and *N. coccinea* to Europe (Thomsen et al. 1949; Castlebury et al. 2006).

The origin and ecological niche of *Neonectria faginata* outside of BBD is unknown. Castlebury et al. (2006) suggested *N. faginata* to be a native fungus based on

an expected level of genetic diversity present among fungal strains isolated across the distribution of BBD. Additionally, surveys conducted throughout the distribution of BBD report a dominant presence of *N. faginata* in comparison to *N. ditissima* (Houston, 1994b; Kasson and Livingston, 2009). This dominance appears to be gained over time as *N. ditissima* appears dominant early in the progression of BBD and is supplanted by *N. faginata* as the dominant pathogen (Houston 1994b). The progression of *N. faginata* across the landscape following the spread of the predisposing scale insect (*C. fagisuga*) may explain this supplantation as the inoculum potential of the native *N. ditissima* would be initially greater if already established on co-occurring hardwood species. Kasson and Livingston (2009) provided evidence supporting this theory as higher densities of non-beech, *N. ditissima*-susceptible species were correlated with higher abundances of *N. ditissima* associated with BBD trees. A number of potentially advantageous characteristics of *N. faginata* have yet to be properly investigated, including its virulence and fecundity on American beech in comparison to *N. ditissima*.

Prior to the advent of molecular phylogenetics, studies relied on the morphological identification of *Neonectria* fungi to species, and as such, the misidentification of *N. faginata* on another host substrate is plausible. *N. faginata* was first morphologically identified as a variety of *N. coccinea* (previously *Nectria coccinea*) as *Nectria coccinea* var. *faginata* (Booth, 1977), but later was determined to be a distinct species based on phylogenetic analyses (Castlebury et al., 2006). Other *Neonectria* species native to eastern North America exhibit a mosaic of morphological attributes, some of which overlap with *N. faginata* (Lohman and Watson, 1943). These factors may have led to an underrepresentation of the overall diversity of Nectriaceae

through the misidentification of species when depending on morphological comparisons. Additionally, given the plausibility of a previous misidentification of *N. faginata* on a non-beech host, additional studies are warranted as such a discovery could provide valuable insight into understanding the ecology of *N. faginata*, including its role in the BBD pathosystem.

Members of Nectriaceae, including *N. ditissima* and *N. faginata*, are often recognized by the production of brightly colored perithecia. These globose fruiting bodies bear ascospores, the products of sexual mating. While perithecia are often products of sexual reproduction between two distinct thalli (heterothallism), a self-fertile condition in fungi known as homothallism can also yield these structures through self-mating (Alexopoulos et al. 1996). Both homothallism and heterothallism have been reported in *N. ditissima* (El-Gholl et. al, 1986; Krüger, 1973), and heterothallism has been reported for *N. faginata* (Cotter and Blanchard, 1978). While the role of ascospores in BBD is limited, there is evidence that ascospore production may affect disease progression. For example, ascospores of *N. ditissima* have been demonstrated as the dominant spore type disseminating from yellow birch infections (Lortie and Kuntz, 1963). BBD progression also may depend on ascospore production and dissemination, and thus, the observed supplantation of *N. ditissima* by *N. faginata* may be in part due to mating strategy as homothallic fungi may more readily produce and disseminate ascospores in nature.

The research presented in this dissertation is intended to provide additional insight into the ecology of *N. faginata* and *N. ditissima*, but these findings are not limited to these two fungi and their associated diseases. In the first chapter, perithecia-forming

nectriaceous fungi occurring on beech and non-beech trees present in forested stands with BBD are surveyed to identify possible alternate substrates of *N. faginata*. This is critical to understanding the ecology of *N. faginata* within and outside of the BBD complex. The second chapter molecularly characterizes the MAT loci of *N. ditissima* and *N. faginata* as to determine the mating strategy. This is important as the progression of BBD may be dependent on the production and dissemination of ascospores and mating strategy could inherently influence this progression. Within Chapters 2 and 3 previously undescribed Nectriaceae or plant x host interactions occurring in eastern North America are investigated. Together these investigations may provide a greater understanding of the ecology of nectriaceous fungi within and outside of BBD.

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CHAPTER 1: Resolving host and species boundaries for perithecia-producing nectriaceous fungi across the central Appalachian Mountains

Abstract

The Nectriaceae contains numerous canker pathogens. Due to scarcity of ascomata on many hosts, comprehensive surveys are lacking. Here the diversity of perithecia-producing nectriaceous fungi across the central Appalachians is characterized. Ten species from twelve hosts were recovered including a novel *Corinectria* sp. from *Picea rubens*. *Neonectria ditissima* and *N. faginata* were most abundant and associated with *Fagus grandifolia* with beech bark disease (BBD). *N. ditissima* also was recovered from additional cankered hardwoods, including previously unreported *Acer spicatum*, *Ilex mucronata*, and *Sorbus americana*. Cross-pathogenicity inoculations of *N. ditissima* confirmed susceptibility of *Acer* and *Betula* spp. *Neonectria magnoliae* was recovered from cankered *Liriodendron tulipifera* and *Magnolia fraseri* and pathogenicity on *L. tulipifera* was confirmed. *Fusarium babinda* was consistently recovered from beech with BBD, although its role remains unclear. This survey provides a contemporary snapshot of Nectriaceae diversity across the Appalachian Mountains. The following nomenclatural changes are proposed: ***Neonectria magnoliae*** comb. nov.

Introduction

Members of the Nectriaceae occupy diverse ecological niches from mycoparasites to phytopathogens, with numerous genera and species implicated in causing annual and perennial cankers on diverse woody plant hosts with varying degrees of host specificity. One such canker disease, beech bark disease (BBD), is a disease complex occurring across the range of American beech (*Fagus grandifolia* Ehrh.) in North America and European beech (*Fagus sylvatica* L.) in Europe. The disease requires prior infestation by an exotic scale insect (*Cryptococcus fagisuga* Lind.), which predisposes the host bark tissues to subsequent invasion by predominantly one of two canker fungi: *Neonectria ditissima* ([Tul. & C. Tul.] Samuels & Rossman) and either *N. faginata* ([Lohman, Watson, & Ayres] Castl. & Watson) on American beech or *N. coccinea* ([Pers.] Rossman and Samuels) (Houston, 1994b; Thomsen et al., 1949). In North America, BBD was first observed in Halifax, Nova Scotia, Canada around 1890 and presently continues to spread throughout the continuous range of American beech (Hewitt, 1914; Erhlich, 1934; Cale et al., 2017). Other members of Nectriaceae have occasionally been associated with BBD, including *Bionectria ochroleuca* ([Schwein.] Schroers & Samuels) and *Fusarium* spp., but their roles, if any, in BBD are not well understood (Cotter and Blanchard, 1982; Houston et al., 1987; Kasson and Livingston, 2009).

In addition to infecting scale-infested beech, *Neonectria ditissima* (formerly *N. galligena*) is well-known perennial target canker pathogen on many hardwood tree species often co-occurring in beech forests impacted by BBD (Lohman and Watson, 1943; Spaulding et al., 1936; Booth, 1967). Unlike BBD, which requires predisposition of

host tissues by beech scale or possibly *Xylococculus betulae* (Cale et al., 2015), no insect partner has yet been established as a causal factor for *N. ditissima* infection on non-beech hosts. Based on previous pathogenicity studies, *N. ditissima* strains occurring in eastern North America does not exhibit host specificity (Plante and Bernier, 1997), allowing unfettered interactions between inoculum produced from cankers on beech and non-beech hosts. Although this has not been confirmed among all dominant host species co-occurring with beech in BBD areas, namely black birch (*Betula lenta* L.) and striped maple (*Acer pensylvanicum* L.), mating barriers do not exist among these strains. This indicates there is likely no host specificity given an outcrossing population of strains occurring on varying host species (Chapter 2). Further investigations are warranted as unknown host specificity and mating barriers could theoretically influence the population dynamics of *N. ditissima* strains participating in the BBD complex by limiting or disrupting gene flow.

In contrast to *N. ditissima*, *N. faginata* (formerly *N. coccinea* var. *faginata*) has only been observed causing cankers following *C. fagisuga* infestation on American beech trees (Castlebury et al., 2006), leaving questions regarding its origin and ecological niche, if any, outside of BBD. Other closely related *Neonectria* species such as *N. punicea* also occur in eastern North America within northern hardwood forests and exhibit morphological attributes that overlap with *N. faginata* including ascospore size (Booth, 1959; Castlebury et al., 2006), which has historically served as a diagnostic measure in previous studies (Houston, 1994a; Kasson and Livingston, 2009; Lohman and Watson, 1943). As such, previous misidentifications of *Neonectria* spp. on beech and non-beech hosts are plausible when relying on morphological attributes, possibly

underestimating diversity (for example see Fig 2 in Kasson and Livingston, 2009). Likewise, the close relationships among *Neonectria* spp., coupled with the lack of sequence data in repositories such as NCBI, has also presented species identification challenges, especially for rarer species, which further complicates identification. For example, our recent phylogenetic analyses of mating type gene sequences for *Nectria magnoliae* from *Liriodendron tulipifera* L. revealed this species formed a genealogically exclusive clade among other *Neonectria* species that was closely allied with *N. faginata* (Chapter 2). This supports previous findings by Gräfenhan and colleagues (2011), who showed but did not discuss that *N. ditissima* and *N. magnoliae* were genealogically exclusive. However, earlier work by Castlebury et al. (2006) concluded *N. magnoliae* was a synonym of *N. ditissima*. These findings emphasize the need for enhanced surveys to recover and phylogenetically resolve cryptic and understudied members of Nectriaceae as well as investigate the potential for cryptic reservoirs of known species including the possibility of *N. faginata* occurring on a non-beech host.

This study sought to provide a greater understanding of the ecology and genetic relationships among *N. ditissima*, *N. faginata*, and allied fungi. The first objective of this study was to survey perithecia-producing members of Nectriaceae in American beech stands to identify possible native reservoirs of *N. faginata*. This is important given the uncertainty surrounding the origin of *N. faginata* and the potential for previous misidentifications of nectriaceous fungi recovered from non-beech hosts. The second objective was to resolve phylogenetic relationships among described and possibly undescribed members of Nectriaceae recovered from forests across the central Appalachian Mountains. This was critical given both the significant undersampling of

nectriaceous fungi in these regions, the resulting the lack of sequence data, and/or contradictory evidence regarding certain known members of Nectriaceae, such as *Nectria magnoliae*. A third objective was to test host specificity of *N. ditissima*, *N. faginata*, and *N. magnoliae* strains isolated from American beech trees and tulip poplar. Together these aims sought to provide contemporary insights into the true diversity, phylogenetic relationships, and ecological niches of perithecia-producing nectriaceous fungi across the central Appalachian Mountains.

Materials and Methods

Site Selection

Sampling locations were selected based on previous reports of cankers and/or perithecia production on woody hosts, accessibility, and ability to secure sampling permits. In total, 13 beech bark disease (BBD) and eight non-BBD sampling locations were selected across WV, MD, VA, PA, TN and NC (Figure 1; Supplemental Table 1). Four of the eight non-BBD sites served for sampling nectriaceous fungi from non-beech hosts, while the other four served as sites where beech tissue samples were collected from stands with no history of BBD. No defined sampling areas (*i.e.* plots) were established, but instead, trees were sampled opportunistically until sampling needs were met based on the sampling procedure described below. Sites GK, MM, and SM had five, two, and four geographically separated discrete sampling areas (Supplemental Table 1). All other sites are represented by a single sampling area.

Three WV sites (BM, GK, FR) were originally confirmed as having BBD at least 35 years prior to the initiation of this study (Mielke et al., 1982, Houston, 1994a). Sites designated BG, DM, and PSF represented more recent BBD outbreaks (~ 15 years) as

determined by various natural resource agency surveys in WV and MD. All other sites were determined to have likely become afflicted with BBD between 5-to-25 years prior (Morin et al., 2007). Four of seven sites lacking BBD (NB, PM, AR, UW) were selected for asymptomatic beech sampling based on a lack of previous reports, and all trees were visually confirmed to be absent of BBD-associated signs and symptoms prior to sampling.

Figure 1: Sampling locations for members of Nectriaceae in the central Appalachian Mountains. Sites with confirmed beech bark disease (BBD) are designated by filled circles, sites lacking BBD are open circles, and counties where BBD has been previously reported are filled. BBD was first discovered in WV at site GK in 1981 (Mielke et al. 1982, Mielke and Houston, 1983). BBD distribution is based on a county level database (Blackburn, 2018).

Sampling Procedure

At each BBD site, American beech trees symptomatic for BBD and non-beech trees with perennial target cankers were identified and sampled. For approximately five trees of each species per site, a maximum of four bark disks harboring fresh perithecia were excised with a 1-cm diameter steel punch and stored in a microtiter plates. Additionally, bark tissue samples (~1 mm diameter plugs; ~8 samples/tree; up to 7 beech trees/site) were taken from symptomatic and asymptomatic trees at BBD and non-BBD sites, respectively. All samples were stored on ice until arriving at the laboratory where samples were stored at -20 °C until processing.

Sample Processing

To process each bark disk, up to five perithecia were removed with a sterile scalpel and cleaned by gently pushing each perithecium through sterile agar to remove debris. Each perithecium was squashed in a 1.5 ml microcentrifuge tube containing 1 ml of sterile H₂O with a micropestle, vortexed for 15 seconds, and then 300 µl of the spore suspension was spread with a cell spreader on glucose-yeast extract agar plates amended with streptomycin sulfate (10 mg/1000 ml) and tetracycline hydrochloride (100 mg/1000 ml) antibiotics (GYE/A). Within 48 hours of plating, five germinating ascospores were subcultured to a new plate, and one isolate was selected for storage at approximately -20 °C on glass filter paper. All micro-sampled bark plugs were surface disinfested by soaking for 14 minutes in a 1:10 commercial bleach-water solution then up to four samples were placed onto each GYE/A agar plate. Resulting fungi with morphologies reminiscent of the Nectriaceae were subcultured individually to new plates then stored as previously described.

Species Identification

Recovered isolates were grouped and tentatively identified based on colony and macroconidia morphology and a subset of isolates were selected to be confirmed by DNA sequencing. Genomic DNA was extracted from isolates using a Wizard® kit (Promega, Madison, WI, USA) and suspended in 75 µl Tris-EDTA (TE) buffer (Amresco, Solon, OH, USA). PCR reactions were performed for the fungal barcoding genes internal transcribed spacer region (ITS), translation elongation factor 1-alpha (EF-1α), 28S rDNA (LSU), and β-tubulin (BTUB). PCR products were generated in 25 µl reactions containing 12.5 µl Bioline PCR Master Mix (Bioline USA Inc, Taunton, MA), 10.0 µl H₂O, 1.5 µl purified DNA, and 1.0 µl each of forward and reverse primers (Integrated DNA Technologies, Coralville, IA, USA). All primers, protocols, and their sources are listed in Supplemental Table 2. Positive reactions identified via gel electrophoresis were prepared for sequencing using ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's recommendations and Sanger sequenced using forward PCR primers (Eurofins, Huntsville, AL, USA). Resultant sequences were used to identify species using BLASTn searches, and the best match in the NCBI database was selected for the isolate's identity.

Phylogenetic Analyses

For all sequences included in the phylogenetic analyses, chromatograms were assessed and clipped using CodonCode Aligner v. 5.1.5. Sequences were then manually corrected for nucleotide misreads by referencing their respective chromatograms. To examine phylogenetic relationships among collected members of Nectriaceae, single-gene and concatenated phylogenetic trees were constructed for all

Corinectria, *Fusarium*, and *Neonectria* species observed in this survey along with additional reference sequences for selected members of Nectriaceae available from NCBI Genbank (Table 3). The other members of the Nectriaceae recovered either lacked sampling depth or adequate reference sequences for the loci of interest to permit meaningful phylogenetic analysis. As such, only BLASTn searches of individual loci were conducted for these species with representative sequences deposited into NCBI Genbank. For *Corinectria*, *Fusarium*, and *Neonectria* species, each gene was aligned using MAFFT (Kato and Stanley, 2013) on the Guidance 2.0 server (Landan and Graur, 2008; Sela et al., 2015). Individual residues with Guidance scores <0.5 were masked (Macias et al., 2020). A concatenated sequence was generated from single gene alignments using the web tool FaBox (Villesen, 2007).

For single gene and concatenated sequences, maximum-likelihood analyses were completed using MEGA v10.1.7 (Stecher et al., 2020), and Bayesian inference (BI) analyses were completed using MrBayes v. 3.2.7 (Ronquist et al., 2012). For ML analyses, the best-fit nucleotide substitution model was chosen using Model Test AICc scores in MEGA and 1000 bootstrap replicates were used. For BI analyses, MrBayes selected the best fit nucleotide selection model, but the rate of substitution was selected from the Model test AICc scores. The BI runs were stopped once the standard deviation of split frequencies fell below 0.01. These were then checked for convergence in Tracer v. 1.7.1 (Rambaut et al., 2018). Trees were prepared for publication using FigTree v. 1.4.4 (Rambaut, 2017) and Adobe Illustrator v. 24.1.

Morphological Measurements

Over the course of this survey, a species molecularly identified as *Nectria magnoliae* was recovered from tulip poplar (*Liriodendron tulipifera* L.) and Fraser magnolia (*Magnolia fraseri* Walter). Based on a recent study in which isolates from both of these hosts were included, this species was shown to form an independent clade with other members of *Neonectria* (Chapter 2). A previously published study also supported these relationships (Gräfenhan et al. 2011). Given these phylogenetic relationships, ascospore and conidia measurements were conducted to further characterize this species to permit comparisons with original descriptions provided by Lohman and Watson (1943).

For ascospore measurements, a total of 15 perithecia were processed. Three perithecia were sampled from each of five bark disks representing two geographically separated sites (FN = 3 disks; SH = 2 disks). A single perithecium was extracted from the bark disk using a sterile scalpel and squash-mounted on a slide with lactophenol plus cotton blue mountant. Length and width measurements were collected for 25 ascospores per perithecium. All measurements were taken with a Nikon Eclipse E600 compound microscope (Nikon Instruments, Melville, NY, USA) equipped with a Nikon Digital Sight DS-Ri1 microscope camera and Nikon NIS-Elements BR3.2 imaging software.

Conidia measurements were similarly conducted for both micro- and macro-conidia. Here, sporodochial masses were harvested from pure four-to six-week-old cultures using a sterile scalpel and mounted onto slides as described above. Both length and width measurements were taken for 50 microconidia were harvested from

two isolates from each of three geographically separated locations (GK, SH, FN). Samples from SH and FN were collected from *L. tulipifera*, and samples from GK were collected from *M. fraseri*. Macroconidia were only found associated with one isolate collected from *M. fraseri* at GK and measured as described.

Pathogenicity Assays

Field inoculations were conducted to further investigate pathogenicity of *N. magnoliae* strains from *L. tulipifera* as well as *N. faginata* and *N. ditissima* strains from beech on birch, striped maple, and beech. For *N. magnoliae*, a single isolate (NmLt001) recovered from a natural infection on *L. tulipifera* was selected. One isolate of both *N. ditissima* (NdFg002) and *N. faginata* (NfFg005) recovered from American beech also were included for cross-pathogenicity testing. All study isolates were grown in pure culture on GYE for two-weeks at room temperature. Prior to inoculations, a sterile 1-cm steel punch was used to cut inoculation plugs along the growing edge of the colony. Negative control plugs were cut from sterile GYE plates.

Six each of tulip poplar (*L. tulipifera*), black birch (*Betula lenta*), yellow birch (*Betula alleghaniensis* Britt.), and striped maple (*Acer pensylvanicum*) trees, located on WVU University Forest were selected for inoculations. Additionally, five American beech trees were selected at this same location. Each tree received an inoculation with *N. ditissima*, *N. faginata*, and *N. magnoliae*. In addition to these fungal inoculations, a negative control inoculation with a sterile GYE agar plug also was performed on each study tree.

For each inoculation, a sterile 1-cm leather punch was used to excise bark tissue and create an inoculum reservoir. A colonized or sterile agar plug was then placed into

the reservoir, and masking tape was applied over the wound to limit inoculum desiccation prior to infection. After six-months, bark tissue was excised from canker margins using a bone-marrow biopsy tool and placed in a 96-well microtiter dish. To ensure accurate canker measurements, a knife was used to remove bark tissue and reveal any underlying necrosis. Length and width measurements were then taken for each canker resulting from inoculation. Recovery of inoculant was achieved as previously described for bark tissue sample processing. All isolate identities were confirmed morphologically.

Statistical analyses

For pathogenicity measures, a one-way ANOVA was completed to check for differences in canker size and a Tukey-HSD post-hoc test was completed identify significant pairwise differences using the stats v3.6.2 package within R v 3.6.3 statistical software (R Core Team, 2020). All p-values <0.05 were considered significant.

