Interactions among fungi, ants, and the ant-plant *Myrmecodia beccarii*

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Cover image: *Myrmecodia beccarii* (water colour painting: Melinda Greenfield)

DECLARATION

I declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any University or other institution of tertiary education.

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Statement of contribution of others

Nature of Assistance	Contribution	Names, Titles and Affiliations of Co-Contributors
Intellectual support	**Collaboration**	My advisors Dr Sandra Abell, Associate Professor Lori Lach, Dr Bradley Congdon, and Emeritus Professor Joseph Holtum collaborated throughout the duration of my PhD. Other collaborators are mentioned at the start of the individual chapters where appropriate. I collaborated with Professor Leho Tedersoo and Dr Sten Anslan from the University of Tartu during this project.
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Nature of Assistance	Contribution	Names, Titles and Affiliations of Co-Contributors
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Abstract

Mutualisms are everywhere in nature and have played a central role in the ecology and evolution of life on Earth. Without mutualisms, major transitions in evolution would not have occurred, including the origin of the eukaryotic cell. Mutualisms are most commonly defined as interactions between species where there is a net benefit to the partners involved and they often include species from different kingdoms. Traditionally they were viewed as bipartite but in the past few decades, technological advancements such as high throughput DNA sequencing are revealing multipartite interactions in mutualisms that involve cryptic microorganisms such as bacteria and fungi that were invisible to earlier researchers.

Myrmecophytes ("ant-plants") are a mutualism between a plant and its resident ants where the plant provides nesting space to the ant colony in specialized hollow structures called 'domatia'. Many ant-plants also provide food rewards to their resident ants and the ants provide protection for the plant against enemies such as herbivores, pathogens, and encroaching vegetation. In other ant-plants, the ant workers defecate inside the domatium, effectively manuring the plant. Traditionally, ant-plant mutualisms were thought to be bipartite, but in the past decade and a half, particular fungi from the order Chaetothyriales have been found in many terrestrial ant-plants, which suggests ant-plants consist of more complex multipartite mutualisms.

Myrmecodia beccarii is an epiphytic ant-plant endemic to tropical Queensland, Australia and listed as vulnerable. The domatium of *M. beccarii* is a tuber-like structure (formed from the hypocotyl) which contains a network of chambers commonly occupied by the native ant, *Philidris cordata*. There are three types of specialised chambers inside the domatium including: (a) dark brown/black waste chambers with wart-like structures that absorb nutrients from waste deposited by ant workers, (b) golden yellow brood ('nursery') chambers where the ant workers keep their brood, and (c) brown ventilation chambers with pores that allow air flow in and out of the domatium. Fungi were first noticed in the domatium chambers of the related epiphytic ant-plant *Myrmecodia tuberosa* in Java over 100 years ago and since then have been mentioned in a few publications but never fully studied. In my thesis, I investigated the long-ignored fungi in the chambers of *M. beccarii.*

In chapter 2, I investigated whether fungal communities differ by chamber type across five locations spanning 675 km of the distribution of *M. beccarii*. I sequenced fungal DNA from the three distinct

domatium chambers of *M. beccarii*. Overall, fungi from the order Chaetothyriales dominated the chambers in terms of the proportion of operational taxonomic units (OTUs; 13.4%) and sequence abundances of OTUs (28% of the total). However, a large portion of OTUs (28%) were unidentified at the order level. Notably, the fungal community in the waste chambers differed consistently from the nursery and ventilation chambers across all five locations. I identified 13 fungal OTUs as dominant/common in the waste chambers that were rare or in very low sequence abundance in the other two chambers. Fungal communities in the nursery and ventilation chambers overlapped more than either did with the waste chambers but were also distinct from each other. Differences in dominance of the common OTUs drove the observed patterns in the fungal communities for each of the chamber types. The results from this survey suggest a long association between fungi and *M. beccarii* and that the role of fungi may differ among chamber types.

In chapter 3, I conducted an ant-exclusion experiment to test the hypothesis that resident *P. cordata* ant workers transport fungi between *M. beccarii* ant-plants. Mature ant-plants (with resident ant colonies) were placed in cages with young *M. beccarii* plantlets not previously colonised by ants. Plantlets were either accessible to, or excluded from, ant workers. Fungal DNA was sequenced from the waste, nursery and ventilation chambers of mature ant-plants and treatment plantlets, and from exoskeletons, heads (including the infrabuccal pocket that may contain fungi that is expelled as a pellet), and abdomens of *P. cordata* ant workers at the end of the three-month experiment. Fungal OTU sequence abundances were 2.6 times higher in waste chambers and 1.9 times higher in nursery chambers of ant-accessible plantlets compared to ant-excluded plantlets. The mature ant-plants shared 60 fungal OTUs with ant-accessible plantlets but only 6 OTUs with ant-excluded plantlets. Seven dominant OTUs in the waste chambers of ant-accessible plantlets and mature ant-plants were absent or occurred rarely in the ant-excluded plantlets and all 7 of these dominant OTUs were found on the bodies or in/on heads of *P. cordata* ant workers. The ant-accessible plantlets and *P. cordata* ant workers shared 31 fungal OTUs but no OTUs were shared solely with the ant-excluded plantlets. My results indicate *P. cordata* ant workers transported chamber fungi between *M. beccarii* ant-plants indirectly on their exoskeletons and heads, and possibly via the infrabuccal pocket.

In chapter 4, I conducted an experiment to test the hypothesis that fungi are involved in the transfer of nitrogen to *M. beccarii* from faeces deposited by ant workers. I placed mature *M. beccarii* ant-plants with their resident ant colonies in cages each with four young ant-plantlets. I used ant-

exclusion and fungicide application as treatments for the four plantlets and fed the ant workers ^{15}N to trace nitrogen from the workers to the plantlets. Fungal DNA was sequenced from the waste chambers of all treatment plantlets and mature ant-plants at the end of the experiment to confirm the fungicide worked and to identify potential fungi that could be involved in nutrient transfer. Leaf samples were collected from all of the plantlets to analyse and compare ^{15}N values. There was a significant but weak effect of fungicide treatment on the fungal OTU community composition in the waste chambers of the plantlets that received fungicide. The ^{15}N values did not differ between ant-accessible plantlets despite fungicide treatment, suggesting fungi might not be involved in the transfer of nitrogen. However, the fungicide treatment did not significantly reduce fungal OTU richness or sequence abundances in the fungicide treated plantlets, and the ant-accessible plantlets (with and without fungicide) were similar in terms of functional guilds and dominant OTUs, making it difficult to conclude whether fungi played a role in nitrogen transfer.

A Chaetothyriales fungal OTU and two saprotrophic OTUs (*Candida* species), were found in this study (in chapters 2, 3 and 4) and are potential candidates for further investigation as to their role in the waste chambers. While I could not conclude whether or not fungi are involved in the transfer of nitrogen from the ant workers to the ant-plantlets, my findings provide some insights. For example, the fungal communities of the waste chambers from the ant-accessible plantlets and mature ant-plants were similar, but not the same, suggesting more time is required for the fungal community to establish in newly occupied *M. beccarii* ant-plants. Also, functional guilds could not be assigned to a large proportion of the fungal community in the mature ant-plants highlighting how little we know about fungi in general and how epiphytic ant-plants are an unexplored niche for fungi.

Before this research was conducted, very little was known about fungi in the domatium chambers of *M. beccarii.* The findings from my three data chapters have advanced our knowledge about the *P. cordata/M. beccarii* ant-plant mutualism despite the role of fungi remaining a mystery. The study of interactions involving microorganisms such as fungi in ant-plants could lead to changes in the way we view mutualistic associations among partners and may influence our understanding of how these relationships evolved and how they are maintained. Mutualisms may be particularly vulnerable to a changing climate so learning about how microorganisms interact with other players within mutualisms could also be useful in conservation efforts for all the species involved.

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Chapter 1 - General Introduction

General Introduction

1.1 Mutualisms

Mutualisms are ubiquitous in nature and have played a central role in the ecology and evolution of life on Earth (Bronstein 2001, 2015). Major evolutionary transitions involving mutualisms include the origin of the eukaryotic cell, which evolved from free-living proteobacteria and cyanobacterial ancestors (Kiers and West 2015), mycorrhizal fungi and the colonization of land by plants (Smith and Read 2008), and leaf-cutter ant agriculture where a non-leaf-cutting higher-attine ant ancestor began to utilise fresh vegetation as substrate for its fungal gardens millions of years ago (Hölldobler and Wilson 1990, Schultz 2022). Mutualisms are defined as interactions between or among species, often from different kingdoms, where there is a net benefit to the partners involved (Bronstein 2015). Another way of defining mutualisms is that they are mutual exploitations in which there is a cost to each organism for contributing to the interaction, but there is a net positive benefit for participating (Herre et al. 1999, Hussa and Goodrich-Blair 2013, Sørensen et al. 2019). The services or rewards exchanged between mutualistic partners usually include nutrition, protection, and/or transportation (Bronstein 1998, 2001, Biedermann and Vega 2020). The partners in mutualisms are often referred to as the host (usually the larger and longer-lived organism) and symbiont (usually the smaller organism) which generally have a shorter generation time and often is a group of multiple individuals such as ants (Ivens 2015).

1.2 Multipartite mutualisms

In recent decades, technological advancements such as high throughput sequencing have revealed multipartite interactions involving microorganisms such as bacteria and fungi in mutualisms traditionally viewed as bipartite. Multipartite mutualisms have been defined as a prolonged association of three or more species in which at least two of the interacting partners receive a net positive benefit (Hussa and Goodrich-Blair 2013). This definition highlights how multipartite mutualisms can include organisms that are beneficial and/or antagonistic. For example, leaf-cutter ants have actinobacteria symbionts in cuticular modifications on their exoskeletons that protect the ants' fungus gardens from *Escovopsis*, a mycoparasitic fungus (Currie et al. 1999a, Li et al. 2018). Benefits received from symbionts in multipartite mutualisms may be required for a particular environmental situation and as such symbionts may be on a continuum which varies ecologically

from mutualism to parasitism (Sørensen et al. 2019). Reef-building corals with their dinoflagellate microalgae interact with other symbionts including microbial representatives from the kingdoms Bacteria, Archaea, and Fungi as well as viruses (Rosenberg et al. 2007, Hussa and Goodrich-Blair 2013). Myrmecophytes ("ant-plants") were long thought to be bipartite mutualisms between a plant and its resident ants but recently, fungi have been found in many ant-plants, suggesting ant-plants consist of more complex multipartite mutualisms (Voglmayr et al. 2011, Mayer et al. 2014).

