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Diversity and function of fungi associated with the fungivorous millipede, *Brachycybe lecontii*

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Thesis submitted to the Davis College of Agriculture, Natural Resources and Design at West Virginia University in partial fulfillment of the requirements for the degree of

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ABSTRACT

Uncovering the diversity and function of fungi associated with the fungivorous millipede, *Brachycybe lecontii*

Angie M. Macias

Brachycybe (Wood) is a genus of fungivorous millipedes. To date, the fungal associates of these millipedes have never been characterized. In an attempt to resolve these relationships, culture-based approaches combined with DNA barcode sequencing were used. Sampling of 313 individuals collected from three of four *B. lecontii* clades and 20 sites across seven states uncovered at least 183 genera in 40 orders from four fungal phyla. At least seven putative new species were recovered in this study, despite the use of more classical culture-based approaches. Three of these fungi were phylogenetically resolved using ITS + LSU and include two new species, aff. *Fonsecaea* sp., *Mortierella* aff. *ambigua,* and a new genus related to *Apophysomyces.* Overall, the results of this study highlight the vast amount of undescribed fungal biodiversity associated with millipedes. Twelve fungal genera from nine orders showed high connectivity across the entire *B. lecontii*-associated fungal network, indicating a central role for these fungi in their association with these millipedes. These twelve include the two putative new species described above. The ecology of these and other fungal associates were also explored, using fungal cohort pairings and entomopathogenicity trials. Over 40% of all fungal pairings resulted in competitive interactions, a majority of which involved inhibition or overgrowth by fungi in the Hypocreales and Polyporales, respectively. The abundance of these competitive interactions in these two orders indicate differing ecological strategies. Hypocreales used chemical warfare to competitively exclude other fungi, while Polyporales physically overgrew their competitors. Mucoromycotan fungi used a similar strategy to the Polyporales. Results of a series of entomopathogenicity trials indicated that *B. lecontii* was less susceptible to entomopathogenic Hypocreales than an insect model (*Galleria mellonella*), even though these fungi are known to attack several classes of arthropods. Furthermore, the absence of a negative interaction between *B. lecontii* and entomopathogenic Hypocreales may indicate a beneficial relationship. When challenged with Polyporales, *B. lecontii* exhibited high mortality, while *G. mellonella* was unaffected. This stands in sharp contrast to previous casual observations of the feeding behavior of *B. lecontii.* Recent discoveries of previously overlooked fungal diversity have been groundbreaking and hint at substantial cryptic fungal biodiversity across the globe. The 200-300 million-year-old association between fungi and the Colobognatha, which includes *Brachycybe lecontii,* provides an ideal system to uncover biodiversity and examine function of these fungi in a highly understudied and ancient association.

ACKNOWLEDGEMENTS

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CHAPTER 1: REVIEW OF LITERATURE

<u>Millipede evolution, biology, and ecology</u>

Millipedes (Arthropoda: Myriapoda: Diplopoda) are non-insect arthropods with many body segments, and two pairs of legs per body segment. Other morphological characteristics include short elbowed antennae, a single fused maxilla, and large mandibles (Hopkin and Read 1992). The earliest fossil evidence of millipedes consists of trace fossils from the Ordovician, though the first body fossil, *Pneumodesmus newmanii*, dates to the Silurian (Wilson and Anderson 2004). This millipede fossil is also the earliest record of air-breathing in any animal. However, a recent molecular clock study of the evolution of air-breathing places the divergence of subphylum Myriapoda (containing millipedes and centipedes) at 554 MYA, in the mid- to late Cambrian (Lozano-Fernandez *et al*. 2016), although the reliability of molecular clock assumptions becomes questionable in deep phylogenetic time.

Millipedes are a relatively diverse group of animals, with approximately 12,000 millipede species described to date, in 16 orders and 140 families (Shear 2011). They are generally solitary animals, interacting with others only to reproduce. Millipede reproductive organs are located near the head, and males clasp the female with their legs while copulating. Some cases of geographic or complete parthenogenesis have been reported (Pinheiro *et al.* 2009). Females typically lay their eggs in an underground brooding chamber constructed of frass, soil, and decayed plant material (Hopkin and Read 1992). Eggs are abandoned after laying, except in a few rare cases of parental care (Kudo et al. 2009, 2011).

The smallest known millipede is 2 millimeters long, while the largest can reach 35 centimeters (Hopkin and Read 1992). The majority are detritivores, feeding on dead and decaying plant material and animal waste on the forest floor, though observations or hypotheses of herbivory (Marek *et al.* 2012), fungivory (Brewer *et al*. 2012, Marek *et al.* 2012), and predation (Srivastava and Srivastava 1967) exist. Detritivorous millipedes play a crucial role in nutrient cycling, particularly in nutrient-poor areas (Mattson 2012, Lawrence and Samways 2003, Bonkowski *et al.* 1998). Nearly all millipedes are burrowers that live underground or inside wet, rotting substrate, and as such, have poor to non-existent vision (Manton 1961). The exoskeleton of millipedes lacks a waxy cuticle, so they must stay in damp environments to maintain body moisture (Cloudsley-Thompson 1950).

The only defenses millipedes possess are curling into a spiral to protect head and legs, and chemical defense through a wide variety of compounds secreted from repugnatorial glands along the body. It is believed that all millipedes synthesize these compounds themselves, but it is possible that some may sequester compounds from fungi (Shear 2015). These compounds include cyanogenics, terpenes, alkaloids, and phenols (Shear 2015). Defensive compounds may also have antibacterial (Williams and Singh 1997) and antifungal (Roncadori *et al.* 1985) properties, in addition to warding off vertebrate and arthropod predators, but some parasites use millipede secretions to home in on suitable hosts (Hash *et al.* 2017). Capuchin monkeys and other primates seek out millipedes to rub onto their bodies to repel insect pests like mosquitos (Valderrama *et al.* 2000).

Millipedes are regularly reported in association with fungi, likely due to their

detritivorous habit. Most records of fungal-millipede interactions are cases where the millipede occasionally grazes on fungi in the environment, or where a parasitic fungus is attacking the millipede (Lilleskov and Bruns 2005, Bultman and Mathews 1996, Hodge *et al.* 2017). Several species of specialist ectoparasitic fungi in the Laboulbeniales have been recorded from millipedes (Santamaria *et al* 2014, Enghoff and Santamaria 2015). In the millipede pet trade, fungal infection due to poor husbandry is a widespread issue, especially of *Archispirostreptus gigas* (McMonigle 2012). Opportunistic saprotrophs may take advantage of weakened millipedes, but true entomopathogenic fungal attack on healthy animals is uncommon in the literature (Brito 1994, Chitty 2006). At least two species of trichomycetes, obligate arthropod gut-associated fungi, have been reported from millipedes (Wright 1979).

Introduction to *Brachycybe*

  Brachycybe (Wood) is a genus of millipedes in the family Andrognathidae (Diplopoda: Platydesmida) with eight described species and two awaiting description (Brewer *et al*. 2012). In the United States, there are two species in the Appalachian Mountains and three (plus two undescribed) in northern California. The remaining three species are in China, Taiwan, Japan, and South Korea (Brewer *et al*. 2012). Interestingly, the two species in the eastern US (*B. petasata* and *B. lecontii*) are most distantly related to each other, with their closest relatives living in California and Japan, respectively (Brewer *et al*. 2012). This phylogenetic evidence indicates that the genus likely originated in the California mountains, and expanded its range at least twice into eastern North America, and at least once into Asia (Brewer *et al*. 2012).

Brachycybe are small millipedes, with some individuals reaching ~40 mm in length (pers. obs.). They are one of the few groups of arthropods that exhibit paternal care of their offspring. Evidence from *B. nodulosa* (Kudo *et al.* 2011) indicates that females choose to mate with the male with the widest body, which would provide the most protection to the eggs and hatchlings. After mating, the female lays her eggs in a sticky mound, which the male then wraps his body around, forming a basket. Males groom the eggs with their legs and mouthparts until they hatch, though it is unclear exactly what role this behavior plays. However, if the males are removed, the eggs quickly become overgrown by fungus and may fail to hatch (Kudo *et al.* 2011, pers. obs. in *B. lecontii*).

Brachycybe millipedes are most frequently found living on rotten logs and branches with visible fungal growth. They are subsocial, forming a pinwheel-like colony around crust-forming fungi such as *Irpex, Peniophora,* and *Ceriporia*. Individuals of all age groups will live in the same colony, and individuals freely move from one colony to another (Paul Marek, pers. comm.). They are believed to be fungivorous, as evidenced by their diminutive mouthparts (Paul Marek, pers. comm.), their clustering around fungi, and the visual damage they leave behind on crust fungi after they move.

This study will focus on the species *Brachycybe lecontii*, which ranges from eastern Oklahoma to Louisiana, and north to Missouri and mid-West Virginia. *B. petasata* overlaps in range with *B. lecontii* in the mountains on the border of North Carolina and Tennessee, and reaching into northern Georgia (Brewer *et al.* 2012). The mitochondrial genome of *B. lecontii* has been sequenced (Spruill 2010). This species has four distinct evolutionary clades, restricted by geography (Brewer *et al.* 2012). Their

defensive secretions have been identified as the chemical deoxybuzonamine (Shear 2015), a heterocyclic nitrogen-containing compound related to buzonamine. Buzonamine has been determined to have antipredator effects, but has only been tested against one ant species (Wood *et al.* 2000).

Historically, only one study reported the identity of a crust fungus fed upon by *Brachycybe*, and it was determined to be a species of *Peniophora* (Gardner 1975). More recently, five Polyporalean fungi have been reported directly from *B. lecontii*, and five others were isolated from *B. lecontii*-associated wood (Kasson *et al.* 2016).

Animal-fungal symbioses

A wide variety of animals depend on fungi for food, shelter, and defense. The following is an exhaustive summary of known cases of animal-fungal symbioses. Eusocial termites and leaf-cutter ants, and subsocial ambrosia beetles are covered in the next section.

Ambrosia beetles (Coleoptera: Curculionidae: Scolytinae) have a nutritional symbiosis with various Ascomycotan fungi (one known exception, see next section). These fungi include *Fusarium* and *Geosmithia* (Hypocreales)*, Raffaelea* and *Afroraffaelea* (Ophiostomatales)*, Ambrosiella, Meredithiella,* and *Phialophoropsis* (Microascales) (Hulcr and Stelinski 2017). These beetles bore tunnels into dead or dying trees, or less commonly, pith, seeds, fruits, and petioles (Mueller *et al.* 2005). These tunnels serve as fertile ground for the beetles' fungal symbiont, which is transported inside a specialized pouch called a mycangium. The beetle inoculates her tunnel and soon after fungal growth appears, lays her eggs. The offspring feed on the

fungus, and once mature, carry it away in their mycangia to start their own galleries (Hulcr and Stelinski 2017). Beetles obtain necessary vitamins, amino acids, and sterols from their fungus, in exchange for helping transport the fungus to new substrate (Mueller *et al.* 2005).

Sirex noctilio wood wasps (Hymenoptera: Siricidae) have a nutritional symbiosis with the fungus *Amylostereum areolatum* (Basidiomycota: Russulales) (Gaut 1969). These wasps oviposit their eggs up to 1.2 cm deep on their host plant, typically a member of the Pinaceae (Coutts and Dolezal 1969). These oviposition sites often have multiple tunnels, the last of which is injected with fungal arthrospores contained in sacs at the base of the female's ovipositor (Coutts and Dolezal 1969). *Amylostereum* is able to kill off a healthy tree, thus making it suitable for larval development, and in fact, the larvae depend on the fungus to condition the wood for their use (Coutts 1969, Coutts and Dolezal 1969). Other members of the Siricidae also have mutualistic fungi that perform similar roles (Coutts and Dolezal 1969).

Ship-timber beetles (Coleoptera: Lymexylidae) have a suspected nutritional symbiosis with the fungus *Endomyces* (Ascomycota: Saccharomycetales) (De Fine Licht and Biedermann 2012). Females carry inoculum in a mycangium near the ovipositor, and eject the fungus when eggs are laid. The larvae then carry spores into the wood as they begin boring (Arnett *et al* 2002). Like ambrosia beetles, ship-timber beetles remove wood dust and frass from their galleries, allowing their symbiotic fungus to grow on the tunnel walls (Henry 1967). Gut dissections have indicated that the larvae are indeed consuming fungus, though wood fragments were present as well (Henry 1967, Casari and Teixeira 2011). No studies on the nutritional content of the fungus, nor

the question of whether or not the larvae required the fungus to survive, have been performed.

The marsh periwinkle, *Littoraria irrorata* (Mollusca: Littorinidae), has a nutritional symbiosis with several fungi, most frequently *Mycosphaerella* (Ascomycota: Mycosphaerellales) and *Phaeosphaeria* (Ascomycota: Pleosporales) (Silliman and Newell 2003, Sieg *et al.* 2013). These snails live on *Spartina* (Poaceae) in salt marshes, where they scrape the grass blades with their radula to create open wounds for fungi to colonize. In addition, these snails defecate onto the wounds, providing extra nitrogen for fungi, and additionally inoculating the wound with fungal spores present in the feces (Silliman and Newell 2003). After the fungus develops on the plant wounds, the snails consume it. Young snails require fungi to survive, and will die if left on *Spartina* with no fungal inoculum (Silliman and Newell 2003).

The Brazilian stingless bee, *Scaptotrigona depilis* (Hymenoptera: Apidae), has a nutritional symbiosis with the fungus *Monascus* sp. (Ascomycota: Eurotiales) (Menezes *et al.* 2015). Worker bees provision nest cells with a liquid food mass prior to egg-laying, and seal the cell when laying is complete (Menezes *et al.* 2015). *Monascus* then grows out from the walls of the cell, coating the food mass before the egg hatches. The larva then eats the fungus and the remaining food mass (Menezes *et al.* 2015). Without fungus, larval survival was only 8% (Menezes *et al.* 2015). When building new nests, bees carry a piece of nest material with them, suggesting that the bees actively transmit a single cultivar of *Monascus* to new nests (Menezes *et al.* 2015).

Some gall midges (Diptera: Cecidomyiidae: Lasiopterini and Asphondyliini) have a nutritional symbiosis with the fungi *Macrophoma* (Ascomycota: Botryosphaeriales),

Ramichloridium (Ascomycota: Mycosphaerellales)*, and *Aureobasidium* (Ascomycota: Dothideales) (Rohfritsch 2008). These midges lay an egg on a plant tissue, and within a few hours of hatching, the host plant's cells become activated and a gall begins to form (Rohfritsch 2008). In most gall midges, the gall is filled with nutritive plant tissue, but in galls caused by the Lasiopterini and Asphondyliini, the gall is lined with fungal tissue. The fungus is deposited by the female at the time of egg-laying (Rohfritsch 1997, 2008). Females pick up new inoculum by rubbing specialized pouches on old, mature galls (Rohfritsch 2008).

Carton-inhabiting ants (Hymenoptera: Formicidae) have a structural symbiosis with several fungi in the Chaetothyriales, Capnodiales, and Pleosporales (Ascomycota), most of which do not yet have names (Voglmayr *et al.* 2011, Schlick-Steiner *et al.* 2008). Each ant species has its own community of structural fungi that help hold the nest wall together and allow the ants to climb the vertical wall surface (Voglmayr *et al.* 2011). It is not yet understood how the fungi are transmitted from nest to nest.

Domatia-inhabiting ants (Hymenoptera: Formicidae) have an unknown type of symbiosis with Chaetothyrialean fungi (Ascomycota) (Voglmayr *et al.* 2011, Defossez *et al.* 2009). Domatia are structures provided by plants as nesting-places for mutualistic ants that defend the plant from herbivory (Voglmayr *et al.* 2011). The fungal communities in domatia are much less diverse than those in ant carton, only containing one or two species of fungi (Defossez *et al.* 2009). These fungi are present in a thick, uniform, cushiony patch inside the domatium, and are never present without the mutualistic ant (Defossez *et al.* 2009). Parasitic ants living in domatia never had a fungus patch (Defossez *et al.* 2009). In addition, the fungi from domatia never

sporulated, suggesting that they may be dependent on the ant for transportation

(Defossez *et al.* 2009). Much more remains to be learned about this relationship

between Chaetothyrialean fungi and their ant partners.

The arboreal ant *Allomerus decemarticulatus* (Hymenoptera: Formicidae) uses

the hyphae of an undescribed fungus to build a trap that captures large insect prey

(Dejean *et al.* 2005). These ants require supplemental nitrogen in their diets, and obtain

it from the trapped prey, while the fungus consumes food material that is bound up in

the trap.

Basidiomycete Fungi and Sociality in Insects

In at least three cases of insect-fungal symbioses, the fungal partner is a

basidiomycete that converts otherwise indigestible plant material into a food source for

the insect. Access to these recalcitrant materials has allowed these animals to

specialize and develop communal social structures that otherwise could not be

supported by their environment.

Ambrosiophilus is an ambrosia beetle (Coleoptera: Curculionidae: Scolytinae)

that cultivates the fungus *Flavodon ambrosius* (Basidiomycota: Polyporales) (Kasson *et*

al. 2016). Ambrosia beetles are well-known for their symbiotic association with fungi, but

the fungal partner has always been reported as a member of the Ascomycota, with the

exception of the sister genus to *Ambrosiophilus*, *Ambrosiodmus* (Li *et al.* 2015,

Simmons *et al*. 2016), which also cultivates *F. ambrosius*. Compared to Ascomycotan

ambrosial fungi like *Fusarium* and *Raffaelea*, *Flavodon* is much more aggressive at

colonizing woody tissue (Kasson *et al.* 2016). Even when compared to known white-rot

fungi, isolates of *Flavodon* caused significantly more loss of mass and hardness in wood tester blocks (Kasson *et al.* 2016). In nature, this results in a large proportion of the recalcitrant nutrients in wood being "claimed" by the beetle and its fungus. As a result of these traits, *Ambrosiophilus* beetles can stay in the same log for more than one generation without running out of resources. This freedom to persist allowed *Ambrosiophilus* to develop large, long-lived, communal colonies with multiple generations dwelling together, a social habit contrary to nearly all other ambrosia beetles (Kasson *et al.* 2016).

Another insect, the termite (Blattodea: Termitoidae: Macrotermitinae), has developed a symbiosis with fungi that allows it to utilize wood, in addition to other plant detritus. Well over 300 species of these widespread insects all cultivate the fungus *Termitomyces* sp. (Basidiomycota: Agaricales: Lyophyllaceae) (Mueller *et al.* 2005), though not all are obligately dependent on it. *Termitomyces* is able to convert wood and decaying plant material brought into the nest by the termites into a concentrated high-nitrogen, high-carbohydrate form (Mueller *et al.* 2005). Termites can have colonies of up to one million individuals divided into two social castes with many-sub-castes, where each individual serves a particular role in the survival of the colony (Abe *et al.* 2000). Access to the nutrients locked away in wood and decaying plant material has allowed termites to inhabit a large suite of ecological niches around the world (Abe *et al.* 2000). Across their range, no other animal utilizes wood to the same extent as termites, with the aid of *Termitomyces*, and it is likely that the near-exclusive access termites enjoy has allowed them to develop the complex social behaviors seen in the obligately mutualistic species (Abe *et al*. 2000).