Results

Survey of nectriaceous fungi

Perithecia of putative nectriaceous fungi were sampled from 180 trees across 17 sites in WV, MD, VA, NC, and TN (Table 1). Sampling included 12 tree species with black birch, red spruce, and mountain ash being the most abundantly sampled species besides America beech. A total of 1,605 sampled perithecia yielded nine fungal species spanning six genera. The majority of these samples were collected from American beech trees (n = 1,257 perithecia). The remaining 348 perithecia were sampled from one of the other 11 host species listed in Table 1. All isolates were grouped initially by

morphology and a subset was identified with NCBI BLASTn searches using ITS barcoding sequences (Table 2; Supplemental Table 3). For NCBI blast hits, a >98% sequence identity was used as a cutoff threshold to confirm identification of previously described species.

Of samples collected from American beech trees, approximately 4.2% (53 out of 1,257) of sampled perithecia yielded isolates of *N. ditissima*, while 93.7% (1,178 out of 1,257) yielded *N. faginata*. The remaining 2.1% (26 out of 1,257) yielded *Bionectria ochroleuca*. Out of the 13 sites where American beech perithecia were sampled, *N. ditissima* was only recovered from locations FR92 and GK and represented 2.8% (5 out of 177) and 10.8% (48 out of 443) of the viable perithecia collected from those sites, respectively. In one instance, both *N. ditissima* and *N. faginata* were isolated from perithecia occurring on the same bark disk. *Bionectria ochroleuca* was recovered from 2 of 13 sites, GK and SM.

N. ditissima also was isolated from conspicuous cankers on a number of additional hardwood hosts including yellow birch (*Betula allegheniensis*), mountain maple (*Acer spicatum* Lam.) (Figure 2C), black birch (*B. lenta*) (Figure 2D), and mountain ash (*Sorbus americana* Marshall) (Figure 2E) (Table 1). Additionally, *N. ditissima* also was recovered from the main stems of striped maple (*A. pensylvanicum*) and mountain holly (*Ilex mucronata* (L.) M.Powell, Savol., & S.Andrews), though the cankers were inconspicuous and mostly lacking callous ridges (Figure 2A and 2F) (Table 1; Figure 2; Figure 3A). *Bionectria ochroleuca* was recovered from American beech and American basswood (*Tilia americanum* L.) (Figure 3G). Three additional members of Nectriaceae also were recovered, albeit less frequently, during this survey

on tree species found co-occurring with beech in BBD areas or nearby. These included *N. magnoliae* on tulip poplar (*L. tulipifera*) and Fraser magnolia (*Magnolia fraseri*) (Figure 6), *Thelonectria veuillotiana* ([Roum. & Sacc.] P. Chaverri & Salgado) on mountain maple (*A. spicatum*) and mountain ash (*S. americana*) (Figure 3F), and *Cosmospora obscura* (Rossman & Samuels) on mountain ash (Figure 3H).

Figure 2: Example *Neonectria ditissima* canker photos for the following host species: A) mountain holly (*Ilex mucronate* at SK), B) American beech (*Fagus grandifolia* at GK), C) mountain maple (*Acer spicatum* at MM), D) black birch (*B. lenta* at UF), E) mountain ash (*Sorbus Americana* at WT), F) striped maple (*A. pensylvanicum* at SK)

Table 1: Survey sample collection summary by host tree species. Values under each fungal species represent the number of sampled perithecia yielding an isolate of that fungus. In total, 1,605 perithecia yielded nine fungal species from ten host tree species at 17 geographically separated sites.

Host Species	Site	General Location	Trees	Peri	<i>Neonectria ditissima</i>	<i>Neonectria faginata</i>	<i>Neonectria magnoliae</i>	<i>Neonectria neomacrospora</i>	<i>Bionectria ochroleuca</i>	<i>Corinectria</i> sp.	<i>Thyronectria balsamea</i>	<i>Cosmopora obscura</i>	<i>Thelonectria veuillotiana</i>
<i>Abies balsamea</i>	MON	Monongalia County, WV	1	5	0	0	0	0	0	0	5	0	0
	MR	Grayson County, VA	1	5	0	0	0	5	0	0	0	0	0
<i>Acer pensylvanicum</i>	GK	Randolph County, WV	3	29	29	0	0	0	0	0	0	0	0
	SM	Cherokee County, NC	2	10	10	0	0	0	0	0	0	0	0
	PSF	Allegany County, MD	3	8	8	0	0	0	0	0	0	0	0
<i>Acer saccharum</i>	GK	Randolph County, WV	1	5	5	0	0	0	0	0	0	0	0
<i>Acer spicatum</i>	SM	Cherokee County, NC	2	8	8	0	0	0	0	0	0	0	0
	MM	Yancey County, WV	4	20	9	0	0	0	0	0	0	0	11
<i>Betula alleghaniensis</i>	UF	Monongalia County, WV	2	9	9	0	0	0	0	0	0	0	0
<i>Betula lenta</i>	BM	Randolph County, WV	1	5	5	0	0	0	0	0	0	0	0
	DM	Allegany County, MD	5	12	12	0	0	0	0	0	0	0	0
	SH	Monongalia County, WV	1	1	1	0	0	0	0	0	0	0	0
	UF	Monongalia County, WV	1	5	5	0	0	0	0	0	0	0	0
<i>Fagus grandifolia</i>	BM	Randolph County, WV	7	107	0	107	0	0	0	0	0	0	0
	DM	Allegany County, MD	6	80	0	80	0	0	0	0	0	0	0
	FR223	Randolph County, WV	5	19	0	19	0	0	0	0	0	0	0
	FR92	Randolph County, WV	12	177	5	172	0	0	0	0	0	0	0
	GK	Randolph County, WV	35	467	48	395	0	0	24	0	0	0	0
	SM	Cherokee County, NC	16	40	0	38	0	0	2	0	0	0	0
	MM	Yancey County, WV	4	10	0	10	0	0	0	0	0	0	0
	MR	Grayson County, VA	2	3	0	3	0	0	0	0	0	0	0
	PSF	Allegany County, MD	16	251	0	251	0	0	0	0	0	0	0

	RH	Mitchell County, NC	5	8	0	8	0	0	0	0	0	0	0
	SK	Pendleton County, WV	5	36	0	36	0	0	0	0	0	0	0
	WT	Grayson County, VA	6	16	0	16	0	0	0	0	0	0	0
	WVB G	Monongalia County, WV	3	43	0	43	0	0	0	0	0	0	0
<i>Ilex mucronata</i>	SK	Pendleton County, WV	3	15	15	0	0	0	0	0	0	0	0
<i>Liriodendron tulipifera</i>	FERN	Tucker County, WV	6	89	0	0	89	0	0	0	0	0	0
	SH	Monongalia County, WV	3	15	0	0	15	0	0	0	0	0	0
<i>Picea rubens</i>	GK	Randolph County, WV	4	27	0	0	0	0	0	27	0	0	0
	WT	Grayson County, VA	5	38	0	0	0	0	0	38	0	0	0
	MR	Grayson County, VA	3	13	0	0	0	0	0	13	0	0	0
<i>Sorbus americana</i>	MM	Yancey County, WV	3	12	7	0	0	0	0	0	0	5	0
	RH	Mitchell County, NC	2	7	7	0	0	0	0	0	0	0	0
	WT	Grayson County, VA	1	5	5	0	0	0	0	0	0	0	0
<i>Tilia americana</i>	SM	Cherokee County, NC	1	5	0	0	0	0	5	0	0	0	0

Table 2: Isolate identification by BLASTn searches using ITS sequences derived from selected representatives of dominant morphologies. Percent identity values represent the lowest identity value among the sequences for each fungal species.

Species	Isolate Count	Closest tblastn match	% identity
<i>Neonectria ditissima</i>	17*	<i>Neonectria ditissima</i>	≥ 98.29%
<i>Neonectria faginata</i>	5	<i>Neonectria faginata</i>	≥ 99.02%
<i>Neonectria magnoliae</i>	11	<i>Nectria magnoliae</i>	≥ 98.38%
<i>Neonectria neomacrospora</i>	2	<i>Neonectria neomacrospora</i>	99.2%
<i>Bionectria ochroleuca</i>	2	<i>Bionectria ochroleuca</i>	≥ 98.5%
<i>Corinectria</i> sp.	5	<i>Corinectria fuckeliana</i>	≥ 94.8%
<i>Thyronectria balsamea</i>	2	<i>Thyronectria balsamea</i>	≥ 99.44%
<i>Cosmospora obscura</i>	2	<i>Cosmospora obscura</i>	≥ 99.04%
<i>Thelonectria veuillotiana</i>	4	<i>Thelonectria veuillotiana</i>	≥ 99.64%
<i>Fusarium babinda</i>	4	<i>Fusarium babinda</i>	≥ 99.8%

*Additional *N. ditissima* samples sequenced to represent isolates from varied host species

Three members of Nectriaceae also were confirmed on conifer hosts. These included *Thyronectria balsamea* ([Cooke & Peck] Seeler) on balsam fir (*Abies balsamea* (L.) Mill.) and *Neonectria neomacrospora* ([C. Booth & Samuels] Mantiri & Samuels) on Fraser fir (*Abies fraseri* (Pursh) Poir.) (Figure 3E and 3C, respectively). Additionally, a novel *Corinectria* sp. on red spruce (*Picea rubens* Sarg.) was recovered in this survey across high elevation spruce forests in two states (Figure 3D; Figure 4).

Figure 3: Diversity of Nectriaceae recovered across the central Appalachian Mountains on natural substrate and in culture: A) *Neonectria ditissima* (shown on striped maple at SK), B) *N. faginata* (on American beech at BG), C) *N. neomacrospora* (on Fraser fir at MR), D) *Corinectria* sp. (on red spruce at GK), E) *Thryonectria balsamea* (on balsam fir at MON), F) *Thelonectria veuillotiana* (on mountain maple at MM), G) *Bionectria ochroleuca* (on American beech at SM), and H) *Cosmospora obscura* (on mountain ash at MM).

In addition to the previously mentioned nectriaceous fungi recovered from perithecia on symptomatic beech trees and nearby co-occurring trees, a member of the *Fusarium babinda* species complex (FBSC) was commonly isolated from wood tissues

of BBD-confirmed American beech trees (31.8% of bark samples), but it was lacking fruiting bodies (Supplemental Figure 1; Supplemental Table 4). To further investigate this finding, five sites without BBD were identified, and American beech bark tissues were sampled and processed. FBSC was recovered from all confirmed BBD sites but was never recovered from non-BBD sites. Aside from *Fusarium babinda*, *N. faginata* and *N. ditissima* were occasionally recovered from beech bark tissues collected but only within BBD sites (Supplemental Table 4).

Phylogenetic Analyses

Phylogenetic analyses were performed for single genes (ITS, TEF1, TUB, LSU) and for a four-gene concatenated sequence to infer relationships among *Neonectria* and *Corinectria* species recovered in the survey. These analyses were supported with the addition of sequence data from several reference strains for both genera (Table 3). Representatives of the *Fusarium concolor* and *Fusarium babinda* species complexes were chosen to serve as outgroup taxa that could confirm the identity of *Fusarium babinda* isolates that were frequently recovered in this study from collected bark tissue samples.

The following results are summarized for relationships with >70% bootstrap support within the concatenated phylogeny (Figure 4). *Neonectria* and *Corinectria* were resolved as strongly supported (100%/1.0) sister genera. All included *Neonectria* species were genealogically exclusive (>83%/1.0). *N. ditissima* isolates recovered from diverse host species formed a monophyletic clade with strong support (98%/1.0). *Neonectria neomacrospora* was sister to *N. ditissima* (72%/1.0) and isolates recovered in this survey were sister to selected NCBI reference sequences.

Isolates identified as *Nectria magnoliae* formed a monophyletic clade within *Neonectria* (99%/1.0) and was sister to a clade containing *N. faginata*, *N. coccinea*, *N. confusa*, and *N. punicea* (92%/1.0). The one isolate recovered from *Magnolia fraseri* (NmMf001) was divergent from all other *N. magnoliae* isolates recovered from *L. tulipifera* (73%/0.91). All single-gene trees available in Supplemental Figure 2.

Table 3: Accession table for sequences used in the phylogenetic analyses of this study. Sequences were either acquired from NCBI or generated as part of this study. Host species and location are also provided where available.

Species	Strain ID	Host	Locality	BT	LSU	EF1	ITS
<i>Fusarium babinda</i>	FbFg001	<i>Fagus grandifolia</i>	Allegany County, MD, USA	TBD	TBD	TBD	TBD
<i>F. babinda</i>	FbFg002	<i>F. grandifolia</i>	Randolph County, WV, USA	TBD	TBD	TBD	TBD
<i>F. babinda</i>	FbFg003	<i>F. grandifolia</i>	Allegany County, MD, USA	TBD	TBD	TBD	TBD
<i>F. babinda</i>	FbFg004	<i>F. grandifolia</i>	Monongalia County, WV, USA	TBD	TBD	TBD	TBD
<i>F. concolor</i>	NRRL 13994	<i>Hordeum</i> sp.	Uruguay	U61549.1	U61653.1	---	U61679.1
<i>F. concolor</i>	BCCM9748	<i>Homo sapiens</i>	Spain	KJ125965.1	KJ126557.1	---	KJ125669.1
<i>Neonectria coccinea</i>	AF3707	<i>F. sylvatica</i>	Slovakia	TBD	TBD	---	TBD
<i>N. coccinea</i>	AR3691	<i>F. sylvatica</i>	Romania	TBD	TBD	---	TBD
<i>N. coccinea</i>	CBS 118916	<i>F. sylvatica</i>	Romania	DQ789840.1	KC660601.1	KC660442.1	KC660505.1
<i>N. coccinea</i>	CBS 119158	<i>Fagus</i> sp.	Germany	KC660727.1	KC660620.1	JF268734.1	KC660521.1
<i>N. confusa</i>	JL2009a-5741	---	---	JF268721.1	---	JF268736.1	JF268760.1
<i>N. confusa</i>	CBS 127484	---	China	KM515886.1	KM515933.1	---	KM515889.1
<i>N. ditissima</i>	NdBa001	<i>Betula alleghaniensis</i>	Monongalia County, WV, USA	TBD	TBD	TBD	TBD
<i>N. ditissima</i>	NdSam001	<i>Sorbus americana</i>	Grayson County, VA, USA	TBD	TBD	TBD	TBD

<i>N. ditissima</i>	NdAp002	<i>Acer pensylvanicum</i>	Swain County, NC, USA	TBD	TBD	TBD	TBD
<i>N. ditissima</i>	NdAs002	<i>Acer spicatum</i>	Yancey County, WV, USA	TBD	TBD	TBD	TBD
<i>N. ditissima</i>	NdII001	<i>Ilex mucronata</i>	Pendleton County, WV, USA	TBD	TBD	TBD	TBD
<i>N. ditissima</i>	NdBI002	<i>Betula lenta</i>	Allegany County, MD, USA	TBD	TBD	TBD	TBD
<i>N. ditissima</i>	NdAsa001	<i>Acer saccharum</i>	Randolph County, WV, USA	TBD	TBD	TBD	TBD
<i>N. ditissima</i>	NdFg002	<i>F. grandifolia</i>	Randolph County, WV, USA	TBD	---	TBD	TBD
<i>N. ditissima</i>	NdFg006	<i>F. grandifolia</i>	Randolph County, WV, USA	TBD	TBD	TBD	TBD
<i>N. ditissima</i>	NdFg001	<i>F. grandifolia</i>	Randolph County, WV, USA	TBD	TBD	TBD	TBD
<i>N. ditissima</i>	CBS 100.316	---	Ireland	HM352864.1	HM364311.1	HM364350.1	HM364298.1
<i>N. ditissima</i>	CBS 226.31	<i>F. sylvatica</i>	Tharandt, Germany	DQ789869.1	AY677330.1	JF735783.1	JF735309.1
<i>N. faginata</i>	CBS 118938	<i>F. grandifolia</i>	PA, USA	DQ789846.1	KC660614.1	KC660446.1	KC660508.1
<i>N. faginata</i>	CBS 217.67	<i>F. grandifolia</i>	New Brunswick, Canada	JF268730.1	HQ840382.1	JF268746.1	HQ840385.1
<i>N. faginata</i>	NfFg014	<i>F. grandifolia</i>	Allegany County, MD, USA	TBD	TBD	TBD	TBD
<i>N. faginata</i>	NfFg007	<i>F. grandifolia</i>	Swain County, NC, USA	TBD	TBD	TBD	TBD
<i>N. faginata</i>	NfFg005	<i>F. grandifolia</i>	Randolph County, WV, USA	TBD	TBD	---	TBD

<i>N. faginata</i>	NfFg015	<i>F. grandifolia</i>	Monongalia County, WV, USA	TBD	TBD	---	TBD
<i>N. magnoliae</i>	NmLt001	<i>Liriodendron tulipifera</i>	Tucker County, WV, USA	TBD	TBD	TBD	TBD
<i>N. magnoliae</i>	NmLt002	<i>L. tulipifera</i>	Tucker County, WV, USA	TBD	---	TBD	TBD
<i>N. magnoliae</i>	NmLt003	<i>L. tulipifera</i>	Monongalia County, WV, USA	TBD	TBD	TBD	TBD
<i>N. magnoliae</i>	NmLt004	<i>L. tulipifera</i>	Monongalia County, WV, USA	TBD	---	TBD	TBD
<i>N. magnoliae</i>	NmMf001	<i>Magnolia fraseri</i>	Randolph County, WV, USA	TBD	TBD	TBD	TBD
<i>N. punicea</i>	CBS 119532	<i>F. sylvatica</i>	Slovakia	KC660728.1	KC660563.1	KC660439.1	KC660503.1
<i>N. punicea</i>	CBS 242.29	<i>Rhamus</i> sp.	Germany	DQ789873.1	MH866520.1	DQ789730.1	MH855054.1
<i>N. neomacrospora</i>	NnAb002	<i>Abies fraseri</i>	Grayson County, VA, USA	TBD	TBD	TBD	TBD
<i>N. neomacrospora</i>	NnAb001	<i>A. fraseri</i>	Grayson County, VA, USA	TBD	TBD	TBD	TBD
<i>N. neomacrospora</i>	CBS 118985	<i>Tsuga heterophylla</i>	Canada	DQ789890.1	HQ840380.1	JF268755.1	HQ840389.1
<i>N. neomacrospora</i>	CBS 118984	<i>Abies balsamea</i>	Canada	DQ789882.1	HQ840379.1	JF735787.1	HQ840388.1
<i>N. hederæ</i>	IMI 058770a	<i>Hedera helix</i>	United Kingdom	DQ789895.1	KC660617.1	KC660461.1	---
<i>N. hederæ</i>	CBS 714.97	<i>H. helix</i>	Netherlands	DQ789878.1	KC660616.1	KC660461.1	QQQB00000000
<i>N. ramulariae</i>	CBS 151.29	<i>Malus sylvestris</i>	United Kingdom	DQ789863.1	AY677333.1	HM054091.1	AY677291.1
<i>N. ramulariae</i>	CBS 182.36	<i>M. sylvestris</i>	---	JF735439.1	MH867276.1	JF735792.1	MH855762.1

<i>Corinectria</i> sp.	CspPr004	<i>Picea rubens</i>	Randolph County, WV, USA	TBD	TBD	TBD	TBD
<i>Corinectria</i> sp.	CspPr007	<i>P. rubens</i>	Randolph County, WV, USA	TBD	TBD	TBD	TBD
<i>Corinectria</i> sp.	CspPr002	<i>P. rubens</i>	Grayson County, VA, USA	TBD	TBD	TBD	TBD
<i>Corinectria</i> sp.	CspPr008	<i>P. rubens</i>	Grayson County, VA, USA	---	TBD	TBD	TBD
<i>C. tsugae</i>	CBS 788.69	<i>T. heterophylla</i>	Canada	KM232020.1	MH871201.1	---	KM231763.1
<i>C. fuckeliana</i>	IMI 342668	<i>Picea</i> sp.	Switzerland	KJ022340.1	KJ022070.1	KJ022404.1	KJ022021.1
<i>C. fuckeliana</i>	CBS 239.29	<i>Picea sitchensis</i>	Scotland	DQ789871.1	HQ840377.1	JF268748.1	HQ840386.1
<i>C. constricta</i>	LASBE 266	<i>Pinus radiata</i>	Chile	KY636417.1	---	KY636410.1	---

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Figure 4: Four-gene (ITS, BTUB, ACTIN, EF1) concatenated phylogeny tree of *Neonectria* spp., *Corinectria* spp. and outgroups. Topology and branch lengths are from the ML analysis. For each node supported in the ML analysis, bootstrap support and posterior probabilities are indicated (ML/BI).

