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Presence and distribution of insect-associated and entomopathogenic fungi in a temperate pine forest soil: An integrated approach

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ABSTRACT

For decades entomopathogenic fungi have garnered interest as possible alternatives to chemical pesticides. However, their ecology outside of agroecosystems demands further study. We assessed the diversity and abundance of entomopathogenic and insect-associated fungi at a loblolly pine forest in North Carolina, USA using culture-dependent and next-generation sequencing libraries. Fungi were isolated using *Galleria mellonella* larvae, as well as from soil dilutions plated on a selective medium. Isolates were identified using Sanger sequencing of the ITS and LSU rRNA gene regions, and represented 36 OTUs including *Metarhizium*, *Lecanicillium*, and *Paecilomyces*. Additionally, we assessed the chitinolytic potential of isolates and found widespread, variable ability to degrade chitin within and between genera. Phylogenetic analyses resolved several isolates to genus, with some forming clades with other insect-associated taxa, as well as with fungi associated with plant tissues. Saprophytes were widely distributed in soil, while entomopathogens were less abundant and present primarily in the top two cm of the soil. The similarity between culture-dependent and next-generation sequencing results demonstrates that both methods can be used concurrently in this system to study the ecology of entomopathogenic fungi.

1. Introduction

Since the advent of agriculture, insect pests have negatively impacted crop yields. Chemical pesticides have helped facilitate the scale of modern agriculture by offering protection from insect-related crop losses in the short term, but an increasing prevalence in insecticide resistance poses threats to a diverse number of crops (Huseth et al., 2018). Unsettlingly, insecticide resistance has also been linked to environmental and public health crises, such as an increase in resistance in vectors of insect-borne illnesses (Reid and McKenzie, 2016). These economic and environmental concerns have prompted research on the use of biopesticides as alternatives to conventional chemical pesticides, with entomopathogenic fungi garnering particular interest in recent years (Vega et al., 2009, 2018).

Historically, research on entomopathogenic fungi has focused on a handful of species in agricultural soils, with less emphasis on the ecology and overall life histories of these fungi in natural systems. However, the recent observation of entomopathogenic taxa as endophytes in tissues of numerous plants has suggested possible mutualistic interactions between these fungi and plant hosts (e.g., entomopathogens protecting plants from insect grazers, either directly or via the activation of immune responses in plant tissues) (Vega et al., 2008, 2018).

Studies using insect-baiting and selective media techniques for fungal isolation have revealed some trends in the relative abundances of common entomopathogens in natural soils. For example, in natural soils, *Beauveria bassiana* appears to be more common than *Metarhizium anisopliae* (Vänninen, 1996; Bidochka et al., 1998). In another study by Sun and Liu (2008), the less-studied species *Paecilomyces farinosus* was the most commonly-isolated entomopathogen in natural soils.

While these advances offer insight into some aspects of entomopathogens' evolution and their ecology outside of agricultural ecosystems, research on their distribution in natural soils is still lacking. Like all soil-dwelling fungi, the abundance and distribution of these taxa are affected by soil physical factors. However, recent studies have shown that genetically distinct populations of

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entomopathogens respond differentially to variables such as temperature and UV radiation, and that the techniques used to isolate entomopathogens can result in the over- and underestimation of the recoveries of certain taxa (Bidochka et al., 2001, 2002; Sharma et al., 2018). As such, we believe that the use of culture-independent surveying methods along with traditional approaches could offer a more accurate picture of entomopathogenic fungal diversity and distribution.

We chose to assess the abundance and distribution of entomopathogenic fungi in a loblolly pine (*Pinus taeda*) forest soil at the Duke Forest in North Carolina, USA. This site has a long history of use in forest ecology studies (e.g., O'Brien et al., 2005; Arnold et al., 2007; Hesse et al., 2016; Weber et al., 2013), but to our knowledge ours is the first study exploring entomopathogenic fungal diversity there. We employed traditional, culture-dependent (*Galleria mellonella* baiting and selective media) approaches in tandem with next-generation sequencing (NGS) techniques to target entomopathogens across multiple soil horizons. In addition, we explored the abundance and distribution in soils of entomopathogens and other insect-associated fungi (e.g., opportunistic pathogens and saprophytes). These taxa exploit closely-related niches, and their study provides a clearer understanding of the types of interactions in which entomopathogens engage in natural systems. Lastly, we hoped to gain insight on any relationships between the metabolic capabilities of these taxa and their role in the environment. Both entomopathogenic and insect-associated fungi are implicated in the breakdown and availability of chitin, one of the most abundant carbohydrates on Earth (Duo-Chuan, 2006). As such, we assessed the ability of unique isolates to degrade chitin using a simple plate assay.

Weber et al. (2013) demonstrated that this site is largely dominated by mycorrhizal fungi in the Basidiomycota. A later study at this site by Torres-Cruz et al. (2018) employed an NGS sequence library in conjunction with culture-dependent selective media approaches to target heavy metal-tolerant fungi, demonstrating that culture-dependent methods yield results comparable to those in NGS approaches. In that study, researchers observed commonly-isolated taxa thriving under stressful conditions, and postulated that these fungi could rely on an ability to withstand harsh environmental stress as an adaptive strategy. As such, we anticipate that culture-dependent and NGS approaches will yield comparable results in both entomopathogenic and insect-associated isolates, and hypothesize that fungi known for success under diverse ecological conditions will comprise the majority of isolates.

2. Methods

2.1. Collection of soil samples

Soil samples were collected from the Duke Free-Air Carbon Dioxide Enrichment (FACE) site in Chapel Hill, NC, USA (35°58′N, 79°06′W; elevation 130 m.a.s.l.). This site is a loblolly pine (*P. taeda*) plantation established in the Blackwood Division of Duke University's Duke Forest in 1983, and its soil is primarily an acidic clay loam (McCarthy et al., 2010). From Aug 1996 to Oct. 2010, circular control test plots (30 m in diameter) were fumigated with ambient air. In 2005, two sites representing ambient conditions were chosen randomly, quartered, and treated with 11.2 g N m^{-2} nitrogen in the form of nitrate pellets (Weber et al., 2013). In Nov. 2012, ten 15 cm depth soil cores were acquired from an N-fertilized and an unfertilized quadrant within ambient CO_2 FACE plot 3; in Oct 2013, another ten 15 cm soil cores were acquired from N-fertilized and unfertilized ambient CO_2 FACE plots representing quadrants 7 and 9. Using aseptic technique, soils were partitioned into four horizons representing the forest floor, 0–2 cm (Oa horizon), 2–5 cm (upper A horizon), and 5–10 cm (lower A horizon). Soil samples were homogenized and stored in the dark at 4 °C. This study assessed soil from control plots and focused on comparison of fungal communities between horizons.