Leafcutter ants (Hymenoptera: Formicidae: Attini) have also developed a similar mutualism with basidiomycete fungi. Members of genera *Atta* and *Acromyrmex* are obligate mutualists with species-specific strains of Agaricaceae fungi (Mueller *et al.* 2005). These ants have some of the most large and complex societies of any animal ever recorded, with counts ranging from over 100,000 individuals per colony to several million (Schultz and Brady 2008, Murakami *et al.* 2000, Hölldobler and Wilson 2008). Each colony can have up to four social castes, each with their own specialized roles in defense, resource-gathering, and offspring care (Mueller *et al.* 2005). While adult ants may supplement their fungus diet with plant sap, the larvae are entirely dependent on the fungus for nutrition (Mueller *et al.* 2005). Because of this mutualism, leaf-cutter ants are able to access the incredibly abundant rainforest resource of fresh leaves, and may utilize up to 17% of this resource in some ecosystems (Aylward *et al.* 2012). Access to this super-abundant but under-utilized resource likely helped fuel the sophistication and complexity seen in leaf-cutter ant societies today (Aylward *et al.* 2012).

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CHAPTER 2: IDENTIFYING FUNGAL ASSOCIATES OF THE MILLIPEDE
BRACHYCYBE LECONTII

ABSTRACT

Brachycybe (Wood) is a genus of millipedes known to be fungivorous, but, to date, their fungal associates have never been characterized. In an attempt to resolve these relationships, culture-based approaches combined with DNA sequencing were used. Sampling of 313 individuals collected from 3 of 4 *Brachycybe lecontii* clades and 20 sites across 7 states revealed at least 183 genera in 40 orders from 4 fungal phyla. Of the genera found, 40% were recovered only once, and only 13% had 10 or more isolates recovered, many of which were found across numerous sampling locations. Twelve fungal genera from nine orders showed high connectivity across the whole fungal network, indicating a central role for these fungi in their association with *B. lecontii*. In the literature, only a single genus of fungus (*Peniophora*) has been reported in association with *Brachycybe,* but systematic examination of photos available on websites such as Flickr.com indicated that many more types of fungus associate with these millipedes. Results of this study do not support the limited previous observations of the feeding behavior of *B. lecontii* — instead, it appears that these millipedes feed on a wide variety of fungi, not just Polyporales. At least seven putative new species were recovered in the study despite the use of more classical culture-based approaches. Overall, the results of this study highlight the vast amount of undescribed fungal biodiversity associated with millipedes.

INTRODUCTION

Millipedes (Arthropoda: Myriapoda: Diplopoda) are non-insect arthropods with many body segments, and two pairs of legs per body segment. The first millipede body fossil, *Pneumodesmus newmanii*, dates to the Silurian (420-440 million years ago), and presents the earliest evidence for air-breathing in any animal (Wilson and Anderson 2004).

Millipedes are a relatively diverse group of animals, with approximately 12,000 millipede species described to date, in 16 orders and 140 families (Shear 2011). The majority are detritivores, feeding on dead and decaying plant material and animal waste on the forest floor, though anecdotal evidence supports herbivorous (Marek *et al.* 2012), fungivorous (Brewer *et al*. 2012, Marek *et al.* 2012), and carnivorous lifestyles (Srivastava and Srivastava 1967). Detritivorous millipedes play a crucial role in nutrient cycling, particularly in nutrient-poor areas (Mattson 2012, Lawrence and Samways 2003, Bonkowski *et al.* 1998). Nearly all millipedes have a subterranean lifestyle, living in decomposing leaf litter, in the soil, or inside wet, rotting wood substrates.

Millipedes are regularly reported in association with fungi, likely due to their detritivorous habit. Most records of fungal-millipede interactions are cases where millipedes occasionally graze on fungi in the environment, or where a parasitic fungus is attacking the millipede (Lilleskov and Bruns 2005, Bultman and Mathews 1996, Hodge *et al.* 2017). Several species of specialist ectoparasitic fungi in the Laboulbeniales have been recorded from millipedes (Santamaria *et al.* 2014, Enghoff and Santamaria 2015). In the millipede pet trade, fungal infection due to poor animal husbandry is a widespread issue, especially in the most notable pet species, the giant African millipede

(*Archispirostreptus gigas*) (McMonigle 2012). Opportunistic saprotrophs may take advantage of weakened millipedes, but confirmed entomopathogenicity of healthy animals is uncommon in the literature (Brito 1994, Chitty 2006). At least two species of trichomycetes, obligate arthropod gut-associated fungi, also have been reported from millipedes (Wright 1979).

    Brachycybe (Wood) is a genus of millipedes in the family Andrognathidae (Diplopoda: Platydesmida) with eight described species and at least two additional new species awaiting description (Brewer *et al*. 2012). In the United States, there are two species in the Appalachian Mountains and three (plus two undescribed) in northern California. All *Brachycybe* are small millipedes, with some individuals reaching 4 cm in length (pers. obs.). They are one of the few groups of arthropods that exhibit paternal care of their offspring (Kudo *et al.* 2011) (Figure 1). *Brachycybe* are most frequently found living on rotten logs and branches with visible fungal growth (Shelley *et al*. 2005). They exhibit various social behaviors including overlapping adult generations and reproductive division of labor. They also form pinwheel-like multigenerational colonies around resupinate fungi underneath downed woody substrates (Gardner 1975) (Figure 1). All members of the millipede subterclass Colobognatha, which includes *Brachycybe*, are believed to be strict fungivores (Gardner 1975), as evidenced by their diminutive mouthparts (Paul Marek, pers. comm.) their clustering around fungi, and the visual damage (i.e. divots) they leave behind on fungi (Figure 1).

FIGURE 1: *Brachycybe lecontii* behaviors. **A**: *B. lecontii* forming a multigenerational partial pinwheel around a food source embedded in bark. **B**: Several adults forming a full pinwheel around a fungus on wood. **C**: Male caring for eggs. **D**: Male transporting eggs to underside of log, after log was flipped. **E**: Millipede feeding on fungus. **F**: Millipedes sharing a feeding site. Several feeding divots visible.

The known geographic range of *Brachycybe lecontii* extends across 13 states from eastern Oklahoma to western South Carolina, south to Louisiana, and north to southern West Virginia (Shelley *et al.* 2005, Brewer *et al.* 2012). Their range encompasses five Level III and nine Level IV Ecoregions (as defined in 2013 report from U.S. EPA). The five Level III Ecoregions include: "Ouachita Mountains" (AR, OK), "Arkansas Valley" (AR), "Piedmont" (SC), "Blue Ridge" (GA, SC), "Ridge and Valley" (VA), and "Central Appalachians" (TN, VA, WV). The nine Level IV Ecoregions were as follows: "Central Mountain Ranges", "Central Hills, Ridges, and Valleys", and "Fourche Mountains" ("Ouachita Mountains"); "Scattered High Ridges and Mountains" ("Arkansas Valley"); "Northern Inner Piedmont" ("Piedmont"); "Southern Crystalline Ridges and

Mountains" ("Blue Ridge"); "Southern Sandstone Ridges" and "Southern Dissected Ridges and Knobs" ("Ridge and Valley"); and "Dissected Appalachian Plateau" ("Central Appalachians"). Within this known range, five subpopulations have been identified (Shelley *et al*. 2005), four of which were recently validated using sequence data (Brewer *et al*. 2012).

Historically, only one study reported the identity of a resupinate fungus fed upon by *Brachycybe*, and it was determined to be a species of *Peniophora* (Russulales) (Gardner 1975). More recently, five Polyporalean fungi (*Irpex lacteus, Phanerochaete sp., Trametopsis cervina, Junghuhnia nitida,* and *Gloeoporus pannocinctus*) have been reported directly from *B. lecontii*, and five other Polyporales were isolated from *B. lecontii* associated wood (Kasson *et al.* 2016). However, the overwhelming number of casual observations in websites such as Arachnoboards.net (2 observations), Bugguide.net (6), Flickr.com (3), Instagram.com (6), and iNaturalist.org (3) of *Brachycybe* interacting with various fungi supports a much more diverse fungal community than has been formally identified.

In an attempt to address this knowledge gap, this study aims to identify fungal associates of *Brachycybe lecontii* across its known geographic range using culture-based approaches. The use of DNA sequencing will permit in-depth examination of culturable fungal communities including taxa with diagnostically informative morphology as well as morphologically cryptic taxa.

MATERIALS AND METHODS

Collection sites and in-field methods

Millipede collection sites were primarily identified through Brewer *et al*. 2012 and Gardner 1975, and additional sites were also identified. Sampling was targeted to collect millipedes from all four *B. lecontii* clades. Based on previous work by Brewer and colleagues (2012), individual sites were expected to contain millipedes from a single clade, with no previously reported overlap. In total, 20 sites were examined, with 18 yielding colonies and individuals, and 2 yielding individuals only. These sites were in Arkansas, Georgia, Oklahoma, South Carolina, Tennessee, Virginia, and West Virginia (Table 1).

TABLE 1: *Brachycybe lecontii* collection sites and associated information for each.

SITE NAME	COLLECTION REFERENCES	MILLIPEDE CLADE	BRIEF DESCRIPTION	LEVEL 3 & 4 ECOREGION[†]
AR1	Gardner 1975	4	Mount Magazine	37A
AR2	None	4	Power Line Elm	36B
AR3	None	4	Serendipity Trail	36C
AR4	Brewer *et al*. 2012	4*	Mount Nebo	37A
AR5	None	4	Lake Ouachita	36B
AR6	None	4	Charlton Campground	36B
GA1	Gardner 1975	1	Moccasin Creek	66D
OK1	Brewer *et al*. 2012	4	Hodgen	36D
SC1	Brewer *et al*. 2012, Gardner 1975	1	Cassidy Bridge	66D
SC2	Gardner 1975	1	Stumphouse Tunnel	45E
TN1	Gardner 1975	3	Caryville	69D
VA1	Brewer *et al*. 2012, Gardner 1975	3	Shortts Road	69D
VA2	None	3	Pinnacle	67I
VA3	Gardner 1975	3	Breaks	69D
VA4	None	1	Pulaski	67H
WV1	Brewer *et al*. 2012, Gardner 1975	3	War	69D
WV2	None	3	Berwind Lake	69D

WV3	None	3	Panther	69D
WV4	None	3	Cabwaylingo	69D
WV5	None	3	Chief Logan	69D

*AR4 was expected to have Clade 2 (Brewer *et al*. 2012) but millipedes collected there were determined to be from Clade 4.
†Ecoregions are defined in Supplemental Table 1.

At each site, decaying logs on the forest floor were overturned until colonies of *B. lecontii* were located. Colonies are defined as groupings of two or more individuals, and were typically found on or near resupinate fungi covering the underside of the logs. When a suitable colony was found, individuals from single colonies were placed together inside 25 ml sterile collection vials, often with a piece of the fungus-colonized wood they were observed feeding on, and stored in a cooler until processing. In addition, cross-sections of wood from which colonies were collected were taken to permit their identification once back in the lab.

In-lab millipede processing & isolate collection

All millipedes were maintained at 4°C until processed, which typically occurred within three days. After surface sterilization in 70% ethanol, individuals were sexed, sectioned with a sterilized scalpel, and tail portions preserved in 70% ethanol for millipede genotyping in the Marek Lab using custom markers previously described by Brewer and colleagues (2012). Gonopods were also preserved from males to permit anatomical study in the Marek Lab. The remainder of the millipede was macerated in 500 µl of sterile distilled water, and 50 µl was spread on glucose yeast extract agar (GYEA) amended with streptomycin sulfate and tetracycline hydrochloride antibiotics to isolate fungi (Appendix A). Cultures were parafilmed and allowed to grow at room

temperature until growth was observed. Each colony-forming unit (CFU) was

categorized by morphotype, counted, and recorded. One representative of each

morphotype from each plate was retained and assigned an isolate number. Culture

plates were retained for up to three weeks to ensure that slow-growing fungi were

counted and sampled. Depending on how rapidly fungi grew in pure culture, isolates

were either grown on potato dextrose broth (Appendix A) prior to DNA extraction, or

mycelium was scraped directly from plates. DNA was extracted from all isolates using a

modified Wizard kit (Promega, Madison, WI, USA) (Appendix B). For long-term storage,

isolates were kept on potato dextrose agar slants (PDA, Appendix A) at 4°C.

Wood samples were dried at room temperature for up to several weeks

depending on the size of the sampled section and sanded for visual identification using

an orbital sander equipped with 220-grit paper. Identifications were made by examining

anatomical features in cross section with the aid of a dissecting microscope, based on

descriptions by Panshin and de Zeeuw (1980).

Isolate identification

All isolates were identified using the universal fungal barcoding gene, the

ribosomal internal transcribed spacer region (ITS), which includes ITS1, 5.8S, and ITS2

(Schoch *et al*. 2012). All primers used in this study were obtained from Integrated DNA

Technologies (IDT, Coralville, IA, USA). PCR was conducted in 25.5 µL reactions with

the following reagents: 12.5 µL Bioline PCR Master Mix (Bioline USA Inc, Taunton, MA),

10 µL sterile distilled water, 1 µL reverse primer (ITS4, 5'-

TCCTCCGCTTATTGATATGC-3'), 1 µL forward primer (ITS5, 5'-

GGAAGTAAAAGTCGTAACAAGG-3'), and 1 μL fungal DNA (White *et al*. 1990).

Reactions were performed on an MJ Research PTC-200 Peltier Thermal Cycler (GMI,

Ramsey, MN). PCR conditions were as follows: initial denaturation at 95°C for 2

minutes, 35 cycles of denaturation at 95°C for 30 seconds, annealing at 56°C for 30

seconds, and elongation at 72°C for 1 minute, and final elongation at 72°C for 7

minutes.

Products were visualized via gel electrophoresis on a 1.5% w/v agarose

(Amresco, Solon, OH, USA) gel with 0.5% Tris-Borate-EDTA buffer (Amresco, Solon,

OH, USA). SYBR Gold (Invitrogen, Grand Island, NY, USA) was used as the nucleic

acid stain, and bands were visualized on a UV transilluminator (Syngene, Frederick,

MD, USA). PCR products were purified using ExoSap-IT (Affymetrix, Santa Clara, CA).

Products were Sanger sequenced with the same primers used for PCR (Eurofins,

Huntsville, AL, USA). Resulting sequences were searched in the NCBI GenBank

BLASTn database, and identifications recorded for each isolate.

New species identification

Isolates were considered to be a putative new species (PNS) if three or more

identical sequences were recovered with identical low percentage (95% or less)

BLASTn matches. When a PNS was identified, the large subunit of the ribosomal ITS

region (LSU) was also sequenced using primers LR0R (5'-ACCCGCTGAACTTAGC-3')

and LR5 (5'-TCCTGAGGGAAACTTCG-3') (Vilgalys and Hester 1990). PCR conditions

were as follows: initial denaturation at 95°C for 2 minutes, 35 cycles of denaturation at

95°C for 30 seconds, annealing at 51.1°C for 45 seconds, and elongation at 72°C for 90

seconds, and final elongation at 72°C for 5 minutes. PCR products were visualized,

purified, and sequenced as above. The LSU gene was also sequenced for known

relatives of the PNS (as identified by BLAST matches of the ITS sequence).

Putative new species were resolved phylogenetically by constructing an

ITS+LSU concatenated maximum likelihood (ML) tree for the new species and its

known relatives based on a combination of BLAST matches and previously published

literature. MEGA7 (Kumar *et al*. 2016) was used to align (CLUSTAL-W, Larkin *et al.*

2007), select a model for estimating evolutionary distance, and construct an ML tree for

each PNS. Reference sequences used for each phylogeny are listed in Supplemental

Tables 2, 3, and 4.

Community and Diversity Analyses

Community and diversity analyses were used to answer two key questions: 1)

Are *B. lecontii* fungal communities stable across millipede clade and/or wood host?; and

2) What fungal genera are core/central in the *Brachycybe* fungal food web, regardless

of clade and/or wood host?

Diversity indices were used to provide information about rarity and commonness

of genera associated with the fungal community of *B. lecontii* by site. Three indices

were used: alpha diversity, Shannon's diversity index, and Shannon's equitability

(evenness) index.

The effects of *B. lecontii* clade and wood host on the community structure were

analyzed by PerMANOVA using the vegan package in R (Oksanen *et al*. 2017). Tukey's

HSD (honest significant difference) test was used as a post-hoc test to permit pairwise

comparisons of significant dependent variables (i.e., clade and wood host).

A co-occurrence network was constructed for fungal isolates obtained from *B. lecontii* based on fungal genus presence/absence data. Betweenness-centrality was used to measure relative contribution of each node (single fungal genus) to connectivity across the whole network. High betweenness-centrality values are typically associated with nodes located in the core of the network, which in this system are defined as fungal genera with multiple edges connecting *Brachycybe* clades and multiple wood hosts. Low betweenness-centrality values indicate fungal genera with a more peripheral location in the network, with fewer edges connecting clades and substrates (Greenblum *et al.* 2011).

To further investigate patterns in the network, network modularity was characterized. A module is a group of nodes (*i.e.* fungal genera) that are highly connected within the module with few connections outside the module (Shi *et al*. 2016). Modularity ranges from -1 to1. Positive values indicate the number of edges inside the group is greater than that expected by chance. In this study, modules were detected using the Louvain algorithm, which aims to determine the optimal number of partitions that maximize the modularity index (Blondel *et al*. 2008).

RESULTS

In total, 313 individuals were collected from 3 of 4 *B. lecontii* clades, distributed among 60 colonies at 20 sites in 7 states. Some sites were not used in the analysis due to containing only singletons (VA1, WV3) or being collected under non-standard sampling methods (VA4). Colonies were obtained from 10 genera of wood hosts. From

these 313 individual millipedes, 5,154 CFUs were counted, and 1,310 isolates retained. Of these, 1,002 isolates were resolved to Kingdom Fungi, and 965 to at least genus. The remaining 308 isolates did not yield usable DNA templates, possibly due to slow growth, contamination, or both, and were excluded from this study. The isolates that were included represent a large amount of diversity, including at least 183 genera in 40 fungal orders from 4 phyla.

Millipede Behavior

In general, *B. lecontii* were found in forested areas under downed woody material of various sizes. Some individuals were found in leaf litter near logs, and these individuals were primarily adult males. Most colonies included 15 or fewer individuals, but numerous logs yielded several colonies. Colonies were multi-generational, containing a mix of hatchlings, juveniles, adults, and older adults that were several years old based on their number of segments. In a few cases, single-sex colonies were found, but most colonies contained a mixture of sexes. Our study recovered 102 males and 146 females.