Pathogenicity Assay

A field pathogenicity assay was conducted to further characterize *N. magnoliae* and test the pathogenicity of *N. faginata* and *N. ditissima* isolates originating from American beech on alternative host species including those not previously included in cross pathogenicity assays. Overall, *N. ditissima* produced significantly larger cankers ($p < 0.05$) than all other treatments on striped maple, yellow birch, and black birch (Figure 5A-C, respectively). *N. faginata* and *N. magnoliae* failed to produce cankers significantly larger than the negative control on any of the aforementioned hosts. On American beech, *N. ditissima* produced larger cankers than *N. magnoliae* and the negative control, but *N. ditissima* and *N. faginata* canker sizes were not significantly different ($p = 0.71$) (Figure 5D). *N. faginata* produced a larger canker than *N. magnoliae*, although not statistically significant, and negative control on American beech ($p = 0.19$ and 0.23 , respectively). *N. magnoliae* produced a significantly larger canker on tulip poplar than all other treatments ($p < 0.0001$) (Figure 5E). The treatment isolate was recovered from all respective inoculations.

Figure 5: Pathogenicity results and representative photos for each host species tested: A) striped maple (*Acer pensylvanicum*), B) yellow birch (*Betula alleghaniensis*), C) black birch (*B. lenta*), D) American beech (*Fagus grandifolia*), and E) tulip poplar (*Liriodendron tulipifera*). Letters designate significant differences at $p < 0.05$. All sample sizes are 6 trees, except for American beech ($n = 5$).

Morphological Characterization of *Neonectria magnoliae*

The morphological features of *Neonectria magnoliae* are summarized here and illustrated in Figure 6. Cankers produced by *N. magnoliae* on tulip poplar (Figure 6A) were reminiscent of the perennial target cankers produced by *N. ditissima*, but these cankers often appeared irregular and less distinct (e.g. lacking concentric rings of callous tissue), especially for infections on *M. fraseri* (Figure 6B-C). Perithecia occur singly or in aggregates on bark tissue or directly on exposed wood surrounding the outer canker margins and typically emerge from thin stroma tissues as reddish-brown globous body with a distinct ostiole then fade to brown as they age (Figure 6D). Asci appear truncated and bear eight ascospores (Figure 6E). Ascospores ((11.3-) 13.1 – 15.3 (-17.7) μm x (4.0-) 5.6 – 7.4 (-9.1) μm) are uniseptate with rounded ends, constricted at the septum, hyaline, and warty on the surface (Figure 6F-G). *Neonectria magnoliae* cultures have a white surface with an orange-red subsurface after 10 d on PDA (Figure 6H). Microconidial sporodochia and conidiophores are regularly produced in culture (Figure 6I). Sporodochia are slimy masses with a cream to buff color. Conidiophores appear white and are penicillately branched. Microconidia ((5.7-) 8.6 – 11.0 (-13.7) μm x (3.0-) 4.0 – 5.3 (-6.4) μm) are hyaline, straight, and aseptate with rounded ends (Figure 6 K). Microconidia are abundantly produced in culture on aerial conidiophores and within sporodochia. Macroconidia ((42.1-) 47.5 – 61.7 (-67.3) μm x (5.5-) 6.8 – 8.4 (-9.4) μm) are primarily 4-5 septate, cylindrical with rounded ends, slightly curved, and hyaline (Figure 6J). Macroconidia, while they do occur, are found less frequently in sporodochia.

A

B

C

D

E

F

H

I

J

K

Figure 6: *Neonectria magnoliae*: A) Example canker on *Liriodendron tulipifera* with perennial target canker morphology; B) Example canker on *L. tulipifera* exhibiting less distinct canker morphology; C) Example canker on *Magnolia fraseri*; D) *N. magnoliae* perithecia erupting from *L. tulipifera* bark tissues; E) Asci containing eight ascospores each; F) Ascospores; G) Ornamented surface of ascospores; H) Top and bottom of 7-day old culture photos on PDA; I) Creamy microconidial sporodochia and aerial conidiophores; J) Macroconidia with four septations; K) Microconidia of varying lengths. Scale bars = 250 μ m for panel D; 20 μ m for panels E, K; 10 μ m for panels F, G, J; 20 mm for panel H.

Figure 7: *Neonectria magnoliae* holotype (BPI 552527 [No. 64184]) submitted by Lohman and Heptig in 1934. Sample consists of *N. magnoliae* fruiting on bark of tulip poplar (*Liriodendron tulipifera*) collected in Richwood, West Virginia, USA. Photos provided by Lisa A Castlebury, Ph.D, Acting Research Leader and Collections Director, USDA ARS MNGDBL.

Taxonomy

Neonectria magnoliae (Lohman & Hepting) C.M. Stauder & M.T. Kasson comb. nov.

≡ *Nectria magnoliae* M.L. Lohman & Hepting, Lloydia 6: 91. 1943.

Similar to *Neonectria coccinea*; ascospores 13.1 – 15.3 x 5.6 – 7.4 µm with regularly scattered warts; Microconidia aseptate; Macroconidia rare except for isolates from *Magnolia fraseri*. When present slightly curved 4-5 septate.

Holotype: UNITED STATES. West Virginia: Richwood. Civilian Conservation Corps Camp Woodbine (currently Camp Richwood) along the Cranberry River, on bark of almost dead *Liriodendron tulipifera*, Nov. 21, 1934, M. L. Lohman, BPI 552527 (No. 64184) (Figure 7).

Specimens examined: UNITED STATES. North Carolina: Asheville. Bent Creek Experimental Forest, on cankered *Liriodendron tulipifera*, October 12, 1934, M. L. Lohman, BPI 552527 (No. 64187; CBS 380.50). MB#288753. ITS and RPB2 sequences available in NCBI Genbank, accessions **MH856671** and **HQ897713**, respectively. West Virginia: Monongalia County. Snake Hill Wildlife Management Area, on cankered *L. tulipifera*, October 1, 2018, C. M. Stauder and M. T. Kasson, NmLt003, NmLt004, ITS sequences available in NCBI Genbank, accessions **TBD**, **TBD**, respectively. Tucker County. Fernow Experimental Forest, on cankered *L. tulipifera*, July 12, 2018, C.M. Stauder, A. M. Macias, M. T. Kasson, NmLt001, NmLt002, ITS sequences available in NCBI Genbank, accessions **TBD**, **TBD**, respectively. Randolph County. Gaudineers Knob, on cankered *Magnolia fraseri*, M.T. Kasson, NmMf001, ITS sequences available in NCBI Genbank, accessions **TBD**.

Habitat and distribution: on bark and exposed wood around cankers on living and dead *Liriodendron tulipifera* L. in Connecticut, Ohio, West Virginia, Virginia, Tennessee and North Carolina, *Magnolia fraseri* Walt. trees in West Virginia and Tennessee, and *M. tripetala* L. in West Virginia. Trees include both overtopped and overstory trees. Often found in forested stands co-occurring with other hosts harboring conspicuous *N. ditissima* target cankers.

Descriptions and illustrations: See Lohman and Watson (1943).

Discussion

In this study, we sampled and characterized the diversity of Nectriaceae across the central Appalachian Mountains. Over the course of the survey, ten species of Nectriaceae belonging to *Bionectria*, *Corinectria*, *Cosmospora*, *Fusarium*, *Neonectria*, *Thelonectria*, and *Thyronectria* were recovered from twelve tree hosts spanning 17 sites across six states (Table 1; Figure 3). The limited recovery of *Cosmospora obscura*, *Neonectria neomacrospora*, *Thelonectria balsamea*, and *Thyronectria veuillotiana* limits the ability to explore their ecology, but follow-up surveys/studies on these species could provide further insight into lifestyles of these fungi.

Other recovered members of Nectriaceae were either widespread or found in sufficient numbers at fewer locations, thereby providing adequate resolution and/or insight into their ecology. These species included the following: *Neonectria magnoliae* from symptomatic tulip poplar and Fraser magnolia; *N. ditissima* from eight hosts; *N. faginata*, *Bionectria ochroleuca*, and *Fusarium babinda* from American beech; and a novel *Corinectria* sp. from red spruce (Table 1; Figure 3).

Neonectria magnoliae was first described as *Nectria magnoliae* by Lohman and Watson (1943) as having some overlapping characteristics of both *Neonectria ditissima* (formerly *Nectria galligena*) and *Neonectria coccinea* (formerly *Nectria coccinea*) but also with notable morphological features including progressive changes in coloration of perithecia and notable spore size differences. Many former members of the genus *Nectria* have since been reclassified into different genera (Brayford et al., 2004; Castlebury et al., 2006; Mantiri et al., 2001; Rossman et al., 1999). However, none of these studies considered *Nectria magnoliae*, and therefore, its taxonomy was never properly resolved with one erroneous exception: Castlebury and colleagues (2006) included one isolate of *Nectria magnoliae* from tulip poplar from Tennessee in a phylogenetic study of *Neonectria*. These authors concluded that *N. magnoliae* was a synonym of *N. ditissima*, based on the data available at the time. However, based on BLASTn searches against NCBI Genbank and pairwise blasting of *N. magnoliae* isolates with *N. ditissima* strain CBS 118919, it's clear that both *N. magnoliae* and *N. ditissima* infect tulip poplar and strain CBS 118919, sequenced by Castlebury et al. (2006) was in fact *N. ditissima*. NCBI BLASTn searches with ITS and RPB2 sequences generated for field-collected *N. magnoliae* isolates had 98.4%-100% sequence similarity with 77-100% sequence coverage with a paratype of *Nectria magnoliae* (CBS 380.50 Genbank Accessions NR_160076 and HQ897713, respectively) (Supplemental Table 3). Additionally, the four-gene concatenated phylogeny placed *N. magnoliae* isolates as a monophyletic ingroup within *Neonectria* (Figure 4).

Ascospore measurements and general morphological descriptions provided in the type description for *N. magnoliae* (Lohman and Watson, 1943) were comparable

with those observed in this study (Figure 6). Notable was the infrequency of macroconidia from fresh cultures on general growth media. Interestingly, this trend did not hold for all isolates as several of the *N. magnoliae* from Fraser magnolia produced abundant macroconidia even in week-old cultures. The only isolate of *N. magnoliae* from magnolia also showed some sequence divergence from all tulip poplar isolates (Figure 4). Further characterization of magnolia and tulip-polar isolates are needed to confirm whether these notable differences are biologically significant. The included pathogenicity trial confirmed the pathogenicity of *N. magnoliae* on tulip poplar but not on other hosts tested (Figure 5). Interestingly, *N. ditissima* did not produce cankers significantly different from the negative control on tulip-poplar despite the previous work by Castlebury et al. (2006) confirming tulip-poplar as a host of *N. ditissima* (Figure 5). Earlier work by Lohman and Watson (1934) also detail cross-pathogenicity experiments in North Carolina where diverse *N. ditissima* strains from various hosts caused significantly larger cankers on tulip poplar compared to *N. magnoliae*. Follow-up studies are needed for cross pathogenicity tests of *N. magnoliae* from different hosts as well as *N. ditissima* isolates from tulip poplar. Together, these results provide evidence to support the transfer of *Nectria magnoliae* into the genus *Neonectria*.

Neonectria ditissima and *N. faginata* represented a majority of isolates recovered during this study (Table 1). Although this study did not uncover cryptic native plant reservoirs of *N. faginata*, *N. ditissima* was confirmed from eight plant hosts including three of which that may represent the first reports: *Acer spicatum*, *Ilex mucronata*, and *Sorbus americana* (Table 1). Interestingly, not all hosts had characteristic perennial target cankers (Figure 2). Instead, *Acer pensylvanicum* and *Ilex mucronata* exhibited

vascular cambium necrosis and perithecia production without an apparent outward host response (e.g. callous ridges, bark malformation). Perithecia production also varied across hosts with black birch cankers consistently yielded some perithecia and mountain ash cankers rarely yielding perithecia.

Cross-pathogenicity assays among *N. ditissima* strains demonstrated the lack of host specificity between isolates recovered from American beech or alternate host species (Figure 5). Additionally, a recent study confirmed the lack of mating barriers among *N. ditissima* strains recovered from varying host tree species (Chapter 2). Together, these results provide additional observational evidence describing the extent of the generalist nature of *N. ditissima* as a ubiquitous phytopathogen in eastern North America capable of infecting many hardwood species.

Neonectria faginata was the most recovered member of the Nectriaceae in this study, though it was exclusive to American beech (Table 1). The identification of a cryptic reservoir may have provided additional evidence supporting the geographic origin of *N. faginata* as eastern North America. Alternatively, *N. faginata* may be an exotic species simply capable of infecting as-yet-unidentified host species given this remains a possibility for American beech. Either way, no evidence of an alternate host for *N. faginata* was uncovered in this survey. Follow-up studies should focus on cankers lacking perithecia and/or asymptomatic host tissue similar to what has been previously described for epiphytic *N. coccinea* on European beech (Chapela and Boddy, 1988; Hendry et al., 2002).

Previous assertions of the eventual dominance of *N. faginata* in the BBD pathosystem (Houston, 1994; Kasson and Livingston, 2009) were confirmed across the

study sites. In total, *N. faginata* was recovered from 93.7% of BBD samples collected in this survey (from 13 of 13 BBD sites) while *N. ditissima* was only recovered from 4.2% of BBD samples (from two of the 13 BBD sites) (Table 1).

Nevertheless, *N. ditissima* still appears to play a minor role in BBD in its more advanced stages. The pathogenicity trial demonstrated the potentially increased pathogenicity of *N. ditissima* on American beech when compared to *N. faginata* (Figure 5). While the interaction of the scale insect may significantly alter the host's physiology and thus, defense responses to fungal infections, these results appear to indicate that an increased virulence of *N. faginata* may not be responsible for its dominance in the BBD pathosystem.

One additional possibility may be related to its production of perithecia and the resulting inoculum potential. For example, taxonomic descriptions of *N. ditissima* and *N. faginata* illustrate differences in their production of perithecia. In general, *N. ditissima* is described as bearing fewer, scattered perithecia while *N. faginata* can be found to produce larger aggregates in higher densities (Castlebury et al., 2006; Lohman and Watson, 1943). Sampling of *N. ditissima* perithecia across eight hosts generally supports these previous observations (Table 1). Seasonal differences in fruiting could explain differential abundances in these two fungi, but *N. ditissima* isolates were recovered from beech and non-beech hosts during the same sampling periods in which sampling of BBD trees resulted in high abundances of *N. faginata*. Therefore, seasonality of fruiting does not appear to have been an apparent factor in this study. Further, several studies report ascospores as the predominant spore type in the environment and more specifically, the dominant infective spore type (Crane et al.,

2009; Lortie and Kuntz, 1963). While these are general descriptive observations and not quantitative comparisons, differences in fruiting habits may explain the eventual abundance of *N. faginata* due to a significantly increased inoculum potential.

Bionectria ochroleuca also was recovered from BBD impacted beech trees at two total sites in West Virginia and North Carolina, representing 2.1% of all perithecia sampled from beech (Table 1; Figure 3). This fungus previously had been implicated in BBD although its geographic range was not well studied (Houston et al., 1987). Here we update the known distribution of this fungus to include West Virginia and North Carolina. Observations of this fungus on successfully colonized trees supports previous studies, which indicate this fungus is mycoparasitic (Barnett and Lilly, 1962; Jager et al. 1979; Turhan, 1993). Isolates of this fungus also were recovered from American basswood in North Carolina during these surveys (Table 1). This appears to be the first report of this fungus on American basswood in the U.S.

An additional fungus recovered consistently from beech with BBD was a member of the *Fusarium babinda* species complex (FBSC), although it did not produce perithecia, like all other Nectriaceae recovered in this study. Instead, members of the FBSC were often found colonizing the base of *Neonectria* perithecia or from necrotic tissue around *Neonectria* fruiting bodies. After its discovery in single spore plates from *Neonectria* perithecia, follow-up studies using micro-sampled bark tissue revealed this fungus was present in 31.8% of BBD-positive bark samples at 100% of BBD sampled sites but not present in any beech bark samples taken from healthy asymptomatic trees outside confirmed BBD epicenters (Supplemental Table 4). Its prevalence from BBD impacted stands, but lack of previous reporting, indicates either a localized (albeit

unclear) role or novel component in BBD pathosystem. However, a re-assessment of raw data and culture photos taken by the senior author during 2005 – 2006 as part of his BBD M.S. research in Maine (Kasson and Livingston, 2009) uncovered a *Fusarium* with identical morphology to *F. babinda* in three of three sites sampled for which culture data was collected (Supplemental Figure 1; See Kasson and Livingston, 2007, Figure 17A). This clearly indicates a more widespread association with BBD and one which has not been observed previously despite over 130 years of research on this important pathosystem. One possibility may be that *F. babinda* is insecticolous, interacting with the scale as populations decline and as *Neonectria* spp. colonize stems, which is likely given this fungus has been recovered from both gypsy moth and hemlock woolly adelgid populations in the eastern United States (Jacobs-Venter et al., 2018). Another plausible, although not mutually exclusive hypothesis based on the observations of sporulation at the base of viable and nonviable perithecia, is that *F. babinda* is a facultative hyperparasite of *Neonectria*. Clearly, more work is needed to test phytopathogenicity and entomopathogenicity and determine its dominant and facultative lifestyles. Preliminary testing of its ability to degrade tannic acid, a proxy assay for lignin degradation (Kasson et al., 2016) clearly shows that it has strong lignin and cellulose degrading activity.

Finally, in repeated attempts to uncover non-conventional hosts of *N. faginata*, we discovered a putatively novel *Corinectria* sp. on red spruce in high elevation spruce-fir forests in close proximity to BBD epicenters in VA and WV (Table 1; Figure 3). Interestingly, the fungus appears to be associated with dead trees but the results of those studies and in-depth investigations into the biology and ecology of this putatively

novel species are forthcoming. However, the *Corinectria* sp. recovered formed a single clade and was divergent from *C. fuckeliana* and *C. tsugae* in the concatenated phylogeny (Figure 4). The third described *Corinectria* species, *C. constricta*, was not included in the concatenated phylogeny due to limited availability of sequence data, but based on EF1, isolates recovered in this study were divergent from *C. constricta* as well. While this appears to be evidence of a novel species of *Corinectria*, further investigation is needed.

Taken together, the results of this broad study emphasize the sheer diversity of Nectriaceae present in the central Appalachian Mountains. Still, this is undoubtedly a gross underestimation of known and novel fungi as this survey intentionally targeted members of the Nectriaceae with viable and conspicuous perithecia. Exclusively anamorphic fungi were largely excluded with the exception of *F. babinda*. The insights gained here into other fungi of the BBD pathosystem opens many opportunities for further investigation. Specifically, what is the dominant lifestyle of *B. ochroleuca*, and what role do members of the FBSC actually play in BBD where it appears to dominate tissues recently colonized by scale insects and/or *Neonectria* fungi? These studies provide an important baseline for how fungal communities assemble, change over time, and affect the forests of the Appalachian Mountains.

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Supplemental Figures

Supplemental Figure 1: Morphological characteristics of *Fusarium babinda*: A) *F. babinda* sporulation on *Neonectria* sp. perithecia; B) 7-day old culture on PDA; C) sporodochia bearing macro- and micro-conidia; D) macroconidia; E) microconidia

BTUB

EF1-alpha

0.010

ITS

0.050

Supplemental Figures 2 - 5: Single-gene (BTUB, EF1, ITS, LSU) phylogenetic tree of *Neonectria* spp., *Corinectria* spp. and outgroups. Topology and branch lengths are from the ML analysis. For each node supported in the ML analysis, bootstrap support and posterior probabilities are indicated (ML/BI).

Supplemental Tables

Supplemental Table 1: Locations of sampling sites (including areas within these sites if applicable). The presence of BBD in each site is noted. Additionally, the general coordinates of the sampling locations are provided.

Site	Location	BBD Present	Coordinates
DM	Allegany County, MD	Y	(39.562432 , -78.948432)
PS	Allegany County, MD	Y	(39.34755 , -79.27962)
SM1	Cherokee County, NC	Y	(35.610143 , -83.421962)
SM2	Cherokee County, NC	Y	(35.598317 , -83.42741)
SMR	Cherokee County, NC	Y	(35.5959 , -83.460056)
SM3	Cherokee County, NC	Y	(35.631206 , -83.467966)
RH	Mitchell County, NC	Y	(36.09984 , -82.09217)
PM	Fayette County, PA	N	(39.740354 , -79.89975)
MR	Grayson County, VA	Y	(36.657625 , -81.520676)
WT	Grayson County, VA	Y	(36.633437 , -81.60132)
BG	Monongalia County, WV	Y	(39.626579 , -79.864538)
SH	Monongalia County, WV	N/A	(39.614379 , -79.829591)
UF	Monongalia County, WV	N/A	(39.669081 , -79.771688)
AR	Monongalia County, WV	N	(39.644653 , -79.976096)
MON	Monongalia County, WV	N/A	(39.619082 , -79.957879)
UW	Monongalia County, WV	N	(39.66875 , -79.934172)
SK	Pendleton County, WV	Y	(38.699077 , -79.533215)
GK1	Randolph County, WV	Y	(38.60741 , -79.84431)
GK2	Randolph County, WV	Y	(38.61129 , -79.84167)
GK3	Randolph County, WV	Y	(38.61768 , -79.84119)
GK5	Randolph County, WV	Y	(38.62928 , -79.8429)
GK6	Randolph County, WV	Y	(38.65584 , -79.83899)
GKT	Randolph County, WV	Y	(38.614709 , -79.842769)
92	Randolph County, WV	Y	(38.66397 , -79.90788)
BM	Randolph County, WV	Y	(38.61641 , -79.72984)
227	Randolph County, WV	Y	(38.62952 , -79.927647)
NB	Ritchie County, WV	N	(39.216486 , -81.09951)
FN	Tucker County, WV	N/A	(39.034 , -79.685)
MM1	Yancey County, WV	Y	(35.71076 , -82.24445)
MM2	Yancey County, WV	Y	(35.724152 , -82.281468)

Supplemental Table 2: Primer sequences, PCR parameters, and sources for each primer pair used to generate sequence data.