2.2. Fungal isolation and identification – 'Galleria bait method'

For each soil horizon, fifteen *G. mellonella* larvae (Speedy Worm, Alexandria, MN, USA) in their 4th or 5th larval instars were distributed between three sterile 9 × 50 mm Petri dishes (five worms per Petri dish). Each of these 12 dishes contained moist soil (enough to cover the plate, approximately 3–6 g) representing one of the four horizons, and larvae were left for one week to allow colonization. Insect tissues were removed from soils and surface-sterilized using autoclaved DI H_2O, 70 % ethanol, and 0.5 % NaClO (from Clorox, 6 %). Surface-sterilized cadavers were incubated in sterile 9 × 50 mm petri dishes containing filter disks moistened with ~0.5 mL autoclaved DI H_2O for up to two weeks until fungal reproductive structures emerged. Seventy-three isolates were removed aseptically using a needle submerged in sterile 20 % sterile glycerol (Fischer Scientific, Waltham, MA, USA) and transferred to Sabouraud's Dextrose Agar (Oxoid Ltd., Hampshire, United Kingdom) containing 50 mg/mL tetracyclin (Amresco Inc., Solon, OH, USA) and 50 mg/mL streptomycin (Calbiotech, Spring Valley, CA, USA). Isolates were sub-cultured as necessary.

2.3. Fungal isolation and identification – selective media

Soil dilutions (10^{-2} and 10^{-3}) were prepared by mixing soil from each horizon with autoclaved DI H_2O. These dilutions were spread on 24 plates of a CTC selective medium (six plates per horizon, with three plates representing 10^{-2} and 3 plates representing 10^{-3}) modified from Fernandes et al. (2010) containing 0.5 g/L chloramphenicol (Sigma–Aldrich, St. Louis, MO, USA), 0.001 g/L thiabendazole (Sigma–Aldrich, St. Louis, MO, USA), 0.25 g/L cycloheximide (Sigma–Aldrich, St. Louis, MO, USA), 50 mg/mL tetracyclin (Amresco Inc., Solon, OH, USA) and 50 mg/mL streptomycin (Calbiotech, Spring Valley, CA, USA). These reagents inhibit bacteria and fast-growing, saprophytic fungi while promoting the growth of entomopathogenic fungi. Plates were stored in the dark at 25 °C and monitored for fungal growth over a period of two weeks. A total of 32 isolates were transferred to Sabouraud's Dextrose Agar (Oxoid Ltd., Hampshire, United Kingdom) containing 50 mg/mL tetracyclin (Amresco Inc., Solon, OH, USA) and 50 mg/mL streptomycin (Calbiotech, Spring Valley, CA, USA) and subcultured as necessary.

2.4. Identification of fungal isolates

Whole genomic DNA was extracted from all fungal isolates using the Wizard DNA Extraction Kit (Promega, Madison, WI, USA). DNA was amplified using primers for the Internal Transcribed Spacer region (ITS rRNA) (White et al., 1990; Gardes and Bruns, 1993), as well as the 28S large subunit (LSU) region (Vilgalys and Hester, 1990; Schoch et al., 2012), using Promega Taq (Promega, Madison, WI, USA) according to Porras-Alfaro et al. (2011). PCR products were treated using an Exonuclease-Shrimp Alkaline Phosphatase (ExoSap) following the manufacturer's protocol (Affymetrix, Santa Clara, CA, USA). All amplified fragments were placed in sequencing reactions and precipitated using a Big Dye protocol (version 1.1, Applied Biosystems, Grand Island, NY, USA). Reactions were sent to the University of New Mexico, Albuquerque, NM and Beckman Coulter Genomics (Danvers, MA, USA) for Sanger sequencing. Sequences were edited and assembled using Sequencher version 5.3. Sequences were used to identify all fungal isolates using the basic

local alignment search tool (BLAST) algorithm (Altshul et al., 1990) and the UNITE database (Abarenkov et al., 2010), and were grouped into unique operational taxonomic units (OTUs) using a 97 % similarity cutoff. All sequences were deposited in GenBank and received accession numbers (Table S1).

2.5. Next-generation sequencing of samples

Twenty-four LSU sequences representing unique OTUs were compared with an NGS sequence dataset (156 samples) obtained at the same site in a previous study (Hesse et al., 2016) for soils representing both ambient and N-amended plots in quadrants 1, 5, and 6. Hesse et al. (2016) extracted DNA from 0.5 g of each soil sample with a FastDNA SPIN kit as per the manufacturer's protocol, which was then purified using the MoBio PowerSoil DNA Isolation kit (Carlsbad, CA) as per the manufacturer's instructions. DNAs were amplified in triplicate with primers LR3 and LR0R for the large subunit (LSU) of the ribosomal RNA gene region via the 454 GS FLX Titanium sequencing platform. Sequence quality assessment was performed as per Weber et al. (2013) as was chimera searching and any necessary sequence trimming. After aligning trimmed sequences to the SILVA global fungal LSU alignment, a distance matrix was generated and sequences were grouped into tentative OTUs using a 97 % similarity cutoff. OTUs were tentatively identified to the genus or species level via a combination of BLAST (Altshul et al., 1990), Bayesian Naïve Classification (Wang et al., 2007), and phylogenetic analyses.

LSU sequences generated from unique cultures isolated in this study were compared to the NGS amplicon dataset. All NGS results mentioned in this study refer to the amplicons generated using primers for LSU with the highest similarity to the LSU sequences generated from insect-associated and entomopathogenic cultures. Sequences were deposited in GenBank and received accession numbers (Table 2; Weber et al., 2013; Hesse et al., 2016).

2.6. Phylogenetic analyses

Multiple sequence alignments were prepared in MEGA5 (Tamura et al., 2011). Both phylogenies included sequences from closely-related taxa representing unique ecological habits; these sequences were chosen based on a minimum 97 % similarity to our sequences, as well as sequences published in previous taxonomic studies. To infer phylogenies, ITS and LSU datasets were analyzed separately in MEGA5 using Maximum Likelihood (ML) analysis. The best evolutionary model was selected using MEGA5. The dataset constructed for ITS contained sequences from this study representing members of the Hypocreales, as well as the outgroup species *Pseudohalonectria lignicola* (Rehner and Samuels, 1995) using ML with the K2+G model. The dataset constructed for LSU contained sequences from this study representing members of the Mucorales and Mortierellales, as well as the outgroup species *Batrychochytrium dendrobatidis* (Lutzoni et al., 2004) using ML with the GTR + G model. Support for branches was estimated using bootstrap analysis based on 1000 replicates. All trees were visualized and edited in MEGA5 and Adobe Illustrator CS6. Alignments were deposited in TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S25102).

Table 1
Frequency of entomopathogenic fungi between upper and lower soil horizons.