Most millipedes were engaged in feeding behavior, with their heads buried in fungus growing on the log. Some millipedes were feeding alone, but most were in a partial or complete pinwheel formation (Figure 1A-B) Multigenerational colonies frequently had adults and hatchlings or juveniles sharing a single feeding site (Figure 1A).

Males were observed caring for eggs in close proximity to the main colony, particularly if collecting was done in early summer (May-June) (Figure 1C). Males with

eggs were not immobile, however, and would transport the eggs to a safe location if the log was overturned (Figure 1D).

Three sites, WV4, SC1, and AR2, had millipedes that displayed atypical behaviors. In WV4, the site as a whole was very moist, and a group of millipedes was observed climbing a standing *Carpinus caroliniana* snag (Figure 2A). The tree was covered in moss and had evidence of fungal colonization. The snag was positioned on a steep west-facing slope. Millipedes were observed as high as fifteen feet off the ground. In SC1, a large fallen *Liriodendron* log was observed with several hundred millipedes gathered on the top of the log (Figure 2B). Conditions at this site were also wet, but the log was not flooded. Aside from the exposed location, these millipedes were forming typical pinwheel aggregations and feeding on fungal mycelium. In AR2, 10 millipedes were found at an extremely arid site, next to a power line clearing without cover or wood debris (Figure 2C). These millipedes were a foot off the ground, in a healthy *Ulmus alatus* tree. There were no signs of moisture or fungus on the tree.

FIGURE 2: *B. lecontii* displaying atypical behaviors. **A**: Colony from site WV4, climbing a rotting snag. **B**: Exposed colony from site SC1, sitting on top of log. **C**: Colony from site AR2, climbing a living tree in an arid site.

Morphotype CFU counts

All colony-forming units on millipede macerate plates were quantified and sorted into one of six broad morphological classes: (non-Hypocreales) white filamentous fungi, entomopathogenic Hypocreales, dematiaceous yeasts, zygomycetes, miscellaneous yeasts, and "Other." The "Other" class included more than a dozen recognizable morphotypes, including *Penicillium* sp., *Acremonium* sp., and *Pestalotiopsis* sp. Many of these genera were quantified separately to ensure that they were not overlooked beneficial fungi, but were later combined.

White filamentous fungi were characterized by white hyphae spread thinly in a loose colony with no other outwardly identifiable characteristics. Entomopathogenic Hypocreales formed a tight, dense colony of white to yellow hyphae, sometimes stained the media red or yellow, or in other cases, formed thin mats that produced crusts of dry conidia. Dematiaceous yeasts had slow-growing, heavily melanized colonies that frequently formed a raised mound in the center of the colony. Zygomycetes were identified by their characteristic sporangia, or by a *Mortierella*-like morphology (roseate growth appressed to the media, garlic odor, rare sporangia). All colonies that appeared creamy and non-hyphal were grouped into the miscellaneous yeast category. All other morphotypes were placed in the "Other" category. Similar to the 'Other' category, morphologically unique yeasts were tallied separately to ensure that these fungi were not inadvertently overlooked, and later combined.

Of the defined morphotypes, miscellaneous yeasts and entomopathogenic Hypocreales dominated across all *Brachycybe lecontii* colonies (18.8% and 17.4% of all

CFUs, respectively). Zygomycetes accounted for 7.6% of all CFUs, followed by white filamentous fungi (7.4%) and dematiaceous yeasts (6.7%). The "Other" category accounted for 41.9% of all CFUs.

At the colony level (Figure 3), entomopathogenic Hypocreales were present in 75% of colonies (mean: 17.2 CFUs, median: 5). Likewise, dematiaceous yeasts were present in 75% of colonies (mean: 6.7 CFUs, median: 3). Miscellaneous yeasts were present in 66.7% of colonies (mean: 21 CFUs, median: 6.5), followed by 61.7% for zygomycetes (mean: 9.1 CFUs, median: 7), and 48.3% for white filamentous fungi (mean: 11.4 CFUs, median: 5). The "Other" morphotype was present in all colonies (mean: 31.6 CFUs, median: 13).
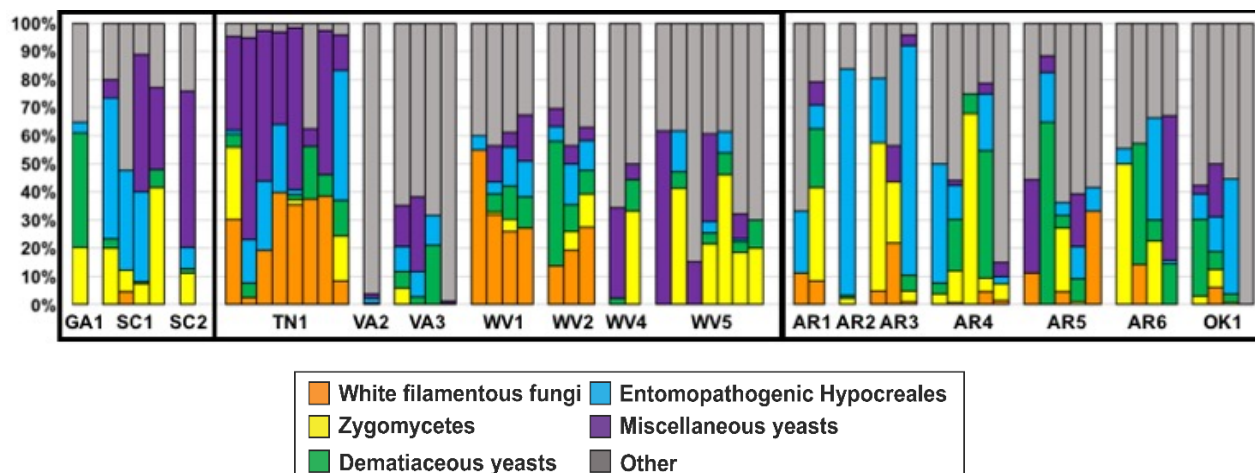
FIGURE 3: Stacked bar chart showing the percentage of CFUs from each colony (=individual bar) from each fungal morphotype. Each cluster of bars represents a site, and larger boxes indicate which millipede clade the sites are from (Clade 1, 3, 4).

At the site level (Figure 4), dematiaceous yeasts and the "Other" morphotype were recovered from all sites, zygomycetes from 94.1% of sites, entomopathogenic Hypocreales from 94.1% of sites, miscellaneous yeasts from 88.2% of sites, and white

filamentous fungi from 58.8% of sites.
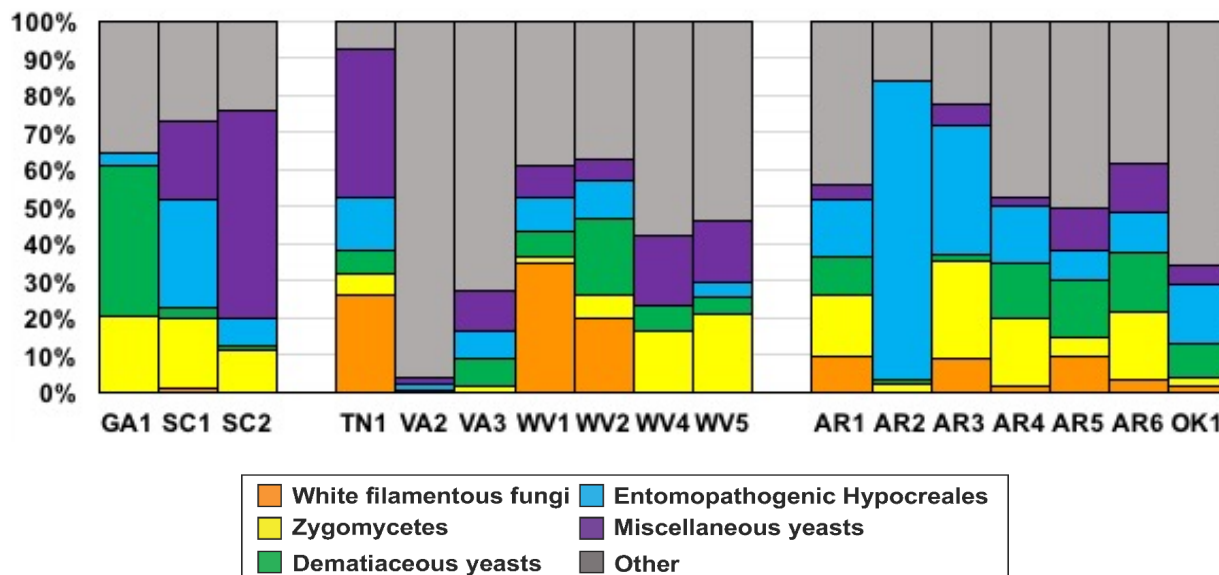
FIGURE 4: Stacked bar chart showing what percentage of CFUs from each site were from each fungal morphotype. Each cluster of bars indicates millipede clade 1, 3 and 4.

Next, the data were partitioned by millipede clade, millipede sex, and wood host, at the colony level. Three millipede clades were sampled. Clade 1 contained 53 individuals from 6 colonies in 3 sites, Clade 3 contained 156 individuals from 29 colonies in 7 sites, and Clade 4 contained 104 individuals from 24 colonies in 7 sites. Incidence of fungal morphotypes varied by clade (Table 2): Entomopathogenic Hypocreales represented almost a third of all CFUs from Clade 4 yet were less than one-tenth of the total CFUs from Clade 3. Likewise, white filamentous fungi varied considerably across clades, representing 12.5% of total CFUs from Clade 3, but below 3% for the other two clades. Miscellaneous yeasts represented one-third to one-fifth of total CFUs from Clade 1 and Clade 3, but less than one-tenth for Clade 4. The remaining fungal morphotypes have, in general, equal representation across clades.

TABLE 2: Table indicating what percentage of CFUs from each millipede clade were from each fungal morphotype.

Morphotype	Clade 1	Clade 3	Clade 4
Wh. fila. Fungi	0.5%	12.5%	2.0%
Entomo. Hypo.	17.7%	9.0%	31.6%
Demat. Yeasts	4.9%	5.5%	9.7%
Zygomycetes	13.4%	4.9%	9.5%
Misc. Yeasts	39.1%	20.8%	6.5%
Other	24.4%	47.3%	40.8%

Millipedes were separated into four life stages: male (gonopods visible), female (adult size, no gonopods), juvenile (not adult size, no gonopods visible), and hatchlings (white colored, extremely small). For analyses by millipede sex, juveniles and hatchlings were excluded because they could belong to either sex. The only dramatic difference between males and females was in the abundance of white filamentous fungi, where 13.1% of CFUs from males were white filamentous fungi, compared to 5.8% of females (Table 3).

TABLE 3: Table indicating what percentage of CFUs from each millipede sex were from each fungal morphotype

Morphotype	Male	Female
Wh. fila. fungi	13.1%	5.8%
Entomo. Hypo.	21.7%	20.0%
Demat. Yeasts	11.4%	6.4%
Zygomycetes	6.5%	9.1%
Misc. Yeasts	15.1%	20.2%
Other	32.1%	38.5%

Millipedes were collected from 10 different genera of wood hosts. The most common wood hosts were *Liriodendron* (23 colonies) and *Quercus* (17), followed by *Acer* (4), *Carya* (4), *Fagus* (3), *Pinus* (3), *Betula* (2), *Ulmus* (2), *Fraxinus* (1), and *Carpinus* (1).

There was only one notable difference between the two most common wood hosts. In

Liriodendron, 21.8% of CFUs were from miscellaneous yeasts, compared to 6.0% in

Quercus. The remaining morphotypes did not vary strongly between the two hosts

(Figure 5).
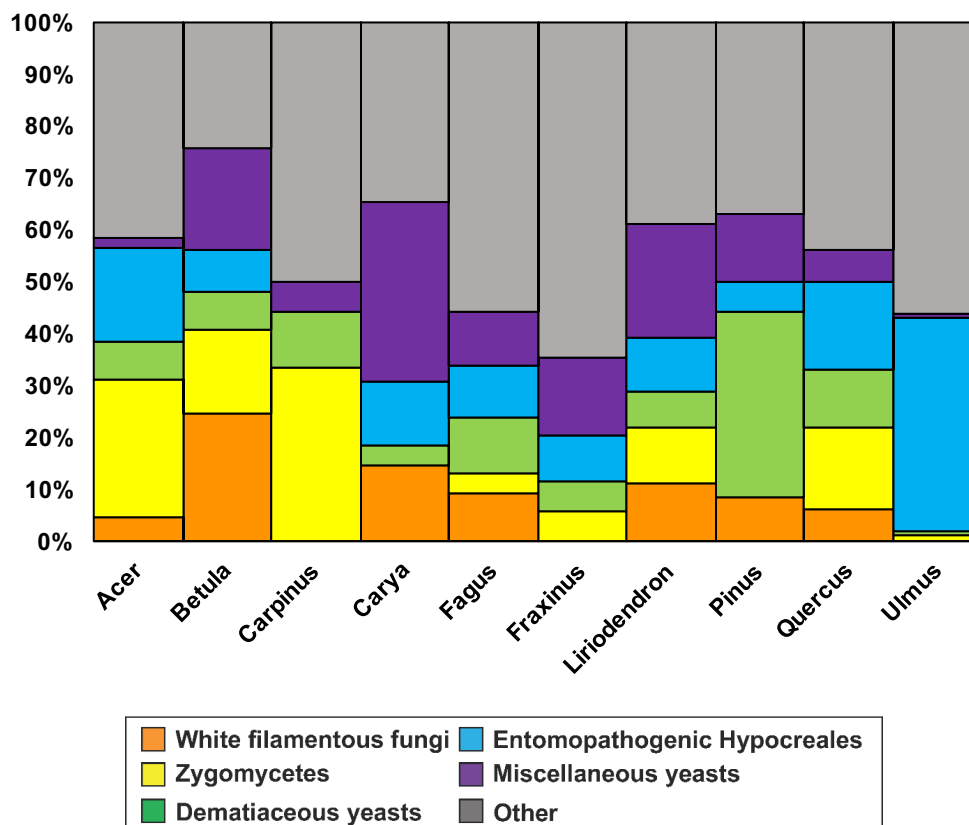
FIGURE 5: Stacked bar chart showing percentages of CFUs from each wood host represented by each fungal morphotype.

The number of colonies sampled across all the uncommon hosts (20) was similar

to the number of colonies sampled from each of the common hosts (23 for *Liriodendron*,

17 for *Quercus*), so the percentages of each morphotype across the three classes

Liriodendron, Quercus, and 'Other hosts' were compared. There were no notable

differences between the three classes for any morphotype (Table 4). Individually, the

uncommon hosts had widely varying percentages for all the morphotypes, as shown in Figure 5.

TABLE 4: Table indicating what percentage of CFUs from *Liriodendron*, *Quercus*, or other substrates (combined) were from each fungal morphotype.

Morphotype	*Liriodendron*	*Quercus*	Other (combined)
Wh. fila. fungi	11.1%	6.2%	7.8%
Entomo. Hypo.	10.6%	16.8%	13.0%
Demat. Yeasts	6.9%	11.1%	10.3%
Zygomycetes	10.7%	16.0%	10.9%
Misc. Yeasts	21.8%	6.0%	12.6%
Other	38.6%	44.0%	45.4%

Sequenced Isolates

Across all millipedes, 1,310 fungal isolates were collected and retained. Of these, 1,002 isolates were sequenced and resolved to Kingdom Fungi, and 965 to at least genus. These isolates represent a large amount of diversity, including at least 183 genera in 40 fungal orders from 4 phyla.

After sequencing, it became clear that the defined morphotypes (see previous section) were not always taxonomically restricted and often contained members from more than one order of fungi. "White filamentous fungi" often included members of the Polyporales, Xylariales, Pleosporales, and occasionally a (non-entomopathogenic) member of the Hypocreales. "Entomopathogenic Hypocreales" only contained fungi from the well-documented entomopathogenic families of the Hypocreales, Cordycipitaceae, Clavicipitaceae, and Ophiocordycipitaceae. "Dematiaceous yeasts" contained members from the Chaetothyriales, Chaetosphaeriales, Capnodiales, Mycosphaerellales, and Coniochaetales. Zygomycetes contained members of the

Mortierellales, Mucorales, and Umbelopsidales. "Misc. yeasts" contained Tremellales, Saccharomycetales, and Dothideales. The remaining 24 orders generally fell into the "Other" morphotype, with individual isolates occasionally falling into the other defined morphotypes. Since the morphotypes were taxonomically heterogeneous, analyses were conducted at the order or genus level.

The most common order was the Hypocreales, containing 25.9% of all isolates resolved to at least order. Of that 25.9%, 42.6% of isolates were from the three entomopathogenic families listed previously. The five next most common orders were the Polyporales (8.6%), Chaetothyriales (8.2%), Xylariales (6.2%), Capnodiales (5.8%), and Eurotiales (5.6%). All other orders contained fewer than 50 isolates (<5%).

The most common genus was *Trichoderma* (45 isolates), followed by *Verticillium* (44), *Umbelopsis* (35), *Penicillium* (34), and *Mortierella* (32). There were 74 genera with only a single isolate, and only 24 genera had 10 or more isolates.

Community and diversity analyses

Alpha diversity was assessed by millipede clade, wood host, and site. Clade 1 included 32 genera, Clade 3 included 148, and Clade 4 included 69. The number of fungal genera obtained from each wood host were as follows: *Liriodendron* (114 genera), *Quercus* (74), *Betula* (54), *Carya* (45), *Fagus* (33), *Ulmus* (19), *Acer* (18), *Pinus* (11), *Carpinus* (10), and *Fraxinus* (10).

At the site level, alpha diversity varied from 3 genera at SC2 to 63 genera at WV1 with a mean of 23 per site (Table 5). In addition to alpha diversity, Shannon's diversity index and Shannon's equitability were also calculated for each site. Shannon's

diversity index ranged from 0.95 in SC2 to 3.79 in WV2. Shannon's equitability ranged

from 0.977 in AR1 and VA2 to 0.848 in TN1 (Table 5). Sites with the five highest

Shannon's diversity index values did not overlap with sites with the five highest site

equitability values. Conversely, sites with the lowest diversity index values did overlap in

at least two of five sites (Table 5).

TABLE 5: Collection information and diversity indices for each site.