1.3 Myrmecophytes ("ant-plants")

The one thing all ant-plants have in common is they provide nesting space to ants in specialized hollow structures called 'domatia' which are formed from modified plant parts such as stems, petioles, thorns, hypocotyls, rhizomes, or leaves (Huxley 1982, Mayer et al. 2014). Ant-plants are almost exclusively tropical and have evolved multiple times across an estimated 681 vascular plant species (159 genera in 50 families) and there are at least 110 ant species from five subfamilies that are either facultative (opportunistic) or obligate occupants of ant-plants (Chomicki and Renner 2015). In the Australasian region, 47% of ant-plants are epiphytic and 40% are trees/shrubs, whereas Africa has no epiphytes and 95% are trees/shrubs, and in the Americas <15% are epiphytes with 79% trees/shrubs (Chomicki and Renner 2015). Estimates of the age of ant-plant lineages in Africa are not much older than 5 million years and for those in Australasia and the Americas, approximately 15 million years (Chomicki and Renner 2015).

Services and rewards exchanged between ant-plants and their resident ants can vary between terrestrial and epiphytic ant-plants. For example, terrestrial ant-plants often provide their resident ants with food rewards such as extrafloral nectaries or food bodies (Beattie 1985, Hölldobler and Wilson 1990, Heil and McKey 2003) and in many ant-plants, the ants protect their host plant against herbivory, pathogenic fungi, and encroaching vegetation (Janzen 1972, Letourneau 1998, Suarez et al. 1998, Frederickson et al. 2005, Rosumek et al. 2009). In the past few decades, studies have shown how ant workers also deposit faeces and other waste inside the domatia of terrestrial ant-plants, effectively fertilising their host plant (Cabrera and Jaffe 1994, Sagers et al. 2000, Fischer et al. 2003, Solano and Dejean 2004, Defossez et al. 2011, Chanam et al. 2014). In epiphytic ant-plants, it has long been known that the resident ant workers deposit faeces, dead insects, and ant corpses in cavities inside the domatium and that this is a vital service because epiphytes live in the nutrient-poor environment of tree canopies (Janzen 1974, Huxley 1978, Rickson 1979, Rico-Gray et al. 1989,

Gay 1993, Treseder et al. 1995, Watkins et al. 2008, Gegenbauer et al. 2012, Chomicki and Renner 2019, Volp et al. 2022). Other services in epiphytic ant-plants include resident ant workers dispersing seeds of their host epiphytic ant-plants (e.g. in rubiaceous ant-plants) effectively ensuring future nesting sites for their colonies (Janzen 1974, Huxley 1978, Sommer 1990, Chomicki and Renner 2016). Also, in species of the Fijian epiphytic ant-plant *Squamalleria*, the resident *Philidris nagasau* ants receive a sugary food reward from their host from post-anthetic flowers (Chomicki et al. 2016) and this might also occur in other rubiaceous epiphytic ant-plants with resident *Philidris* species including species of *Myrmecodia*.

1.4 Ant-plants and fungi

Historically, fungi were first noticed in the epiphytic ant-plant *Myrmecodia tuberosa* Jack (Gentianales: Rubiaceae) over 100 years ago in Java by Miehe (1911) who isolated a dominant fungus from the 'warted' chambers of the domatium and concluded the ants were feeding the fungus by defecating on the fungal mat. Miehe (1911) also concluded that the fungal mats were trimmed by resident ant workers to prevent fungal overgrowth which could lead to obstacles for the ants in the domatium. Huxley (1978, 1982) found two types of fungi in domatia of *M. tuberosa* and *M. beccarii* in Papua New Guinea and Australia respectively and hypothesised that the fungus in the warted chambers might be involved in breaking down waste deposited by ants, and the fungus in the brood chambers was possibly food for ant larvae. However, fungi were not the main focus of Huxley's studies and were not further investigated. Fungi in epiphytic ant-plants have been mentioned in a few other studies but only briefly (Janzen 1974, Gay 1993, Gegenbauer et al. 2012). This has left questions unanswered about the identity and possible role of these fungi in epiphytic ant-plant mutualisms.

More recently, fungi from the order Chaetothyriales (also known as "black yeasts") have been found in terrestrial ant-plant-fungus associations. Chaetothyriales fungi were independently acquired on different continents by different ant lineages in plants from different families (Voglmayr et al. 2011). Chaetothyriales fungi are usually characterised by dark, melanized, slow-growing hyphae and are known for their tolerance of extreme environments (Mayer et al. 2014). For example, Chaetothyriales fungi have been found growing in environments polluted with aromatic hydrocarbons, (Zhao et al. 2010, Baron et al. 2021), on rocks in Antarctica (Selbmann et al. 2015), and on the walls of an underground metro station (Réblová et al. 2016). Ants produce a diversity of

compounds from their exocrine glands (Hölldobler and Wilson 1990) (e.g. for communication and defence) and it has been suggested Chaetothyriales fungi might tolerate and possibly metabolise ant-produced toxic compounds (Voglmayr et al. 2011, Moreno et al. 2019). In another study, it was suggested Chaetothyriales fungi may play a role in reducing volatile organic compounds inside domatia (Mayer et al. 2021). In other studies of terrestrial ant plants, it has been found that Chaetothyriales fungi are manured by their resident ant workers (Defossez et al. 2011), used as food for ant larvae (Blatrix et al. 2012, Mayer et al. 2018), and are transmitted by founding queens from their natal nests to start new colonies (Mayer et al. 2018). There has been one other recent study of fungi in an epiphytic ant-plant in Thailand in which algae were found along with Chaetothyriales and Capnodiales fungi highlighting the presence of other possible symbionts in ant-plant mutualisms (Blatrix et al. 2021). Patterns of specificity among these ant-plants, their symbiont ants, and Chaetothyriales fungi are not fully understood with some fungal strains from domatia showing ant-host specificity while other strains are ubiquitous and display no plant or ant specificity (Voglmayr et al. 2011, Blatrix et al. 2013, Kokolo et al. 2016, Nepel et al. 2016, Vasse et al. 2017).

1.5 Study species

Myrmecodia beccarii Hook.f. (Gentianales: Rubiaceae) is an epiphytic ant-plant endemic to tropical Queensland, Australia where it has a fragmented distribution in coastal lowland *Melaleuca* woodlands (Fig. 1.1) and mangroves from Bamaga at the top of Cape York Peninsula south to Lucinda (Fig. 1.2). *Myrmecodia beccarii* is listed as vulnerable under the Environment Protection and Biodiversity Conservation Act 1999 (Commonwealth of Australia), the Nature Conservation Act 1992 (State of Queensland) and is listed in the species profile and threats database (Department of the Environment 2022). The main threats to *M. beccarii* are habitat loss due to the clearing of *Melaleuca* woodlands for forestry plantations, agriculture, housing developments, and road expansions. It has been estimated that only 20% of the original coastal *Melaleuca* woodlands remain in northern Queensland (Braby 1992).

Figure 1.1 Mature *Myrmecodia beccarii* ant-plant on a *Melaleuca* tree in an open woodland near Cardwell, Queensland, Australia

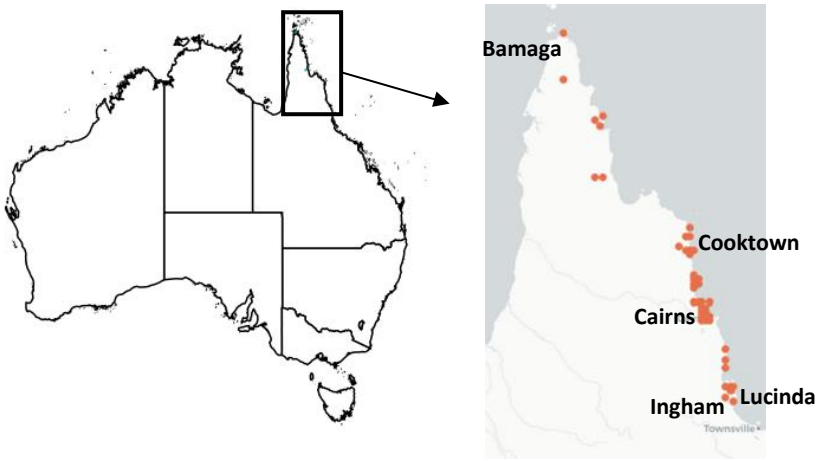

Figure 1.2 Map of Australia with enlarged inset for northern Queensland showing distribution of Myrmecodia beccarii along the coast from Lucinda to Bamaga (red dots).The distribution of the Apollo Jewell Butterfly, Hypochrysops apollo apollo, is from Ingham to Cooktown. From Atlas of Living Australia, Map data© OpenStreetMap, imagery© CartoDB.The domatium of *M.*

beccarii is a tuber-like structure (formed from the hypocotyl) which contains a network of chambers commonly occupied by the native ant, *Philidris cordata* Smith F. (Hymenoptera: Formicidae) (Huxley 1982). There are three types of specialised chambers inside the domatium which develop regardless of occupation by ants including: (a) dark brown/black waste chambers with wart-like structures that absorb nutrients from waste deposited by the ant workers, (b) golden yellow brood ('nursery') chambers where the resident *P. cordata* ant workers keep their brood, and (c) brown ventilation chambers that allow air flow in and out of the domatium (Fig. 1.3) (Huxley 1978, Jebb 1991, Volp et al. 2022).

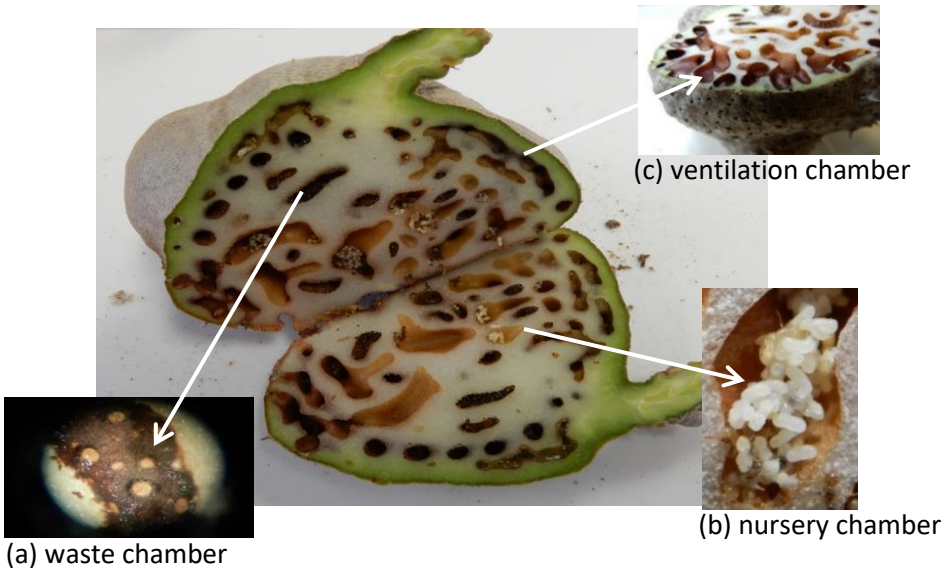

Figure 1.3 *Myrmecodia beccarii* ant-plant cut in half to show the three chamber types.
(a) waste chambers with wart-like structures that absorb nutrients from waste deposited by ant workers; (b) nursery chambers with smooth surface where the brood of the colony is kept (eggs, larvae, and pupae visible in this photo); and (c) ventilation chambers, which allow air to flow into the domatium chambers through pores on the surface of the ant-plant.