Sample	BLAST (ITS)	Next Generation Sequencing		Culture	
		0–2 cm	2–10 cm	0–2 cm	2–10 cm
REBM-027	*Lecanicillium*	92	8	100	0
REBM-022	*Paecilomyces*	88	12	100	0
REBM-056	*Metapochonia*	88	12	50	50
REBM-050	*Metarhizium*	85	15	100	0
REBM-059	*Verticillium*	95	5	100	0

2.7. Chitinolytic enzyme assay

Modified protocols from Agrawal and Kotasthane (2012) as well as Roberts and Selitrennikoff (1988) were used to conduct assays for the production of chitinolytic enzymes by fungal isolates. Colloidal chitin was prepared by digesting 20 g of powdered crab-shell chitin (Sigma Aldrich, St. Louis, MO) in 6M HCl for 48 h at 4 °C with rapid stirring. The HCl/chitin solution was filtered through glass wool into 2 L of 95 % ethanol chilled to −20 °C with rapid stirring; the neutralized solution was centrifuged at 10,000 g for 20 min, and the supernatant was discarded. Chitin pellets were rinsed with distilled water, then centrifuged at 3000 g for 5 min, and the supernatant was discarded. This rinse step was repeated until pellets no longer smelled like ethanol. The digested chitin pellets were squeezed through cheese cloth to remove excess moisture, and the colloidal chitin was homogenized and stored at 4 °C until use. The chitinase detection medium contained (1 L): 0.3 g of $MgSO_4 7H_2O$, 3.0 g of $(NH_4)_2SO_4$, 2.0 g of KH_2PO_4, 1.0 g of citric acid monohydrate, 15 g of agar, 200 μl of Tween-80, 4.5 g of colloidal chitin, and 0.15 g of bromocresol purple (pH adjusted to 4.7); the medium was autoclaved at 121 °C for 15 min. Plates were inoculated in triplicate with agar plugs containing each of the 34 fungal isolates tested, and stored in the dark at 25 °C.

In the presence of chitinolytic enzymes, the medium experiences a pH shift towards alkalinity resulting in a color change from yellow to purple (Fig. 5A). The diameter of the purple zone of clearing corresponds to the diameter of the spreading fungal tissue, and as such the strength of the chitinolytic activity of each isolate was delineated into one of four categories based on the radius of the purple zone relative to the center of the agar plug three days after inoculation: no activity, low activity (0–10 mm), medium activity (10–20 mm), and high activity (20 mm or more). The degradation visualized in the chitin assay provided indirect evidence of isolates' abilities to degrade *G. mellonella* cadavers, which helped support our characterization of those isolates as entomopathogenic or insect-associated.

3. Results

3.1. Isolation and identification of fungi

A total of 105 fungal isolates were obtained from soil samples. Isolates included entomopathogenic fungi, as well as opportunistic pathogens and saprophytes. Opportunistic pathogens were identified as fungi that have exhibited some pathogenicity as reported in the literature, but are not well-known entomopathogenic fungi; saprophytes were identified as taxa that have not exhibited pathogenicity based on Sun and Liu (2008). Seventy-three isolates were obtained from *G. mellonella* cadavers, and a total of 32 isolates were obtained from dilutions plated on CTC selective medium (Table S1). While opportunistic and saprophytic fungi were isolated from both treatments, entomopathogens were only isolated with the selective media. Thirty-six OTUs comprising 20 genera were identified using the ITS rRNA gene region. These isolates were primarily in the Ascomycota and Mucoromycota, with each representing 62 % and 35 % of total isolates, respectively; only three isolates (all three were identified as the genus *Vanrija*) were in the Basidiomycota. Isolates represented six orders including Hypocreales, Mucorales, Mortierellales, Eurotiales, Sordariales, and Tremellales, with a majority of isolates within Hypocreales and Mucorales (Fig. 1A).

Table 2
Mean sequence abundance detected using next generation sequencing and frequency in soil samples.

a. Fungal isolates

OTU	Code	Accession Number	Mean ± StDev	Frequency
Trichoderma virens	REB-010C	MK164237	115 ± 328	99
Umbelopsis	REBM-044	MK164262	74 ± 111	98
Penicillium	REB-005X	MK164233	27 ± 43	96
Mortierella	REB-008A	MK164234	17 ± 29	92
Ilyonectria	REB-040X	MK164250	11 ± 19	81
Lecanicillium psalliotae	REBM-027	MK164259	10 ± 18	82
Metapochonia bulbillosa	REBM-056	MK164263	10 ± 12	94
Clonostachys rosea	REB-020X	MK164241	8 ± 24	49
Verticillium	REBM-059	MK164264	2 ± 5	35
Gongronella	REBM-036	MK164261	1 ± 4	27
Paecilomyces	REBM-022	MK164257	1 ± 3	26
UF-Hypocreales	REBM-060	MK164265	1 ± 3	17
Mortierella globulifera	REB-010B	MK164236	1 ± 2	19
Mucor moelleri	REB-031X	MK164244	<1	5
Fusarium	REB-001A	MK164231	<1	4
Vanrija	REBM-024	MK164258	<1	5
Phialemoniopsis pluriloculosa	REB-040B	MK164249	<1	5
Absidia	REB-022A	MK164242	<1	5
Mucor abundans	REB-057A	MK164256	<1	3
Mortierella	REB-048A	MK164253	0	0
Mucor genevensis	REB-039X	MK164248	0	0
Backusella circina	REB-033A	MK164245	0	0

b. Other entomopathogens not isolated in pure culture

OTU	Accession Numbers	Mean ± StDev	Frequency
Beauveria bassiana	KF747135, KF747161	3 ± 9	53
Ophiocordyceps	JX968033, KJ130992	<1	4
Metarhizium anisopliae	GU979951, DQ067301	<1	1

Entomopathogens comprised 9 % of total isolates (9 of 105). Fungi in this group belonged to the genera *Metarhizium* (4 %), *Lecanicillium* (3 %), *Metapochonia* (6 %), *Verticillium* (1 %), and *Paecilomyces* (1 %), and were isolated exclusively from the topmost (forest floor) soil horizon, with the exception of a single *Metarhizium carneum* isolated from the Oa horizon (Table 1). Notably, the only cultures isolated from all four horizons were in the genus *Metapochonia*. Fungi traditionally characterized as opportunistic pathogens were the second most abundant group isolated in this study, comprising 42 % of total isolates. The most abundant taxa in this group belonged to the genus *Mucor* (26 %), followed by *Mortierella* (9 %), *Clonostachys* (5 %), *Absidia* (4 %), and *Fusarium* (4 %). Of the samples represented by *Mucor*, seventeen were isolated from the forest floor, two were isolated from the Oa horizon, and seven were isolated from the upper A horizon; none were isolated from the lower A horizon. *Mortierella* was isolated from the top two horizons, *Clonostachys* was isolated from the bottom two horizons, and *Absidia* was isolated from the top three horizons. All four *Fusarium* isolates were isolated only from the Oa soil horizon (Fig. 1B).