Site	No. of Millipedes sampled	Alpha diversity	Shannon's diversity index (H)	Shannon's equitability (EH)
AR1	6	11	2.342	0.977
AR2	6	14	2.497	0.946
AR3	13	17	2.590	0.914
AR4	22	26	3.064	0.941
AR5	14	19	2.801	0.951
AR6	12	18	2.583	0.894
GA1	9	13	2.378	0.927
OK1	14	18	2.737	0.947
SC1	22	14	2.262	0.857
SC2	4	3	0.950	0.865
TN1	42	42	3.170	0.848
VA1	4	34	3.301	0.936
VA2	8	11	2.342	0.977
VA3	15	24	3.043	0.957
VA4	5	7	1.787	0.918
WV1	29	63	3.784	0.913
WV2	24	57	3.790	0.938
WV3	4	16	2.599	0.937
WV4	12	24	3.033	0.954
WV5	36	34	3.045	0.864

Next, PerMANOVAs were used to check for significant differences among fungal

communities by clade and wood host. For clade, pairwise comparisons indicated

significant differences in communities between Clade 1 and Clade 3 (p < 0.001) and

Clade 3 and 4 (p < 0.05), but not between Clade 1 and Clade 4 (p > 0.05). For wood

host, pairwise comparisons indicated significant (p < 0.05) differences among 18 host

combinations (Supplemental Table 5). Twenty additional pairwise comparisons were not

significant (Supplemental Table 5). Two hosts, *Ulmus* sp., and *U. alatus*, accounted for

15 of 18 significant differences, and both hosts were significantly different (p < .001)

from *Acer, Betula, Carya, Fagus, Liriodendron, Pinus,* and *Quercus.* The remaining

three significant (p < 0.05) interactions included pairings of *Acer, Betula, Fagus,*

Liriodendron, and *Quercus.*

      The network inferred from co-occurrence data revealed a complex topology with

several co-dominant foci (Figure 6). As a whole, community structure was

heterogeneous across millipede clades and wood hosts. However, some genera were

consistently present in most clades and wood hosts as indicated by betweenness-

centrality results. Twelve fungal genera showed high connectivity across the whole

network (betweenness-centrality values > 0.5) (Figure 7). These included *Phialophora*

(1.55)*, Ramichloridium* (1.44)*, Mortierella* (1.28)*, Trichoderma* (1.03)*, Mucor* (1.02)*,*

Verticillium (0.90)*, Phanerochaete* (0.89)*, Fonsecaea* (0.84)*, Penicillium* (0.75)*,*

Umbelopsis (0.73)*, Cosmospora* (0.68)*,* and *Xylaria* (0.63). All other fungal genera fell

below the 0.5 threshold including 144 genera with a betweenness-centrality values of

0.0 (Figure 7).
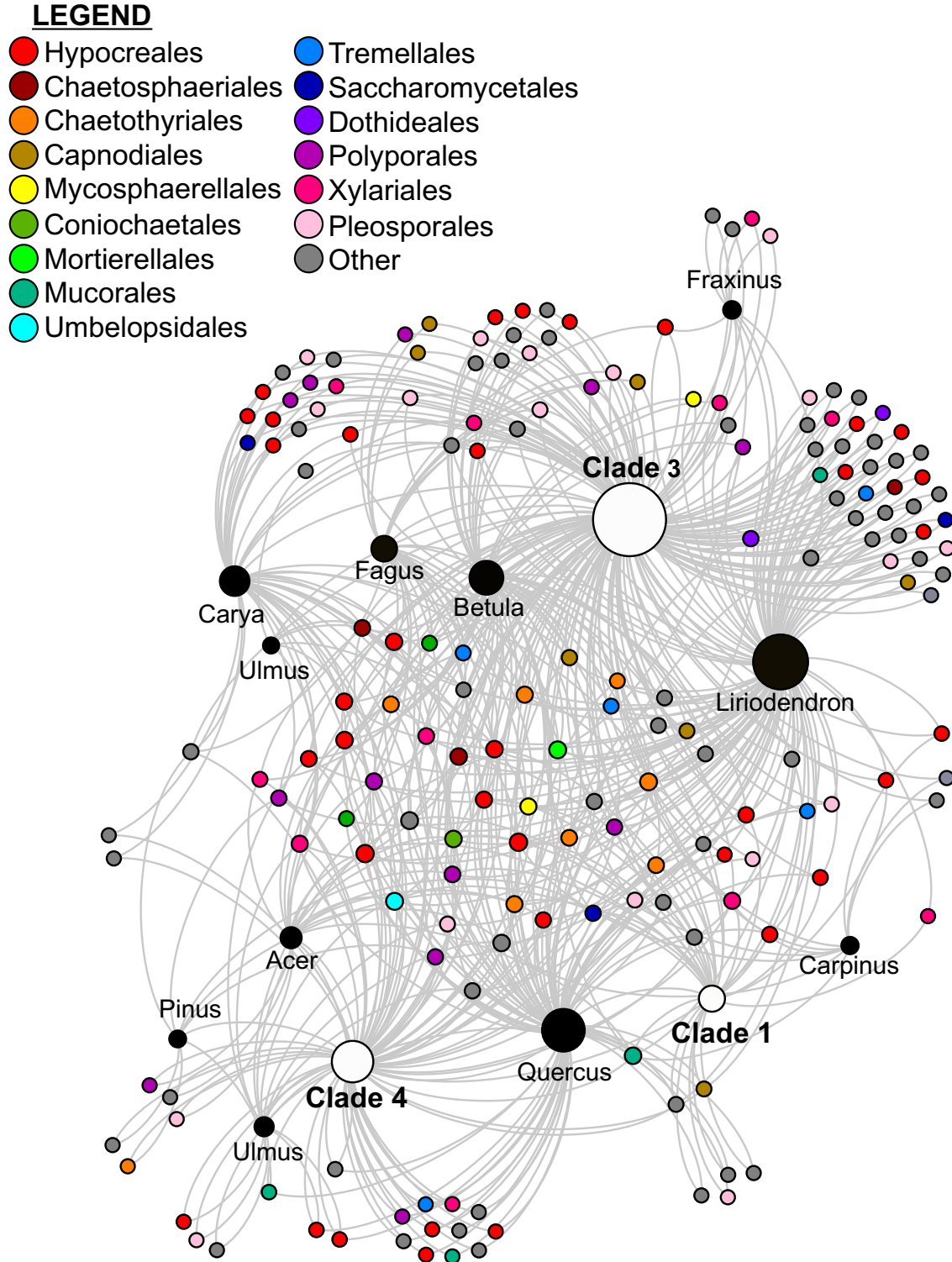
FIGURE 6: Fungal community connectivity network across *B. lecontii* clades and wood hosts. Unlabeled nodes represent unique fungal genera, color-coded by fungal order. White and black nodes represent clades and wood hosts. For these, the size of the circle represents the relative sample size for that group.
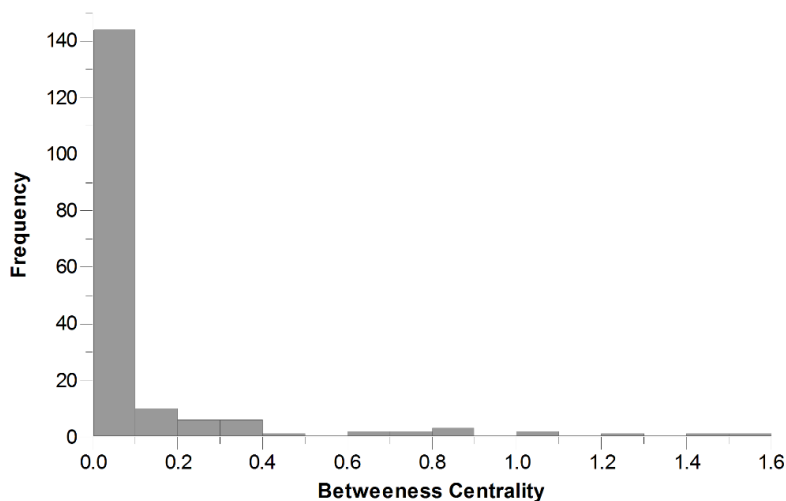
FIGURE 7: Histogram showing distribution of betweenness-centrality values for 183 fungal genera.

Modularity was assessed for the entire network and yielded a score of 0.239. Six distinct modules were detected.

Putative new species

At least seven putative new species were identified based on previously established criteria, but only three were investigated. The four not examined are "aff. *Coniochaeta"* (Coniochaetales), "aff. *Leptodontidium*" (Helotiales), "*Pseudonectria* aff. *buxi*" (Hypocreales), and "aff. *Oidiodendron*" (Onygenales).

"Aff. *Fonsecaea"* is represented by 11 isolates from six collection sites (AR3, AR4, OK1, SC2, VA4, and WV1). These isolates are 95% identical to strain "*Fonsecaea* sp. CBS 102252". The phylogenetic tree shown in Figure 8 was generated in MEGA7 (Kumar *et al*. 2016) and inferred by using the maximum likelihood method based on the Kimura 2-parameter model (Kimura 1980). The alignment contained 1,391 positions, but only 591 were retained in the final dataset. Fewer than 5% alignment gaps, missing

data, and ambiguous bases were allowed at any position. Although there are 11 isolates

of this PNS, only 9 were used in this phylogeny.
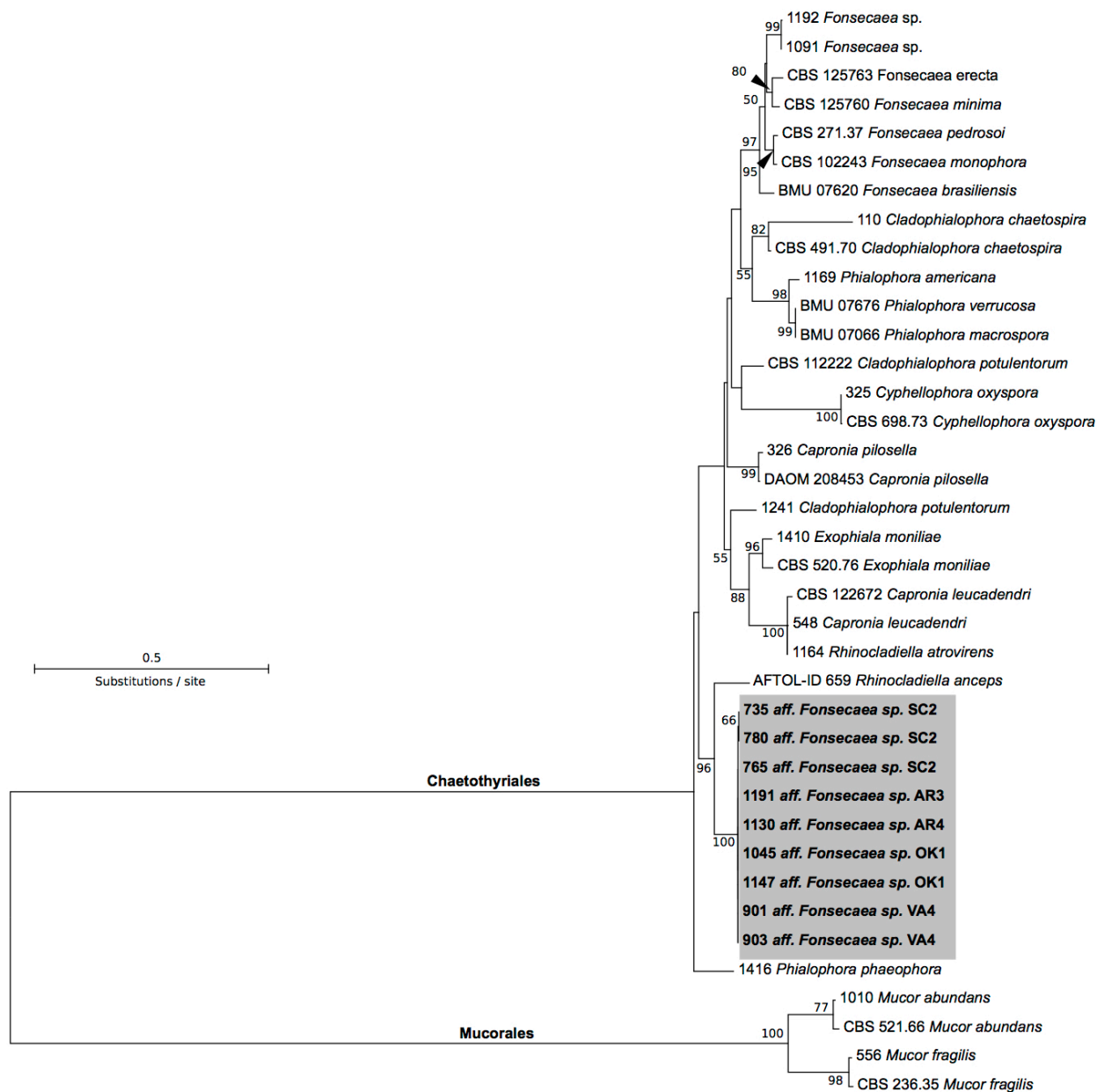
FIGURE 8: Concatenated ITS+LSU maximum likelihood phylogenetic tree for "aff. *Fonsecaea*," a putative new species. Bootstrap support is indicated near each node, and only values greater than 50% are shown. The grey box indicates the isolates belonging to aff. *Fonsecaea* sp.

"*Mortierella* aff. *ambigua*" is represented by 27 isolates from seven collection

sites (AR1, AR3, AR4, VA3, WV2, WV4, and WV5). These isolates are 92% identical to

strain "*Mortierella ambigua* CBS 450.88". All isolates of "*Mortierella* aff. *ambigua*"

produced large, granular, firm, lipid-rich structures as the cultures aged past ~10 days.

These structures grew up to at most half a centimeter across, and were present on the

surface and embedded in the media. When crushed, these structures appeared to be

filled with large thin-walled cells, each filled with lipids and vacuoles (Figure 9). The

function of these structures is unknown, and their presence has not been previously

reported in the literature.
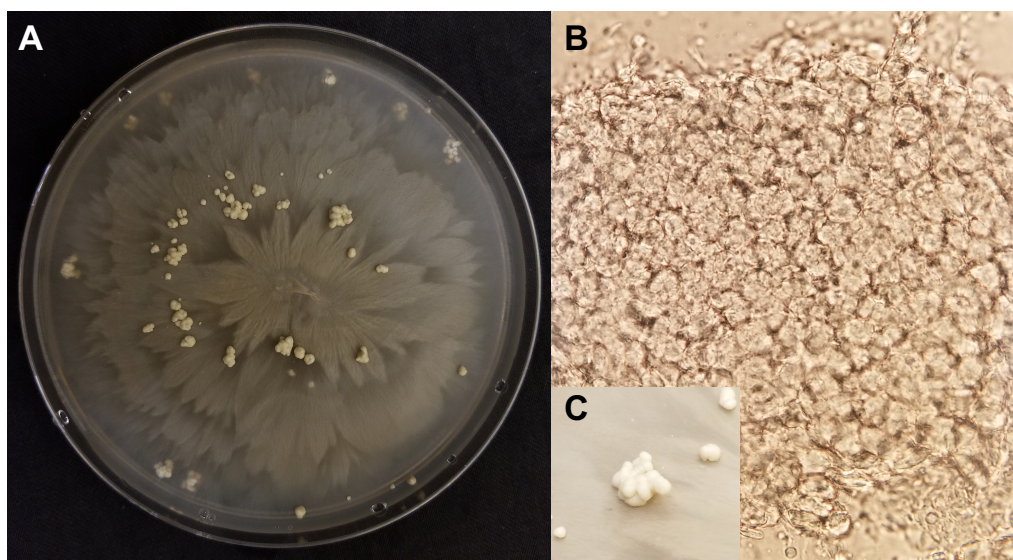
FIGURE 9: Colony morphology of PNS "*Mortierella* aff. *ambigua*", isolate 1150 after six weeks of growth on GYEA. **A**: Overall colony morphology, with surface and submerged structures. **B**: Magnification 40x, squash mount. **C**: Magnification 5x.

The phylogenetic tree shown in Figure 10 was generated in MEGA7 (Kumar *et al*. 2016) and inferred by using the maximum likelihood method based on the general time-reversible model (Nei and Kumar 2000). The alignment contained 1,234 positions, but only 918 were retained in the final dataset. Fewer than 5% alignment gaps, missing

data, and ambiguous bases were allowed at any position. Although there are 27 isolates

of this PNS, only 16 were used in this phylogeny.
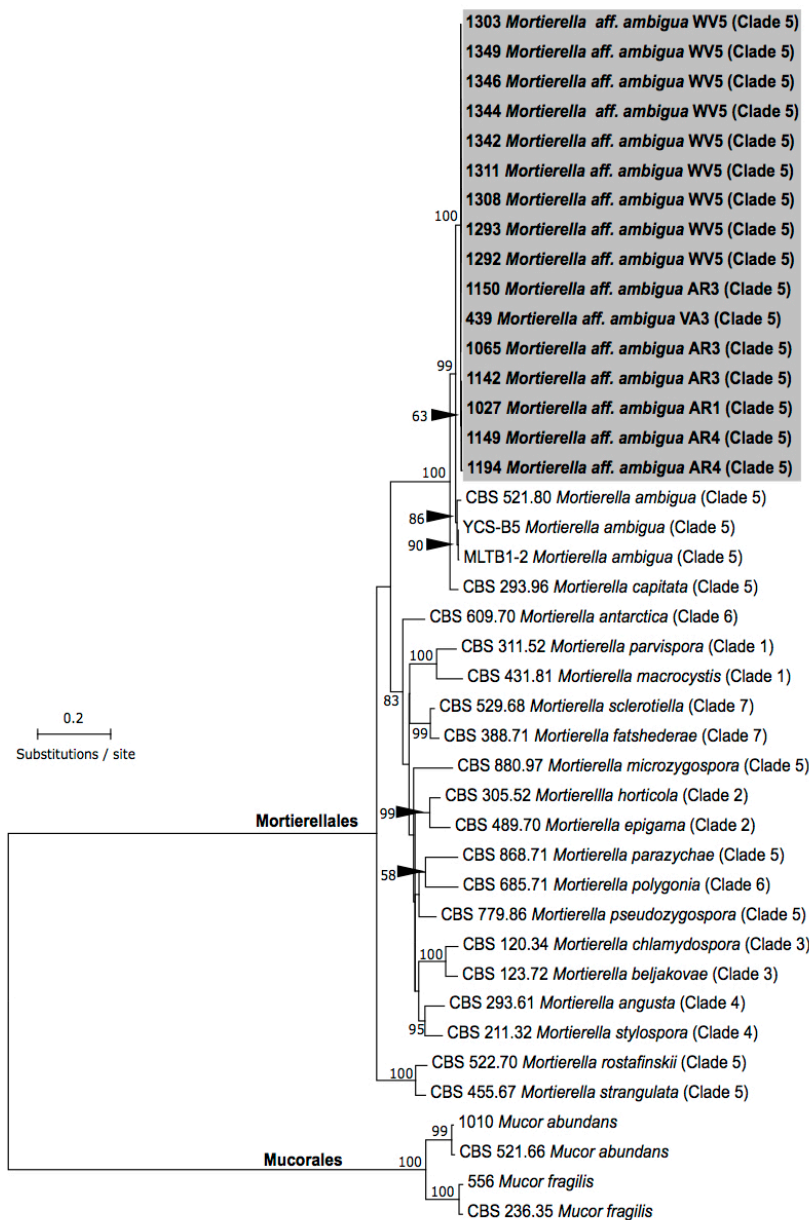
FIGURE 10: Concatenated ITS+LSU maximum likelihood phylogenetic tree for "*Mortierella* aff. *ambigua,*" a putative new species. Bootstrap support is indicated near each node, and only values greater than 50% are shown. The grey box indicates the isolates belonging to *Mortierella* aff. *ambigua*.

