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

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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 comfirmed 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 our limited sampling and, as such, thallism cannot help explain the inevitable dominance of *N. faginata* in the BBD pathosystem.

Keywords: mating type, heterothallic, *Nectriaceae***;** beech bark disease, Nectria canker, *Nectria galligena, Corinectria fuckeliana,* genome

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 interdependent 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 a 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

106 solely on culture-based observations via *in vitro* mating assays. No molecular
107 characterization of the MAT locus has been completed for any member of *Neonectria*
108 despite the publicly available genomes of several species in the genus (Salgado-
109 Salazar and Crouch 2019; Gómez-Cortecero et al. 2015; Deng et al. 2015).

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

119 Characterizing thallism can inform our understanding of disease cycle dynamics
120 including propagule dissemination and mode of infection. One study investigating the
121 production and dissemination of spores by *N. ditissima* infecting yellow birch (*Betula*
122 *alleghaniensis* Britton) determined ascospores to be the dominant spore type in the
123 environment throughout the year (Lortie and Kuntz 1963). Additionally, ascospores of a
124 related species *Corinectria fuckeliana* (C. Booth) C. González & P. Chaverri (formerly
125 *Neonectria fuckeliana*) have also been shown to be dominant relative to asexual conidia
126 when infecting *Pinus radiata* D. Don in New Zealand (Crane et al. 2009). Together,
127 these results indicate the progression of diseases caused by *Neonectria* and *Corinectria*
128 fungi may depend on ascospore production and dissemination given the proposed

129 limited dissemination of asexual conidia by these fungi (Lortie and Kuntz 1963; Crane et
130 al. 2009).

131 In addition to the potential importance of mating strategy on disease
132 epidemiology, fungal population density assessments that depend on fruiting structure
133 detection can be influenced by the relative rates of reproduction. Rates of visual
134 detection are also likely influenced by fruiting structure type, where bright red perithecia
135 are far easier to see and positively identify than the small, whitish conidiophores and
136 sporodochia. Nearly all BBD studies that investigated interactions among these two
137 *Neonectria* fungi have depended on the sampling and processing of perithecia to
138 measure ascospores directly or culture the associated fungi for identification (Houston
139 1994; Kasson and Livingston 2009). These studies have indicated *N. faginata,* over
140 time, supplants *N. ditissima* as the dominant pathogen in the BBD pathosystem. As with
141 differences in optimal abiotic conditions (temperature, relative humidity) or seasonality
142 of fruiting, differences in thallism – if they exist – could likewise influence the frequency
143 of perithecia production and therefore, detection rates. Thus, determining thallism for *N.*
144 *ditissima, N. faginata* and *N. coccinea* could enhance our ability to interpret patterns of
145 relative abundance in the BBD system.

146 The objectives of this study were as follows: 1) Determine thallism among
147 members of *Neonectria* with emphasis on BBD-associated fungi: *N. ditissima*, *N.*
148 *faginata,* and *N. coccinea*. This is important as studies to determine the mating
149 strategies of *Neonectria* fungi are limited. Furthermore, genomic data for many
150 *Neonectria* spp. are lacking, and therefore, more general primers would be useful to
151 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) was also used in this study.

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

204

205 *Characterizing the structure of MAT loci*

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

215

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

217 Once we had identified the MAT locus in each genome, we designed forward and
218 reverse MAT1-1-1 and MAT1-2-1 specific primers for rapid characterization of mating
219 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 (Intergrated 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 H_2O, 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.

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: QGQA00000000.1), and *N. hederae* genomes ([C. Booth] Castl. & Rossman) (MAT1-2-1; GenBank accession: QGQB00000000.1). 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 amplifying 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 μl SYBR gold (Invitrogen, Grand Island, NY, USA) and 4 μl 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) were also 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 purified 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.