Secondary colonizers were the most abundant fungi isolated in this study, representing 49 % of total isolates (52 of 105). The majority of these fungi were represented by the genus *Trichoderma* (24 %). Isolates also included the genera *Umbelopsis* (4 %), *Vanrija* (3 %), and *Ilyonectria* (2 %). *Trichoderma* and *Umbelopsis* were both isolated from three horizons. Both *Ilyonectria* samples were only found in the upper A horizon. Two secondary colonizers were isolated only once, and represented the genera *Backusella* and *Gongronella*; together, these taxa represent less than 2 % of total isolates (2 of 105) (Fig. 1B).

3.2. Phylogenetic analyses

Maximum likelihood analyses of the aligned ITS sequence dataset showed similar tree topology as described in previous studies using the same marker for Hypocreales (Rehner and Samuels, 1995; Zhang et al., 2006). Maximum likelihood analysis of the aligned LSU sequence dataset also showed similar topology to previous studies (O'Donnell et al., 2001; White et al., 2006; Wagner et al., 2013). Analyses of the ITS dataset placed the sixteen sequences representing samples from this study in twelve distinct clades (Fig. 2). Samples REB-002A and REB-061A formed a clade with sequences representing the *F. fujikuroi* species complex, sequences deposited as *Gibberella* spp. (the teleomorph of *Fusarium*) and an endophytic *Fusarium* isolate from *Withania somnifera* (ashwagandha Solanaceae). Isolates REB-020X and REB-008C clustered with sequences representing species in the genus *Clonostachys* as well as a sequence from a rhizome endophyte of *Gastrodia sesamoises* (Orchidaceae), also most likely representing *Clonostachys*. Isolate REBM-060 clustered with sequences for a *Mabea occidentalis* leaf endophyte (Euphorbiaceae). In addition, several isolates formed clades with sequences representing entomopathogenic genera including *Paecilomyces* and *Lecanicillium*. Other isolates formed clades with taxa representing *Verticillium*, *Trichoderma*, and uncharacterized clones from studies on soil fungi and colonizers of dead plants roots (Fig. 2).

Analyses of the LSU dataset for the orders Mucorales and Mortierellales placed the thirteen sequences representing samples from this study in eight distinct clades (Fig. 3), all comprised primarily of soil-dwelling saprobes and opportunistic pathogens. Six isolates from this study formed three well-supported clades with sequences representing the genus *Mucor*. Isolates REB-048A and REB-043A formed a clade with sequences representing *Mucor fragilis*, *Mucor circinelloides*, and *Mucor racemosus*, with low support for the species-level relationships. Similarly, a clade containing sequences representing *Mucor abundans* and *Mucor durus* was nested within a larger clade containing isolates REB-055A, REB-019A, and REB-057A. Isolate REB-031X formed a clade with sequences for *Mucor moelleri*, *Dicranophora fulva*, and an uncharacterized fungal clone from the

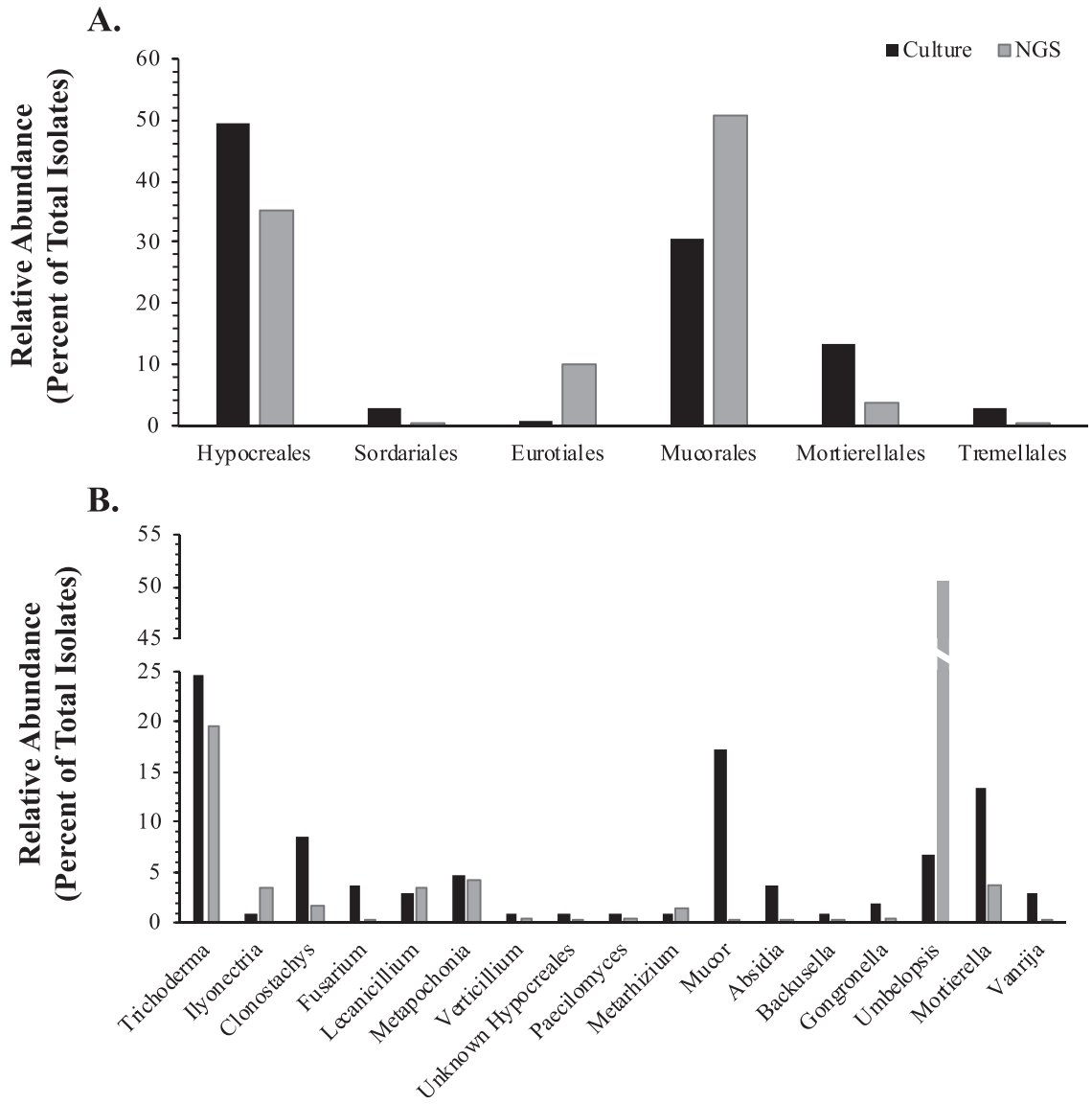

Fig. 1. Relative abundance of fungal taxa in culture collection versus next generation sequence (NGS) library based on orders (**A**) and genera (**B**).

pine site soil. Several isolates were closely related to taxa in the genus *Mortierella*. Isolate REB-010B formed a clade with sequences in the *Mortierella globulifera* group, while isolates REB-008A and REB-025A clustered with sequences representing *Mortierella verticillata* and *Mortierella minutissima* species complexes. Isolates from this study also clustered with sequences representing the genera *Backusella*, *Absidia*, and *Umbelopsis*.