Last, "aff. *Apophysomyces* sp." is represented by five isolates from one site

(OK1). These isolates are 84% identical to strain "*Apophysomyces ossiformis* strain

UTHSC 04-838". Sporangial morphology of these isolates aligns with described features

for this genus (Alvarez *et al*. 2010). The phylogenetic tree shown in Figure 11 was

generated in MEGA7 (Kumar *et al*. 2016) and inferred by using the maximum likelihood

method based on the Tamura 3-parameter model (Tamura 1992). The alignment

contained 1,389 positions, but only 814 were retained in the final dataset. Fewer than

5% alignment gaps, missing data, and ambiguous bases were allowed at any position.

Although there are 5 isolates of this PNS, only 3 were used in this phylogeny.
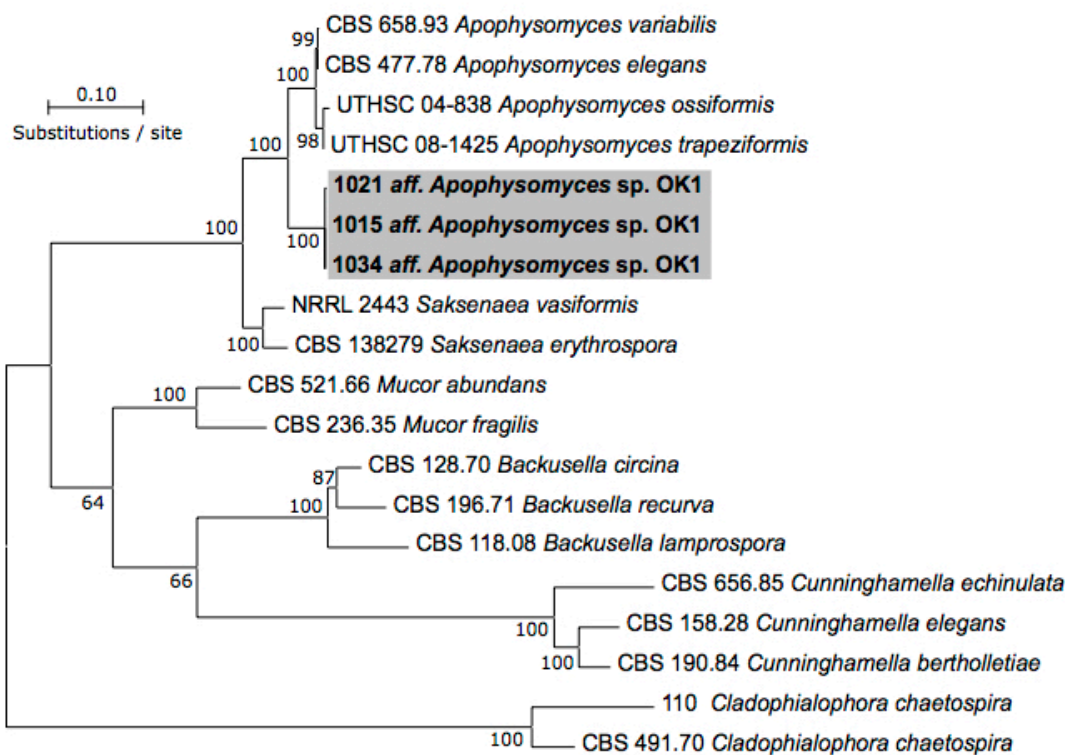
FIGURE 11: Concatenated ITS+LSU maximum likelihood phylogenetic tree for "aff. *Apophysomyces,*" a putative new species. Bootstrap support is indicated near each node, and only values greater than 50% are shown. The grey box indicates the isolates belonging to aff. *Apophysomyces*.

DISCUSSION

Who's who in the fungal community?

Brachycybe-associated fungi were sorted into five morphotypes, three of which were not taxonomically restricted. "White filamentous fungi" included members of four fungal orders, "Dematiaceous yeasts" had five orders and "Miscellaneous yeasts" had three orders spanning two fungal phyla. Quantification of CFUs provides a simplified coarse-scale approach to characterizing fungal communities. However, this method has several limitations, including morphological crypsis among distantly related taxa (Ko *et al.* 2011).

Based on sequencing results, *Brachycybe lecontii* associates with a large, diverse community of fungi, including at least 183 genera in 40 fungal orders from 4 phyla. Of all the genera of fungi found in this study, 40% were represented by a single isolate, and only 13% had 10 or more isolates. These genera are not evenly distributed among different sites, wood hosts, and millipede clades. But, across large groups of millipedes, certain genera appear more frequently than expected by chance.

Betweenness-centrality scores from the network analysis reveal that a small group of fungal genera make up the core of the fungal network. This core included two members of the Chaetothyriales, one Mycosphaerellales, one Mortierellales, three Hypocreales, one Mucorales, one Polyporales, one Eurotiales, one Umbelopsidales, and one Xylariales. The network analysis revealed that these fungi are consumed by many individuals across different lineages of *B. lecontii* and across many wood hosts, indicating that they may be the preferred fungal food source for these millipedes.

The role of white filamentous fungi

Casual observations of naturally-occurring *Brachycybe* colonies led to the hypothesis that white filamentous fungi, mostly in the order Polyporales, are the primary food source for *B. lecontii*. However, both morphotype CFU counts and isolate sequencing data do not support this hypothesis. This morphotype was in extremely low incidence, compared to the other dominant morphotypes, and was recovered from only 48% of colonies. Since the Polyporales are only a subset of fungi in this morphotype, the true incidence of Polyporales is even lower. When investigated with culture-independent sequencing, the proportion of Polyporales remained low at *ca.* 2% (Michael Brewer, pers. comm.).

Although it is clear that *B. lecontii* are not consuming much of the white filamentous morphotype, that does not discount the possibility that these fungi may play a different but vital role during part of or throughout in the millipede life cycle. This study is designed to capture the fungi on or in *B. lecontii*, so fungi used in other ways, and not present on or in the millipede, would not be detected.

Unlike most fungi recovered during this study, Polyporales are perennial and can persist across many years, surviving through harsh conditions (Halme *et al.* 2009). In the field and in the laboratory colonies, *B. lecontii* displayed a marked preference for moister logs, and it is possible that in times of reduced moisture, the millipedes rely on moisture from Polyporales. Other arthropods use Polyporalean fruiting bodies for protection from changes in moisture (Mitgaard *et al.* 1998, Jonsell *et al.* 2001) Another possibility is that *B. lecontii* may only consume a liquid filtered extract of Polyporales. Ongoing studies by Wong et al. (*in prep*) support this hypothesis in that *Brachycybe*

have a brush-like labrum, which would likely prevent ingestion of fungal hyphal

fragments, but not spores. If no viable fungal propagules are present in the millipede, no

fungi will be detected using the methods of this study. In addition, little is known of

where the millipedes go or what they do in the winter (Gardner 1975, Shelley *et al.*

2005), and it is also possible that Polyporales and other white filamentous fungi may

play some role during this period of time.

Millipede Behavior

Pinwheel formations were abundant at all sites in the field and in laboratory

colonies. While documented in the literature for a half-century (referred to as star-

clusters, pinwheels or stellate arrangements) their function remains unknown (Gardner

1975, Manton 1961). These self-assembled formations may be a result of merely

gathering around a common feeding site, or they may have another role, such as aiding

in physical defense through aggregation (Dury et al. 2014, Curley et al. 2015) and/or

concentrating millipede defensive compounds (Wood et al. 2000; Shear 2015). Another

possibility is adults facilitate cooperative feeding that may benefit juveniles and

hatchlings, who have smaller mouthparts. These hypotheses remain to be explored.

Observations made at three sites indicated atypical "summit-like" behavior

(Hodge *et al.* 2017) in *B. lecontii,* with individuals exposing themselves to the elements

and various predators. At two of those three sites, it was found that entomopathogenic

Hypocreales dominated the fungal community. In site SC1, 29.3% of all CFUs were

entomopathogenic Hypocreales, and the site had an abundance of one genotype of

Lecanicillium. Eighty-one percent of millipedes at SC1 harbored *Lecanicillium*, some of

which were confirmed to be infected with the entomopathogenic nematode,

Heterohabditis sp. (data not shown). In AR2, 80.2% of all CFUs were

entomopathogenic Hypocreales, including *Verticillium, Metarhizium, Pochonia,* and

Purpureocillium. Whether or not these fungi are the cause of the atypical behavior

observed at these sites is unclear, but studies of entomopathogenic Hypocreales in

insects indicate behavioral modification of the host is a possibility. For example, an

arboreal carpenter ant (*Camponotus leonardi*) is occasionally parasitized by the

Hypocrealean fungus *Ophiocordyceps unilateralis.* This fungus manipulates its host to

travel to an optimum microclimate and clamp its mandibles onto a plant before death,

improving the ability of the fungus to grow and reproduce (Andersen *et al*. 2009). Sexual

behavior may also be manipulated by entomopathogens: male *Ceratitis* fruit flies

infected with *Metarhizium anisopliae* delayed mating to groom, an activity that spreads

spores all over the body (Dimbi *et al.* 2009). When females were infected, males found

them highly attractive and attempted mating, even if the female was deceased.

Future studies should attempt to fulfull Koch's Postulates with fungi isolated from

millipedes displaying unusual behaviors, to determine if these isolated fungi are the

cause of the behaviors.

Putative New Species

In this study, evidence was found for at least seven putative new species (PNS),

but only three were studied in detail. One of the three PNS studied was from the

Ascomycota, in the order Chaetothyriales, and two were from the Mucoromycota, in the

orders Mortierellales and Mucorales.

Based on NCBI BLAST results, the PNS from the Chaetothyriales was called "aff. *Fonsecaea* sp." The *Fonsecaea* isolate most similar to isolates from this study matched at 95% of loci, but there was only a single isolate of *Fonsecaea* in the top matches. Most of the next closest matches were to *Rhinocladiella/Ramichloridium anceps*, and were accompanied by a significant drop in percent match from the *Fonsecaea* isolate. In addition, the fungus *R. anceps* belongs to a different order, either the Mycosphaerellales or the Capnodiales (still under debate, Crous *et al.* 2009). Many of the orders that contain dematiaceous fungi, like Chaetothyriales, Chaetosphaeriales, Capnodiales, and others, are not resolvable with just the ITS region, further complicating the issue (Crous *et al.* 2009). Many phylogenetic studies in the literature appear to have found some resolution of these orders, but other papers using fungi supposedly in the same genus as those in the first yield a different phylogenetic structure (Arzanlou *et al.* 2007).

Regardless of the exact identification and higher taxonomy for the PNS "aff. *Fonsecaea* sp.," our phylogenetic study indicates that it is not a close relative of *Fonsecaea*, and instead a close relative of *Rhinocladiella anceps*. All 11 isolates of "aff. *Fonsecaea* sp." are clonal and come from at least 6 sites and 3 wood hosts, spanning all three millipede clades. Future work will focus on morphological studies and phylogenetic analyses that will explicitly include additional loci and members of the Mycosphaerellales and Capnodiales. Once completed, a formal description will follow.

NCBI BLAST results from the two other PNS were not ambiguous, and the higher taxonomy for the Mucoromycota has already been clearly established (Spatafora *et al.* 2017). The PNS "*Mortierella* aff. *ambigua*" is represented by 27 clonal isolates from 7

sites and 5 wood hosts, spanning 2 millipede clades (Clade 3 and 4). This PNS forms a clade with known reference sequences of *Mortierella ambigua*, but is also in its own clade sister to *M. ambigua*, indicating that "*Mortierella* aff. *ambigua*" is a new species of *Mortierella* closely related to *Mortierella ambigua*.

An attempt was made to use a subset of *Mortierella* references representing the seven identified clades in the genus (Wagner *et al.* 2013), but our phylogeny failed to recover the same topology. Future work for "*Mortierella* aff. *ambigua*" will focus on morphological studies, particularly the unique structures produced in pure culture. Once completed, a formal description will follow.

The last PNS, "aff. *Apophysomyces* sp.," is represented by five clonal isolates from one site, OK1, in Clade 4. The genus *Apophysomyces* includes four species, and is sister to the genus *Saksenaea* (Alvarez *et al.* 2010). This PNS forms a clade with known reference sequences of all four species known to exist in *Apophysomyces*, but is also in its own clade sister to the known species, indicating that "aff. *Apophysomyces* sp." is likely a new genus of closely related to *Apophysomyces*. The amount of divergence between the PNS and known species of *Apophysomyces* is greater than the divergence within known species of *Apophysomyces,* further supporting the PNS as a new genus.

Future work for "aff. *Apophysomyces* sp." will focus on morphological studies. Once completed, a formal description will follow.

Despite the use of more classical culture-based approaches, the recovery of seven putative new species highlights the vast amount of undescribed fungal biodiversity associated with millipedes. Culture-independent approaches will likely

uncover many more new species, possibly including some from unculturable lineages of fungi.

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SUPPLEMENTAL TABLES

SUPPLEMENTAL TABLE 1: Level III and IV ecoregions used. Source: EPA, 2013.

Number	Level III	Level IV
36B	Ouachita Mountains	Central Mountain Ranges
36C	Ouachita Mountains	Central Hills, Ridges, and Valleys
36D	Ouachita Mountains	Fourche Mountains
37A	Arkansas Valley	Scattered High Ridges and Mountains
45E	Piedmont	Northern Inner Piedmont
66D	Blue Ridge	Southern Crystalline Ridges and Mountains
67H	Ridge and Valley	Southern Sandstone Ridges
67I	Ridge and Valley	Southern Dissected Ridges and Knobs
69D	Central Appalachians	Dissected Appalachian Plateau

SUPPLEMENTAL TABLE 2: NCBI reference numbers for reference sequences used in the "aff. *Fonsecaea* sp." phylogenetic tree.

Name	Strain number	ITS	LSU
Fonsecaea erecta	CBS 125763	KC886414.1	KF155186.1
Fonsecaea minima	CBS 125760	KC886416.1	KF155187.1
Fonsecara pedrosoi	CBS 271.37	NR_130652.1	KJ930166.1
Fonsecaea monophora	CBS 102243	EU938579.1	FJ358247.1
Fonsecaea brasiliensis	BMU 07620	KJ701015.1	KJ930163.1
Cladophialophora chaetospira	CBS 491.70	EU035405.1	EU035405.1
Phialophora verrucosa	BMU 07676	KJ701006.1	KJ930157.1
Phialophora macrospora	BMU 07066	KF881933.1	KJ930071.1
Cladophialophora potulentorum	CBS 112222	EU035409.1	EU035409.1
Cyphellophora oxyspora	CBS 698.73	NR_132883.1	KC455262.1
Capronia pilosella	DAOM 208453	AF050255.1	AF050255.1
Exophiala moniliae	CBS 520.76	NR_111448.1	KJ930162.1
Capronia leucadendri	CBS 122672	EU552108.1	EU552108.1
Rhinocladiella anceps	AFTOL-ID 659	DQ826740.1	DQ823102.1
Mucor abundans	CBS 521.66	JN206110.1	JN206457.1
Mucor fragilis	CBS 236.35	JN205979.1	FN650671.1

SUPPLEMENTAL TABLE 3: NCBI reference numbers for reference sequences used in the "*Mortierella* aff. *ambigua*" phylogenetic tree.

Name	Strain number	ITS	LSU
Mortierella ambigua	CBS 521.80	JX976120.1	KC018423.1
Mortierella ambigua	YCS-B5	KP744427.1	KP744409.1
Mortierella ambigua	MLTB1-2	KP744425.1	KP744407.1
Mortierella capitata	CBS 293.96	JX976123.1	KC018334.1
Mortierella antarctica	CBS 609.70	NR_111580.1	NG_042563.1
Mortierella parvispora	CBS 311.52	NR_077185.1	HM849689.1
Mortierella macrocystis	CBS 431.81	JX975897.1	KC018437.1
Mortierella sclerotiella	CBS 529.68	NR_145298.1	HQ667387.1
Mortierella fatshederae	CBS 388.71	JX976003.1	JX976136.1
Mortierella microzygospora	CBS 880.97	NR_111569.1	HQ667394.1
Mortierella horticola	CBS 305.52	NR_111572.1	NG_042556.1
Mortierella epigama	CBS 489.70	NR_077210.1	HQ667367.1
Mortierella parazychae	CBS 868.71	HQ630283.1	HQ667362.1
Mortierella polygonia	CBS 685.71	NR_111562.1	NG_042546.1
Mortierella pseudozygospora	CBS 779.86	JX975960.1	KC018353.1
Mortierella chlamydospora	CBS 120.34	HQ630354.1	HQ667430.1
Mortierella beljakovae	CBS 123.72	NR_111584.1	NG_042568.1
Mortierella angusta	CBS 293.61	NR_111555.1	HQ667358.1
Mortierella stylospora	CBS 211.32	NR_111556.1	HQ667359.1
Mortierella rostafinskii	CBS 522.70	NR_111586.1	NG_042570.1
Mortierella strangulata	CBS 455.67	HQ630359.1	HQ667437.1
Mucor abundans	CBS 521.66	JN206110.1	JN206457.1
Mucor fragilis	CBS 236.35	JN205979.1	FN650671.1

SUPPLEMENTAL TABLE 4: NCBI reference numbers for reference sequences used in the "aff. *Apophysomyces* sp." phylogenetic tree.

Name	Strain number	ITS	LSU
Apophysomyces variabilis	CBS 658.93	NR_130683.1	HM849695.1
Apophysomyces elegans	CBS 477.78	JN206280.1	JN206536.1
Apophysomyces ossiformis	UTHSC 04-838	NR_137035.1	FN554252.1
Apophysomyces trapeziformis	UTHSC 08-1425	NR_137034.1	FN554261.1
Saksenaea vasiformis	NRRL 2443	FR687327.1	HM776679.1
Saksenaea erythrospora	CBS 138279	KM102733.1	KM102734.1
Mucor abundans	CBS 521.66	JN206110.1	JN206457.1
Mucor fragilis	CBS 236.35	JN205979.1	FN650671.1
Backusella circina	CBS 128.70	NR_103649.1	JN206529.1
Backusella recurva	CBS 196.71	JN206265.1	JN206523.1
Backusella lamprospora	CBS 118.08	NR_145291.1	JN206531.1

Cunninghamella echinulata	CBS 656.85	JN205896.1	JN206598.1
Cunninghamella elegans	CBS 158.28	JN205888.1	JN206602.1
Cunninghamella bertholletiae	CBS 190.84	JN205878.1	HM849701.1
Cladophialophora chaetospira	CBS 491.70	EU035405.1	EU035405.1

SUPPLEMENTAL TABLE 5: Results of Tukey's HSD post-hoc test comparing the fungal communities between wood hosts where more than one colony was sampled.