289 To confirm the specificity of species-specific primer sets, mating type PCR
290 reactions were performed using *N. ditissima* MAT primers for *N. faginata* isolates and
291 vice versa. Additionally, a number of other members of the Nectriaceae were also
292 tested using both *N. ditissima* and *N. faginata* MAT primers as described above. These
293 included isolates of *N. coccinea, N. neomacrospora* ([C. Booth & Samuels] Mantiri &
294 Samuels)*, Fusarium concolor* (Reinking, 1934)*, Nectria magnoliae* (M.L. Lohman &
295 Hepting, 1943) and an additional unresolved species for which additional data is needed
296 to confirm identity: *Corinectria* aff. *fuckeliana* (99.37% EF1-α sequence similarity to
297 NCBI Genbank accession MK911707.1) (Table 1). Genus-level *Neonectria* MAT primer
298 sets were similarly tested using representatives from all five Nectriaceae species tested
299 in this study.
300 All resulting sequences were aligned as described above and compared to
301 genome-derived MAT1-1-1 or MAT1-2-1 sequences to confirm their identity based on a
302 sequence similarity. All sequences having greater than 70% sequence similarity were
303 selected for further analysis. This threshold was determined by comparing MAT1-1-1
304 and MAT1-2-1 genome-derived sequences from *N. ditissima* to the more distantly
305 related *Fusarium anguioides* (MAT1-1-1; Genbank Accession: MH742713) and
306 *Fusarium tucumaniae* (MAT1-2-1; Genbank Accession: KF706656) sequences.
307

308 *Phylogenetic analysis and protein alignment*

309 To examine evolutionary patterns and divergence in mating type genes, we
310 constructed phylogenetic trees using the mating type gene sequence data produced in
311 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 the media along the center point of the petri dish (Figure 2A). 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 2B).

As perithecia were produced, squash mounts were used to check for ascospores using light microscopy (Figure 2C). Additionally, ascospores were checked for viability by removing a single perithecium, macerating in 1 ml of sterile dH_2O, 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 *Neonectria ditissima* (MAT1-1: NdBl001; 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 *Neonectria ditissima* isolates were paired three times with the *N. faginata* isolate of the opposite mating type. Plates were weekly checked 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 1). The MAT1-1-1 ORF 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 ORF for *N. faginata* was 1204 bp (371 aa; GenBank accession XXXXX) with two introns of 46 bp and 44 bp. The MAT1-2-1 ORF for *N. ditissima* was 827 bp (243 aa; GenBank accession XXXXX) with two introns of 48 bp and 49 bp, while the ORF 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 ORF 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 1;

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.

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 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 of *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.

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 confirmed a putative 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 concolor* isolate. Non-target amplification of a hypothetical protein (~590 bp > target) was observed when

425 the NeoM2f/r primer pair was applied to MAT1-1 *N. coccinea* and both MAT1-1 and
426 MAT1-2 *N. neomacrospora* isolates.

427

428 *Phylogenetic analyses and protein alignments*

429 Phylogenetic analyses of MAT1-1-1 and MAT1-2-1 sequences resulted in similar
430 tree topologies (Figures 3 and 4). Analysis of MAT1-1-1 grouped all *Neonectria* species
431 into a strongly supported monophyletic clade sister to *Corinectria* (formerly *Neonectria*)
432 (González and Chaverri 2017) (Figure 3). Within *Neonectria*, a clade containing *N.*
433 *coccinea, N. faginata* and *N. punicea* was resolved as sister to *Nectria magnoliae* from
434 *Liriodendron tulipifera* and *Magnolia fraseri*. For MAT1-2-1, all included *Neonectria*
435 species resolved to a monophyletic clade that was sister to a clade containing
436 *Corinectria* aff. *fuckeliana* and the three species of *Fusarium* (Figure 4). Additionally,
437 two sister clades within *Neonectria* were resolved: 1) *N. faginata* was sister to a clade
438 containing *N. coccinea* and *Nectria magnoliae*, and 2) *Neonectria ditissima* and *N.*
439 *neomacrospora* formed a monophyletic clade sister to *N. hederae*. All isolates of *N.*
440 *ditissima* formed a single lineage regardless of plant host for both MAT1-1-1 and MAT1-
441 2-1.