Target Locus	Primer	Primer Sequence	Initial Denature		Cycles	Denature		Anneal		Extension		Final Extension		Source
ITS	ITS5	GGAAGTAAAAGTCGTAACAAGG	95C	2:00	35	95C	0:30	56C	0:30	72C	1:00	72C	7:00	White et al., 1990
	ITS4	TCCTCCGCTTATTGATATGC												
tef-1α	EF1-728	CATCGAGAAGTTCGAGAAGG	95C	2:00	35	95C	0:30	56C	0:30	72C	1:00	72C	7:00	Carbone and Kohn, 1999 Rehner 2001
	EF1-1576	ACHGTRCCRATACCACCRATCTT												
nrLSU	LR5	ATCCTGAGGGAAACTTC	95C	2:00	35	95C	0:30	51.1C	0:45	72C	1:30	72C	7:00	Vilgalys and Hester, 1990 Rehner and Samuels, 1994
	LROR	GTACCCGCTGAACTTAAGC												
Beta-tublin	T1	AACATGCGTGAGATTGTAAGT	95C	2:00	35	95C	0:30	64C	0:30	72C	1:00	72C	7:00	O'Donnell and Cigelnik, 1997
	T2	TAGTGACCCTTGGCCCAGTTG-												

Supplemental Table 3: All BLASTn results from representative isolates recovered in this survey. The accession number, query coverage, and percent identity are provided for the top hit for each sequence.

Isolate	Closest Match	Accession	ITS	
			% ID	Query Cover (%)
F10121	<i>Nectria magnoliae</i>	NR_160076.1	100	100
F10222	<i>Nectria magnoliae</i>	NR_160076.1	99.8	78
F10511	<i>Nectria magnoliae</i>	NR_160076.1	98.38	77
F10521	<i>Nectria magnoliae</i>	NR_160076.1	99.2	81
F10522	<i>Nectria magnoliae</i>	NR_160076.1	99.6	100
AM52	<i>Nectria magnoliae</i>	NR_160076.1	99	94
AM57	<i>Nectria magnoliae</i>	NR_160076.1	98.39	99
Neomag21	<i>Nectria magnoliae</i>	NR_160076.1	99.4	98
Neomag32	<i>Nectria magnoliae</i>	NR_160076.1	99.6	100
GKMAG1	<i>Nectria magnoliae</i>	NR_160076.1	98.6	83
GKMAG2	<i>Nectria magnoliae</i>	NR_160076.1	99.6	86
BG1423	<i>Neonectria faginata</i>	KR019361.1	99.6	100
GK311	<i>Neonectria faginata</i>	KR019361.1	99.4	99
SM223	<i>Neonectria faginata</i>	KR019361.1	99.02	96
SM225	<i>Neonectria faginata</i>	KR019361.1	99.2	100
DM731	<i>Neonectria faginata</i>	KR019361.1	100	96
DM1_FCSC	<i>Fusarium babinda</i>	NR_159861.1	99.8	100
PSF164	<i>Fusarium babinda</i>	NR_159861.1	100	99
BG10a	<i>Fusarium sp.</i>	MK102657.1	99.8	96
GK554	<i>Fusarium babinda</i>	NR_159861.1	99.8	94
GK581	<i>Neonectria ditissima</i>	MN843954.1	98.9	98
UFBEAL	<i>Neonectria ditissima</i>	JF735309.1	98.55	100
SOAMRED2	<i>Neonectria ditissima</i>	JF735309.1	99.1	100
SOAMRED5	<i>Neonectria ditissima</i>	JF735309.1	99.7	100
MM2AS512	<i>Neonectria ditissima</i>	MN843954.1	99.7	100
MM2AS614	<i>Neonectria ditissima</i>	MN843954.1	99.7	100
RHS142	<i>Neonectria ditissima</i>	JF735309.1	99.7	100
SM214	<i>Neonectria ditissima</i>	JF735309.1	99.7	100
SMRAS22	<i>Neonectria ditissima</i>	JF735309.1	99.7	100
SMRAS25	<i>Neonectria ditissima</i>	JF735309.1	99.4	100
SMRAS3w	<i>Neonectria ditissima</i>	JF735309.1	99.7	100
SMRAS3w5	<i>Neonectria ditissima</i>	JF735309.1	98.7	99
WTS125	<i>Neonectria ditissima</i>	MN843954.1	99.7	100
SKI12A	<i>Neonectria ditissima</i>	JF735309.1	99.23	96

SKI31A	<i>Neonectria ditissima</i>	JF735309.1	98.29	94
SKI23A	<i>Neonectria ditissima</i>	MN843954.1	99.1	93
SKI31B	<i>Neonectria ditissima</i>	JF735309.1	99.01	89
MM2S313y	<i>Cosmospora obscura</i>	KC291719.1	100	99
MM2S31p	<i>Cosmospora obscura</i>	KC291719.1	99.04	99
GK61053	<i>Bionectria ochroleuca</i>	HQ328025.1	98.85	100
SM2TIAM3	<i>Bionectria ochroleuca</i>	HQ328025.1	98.5	100
MM2AS113	<i>Thelonectria veuillotiana</i>	JQ734922.1	99.64	100
MM2AS115	<i>Thelonectria veuillotiana</i>	JQ734922.1	99.8	99
MM2AS211	<i>Thelonectria veuillotiana</i>	JQ734922.1	99.64	99
MM2AS215	<i>Thelonectria veuillotiana</i>	JQ734922.1	100	97
MR12	<i>Corinectria fuckeliana</i>	MH859658.1	99.1	100
PIRUPERI	<i>Corinectria fuckeliana</i>	MF593148.1	95.7	100
PIRUSCR1	<i>Corinectria fuckeliana</i>	MF593148.1	97.04	93
PIRUSCR2	<i>Corinectria fuckeliana</i>	MF593148.1	98.8	94
WTPIRU4	<i>Corinectria fuckeliana</i>	MF593148.1	94.8	99
MR13ODD	<i>Neonectria neomacrospora</i>	MH580206.1	99.21	93
MR13ODDA	<i>Neonectria neomacrospora</i>	MH580206.1	99.21	93
ABBA1N2	<i>Thyronectria balsamea</i>	JN995618.1	99.64	97
ABBA1N1	<i>Thyronectria balsamea</i>	JN995618.1	99.44	73

Isolate	Closest Match	Accession	RPB2	
			% ID	Query Cover (%)
AM57	<i>Nectria magnoliae</i>	HQ897713	99.77	91
F10521	<i>Nectria magnoliae</i>	HQ897713	99.9	89
F10522	<i>Nectria magnoliae</i>	HQ897713	99.1	90

Supplemental Table 4: Recovery of *Fusarium babinda* isolates from bark tissue samples collected from asymptomatic American beech trees (shaded grey) or American beech trees with signs or symptoms associated with beech bark disease. Values represent the number of bark samples yielding *Fusarium babinda* at the designated sampling location.

Site	Trees Sampled	Host	Total Bark Samples	Scale Present	Perithecia Present	<i>N. faginata</i>	<i>N. ditissima</i>	<i>F. babinda</i>
ARB	12	FAGR	96	N	N	-	-	-
BG	4	FAGR	32	N	N	-	-	-
FH	12	FAGR	96	N	N	-	-	-
UWL	15	FAGR	120	N	N	-	-	-
NBB	12	FAGR	96	N	N	-	-	-
BG	5	FAGR	96	Y	Y	-	-	27
DM	5	FAGR	60	Y	Y	6	-	21
GK	25	FAGR	248	Y	Y	30	-	83
PSF	7	FAGR	72	Y	Y	-	-	29
MM	5	FAGR	40	Y	Y	2	-	2
MR	2	FAGR	12	Y	Y	-	-	1
PSF	7	FAGR	72	Y	Y	-	-	29
SK	2	FAGR	36	Y	Y	28	-	3
SM	14	FAGR	112	Y	Y	5	-	44
WT	1	FAGR	8	Y	Y	-	-	1

CHAPTER 2: Characterization of mating type genes in heterothallic *Neonectria* species with emphasis on *N. coccinea*, *N. ditissima*, and *N. faginata*

Abstract

Neonectria ditissima and *N. faginata* are canker pathogens involved in an insect-fungus disease complex of American beech (*Fagus grandifolia*) commonly known as beech bark disease (BBD). In Europe, both *N. ditissima* and *N. coccinea* are involved in BBD on European beech (*Fagus sylvatica*). Field observations across the range of BBD indicate that new infections occur primarily via ascospores. Both heterothallic (self-sterile) and homothallic (self-fertile) mating strategies have been reported for *Neonectria* fungi. As such, investigations into mating strategy are important for understanding both the disease cycle and population genetics of *Neonectria*. This is particularly important in the U.S. given that over time *N. faginata* dominates the BBD pathosystem despite high densities of non-beech hosts for *N. ditissima*. This study utilized whole-genome sequences of BBD-associated *Neonectria* spp. along with other publicly available *Neonectria* and *Corinectria* genomes and *in vitro* mating assays to characterize mating type (MAT) loci and confirm thallism for select members of *Neonectria* and *Corinectria*. MAT gene-specific primer pairs were developed to efficiently characterize the mating types of additional single ascospore strains of *N. ditissima*, *N. faginata*, and *N. coccinea* and several other related species lacking genomic data. *In vitro* mating assays were used in combination with molecular results to confirm thallism. These assays also confirmed the sexual compatibility among *N. ditissima* strains from different plant hosts. Maximum likelihood phylogenetic analysis of both MAT1-1-1 and MAT1-2-1 sequences

recovered trees with similar topology to previously published phylogenies of *Neonectria* and *Corinectria*. The results of this study indicate that all *Neonectria* and *Corinectria* tested are heterothallic based on the limited sampling and, as such, thallism cannot help explain the inevitable dominance of *N. faginata* in the BBD pathosystem.

Introduction

Beech bark disease (BBD), a canker disease complex arising from interactions among insect and fungal causal agents, has significantly impacted the health of American (*Fagus grandifolia* Ehrh.) and European (*Fagus sylvatica* L.) beech forests throughout North America and Europe over the last century (Cale et al., 2017; Ehrlich, 1934; Thomsen et al., 1949). More recently, BBD has intensified in areas where historic cold temperatures have kept the disease in check, raising concerns for the impact that global climate change may have on the expansion of this disease and other plant pathogens (Dukes et al., 2009; Kasson and Livingston, 2012; McCullough and Wieferich, 2001; McLaughlin and Greifenhagen, 2012).

Beech bark disease requires prior infestation by a non-native scale insect *Cryptococcus fagisuga* Lind., which predisposes the host's bark tissues to subsequent invasion by one or more closely related canker fungi: *Neonectria ditissima* ([Tul. & C. Tul.] Samuels & Rossman), *N. faginata* ([Lohman, Watson, & Ayres] Castl. & Watson), and *N. coccinea* ([Pers.] Rossman and Samuels) (Houston, 1994b; Thomsen et al., 1949). *Neonectria ditissima* (formerly *N. galligena* Bres.) has been implicated in BBD both in the U.S. and in Europe while *N. faginata* (formerly *N. coccinea* var. *faginata* (Pers.:Fr.) Fr. Var. Lohman, A. M. Watson, & Ayers) appears restricted to American beech in the U.S. and *N. coccinea* to European beech in Europe (Thomsen et al., 1949;

Castlebury et al., 2006). *Bionectria ochroleuca* ([Schwein.] Schroers & Samuels) has also been implicated in the U.S., but its role in BBD is not well understood (Houston et al., 1987).

Neonectria ditissima is perhaps best known as the causal agent of perennial target canker on many non-beech hosts including birch, maple, and walnut among others (Lohman and Watson, 1943; Spaulding et al., 1936; Booth, 1967). *Neonectria faginata* is unique to the BBD pathosystem in that it has only been observed causing annual cankers on American beech trees following *C. fagisuga* infestation (Castlebury et al., 2006). Other native plant hosts of *N. faginata* have not been detected. Unlike *N. ditissima* and *N. faginata*, *N. coccinea* is known to persist endophytically (asymptomatically) in the bark of European beech, with the ability to initiate disease following wounding, including but not limited to damage inflicted by *C. fagisuga* (Chapela and Boddy, 1988; Hendry et al., 2002). When present, fruiting structures of *Neonectria* species are easily recognizable as bright red or orange, globose sexual ascocarps (perithecia) bearing uniseptate, hyaline ascospores. While perithecia are often products of mating between two distinct thalli of the opposite mating type, self-fertility (homothallism) can lead to the completion of the sexual cycle through selfing, which has been previously confirmed for members of the Nectriaceae (Alexopolous et al., 1996; Yun et al., 2000).

Sexual reproduction in ascomycetes is generally understood to be regulated by the presence of one or both mating-type (MAT) idiomorphs (MAT1-1 and MAT1-2) at a mating type locus (Coppin et al., 1997; Turgeon, 1998). The term “idiomorph” refers to sequences which encode different functional proteins but are found occupying the same

locus in different strains. For heterothallic ascomycetes, three genes (MAT1-1-1, MAT1-1-2, and MAT1-1-3) are commonly found at the MAT locus for the MAT1-1 mating type while two genes (MAT1-2-1 and MAT1-2-2) often occur at this locus for the MAT1-2 mating type (Coppin et al., 1997; Pöggeler and Kück, 2000).

These mating type idiomorphs encode polypeptides responsible for the regulation of the sexual mating cycle in filamentous fungi (Kronstad and Staben, 1997). For the MAT1-1 idiomorph, MAT1-1-1, MAT1-1-2, and MAT1-1-3 encode proteins with an α domain, a PPF domain, and an HMG (high-mobility group) domain, respectively (Debuchy et al., 2010). Of these, the α domain protein encoded by MAT1-1-1 is responsible for MAT identity and sexual development (Saupe et al., 1996). The PPF and HMG domain proteins appear to have redundant roles in fertility as the deletion of one or the other has no apparent effect, while the deletion of both has been shown to decrease fertility (Ferreira et al., 1998).

For the MAT1-2 idiomorph, MAT1-2-1 encodes a protein with an HMG domain responsible for the establishment of MAT identity (Chang and Staben, 1994; Coppin et al., 1997). MAT1-2-2 encodes a small open reading frame (ORF) that does not have an apparent function (Pöggeler and Kück, 2000), but this ORF appears to be absent in some filamentous fungi (Debuchy and Coppin, 1992). Both MAT proteins act as transcription factors and are required for the initiation and regulation of the sexual cycle. Self-fertile (homothallic) fungi contain both MAT genes critical for sexual development (MAT1-1-1 and MAT1-2-1) at the mating type locus. In this case, reliance on a complementary mating type to complete the sexual cycle is not required.

Both homothallism and heterothallism have been previously reported for *N. ditissima* (El-Gholl et al., 1986; Krüger, 1973) while only heterothallism has been reported for *N. faginata* (Cotter and Blanchard, 1978). However, these determinations relied solely on culture-based observations via *in vitro* mating assays. No molecular characterization of the MAT locus has been completed for any member of *Neonectria* despite the publicly available genomes of several species in the genus (Salgado-Salazar and Crouch, 2019; Gómez-Cortecero et al., 2015; Deng et al., 2015).

Confirming the thallism of the BBD pathogens is important for several reasons: 1) thallism is predicted to affect expected patterns of genomic diversity via obligate or facultative outcrossing (Glémin and Galtier, 2012); and 2) relative rates of ascospore production among species linked to potential differences in thallism could influence patterns of dominance in the BBD pathosystem and/or the perception of dominance where species-level determination are primarily made using ascospores from field-collected perithecia. In addition, MAT genes have been demonstrated as highly useful for determining phylogenetic relationships among species within a genus or clade (Lopes et al., 2018; O'Donnell et al., 2004; Turgeon, 1998).

Characterizing thallism can inform our understanding of disease cycle dynamics including propagule dissemination and mode of infection. One study investigating the production and dissemination of spores by *N. ditissima* infecting yellow birch (*Betula alleghaniensis* Britton) determined ascospores to be the dominant spore type in the environment throughout the year (Lortie and Kuntz, 1963). Additionally, ascospores of a related species *Corinectria fuckeliana* (C. Booth) C. González & P. Chaverri (formerly *Neonectria fuckeliana*) have also been shown to be dominant relative to asexual conidia

when infecting *Pinus radiata* D. Don in New Zealand (Crane et al., 2009). Together, these results indicate the progression of diseases caused by *Neonectria* and *Corinectria* fungi may depend on ascospore production and dissemination given the proposed limited dissemination of asexual conidia by these fungi (Lortie and Kuntz, 1963; Crane et al., 2009).

In addition to the potential importance of mating strategy on disease epidemiology, fungal population density assessments that depend on fruiting structure detection can be influenced by the relative rates of reproduction. Rates of visual detection are also likely influenced by fruiting structure type, where bright red perithecia are far easier to see and positively identify than the small, whitish conidiophores and sporodochia. Nearly all BBD studies that investigated interactions among these two *Neonectria* fungi have depended on the sampling and processing of perithecia to measure ascospores directly or culture the associated fungi for identification (Houston, 1994; Kasson and Livingston, 2009). These studies have indicated *N. faginata*, over time, supplants *N. ditissima* as the dominant pathogen in the BBD pathosystem. As with differences in optimal abiotic conditions (temperature, relative humidity) or seasonality of fruiting, differences in thallism – if they exist – could likewise influence the frequency of perithecia production and therefore, detection rates. Thus, determining thallism for *N. ditissima*, *N. faginata* and *N. coccinea* could enhance our ability to interpret patterns of relative abundance in the BBD system.

The objectives of this study were as follows: 1) Determine thallism among members of *Neonectria* with emphasis on BBD-associated fungi: *N. ditissima*, *N. faginata*, and *N. coccinea*. This is important as studies to determine the mating

strategies of *Neonectria* fungi are limited. Furthermore, genomic data for many *Neonectria* spp. are lacking, and therefore, more general primers would be useful to permit broader characterization of mating type genes across species. 2) Confirm existing intra- and interspecies mating barriers using *in vitro* pairing assays. This is important as *N. ditissima* is reported from many plant hosts and the compatibility of strains from these various hosts remains unclear. Together, these results offer insight into mating regulation of *Neonectria* and allied fungi, thus providing an enhanced understanding of gene flow within and outside of the BBD system.

Materials and Methods

Genome sequencing and identification of MAT loci

Two genomes for *N. faginata* isolates (SK113 and MES1_34.1.1) were produced in association with this work and allowed for MAT gene discovery (Table 1). Full genome details will be provided in a forthcoming publication (Morrison and Garnas unpublished). Genomic data for the two *N. faginata* isolates of putatively opposite mating types as determined using the *in vitro* pairing assay described below were generated by a combination of Oxford Nanopore Technologies (ONT) and Illumina HiSeq sequencing. Genomic DNA was extracted from a *N. faginata* MAT1-1 isolate using a CTAB-chloroform DNA extraction method (van Diepen et al., 2017) and a MAT1-2 isolate using a Wizard® kit (Promega, Madison, WI, USA) and suspended in 75 µl Tris-EDTA (TE) buffer (Amresco, Solon, OH, USA) with RNase treatment to remove co-extracted RNA. The MAT1-1 isolate was sequenced using a MinION sequencer (Oxford Nanopore Technologies MIN-101B) using the unmodified 1D factory protocol (SQK-LSK109 protocol version DE_9062_v109_revD_23May2018) and a MIN-106 flow

cell (FLOW MIN-106 R9 version). Both MAT1-1 and MAT1-2 isolates were subjected to Illumina HiSeq 2 x 250 PE sequencing at the University of New Hampshire Hubbard Center for Genome Studies. The ONT signal-level data was translated to FASTQ files using the Albacore v. 2.3.4 ONT proprietary basecaller resulting in >960,000 reads of which 50% were greater than 1 Kb. ONT reads were quality controlled and assembled using the Canu assembler v. 1.8 (Koren et al., 2017) with initial genome size estimate of 45 Mb. Signal level ONT data was used to polish the assembly to correct major assembly errors using Nanopolish v. 0.10.2 (Loman et al., 2015). Illumina HiSeq data was trimmed for adapter sequences and quality filtered using BBDuk (BBMap v. 38.58; Bushnell B, sourceforge.net/projects/bbmap/) resulting in 2.5 million and 11.8 million high-quality paired-end sequence reads for the MAT1-1 and MAT1-2 isolates, respectively. The MAT1-1 isolate Illumina sequences were subsequently used to further polish the Canu assembly using Pilon v. 1.22 (Walker et al., 2014). The MAT1-2 isolate sequence reads were assembled using SPAdes 3.13.1 with default settings. The resulting assemblies were assessed for contiguity using QUAST (Gurevich et al., 2013) and were checked for universal single-copy ortholog content using BUSCO v. 3.0.0 (Simão et al., 2015) with lineage Sordariomycetes. Assembly summary statistics are presented in Table S1, and complete code is available on request. Draft genome sequences are available on request.