Wood host #1	Wood host #2	Adj. p-value
Ulmus sp.	*Acer*	0.0000*
Ulmus sp.	*Betula*	0.0000*
Ulmus sp.	*Carya*	0.0000*
Ulmus sp.	*Fagus*	0.0000*
Ulmus sp.	*Liriodendron*	0.0000*
Ulmus sp.	*Pinus*	0.0000*
Ulmus sp.	*Quercus*	0.0000*
Ulmus alatus	*Acer*	0.0000*
Ulmus alatus	*Betula*	0.0000*
Ulmus alatus	*Carya*	0.0000*
Ulmus alatus	*Fagus*	0.0000*
Ulmus alatus	*Fraxinus*	0.0000*
Ulmus alatus	*Liriodendron*	0.0000*
Ulmus alatus	*Pinus*	0.0000*
Ulmus alatus	*Quercus*	0.0000*
Fagus	*Betula*	0.0120*
Liriodendron	*Acer*	0.0338*
Liriodendron	*Fagus*	0.0073*
Betula	*Acer*	0.0596
Carya	*Acer*	0.8868
Carya	*Betula*	0.7778
Fagus	*Acer*	0.9954
Fagus	*Carya*	0.3800
Liriodendron	*Betula*	1.0000
Liriodendron	*Carya*	0.6862
Pinus	*Acer*	1.0000
Pinus	*Betula*	0.2002
Pinus	*Carya*	0.9438
Pinus	*Fagus*	0.9990
Pinus	*Liriodendron*	0.1609
Quercus	*Acer*	0.3860
Quercus	*Betula*	0.9443
Quercus	*Carya*	0.9997
Quercus	*Fagus*	0.0878

Quercus	Liriodendron	0.8753
Quercus	Pinus	0.6380
Ulmus sp.	Carpinus	1.0000
Ulmus sp.	Ulmus alatus	1.0000

CHAPTER 3: IDENTIFYING FUNCTIONAL ROLES OF *BRACHYCYBE LECONTII-* ASSOCIATED FUNGI

ABSTRACT

Brachycybe (Wood) is a genus of fungivorous millipedes, whose fungal associates have only recently been described. The ecology of these fungi and their interactions with *B. lecontii* remain unclear. In an attempt to resolve these relationships, fungal cohort pairings and entomopathogenicity trials were conducted. Of the 156 pairings tested, 67 resulted in competitive interactions. A majority of these interactions involved inhibition or overgrowth by fungi in the Hypocreales and Polyporales, respectively. Results of a series of entomopathogenicity trials indicated that *B. lecontii* was less susceptible to entomopathogenic fungi than an insect model, *Galleria mellonella.* Conversely, *B. lecontii* exhibited high mortality when challenged with Polyporales, while *G. mellonella* was unaffected. Recent work involving network analysis shows that a dozen fungal genera play a central role in millipede-fungus associations, and results of this study indicate that individual roles vary in unexpected ways.

INTRODUCTION

Millipedes (Arthropoda: Myriapoda: Diplopoda) are non-insect arthropods with many body segments and two pairs of legs per body segment. The oldest known millipede body fossil, *Pneumodesmus newmanii*, dates to the Silurian (420-440 million years ago), and presents the earliest evidence for air-breathing in any animal (Wilson and Anderson 2004).

Millipedes are a relatively diverse group of animals, with approximately 12,000

millipede species described to date, in 16 orders and 140 families (Shear 2011). The

majority are detritivores, feeding on dead and decaying plant material and animal waste

on the forest floor, though anecdotal evidence supports herbivorous (Marek *et al.* 2012),

fungivorous (Brewer *et al*. 2012, Marek *et al.* 2012), and carnivorous lifestyles

(Srivastava and Srivastava 1967). Detritivorous millipedes play a crucial role in nutrient

cycling, particularly in nutrient-poor areas (Mattson 2012, Lawrence and Samways

2003, Bonkowski *et al.* 1998). Nearly all millipedes have a subterranean lifestyle, living

in decomposing leaf litter, in the soil, or inside wet, rotting wood substrates.

Millipedes are regularly reported in association with fungi, likely due to their

detritivorous habit. Most records of fungal-millipede interactions are cases where

millipedes occasionally graze on fungi in the environment, or where a parasitic fungus is

attacking the millipede (Lilleskov and Burns 2005, Bultman and Mathews 1996, Hodge

et al. 2017). Several species of specialist ectoparasitic fungi in the Laboulbeniales have

been recorded from millipedes (Santamaria *et al*. 2014, Enghoff and Santamaria 2015).

In the millipede pet trade, fungal infection due to poor animal husbandry is a widespread

issue, especially in the most notable pet species, the giant African millipede

(*Archispirostreptus gigas*) (McMonigle 2012). Opportunistic fungi may take advantage of

weakened millipedes, but confirmed pathogenicity on healthy animals is uncommon in

the literature (Brito 1994, Chitty 2006). At least two species of trichomycetes, obligate

arthropod gut-associated fungi, also have been reported from millipedes (Wright 1979).

Brachycybe (Wood) is a genus of millipedes in the family Andrognathidae

(Diplopoda: Platydesmida) with eight described species and at least two additional

undescribed species awaiting description (Brewer *et al*. 2012). In the United States,

there are two species in the Appalachian Mountains and three (plus two undescribed) in

northern California. All *Brachycybe* are small millipedes, with some individuals reaching

4 cm in length (pers. obs.). They are one of the few groups of arthropods that exhibit

paternal care of their offspring (Kudo *et al.* 2011). *Brachycybe* are most frequently found

living on rotten logs and branches with visible fungal growth (Shelley *et al*. 2005). They

exhibit various social behaviors including overlapping adult generations and

reproductive division of labor. They also form pinwheel-like multigenerational colonies

around resupinate fungi underneath downed woody substrates (Gardner 1975). All

members of the millipede subterclass Colobognatha, which includes *Brachycybe*, are

believed to be strict fungivores (Gardner 1975), as evidenced by their diminutive

mouthparts (Paul Marek, pers. comm.) their clustering around fungi, and the visual

damage (i.e. divots) they leave behind on fungi.

The known geographic range of *Brachycybe lecontii* extends across 13 states

from eastern Oklahoma to western South Carolina, south to Louisiana, and north to

southern West Virginia (Shelley *et al.* 2005, Brewer *et al.* 2012). Their range

encompasses five Level III and nine Level IV Ecoregions (as defined in 2013 report

from U.S. EPA). The five Level III Ecoregions include: "Ouachita Mountains" (AR, OK),

"Arkansas Valley" (AR), "Piedmont" (SC), "Blue Ridge" (GA, SC), "Ridge and Valley"

(VA), and "Central Appalachians" (TN, VA, WV). The nine Level IV Ecoregions were as

follows: "Central Mountain Ranges", "Central Hills, Ridges, and Valleys", and "Fourche

Mountains" ("Ouachita Mountains"); "Scattered High Ridges and Mountains" ("Arkansas

Valley"); "Northern Inner Piedmont" ("Piedmont"); "Southern Crystalline Ridges and

Mountains" ("Blue Ridge"); "Southern Sandstone Ridges" and "Southern Dissected

Ridges and Knobs" ("Ridge and Valley"); and "Dissected Appalachian Plateau" ("Central Appalachians"). Within this known range, five subpopulations have been identified (Shelley *et al*. 2005), four of which were recently validated using sequence data (Brewer *et al*. 2012).

In the previous chapter, we determined that *Brachycybe lecontii* associates with a large, diverse community of fungi, including at least 183 genera in 40 fungal orders from 4 phyla. This diversity includes *Peniophora*, the only fungus historically reported in association with *Brachycybe* (Gardner 1975). A majority of the fungal associates were in five broad morphological classes that encompassed 14 fungal orders. The Hypocreales were the most common order across all *B. lecontii* colonies. Community and diversity analyses confirmed high alpha diversity averaging 83 genera across three *B. lecontii* clades, and 39 genera across ten wood hosts. Overall, significant differences were found among clades and wood hosts, although twelve genera displayed high connectivity across the whole network. These genera included *Fonsecaea* and *Phialophora* (Chaetothyriales), *Cosmospora*, *Trichoderma*, *Verticillium* (Hypocreales), *Ramichloridium* (Mycosphaerellales), *Mortierella* (Mortierellales), *Mucor* (Mucorales), *Phanerochaete* (Polyporales), *Penicillium* (Eurotiales), *Umbelopsis* (Umbelopsidales), and *Xylaria* (Xylariales). In addition, some fungi were associated with atypical millipede behavior, indicating a sub-optimal fungal community.

The objective of this study is to identify the functional roles of *B. lecontii*-associated fungi, through laboratory assays of pathogenicity on millipedes, and assays to identify the interactions occurring between co-cultured fungi.

MATERIALS AND METHODS

Fungal cohort pairings

Pairings were conducted at the site level, and only isolates recovered from an individual site were paired with each other. The purpose of this experimental design was to only test interactions between fungi that could interact in nature. Five sites were selected across the study area. Two of these sites were chosen because the millipedes had exhibited atypical behavior (Chapter 2). Representatives of the dominant fungi at each site were paired (Table 1).

TABLE 1: Isolates used in fungal cohort pairings. Asterisk denotes putative new species.

SITE	FUNGAL ORDER	ISOLATE ID
TN1	Amphisphaeriales	630-*Pestalotiopsis microspore*
	Chaetosphaeriales	524-*Chaetosphaeria chloroconia*
	Chaetothyriales	626-*Cladophialophora* sp.
	Hypocreales	482-*Verticillium insectorum*
	Mucorales	556-*Mucor fragilis*
	Polyporales	523-*Irpex lacteus*
	Umbelopsidales	529-*Umbelopsis angularis*
SC1	Chaetothyriales	717-*Phialophora americana*
	Hypocreales	678-*Lecanicillium attenuatum*
	Hypocreales	701-*Verticillium insectorum*
	Mucorales	695-*Mucor fragilis*
	Polyporales	709-*Phanerochaete cumulodentata*
	Umbelopsidales	702-*Umbelopsis isabellina*
	Umbelopsidales	683-*Umbelopsis ramanniana*
WV5	Amphisphaeriales	1283-*Pestalotiopsis jesteri*
	Chaetosphaeriales	1318-*Codinaea acacia*
	Chaetothyriales	1403-*Phialophora americana*
	Hypocreales	1310-*Verticillium insectorum*
	Mortierellales	1349-*Mortierella* aff. *ambigua**
	Mucorales	1285-*Mucor circinelloides*
	Polyporales	1316-*Bjerkandera adusta*
	Umbelopsidales	1290-*Umbelopsis isabellina*
AR2	Amphisphaeriales	1217-*Pestalotiopsis* sp.
	Hypocreales	1163-*Metarhizium flavoviride*
	Hypocrelaes	1165-*Pochonia chlamydosporia*

	Hypocreales	1113-*Tolypocladium album*
	Hypocrelaes	1084-*Verticillium* sp.
	Mucorales	1001-*Mucor genevensis*
	Umbelopsidales	1024-*Umbelopsis isabellina*
AR4	Amphisphaeriales	1212-*Pestalotiopsis jesteri*
	Chaetothyriales	1091-*Fonsecaea* sp.
	Chaetothyriales	1130-aff. *Fonsecaea* sp.*
	Hypocreales	1172-*Verticillium insectorum*
	Mortierellales	1148-*Mortierella* aff. *ambigua**
	Mucorales	1016-*Mucor abundans*
	Polyporales	1143-*Trametopsis cervina*
	Umbelopsidales	1023-*Umbelopsis ramanniana*

In total, 37 isolates were tested in 156 pairings. Single 0.9-cm diameter plugs were placed 3 cm from the edge of a potato dextrose agar plate, and the paired isolate placed opposite. Each pairing was done in triplicate. Plates were allowed to grow at 20°C for 1-week intervals for up to 4 weeks total for slow-growing fungi. At the end of each 1-week period, observations of fungal interactions were taken. These interactions included overgrowth of one fungus by another, inhibition of one fungus by another, or no interaction between the paired fungi (Figure 1).
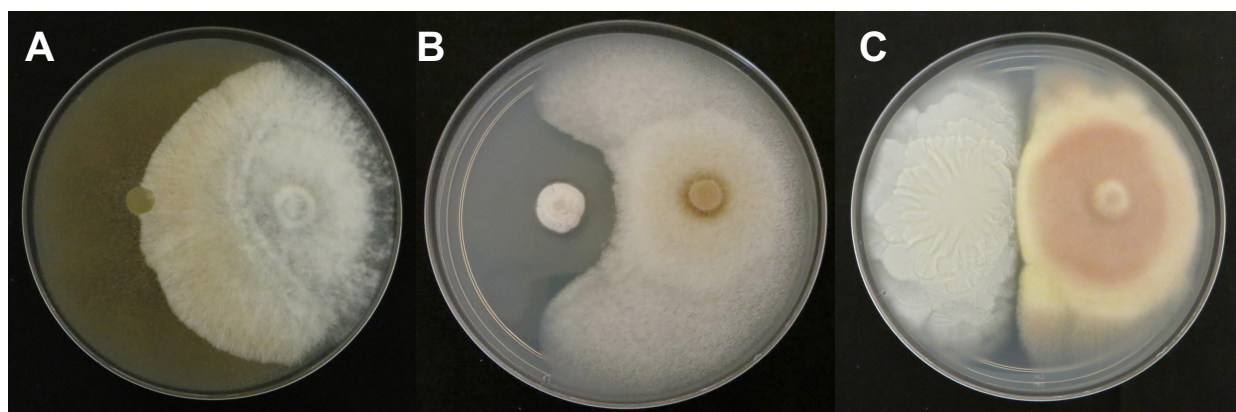

FIGURE 1: Outcomes of fungal-fungal interactions. **A**: Overgrowth; *Mucor fragilis* 556 (left) is being overgrown by *Irpex lacteus* 523 (right). **B**: Inhibition; *Pochonia chlamydosporia* 1165 (left) is inhibiting *Mucor genevensis* 1001 (right). **C**: Tie/no interaction; "*Mortierella* aff. *ambigua*" 1148 (left) has no interaction with *Umbelopsis ramanniana* 1023 (right).

Entomopathogenicity testing: live plating

Twenty-two representative isolates (Table 2) were chosen for live-plating

entomopathogenicity assays on *Galleria mellonella* (waxworms, from New York Worms

http://www.nyworms.com/) and *Brachycybe lecontii* (collected in confirmed sampling

locations in WV)*.* Waxworms were used in this assay because they are model

organisms for studies of pathogenicity/toxicity in arthropods in general (Panaccione and

Arnold 2017).

TABLE 2: Isolates used in live plating pathogenicity assays. Asterisk denotes putative new species.

FUNGAL ORDER	ISOLATE ID
Amphisphaeriales	630-*Pestalotiopsis microspora*
Chaetosphaeriales	320-*Chaetosphaeria myriocarpa*
Chaetothyriales	1244-*Capronia dactylotricha*
Chaetothyriales	1147-aff. *Fonsecaea* sp.*
Chaetothyriales	1193-*Phialophora americana*
Hypocreales	678-*Lecanicillium attenuatum*
Hypocreales	1163-*Metarhizium flavoviride*
Hypocreales	29-*Pochonia bulbillosa*
Hypocreales	1216-*Trichoderma viride*
Hypocreales	482-*Verticillium insectorum*
Mortierellales	1150-*Mortierella* aff. *ambigua**
Mortierellales	530-*Mortierella* sp.
Mucorales	1015-aff. *Apophysomyces* sp.*
Mucorales	1010-*Mucor abundans*
Mycosphaerellales	329-*Ramichloridium anceps*
Polyporales	310-*Bjerkandera adusta*
Polyporales	1158-*Ceriporia lacerata*
Polyporales	523-*Irpex lacteus*
Polyporales	494-*Trametopsis cervina*
Umbelopsidales	529-*Umbelopsis angularis*
Umbelopsidales	1290-*Umbelopsis isabellina*
Umbelopsidales	1028-*Umbelopsis ramanniana*

Isolates were grown on glucose yeast extract agar (GYEA) and scraped to generate inoculum suspensions in sterile water. An aliquot of suspension was spread on six GYEA plates per fungus (three for millipedes, three for waxworms). After all plates were covered by fungal growth (~3 weeks), the arthropods were introduced for 7-day pathogenicity trials. Five individuals were placed on each plate, for a total of 15 animals of each type for each fungal treatment. For a negative control, animals were placed on clean GYEA plates that were changed each time contaminating fungal growth was observed. Plates required replacement due to inadvertent inoculation by phoretic contaminants and gut community members. Observations were made every 12 hours for the first 36 hours (until mortality began to appear), and every 4 hours for an additional 108 hours until 7 days were complete. At the end of the assay, samples of deceased individuals were preserved for future chemical analyses. Surviving waxworms were euthanized by freezing for 24 hours, and surviving millipedes were placed into laboratory colonies for future studies.

Statistical analysis of survivorship was performed using the "Survival / Reliability" function in JMP 13.1.0. Post-hoc pairwise comparisons to the control treatment were performed in descending order from highest mortality to lowest mortality, for each arthropod type, until such comparisons were no longer significant. Both log-rank and Wilcoxon tests were used. Log-rank tests score mortality at all time points evenly, while Wilcoxon tests score early mortality more heavily. Significance thresholds were Bonferroni-corrected.

Entomopathogenicity testing: injections

Thirty-one representative isolates (Table 3) were chosen for injection

entomopathogenicity assays on *Galleria mellonella*. Waxworms were used in this assay

because they are model organisms for studies of pathogenicity/toxicity in arthropods

(Panaccione and Arnold 2017). In addition, *Brachycybe lecontii* could not be tested due

to their small size and the difficulty of injecting through thick cuticle.

TABLE 3: Isolates used in *Galleria mellonella* larvae injections. Asterisk denotes
putative new species.

FUNGAL ORDER	ISOLATE ID	CONIDIA CONCENTRATION (CONIDIA/ML)
Chaetothyriales	1244-*Capronia dactylotricha*	2.5×10^7
Chaetothyriales	626-*Cladophialophora* sp.	3.9×10^7
Chaetothyriales	325-*Cyphellophora* sp.	1.3×10^7
Chaetothyriales	1091-*Fonsecaea* sp.	1.5×10^7
Chaetothyriales	1147-aff. *Fonsecaea* sp.*	3.0×10^7
Chaetothyriales	1193-*Phialophora americana*	2.6×10^7
Chaetothyriales	1164-*Rhinocladiella atrovirens*	2.2×10^7
Hypocreales	1250-*Beauveria caledonica*	1.1×10^7
Hypocreales	678-*Lecanicillium attenuatum*	3.5×10^7
Hypocreales	1163-*Metarhizium flavoviride*	2.5×10^7
Hypocreales	587-*Pochonia chlamydosporia*	6.2×10^7
Hypocreales	17-*Pochonia suchlasporia*	1.9×10^7
Hypocreales	1070-*Purpureocillium lilacinum*	3.6×10^7
Hypocreales	1113-*Tolypocladium album*	3.4×10^7
Hypocreales	1216-*Trichoderma viride*	4.8×10^7
Hypocreales	488-*Verticillium fungicola*	3.5×10^7
Hypocreales	482-*Verticillium insectorum*	2.9×10^7
Hypocreales	1005-*Verticillium insectorum*	3.6×10^7
Mortierellales	1150-*Mortierella* aff. *ambigua* *	6.2×10^6
Mortierellales	530-*Mortierella* sp.	4.3×10^7
Mucorales	1015-aff. *Apophysomyces* sp.*	0**
Mucorales	1267-*Backusella circina*	1.7×10^7
Mucorales	1044-*Cunninghamella elegans*	1.2×10^6
Mucorales	1010-*Mucor abundans*	4.4×10^7
Mucorales	1029-*Mucor genevensis*	5.2×10^7

Mucorales	1009-*Mucor luteus*	1.4×10^6
Mycosphaerellales	329-*Ramichloridium anceps*	4.8×10^7
Polyporales	305-*Bjerkandera adusta*	4.7×10^7
Umbelopsidales	529-*Umbelopsis angularis*	1.7×10^7
Umbelopsidales	1290-*Umbelopsis isabellina*	4.7×10^7
Umbelopsidales	1028-*Umbelopsis ramanniana*	2.4×10^7

**"aff. *Apophysomyces* sp." did not produce spores in culture, but the suspension generated contained enough hyphal fragments to attempt the trial.