442 Protein alignments included approximately 100 amino acids for MAT1-1-1
443 sequences and approximately 75 amino acids for MAT1-2-1 sequences within the
444 conserved region of these genes (Figure 5). *Neonectria coccinea, Nectria magnoliae,*
445 *Corinectria* aff. *fuckeliana*, and *Fusarium concolor* were missing the first five MAT1-2-1
446 amino acids, and therefore, results reported for MAT1-2-1 below represent values with
447 (full) and without (partial) the first five amino acids. Protein alignments for both MAT1-1-

448 1 and MAT1-2-1 were most similar among *Neonectria* spp. with 28% and 29.3% (or
449 36% for full sequence comparisons) of amino acids conserved across all species
450 considered, respectively. Of these shared amino acids, 19% MAT1-1-1 and 25.3%
451 MAT1-2-1 amino acids (partial seqs) were shared with included *Corinectria* spp. Across
452 all included fungi, only 5% of amino acids were shared among MAT1-1-1 sequences
453 and 21.3% (or 28% for full sequence comparisons) amino acids were shared among
454 MAT1-2-1 sequences.

455

456 *In vitro pairing assay results*

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

465 Each progeny included in the subsequent PCR screening resulted in the
466 amplification of a single MAT gene. Both MAT genes were found to be segregated
467 among progeny at a ratio not significantly divergent from the expected 1:1 for *N.*
468 *ditissima* (χ^2 = 0.07, df = 1,60; *P* = 0.80) and *N. faginata* (χ^2 = 0.27, df = 1,60; *P* = 0.606)
469 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” 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.

493 Despite having resolved the mating strategy of these fungi, the potential
494 overrepresentation of either *N. ditissima* or *N. faginata* in previous studies may be due
495 to perithecia-dependent sampling bias. For example, environmental conditions (*e.g.* RH,
496 temperature) and time required for perithecia production may significantly differ between
497 these two fungi. Given potential seasonal differences in fruiting, sampling at a single
498 time point in the year could potentially yield a community not representative of the
499 relative abundance of the *Neonectria* spp. present. Additionally, casual observations of
500 *N. ditissima* on non-beech hosts can reveal very limited to no perithecia occurring at a
501 given infection site. This may be in part due to host susceptibility, resulting in more or
502 less necrotic tissue, which is generally considered favorable for perithecia production by
503 necrotrophic fungi.

504 Host specificity may develop through the evolution of mating barriers among
505 strains of a single species occurring on differing hosts. Results from this study have
506 demonstrated a lack of reproductive barriers among *N. ditissima* strains from several
507 plant hosts. All pairings of either MAT1-1-1 or MAT1-2-1 strains from different plant
508 hosts resulted in perithecia formation. While a lack of host specificity by *N. ditissima* has
509 been previously demonstrated using pathogenicity assays (Lortie 1969; Ng and Roberts
510 1974; Barnard et al. 1988; Plante and Bernier 1997), mating among *N. ditissima* strains
511 infecting co-occurring tree species had not been previously tested until now. Given the
512 lack of reproductive barriers, evolution of host specificity is limited by obligate
513 outcrossing among these co-occurring strains.

514 The phylogenetic analyses using MAT gene sequences were found to be in
515 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, limiting the possibility the development of host specificity within *N. ditissima* populations co-occurring across multiple hosts. 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 product size. 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.

Conclusion

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 understand the fungal dynamics of *N. ditissima, N. faginata,* and *N. coccinea* in their respective BBD systems. These efforts include: 1)

539 identifying differing environmental factors required for perithecia production among
540 *Neonectria* spp.; 2) characterizing variability in sexual reproduction by *N. ditissima*
541 across host substrates; 3) assessing the potential for interspecific hybridization between
542 closely related *Neonectria* spp. found co-occurring on beech and other hosts but
543 excluded from this study; and 4) comprehensive screening of additional isolates from
544 populations not sampled in this study to assess intraspecies MAT gene diversity and
545 uncover possible intraspecies mating barriers.