3.3. Comparison of cultures with NGS dataset

A library of 83,649 sequences meeting the criteria for length and quality from a larger NGS dataset generated by Hesse et al. (2016) was used to determine the abundance of unique entomopathogenic and insect-associated taxa identified in this study (Table 2). While differences existed between the relative abundances of many taxa in culture-based versus environmental sequencing approaches, both methods showed similar overall patterns. The two most abundant orders in the NGS dataset—Hypocreales and Mucorales—were also the most abundant taxa in culture-based approaches (Fig. 1A). Furthermore, most taxa isolated in the laboratory were at least present in some number in the NGS dataset.

Of the sequences evaluated, those representing *Umbelopsis* were the most abundant in the NGS dataset with 50 % of all the sequences. The second and third most abundant OTUs were represented by *Trichoderma* and *Penicillium* with 20 % and 10 % of total sequences, respectively. Three OTUs representing cultures of *Mucor*, *Mortierella*, and *Backusella* were not present at all in the NGS dataset.

The vast majority of entomopathogenic fungi in this study were present in the topmost soil horizons, with 95 % of sequences for *Verticillium*, 92 % for *Lecanicillium*, 88 % for *Paecilomyces*, 88 % for *Metapochonia*, and 85 % for *Metarhizium* found only in the top two cm of the soil (Table 1). However, the majority of the other insect-associated fungi assessed showed very large variation across samples, illustrating the patchy distribution of fungal communities (Fig. 4).

The frequency of occurrence across sample plots also varied between taxa. Isolates representing *Trichoderma* and *Umbelopsis* were more abundant (115 ± 328 and 74 ± 111 average sequences per sample, respectively) and occurred in high frequency (99 % and 97 % of samples, respectively) in most plots. Not surprisingly, isolates occurring in low abundance in the plots were recovered infrequently in culture. However, in some cases isolates low in mean overall abundance were recovered in a large number of sample plots. For

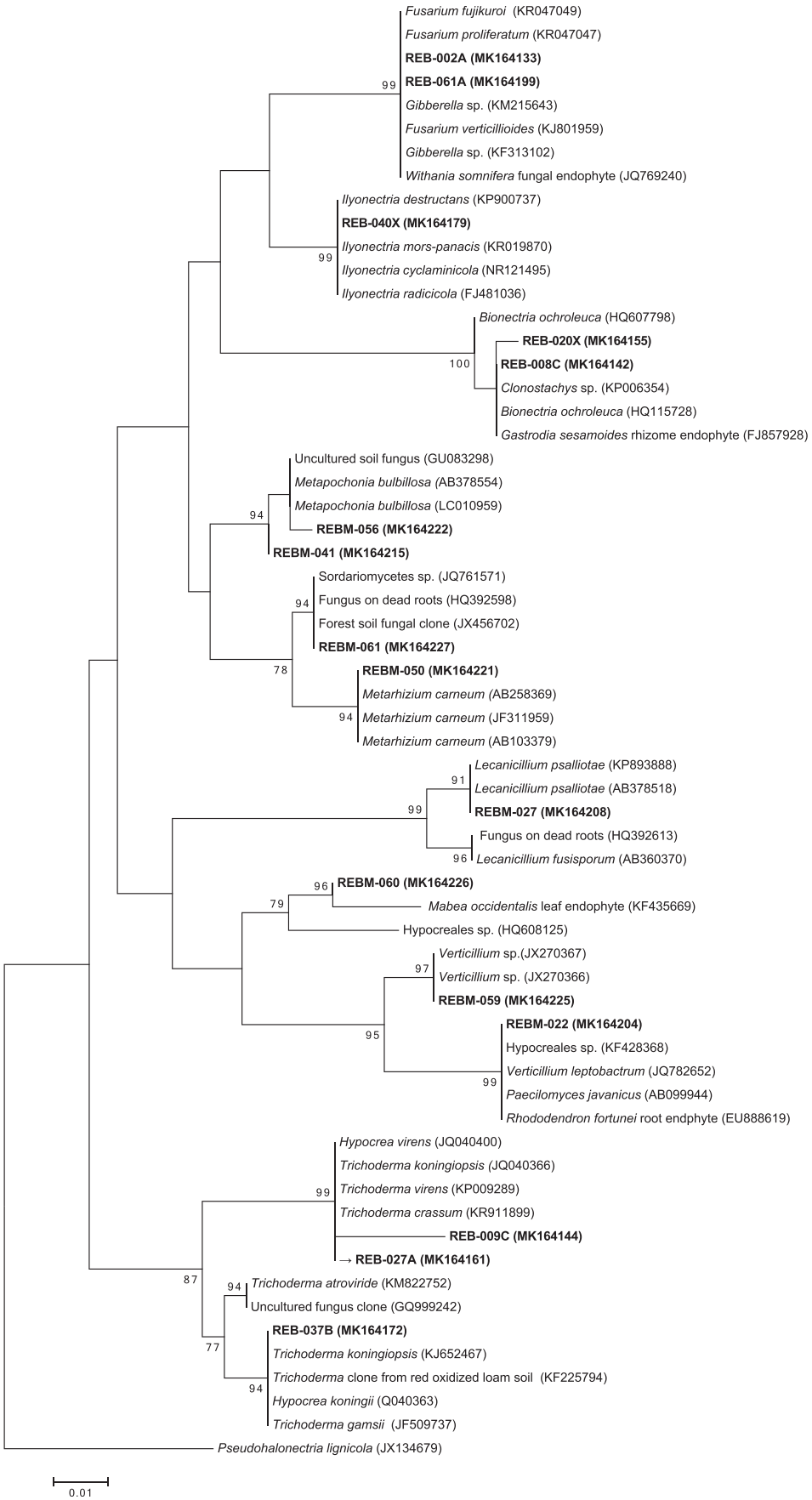

Fig. 2. Maximum likelihood (ML) phylogeny of isolates representing the order Hypocreales, rooted to the outgroup species *Pseudohalonectria lignicola*. Bootstrap support values per 1000 replicates greater than 70 % are included at branch internodes. Bar length indicates number of substitutions.