Myrmecodia beccarii is also habitat for the larval and pupal stages of the Apollo jewel butterfly *Hypochrysops apollo apollo* Miskin (Lycaenidae) (Fig. 1.4). The larvae of *H. apollo apollo* feed exclusively on *M. beccarii* and this butterfly has a restricted distribution between Cooktown and Ingham (Fig. 1.2). The butterfly lays its eggs on the lower surface of the domatium of *M. beccarii* (usually one egg per ant-plant) and the larvae mostly feed on the inside of the domatium but will also feed on the underside of *M. beccarii* leaves at night (Lane 1985, Sommer 1990, Braby 1992). The *P. cordata* ant workers tend the larvae and receive a food reward in the form of excretions from

the larvae (Braby 1992, Forster 2000). The abundance of *H. a. apollo* has been declining for many decades because of habitat loss for *M. beccarii* ant-plants (Braby 1992).

Figure 1.4 The Apollo Jewel butterfly, *Hypochrysops apollo apollo*
(a) female top view, (b) underneath dorsal view, and (c) male top view

1.6 Why study fungi in *Myrmecodia beccarii*?

Fungi have long-been ignored in epiphytic ant-plants in the Australasian region, so we know very little about the fungi that inhabit the domatium chambers of these ant-plants. Studying epiphytic ant-plants could reveal multipartite mutualisms involving fungi and also other microorganisms. Knowledge gained about these interactions will provide valuable insights that add to the growing body of research on ant-plants around the world. In turn, this could ultimately lead to a better understanding of how these ant-plant mutualisms have evolved, how they are maintained, and what may destabilise them under changing environmental circumstances. *Myrmecodia beccarii* is an ideal model for studying interactions with fungi in an ant-plant mutualism because it has specialised chambers that serve different ant-associated functions. If fungi differ among the three types of chambers in *M. beccarii*, it will suggest fungi play different roles in the different chambers such as involvement in the transfer of nutrients, or as antibiotics for the ant colony. To be able to use *M. beccarii* as a model, first we need to identify the fungi in the chambers of *M. beccarii,* determine how fungi come to be in the chambers of *M. beccarii,* and establish whether or not fungi play a role in *M. beccarii.* The results of this research will also help conservation efforts for *M. beccarii.*

1.7 Thesis structure

I have set out this thesis following the formatting and thesis preparation guidelines provided by the Graduate Research School at James Cook University. This thesis is presented as three research chapters (Chapters 2, 3 and 4) which stand-alone except for the thesis wide consolidated reference list. Each chapter contains an introduction, methods, results, and discussion. The abstracts have been consolidated and the fifth chapter is a general discussion/synthesis of the main results and the conclusions of my research.

1.8 Key research questions and Thesis overview

I investigated fungi in the different domatium chambers of the ant-plant *M. beccarii* occupied by *P. cordata* ant colonies. Specifically, I set out to answer the following questions:

1. What fungi are in the domatium chambers of *M. beccarii*?
2. Does the fungal community differ with chamber type?
3. Does the fungal community differ across locations within the distribution of *M. beccarii*?
4. Are fungi transported by *P. cordata* ant workers and if so, how?
5. Do fungi play a nutritional role in the *P. cordata/M. beccarii* mutualism?

In chapter 2 of this thesis, to answer questions 1, 2 and 3 above, I conducted a survey to determine the identity of fungi in the domatium chambers of *M. beccarii*. I collected mature *M. beccarii* specimens from five locations across approximately 675 km of northern Queensland coastline. This survey included locations from State Forests, National Parks, and one location from a residential suburban area. I dissected the *M. beccarii* ant-plants and collected chamber surfaces from which I extracted DNA and sequenced the fungal communities in the different chambers using high throughput sequencing. This survey revealed the fungal communities in the three different domatium chambers of *M. beccarii* across a large portion of its fragmented distribution. This chapter has been published in Mycological Progress and is referenced in my thesis as Greenfield *et al.* 2021.

For chapters 3 and 4, to answer questions 4 and 5, I conducted a 3-month experiment using a 2 x 2 factorial design with ant-exclusion and fungicide application as treatments. I placed mature *M. beccarii* ant-plants with their *P. cordata* ant colonies (collected from the wild) into cages with four young *M. beccarii* plantlets (grown by me ant-free). In each cage, two of the plantlets were

placed in moats to exclude ant workers and two were accessible to the ant workers. I applied fungicide to one of the ant-accessible plantlets and one of the ant-excluded plantlets in each cage.

In chapter 3, to answer question 4, I extracted and sequenced fungal DNA from the chamber samples of the ant-accessible plantlets and ant-excluded plantlets (both non-fungicide treatment plantlets) as well as the mature ant-plants at the end of the experiment described above. I also sequenced fungal DNA from samples collected from the exoskeletons, heads, and abdomens of ant workers from each of the cages. The sequencing data generated from this experiment provided strong evidence for how fungi are transported from an established mature *M. beccarii* ant-plant to younger *M. beccarii* ant-plantlets by the resident ant workers. This experiment also provided insight into how fungi are moved by ant workers from comparisons of the abundances of fungi on the exoskeletons, heads, and abdomens.

In chapter 4, to answer question 5, I fed the ant workers the stable isotope ^{15}N at the beginning of the experiment to trace nitrogen from the ant workers to the leaves of the experimental plantlets. At the end of the experiment, I extracted and sequenced fungal DNA from the chambers of the ant-accessible plantlets with and without fungicide and compared the ^{15}N values in the leaves of those same ant-accessible plantlets. The experiment confirmed that the ant workers feed *M. beccarii* by depositing faeces in the waste chambers, and the ^{15}N values were not different between the ant-accessible plantlets despite fungicide treatment. However, the fungicide treatment was not entirely effective, and I discuss different scenarios to explain why the results for this chapter are inconclusive.

In chapter 5, I conclude with a summary of my three data chapters and synthesise main results and conclusions. I also include limitations of my research and opportunities for future research.

Chapter 2 - Consistent patterns of fungal communities within ant-plants across a large geographic range strongly suggest a multipartite mutualism

Chapter 2 - Consistent patterns of fungal communities within ant-plants across a large geographic range strongly suggest a multipartite mutualism

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Statement of contribution of others:

M.J.G. designed the study, conducted the field and lab work, collected the data, analysed the data, created the figures and tables, and wrote the manuscript. S.A. performed the bioinformatics and edited the manuscript. L.T. provided laboratory space, primers and reagents, advice on high-throughput sequencing, and edited the manuscript. M.F. provided advice on data analysis and edited the manuscript. B.C.C. provided advice on interpreting the data and edited the manuscript. S.E.A. and L.L. provided advice on the design of the study, interpretation of results, and edited the manuscript.

2.1 Introduction

Microorganisms such as fungi and bacteria engage in symbioses with other organisms that can have antagonistic (negative) or mutualistic (positive) effects on their hosts. Multipartite mutualisms consist of a prolonged association of more than two partners in which at least two of the interacting organisms receive a net positive benefit (Hussa and Goodrich-Blair 2013). Microorganisms have been found in many mutualisms previously thought of as bipartite including the coral-algae association that also involves bacteria, archaea and viruses (Rosenberg et al. 2007); the fungus-farming attine ants and antibiotic-producing bacteria that control fungal garden parasites (Currie et al. 1999b); and lichen-forming fungi that have complex associations with a green algal photobiont and cyanobacteria (Nelsen et al. 2020). In tropical regions, complex mutualisms have evolved in plants known as myrmecophytes ("ant-plants"). Ant-plant mutualisms were long thought of as bipartite - between the plant and its resident ants - but recent studies suggest that these interactions are more complex and involve hidden microorganisms such as fungi (Voglmayr et al. 2011, Mayer et al. 2014).

Ant-plants provide nesting space to ants in specialised structures called domatia, which are formed from modified plant parts such as stems, thorns, hypocotyls, or leaves (Chomicki and Renner 2015). In some ant-plants, the resident ants obtain food rewards from their host (e.g. extrafloral nectar or food bodies) (Hölldobler and Wilson 1990) and others obtain honeydew from hemipterans they rear in the domatium (Beattie 1985). The resident ants usually defend the plant against enemies such as herbivores (Janzen 1972, Rosumek et al. 2009), and some ant workers feed their host plant by depositing waste on domatium surfaces (Huxley 1978, Rickson 1979, Rico-Gray et al. 1989, Gay 1993, Treseder et al. 1995, Defossez et al. 2011).

Multipartite mutualisms involving fungi, ants and ant-plants have been identified relatively recently. For example, domatium fungal patches are used as a source of food by ants in three independently evolved and geographically distinct terrestrial ant-plant associations (Defossez et al. 2009, Defossez et al. 2011, Blatrix et al. 2012, Blatrix et al. 2013). In one of these ant-plants, the resident ants were observed defecating and depositing detritus on their fungal patch, transporting fragments of the fungus, and chewing hyphae (Defossez et al. 2009). Other ants build traps to capture insects by combining fungi with plant trichomes (hairs) in ant-carton (a combination of vegetative material and soil held together by sugary secretions) on the stems of their host myrmecophyte tree (Dejean

et al. 2005). The fungi play a structural role in the trap, receive nutrients from the ants, and facilitate the transfer of nutrients to the plant (Dejean et al. 2005, Mayer and Voglmayr 2009, Leroy et al. 2011, Nepel et al. 2014, Leroy et al. 2017). The dominant fungi isolated from ant-plant systems studied so far are "black yeasts" from the orders Chaetothyriales (class Eurotiomycetes) and Capnodiales (class Dothideomycetes) of phylum Ascomycota (Voglmayr et al. 2011). A recent phylogenetic study found sufficient support for a clade of Chaetothyriales fungi obtained from ant domatia to be recognised as a separate family (Quan et al. 2020).

Epiphytic ant-plants usually grow on trees for support and are typically nutrient-limited, because, like other epiphytes, they do not obtain nutrients or water directly from soil or from their host tree. For this reason, waste deposition by ant workers in the domatium is believed to be particularly important for epiphytic ant-plants (Janzen 1974). In the Australasian region, 47% of ant-plants are epiphytic, whereas most ant-plants are trees or shrubs in Africa (no epiphytes) and the Americas (15% epiphytes) (Chomicki and Renner 2015). Fungi were first noticed in the epiphytic ant-plant *Myrmecodia tuberosa* Jack (Gentianales: Rubiaceae) (Miehe 1911), but have since been mentioned rarely in the literature (Bailey 1920, Janzen 1974, Huxley 1978) or dismissed as opportunistic (Miehe 1911, Bailey 1920).