546

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558

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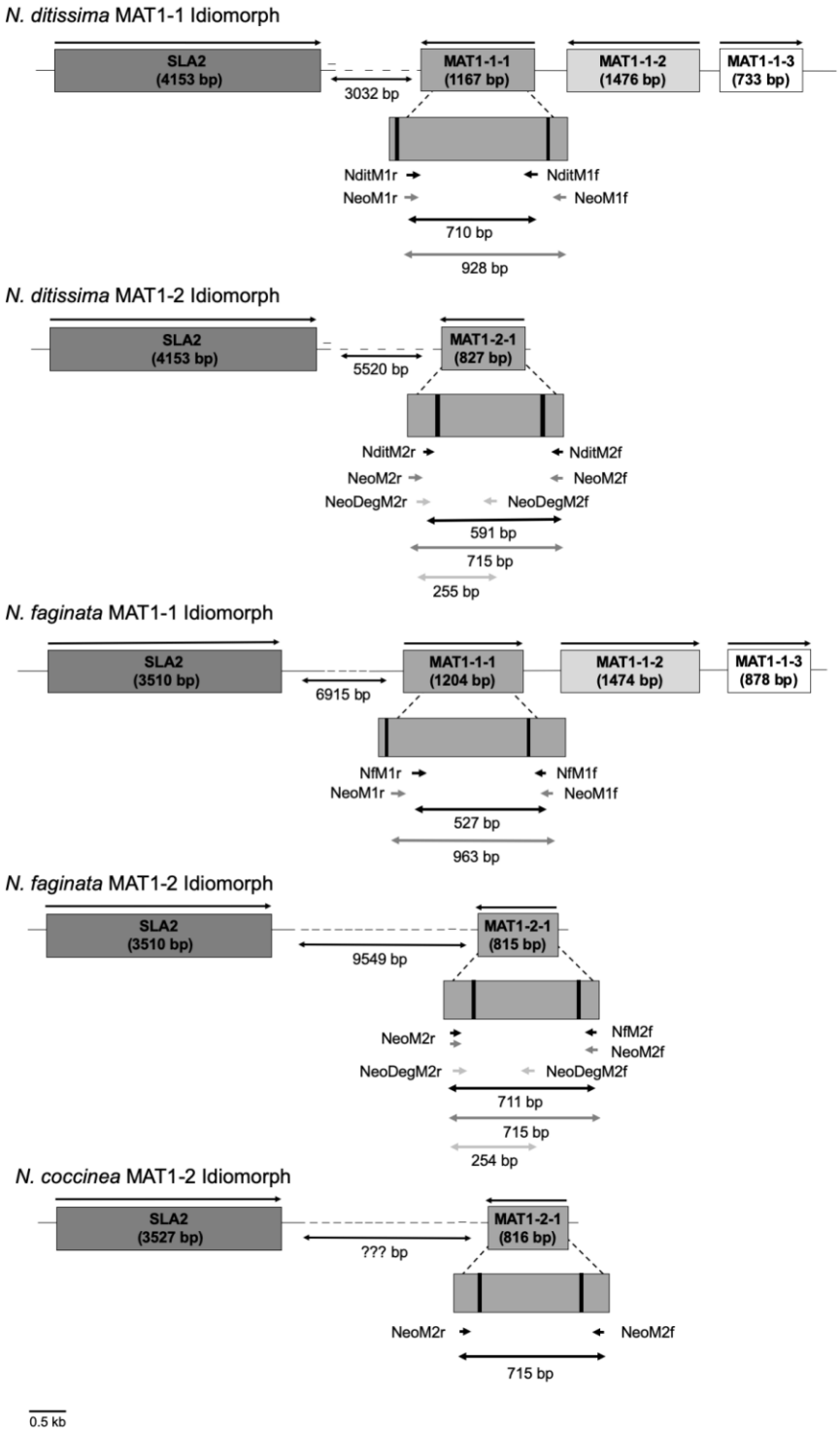


724

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

726 *ditissima*, *Neonectria faginata,* and *N. coccinea* (MAT1-2 only). Arrows above the genes