Additionally, draft genomes of one European *N. ditissima* isolate (GenBank accession: LKCW01000000) (Gómez-Cortecero et al., 2015) and two New Zealand *N. ditissima* isolates (GenBank accessions: LDPK00000000; LDPL01000000) (Deng et al., 2015) were used to identify putative *N. ditissima* MAT idiomorphs. An unpublished *N.*

coccinea draft genome (GenBank accession WPDF00000000, Castlebury et al., unpublished) also was used in this study.

To locate MAT loci within these genomes, we used NCBI GenBank tBLASTn algorithms with predicted MAT amino acid sequences derived from available MAT1-1 and MAT1-2 nucleotide sequences of two *Fusarium* (Nectriaceae) NCBI accessions: *Fusarium anguioides* (MH742713) and *Fusarium tucumaniae* (KF706656), respectively. Contigs containing sequences with an arbitrarily chosen similarity cutoff equal to or greater than 50% were selected for further examination.

Characterizing the structure of MAT loci

Genomic data were used to create genetic maps of the MAT1-1 and MAT1-2 loci for *Neonectria ditissima* and *N. faginata* as well as the MAT1-2 locus for *N. coccinea*. AUGUSTUS 3.3.1 (Stanke et al., 2008) was used to predict potential gene coding regions and their resulting amino acid sequences within putative MAT loci as well as to search for conserved genes within flanking regions up to 15,000 bp upstream and downstream of the MAT idiomorphs. The selected reference genome for this prediction was *Fusarium graminearum*, which is embedded in the AUGUSTUS software. NCBI GenBank BLASTp search algorithms were used identify genes by comparing predicted amino acid sequences to the NCBI protein database.

Species-specific MAT 1-1-1 and MAT 1-2-1 primer design and PCR amplification

Once we had identified the MAT locus in each genome, we designed forward and reverse MAT1-1-1 and MAT1-2-1 specific primers for rapid characterization of mating

type for *N. ditissima* and *N. faginata* isolates (Table 2). Excluding the primer binding sites, amplicon lengths were 710 bp (MAT1-1-1) and 591 bp (MAT1-2-1) for *N. ditissima*. Amplicon lengths for *N. faginata* were 527 bp (MAT1-1-1) and 612 bp (MAT1-2-1) (Table 2). Primers were manually designed in polymorphic regions distinct to each *Neonectria* species with limited repeats and approximately 60% G/C content. Primer dimer and hairpin formation among primer pairs was assessed using AutoDimer (www-s.nist.gov/dnaAnalysis; Vallone and Butler, 2004). Melting temperatures were calculated using OligoAnalyzer Tool (Integrated DNA Technologies, Coralville, IA, USA) for standard Taq polymerase. PCR products were generated in 25 µl reactions containing 12.5 µl Bioline PCR Master Mix (Bioline USA Inc, Taunton, MA), 10.0 µl MG H₂O, 1.5 µl purified DNA, and 1.0 µl of both MAT1-1 or MAT1-2 primers (25 nM; Integrated DNA Technologies, Coralville, IA, USA). PCR conditions are outlined in Table 2 for each primer set.

Table 1: Identity, source location, and host substrate of fungal isolates used in this study. GenBank accession numbers for derived and submitted sequences are included.

Species	Isolate No.	Geographic origin	Host	GenBank Accession Numbers ^b		
				ITS (tef1- α)	MAT1-1-1	MAT1-2-1
<i>Neonectria ditissima</i>	NdAp001	Randolph County, West Virginia, USA	<i>Acer pensylvanicum</i>			
	NdSam001	Grayson County, Virginia, USA	<i>Sorbus americana</i>			
	NdBI001	Monongalia County, West Virginia, USA	<i>Betula lenta</i>			
	NdBa001	Monongalia County, West Virginia, USA	<i>Betula alleghaniensis</i>	#####	#####	
	NdSa001	Monongalia County, West Virginia, USA	<i>Sassafras albidum</i>			
	NdFg001	Randolph County, West Virginia, USA	<i>Fagus grandifolia</i>	#####	#####	
	NdFg002	Randolph County, West Virginia, USA	<i>Fagus grandifolia</i>		#####	
	NdAs001	Swain County, North Carolina, USA	<i>Acer spicatum</i>			####
	NdSam002	Pendleton County, West Virginia, USA	<i>Sorbus americana</i>			
	NdFg003	Randolph County, West Virginia, USA	<i>Fagus grandifolia</i>			
	NdSam003	Mitchell County, North Carolina, USA	<i>Sorbus americana</i>	#####		####
<i>Neonectria faginata</i>	NdFg004	Randolph County, West Virginia, USA	<i>Fagus grandifolia</i>			
	NdFg005	Randolph County, West Virginia, USA	<i>Fagus grandifolia</i>	#####		####
	SK113	Pendleton County, West Virginia, USA	<i>Fagus grandifolia</i>			
	MES13411	Penobscot County, Maine, USA	<i>Fagus grandifolia</i>			
	NfFg001	Allegany County, Maryland, USA	<i>Fagus grandifolia</i>			
	NfFg002	Pocahontas County, West Virginia, USA	<i>Fagus grandifolia</i>			
	NfFg003	Randolph County, West Virginia, USA	<i>Fagus grandifolia</i>			
	NfFg004	Monongalia County, West Virginia, USA	<i>Fagus grandifolia</i>		####	
	NfFg005	Randolph County, West Virginia, USA	<i>Fagus grandifolia</i>	#####	####	
	NfFg006	Pendleton County, West Virginia, USA	<i>Fagus grandifolia</i>			
	NfFg007	Swain County, North Carolina, USA	<i>Fagus grandifolia</i>	#####		####
	NfFg008	Pendleton County, West Virginia, USA	<i>Fagus grandifolia</i>			
<i>Neonectria coccinea</i>	NfFg009	Allegany County, Maryland, USA	<i>Fagus grandifolia</i>			
	NfFg010	Allegany County, Maryland, USA	<i>Fagus grandifolia</i>			
	NfFg011	Randolph County, West Virginia, USA	<i>Fagus grandifolia</i>			####
	AR3691	Slovakia	<i>Fagus sylvatica</i>			####
	AR3707	Romania	<i>Fagus sylvatica</i>		####	
	AR3700	Slovakia	<i>Fagus sylvatica</i>	(HM054087)		####
<i>Neonectria neomacro-spora</i>	AR3696	France	<i>Fagus sylvatica</i>			
	AR3712	Slovakia	<i>Fagus sylvatica</i>		####	
	GJS92-33	Scotland	<i>Fagus sp.</i>			
	NnAb001	Grayson County, Virginia, USA	<i>Abies fraseri</i>	#####		####
	NnAb002	Grayson County, Virginia, USA	<i>Abies fraseri</i>	#####	#####	
	NnAb003	Grayson County, Virginia, USA	<i>Abies fraseri</i>		####	
<i>Neonectria magnoliae</i>	NnAb004	Grayson County, Virginia, USA	<i>Abies fraseri</i>			####
	NnAb005	Grayson County, Virginia, USA	<i>Abies fraseri</i>			
	NecmLt001	Monongalia County, West Virginia, USA	<i>Liriodendron tulipifera</i>		####	
	NecmLt002	Monongalia County, West Virginia, USA	<i>Liriodendron tulipifera</i>			#####
	NecmLt003	Monongalia County, West Virginia, USA	<i>Liriodendron tulipifera</i>			

	NecmLt004	Tucker County, West Virginia, USA	<i>Liriodendron tulipifera</i>	#####		####
	NecmLt005	Tucker County, West Virginia, USA	<i>Liriodendron tulipifera</i>			
	NecmMf001	Randolph County, West Virginia, USA	<i>Magnolia fraseri</i>	#####	####	
<i>Neonectria hederæ</i>	CBS714.97	Netherlands	<i>Hedera helix</i>			
<i>Neonectria punicea</i>	CBS119724	Austria	<i>Frangula alnus</i>			
<i>Corinectria fuckeliana</i>	IMI342667	Switzerland	<i>Picea sp.</i>			
	CafPr001	Randolph County, West Virginia, USA	<i>Picea rubens</i>		####	
	CafPr002	Grayson County, Virginia, USA	<i>Picea rubens</i>			#####
	CafPr003	Grayson County, Virginia, USA	<i>Picea rubens</i>			
<i>Corinectria aff. fuckeliana</i>	CafPr004	Randolph County, West Virginia, USA	<i>Picea rubens</i>	#####	####	
	CafPr005	Grayson County, Virginia, USA	<i>Picea rubens</i>	#####		####
	CafPr006	Pocahontas County, West Virginia, USA	<i>Picea rubens</i>			
<i>Fusarium babinda</i>	FbFg001	Monongalia County, West Virginia, USA	<i>Fagus grandifolia</i>	(#####)		#####
<i>Gibberella fujikuroi</i>	A00149				AF100925	
	G-2					AB005041
<i>Fusarium oxysporum</i>	F23				EU841022	
	F48					EU841047
<i>Ustilaginoidea virens</i>	UVas2-26				KM096573	
	UVas2-23					KM096575
<i>Ophiocordyceps xuefengensis</i>	MHHNU7968	Hunan Province, China			MH176304	
	MHHNU7966	Hunan Province, China				MH176301

Table 2: MAT1-1-1 and MAT1-2-1 primers designed in this study, including the corresponding amplicon sizes and PCR protocols.

Species	Target	Primer	bp	Primer Sequence (5' - 3')	Cycles	Initial Denaturation	Denaturation	Annealing	Extension	Final Extension
<i>N. ditissima</i>	MAT1	NdM1f NdM1r	710	TCAAGCTGTTCCAGACG GGCTTCGAACGCTTGAG	35	95C 2:00	95C 0:30	53C 0:30	72C 1:00	72C 7:00
<i>N. ditissima</i>	MAT2	NdM2f NdM2r	590	TCATGCCTTCTGAAGCC AAGCGCGACCGAGAATT	35	95C 2:00	95C 0:30	52C 0:30	72C 1:00	72C 7:00
<i>N. faginata</i>	MAT1	NfM1f NfM1r	530	GCTAAGCGGCCTCTCAACG GAGATACCGTACGTAGCCAGC	35	95C 2:00	95C 0:30	54C 0:30	72C 1:00	72C 7:00
<i>N. faginata</i>	MAT2	NeoM2f NfM2r	610	GCATGGACAAAGGCGTCG GGAGCGGATGAAGGCCA	35	95C 2:00	95C 0:30	57C 0:30	72C 1:00	72C 7:00
<i>N. ditissima</i> / <i>N. faginata</i>	MAT1	NeoM1f NeoM1r	800	CGAGGTnGCTGCCAnGT AATGGGTTGTCGATGTCAATGG	35	95C 2:00	95C 0:30	57C 0:30	72C 1:00	72C 7:00
<i>N. ditissima</i> / <i>N. faginata</i>	MAT2	NeoM2f NeoM2r	610	GCATGGACAAAGGCGTCG TCTGGGAnCGGATnAAGG	35	95C 2:00	95C 0:30	57C 0:30	72C 1:00	72C 7:00
<i>Neonectria</i> spp.	MAT2	NeoM2Df NeoM2Dr	300	CGAGGKCGRTACTKG TAGTCRG G CCMAAYGCSTACATYCTKTACC GYAA	35	95C 2:00	95C 0:30	57C 0:30	72C 0:45	72C 7:00

Genus-level MAT 1-1-1 and MAT 1-2-1 primer design and PCR amplification

Genus-level primer pairs intended to amplify MAT1-1-1 (NeoM1f and NeoM1r) and MAT1-2-1 (NeoM2f and NeoM2r) for all included *Neonectria* species were designed as described above using available *Neonectria* genomic data (Table 2). Primer development for *Neonectria* spp. was completed using MAT1-1-1 and MAT1-2-1 sequences derived from *N. ditissima*, *N. faginata*, *N. coccinea* (MAT1-2-1), *N. punicea* ([J.C. Schmidt] Castl. & Rossman) (MAT1-1-1; GenBank accession: QGQA000000000.1), and *N. hederæ* genomes ([C. Booth] Castl. & Rossman) (MAT1-2-1; GenBank accession: QGQB000000000.1). MAT1-1-1 and MAT1-2-1 nucleotide sequences were separately aligned using CLUSTAL-W (Larkin et al., 2007) within MEGA v10.1.7 (Stecher et al., 2018), and primers were designed within conserved regions to potentially increase the utility of these primers for other *Neonectria* and allied fungi. MAT1-2-1 exhibited a higher level of polymorphism among the included *Neonectria* species and therefore, a second set of degenerate primers were designed to amplify the MAT1-2-1 gene (Table 2; NeoM2Df and NeoM2Dr). All the sequences generated were deposited in GenBank (Table 1).

Selection of Isolates

Single ascospore-derived isolates of *N. ditissima* and *N. faginata* recovered from various geographic locations and host substrates (Table 1) were generated by squashing a single perithecium in 1ml of sterile water within a 1.5 ml Eppendorf tube, vortexing for 15 seconds to disperse ascospores, and spreading 100 µl onto a glucose-yeast extract (GYE) medium. Five germinating ascospores were transferred to a new GYE plate using a sterile needle after 24 hours and were allowed to grow for one week

before replating. Each isolate was grown on GYE for two weeks and then identified using colony morphology based on type descriptions (Castlebury et al., 2006).

Genomic DNA was extracted from isolates or directly from perithecia as described above. For each species identified morphologically, three random isolate identifications were confirmed by sequencing the ribosomal internal transcribed spacer region (ITS) using primers ITS5 (5' – GGAAGTAAAAGTCGTAACAAGG – 3') and ITS4 (5' – TCCTCCGCTTATTGATATGC – 3') (White et al., 1990). The PCR protocol was as follows: 95 °C for 2 min followed by 35 cycles of 95 °C for 30 sec, 56 °C for 30 sec, 72 °C for 1 min with a final extension at 72 °C for 7 min. EF1- α sequencing was completed using primers EF1728F (5' – CATCGAGAAGTTCGAGAAGG – 3') (Carbone and Kohn, 1999) and EF1-1567R (5' – ACHGTRCCRATACCACCRATCTT – 3') (Rehner 2001) with the following PCR protocol: 95 °C for 2 min followed by 35 cycles of 95 °C for 30 sec, 55 °C for 1 min, 72 °C for 1 min with a final extension at 72 °C for 7 min.

PCR products were visualized with gel electrophoresis by adding 4 μ l SYBR gold (Invitrogen, Grand Island, NY, USA) and 4 μ l loading dye (5Prime, Gaithersburg, MD) to products. Samples were then loaded into a 1.5%, wt/vol, agarose gel (Amresco, Solon, OH, USA) made with 0.5% Tris-borate-EDTA buffer (Amresco, Solon, OH, USA). To compare sizes, 100-bp and 1-kb molecular ladders (Omega Bio-tek, Norcross, GA, USA) also were added to gels. Electrophoresis was performed at 90V for 75 minutes. Bands were visualized on a UV transilluminator (Syngene, Frederick, MD, USA).

Positive reactions were prepared for sequencing using ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's recommendations. Purified PCR products were Sanger sequenced in forward and reverse directions using the

same PCR primers (Eurofins, Huntsville, AL, USA). BLASTn searches were then used to identify species based on the best match in the NCBI database.

Mating type gene screening

All selected *N. ditissima* and *N. faginata* (22 and 18 isolates, respectively) were screened for the presence of MAT1-1-1 and/or MAT1-2-1 using species-specific and genus-level primer sets with the PCR protocols listed in Table 2. All PCR products were visualized, and a subset of positive reactions were sequenced as described for ITS and EF1- α amplicons.

To confirm the specificity of species-specific primer sets, mating type PCR reactions were performed using *N. ditissima* MAT primers for *N. faginata* isolates and vice versa. Additionally, a number of other members of the Nectriaceae also were tested using both *N. ditissima* and *N. faginata* MAT primers as described above. These included isolates of *N. coccinea*, *N. neomacrospora* ([C. Booth & Samuels] Mantiri & Samuels), *Fusarium babinda* (Summerell, C.A. Rugg & L.W. Burgess, 1995), *Nectria magnoliae* (M.L. Lohman & Hepting, 1943) and an additional unresolved species for which additional data is needed to confirm identity: *Corinectria* aff. *fuckeliana* (99.37% EF1- α sequence similarity to NCBI Genbank accession MK911707.1) (Table 1). Genus-level *Neonectria* MAT primer sets were similarly tested using representatives from all five Nectriaceae species tested in this study.

All resulting sequences were aligned as described above and compared to genome-derived MAT1-1-1 or MAT1-2-1 sequences to confirm their identity based on a sequence similarity. All sequences having greater than 70% sequence similarity were selected for further analysis. This threshold was determined by comparing MAT1-1-1

and MAT1-2-1 genome-derived sequences from *N. ditissima* to the more distantly related *Fusarium anguioides* (MAT1-1-1; Genbank Accession: MH742713) and *Fusarium tucumaniae* (MAT1-2-1; Genbank Accession: KF706656) sequences.

Phylogenetic analysis and protein alignment

To examine evolutionary patterns and divergence in mating type genes, we constructed phylogenetic trees using the mating type gene sequence data produced in this study together with comparable sequences for other Nectriaceae available in NCBI Genbank, including 11 strains representing 7 species (Table 1). All analyses were completed using MEGA v10.1.7 (Stecher et al., 2018). MAT nucleotide sequences were aligned using CLUSTAL-W (Larkin et al. 2007) and the best-fit nucleotide substitution model was chosen using Model Test AICc scores in MEGA v10.1.7. MAT1-1-1 and MAT1-2-1 maximum-likelihood trees were constructed independently using the Kimura 2-parameter model with gamma distribution (K2+G) and 1000 bootstrap replicates. For both trees, MAT1-1-1 and MAT1-2-1 sequences from *Ophiocordyceps xeufengensis* and *Ustilaginoidea virens* served as outgroup taxa (Table 1).

We performed protein alignments to characterized divergence among species that could play a role in the maintenance of mating barriers. Protein sequences were predicted from one MAT1-1-1 and one MAT1-2-1 coding sequence from each species using ExPASy-Translate tool (<https://web.expasy.org/translate/>). Resulting protein sequences were aligned using CLUSTAL-W within MEGA v10.1.7. Boxshade Server v.3.21 (https://embnet.vital-it.ch/software/BOX_form.html) was used to visualize shared amino acids within each sequence.

***In vitro* mating assay**

To demonstrate mating among MAT1-1 and MAT1-2 strains, an *in vitro* mating assay was performed with six MAT1-1-1 and six MAT1-2-1 isolates of both *N. faginata* and *N. ditissima*. These isolates varied in geographic origin and for *N. ditissima*, host substrate as to test compatibility among representatives from allopatric populations and potentially host-specific *N. ditissima* isolates. This pairing assay did not include *N. coccinea* as only genomic DNA was available. Selected isolates were grown on glucose-yeast extract agar for two weeks. Each selected isolate was then paired three times with itself, with an isolate of the same mating type, and with two isolates of the opposite mating type for a total of twelve pairings per isolate. For each pairing, a 5x5-mm fungus-colonized agar plug from each isolate was placed on opposite sides of a sterile, flat toothpick placed atop GYE media along the center point of the petri dish (Figure 1A). All plates were parafilmed and placed at 20 °C with a 16 h/8 h light/dark cycle under cool fluorescent lamps. Plates were checked weekly for perithecia formation for up to 12 weeks (Figure 1B).

Figure 1: *In vitro* mating assay for *Neonectria* spp. and related fungi: A) Approximately 5mm x 5mm colonized plugs of confirmed MAT1-1 and/or MAT1-2 strains placed on either side of a sterile wooden toothpick placed upon GYE media; B) Perithecia exuding ascospores on toothpick after approximately 12 weeks; C) Perithecia squash mount showing ascospores within asci. *Neonectria magnoliae* shown in figure. Individual scale bars are included in each panel.