My study investigated fungi in the epiphytic ant-plant *Myrmecodia beccarii* Hook.f. (Gentianales: Rubiaceae), endemic to northern Queensland, Australia. *Myrmecodia beccarii* is listed as vulnerable under the Environment Protection and Biodiversity Conservation Act 1999 (Commonwealth of Australia) and the Nature Conservation Act 1992 (State of Queensland) with the main threat being habitat loss due to the destruction of forests containing its host trees (Kemp et al. 2007). The domatium of *M. beccarii* contains a network of multiple chambers commonly occupied by the native ant *Philidris cordata* Smith F. (Hymenoptera: Formicidae) (Huxley 1982, Volp and Lach 2019). The chambers include smooth-walled ('nursery') chambers where *P. cordata* keeps its brood (eggs, larvae, and pupae), warted ('waste') chambers that absorb nutrients from faeces and waste deposited by ant workers (Huxley 1978, Volp et al. 2022), and superficial ('ventilation') chambers that allow air-flow into the system (Huxley 1978, 1982, Jebb 1991). While Huxley (1982) noted two fungal taxa in the waste and nursery chambers of *M. beccarii*, they were not investigated in detail.

In this chapter, I hypothesized that epiphytic ant-plants have consistent associations with fungi that correspond with chamber type. To test this, I sampled the fungal communities in the three

domatium chambers of *M. beccarii* across five geographic locations to answer the questions: (a) which fungi dominate the domatium chambers of *M. beccarii*? (b) are fungi unique to each of the three chamber types or shared among the chambers? (c) do fungal communities differ among the three chamber types, and if so, is this consistent across geographic sites? If fungal communities are consistently distinct in each of the three chamber types across the five locations, it would suggest fungi play different roles in the different chambers because the three chamber types serve different ant-associated functions.

2.2 Methods

2.2.1 Study sites and sampling

I collected 46 whole *Myrmecodia beccarii* plants from *Melaleuca* trees at five locations spanning 675 km in northern Queensland, Australia (Fig. 2.1) from March 2016 until March 2017. I collected from two sites at Cardwell (10 plants total), one site at Cowley Beach (10 plants), residential *Melaleuca* trees in Port Douglas (10 plants), one site at Annan River National Park (9 plants), and two sites at Kutini-Payamu National Park (7 plants total). I aimed to analyse the fungal communities from 10 *M. beccarii* plants from each location, but the plants were rare (and often too high in the canopy to be accessible) in Kutini-Payamu National Park, and one plant collected from Annan River National Park was decomposing inside its domatium at time of dissection and was excluded. The study area represents a large part of the known distribution of *M. beccarii* from Cardwell in the south (18°19’09.5”, 146°02’58.9”E) to Kutini-Payamu National Park in the north (12°41'11.7"S, 143°20'03. 0"E) (Fig. 2.1).

I selected *M. beccarii* ant-plants for collection based on size (circumference of domatium approximately 300 mm around the base), presence of ant workers, and accessibility. All ant-plants collected were from *Melaleuca* trees at least 20 m distant from any other ant-plant collected. Each *M. beccarii* was removed from the host tree and placed immediately into a 27 L plastic box that had been lined with Fluon® (Livefoods Unlimited, Tinbeerwah, Qld) on its sides and Tangle-Trap® (Australian Entomological Supplies Pty. Ltd, Murwillumbah, NSW, Australia) in a 25 mm strip around the upper edge of the containers to prevent the resident ant colonies from escaping. The collected *M. beccarii* ant-plants were kept in their plastic boxes in a greenhouse at James Cook University, Cairns Campus (16°48’58.83”S, 145°41’16.73”E) until dissection (1 to 21 days after collection). During this time, I sprayed the roots of the ant-plants with tap water three to four times per week

with a garden pressure sprayer to the point of run-off. Each ant colony was provided with two meal worms once per week and approximately 15 mL of 25% sucrose solution divided into two plastic 8 mL vials (plugged with a small ball of cotton wool) twice per week.

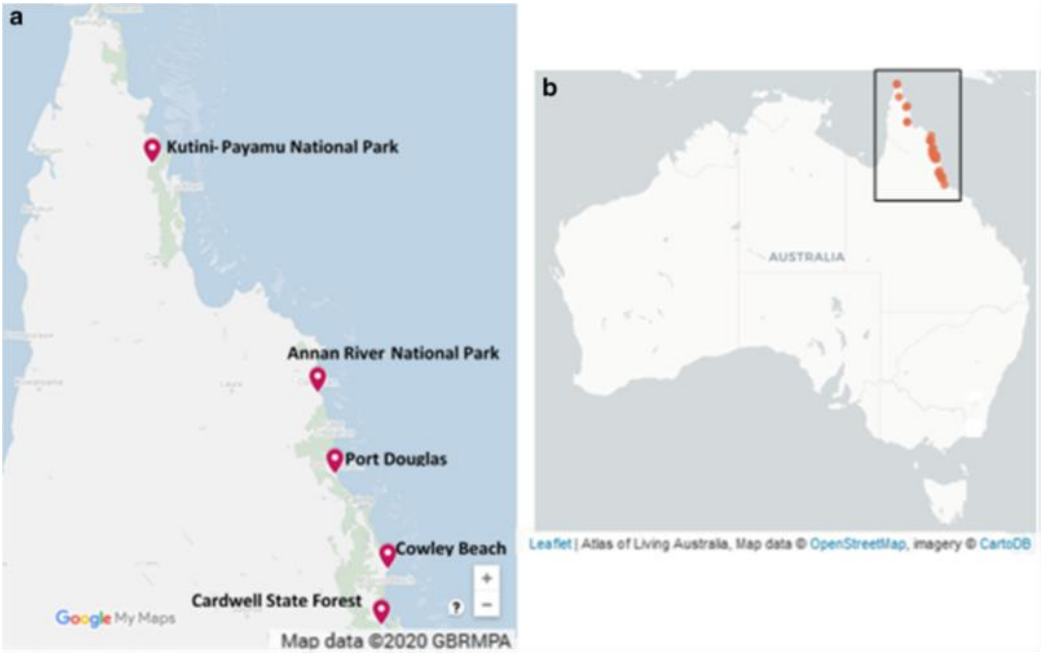


Figure 2.1 Map of northern Queensland, Australia (a) the five locations where *Myrmecodia beccarii* ant-plants were collected during the study and (b) known distribution of *M. beccarii* - red dots indicate where *M. beccarii* has previously been found**.**

2.2.2 Dissection of *Myrmecodia beccarii* ant-plants and collection of chambers

At time of dissection, I placed each whole *M. beccarii* ant-plant into an 8.5 L sealed plastic container with five cotton balls soaked in approximately 10 mL total ethyl acetate (Sigma Aldrich, St. Louis, MO, USA) to euthanise the ant colony. The stems, leaves, and roots of the ant-plants were removed from the domatium and discarded. Each domatium was sliced vertically into approximately 10 mm cross-sections with a knife (flame-sterilised using 99.5% ethanol between slices). Three slices were selected for sample collection including one slice from each side/end of the domatium and one slice from the middle of the domatium. For each slice, 4-5 chambers of each chamber type (waste, nursery, and ventilation) were collected using a scalpel to lift the chambers away from the domatium and placed into individual 1.5 mL tubes (total sample weight of 25 mg ± 5 mg for each chamber type for each slice). I identified the different chambers based on their characteristic features: waste chambers being dark brown/black with wart-like surface structures; nursery

chambers being yellow coloured with smooth (wart-free) surfaces, and ventilation chambers being brown-coloured with smooth surfaces and a honeycomb type structure near the outer edge of the domatium (Fig. 2.2). I flame-sterilised the scalpel and forceps during dissections using 99.5% ethanol between each sample collected. A 1.5 mL control tube was left open during each ant-plant dissection to account for potential contaminants during sample processing for DNA extraction. Nine chamber samples per ant-plant (three of each chamber type) were collected during each ant-plant dissection except for Cowley Beach which had only three chamber samples (one of each type of chamber) per ant-plant (being a combination of the chambers collected from three slices as above). The Cowley Beach *M. beccarii* ant-plants were the first set of ant-plants to be dissected (pilot study).

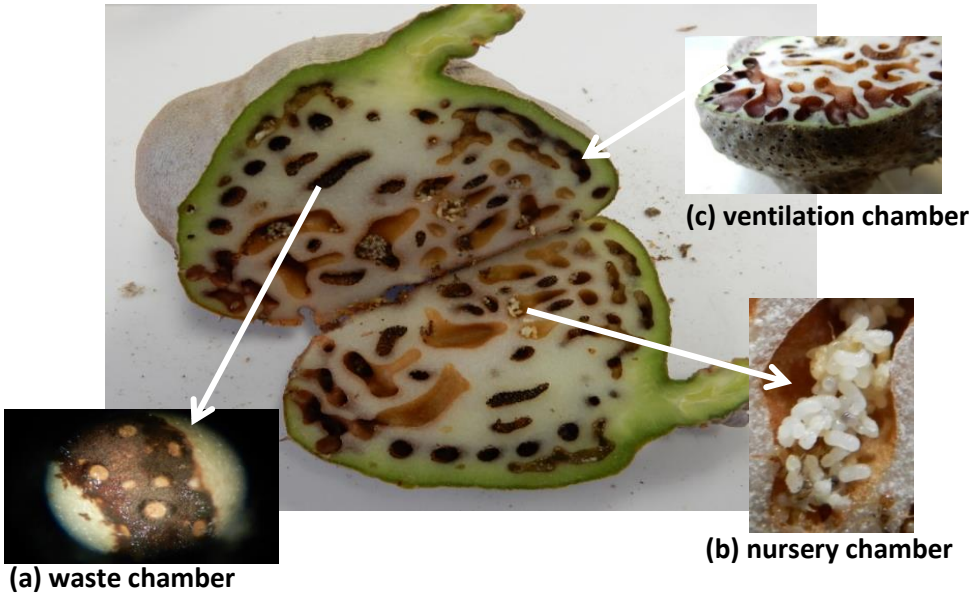


Figure 2.2 *Myrmecodia beccarii* specimen showing the three different domatium chambers (a) waste chambers with wart-like structures that absorb nutrients from waste deposited by ant workers; (b) nursery chamber with smooth surface where the brood of the colony is kept (eggs, larvae and pupae visible in this photo); and (c) ventilation chambers, which allow air to flow into the domatium chambers through pores on the surface of the ant-plant.