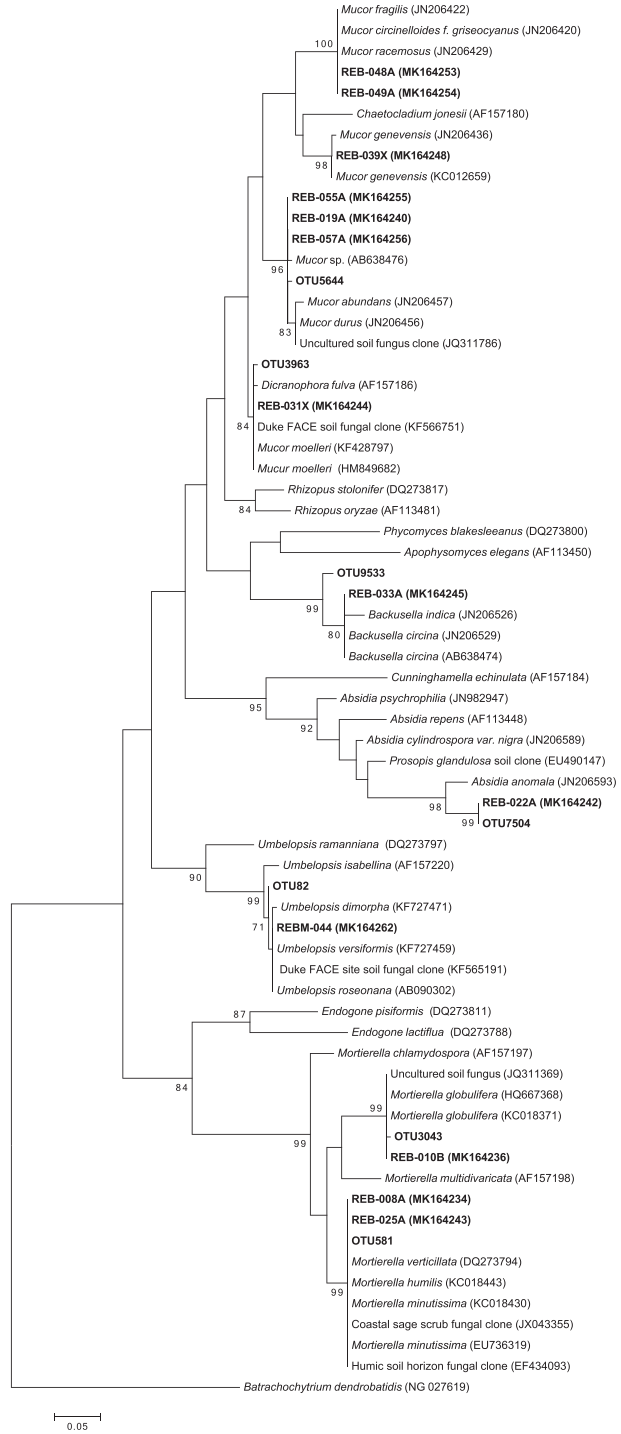

Fig. 3. Maximum likelihood (ML) phylogeny of isolates representing the orders Mucorales and Mortierellales, rooted to the outgroup species *Batrychochytrium dendrobatidis*. Bootstrap support values greater than 70 % are included at branch internodes. Branch length indicates number of substitutions.

example, only 10 ± 11 average sequences per sample were recovered for an isolate representing *Metapochonia*; however, this isolate (REBM-056) was found in 94 % of the soil samples (Table 2).

3.4. Chitin bioassay

Isolates representing all 20 genera exhibited some degree of chitinolytic activity; however, several isolates within those genera failed to degrade chitin. For example, of the isolates tested, eight isolates in the genera *Mucor*, *Ilyonectria*, and *Mortierella* demonstrated no chitinolytic activity. Of the isolates that demonstrated chitin degradation, 12 exhibited low chitinolytic activity, 10 exhibited medium chitinolytic activity, and 11 exhibited high chitinolytic activity. The greatest disparity in chitinolytic activity within genera was evident in *Mucor* and *Mortierella*, which each contained isolates exhibiting high activity as well as no activity (Fig. 5B).

4. Discussion

4.1. Diversity and distribution of isolates in culture and NGS libraries

This study demonstrated that entomopathogenic taxa—notably *Metarhizium*, *Paecilomyces* and *Lecanicillium*—are present and common at this site, and are predominantly found in the topmost soil horizons. Our isolates showed similarity in community composition between this study and previous studies in agricultural and natural soils (Sun et al., 2008; Sun and Liu, 2008). The taxa isolated by Sun et al. (2008) using the *Galleria* baiting technique included entomopathogens, opportunistic pathogens, and secondary colonizers; many of these taxa were isolated in this study, including *Paecilomyces*, *Lecanicillium*, *Mucor*, *Mortierella*, *Absidia*, *Fusarium*, and *Trichoderma* species.

While estimations of the relative abundances of entomopathogenic fungi in soil communities can vary in response to factors that include geography and temporal variation (Tarasco et al., 1997; Chandler et al., 1997; Meyling and Eilenberg, 2006), some trends in the distribution of these fungi appear to exist between natural and agricultural soils (Quesada-Moraga et al., 2007). In many studies in agricultural soils, *M. anisopliae*, *B. bassiana*, and *Lecanicillium* spp. are the most abundantly isolated entomopathogenic taxa, with few isolates representing the genus *Paecilomyces* (Klingen et al., 2002; Meyling and Eilenberg, 2006; Sun et al., 2008). In contrast to trends in agricultural soils, Sun and Liu (2008) found that *P. farinosus* was the most abundant entomopathogenic taxon isolated from natural forest soils. Our isolates included two OTUs representing *Paecilomyces* and two OTUs representing *Lecanicillium*; *Lecanicillium* was also relatively abundant in the NGS library analyzed in this study (Table S1; Table 2).

In contrast, other entomopathogens in the Hypocreales such as *Beauveria* and *Ophiocordyceps* were not recovered in our culture-based approaches at all. As such, we assessed the NGS sequence libraries from Hesse et al. (2016) for the presence of these taxa. *B. bassiana* and *Ophiocordyceps* represented on average less than 1 % of sequences per sample, illustrating their low abundance and seemingly supporting the results of culture-dependent approaches, in which neither of these fungi were recovered. However, environmental sequences representing *Metarhizium* were considerably less abundant (<1 per plot in fewer than 1 % of samples) than those of *Beauveria* (3 per plot in 53 % of samples) (Table 2). Despite this relative scarcity of *Metarhizium* in the NGS dataset, *Metarhizium* was isolated in culture while *Beauveria* was not. The trends in our NGS data support the results of foundational studies on entomopathogen ecology conducted using culture-dependent approaches, such as Bidochka et al. (1998), that demonstrated that *B. bassiana* is more common in forest soils than *M. anisopliae*. Similarly, Quesada-Moraga et al. (2007) concluded that *M. anisopliae* was less abundant in natural soils than in cultivated soils, and that the occurrence of both *M. anisopliae* and *B. bassiana* were affected by soil physical factors including pH and substrate type. Bidochka et al. (2002) found that genetically-distinct populations of *B. bassiana* were present in forest soils and responded differently to physical variables such as