Selected isolates were grown on GYEA and scraped to generate spore suspensions in sterile distilled water. Spore concentrations were checked via hemocytometer and diluted to a concentration between 1×10^7 and 5×10^7 conidia/mL, except for four Mucoromycotan isolates that did not produce enough conidia and were assayed at the concentrations given in Table 3. Due to the use of conidia for inoculum, several groups of non-sporulating isolates could not be tested, most notably many Polyporales. Injections were performed with a disposable 29.5-gauge hypodermic needle, and a fresh needle was used for each treatment. In each treatment, 20 waxworms were injected behind the left rear proleg with 20 µL of conidial suspension supplemented with 10 µg/mL rifampicin, an antibiotic to combat wound infections (Panaccione and Arnold 2017). For a negative control, 20 worms were injected with only sterile water and rifampicin. To test for a negative effect from injections, the negative control group was compared to 20 non-injected worms. Observations were made every four hours for the first 48 hours, and every 12 hours for an additional 96 hours, for a total of 144 hours. At the end of the assay, samples of deceased individuals were preserved for future chemical analyses. Surviving waxworms were euthanized by freezing for 24 hours.

Injections were performed in three separate batches of 7 to16 treatments, and each batch had a water + rifampicin injection treatment and a no-injection treatment as independent controls.

Statistical analysis of survivorship was performed using the "Survival / Reliability" function in JMP 13.1.0. Waxworms that pupated during the studies were censored. Post-hoc pairwise comparisons to the control treatment were performed in descending order from highest mortality to lowest mortality, for each arthropod type, until such comparisons were no longer significant. Both log-rank and Wilcoxon tests were used. Log-rank tests score mortality at all time points evenly, while Wilcoxon tests score early mortality more heavily. Significance thresholds were Bonferroni-corrected.

RESULTS

<u>Fungal cohort pairings</u>

A majority of cohort pairings did not result in inhibition. There were 156 pairings, and of these, 49 resulted in one fungus overgrowing another, and 18 resulted in one fungus inhibiting the growth of another (Figure 2).

Of the 42 pairings involving entomopathogenic Hypocreales (excluding Hypo. X Hypo. pairings), one overgrew the other fungus and eight inhibited the paired non-Hypocreales isolate. In AR2, six Hypo. X Hypo. interactions were tested and four of six resulted in inhibition. In these pairings, a pattern of inhibition emerged: *Pochonia chlamydosporia* inhibited *Verticillium* sp., which inhibited *Tolypocladium album*, which inhibited *Metarhizium flavoviride* (Figure 2).

Of the 40 pairings involving a dematiaceous yeast (Chaetothyriales or Chaetosphaeriales, and excluding Dem. X Dem. pairings), zero overgrew the other fungus and four inhibited the other non-dematiaceous-yeast isolate. Only three Dem. X Dem. interactions were tested and two of three had no interaction. In the interacting pair, *Cladophialophora* sp. inhibited *Chaetosphaeria chloroconia* (Figure 2).

Of the 62 pairings involving a zygomycete (*Umbelopsis, Mucor*, or *Mortierella*, and excluding zygo. X zygo. pairings), 14 overgrew the other fungus, and one inhibited the other non-zygomycete isolate. Eleven zygo. X zygo. interactions were tested and six resulted in no interaction. In all five other cases, a *Mucor* sp. overgrew the other fungus (*Umbelopsis* or *Mortierella*) (Figure 2).

Of the 46 pairings involving a white filamentous fungus (Amphisphaeriales and Polyporales, and excluding W.F.F. X W.F.F. pairings), 28 overgrew the other fungus, and zero inhibited the other non-white-filamentous isolate. Only three W. F. F. x W. F. F. interactions were tested, and one of three had no interaction. The remaining two interactions involved overgrowth: in one, *Pestalotiopsis jesteri* overgrew *Bjerkandera adusta,* and in the other, *Pestalotiopsis jesteri* was overgrown by *Trametopsis cervina* (Figure 2).
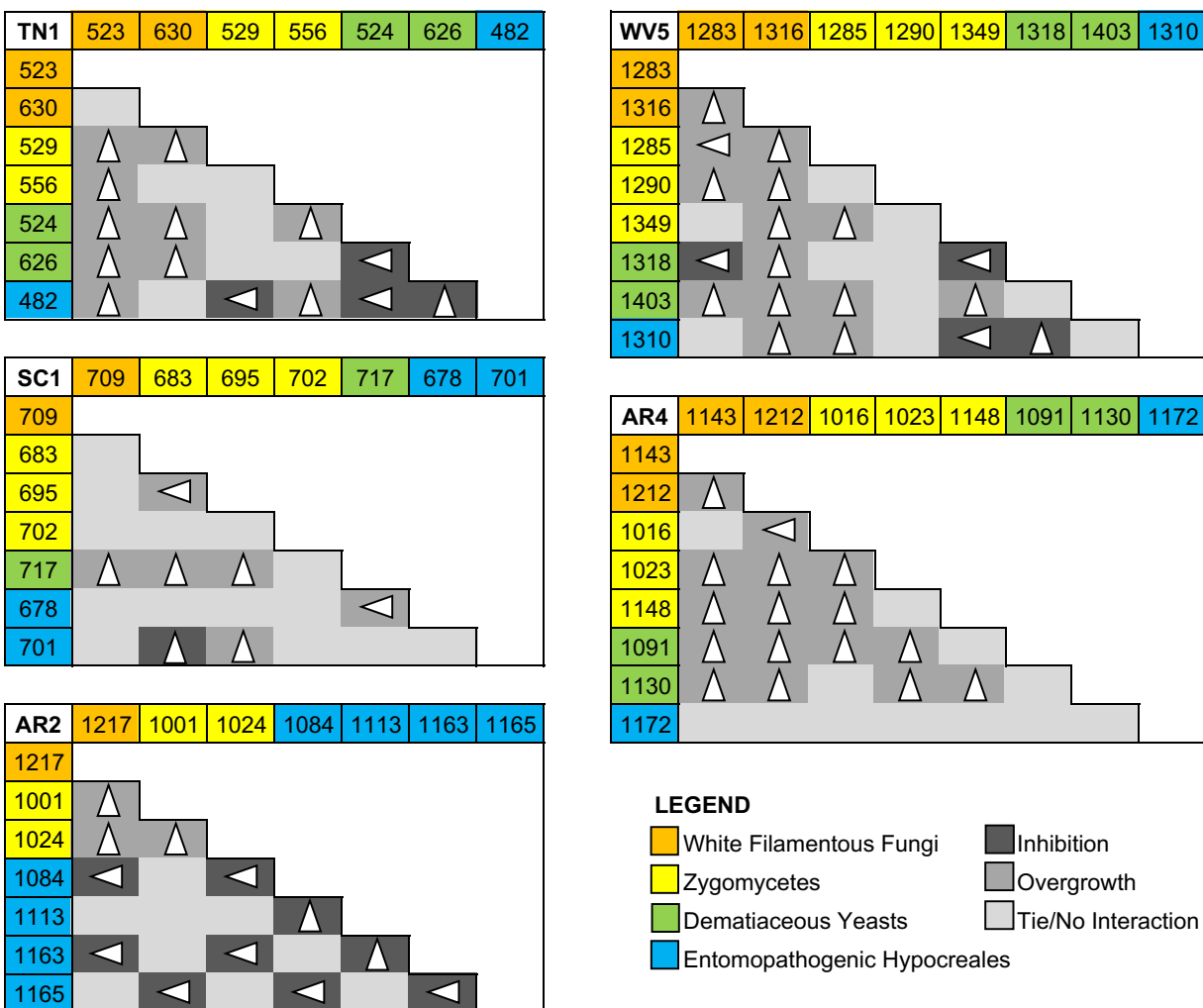
FIGURE 2: Interaction tables showing the results of fungal cohort pairings, by site. The color of each isolate label indicates its fungal morphotype, and the shade of grey inside the chart indicates if the interaction resulted in inhibition, overgrowth, or a tie/no interaction. Arrows indicate the "winners" of inhibition or overgrowth interactions.

Entomopathogenicity testing: live plating

A majority of the fungi used in live plating entomopathogenicity tests did not cause significant mortality in *Galleria mellonella* or *Brachycybe lecontii*. For *B. lecontii*, only treatments that are significantly different from the control are shown in the survivorship curve in Figure 3.

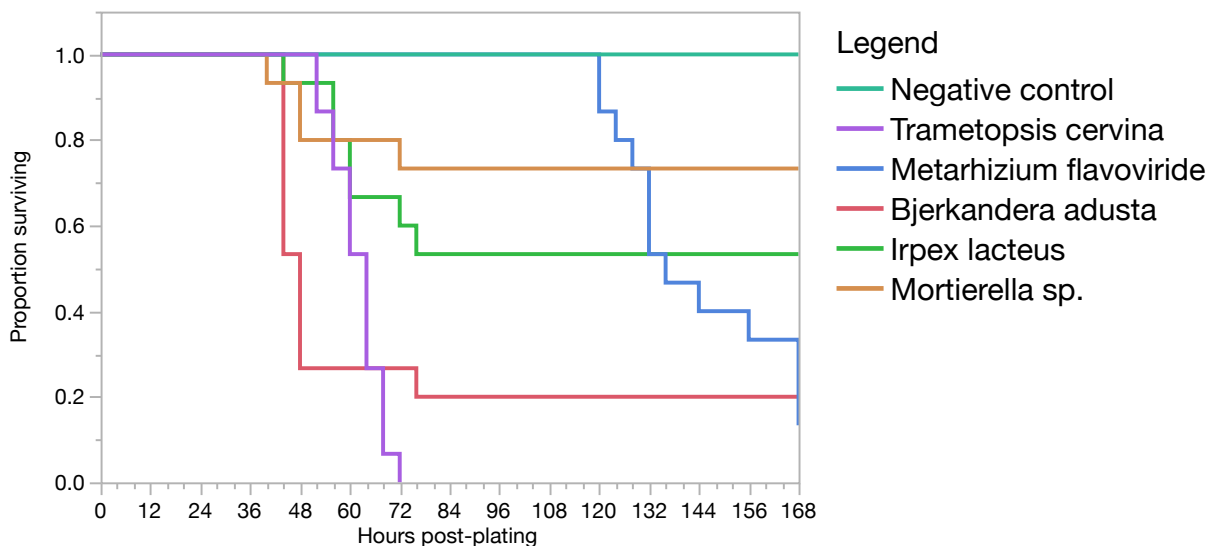**FIGURE 3**: Survivorship curves for *Brachycybe lecontii* live-plating trials. Only fungal treatments that were significantly different from the control are shown.

In *Brachycybe lecontii,* white filamentous fungi caused the most severe damage, with 50% mortality at 48 hours post-plating for *Bjerkandera adusta* and at 64 hours post-plating for *Trametopsis cervina*. In addition to the white filamentous fungi, one entomopathogenic Hypocrealean fungus, *Metarhizium flavoviride,* caused 50% mortality at 136 hours post-plating. No other treatment reached 50% mortality. Fungi from all other groups did not appear to have any negative effect on *Brachycybe*.

A survival analysis was performed for the live-plating assay on *Brachycybe lecontii.* Of the 345 millipedes used in this study, 266 were unaffected by fungi. Both log-rank and Wilcoxon tests were performed to determine if the treatment received had an effect on survivorship. Both tests yielded a p-value less than 0.0001. All millipedes in the control treatment (plating with no fungus) survived, in addition to all millipedes in the 13 following treatments: *Chaetosphaeria myriocarpa,* "*Mortierella* aff. *ambigua*", "aff. *Fonsecaea* sp.", "aff *Apophysomyces* sp.", *Verticillium insectorum, Lecanicillium*

attenuatum, Umbelopsis ramanniana, Mucor abundans, Capronia dactylotricha, Umbelopsis isabellina, Pochonia bulbillosa, Ramichloridium anceps, and *Ceriporia lacerata*. Three additional treatments lost no more than two of 15 millipedes over the course of the experiment. The remaining six treatments, *Trametopsis cervina, Metarhizium flavoviride, Bjerkandera adusta, Irpex lacteus, Mortierella* sp., and *Pestalotiopsis microspora* lost 15, 13, 12, seven, four, and three millipedes, respectively. Pairwise comparisons were made for treatments starting with those that resulted in the most severe mortality. Comparisons were halted after the first non-significant comparison.

Four fungal treatments, *Trametopsis cervina, Metarhizium flavoviride, Bjerkandera adusta,* and *Irpex lacteus*, were significantly different from the control based on Log-rank and Wilcoxon tests (Supplemental Table 1).

In *Galleria mellonella,* entomopathogenic Hypocreales caused the most severe damage, with 50% mortality at 44 hours post-plating for *Metarhizium flavoviride*, and at 132 hours post-plating for *Pochonia bulbillosa*. No other treatment reached 50% mortality (Figure 4), but the other treatments in the entomopathogenic Hypocreales (*Lecanicillium attenuatum, Verticillium insectorum*) resulted in dermal lesions with active sporulation*.* Fungi from all other groups did not appear to have any effect on *Galleria.*
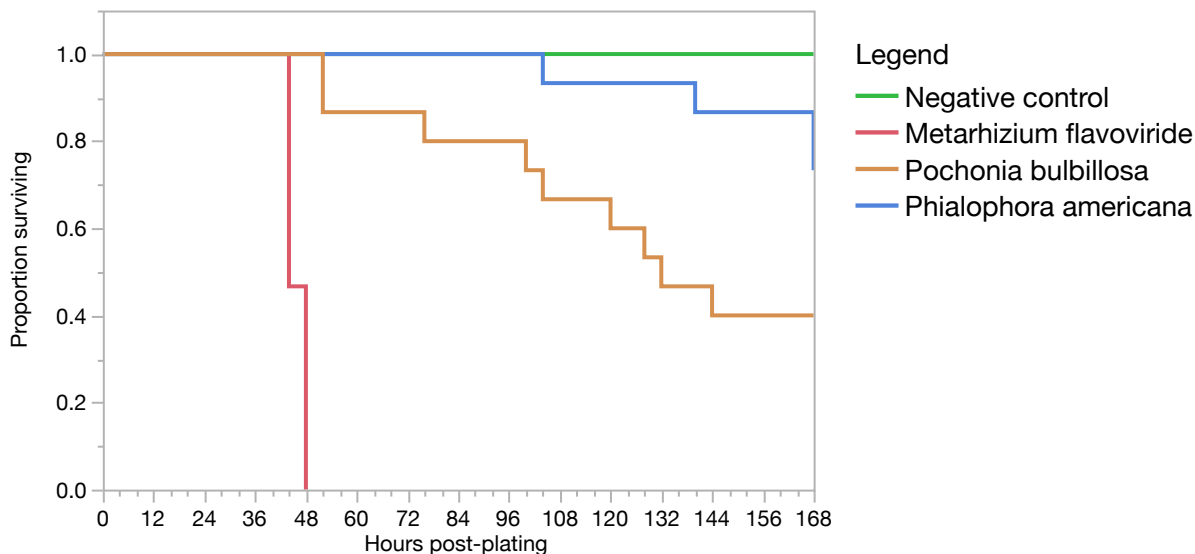
FIGURE 4: Survivorship curves for *Galleria mellonella* live-plating trials. Only fungal treatments that were significantly different from the control are shown.

A survival analysis was performed for the live-plating assay on *Galleria mellonella.* Of the 345 waxworms used in this study, 299 were unaffected by fungi or had pupated by the end of the study. Both log-rank and Wilcoxon tests were performed to determine if the treatment received had an effect on survivorship. Both tests yielded a p-value less than 0.0001. All waxworms in the control treatment (plating with no fungus) survived, in addition to all waxworms in "aff. *Fonsecaea* sp.", *Pestalotiopsis microspora, Irpex lacteus, Ramichloridium anceps,* and *Bjerkandera adusta*. Fourteen additional treatments lost no more than two waxworms over the course of the experiment. The remaining three treatments, *Metarhizium flavoviride, Pochonia bulbillosa,* and *Phialophora americana,* lost 15, nine, and four waxworms, respectively. Two fungal treatments, *Metarhizium flavoviride* and *Pochonia bulbillosa*, were significantly different from the control based on Log-rank and Wilcoxon tests (Supplemental Table 2).

Entomopathogenicity testing: injections

Most fungi used in injection entomopathogenicity tests were harmful to *Galleria mellonella* (Figure 5). Only 8 of 31 treatments did not cause at least 50% mortality.

The Mucorales had the most rapid effect, causing complete melanization of the larvae within two hours of injection and 50% mortality by 24-60 hours post-injection. The only Mucorales without this effect was aff. *Apophysomyces* sp., which did not reach 50% mortality by the end of the experiment (Figure 5).

The dematiaceous yeasts exhibited the same larval melanization effect as the Mucorales but did not always result in severe mortality. Only five of eight treatments involving dematiaceous yeasts resulted in 50% mortality by the end of the assay (Figure 5).

Among all Hypocreales treatments, nine of 10 resulted in 50% mortality by the end of the study, and all nine were from families of known entomopathogenic Hypocreales. Among these treatments, 50% mortality was reached by 28-72 hours post-injection (mean and median 44 HPI) (Figure 5).

The remaining treatments in orders Umbelopsidales and Mortierellales did not cause significant mortality by the end of the study (Figure 5).