As perithecia were produced, squash mounts were used to check for ascospores using light microscopy (Figure 1C). Additionally, ascospores were checked for viability by removing a single perithecium, macerating in 1 ml of sterile dH₂O, spreading 300 μl of the spore suspension on GYE, and observing growth. For one progeny plate from each paired isolate set, ten germinating ascospores were sub-cultured onto a new GYE plate after 24 hours and allowed to grow for one week. Genomic DNA was extracted from the ten subcultured progeny and screened for MAT genes as described above. Given that progeny should segregate 1:1 for MAT1-1 and MAT1-2, all ascospore suspension plates were incubated for up to 12 weeks to observe mating among the progeny.

A subsequent interspecies mating assay was performed to test mating capability among *N. ditissima*, *N. faginata*, and other Nectriaceous fungi. Here, MAT1-1- and MAT1-2-positive isolates of *N. ditissima* (MAT1-1: NdBI001; MAT1-2: NdSam003) and *N. faginata* (MAT1-1: NfFg005; MAT1-2: NfFg008) were paired three times each with an isolate of *Nectria magnoliae* (isolate no. NecmLt005) and *Corinectria* aff. *fuckeliana* (isolate no. CafPr004) as described above. Additionally, both *N. ditissima* isolates were paired three times with the *N. faginata* isolate of the opposite mating type. Plates were checked weekly for perithecia formation for up to 12 weeks and processed as described above.

Results

Identification and structure of MAT loci in *N. ditissima* and *N. faginata*

Each of the *N. faginata* and *N. ditissima* genomes contained either the MAT1-1 or MAT 1-2 idiomorph, and the single *N. coccinea* genome contained only a MAT1-2 idiomorph. MAT genes of the opposite mating type were not found within the genomes. AUGUSTUS analyses of the MAT loci and flanking genes revealed a similar genetic structure for *N. coccinea*, *N. ditissima* and *N. faginata* with only minor differences in the MAT gene open reading frame (ORF) and intron lengths (Figure 2). The MAT1-1-1 coding sequence for *N. ditissima* was 1,167 bp (357 amino acids [aa]; GenBank accession XXXXX) with two introns of 48 bp and 46 bp, while the MAT1-1-1 coding sequence for *N. faginata* was 1204 bp (371 aa; GenBank accession XXXXX) with two introns of 46 bp and 44 bp. The MAT1-2-1 coding sequence for *N. ditissima* was 827 bp (243 aa; GenBank accession XXXXX) with two introns of 48 bp and 49 bp, while the coding sequence for *N. faginata* was 815 bp (239 aa; GenBank accession XXXXX) with

two introns of 47 bp and 50 bp. Additionally, the genetic structure of MAT1-2-1 of *N. coccinea* was found to be similar to *N. ditissima* and *N. faginata* with an coding sequence of 816 (238 aa; GenBank accession XXXXX) with two introns of 50 bp and 48 bp.

Two commonly co-occurring MAT associated genes were found near MAT1-1-1 in both *N. ditissima* and *N. faginata*, including MAT1-1-2 and MAT1-1-3 (Figure 2; Coppin et al., 1997). For each of the three *Neonectria* spp., evidence of co-occurrence of MAT1-2-1 and MAT1-2-2 was not found. Both *N. ditissima* and *N. faginata* MAT loci were flanked by the conserved SLA2 gene previously described as being associated with MAT loci (Debuchy and Turgeon, 2006). SLA2 was found to occur in a separate contig of the *N. coccinea* de novo genome assembly, and although present, could not be shown to be part of the MAT locus without further assembly. APN2 is a second conserved gene often flanking the MAT loci of other Ascomycetes (Debuchy and Turgeon, 2006), but this gene was not identified in the regions flanking the MAT loci using the de novo genome assemblies analyzed in this study.

N. ditissima MAT1-1 Idiomorph

N. ditissima MAT1-2 Idiomorph

N. faginata MAT1-1 Idiomorph

N. faginata MAT1-2 Idiomorph

N. coccinea MAT1-2 Idiomorph

0.5 kb

Figure 2: Structure of the MAT1-1 and MAT1-2 loci of the heterothallic fungi:

Neonectria ditissima, *Neonectria faginata*, and *N. coccinea* (MAT1-2 only). Arrows above the genes indicate the 5' – 3' orientation. Coding sequence lengths are included below each gene identifier. For MAT1-1-1 and MAT1-2-1 genes, introns are represented by vertical black lines, and approximate primer binding locations are illustrated for each primer pair below the gene illustration. The approximate amplicon size for each primer pair is shown below the primer binding locations and is shaded based on each primer pair. All distances and sizes are estimations and not drawn to scale.

Species specific MAT1-1-1 and MAT1-2-1 primers

Neonectria ditissima (NdM1f/r) and *N. faginata* (NdM2f/r) MAT primer pairs amplified a single product of the expected size for their target species (Table 2). Sequencing confirmed the identity of all PCR products, each of which exhibited 99% or greater sequence identity with the target MAT sequences. All of the 160 (40 initially screened or 120 progenies from *in vitro* crosses) single-spore derived isolates tested yielded either MAT1-1-1 or MAT1-2-1 amplicons while DNA extractions from perithecia (N = 7) containing ascospores of both mating types yielded both MAT products. Species-specific primers designed for *N. faginata* did not amplify either MAT gene in *N. ditissima*. Likewise, *N. ditissima*-specific primers did not amplify either MAT gene in *N. faginata*.

MAT1-1-1 and MAT1-2-1 primer pairs designed for *N. ditissima* did not amplify DNA in any of the other tested species (Table 3). In contrast, the *N. faginata* MAT1-1-1 primer pair amplified MAT1-1-1 in MAT1-1 isolates in *N. coccinea*, *N. neomacrospora*,

and *Nectria magnoliae*. In contrast, MAT1-2-1 primers for *N. faginata* did not amplify MAT1-2-1 for any other tested species.

Table 3: Summary PCR results for each included species and primer pair with ‘+’ denoting successful amplification of the target sequence and “*” indicating non-target amplification when primer pair is applied to an isolate of the opposite mating type. Empty cells indicate no PCR product was amplified.

Species	Primer Set						
	NdM1	NdM2	NfM1	NfM2	NeoM1	NeoM2	NeoDegM2
<i>Neonectria ditissima</i>	+	+			+	+	+
<i>Neonectria faginata</i>			+	+	+	+	+
<i>Neonectria coccinea</i>			+	*		*	+
<i>Neonectria neomacrospora</i>			+			*	+
<i>Nectria magnoliae</i>			+				+
<i>Corinectria</i> aff. <i>fuckeliana</i>				*	+		+
<i>Fusarium concolor</i>							+

* = non-target amplification when primer pair is applied to isolates containing the opposite MAT idiomorph

Amplification of non-mating type associated proteins was observed when applying *N. faginata* MAT1-1-1 primers to MAT1-2 strains of *N. coccinea*, *Corinectria* aff. *fuckeliana*, and *N. neomacrospora*. Sequencing of these amplicons revealed a putative major facilitator superfamily (MFS-type) transporter protein (~120 bp > target) in *N. coccinea* and two undescribed hypothetical proteins in *N. neomacrospora* (~470 bp > target) and *C. aff. fuckeliana* (~470 bp > target) (Table S2). Additionally, amplification of non-mating type associated proteins was observed when *N. faginata* MAT1-2-1 primers were applied to MAT1-1 *N. coccinea* and *C. aff. fuckeliana* isolates

including hypothetical proteins in *N. coccinea* (~540 bp > target) and *C. aff. fuckeliana* (~190 bp > target) (Table S2).

Genus-level *Neonectria* MAT1-1-1 and MAT1-2-1 primers

Genus-level MAT primer pairs (NeoM1f/r and NeoM2f/r) successfully amplified both MAT1-1-1 and MAT1-2-1 gene for *N. ditissima* and *N. faginata* (Supplemental Figure 1). These same primers also amplified MAT1-1-1 for *Corinectria* aff. *fuckeliana* (Table 3) but failed to amplify MAT1-1-1 and MAT1-2-1 for all other non-target fungi.

The MAT1-2-1 degenerate primer pair (NeoM1df/r) successfully amplified MAT1-2-1 for all species tested, including a more distantly related *Fusarium babinda* isolate. Non-target amplification of a hypothetical protein (~590 bp > target) was observed when the NeoM2f/r primer pair was applied to MAT1-1 *N. coccinea* and both MAT1-1 and MAT1-2 *N. neomacrospora* isolates.

Phylogenetic analyses and protein alignments

Phylogenetic analyses of MAT1-1-1 and MAT1-2-1 sequences resulted in similar tree topologies (Figures 3 and 4). Analysis of MAT1-1-1 grouped all *Neonectria* species into a strongly supported monophyletic clade sister to *Corinectria* (containing several species formerly classified in *Neonectria*) (González and Chaverri, 2017) (Figure 3). Within *Neonectria*, a clade containing *N. coccinea*, *N. faginata* and *N. punicea* was resolved as sister to *Nectria magnoliae* from *Liriodendron tulipifera* and *Magnolia fraseri*. For MAT1-2-1, all included *Neonectria* species resolved to a monophyletic clade that was sister to a clade containing *Corinectria* aff. *fuckeliana* and the three species of *Fusarium* (Figure 4). Additionally, two sister clades within *Neonectria* were resolved: 1)

N. faginata was sister to a clade containing *N. coccinea* and *Nectria magnoliae*, and 2) *Neonectria ditissima* and *N. neomacrospora* formed a monophyletic clade sister to *N. hederæ*. All isolates of *N. ditissima* formed a single lineage regardless of plant host for both MAT1-1-1 and MAT1-2-1.

Figure 3: Phylogenetic relationships of *Neonectria* spp. and related fungi based on MAT1-1-1 gene sequence data. The phylogeny was inferred using a Maximum Likelihood analysis based on the Kimura 2-parameter model with gamma distribution (K2+G) and 1000 bootstrap replicates. Bootstrap values > 70% are given at the nodes. Branch lengths represent the number of substitutions per site. Outgroup includes *Ophiocordyceps xuefengensis* and *Ustilaginoidea virens*.

Figure 4: Phylogenetic relationships of *Neonectria* spp. and related fungi based on MAT1-2-1 gene sequence data. The phylogeny was inferred using a Maximum Likelihood analysis based on the Kimura 2-parameter model with gamma distribution (K2+G) and 1000 bootstrap replicates. Bootstrap values > 70% are given at the nodes. Branch lengths represent the number of substitutions per site. Outgroup includes *Ophiocordyceps xuefengensis* and *Ustilaginoidea virens*.

Protein alignments included approximately 100 amino acids for MAT1-1-1 sequences and approximately 75 amino acids for MAT1-2-1 sequences within the conserved region of these genes (Figure 5). The protein sequences deduced from *Neonectria coccinea*, *Nectria magnoliae*, *Corinectria* aff. *fuckeliana*, and *Fusarium babinda* MAT1-2-1 PCR products were missing the first five amino acids due to lacking primer binding locations, and therefore, results reported for MAT1-2-1 below represent values with (full) and without (partial) the first five amino acids. Protein alignments for both MAT1-1-1 and MAT1-2-1 were most similar among *Neonectria* spp. with 28% and 29.3% (or 36% for full sequence comparisons) of amino acids conserved across all species considered, respectively. Of these shared amino acids, 19% MAT1-1-1 and 25.3% MAT1-2-1 amino acids (partial seqs) were shared with included *Corinectria* spp. Across all included fungi, only 5% of amino acids were shared among MAT1-1-1 sequences and 21.3% (or 28% for full sequence comparisons) amino acids were shared among MAT1-2-1 sequences.

MAT1-1-1

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Nef 1 LLPEDFPGTSDENCFLGSHDFSLVEGHFQKPVGTCTROGLLLRARRNWSGOHFPATLPRHRLFHGHGHPSSFLAVLRMD-CVROGWPLRCHQGRV
Nec 1 LLPEDFPGTSDENCFLGSHDFSLVEGHFQKPVGTCTROGLLLRARRNWSGOHFPANLPRHRWLHGGPHPPFSLVGLDLMD-CVRR-WPQRCQGRV
Nep 1 LLPEGFPGTSDENCFLGSHDFSLVEGHFQKPVGTCTROGLLLRARRNWSGOHFPVNLPRRCLLHGHGHPSPSTVEDDLMD-CVRRQWPLRRHQGRV
Necm 1 LLPENLPGAPDENCFLGSHDFSLVEGHFQKPVGTCTROGLVLRAGDRDRPEOHFPDILPRHCVSHHGHGHPSSPVECGLRMDGAVRRGRPRRRHQGRV
Ned 1 LLPEAVPRRPENECFLGSHDFSLVAGPFQKQVGTCTROGLLLRAGRWPNOHIFVALSRCLLPRDENHSSCFLLGLRLVDCSVRG-RSLRSCSGRR
Nen 1 LLLEAVPRPPAENECFLGSHDFSLVAGPFQKQVGTCTROGLLLRAGRWPNOHIFVALSRCLLPRDENHSSCFLLGLRLVDCSVRG-RSLRSCSGRR
Cof 1 LLHETSPGPPAEDCISPLNPSLEPRHFQKQVGTCTROGLLLRREG-DWQEOHILGTLSRCFLSCHEHCPPYPLFFCLRVDSARR-RSYP-RSGTA
Coaf 1 LLHETSPGPPAEDCISPLNPSLEPRHFQKQVGTCTROGLLLRREG-DWQEOHILVTLSRCFLSCHEHCSPYPLFFCLRVDSARR-RSYP-RSGTA
Fuo 1 LLSEATPRHPAEECLRFPSALGRRPSFKMGDCS-LFLSPR-STROEYRLVRFPWYRLPFDEHG-TLLVYGPWLAANCFSRHRDISAKHCHEG
Usv 1 LLSQDVP-CSAKNCVWIFDHPVEQGSVEK-MGVDSLLLRSR--NRQSFASIFPWVLLPCGNCRTSCVCSPPMVSSGNRLSKTGSSSNSS
Opx 1 LLFEAVSRRAAKDCFWVSHHLAQRPVQKQVGTCTROGLLLRAGRWPNOHIFVALSRCLLPRDENHSSCFLLGLRLVDCSVRG-RSLRSCSGRR
cons 1 LL E vpg paenCfgs d sl gpfqKqvgtr qglllr r w qhf lpr l rhehh vp lrmd vrr r r h gr r

```

MAT1-2-1