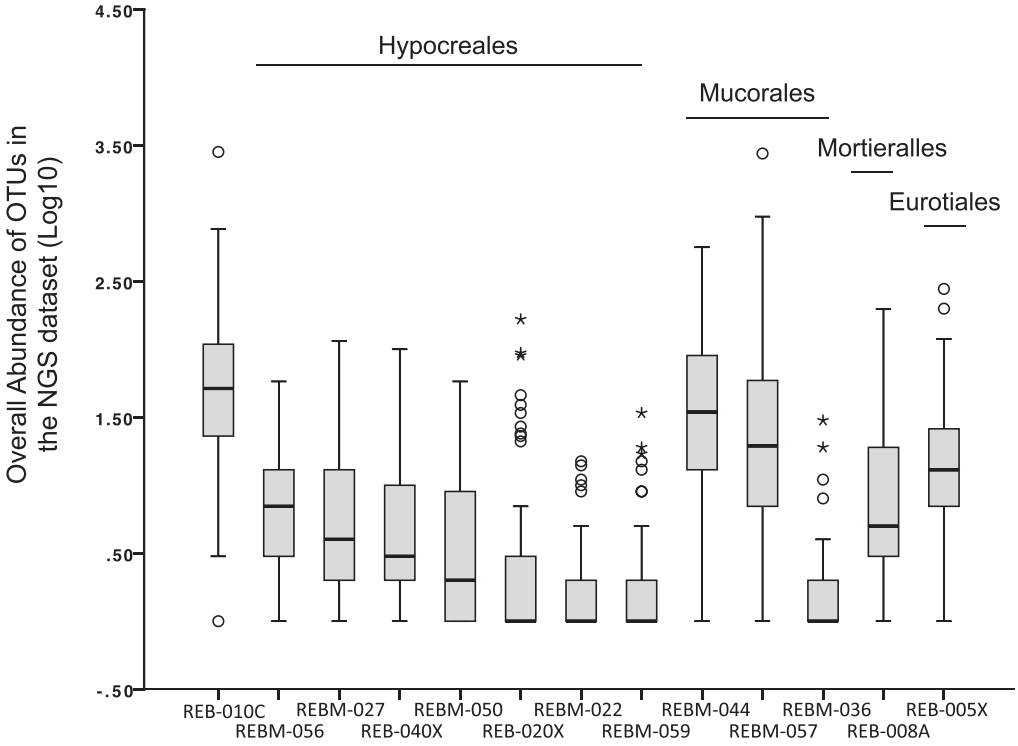

Fig. 4. Box plots of abundance of most common unique OTUs in next generation sequence library. Data are transformed (Log_{10}). OTUs are delineated, from left to right, into the orders Hypocreales, Mucorales, Mortierellales, and Eurotiales. Circles represent outliers, while stars represent extreme outliers. Middle lines represent mean abundance, while boxes represent lower (25 %) and upper (75 %) interquartile range. Vertical lines represent the overall range of the data, excluding outliers and extreme outliers.

UV radiation and temperature, and as such, it is possible that the abundance of *Beauveria* in our NGS data and its absence in culture stems in part from the conditions that we chose for our culture-dependent methods. A more detailed analysis exploring the different genetic populations of *Beauveria* at this site, along with different isolation parameters, might help to answer these questions.

Opportunistic fungi and secondary colonizers comprised the majority of isolates and represented a diverse range of taxa. However, their distribution appears patchy and subject to considerable spatial heterogeneity. Studies examining soil microbial communities have suggested that spatial variation, often on the scale of centimeters, plays a major role in data variation (Morris, 1999; Quesada-Moraga et al., 2007; Goble et al., 2010; Uzman et al., 2019). As such, the abundances of many of our isolates varied greatly between horizons, both within and between taxa. However, those differences appear to be reflected proportionally in both approaches, particularly at the order level and higher (Fig. 1A). For example, the Hypocreales was the most abundant order represented in culture-based approaches, as well as in the NGS dataset for this taxon. Similarly, Mucorales and Mortierellales were the second and third most abundant orders, respectively, in both culture and NGS datasets. As such, it appears that the findings from both approaches indicate common trends in terms of large-scale abundances of the fungi studied.

4.2. Phylogenetic analyses provide insights on trends in fungal evolution

Phylogenetic analyses resolved the species-level relationships of several isolates in the Hypocreales and placed several isolates into clades representing the Mucorales and Mortierellales (Figs. 2 and 3). While most clades were strongly supported, the analysis of isolates in the Mucorales and Mortierellales using LSU produced some poorly-supported clades. As such, future work with these taxa might benefit from the use of a combination of markers, such as RPB2 or EF-1a. Of particular interest were isolates that formed clades with sequences representing entomopathogenic taxa, as well as with endophytes and other sequences from unique environments, reinforcing previous findings suggesting complex ecological interactions between entomopathogenic taxa and plants (Fig. 2). One isolate in particular (REBM-022) formed a clade with sequences representing *Paecilomyces javanicus*, as well as a root endophyte of *Rhododendron fortunei* (Ericaceae). *B. bassiana* has been recovered as an endophyte of maize (*Zea mays*), cotton (*Gossypium hirsutum*) and cocoa (*Theobroma cacao*) (Wagner and Lewis, 2000; Jones, 1994; Akello et al., 2007), while *Paecilomyces* have been isolated from banana (*Musa acuminata*) and rice (*Oryza sativa*) (Cao et al., 2002; Tian et al., 2004). Although entomopathogenic taxa related to our isolates have demonstrated endophytism, additional experimentation is necessary to establish definitive links between these fungi and plant hosts in this site. Some of the limitations to establishing clear links between plant symbioses and entomopathogens stem from the logistical challenges posed by field and laboratory experiments in which these links can be established, as well as the availability of suitable molecular markers. For example, Gazis et al. (2011) demonstrated that the choice of molecular marker(s) and the identity of the taxa being evaluated can skew interpretations when delimiting species of fungal endophytes, especially when using the ITS rRNA gene region.

4.3. Chitinolytic activity is widespread in isolates but varies in strength within and between taxa

Chitinolytic enzyme assays confirmed that the majority of fungi in this study were capable of some degree of chitinolysis.