727 indicate the 5’ – 3’ orientation. Coding sequence lengths are included below each gene
728 identifier. For MAT1-1-1 and MAT1-2-1 genes, introns are represented by vertical black
729 lines, and approximate primer binding locations are illustrated for each primer pair
730 below the gene illustration. The approximate amplicon size for each primer pair is
731 shown below the primer binding locations and is shaded based on each primer pair. All
732 distances and sizes are estimations and not drawn to scale.
733

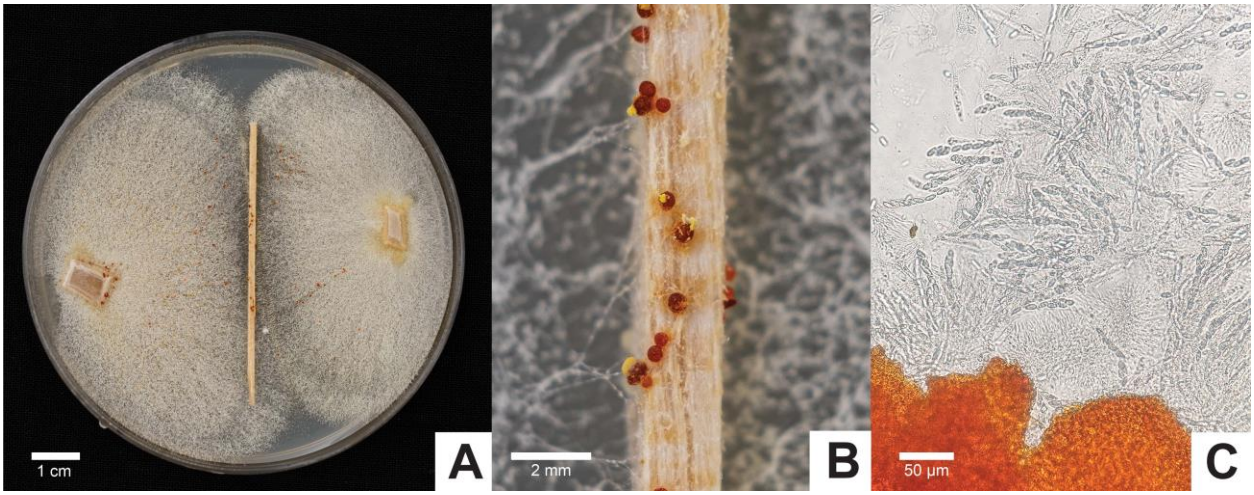


Figure 2: *In vitro* mating assay for *Neonectria* spp. and related fungi: A) Approximately 5mm x 5mm colonized plugs of confirmed MAT1-1 and/or MAT1-2 strains placed on either side of a sterile wooden toothpick placed upon GYE media; B) Perithecia exuding ascospores on toothpick after approximately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