2.2.3 DNA Extraction and Sequencing

I extracted DNA from the samples to determine the identity of fungi in the different chamber samples, using the Qiagen DNeasy Plant Mini Kit (Qiagen Pty Ltd, Victoria, Australia) following

manufacturer's instructions, except at the final step where I eluted 50 µL of purified DNA instead of 100 µL. I performed polymerase chain reaction (PCR) using the forward primer ITS1Fngs (GGTCATTTAGAGGAAGTAA) (Tedersoo et al. 2015) and reverse primer ITS4ngs (TTCCTSCGCTTATTGATATGC) (Tedersoo et al. 2014) to target the full internal transcribed spacer (ITS) region (ITS1-5.8S-ITS2), the formal barcode for identification of fungi in molecular studies (Schoch et al. 2012). The primers were tagged with 10-11 base pair unique identifiers for multiplexing (Supplementary Table S2.1). The PCR cocktail consisted of 2 µL DNA extract, 0.5 µL each of the primers (20 µM), 5 µL of 5 x HOT FIREPol® Blend Master Mix (Solis Biodyne, Tartu, Estonia) and 17 µL of double-distilled water. The HOT FIREPol Blend® Master Mix contains HOT FIREPol DNA polymerase (modified Taq polymerase, 99.5% units, error rate 0.011% per base) and a modified proofreading polymerase (0.5% units, estimated 5 x error rate reduction). This enzyme mixture has both 5'-3' exonuclease activity and 3'-5' proofreading activity. All samples were amplified in duplicate and PCRs were carried out in the following thermo-cycling conditions: an initial 15 minutes at 95°C, followed by 25 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute and a final cycle of 10 minutes at 72°C. PCR products for each of the duplicate samples were combined and the relative quantity of the product was estimated by running gel electrophoresis of 5 µL of DNA sample on 1% agarose gel for approximately 20 minutes. DNA samples yielding no visible band were reamplified by using 30 or 35 cycles to obtain enough PCR product. Based on visual estimates, the product quantity was categorised into three groups and, accordingly, 5 to 10 µL of PCR products were subjected to library preparation. Negative controls and positive controls (*Cantharellus* sp. from Africa that does not occur naturally in Australia) were used throughout all procedures (for DNA extraction, PCRs, and sequencing). Pooled amplicons were purified using a FavorPrep™ PCR Purification Kit (FavorGen® Biotech Corp., Vienna, Austria). The amount of DNA in each tube was quantified using Qubit®. Purified PCR products were arranged in four libraries and subjected to SMRTbell library preparation following Pacific Biosciences Amplicon library preparation protocol. The libraries were loaded to 8 SMRT cells using the Diffusion method and sequenced on a PacBio Sequel instrument using Sequel Polymerase v2.1, Sequencing chemistry v2.1 and movie time of 600 min following the manufacturer's recommendations. Circular consensus sequences (CCS) pipeline on SMRT Link (v5.1.0.26412, SMRT Link Analysis Services and GUI v5.1.0.26411) with default settings (minPasses=3, minAccuracy=0.9) was used for generating CCS reads.

2.2.4 Bioinformatics

The 8 SMRT cells yielded CCS reads totalling 50461 (library 1), 49839 (library 2), 44336 (library 3) and 52476 (library 4). Bioinformatics analyses of the PacBio sequencing data (for full ITS region) were performed using PipeCraft (v1.0) (Anslan et al. 2017). This analysis platform incorporates required tools for quality filtering, demultiplexing, chimera filtering, clustering, and taxonomy annotation. Quality filtering of the CCS reads were conducted using vsearch (v1.11.1) (Rognes et al. 2016) (fastq_maxee = 1, fastq_minlen = 50, fastq_maxns = 0). The filtered data was demultiplexed based on unique identifiers using mothur (v1.36.1) (Schloss et al. 2009) (allowed barcode differences = 1, primer differences = 2). Potential chimeric sequences were detected and removed with vsearch (v1.11.1) using *de novo* and reference database-based filtering (against UNITE UCHIME release v7.2) (Abarenkov et al. 2010). Multiprimer artifacts (chimeric reads where full primer sequences were found in the middle of the read) were also removed using PipeCraft built-in module. Full ITS region sequences (without flanking genes of 18S and 28S; primer binding sites) were extracted with ITSx (v1.0.9) (Bengtsson-Palme et al. 2013). The full ITS reads were clustered to Operational Taxonomic Units (OTUs) with CD-HIT (v4.6) (Li and Godzik 2006) at a threshold of 97% similarity, as commonly set in fungal molecular ecology (Kõljalg et al. 2013, Taylor et al. 2014). I used BLASTn search for the most abundant sequence of each non-singleton cluster (i.e. OTU) against GenBank and UNITE (v7.2) reference databases for taxonomic assignment of OTUs (e-value = 0.001, word size=7, reward = 1, penalty = -1, gap opening cost = 1, gap extension cost = 2).

I further manually filtered the remaining OTUs based on BLASTn values where e-values of $<e^{-50}$ were used to assign sequences as reliable to the fungal kingdom and e-values $>e^{-20}$ were considered unknown and removed from the dataset. E-values between e^{-20} and e^{-50} were manually checked against the ten best matches for assignment to kingdom Fungi or removal (resulting in another 33 OTUs being detected as chimeric sequences and removed). A further 16 OTUs were detected as chimeric sequences (artefacts of PCR amplification). Two non-fungal OTUs were removed (one insect and one plant). Global singletons (394 OTUs in total, each with only one occurrence in the dataset) were removed to avoid potentially erroneous sequences. The single positive PCR control OTU was removed along with five OTUs (all singletons) that were found only in the positive controls. I also removed two OTU doubletons (only 2 occurrences across the dataset) with low sequence coverage and low sequence similarity. This left a dataset containing 374 OTUs with minimum read abundances of 2 (Greenfield 2020) which I further filtered to remove any OTUs with total read

abundances <10, leaving a dataset with a total of 164 fungal OTUs (Supplementary Table S2.2). This final step was performed because the focus of this study is on the dominant fungal taxa inside the domatium chambers of *M. beccarii* and, according to previous studies, excluding rare species makes the community matrix more coherent and less noisy and hence strengthens the statistical power (Tedersoo et al. 2015). I used sequence similarity thresholds of >97%, >90%, >85%, >80%, >75%, and >70% to match OTUs roughly to species, genus, family, order, class, and phylum levels, respectively (Nilsson et al. 2019). Of the 164 OTUs, 70 OTUs (42.7%) matched the taxonomic identity of >97% to pre-existing fungal ITS sequences in existing databases (GenBank and UNITE). A further 54 OTUs (32.9%) matched at 90-97% and the remaining 40 OTUs (24.4%) matched to closest taxa at <90% sequence similarity.

I sequenced 371 samples including 335 fungal DNA amplicon samples collected from the domatium chambers of *M. beccarii* and 36 laboratory controls (4 positive controls and 4 negative PCR controls, and 28 dissection/extraction controls). The 335 chamber samples were comprised of 116 nursery chambers (27 from Annan River, 26 from Cardwell, 13 from Cowley Beach, 20 from Kutini-Payamu National Park, and 30 from Port Douglas), 97 ventilation chambers (26 from Annan River, 15 from Cardwell, 9 from Cowley Beach, 17 from Kutini-Payamu, and 30 from Port Douglas) and 119 waste chambers (27 from Annan River, 11 from Cowley Beach, 30 from Cardwell, 21 from Kutini-Payamu, and 30 from Port Douglas). Three chamber samples failed to amplify fungi, so they were excluded from the dataset. I pooled the remaining 332 chamber samples to reduce the multiple number of chambers per plant to 3 samples per plant – resulting in there being one nursery chamber sample, one ventilation chamber sample and one waste chamber sample for each plant collected. This was necessary because multiple samples for each chamber type from the same plant would not be independent due to the interconnectedness of the chambers. This left a total of 135 samples comprising 46 nursery chambers, 44 ventilation chambers, and 45 waste chambers from the 46 ant-plants (Greenfield 2020). Twenty-seven of the 28 dissection/extraction controls contained no contamination and were removed. One dissection control tube (open in the lab during the dissection of the plant AN03 from Annan River National Park) contained a single occurrence of OTU1029 (*Tremellomycetes* sp.) so this OTU was removed from the AN03 chamber samples. OTU1029 was not removed from any other plant chamber samples because it had not contaminated any other dissection or extraction controls. The positive and negative controls for the PCRs (8 in total) were also removed.

2.2.5 Statistical analyses

Statistical analyses were conducted in R version 3.6.1 (R Core Team 2019) using the dataset containing sequence abundance data for the 164 fungal OTUs from 135 samples (Supplementary Table S2.2). Unique and shared fungal OTUs were investigated by creating a Venn diagram using the R package 'VennDiagram' (v1.6.20) (Chen 2018). I used the multivariate abundance analysis package 'mvabund' (v4.0.1) (Wang et al. 2012, Wang et al. 2020) to test for significant differences among the fungal OTU communities in the three chambers across the five locations. The manyglm function in mvabund was used to fit a model which included chamber type, location, and an interaction term for chamber type and location, with default arguments including family = "negative binomial", test = "LR" (likelihood-ratio-test), and resamp = "pit.trap". I used the anova function in mvabund to compute an analysis of deviance table for the model with pairwise comparisons among the three chamber types (all locations combined). To identify fungal OTUs that were significantly abundant in the chambers and across locations, I used the "p.uni" argument to calculate univariate test statistics and their p-values (adjusted for multiple testing using a step-down resampling procedure). I also ran separate models for each of the three chamber types to test for differences in fungal communities across the five locations for each chamber type using pairwise comparisons.

I used the packages 'phyloseq' (v1.28.0) (McMurdie and Holmes 2013), 'vegan' (v.2.5.6) (Oksanen et al. 2019) and 'ggplot2' (v.3.3.0) (Wickham 2016) to create ordination plots in order to visualise differences in the fungal communities in the three different chambers across the five locations. First, I standardized my OTU matrix with a Hellinger-transformation (to account for varying sampling and sequencing depth) and then performed non-metric multidimensional scaling (NMDS) with Bray-Curtis distance measure on the whole dataset (all chambers), and then each chamber type separately to examine differences across locations.

The packages 'DESeq2' (v1.24.0) (Love et al. 2014) and 'phyloseq' were used to further investigate differentially abundant fungal OTUs in the different chambers. Abundance OTU data was first loaded into Phyloseq and imported into DESEq2 using the phyloseq_to_deseq2 function. The DESeq2 model included both chamber type and location with significance test set to "Wald", fitType set to "local" and multiple inference correction set to "Benjamini-Hochberg". Pairwise contrasts on chamber type were then carried out with DESeq2 to identify differentially abundant OTUs. The bar plots were created with the package 'phyloseq' and 'ggplot2'. I used FUNGuild (v1.0) (Nguyen et al. 2016) to assign trophic modes to the significantly abundant fungal OTUs.