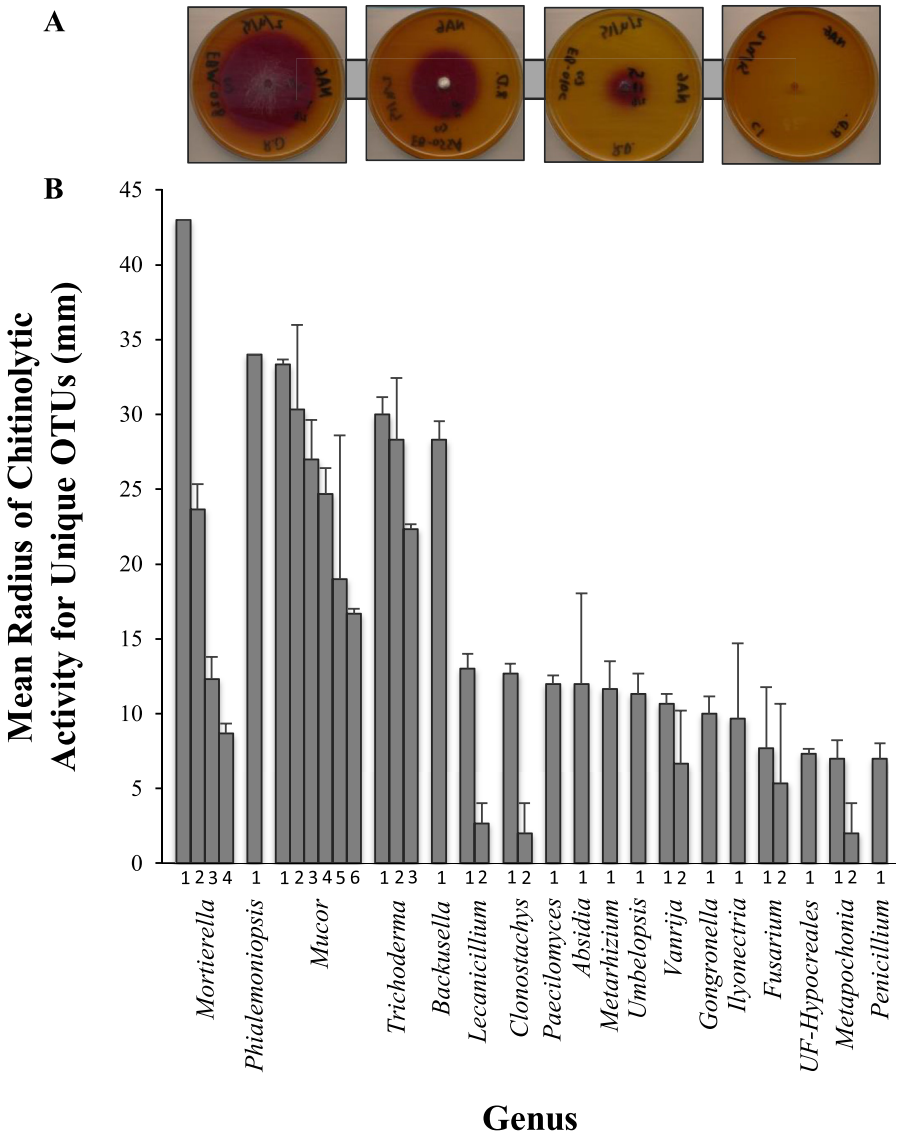

Fig. 5. Chitinolytic activity of fungal isolates. Examples of high, medium, low, and no chitinolytic activity in representative isolates after 72 h (**A**); mean radius of chitinolytic activity for unique OTUs on NAG medium after 72 h (**B**). Lines above bars represent standard deviation.

Pathogenic taxa such as *Metarhizium*, *Lecanicillium*, *Paecilomyces*, and *Metapochonia* appear to degrade chitin at a lower rate than saprobic fungi such as *Mortierella* and *Mucor*. Several isolates within the same genera showed high disparity in chitinolytic activity. In the case of *Mortierella*, one isolate (REB-008A; *Mortierella* 1) illustrated the highest capability for chitin degradation of any isolate in this study, while another isolate (REBM-044; *Mortierella* 4) illustrated some of the lowest capacity to degrade chitin; several other *Mortierella* isolates failed to degrade chitin entirely. Similar trends were observed in isolates within the genus *Mucor* (Fig. 5B). Chitin is one of the most abundant polysaccharides on the planet (Muzzarelli, 1977), and opportunistic and saprobic taxa (such as *Mortierella* and *Mucor*) likely receive much of their carbon and nitrogen from sources other than insect cadavers (such as plant litter) (Patil et al., 2000). Opportunistic and saprobic isolates recovered from *G. mellonella* tissues in this study are frequently recovered from insect tissues (Sun et al., 2008), suggesting that these fungi might occur frequently on decomposing insects as well as in leaf litter and fungal biomass (Burns and Dick, 2002).

Differences in chitinolytic ability between strains of a single species, as well as between different species within a genus are well-documented (de la Cruz et al., 1992; Lorito et al., 1993; Duo-Chuan, 2006). The differences observed in degradation rates could be a result of different responses to the chitin source provided and growth rates, since multiple genes for chitinolytic enzymes are present and active in varying proportions in entomopathogenic and insect-associated fungi (St. Leger et al., 1993; Patil et al., 2000; Barretto et al., 2004). As such, one might expect different sources of chitin to be better or worse suited for different fungi. Different substrates containing chitin could differentially induce or repress genes for chitinolytic enzymes, suggesting that a one-size-fits all assay might not detect sensitive differences in chitinolytic potential (de la Cruz et al., 1993; Barretto et al., 2004). It is possible that limitations presented by the use of only one chitin medium in this study could account for some of the differences observed between fungi (Fig. 5B). While the technique employed in this study is useful for screening large numbers of isolates for general trends in chitinolytic ability, more sensitive colorimetric assays should be conducted on isolates of special interest.

5. Conclusions

Studies on the diversity and relative abundance of entomopathogens in soils have focused primarily on evaluating species of *Metarhizium* and *Beauveria*, with few studies on genera such as *Lecanicillium* and *Paecilomyces* (Sun et al., 2008; Rudeen et al., 2013; Kepler et al., 2015). Our study demonstrates that the combination of NGS and culture-dependent approaches could help target less-studied genera, given that *Lecanicillium* and *Paecilomyces* occur more frequently at this site than the better-studied members of *Metarhizium* and *Beauveria*.

Interestingly, our results mirror the findings of similar studies in the same system, notably Torres-Cruz et al. (2018). In their study, isolates with low abundances in the NGS library were successfully isolated using targeted approaches (specifically, media doped with heavy metals). Moreover, they demonstrated that the most abundant taxa described in culture-dependent approaches in soil systems in general (e.g., *Penicillium*, *Trichoderma*, *Fusarium*, and *Umbelopsis*) were also abundant in their culture collections, despite having been isolated and cultured in the presence of harsh selective reagents.

Similarly, we observed cosmopolitan fungi such as *Umbelopsis*, *Absidia*, and *Mucor* thriving in the presence of selective reagents, reinforcing the assertion that some fungi might benefit from intermittent environmental stress as an adaptive advantage. The ability of members of the Hypocreales to grow in the presence of chloramphenicol, thiabendazole, and cycloheximide is well-documented (Fernandes et al., 2010). Moreover, Uzman et al. (2019) reported entomopathogenic fungi thriving in the presence of copper-based fungicides in vineyard soils. As such, we propose that the hardiness of entomopathogens in the presence of stressful conditions, along with the growing body of research on plant endophytism in these taxa, suggests that these fungi, like other, more cosmopolitan taxa in the Hypocreales, might depend on strenuous environmental conditions as determinants of their ecological function and success.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2019.09.006.

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