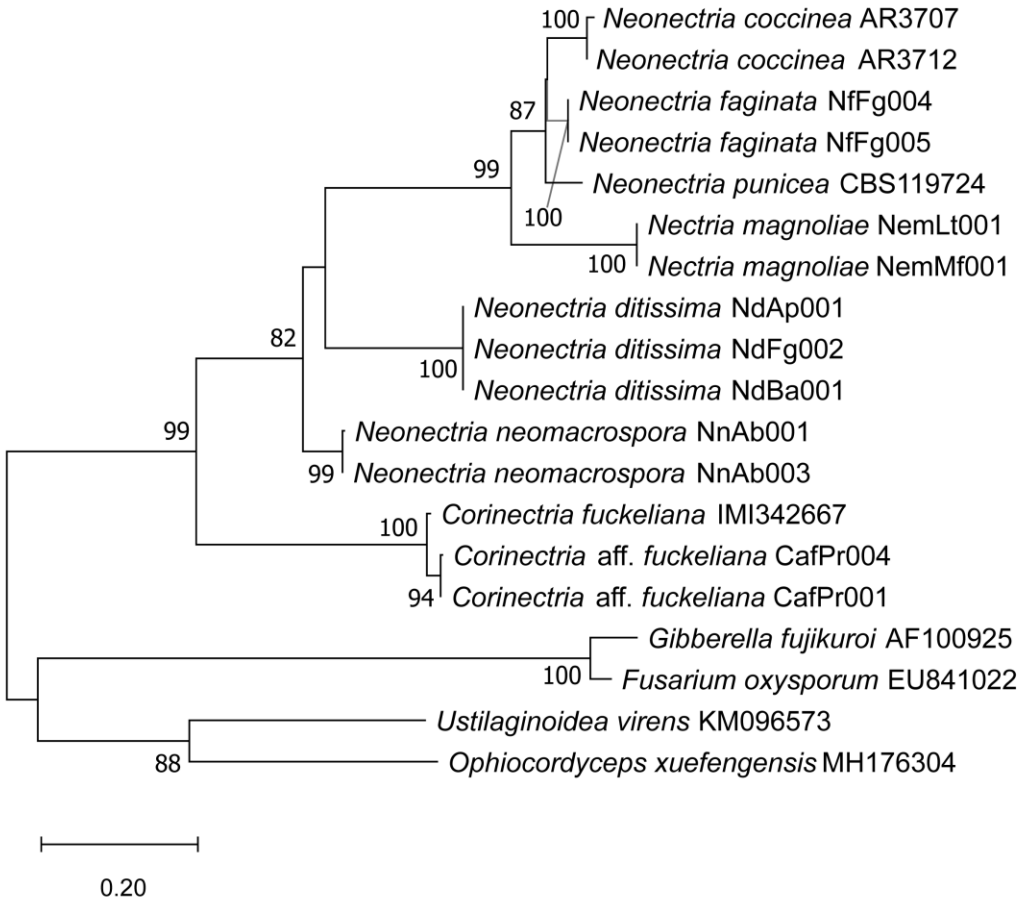


Figure 3: Phylogenetic relationships of *Neonectria* spp. and related fungi based on MAT1-1-1 gene sequence data. The phylogeny was inferred using a Maximum Likelihood analysis based on the Kimura 2-parameter model with gamma distribution (K2+G) and 1000 bootstrap replicates. Bootstrap values > 70% are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