I used the dataset containing 374 OTUs (minimum read abundances of 2) (Greenfield 2020) to compare fungal OTU richness among chambers across geographic locations. First, I tested if OTU richness is dependent on sample sequencing depth using the packages 'car' (v.3.0.10) (Fox and Weisberg 2018) and 'stats' (v3.6.3) (R Core Team 2020). I found OTU richness was significantly positively correlated to sequencing depth (Supplementary Table S2.3, Fig. S2.1), so I calculated the residuals for richness for each sample from the regression to account for the relationship in my subsequent analysis. I used the packages 'lme4' (v.1.1.26) (Bates et al. 2015) and 'lmerTest' (v.3.1.3) to determine whether fungal OTU richness (residuals) varies with chamber type and geographic location. Fixed effects included location and chamber type and an interaction term for location and chamber type, and I included plant ID as a random effect (i.e., the individual plant from which the chamber samples were collected). I used the emmeans package (v1.5.3) (Lenth 2020) for pairwise comparisons between locations for each of the chamber types.

2.3 Results

2.3.1 Dominant fungal orders in the domatium chambers

I detected a total of 42,747 sequences from 164 distinct fungal operational taxonomic units (OTUs) across the 135 samples collected from the domatium chambers of *M. beccarii* (Supplementary Table S2.2). Seventy percent of the 164 OTUs were classified into 25 fungal taxonomic orders. Below the order level, 33.5% of the 164 OTUs were classified to a family, 32.3% to genus, and 15.9% were classified to species level using available public databases GenBank and UNITE. Sixteen of the 25 orders were from phylum Ascomycota, eight from phylum Basidiomycota, and one from phylum Mortierellomycota. The orders with the highest number of OTUs were Chaetothyriales, Capnodiales, and Eurotiales, which collectively accounted for 57 of the total 164 OTUs (Fig. 2.3a). Of the 164 OTUs, 46 were unidentifiable at the order level and 15 of these could not be assigned beyond kingdom Fungi. Chaetothyriales was the dominant order in terms of sequence abundance making up 28% of the total (Fig. 2.3b).

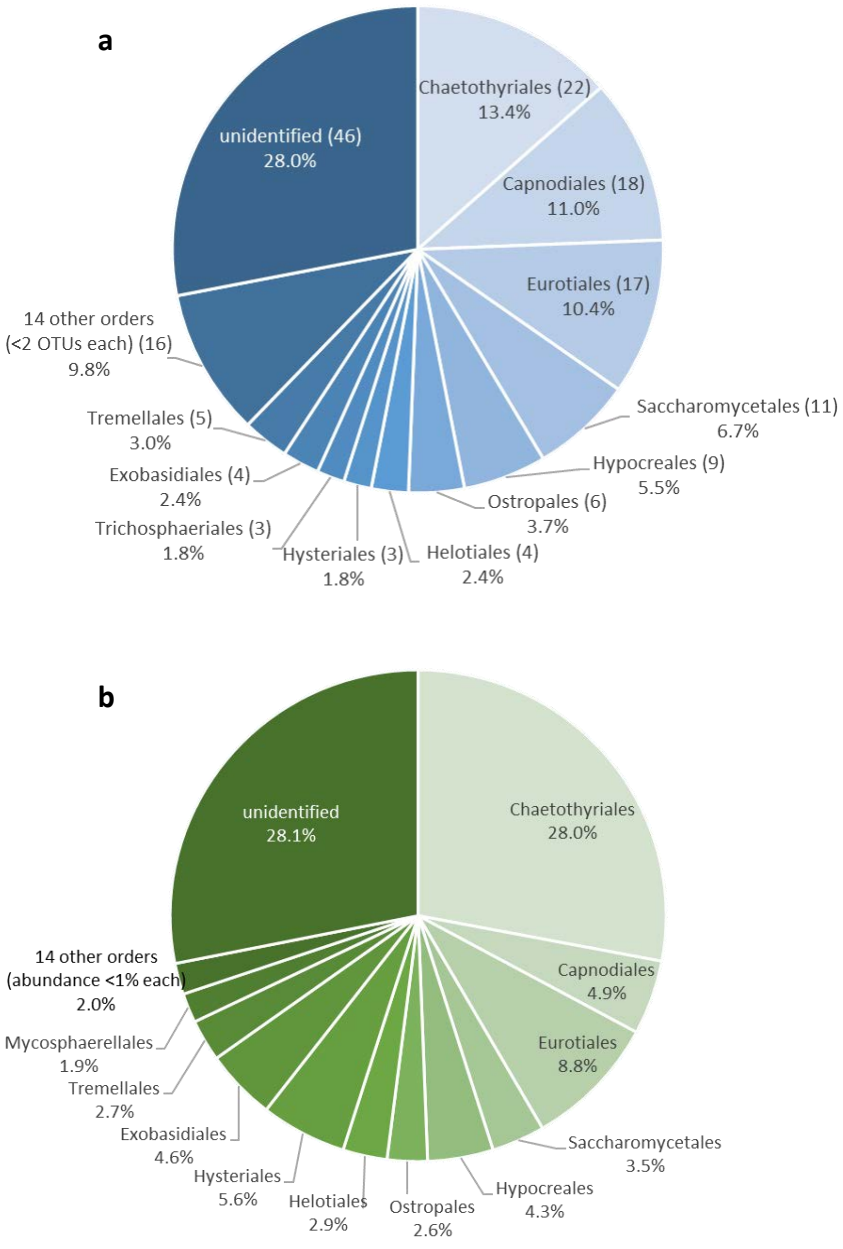


Figure 2.3 Dominant fungal taxonomic orders in the chambers of *Myrmecodia beccarii* (a) Relative proportion of fungal operational taxonomic units (OTUs) found in the chambers of *M. beccarii* and assigned to fungal orders, showing Chaetothyriales, Capnodiales, and Eurotiales are dominant. Numbers in brackets indicate the number of fungal OTUs assigned to each order (total 164 OTUs). (b) Relative abundances of the 164 fungal OTUs found in the chambers of *M. beccarii* and assigned to fungal orders, showing Chaetothyriales is the most abundant order.

2.3.2 Unique and shared fungal OTUs in the domatium chambers

Of the total 164 OTUs detected, there were 125 OTUs in the waste chambers, 142 OTUs in the nursery chambers, and 138 OTUs in the ventilation chambers. Ninety-four OTUs (57.3% of the 164 OTUs) were shared among the three chamber types (Fig. 2.4). The read abundances of these 94 shared OTUs made up 88.8% of the total 42747 sequences. The nursery and ventilation chambers shared 28 OTUs which made up 3.9% of the sequence abundances. The nursery and waste chambers shared 16 OTUs, comprising 5.6% of the sequences and the ventilation and waste chambers shared the fewest OTUs (9 in total) making up only 0.7% of the abundances. The 17 OTUs unique to one of the chamber types collectively accounted for 1.1% of sequences (Fig. 2.4).

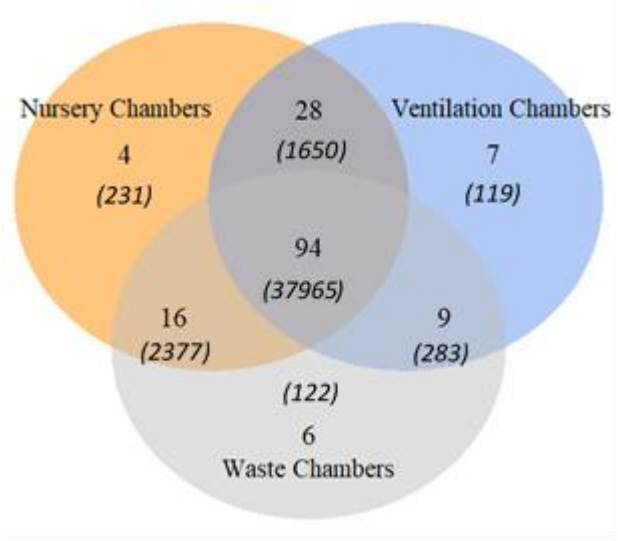


Figure 2.4 Venn diagram of unique and shared fungal operational taxonomic units (OTUs) among the three different chambers of *Myrmecodia beccarii.* The numbers displayed in plain font represent the number of fungal OTUs shared between and among chambers (overlapping shaded areas) and unique (not overlapping) to each chamber type (total 164 fungal OTUs). The numbers in italics in brackets are the corresponding OTU sequence abundances (totalling 42747). The total number of OTUs associated with each of the chambers is: waste chambers 125 OTUs; nursery chambers 142 OTUs; and ventilation chambers 138 OTUs.

2.3.3 Fungal communities among domatium chambers

My analysis indicated significant differences in the fungal communities among the chamber types (LRT = 2546, p<0.001). Pairwise comparisons of the chamber types showed that the fungal community in the waste chambers was different from the fungal communities in both the nursery (LRT = 1300, p<0.001) and ventilation chambers (LRT = 1872, p<0.001). Fungal communities in the waste chambers formed a cluster which was distinct from the other two chambers (Fig. 2.5a). Significantly different fungal communities were also found between the nursery and ventilation chambers (LRT = 609, p<0.001) (Fig. 2.5a).

2.3.4 Fungal community differences across the geographic distribution of *M. beccarii*

I analysed each of the three chambers of *M. beccarii* separately to determine if the fungal OTU communities differed among locations for each of the chambers. The fungal OTU community composition varied with location for the waste chambers (LRT = 939.9, p<0.001), nursery chambers (LRT = 990.5, p<0.002), and ventilation chambers (LRT = 1165, p<0.001) (Fig. 2.5b-d). My analysis also indicated an interaction between chamber type and location (LRT=887, p<0.001). Pairwise comparisons for the waste chambers differed across locations for all but one of the pairwise comparisons, and for the nursery chambers, the fungal OTU communities differed for three of the pairwise comparisons of locations (Table 2.1). The fungal OTU community composition in the ventilation chambers differed across all locations (Table 2.1). Fungal OTU richness was significantly higher in the ventilation chambers at Port Douglas (mean 40.0 ± 3.27 SE) compared to the ventilation chambers at Cardwell (mean 22.3 ± 2.13 SE, p=0.002) (Supplementary Tables S2.4, S2.5 and S2.6, Fig. S2.2). All other pairwise comparisons of fungal OTU richness between the five locations for each of the three chamber types were not significant (Supplementary Table S2.6).

To better understand why there were differences in the fungal community compositions across locations, I identified fungal OTUs individually that were significantly abundant in one or more of the three different chamber types. My DESeq2 analysis found 41 OTUs to be significantly abundant in one or more chambers and of these, the mvabund analysis identified 22 OTUs significant for chamber type only, 10 OTUs significant for chamber type and location, 6 OTUs significant for location only, and 2 OTUs significant for chamber type, location and an interaction effect (Table 2.1).