```

Nef 1 KIPRPPNAYILYRKDRHRLKASRPDIHNNICESLDLFDTTIDFLSARILGRAWNKESAEIRLYKYLRADEI
Nec 1 -----PNAYILYRKDRHRLKASRPDIHNNICESLDLFDGN-NHLFFSS--HSWPGLEQGVRRDQTQVQAQS
Necm 1 -----PNAYILYRKDRHRLKASQPGISNNIDICASP-FRVTILTWAARVLGRAWNQESAEVRLKYKLRADEI
Ned 1 KIPRPPNAYILYRKDRHRLVKAGRPDIHNNICESDEVLCNGIKTDFFS--NSRSRLQGVRC-CQTQVQDQS
Nen 1 -----PNAYILYRKDRHRLVKAGRPDIHNNICES--VFNRG-TTNFFSP--NSRSRLQGVGRCQTEVQDQS
Neh 1 KIPRPPNAYILYRKDRHRLVKAGRPDIHNNICESHYDFNREITITDFYS--NPWSCLEQGVG-CQTQVQDQS
Coaf 1 -----PNAYILYRKDRHRLTRAGNPGIHNNEICECLNVLNCETILTFFISD-NPWTSLB-----PRDQSQT
Fub 1 -----PNAYILYRKDRHRLVKAGRPDIHNNICESKCVMTCTRTPLKLCPRS-----LADFGTLRPVRFEPPTSTG
Fuo 1 KIPRPPNAYILYRKDRHRLKASRPDIHNNICESKFMPIYKTSITSFPRS-----LAASGTPRPVRFHSTSRW
Usv 1 KIPRPPNAYILYRKDRHRLVKAGRPDIHNNICESKFMPIYKTSITSFPRS-----LAASGTPRPVRFHSTSRW
Opx 1 KIPRPPNAYILYRKDRHRLVKAGRPDIHNNICESKFMPIYKTSITSFPRS-----LAASGTPRPVRFHSTSRW
cons 1 kiprpPNAYILYRKdRHrlvkagrPdIhNNeIces lfn i s w le i k

```

Figure 5: MAT1-1-1 and MAT1-2-1 amino acid alignments including a selection of study fungal species. Name abbreviations are as follows: *Nef* = *Neonectria faginata*; *Nec* = *Neonectria coccinea*; *Nep* = *Neonectria punicea*; *Necm* = *Nectria magnoliae*; *Ned* = *Neonectria ditissima*; *Nen* = *Neonectria neomacrospora*; *Neh* = *Neonectria hederiae*; *Cof* = *Corinectria fuckeliana*; *Coaf* = *Corinectria* aff. *fuckeliana*; *Fub* = *Fusarium babinda*; *Fuo* = *Fusarium oxysporum*; *Usv* = *Ustilaginoidea virens*; *Opx* = *Ophiocordyceps xuefengensis*; Cons = consensus. Uppercase letters within the consensus sequence represent positions with identical amino acids. Lower case letters represent positions with similar amino acids. Black shading represents conserved amino acids among 50% or more species. Grey shading represents shared amino acids with shared characteristics/properties among species.

In vitro pairing assay results

For *N. faginata* and *N. ditissima* intraspecies pairings, perithecia with viable ascospores were produced for all pairings between MAT1-1 and MAT1-2 strains with one exception: one pairing between isolates NfFg006 and NfFg012 failed to produce perithecia within the 12-week assay (Table 4). All ascospore suspension plates containing ascospore progeny from single, squashed perithecia resulting from MAT1-1 x MAT1-2 pairings also yielded perithecia. No pairings with an isolate of the same mating type, including self-pairings, yielded perithecia. Additionally, no evidence of mating was observed in interspecies pairings.

Table 4: In vitro pairing assay results for *N. ditissima* and *N. faginata*. PCR confirmed MAT identities are included for each isolate. Three pairings were completed for each isolate set. Result format = number of positive results out of the three total pairings. Results categories include the following: 1. Production of perithecia; 2. Presence of ascospores within perithecia 3. Viability of ascospores from ascospore suspension plates; 4. Production of perithecia on ascospore suspension plates used for viability confirmation.

	Isolate A		Isolate B		Perthecia	Ascospores	Viability	Progeny Perithecia
	ID	MAT	ID	MAT				
<i>Neonectria ditissima</i>	NdAp001	MAT1	NdAs001	MAT2	3	3	3	3
	NdSam001	MAT1	NdSam002	MAT2	3	3	3	3
	NdBI001	MAT1	NdFg003	MAT2	3	3	3	3
	NdBa001	MAT1	NdSam003	MAT2	3	3	3	3
	NdSa001	MAT1	NdFg004	MAT2	3	3	3	3
	NdFg002	MAT1	NdFg005	MAT2	3	3	3	3
	NdAp001	MAT1	NdSam001	MAT1	0	-	-	-
	NdBI001	MAT1	NdBa001	MAT1	0	-	-	-
	NdSa001	MAT1	NdFg002	MAT1	0	-	-	-

NdAs001	MAT2	NdSam002	MAT2	0	-	-	-
NdFg003	MAT2	NdSam003	MAT2	0	-	-	-
NdFg004	MAT2	NdFg005	MAT2	0	-	-	-
NdAp001	MAT1	NdAp001	MAT1	0	-	-	-
NdSam001	MAT1	NdSam001	MAT1	0	-	-	-
NdBI001	MAT1	NdBI001	MAT1	0	-	-	-
NdBa001	MAT1	NdBa001	MAT1	0	-	-	-
NdSa001	MAT1	NdSa001	MAT1	0	-	-	-
NdFg002	MAT1	NdFg002	MAT1	0	-	-	-
NdAs001	MAT2	NdAs001	MAT2	0	-	-	-
NdSam002	MAT2	NdSam002	MAT2	0	-	-	-
NdFg003	MAT2	NdFg003	MAT2	0	-	-	-
NdSam003	MAT2	NdSam003	MAT2	0	-	-	-
NdFg004	MAT2	NdFg004	MAT2	0	-	-	-
NdFg005	MAT2	NdFg005	MAT2	0	-	-	-
NfFg001	MAT1	NfFg007	MAT2	3	3	3	3
NfFg002	MAT1	NfFg008	MAT2	2	2	2	2
NfFg003	MAT1	NfFg009	MAT2	3	3	3	3
NfFg004	MAT1	NfFg010	MAT2	3	3	3	3
NfFg005	MAT1	NfFg011	MAT2	3	3	3	3
NfFg006	MAT1	NfFg012	MAT2	2	2	2	2
NfFg001	MAT1	NfFg002	MAT1	0	-	-	-
NfFg003	MAT1	NfFg004	MAT1	0	-	-	-
NfFg005	MAT1	NfFg006	MAT1	0	-	-	-
NfFg007	MAT2	NfFg008	MAT2	0	-	-	-
NfFg009	MAT2	NfFg010	MAT2	0	-	-	-
NfFg011	MAT2	NfFg012	MAT2	0	-	-	-

NfFg001	MAT1	NfFg001	MAT1	0	-	-	-
NfFg002	MAT1	NfFg002	MAT1	0	-	-	-
NfFg003	MAT1	NfFg003	MAT1	0	-	-	-
NfFg004	MAT1	NfFg004	MAT1	0	-	-	-
NfFg005	MAT1	NfFg005	MAT1	0	-	-	-
NfFg006	MAT1	NfFg006	MAT1	0	-	-	-
NfFg007	MAT2	NfFg007	MAT2	0	-	-	-
NfFg008	MAT2	NfFg008	MAT2	0	-	-	-
NfFg009	MAT2	NfFg009	MAT2	0	-	-	-
NfFg010	MAT2	NfFg010	MAT2	0	-	-	-
NfFg011	MAT2	NfFg011	MAT2	0	-	-	-
NfFg012	MAT2	NfFg012	MAT2	0	-	-	-

Each progeny included in the subsequent PCR screening resulted in the amplification of a single MAT gene. Both MAT genes were found to be segregated among progeny at a ratio not significantly divergent from the expected 1:1 for *N. ditissima* ($\chi^2 = 0.07$, df = 1,60; $P = 0.80$) and *N. faginata* ($\chi^2 = 0.27$, df = 1,60; $P = 0.606$) progeny (Table 4).

Discussion

Beech bark disease was first reported in Halifax, Nova Scotia, Canada around 1890 and continues to spread throughout the range of American beech (Hewitt, 1914; Erhlich, 1934). Likewise, BBD continues to impact European Beech throughout Europe (Cicák et al., 2006; Cicák and Mihál, 2008). Despite the ecological importance of this disease (Houston, 1994b; Garnas et al., 2011a,b), certain aspects of BBD biology and epidemiology remain largely unexplored, including the mating strategies of *N. ditissima*,

N. faginata, and *N. coccinea*. Previous studies regarding the thallism of *N. ditissima* and *N. faginata* (El-Gholl et al., 1986; Krüger, 1973; Cotter and Blanchard, 1978) were either inconclusive or sample limited, and as such, the role of mating strategy in the BBD pathosystem in the U.S. has historically been uncertain. Thallism is particularly important to the BBD pathosystem given that at least two dominant, sexually reproducing pathogens are causal agents of the disease in both the U.S. and Europe. Both can colonize single trees and, in some instances, have been found to co-occur within the same 2.5 inch diameter bark disk (Kasson and Livingston, 2009).

Neonectria faginata has been previously reported as the dominant pathogen in the BBD system in North America (Houston, 1994; Kasson and Livingston, 2009). Mating strategy may have provided one explanation for its dominance, but as shown in this study, both *N. faginata* and *N. ditissima* are heterothallic fungi based on limited sampling. Therefore, the possibility of any advantage that homothallism might confer is eliminated, indicating that *N. faginata* is likely dominant due to an increased level of virulence, other advantageous traits, or some combination of these factors.

Despite having resolved the mating strategy of these fungi, the potential overrepresentation of either *N. ditissima* or *N. faginata* in previous studies may be due to perithecia-dependent sampling bias. For example, environmental conditions (e.g. RH, temperature) and time required for perithecia production may significantly differ between these two fungi. Given potential seasonal differences in fruiting, sampling at a single time point in the year could potentially yield a community not representative of the relative abundance of the *Neonectria* spp. present. Additionally, casual observations of *N. ditissima* on non-beech hosts can reveal very limited to no perithecia occurring at a

given infection site. This may be in part due to host susceptibility, resulting in more or less necrotic tissue, which is generally considered favorable for perithecia production by necrotrophic fungi.

Host specificity may develop through the evolution of mating barriers among strains of a single species occurring on differing hosts. Results from this study have demonstrated a lack of reproductive barriers among *N. ditissima* strains from several plant hosts. All pairings of either MAT1-1-1 or MAT1-2-1 strains from different plant hosts resulted in perithecia formation. While a lack of host specificity by *N. ditissima* has been previously demonstrated using pathogenicity assays (Lortie, 1969; Ng and Roberts, 1974; Barnard et al., 1988; Plante and Bernier, 1997), mating among *N. ditissima* strains infecting co-occurring tree species had not been previously tested until now. Given the lack of reproductive barriers, evolution of host specificity is limited by obligate outcrossing among these co-occurring strains.

The phylogenetic analyses using MAT gene sequences were found to be in agreement with previously resolved relationships demonstrated using EF1- α , RPB2, and β -tubulin (Castlebury et al., 2006). This finding confirms the utility of MAT genes in resolving relationships among *Neonectria* and allied fungi. Additionally, *N. ditissima* isolates from different plant hosts included in the phylogeny form a single lineage providing additional evidence for the lack of reproductive barriers. The amino acid alignments visualized protein sequence divergence potentially contributing to mating barriers among these closely related species that were confirmed by inter-species mating assays. Using such methods to test intraspecies MAT gene diversity among these and closely allied fungi may prove valuable for broader surveys.

Limited amplification of non-mating type proteins was observed among several MAT primer – species combinations for isolates of the opposite mating type. However, in all cases, the sequences were found to be larger than the expected size of the target product. Given that such issues can lead to erroneous conclusions, electrophoresis gels should be run to 100 bp resolution and additionally, PCR conditions should be optimized for the target species.

In this study, we confirm heterothallism and characterized the MAT idiomorphs of *N. ditissima*, *N. faginata*, *N. coccinea* and several other members of Nectriaceae. These findings provide additional insight into characteristics that may shape the community and population dynamics of the beech bark disease complex and its causal agents. Additional studies are needed to further characterize sexual reproduction of *N. ditissima*, *N. faginata*, and *N. coccinea* in their respective BBD systems. These efforts include: 1) identifying differing environmental factors required for perithecia production among *Neonectria* spp.; 2) characterizing variability in sexual reproduction by *N. ditissima* across host substrates; 3) assessing the potential for interspecific hybridization between closely related *Neonectria* spp. found co-occurring on beech and other hosts but excluded from this study; and 4) comprehensive screening of additional isolates from populations not sampled in this study to assess intraspecies MAT gene diversity and uncover possible intraspecies mating barriers.

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Supplemental Figure

Supplemental Figure 1: Electrophoresis gel photo demonstrating specific amplification of MAT1-1-1 and MAT1-2-1 by the genus-level primer pair (NeoM1f/r and NeoM2f/r) for both *N. ditissima* and *N. faginata*.

Supplemental Table

Supplemental Table 1: Non-target amplification identities for primer x species pairings: MAT identities are given with the species names. The approximate product sized expected for specific amplification of the target MAT gene are provided. For instances of multiple non-target amplicons among tested species, the lowest sequence similarity value is given. Non-target protein blastx results with the greatest percent sequence coverage are provided including: query coverage, percent identity, and NCBI accession numbers.

DNA Template (MAT ID)	Primer Pair	Approx. Target Product Size (bp)	Approx. Non-Target Product Size (bp)	Sequence Similarity Among Non-Target Amplicons	Non-Target blastx Results		
					Query Coverage	% Identity	NCBI Accession
<i>N. coccinea</i> (M2)	NfM1f/r	530	650	94%	88.0%	90.0%	KPM41412
<i>N. neomacrospora</i> (M2)	NfM1f/r	530	1000	N/A	29.0%	83.5%	KPM36460
<i>C. aff. fuckeliana</i> (M2)	NfM1f/r	530	1000	N/A	32.0%	75.2%	KPM45843
<i>N. coccinea</i> (M1)	NfM2f/r	610	1150	81%	51.0%	68.2%	KPM37850
<i>C. aff. fuckeliana</i> (M1)	NfM2f/r	610	800	82%	45.0%	83.5%	KPM46131
<i>N. coccinea</i> (M1)	NeoM2f/r	610	1200	87%	44.0%	74.8%	KPM37850
<i>N. neomacrospora</i> (M1)	NeoM2f/r	610	1200	76%	50.0%	35.7%	KPM37850

CHAPTER 3: *Corinectria gaudineerii* sp. nov., a novel nectriaceous fungus associated with high elevation *Picea rubens* in the Appalachian Mountains

Abstract

The Nectriaceae is a family of fungi including many phytopathogens capable of producing cankers on hardwood and conifer host species. Among these nectriaceous fungi are members of the recently circumscribed genus *Corinectria*. Members of *Corinectria* are closely related to *Neonectria*, but unlike *Neonectria* whose members primarily infect hardwood tree species, *Corinectria* spp. are found exclusively on conifer tree species as canker pathogens or hemibiotrophs. In this study, *Corinectria gaudineerii* sp. nov. is described as a new member of the recently circumscribed genus *Corinectria*. *C. gaudineerii* is the fourth species described for this genus but represents the first to be described occurring in the central Appalachian Mountains on the bark of dead *Picea rubens* (red spruce). In this study, we provide phylogenetic evidence using five loci (ITS, LSU, BTUB, EF1, ACT) to characterize *C. gaudineerii* as a new species. Additionally, field surveys recovered *C. gaudineerii* from four geographically separated spruce forests in WV and VA. Finally, live- and excised-stem assays were conducted to determine the pathogenic and saprophytic potential, revealing *C. gaudineerii* to be a hemibiotroph and possibly, a weak canker pathogen. Together, these results provide evidence to support the description of a novel species of *Corinectria* associated with dead red spruce in central Appalachia.

Introduction

Corinectria is a recently circumscribed genus in the Nectriaceae that formerly belonged to but was phylogenetically distinct from the closely related *Neonectria* (González and Chaverri, 2017). This genus consists of three formally described species: *C. fuckeliana* ([C. Booth] C. González & P. Chaverri), *C. constricta* (C. González & P. Chaverri), and *C. tsugae* ([W. Gams] C. González & P. Chaverri). All members of *Corinectria* are hemibiotrophic and are associated with stem cankers on diverse coniferous hosts including *Picea*, *Abies*, *Pinus*, and *Larix* with distribution in both the northern and southern hemispheres. *C. fuckeliana* (formerly *Neonectria fuckeliana*), which is among the most well studied *Corinectria* species, has been associated with species of the aforementioned host genera as a weak pathogen, saprophyte, and endophyte (Huse, 1981, Rolls-Hansen and Rolls-Hansen, 1979, Vasiliauskas and Stenlid, 1998). *C. tsugae* has been only reported on *Tsuga heterophylla* (Raf.) Sarg. in western North America (Williams, 1987). More recently, previous reports of *C. fuckeliana* infecting *Pinus radiata* D.Don in Chile (Gadgil et al., 2003, Dick and Crane, 2009, Crane et al., 2009) were re-evaluated and determined to be a separate species leading to the circumscription of *C. constricta* (González and Chaverri, 2017). To date, all species infected and/or associated with *Corinectria* members have been coniferous species, and none have been confirmed on any trees native to eastern North America.

In December of 2018, perithecia were observed extruding from bark along the trunk of toppled dead understory red spruce (*Picea rubens* Sarg.) trees on Gaudineer Knob in the Monongahela National Forest of West Virginia, USA. This discovery was

reported in a Chapters 1 and 2 where phylogenetic results indicated this species to be divergent from all previously described *Corinectria* spp. Further, this putatively novel *Corinectria* sp. was exclusive to red spruce trees at three geographically separate locations in West Virginia and Virginia, but no formal description of this species was made nor were any morphological characters compared with previously described *Corinectria* spp.

Red spruce has been a primary target for restoration in the Appalachian Mountains over the last few decades following several decades of decline. Extensive surveys of declining or dead red spruce trees were conducted to identify plant pathogens potentially contributing to this decline, but none reported “Nectria”-like fungi associated with red spruce (Johnson and McLaughlin, 1986, Bruck, 1989). Given that this *Corinectria* species was identified at several locations in central Appalachia, this fungus may represent a newly introduced pathogen that has only recently encountered native red spruce. Otherwise, a native fungus has significantly increased in incidence from historic undetectable levels due to unique stressors that are predisposing trees to fungal invasion, similar to BBD following the introduction of beech scale although no scale insect has been observed.

To further investigate this putatively novel *Corinectria* sp., we sought to determine the identity of this *Corinectria* sp. using phylogenetic and morphological comparisons with previously described species. This was important as the lack of previous reports of this fungus despite extensive red spruce surveys might indicate the introduction of a new phytopathogen. We next sought to conduct surveys to determine the ecology of this fungus by screening red spruce stands throughout the central

Appalachian Mountains, and by doing so, determine the threat this new species might pose to the red spruce forests. Finally, we sought to test the pathogenic and saprophytic capacity of this fungus using live- and excised-stem assays. Together these provide the baseline for characterizing the biology and ecology of this novel *Corinectria* sp. across red spruce forests of central Appalachia. Such investigations are vital to identify potential threats to management and restoration efforts of red spruce.

Materials and Methods

Survey approach

Sampling areas with large populations of red spruce were selected in West Virginia and Virginia. These sites were part of a broader survey of Nectraiaceae across the central Appalachian Mountains (Chapter 1). These sites included sampling areas near Mount Rogers (MR) and Whitetop Mountain (WT) of the Jefferson National Forest in VA and areas near Cranberry Wilderness (CR) and Gaudineer Knob (GK) of the Monongahela National Forest in WV (Figure 1; Supplemental Table 1). The United States Forest Service provided a permit for sampling on the Mon. National Forest (FS 2400-8) and written permission were given to collect samples without a special use permit on the Jefferson National Forest. At each location, declining red spruce stands were identified by the occurrence of five or more outwardly stressed or dead understory or intermediate red spruce trees within a 1/10th acre plot. Within each plot at WT, MR, and CR, tree health status, size class, canopy position, and the presence of perithecia were recorded for all red spruce trees. No data were collected at GK. Tree health statuses included alive (A; < 50% dieback), declining (V; > 50% dieback), and dead (D; loss of fine branches and bark). Size classes were recorded in ten-centimeter

increments of 0-10 cm, 10-20 cm, and >20 cm diameter at breast height (DBH). Canopy positions included dominant, co-dominant, intermediate, and suppressed.

Figure 1: Sampling locations for members of Nectriaceae on red spruce in WV and VA. These include Mount Rogers (MR) and Whitetop Mountain (WT) of the Jefferson National Forest in VA and areas near Cranberry Wilderness (CR) and Gaudineer Knob (GK) of the Monongahela National Forest in WV. Shaded areas represent the natural range of red spruce (Prasad and Iverson 2003).

Sampling method and processing

A maximum of four bark disks harboring perithecia were excised from red spruce trees with a 1-cm steel punch and stored in 96-well microtiter dishes. All samples were

stored on ice until they were stored at -20 °C in the laboratory until being processed. Sample processing was completed as previously described in Chapter 2. In summary, five perithecia were removed from each bark disk, macerated in 1 ml of sterile H₂O within a microcentrifuge tube, vortexed for 15 seconds, then 300 µl of the spore suspension was spread on glucose-yeast extract agar plates (GYE/A) amended with streptomycin sulfate (10 mg/1000 ml) and tetracycline hydrochloride (100 mg/1000 ml) antibiotics. Five single ascospore colonies were transferred to a new GYE/A plate within 48 hours. Selected isolates were stored at approximately -20 °C on glass filter paper.

DNA Extraction and PCR Protocol

Suspect *Corinectria* isolates recovered from field-collected perithecia were grouped and tentatively identified based on colony morphology. Genomic DNA extractions were performed for selected isolates using a modified Wizard® kit (Promega, Madison, WI, USA) and suspended in 75 µl Tris-EDTA (TE) buffer (Amresco, Solon, OH, USA) (Short et al. 2015). The fungal bar-coding genes internal transcribed spacer region (ITS), translation elongation factor 1-alpha (EF1), 28S rDNA (LSU), β-tubulin (BTUB), and actin (ACT) were PCR amplified in 25 µl reactions containing 12.5 µl Bioline PCR Master Mix (Bioline USA Inc, Taunton, MA), 10.0 µl H₂O, 1.5 µl purified DNA, and 1.0 µl each of forward and reverse primers (Integrated DNA Technologies, Coralville, IA, USA). All primers, protocols, and their sources are outlined in Supplemental Table 2. PCR products were visualized via gel electrophoresis, and positive reactions were purified using ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's recommendations. Purified PCR products were Sanger

sequenced with both forward and reverse PCR primers (Eurofins, Huntsville, AL, USA).

Phylogenetic analyses

Phylogenetic analyses were conducted as described in Chapter 2 and are summarized below. Assessment and clipping of sequences were completed with CodonCode Aligner v. 5.1.5, then manual corrections for nucleotide misreads were performed. To resolve the identity of recovered *Corinectria* isolates, single-gene and concatenated five-gene phylogenetic trees were constructed using additional reference sequences for all described members of *Corinectria* along with selected *Neonectria* members to serve as outgroup taxa (Table 1). Alignments were completed via MAFFT (Kato and Stanley, 2013) on the Guidance 2.0 server (Landan and Graur, 2008, Sela et al., 2015), and all residues with Guidance scores <0.5 were masked (Macias et al., 2020). Aligned single gene sequences were concatenated using the web tool FaBox (Villesen 2007).

Maximum-likelihood analyses were performed in MEGA v10.1.7 (Stecher et al., 2020), and Bayesian inference (BI) analyses were performed using MrBayes v. 3.2.7 (Ronquist et al., 2012). Model Test AICc scores in MEGA were used to select the best fit substitution model for ML analyses. All ML analyses included 1000 bootstrap replicates. For BI analyses, MrBayes was allowed to select the best fit nucleotide selection model, but Model test AICc scores were used to select the rate of substitution. All BI analyses ran until the standard deviation of split frequencies fell below 0.01 and then checked for convergence in Tracer v. 1.7.1 (Rambaut et al., 2018). Trees were edited using FigTree v. 1.4.4 (Rambaut, 2017) and Adobe Illustrator v. 24.1

Table 1: Accession table for sequences used in this study. Sequences were either generated here or acquired from NCBI. Host species and location are also provided where available.