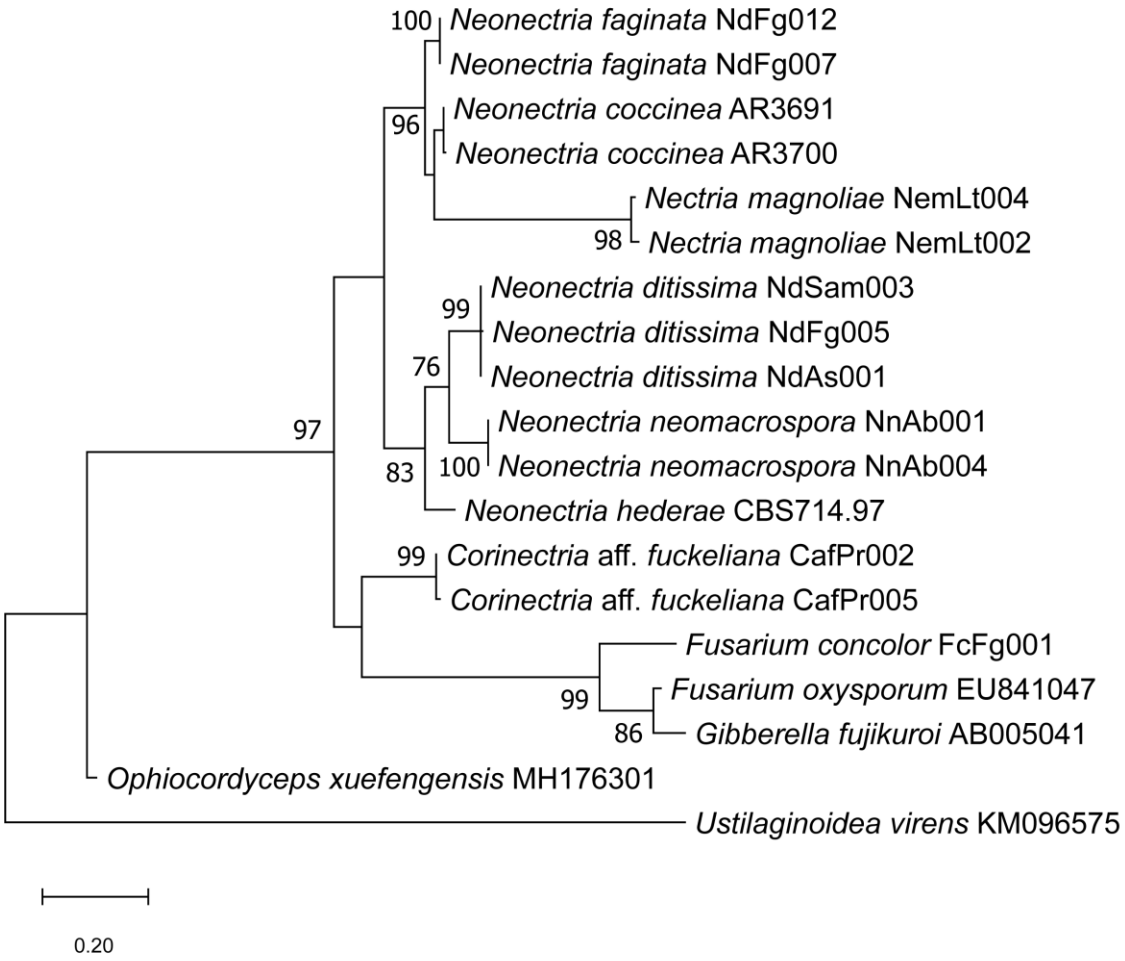


Figure 4: Phylogenetic relationships of *Neonectria* spp. and related fungi based on MAT1-2-1 gene sequence data. The phylogeny was inferred using a Maximum Likelihood analysis based on the Kimura 2-parameter model with gamma distribution (K2+G) and 1000 bootstrap replicates. Bootstrap values > 70% are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