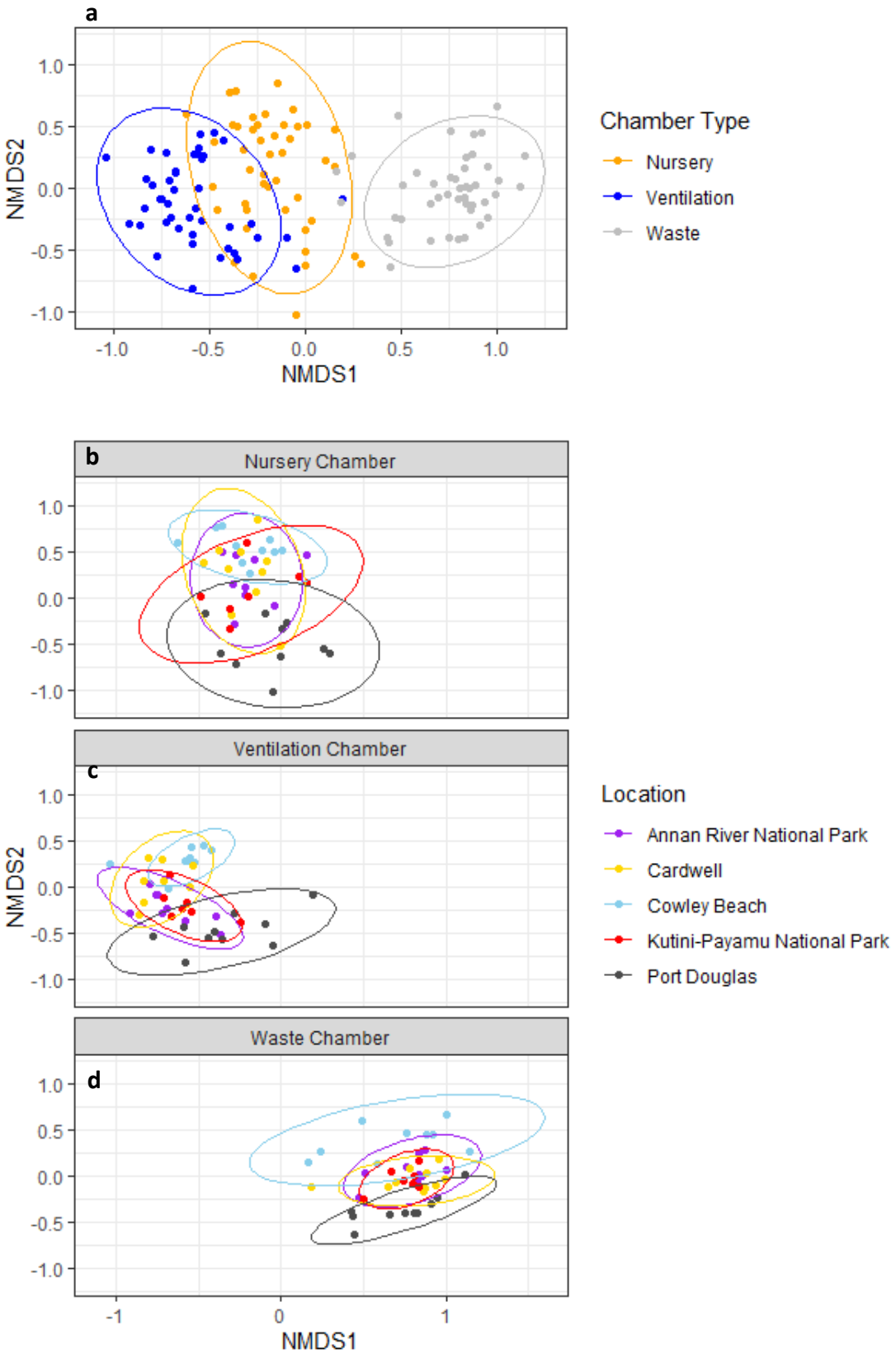

a
NMDS2
NMDS1
1.0
0.5
0.0
-0.5
-1.0
-1.0
-0.5
0.0
0.5
1.0
Chamber Type
Nursery
Ventilation
Waste
b
Nursery Chamber
c
Ventilation Chamber
d
Waste Chamber
-1
0
1
Location
Annan River National Park
Cardwell
Cowley Beach
Kutini-Payamu National Park
Port Douglas

Figure 2.5 Non-metric multidimensional scaling (NMDS) ordinations displaying fungal OTU community compositions in the chambers of *Myrmecodia beccarii* across five locations.
Plot (a) is for the three chamber types for all locations combined, showing the fungal communities in the waste chambers (grey) are distinct from the fungal communities in the nursery (orange) and ventilation (blue) chambers. Plots (b), (c) and (d) are separate NMDS plots for the nursery, ventilation, and waste chambers respectively, showing differences across the five locations surveyed: Annan River National Park (purple), Cardwell (yellow), Cowley Beach (light blue), Kutini-Payamu National Park (red), and Port Douglas (dark grey). Each point on a plot is a sample of a fungal community collected from one of the three chambers from one of the five locations. This ordination plot includes 164 fungal operational taxonomic units (OTUs). A Hellinger transformation was used to account for varying sampling and sequencing depth. Bray Curtis distance was used with k=3 dimensions.

Table 2.1 Pairwise comparisons of fungal OTU community composition in the domatium chambers of *Myrmecodia beccarii* across five locations (the abbreviation NP = National Park). Chambers include waste, nursery, and ventilation. The LRT is the likelihood ratio test. p-value is the adjusted p-value calculated using 999 resampling iterations via PIT-trap resampling (to account for correlation in testing).

Pairwise contrasts	Chamber Type					
	Waste		Nursery		Ventilation	
	LRT	p-value	LRT	p-value	LRT	p-value
Annan River NP vs Cardwell	207.6	0.034 *	195.3	0.177	258.1	0.023 *
Annan River NP vs Cowley Beach	195.3	0.049 *	251.5	0.046 *	293.2	0.015 *
Annan River NP vs Kutini-Payamu NP	199.7	0.043 *	186.7	0.177	258.5	0.023 *
Annan River NP vs Port Douglas	235.2	0.023 *	198.6	0.177	258.6	0.023 *
Cardwell vs Cowley Beach	241.9	0.023 *	216.5	0.112	164.7	0.023 *
Cardwell vs Kutini-Payamu NP	142.1	0.075	165.3	0.177	204.1	0.023 *
Cardwell vs Port Douglas	231.8	0.023 *	250.6	0.046 *	328.2	0.006 **
Cowley Beach vs Kutini-Payamu NP	237.5	0.023 *	237.2	0.061	252.6	0.023 *
Cowley Beach vs Port Douglas	280.7	0.006 **	360.3	0.002 **	386.1	0.003 **
Kutini-Payamu NP vs Port Douglas	191.4	0.049 *	191.9	0.177	280.6	0.023 *

Significance codes: ***= 0.001, **= 0.01, *= 0.05

2.3.5 Dominant fungal taxa

I identified 27 dominant/common OTUs (from the dataset of 164 OTUs) by selecting those OTUs that occurred in at least 50% of the samples for a chamber type (Fig. 2.6, Table 2.2 - grey shaded values). These 27 OTUs were a subset of the 41 fungal OTUs previously identified as significantly differentially abundant (Table 2.3) and 10 of these 27 fungal OTUs recorded the highest sequence abundances (Supplementary Fig. S2.3). Each of the 27 OTUs occurred in more than one chamber type but with significantly different abundances (Fig. 2.6, Tables 2.2 and 2.3). For example, OTU0283 (Eurotiomycetes sp) was found in all waste chamber samples, 34.8% of nursery chambers and 9.1%

of ventilation chambers but in terms of abundance, the waste chambers contained most (96.4%) of the total abundance for this OTU (Table 2.2).

In the waste chambers, I found 13 dominant/common fungal OTUs from the orders Chaetothyriales (4), Eurotiales (1), Saccharomycetales (2), Mycosphaerellales (2), and from the class Eurotiomycetes (1) and Tremellomycetes (3). The percentage abundances of each of these 13 common waste chamber OTUs were more than 90% of the total abundances for each of these OTUs across all chambers (Table 2.2). The 13 common OTUs were either very low in abundance or absent in the nursery and ventilation chambers (Table 2.2, Fig. 2.6). Five of the 13 common fungal OTUs in the waste chambers were assigned to trophic modes by FUNGuild with three assigned as saprotrophs and two as symbiotrophs (Table 2.2). The nursery and ventilation chambers had four fungal OTUs that were common to both these chambers. These four OTUs were from the orders Chaetothyriales (1), Hysteriales (1), Hypocreales (1) and one OTU classified to class Eurotiomycetes (Table 2.2). Chaetothyreales OTU0347 was found in 71.7% of nursery chambers and 56.8% of ventilation chambers but the abundance of this OTU in the nursery chambers was 83.3% of the total abundance. Likewise, the Hysteriales OTU0563 was found in just over half of both the nursery and ventilation chambers but with highest abundance in the nursery chambers (72.8%). Despite occurring in at least 50% of both nursery and ventilation chambers, the Eurotiomycetes OTU0438 and the Hypocrealeas OTU0544 (*Fusarium* sp.) occurred in low abundances in the nursery chambers (<27%) compared to the ventilation chambers (>70% of the total abundances) (Table 2.2). The nursery chambers also contained two common OTUs, both from the order Eurotiales, that were found in >50% of the nursery chambers with relatively high abundances of 84.5% (OTU0281) and 77.2% (OTU0300). The ventilation chambers contained eight other common fungal OTUs belonging to the orders Exobasidiales (3), Capnodiales (3), and Tremellales (2). These eight fungal OTUs had the highest percentage abundances in the ventilation chambers (Table 2.2).

The 13 dominant/common OTUs in the waste chambers were significantly abundant (for chamber type), and five (OTU0202, OTU0214, OTU0263, OTU0302 and OTU0469) were also significantly abundant for location, (Table 2.2). Also, two OTUs were significant for location and had an interaction effect with chamber type including OTU0283 and OTU1029. Of the six common OTUs in the nursery chambers and 12 OTUs common to ventilation chambers, two were also significant for location including OTU0347 and OTU0898 (Table 2.2, Fig. 2.6).

Table 2.2 Fungal Operational Taxonomic Units (OTUs) that are significantly abundant in at least one of the chamber types of *M. beccarii*. Included are fungal taxon which is the closest match in online databases GenBank and UNITE, GenBank accession number, UNITE species hypothesis (SH) number, sequence similarity (Seq Sim%), sequence abundance (Seq abund), percentage sequence abundance in chamber samples, and percentage of chambers with the OTU. Grey shading indicates the OTU was dominant/common (in at least 50% of samples for that chamber type). Chambers: NC = nursery, VC = ventilation, WC = waste. FUNGuild trophic modes: SYM=symbiotroph, SAP=saprotroph and PATH=pathotroph and symbols represent confidence ranking: †probable and ‡possible.