A survival analysis was performed for the injection assays on *Galleria mellonella.* The injection assay was performed in three separate batches, each with their own controls, so survival analysis was performed for each batch separately. Of the 740 waxworms used in injection trials, 279 were unaffected by fungi.
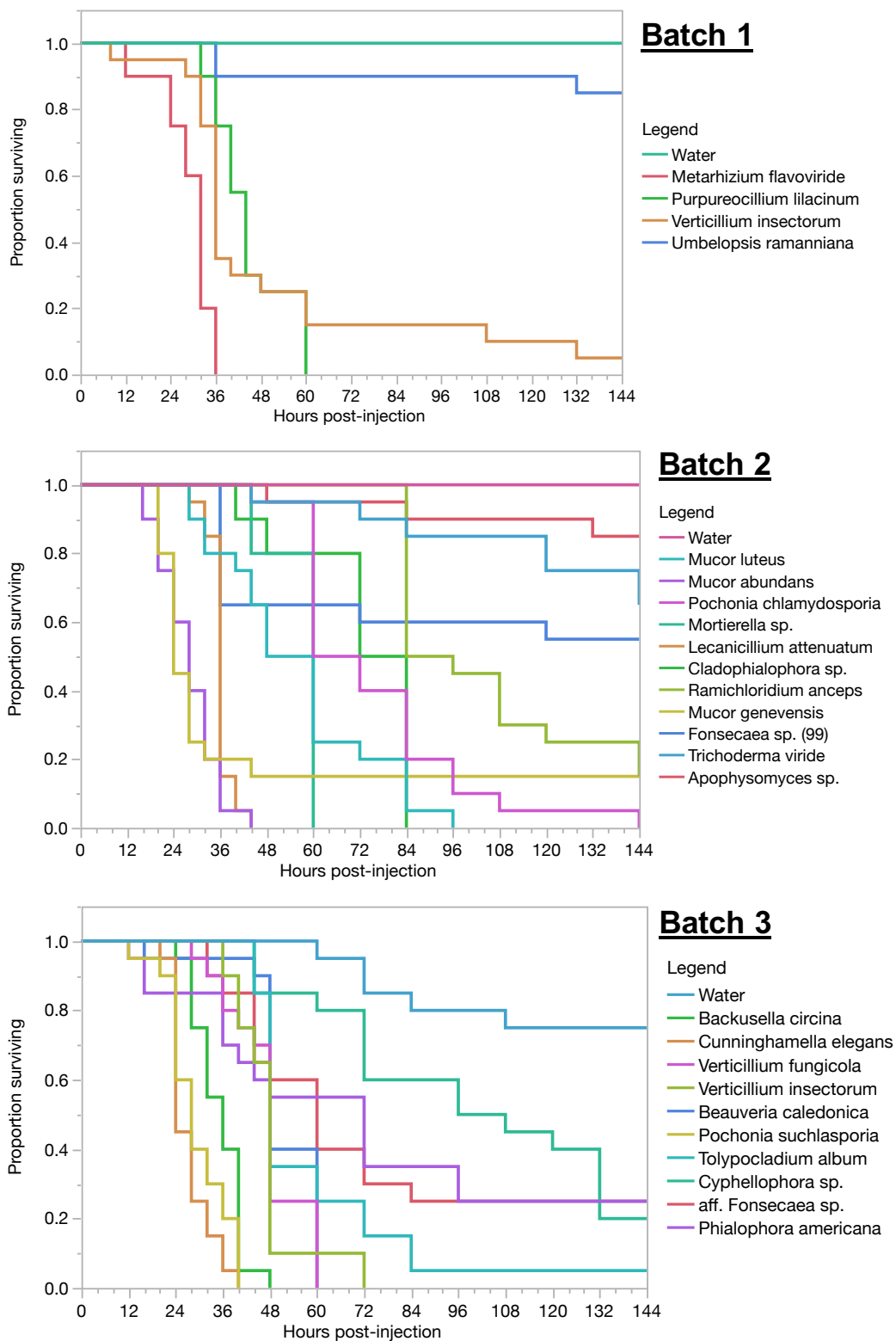
FIGURE 5: Survivorship curves for *Galleria mellonella* injection trials, grouped by the batch in which each fungus was tested. Only fungal treatments that were significantly

different from the control are shown.

Batch 1 had four fungal treatments and the two controls (no injection, water injection+antibiotic). Only the control groups had no mortality. Both log-rank and Wilcoxon tests were performed to determine if the treatment received had an effect on survivorship. Both tests yielded a p-value less than 0.0001. The four treatments, *M. flavoviride, P. lilacinum, V. insectorum,* and *U. ramanniana,* lost 20, 20, 19, and three waxworms, respectively (Figure 5). Since there was no mortality recorded in the control groups (no injection, water + antibiotic), they were not statistically compared. Fungal treatments were then only compared to the water + antibiotic control. Three fungal treatments, *M. flavoviride, P. lilacinum*, and *V. insectorum* were significantly different from the control based on Log-rank and Wilcoxon tests (Supplemental Table 3).

Batch 2 had fourteen fungal treatments and the two controls. No mortality was observed in the control groups and in "*Mortierella* aff. *ambigua.*" Both log-rank and Wilcoxon tests were performed to check for differences between all treatments. Both tests yielded a p-value less than 0.0001. Only eight of the fourteen treatments lost at least 10 waxworms by the end of the trial: *Mucor luteus* (20 waxworms deceased), *Ramichloridium anceps* (17), *Mucor abundans* (20)*, Pochonia chlamydosporia* (20)*, Mortierella sp.* (20)*, Mucor genevensis* (17)*, Lecanicillium attenuatum* (20)*,* and *Cladophialophora* sp. (20) (Figure 5). Similar to Batch 1, there was no mortality recorded in either control group (no injection, water + antibiotic), so fungal treatments were then only compared to the water + antibiotic control. Ten fungal treatments, *Mucor luteus, Mucor abundans, Pochonia chlamydosporia, Mortierella* sp.*, Lecanicillium attenuatum, Cladophialophora* sp.*, Ramichloridium anceps, Mucor genevensis,*

Fonsecaea sp.*,* and *Trichoderma viride* were significantly different from the control based on Log-rank and Wilcoxon tests (Supplemental Table 4).

Batch 3 had thirteen fungal treatments and the two controls. The control groups had mild mortality: the no-injection treatment lost one worm by the end of the trial, and the water + antibiotic treatment lost five. No treatment had all worms surviving at the end of the trial. Both log-rank and Wilcoxon tests were performed to determine if the treatment received had an effect on survivorship. Both tests yielded a significant p-value. Twelve of the thirteen fungal treatments lost at least 10 waxworms by the end of the trial: *Cyphellophora oxyspora* (16)*, Backusella circina* (20)*, Cunninghamella elegans* (20)*, Umbelopsis angularis* (10)*, Verticillium fungicola* (20)*, Verticillium insectorum* (20), *Beauveria caledonica* (20)*, Pochonia suchlasporia* (20)*, Tolypocladium album* (19)*,* "aff. *Fonsecaea* sp." (15), *Phialophora americana* (15)*,* and *Capronia dactylotricha* (12) (Supplemental Table 5)*.* Because mortality was observed in both control groups, they were statistically compared but were not significantly different. Only the water + antibiotic control was used for pairwise comparisons with fungal treatments. Nine fungal treatments, *Backusella circina, Cunninghamella elegans, Verticillium fungicola, Beauveria caledonica, Pochonia suchlasporia, Tolypocladium album, Cyphellophora oxyspora,* aff. *Fonsecaea* sp.*,* and *Phialophora americana* were significantly different from the control based on Log-rank and Wilcoxon tests (Supplemental Table 5).

DISCUSSION

Fungal cohort pairings

Overall, most fungi paired up in cohort pairings did not inhibit or overgrow other fungi. Out of 156 pairings, 12% resulted in one fungus inhibiting the growth of another, and 31% resulted in one fungus overgrowing another.

Entomopathogenic Hypocrealean fungi displayed the most inhibitory effects on other fungi (17%) and amongst each other (66%), followed by dematiaceous yeasts (10% for other / 33% amongst each other), zygomycetes (2% / 0%), and white filamentous fungi (0% / 0%). White filamentous fungi displayed the most overgrowth in response to competition (61% / 66%), followed by zygomycetes (23% / 45%), entomopathogenic Hypocreales (2% / 0%), and dematiaceous yeasts (0% / 0%).

Based on these results, entomopathogenic Hypocreales invest in the competitive strategy of chemical warfare, where they produce diffusible compounds into their growth substrate that prevent or slow the growth of other fungi. Several studies have characterized numerous secondary metabolites that inhibit the growth and reproduction of other fungi (Molnar *et al*. 2010, Vicente *et al*. 2016, Schulz *et al.* 2002). Some dematiaceous yeasts may also invest in this strategy to a lesser degree. It has been hypothesized previously (Shear 2015) that millipedes may sequester chemicals that they then use for their own defenses, and it is possible that the preference *B. lecontii* displays for entomopathogenic Hypocreales (17% of all CFUs, Chapter 2) is a result of this. Another possibility is that *B. lecontii* selectively harbors and inoculates substrate with entomopathogenic Hypocrealean fungi, which help prevent the growth of fungi that are not desired. Having wood substrate preferentially filled with entomopathogenic fungi

may also help keep the worst *B. lecontii* predators (ants) and competitors (termites) away (Kudo *et al.* 2011). If this hypothesis is correct, resistance to entomopathogenic fungi in *B. lecontii* would offer evidence to support this assertion.

Zygomycetes and white filamentous fungi (especially *Irpex lacteus*), however, invest in a completely different strategy, physical overgrowth of the competition. These fungi rapidly colonize all available substrates, and will overgrow other fungi that are present, killing them and using them as a food source (Kasson *et al*. 2015, Mikeskova *et al*. 2012). These fungi represent 8% and 7% of all CFUs identified in Chapter 2, respectively, and may represent preferred fungal community members. However, it is also possible that these fungi are "weeds" to *B. lecontii.* This is unlikely for white filamentous fungi, since *B. lecontii* are nearly always observed in the field on or near fruiting Polyporalean fungi (Chapter 2). However, in contrast, white filamentous fungi were only identified from 48.3% of *B. lecontii* colonies (Chapter 2). Members of the Polyporales are also known to produce many secondary metabolites (Yao et al. 2016), and it is possible that *B. lecontii* sequesters those compounds and uses them for their own purposes. If any of these hypotheses are correct, the absence of pathogenicity in *B. lecontii* would offer evidence to support this assertion.

Entomopathogenicity testing: live plating

Galleria mellonella live plating trials were used to determine which fungi isolated from *B. lecontii* can be considered general entomopathogens. Most fungal treatments resulted in no mortality over 7-day trials, except for the entomopathogenic Hypocreales. *M. flavoviride* and *P. bulbillosa* caused mortality in >60% of all tested individuals. No

other treatment reached 50% mortality, but the other treatments in the
entomopathogenic Hypocreales (*L. attenuatum, V. insectorum*) resulted in dermal
lesions with active sporulation on a majority of individuals. These results indicate that,
as expected, only entomopathogenic Hypocrealean fungi are general
entomopathogens.

When the same fungi were tested in *B. lecontii*, however, very different results
were obtained. Only one entomopathogenic Hypocrealean fungus caused 100%
mortality in *B. lecontii* (*Metarhizium flavoviride*). The most severe mortality came from
white filamentous fungi, especially the Polyporales *Bjerkandera adusta* (50% mortality
at 48 hours post-plating) and *Trametopsis cervina* (50% mortality at 64 hours post-
plating). This result was highly unexpected, given the abundance of Polyporales in the
field and the apparent behavioral preference for them. It is possible that the life stage of
these fungi may affect their toxicity/pathogenicity to *B. lecontii* (Lu *et al*. 2014, Calvo *et
al*. 2002), though a similar pattern ought to have appeared in the waxworm trials, since
they were exposed to fungi at the same life stage. When a Polyporalean fungus first
enters a log, it must actively compete with other fungi already present in the log (Kasson
et al. 2016, Ottosson *et al*. 2015). This competition may result in the production of
chemicals that inadvertently harm millipedes. In the field, *B. lecontii* is seen in
association with fungal fruiting bodies. If a fungus has grown enough to be able to
produce a fruiting body, it is possible that these harmful chemicals may no longer be
produced. To a fungus, an uncolonized agar plate may more closely resemble the early
stages of growth in a log, causing it to produce harmful compounds.

Entomopathogenicity testing: injections

In addition to live platings, *Galleria mellonella* fungal injection trials were used to determine ability of fungi isolated from *B. lecontii* to opportunistically colonize arthropods. The body wall provides a strong defense against pathogens for all animals, so injection of spores through the body wall could permit weakly pathogenic fungi to cause disease. Conversely, if direct injection of spores does not result in disease, this may rule out pathogenicity all together (Kasson et al. 2015).

Most fungi (23 of 31 treatments) caused at least 50% mortality in waxworms by the end of the 6-day pathogenicity trials. The Mucorales and the dematiaceous yeasts caused a melanization response within 2 hours of injection, indicating that the fungi were recognized by the larval immune system as attackers (Loh *et al*. 2013). All Mucorales except "aff. *Apophysomyces* sp." caused 50% mortality within 24-60 hours post-injection. The result for "aff. *Apophysomyces* sp." is not unexpected, since few to no conidia were present in the inoculum (Table 3). This pattern of rapid colonization is consistent with the results from the fungal co-plate assays, where Mucoralean fungi overgrew their competition in 26% of interactions with other fungi.

While dematiaceous yeasts did cause melanization, they did not always cause mortality. Only 5 of 8 treatments involving dematiaceous yeasts resulted in 50% mortality by the end of the study, and this mortality all occurred near the end of the study. These results indicate that even following injection, waxworms are able to hold off attacks from dematiaceous yeasts, at least for a time.

As expected, all entomopathogenic Hypocrealean fungi tested resulted in 50% mortality by the end of the study, within 28-72 hours post-injection.

The remaining treatments in Umbelopsidales and Mortierellales did not cause significant mortality in waxworms, even following direct injections. Future work should investigate potential positive effects for these fungi, especially since results from Chapter 2 place these fungi in the core of the preferred *B. lecontii* fungal community.

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SUPPLEMENTAL TABLES

SUPPLEMENTAL TABLE 1: Results of pairwise comparisons for *B. lecontii* live-plating trials. All listed fungal isolates are compared to the negative control.

Fungal isolate	Log-Rank P	Wilcoxon P	Bonferroni-corrected significance threshold
Trametopsis cervina	<.0001*	<.0001*	0.05
Metarhizium flavoviride	<.0001*	<.0001*	0.025
Bjerkandera adusta	<.0001*	<.0001*	0.0167
Irpex lacteus	0.0028*	0.0031*	0.0125
Mortierella sp.	0.0346	0.035	0.01

SUPPLEMENTAL TABLE 2: Results of pairwise comparisons for *G. mellonella* live-plating trials. All listed fungal isolates are compared to the negative control.

Fungal isolate	Log-Rank P	Wilcoxon P	Bonferroni-corrected significance threshold
Metarhizium flavoviride	<.0001*	<.0001*	0.05
Pochonia bulbillosa	<.0004*	.0005*	0.025
Phialophora americana	0.0348	0.0351	0.0167

SUPPLEMENTAL TABLE 3: Results of pairwise comparisons for *G. mellonella* injection trials (Batch 1). All listed fungal isolates are compared to the water + antibiotic negative control.

Fungal isolate	Log-Rank P	Wilcoxon P	Bonferroni-corrected significance threshold
Metarhizium flavoviride	<.0001*	<.0001*	0.05
Purpureocillium lilacinum	<.0001*	<.0001*	0.025
Verticillium insectorum	<.0001*	<.0001*	0.0167
Umbelopsis ramanniana	0.0753	0.0755	0.0125

SUPPLEMENTAL TABLE 4: Results of pairwise comparisons for *G. mellonella* injection trials (Batch 2). All listed fungal isolates are compared to the water + antibiotic negative control.

Fungal isolate	Log-Rank P	Wilcoxon P	Bonferroni-corrected significance threshold
Mucor luteus	<.0001*	<.0001*	0.05
Mucor abundans	<.0001*	<.0001*	0.025
Pochonia chlamydosporia	<.0001*	<.0001*	0.0167
Mortierella sp.	<.0001*	<.0001*	0.0125
Lecanicillium attenuatum	<.0001*	<.0001*	0.01
Cladophialophora sp.	<.0001*	<.0001*	0.0083
Ramichloridium anceps	<.0001*	<.0001*	0.0071
Mucor genevensis	<.0001*	<.0001*	0.0063
Fonsecaea sp.	0.0007*	0.0008*	0.0056
Trichoderma viride	0.004*	0.0041*	0.005
aff. *Apophysomyces* sp.	0.0754	0.0756	0.0045

SUPPLEMENTAL TABLE 5: Results of pairwise comparisons for *G. mellonella* injection trials (Batch 2). All listed fungal isolates are compared to the water + antibiotic negative control.

Fungal isolate	Log-Rank P	Wilcoxon P	Bonferroni-corrected significance threshold
(No injection)	0.011	0.1025	0.05
Backusella circina	<.0001*	<.0001*	0.025
Cunninghamella elegans	<.0001*	<.0001*	0.0167
Verticillium fungicola	<.0001*	<.0001*	0.0125
Beauveria caledonica	<.0001*	<.0001*	0.01
Pochonia suchlasporia	<.0001*	<.0001*	0.0083
Tolypocladium album	<.0001*	<.0001*	0.0071
Cyphellophora oxyspora	0.0011*	.0026*	0.0063
aff. Fonsecaea sp.	.0003*	.0001*	0.0056
Phialophora americana	0.0005*	0.0003*	0.005
Capronia dactylotricha	0.0312	0.0402	0.0045

Appendix A: Media Used

Glucose Yeast Extract Agar with Antibiotics (GYEA):

Agar	20g
Dextrose	10g
Yeast extract	2g
KH_2PO_4	1g
$MgSO_4$	0.5g
Thiamine	50µg
Biotin	10µg
Microelements	
Fe^{3+}	500µg
Mn^{2+}	439µg
Zn^{2+}	154µg
Distilled water	1L

Autoclave 22 minutes, cool to ~50C, add antibiotics, mix & pour

Tetracycline hydrochloride	100mg
Streptomycin sulfate	10mg

Potato Dextrose Agar (PDA):

Difco® potato dextrose agar	39g
Distilled water	1L

Autoclave 22 minutes, cool to ~50C, pour

Appendix B: DNA Extraction Protocol

1. Harvest mycelium and dry between filter paper. Transfer to 1.5 mL Eppendorf tubes.
2. Add 600 µL Nuclei Lysis Solution, macerate.
3. Incubate at 65C for 15 minutes, vortex for 10 seconds, and incubate for another 15 minutes.
4. Cool to room temperature for 5 minutes.
5. Add 200 µL Protein Precipitation Solution, vortex for 20 seconds, and centrifuge at 13000xg for 3 minutes.
6. Transfer supernatant to a new 1.5 mL Eppendorf tube containing 600 µL isopropanol. Mix by inversion and centrifuge at 13000xg for 1 minute.
7. Decant supernatant and add 600 µL 70% ethanol. Centrifuge at 13000xg for 1 minute. Decant supernatant.
8. For extra purification, repeat step 7.
9. Air-dry the pellet for approximately 30 minutes. Add 100 µL Elution Buffer and store in freezer.