Species	Strain ID	Host	Locality	BT	LSU	EF1	ITS	ACT
<i>Neonectria ditissima</i>	NdFg001	<i>Fagus grandifolia</i>	Randolph County, WV, USA	TBD	TBD	TBD	TBD	TBD
<i>Neonectria faginata</i>	NfFg007	<i>Fagus grandifolia</i>	Swain County, NC, USA	TBD	TBD	TBD	TBD	TBD
<i>Neonectria magnoliae</i>	NmMf001	<i>Magnolia fraseri</i>	Randolph County, WV, USA	TBD	TBD	TBD	TBD	TBD
<i>Corinectria gaudneerii</i>	CgPr004	<i>Picea rubens</i>	Randolph County, WV, USA	TBD	TBD	TBD	TBD	-
<i>Corinectria gaudneerii</i>	CgPr007	<i>Picea rubens</i>	Randolph County, WV, USA	TBD	TBD	TBD	TBD	TBD
<i>Corinectria gaudneerii</i>	CgPr009	<i>Picea rubens</i>	Randolph County, WV, USA	TBD	TBD	TBD	TBD	TBD
<i>Corinectria gaudneerii</i>	CgPr002	<i>Picea rubens</i>	Grayson County, Virginia, USA	TBD	TBD	TBD	TBD	TBD
<i>Corinectria gaudneerii</i>	CgPr010	<i>Picea rubens</i>	Pocahontas County, WV, USA	TBD	TBD	TBD	TBD	TBD
<i>Corinectria gaudneerii</i>	CgPr011	<i>Picea rubens</i>	Pocahontas County, WV, USA	TBD	TBD	TBD	TBD	TBD
<i>Corinectria gaudneerii</i>	CgPr008	<i>Picea rubens</i>	Grayson County, Virginia, USA	-	TBD	TBD	TBD	-
<i>Corinectria tsugae</i>	CBS 788.69	<i>Tsuga heterophylla</i>	Canada	KM232020.1	MH871201.1	-	KM231763.1	KM231147.1
<i>Corinectria tsugae</i>	CBS 290.70	<i>Tsuga heterophylla</i>	Canada	-	MH871386.1	-	MH859632.1	-
<i>Corinectria fuckeliana</i>	IMI 342668	<i>Picea</i> sp.	Switzerland	KJ022340.1	KJ022070.1	KJ022404.1	KJ022021.1	KJ022285.1
<i>Corinectria fuckeliana</i>	CBS 239.29	<i>Picea sitchensis</i>	Scotland	DQ789871.1	HQ840377.1	JF268748.1	HQ840386.1	-
<i>Corinectria constricta</i>	LASBE 266	<i>Pinus radiata</i>	Chile	KY636.417.1	-	KY636410.1	-	KY636427.1
<i>Corinectria constricta</i>	LASBE 340	<i>Pinus radiata</i>	Chile	KY636423.1	-	KY636414.1	-	KY636433.1

Morphological characterization of *Corinectria gaudineerii*

Ascospore and conidia measurements of *C. gaudineerii* were conducted and compared to previously described *Corinectria* members (González and Chaverri, 2017). Ascospore measurements were completed for three perithecia from each of five bark disks collected from two geographically separated sites (WT = 3 disks; GK = 2 disks). Spore measurements were completed by squash-mounting a single perithecium on a slide with lactophenol plus cotton blue mountant. Length and width measurements were taken with a Nikon Eclipse E600 compound microscope (Nikon Instruments, Melville, NY, USA) equipped with a Nikon Digital Sight DS-Ri1 microscope camera and Nikon NIS-Elements BR3.2 imaging software for 25 ascospores per perithecium.

Length and width measurements were similarly conducted for microconidia harvested from sporodochial masses on four-to-six week old pure-cultures grown on GYE. Measurements were taken for 50 microconidia harvested from four isolates from WT and two isolates from GK. Macroconidia were not found associated with any included isolates.

Pathogenicity of *C. gaudineerii* on *Picea rubens*

To test the pathogenicity of *C. gaudineerii*, a pathogenicity assay was conducted on 2.5-3.5 year old red spruce seedlings grown from seed collected near Spruce Knob, WV in 2011 and 2015. *Corinectria* sp. isolates included one recovered from WT in VA (CspPr002) and a second from GK in WV (CspPr004). Isolates were grown in pure-culture on GYE media for 10 days at 20°C under a 16-8 h light-dark cycle. Inoculum plugs were cut along the growing edge of the fungal colonies using a 5 mm diameter

steel punch. Negative control plugs were similarly cut from a sterile GYE plate. A 1-cm incision was made in the bark tissue with a sterile scalpel to expose the vascular cambium as an inoculation site upon which an inoculum plug was placed. Parafilm was used to cover the inoculation site to minimize desiccation of the inoculum prior to infection. In total, five seedlings were inoculated with isolate CspPr002, five with isolate CspPr004, and five with a sterile agar plug to serve as a negative control. Seedlings were placed on a window seal providing ambient light. Every other day, the seedlings were watered, and their positions were rotated.

At the end of eight weeks, a scalpel was used to remove the bark tissue surrounding the inoculation site. Length and width measurements were taken for all necrotic lesions associated with the inoculation sites. Canker growth was calculated as $= (\text{length} + \text{width})/2$. Four bark samples were excised from each canker margin for reisolation of inoculum. All bark samples were surface disinfested by soaking for 14 minutes in a 1:10 commercial bleach-water solution then placed onto GYE/A media. Recovery of inoculation isolates was confirmed morphologically.

Saprophytic capacity of *C. gaudineerii* on *Picea rubens*

Over the course of the survey, perithecia of *C. gaudineerii* were found exclusively on dead red spruce. While this does not exclude the possible pathogenicity of *C. gaudineerii*, the saprophytic state of this fungus was the only state in which the fungus was observed. As such, an excised-stem assay was completed using freshly cut red spruce stems. The ends of each stem were coated in parafilm to limit stem desiccation. Prior to inoculation, each stem was disinfested in a 1:10 commercial bleach solution for

14 minutes. Inoculum was prepared as previously described, but a 1-cm steel punch was used to cut inoculation plugs.

The assay included four treatments: 1) *C. gaudineerii*; 2) *Neonectria faginata*; 3) *N. ditissima*; and 4) negative control. The two *Neonectria* treatments served as comparison fungi capable of saprotrophy, although exclusively on hardwood tree species. Inoculations proceeded as a 1-cm diameter steel punch was used to create two inoculum reservoirs on each stem. An inoculum plug was then placed in the reservoir and tape was applied to limit desiccation of the inoculum. Each stem had a single treatment for a total of 8 inoculations for *C. gaudineerii* and 6 inoculations for all other treatments. Once inoculated, stems were placed into separate loosely tied autoclave bags with three tri-fold paper towels soaked with approximately 100 ml of H₂O. All stems were stored in a dark closet. After approximately 4 weeks, stems were removed and processed as described above for measurements and inoculum recovery.

Statistical analyses

One-way ANOVAs were completed to check for differences in canker size measurements taken in pathogenicity assays with a Tukey-HSD post-hoc test to identify significant pairwise differences. All analyses were completed using the stats v3.6.2 package within R v 3.6.3 statistical software (R Core Team, 2020). P-values <0.05 were considered statistically significant.

Results

Survey of *C. gaudineerii* on *Picea rubens* in central Appalachia

Across all sites, a total of 312 red spruce trees were surveyed, with 6.7% (21 of 312) of these trees harboring perithecia that yielded *C. gaudineerii*, hereafter referred to as *Cg*. No other perithecia-producing fungi were recovered from collected samples. Specifically, these positive samples represented 11.5% (17 of 148), 4.2% (2 of 48), and 1.7% (2 of 116) of surveyed trees at WT, MR, and CR, respectively. Additionally, three trees were sampled at GK, but no formal plots were established at this site.

Site data were collected at WT, MR, and CR and included size class, tree health, and crown class. All red spruce tree harboring perithecia were dead, but a majority of these trees (85.7%; 18 of 21) were not significantly decayed as the bark and fine branches of the crown were still intact. Additionally, a majority of *Cg*-positive trees were found to be suppressed underneath a closed canopy (95.2%; 20 of 21), and all were of the smallest size class.

Phylogenetic resolution of *C. gaudineerii*

Phylogenetic analyses of single genes (ACT, BT, EF1, ITS, LSU) and a five-gene concatenated sequence containing 2,526 positions were conducted to infer the relationship of *Cg* with other members of *Corinectria*. As previously mentioned, these analyses were bolstered by the inclusion of the following three previously described *Corinectria* species: *C. constricta*, *C. fuckeliana*, and *C. tsugae* (Table 1). Sequence data for all five genes were not available for every included strain, and this is reflected in the single gene phylogenies. All available sequence data were used for concatenated sequences, and all strains were represented by ≥ 3 single gene sequences with one

exception, *C. tsugae* strain CBS 290.70 was only represented by LSU and ITS sequences.

The following results are for relationships with >70% bootstrap support within the concatenated phylogeny, and both ML and BI values are reported for those relationships (ML/BI) (Figure 2). Disagreements between single-gene and concatenated phylogenies are also noted (Figure 3). All *Corinectria* spp. were monophyletic (100%/1.0) and sister to included *Neonectria* spp. *C. gaudineerii* isolates recovered in this survey formed a monophyletic clade sister to all other previously described *Corinectria* spp. (98%/1.0), and this was strongly supported (>77% / >0.77) in all single gene trees aside from the BT phylogeny in which no *Corinectria* spp. were genealogically exclusive (Figure 3). The only other genealogically exclusive *Corinectria* spp. in the concatenated tree was *C. constricta* (97%/0.98), but this was not strongly supported in any of the five single gene trees.

Figure 2: Concatenated sequence (ACT, BTUB, EF1, ITS, LSU) phylogenetic tree of *Corinectria* spp. and *Neonectria* spp. outgroups. Topology and branch lengths are from the ML analysis. For each node supported in the ML analysis, bootstrap support and posterior probabilities are indicated (ML/BI).

Figure 3: Single-gene (ACT, BTUB, EF1, ITS, LSU) phylogenetic trees of *Corinectria* spp. and *Neonectria* spp. outgroups. Topology and branch lengths are from the ML analysis. For each node supported in the ML analysis, bootstrap support and posterior probabilities are indicated (ML/BI).

Pathogenicity and saprotrophy of *C. gaudineerii* on *Picea rubens*

In the pathogenicity assay on red spruce seedlings, all *Cg* inoculations produced a necrotic lesion from which the inoculation isolate was recovered (Figure 4A). Both *Cg* treatments produced similar canker sizes ($p = 0.208$) that were significantly greater than the negative control ($p < 0.00001$). In this case, the negative control had no measurable necrosis, and therefore, was set at 0 cm. While not an included metric in this study, no obvious dieback was observed, and all trees survived to the end of the study.

In the excised stem assay, two additional treatments using isolates of *Neonectria faginata* and *N. ditissima* were included in addition to a single *Cg* and a negative control (Figure 4B). In this case, both *Cg* and *N. ditissima* produced necrotic lesions larger than *N. faginata* and the negative control ($p < 0.001$), which both similarly produced little to no necrosis larger than the inoculation wound (~ 1 cm) ($p = 0.988$). Each treatment's inoculation isolate was recovered from all respective areas of necrosis.

Figure 4: A: Live red spruce stem assay results for inoculations of *Corinectria gaudineerii* from two separate locations (WV and VA) and a negative control. B: Excised red spruce stem assay for inoculations with *C. gaudineerii*, *Neonectria ditissima*, *N. faginata*, and negative control. Letters designate significant differences at $p < 0.05$.

Morphological characteristics of *C. gaudineerii*

Morphological characteristics of *Corinectria gaudineerii* sp. nov. are summarized here and illustrated in Figure 5. Perithecia protrude singly or in aggregates (up to >25) through the thin, intact bark tissue of dead red spruce trees and are typically found dispersed along the trunk or possibly larger branches of the tree. Distinct color transitions occur over the life of a perithecium as they typically emerge from thin stroma tissues as smooth, shiny yellowish-orange globose bodies, become bright red with a

distinct dark-red to black papillate ostiole as they mature, then fade to a dark reddish-purple (Figure 5B). Asci are cylindrical and bear eight ascospores (Figure 5C). Hyaline ascospores (12.0 - 18.8 x 4.6 - 9.8 μm (avg. 15.2 x 7.5 μm)) are uniseptate with tapered-ends, constricted at the septum, and warty on the surface (Figure D-E). *C. gaudineerii* cultures have cream-to-buff colored surface with a yellow-to-orange subsurface after 10 d on PDA (Figure F-G). Microconidial sporodochia are abundantly produced in culture, typically underneath aerial conidiophores (Figure 5H). Sporodochia are slimy masses with a cream-to-white color. Microconidia (6.4 - 15.7 x 3.3 - 7.2 μm (avg. 9.3 x 4.9 μm)) are hyaline, fusiform, and aseptate (Figure I-K). As with previously described *Corinectria* spp., *C. gaudineerii* does not appear to produce macroconidia in culture, although only GYE and PDA media were tested here.

Figure 5: *Corinectria gaudineerii*: A) Example of abundant fruiting on *Picea rubens*; B) *C. gaudineerii* perithecia erupting from *P. rubens* bark tissues; C) Ascus containing eight ascospores; D) Ascospore; E) Ornamented surface of ascospore; F) Top of 10-day old culture photos on PDA; G) Bottom of culture; H) Microconidial sporodochia underneath aerial conidiophores; I) Microconidia of varying lengths; J) Close-up of microconidia; Scale bars = 250 µm for panel D; 20 µm for panels E, K; 10 µm for panels F, G, J; 20 mm for panel H.

Taxonomy

Corinectria gaudineerii C. Stauder & M. Kasson **sp. nov.**

Etymology: Inspired by the first location *C. gaudineerii* was observed: Gaudineer Mountain, Monongahela National Forest, Randolph County, West Virginia, USA.

Diagnosis: Differs from *C. tsugae* by forming perithecia; from *C. fuckeliana* by forming ascospores with a constricted septum; from *C. constricta* by producing overlapping ascospores in asci.

Sexual morph: Perithecia formed singly or in clusters of up to 25 or more on thin stromata erumpent through bark tissue of dead red spruce, typically found dispersed along the trunk thus potentially on larger branches too, first yellowish-orange, then bright red, dark reddish-purple when past maturity, globose, forming a papillate, dark red-to-black ostiole, not collapsing with dry. Asci cylindrical bearing eight overlapping ascospores. Ascospores (12.0 - 18.8 x 4.6 - 9.8 μm (avg. 15.2 x 7.5 μm)) uniseptate with tapered-ends, slightly constricted at the septum, hyaline, and warty on the surface.

Asexual morph: Colonies on PDA or GYE cream-to-buff with a yellow-to-orange subsurface after 10 d at room temperature (~20 °C). Microcondial sporodochia and aerial conidiophores abundant in culture on PDA and GYE media. Microconidia (6.4 - 15.7 x 3.3 - 7.2 μm (avg. 9.3 x 4.9 μm) hyaline, fusiform, and aseptate. Macroconidia not observed in culture or nature.

Geographic distribution: UNITED STATES: central Appalachian Mountains.

Habitat: On bark of dead *Picea rubens* (red spruce) as saprophyte or possibly, hemibiotroph

Type: **United States**, West Virginia, Gaudineer Knob (38.62928, -79.8429), on dead bark tissue of *Picea rubens* (red spruce), 28 Dec 2019. M. T. Kasson.

Additional specimens examined: All on bark on dead *Picea rubens*. **United States**, Randolph County, Gaudineer Knob, Monongahela National Forest (38.60741, -79.84431), 29 Dec 2018, M. T. Kasson, (GK1, strain CgPr004, strain CgPr007, strain CgPr009). Virginia, Grayson County, Whitetop Mountain, Jefferson National Forest (36.633437, -81.60132), 18 Apr 2019, C. M. Stauder, (WTPIRU4, strain CgPr002). Virginia, Grayson County, Mount Rogers, Jefferson National Forest (36.657625, -81.520676), 19 Apr 2019, C. M. Stauder (MR12, strain CgPr008). West Virginia, Pocahontas County, Cranberry Wilderness, Monongahela National Forest (38.301702, -80.230349), 20 Apr 2019, C. M. Stauder, (CRAN14, strain CgPr010; CRAN32, strain CgPr011).

Notes: *C. gaudineerii* has morphological features similar to those previously described for *C. fuckeliana*, *C. constricta*, and *C. tsugae*. *Cg* asci contain overlapping, constricted ascospores which can serve to distinguish it from *C. fuckeliana* and *C. tsugae*.

Geographic distribution and host range also may be informative for rudimentary identification. Additionally, internal transcribed spacer region (ITS), translation elongation factor 1-alpha (EF1), 28S rDNA (LSU), and actin (ACT) have informative nucleotide substitutions capable of distinguishing this species from *C. fuckeliana*, *C. constricta*, and *C. tsugae*.

Discussion

Red spruce experienced several decades of decline, since at least the 1960s (Cook and Zedaker, 1992). Although this decline was eventually associated with anthropogenic activities resulting in acid rain deposition, plant pests and pathogens were initially considered as a major contributor if not the sole cause of this decline. Extensive surveys of declining red spruce stands provided evidence supporting the contribution of these associated pests and pathogens, but not as main causal agents of the decline. In Chapter 2, a novel *Corinectria* sp. was reported on dead red spruce in central Appalachia, and motivated the investigations included in this study. In the extensive surveys previously conducted in declining red spruce stands a number of fungi associated with dead red spruce were included. Interestingly, none reported the occurrence of any nectriaceous fungi. Therefore, we believe this is the first report of a nectriaceous fungus associated with red spruce in the central Appalachian Mountains.

In this study, we described *Corinectria gaudineerii* sp. nov. C. Stauder & M. Kasson through the use of phylogenetic and morphological comparisons among previously described *Corinectria* spp. Among these data, phylogenetic analyses using concatenated sequence data of the conserved loci BTUB, EF1, ITS, ACT, and LSU support the description of *Cg* as a new species (Figure 2A). Additionally, this was supported by nucleotide substitutions in ACT, EF1, ITS, and LSU sequences (Figures 2B,D-F), but BTUB sequences were uninformative as these data did not support the monophyly of any of the previously described *Corinectria* species (Figure 2C). Together, these analyses provide clear evidence that isolates recovered from red spruce in central Appalachia represent a novel species considerably divergent from previously described

Corinectria sp. As such, *Cg* represents the first *Corinectria* species to be described associated with red spruce in eastern North American forests.

The *Corinectria* genus was circumscribed as a distinct genus sister to *Neonectria* (González and Chaverri, 2017). A number of characters can be found to distinguish members of *Corinectria* from *Neonectria* (González and Chaverri, 2017). The most obvious of these involves the ecology of these fungi as *Corinectria* members have only been described from coniferous tree species while nearly all *Neonectria* host species are solely associated with hardwood tree species. Generally, members of both genera are considered weak pathogens or saprophytes associated with woody tree species. Here, we provide evidence using a live-stem inoculation trial that supports *Cg* to be weakly pathogenic on red spruce (Figure 3). This conclusion is made based on the presence of expanded necrosis associated with inoculation sites when compared to the negative control in which no necrotic tissue was observed. The excised stem assay demonstrated the saprophytic capacity of *Cg* on red spruce (Figure 4). Additionally, *Cg* was only found on dead red spruce trees with no evidence of symptomology characteristic of canker diseases (e.g. swollen canker margins due to compartmentalization attempts). As such, *Cg* would be best described as a hemibiotrophic fungus of red spruce.

In a publication by González and Chaverri (2017), the morphological characters capable of distinguishing among *C. constricta*, *C. fuckeliana*, and *C. tsugae* are provided. The morphology of these species was not directly compared to *Cg* isolates as part of this study, but a few diagnostic differences are highlighted here. First, perithecia of *C. tsugae* have not been observed (Williams, 1987, González and Chaverri, 2017)

while the sexual state of *Cg* is dominant on red spruce. In asci of *C. fuckeliana*, ascospores overlap and are unconstricted at the septum, while ascospores in asci of *C. constricta* are constricted and maintain an end-to-end arrangement. Here, *Cg* asci appear to contain overlapping, constricted ascospores. Therefore, ascospore arrangement and morphology may serve as a diagnostically informative feature in addition to its apparent ecology and geographic distribution. It is worth noting that the latter represents findings from a limited survey in central Appalachia, and while *Cg* was not observed in more northern red spruce forests, the true geographic distribution and host range remains unclear.

Together these results provide evidence supporting *Corinectria gaudineerii* as a novel hemibiotrophic species on red spruce of central Appalachia. Phylogenetic, morphological, and ecological characteristics capable of discerning this species from previously described *Corinectria* spp. are also provided. While its pathogenicity on red spruce was not apparent, the fact that this species was not observed in historic surveys of declining red spruce stands is interesting. These surveyed areas may or may not overlap with those included in this study, and as such, expanded surveys are needed to determine the geographic extent of *Cg*. This is vital to determine the geographic distribution of *Cg* within and outside of central Appalachia. Otherwise, the geographic distribution of this species may be greater than that observed in this study. As such, further investigations are warranted to further describe the biology and ecology of *C. gaudineerii* in red spruce forests of eastern North America.

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Supplemental Tables

Supplemental Table 1: Locations of survey sites. The presence of *Corinectria gaudineerii* is noted for each site. Additionally, the general coordinates of the sampling locations are provided. Samples were only collected within permitted areas.

Site	Location	BBD Present	Coordinates
MR	Grayson County, VA	Y	(36.657625 , -81.520676)
WT	Grayson County, VA	Y	(36.633437 , -81.60132)
GK	Randolph County, WV	Y	(38.60741 , -79.84431)
CR	Pocahontas County, WV	Y	(38.301702, -80.230349)
DS	Tucker County, WV	N	(39.015311, -79.343767)
CS	Preston County WV	N	(39.537414, -79.482550)
SK	Pendleton County, WV	N	(38.699077 , -79.533215)

Supplemental Table 2: Primer sequences, PCR protocols, and sources for each primer pair used to generate sequence data within this study.

Target Locus	Primer	Primer Sequence	Initial Denature		Cycle s	Denature		Anneal	Extension		Final Extension		Source	
ITS	ITS5	GGAAGTAAAAGTCGTAACAAG G	95C	2:0 0	35	95 C	0:3 0	56C	0:3 0	72 C	1:0 0	72C	7:0 0	White et al., 1990
	ITS4	TCCTCCGCTTATTGATATGC												
tef-1α	EF1-728	CATCGAGAAGTTCGAGAAGG	95C	2:0 0	35	95 C	0:3 0	56C	0:3 0	72 C	1:0 0	72C	7:0 0	Carbone and Kohn, 1999
	EF1-1576	ACHGTRCCRATACCACCRATC TT												Rehner 2001
nrLSU	LR5	ATCCTGAGGGAAACTTC	95C	2:0 0	35	95 C	0:3 0	51.1 C	0:4 5	72 C	1:3 0	72C	7:0 0	Vilgalys and Hester, 1990
	LROR	GTACCCGCTGAACTTAAGC												Rehner and Samuels, 1994
ACTIN	Tact1	TGGCACCACACCTTCTACAAT GA	95C	2:0 0	35	95 C	0:3 0	55C	0:4 5	72 C	1:0 0	72C	7:0 0	Samuels et al., 2006
	Tact2_1	TCTCCTTCTGCATACGGTCGG A												
Beta-tublin	T1	AACATGCGTGAGATTGTAAGT	95C	2:0 0	35	95 C	0:3 0	64C	0:3 0	72 C	1:0 0	72C	7:0 0	O'Donnell and Cigel'nik, 1997
	T2	TAGTGACCCTTGGCCCAGTT G-												