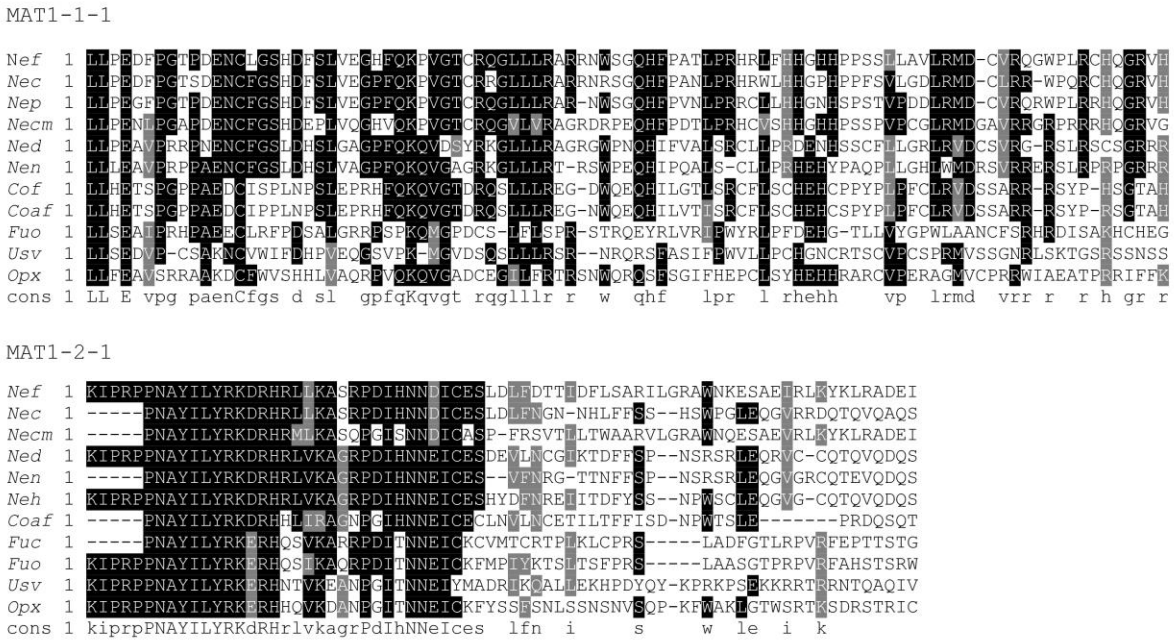
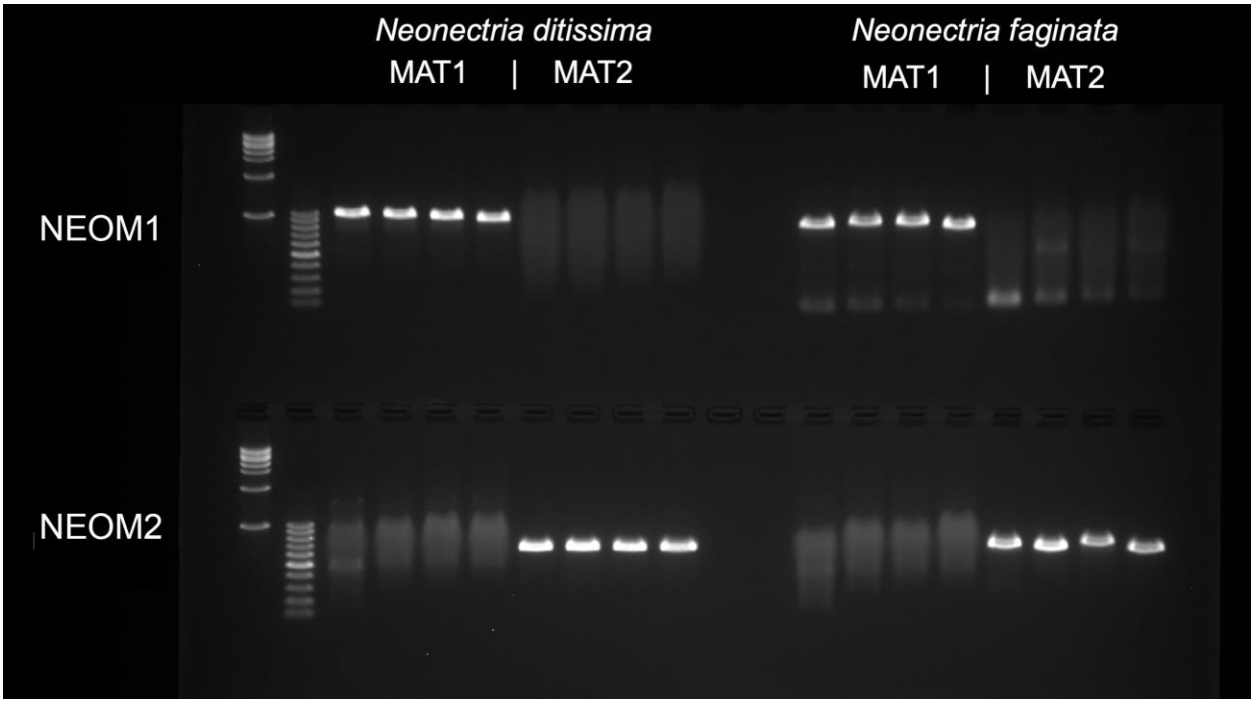


Figure 5: MAT1-1-1 and MAT1-2-1 amino acid alignments including a selection of study fungal species. Name abbreviations are as follows: *Nef* = *Neonectria faginata*; *Nec* = *Neonectria coccinea*; *Nep* = *Neonectria punciea*; *Necm* = *Nectria magnoliae*; *Ned* = *Neonectria ditissima*; *Nen* = *Neonectria neomacrospora; Neh* = *Neonectria hederae*; *Cof* = *Corinectria fuckeliana*; *Coaf* = *Corinectria* aff. *fuckeliana*; *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.



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*.