OTUID	Fungal taxon	GenBank Accession number	UNITE SH number	Seq Sim (%)	Seq abund	% sequence abundance in chamber samples			% of chamber samples with OTU			FUNGuild trophic mode
						NC	VC	WC	NC	VC	WC	
OTU0170	Trichomeriaceae sp	KU195499	SH491217.07FU	95	2308	4.7	1.4	93.9	32.6	9.1	68.9	SYM†
OTU0171	Candida fluviatilis	HQ652068	SH200664.07FU	98	472	7.2	0.4	92.4	19.6	4.5	66.7	SAP‡
OTU0202[a]	Chaetothyriales sp	HQ634649	SH196444.07FU	99	1678	1.8	3.2	94.9	17.4	9.1	80.0	-
OTU0214	Trichomeriaceae sp	KU195499	SH491217.07FU	94	829	4.3	2.2	93.5	21.7	9.1	68.9	SYM†
OTU0263	Debaryomycetaceae sp	KP109748	SH192552.07FU	94	255	0.8	0.0	99.2	2.2	0.0	62.2	SAP‡
OTU0283	Eurotiomycetes sp	DQ914677	SH206547.07FU	83	3977	1.4	2.3	96.4	34.8	9.1	100.0	-
OTU0302	Mycosphaerellales sp*	GU117898	SH1541673.08FU	84	731	0.5	0.3	99.2	4.3	2.3	57.8	-
OTU0313	Mycosphaerellales sp	GU117898	SH1541673.08FU	83	97	1.0	0.0	99.0	2.2	0.0	60.0	-
OTU0469	Talaromyces sp	KP143766	SH209380.07FU	99	602	7.1	0.8	92.0	19.6	9.1	64.4	SAP†
OTU0518	Chaetothyriales sp	HM239979	SH212163.07FU	93	1283	0.1	0.0	99.9	2.2	0.0	57.8	-
OTU1026	Tremellomycetes sp	JX999048	SH477174.07FU	87	1397	1.4	2.6	96.1	6.5	4.5	71.1	-
OTU1028	Tremellomycetes sp	JX999048	SH477174.07FU	86	81	4.9	0.0	95.1	4.3	0.0	60.0	-
OTU1029	Tremellomycetes sp	JX999048	SH477174.07FU	87	2650	5.9	3.4	90.7	13.0	11.4	97.8	-
OTU0281	Talaromyces sp	KU141384	SH194198.07FU	100	375	84.5	3.2	12.3	52.2	13.6	26.7	SAP†
OTU0300	Talaromyces sp	KJ608116	SH194198.07FU	99	859	77.2	2.8	20.0	69.6	25.0	44.4	SAP†
OTU0347[b]	Chaetothyriales sp	KC951221	SH212029.07FU	97	4070	83.3	14.3	2.4	71.7	56.8	37.8	-
OTU0438	Eurotiomycetes sp	KX908623	-	86	679	26.7	70.1	3.2	54.3	59.1	15.6	-
OTU0544	Fusarium sp	JQ905732	SH025137.07FU	98	1606	24.2	72.6	3.2	73.9	100	22.2	PATH-SAP-SYM

OTUID	Fungal taxon	GenBank Accession number	UNITE SH number	Seq Sim (%)	Seq abund	% sequence abundance in chamber samples			% of chamber samples with OTU			FUNGuild trophic mode
						NC	VC	WC	NC	VC	WC	
OTU0563	Hysteriales sp	KF675741	SH205606.07FU	97	1637	72.8	21.9	5.3	56.5	52.3	20.0	-
OTU0648	Exobasidiales sp	KP730059	-	88	1190	26.1	73.4	0.5	41.3	84.1	8.9	-
OTU0670	Exobasidiales sp	KP730059	-	87	167	16.2	83.2	0.6	19.6	63.6	2.2	-
OTU0677	Exobasidiales sp	KP730059	-	88	605	26.8	73.1	0.2	37.0	88.6	2.2	-
OTU0780	Capnodiales sp	KC222753	SH025821.07FU	95	355	44.5	52.1	3.4	28.3	61.4	13.3	-
OTU0782	Capnodiales sp	KC222753	SH025821.07FU	99	205	4.9	94.1	1.0	13.0	52.3	2.2	-
OTU0783	Capnodiales sp	KC222753	SH025821.07FU	96	176	26.1	69.3	4.5	21.7	56.8	13.3	-
OTU0898	Cuniculitremaceae sp	KY103846	SH1523569.08FU	91	579	40.4	56.5	3.1	43.5	59.1	6.7	-
OTU0938	Fellomyces sp	AJ608646	SH204460.07FU	100	270	33.7	64.8	1.5	43.5	75.0	8.9	PATH-SAP-SYM‡
OTU0221	Sporothrix eucalyptigena	KU865592	-	99	309	61.5	0.3	38.2	23.9	2.3	44.4	PATH-SAP-SYM
OTU0291	Ustilaginomycotina sp	AB180368	SH025674.07FU	91	580	20.9	78.3	0.9	34.8	43.2	6.7	-
OTU0372[c]	Chaetothyriales sp	HQ634648	SH212029.07FU	100	424	89.9	4.5	5.7	30.4	11.4	15.6	-
OTU0373[d]	Chaetothyriales sp	HQ634653	SH025817.07FU	98	767	94.0	2.6	3.4	45.7	22.7	26.7	-
OTU0457	Talaromyces sp	KF366489	SH209380.07FU	100	512	70.5	14.3	15.2	47.8	34.1	40.0	SAP†
OTU0561	Hysteriales sp	KF675741	SH021234.07FU	98	728	57.8	38.7	3.4	34.8	25.0	17.8	-
OTU0567	Hysteriales sp	KF675741	SH021234.07FU	98	600	60.0	37.0	3.0	47.8	27.3	8.9	-
OTU0623	Cryptodiscus sp	AJ877182	SH210980.07FU	99	536	48.3	51.5	0.2	26.1	47.7	2.2	SAP†
OTU0667	Pezicula radicicola	HQ889715	SH201622.07FU	100	619	50.2	48.5	1.3	19.6	20.5	8.9	PATH-SAP†
OTU0674	Capnodiales sp	KC222753	SH025821.07FU	94	198	37.4	59.1	3.5	37.0	45.5	15.6	-
OTU0746	Capnodiales sp	KC222753	SH025821.07FU	98	279	2.5	96.8	0.7	8.7	47.7	2.2	-
OTU0815	Capnodiales sp	JQ760724	SH025821.07FU	95	263	46.8	48.7	4.6	39.1	38.6	11.1	-

OTUID	Fungal taxon	GenBank Accession number	UNITE SH number	Seq Sim (%)	Seq abund	% sequence abundance in chamber samples			% of chamber samples with OTU			FUNGuild trophic mode
						NC	VC	WC	NC	VC	WC	
OTU0821	Candida sp	JQ683772	SH203686.07FU	96	157	0.6	0.0	99.4	2.2	0.0	37.8	SAP‡
OTU0981	Kockovaella sp	KY103848	SH176359.07FU	95	285	48.8	46.0	5.3	34.8	45.5	11.1	PATH-SAP-SYM

[a] Fungal OTU0202 – closest match: domatia of ant-plant *Keetia hispida* (Rubiaceae) in Cameroon (ant species: *Crematogaster* sp. (Myrmicinae)) KhNk3-2 (Voglmayr et al., 2011)
[b] Fungal OTU0347 – closest match: domatia of ant-plant *Leonardoxa africana letouzeyi* (Fabaceae: Caesalpinioideae) (ant species: *Aphomomyrmex afer* (Formicinae)) Kh-1 (Blatrix et al., 2013)
[c] Fungal OTU0372 – closest match: domatia of ant-plant *Keetia hispida* (Rubiaceae) in Cameroon (ant species: *Crematogaster margaritae*) KhNk2-2b (Voglmayr et al., 2011)
[d] Fungal OTU0373 – closest match: domatia of ant-plant *Saraca thaipingensis* (Fabaceae: Caesalpinioideae) in Malaysia (ant species: *Cladomyrma petalae* (Formicinae)) MACP1 (Voglmayr et al., 2011)

Table 2.3 Significantly abundant fungal operational taxonomic units (OTUs) in the waste, nursery and ventilation chambers of *M. beccarii.* The symbol † indicates the OTU is included in the Top 10 most abundant fungal OTUs. Fungal taxon is the best match found in GenBank/UNITE databases to the fungal OTUs collected in this study. Pairwise contrasts found 41 OTUs to be significantly differentially abundant (DESeq2 results). Base Mean is the average of the normalized count values, divided by size factors, taken over all samples, log2Fold Change is the effect size estimate (how much the OTU abundance changed in the pairwise contrast of chamber types) and the value is reported on a logarithmic scale to base 2. LFC SE is the standard error estimate for the log2 fold change estimate. The adjusted p-value is the Benjamini-Hochberg adjustment for multiple testing (to control the false discovery rate). Of these 41 OTUs, 34 were found to be significantly differentially abundant from the multivariate abundance analysis (mvabund results). The mvabund analysis identified which fungal OTUs were significantly abundant for chamber type and/or location and where there was an interaction, but it does not identify which chamber type or which location. The abbreviation "ns" refers to non-significance.

OTUID	Fungal taxon	DESeq2 results					mvabund results		
		Base Mean	log2 Fold Change	LFC SE	adjusted p-value	contrasts between chamber types	chamber type	location	inter-action
OTU0170†	Trichomeriaceae sp	15.8755	4.5244	0.7261	<0.0001	waste vs. nursery	<0.001	ns	ns
			6.7255	0.7564	<0.0001	waste vs. ventilation			
OTU0171	Candida fluviatilis	3.4609	3.4016	0.7408	<0.0001	waste vs. nursery	<0.001	ns	ns
			5.2003	0.7660	<0.0001	waste vs. ventilation			
OTU0202†	Chaetothyriales sp	10.9030	5.3425	0.7441	<0.0001	waste vs. nursery	<0.001	<0.01	ns
			5.5793	0.7509	<0.0001	waste vs. ventilation			
OTU0214	Trichomeriaceae sp	5.9390	4.2244	0.7087	<0.0001	waste vs. nursery	<0.001	<0.01	ns
			5.2024	0.7237	<0.0001	waste vs. ventilation			
OTU0263	Debaryomycetaceae sp	1.4426	3.6296	0.8178	<0.0001	waste vs. nursery	<0.001	<0.001	ns
			4.1561	0.8289	<0.0001	waste vs. ventilation			
OTU0283†	Eurotiomycetes sp	28.2797	6.1078	0.5795	<0.0001	waste vs. nursery	<0.001	<0.05	<0.001
			7.1054	0.5974	<0.0001	waste vs. ventilation			
OTU0302	Mycosphaerellales sp	7.3770	5.4152	0.9331	<0.0001	waste vs. nursery	<0.001	<0.05	ns
			5.9907	0.9461	<0.0001	waste vs. ventilation			
OTU0313	Mycosphaerellales sp	0.6314	2.7155	0.9049	<0.001	waste vs. nursery	<0.001	ns	ns
			3.1878	0.9169	<0.001	waste vs. ventilation			
OTU0469	Talaromyces sp	5.3789	4.2540	0.7708	<0.0001	waste vs. nursery	<0.001	<0.05	ns
			5.8557	0.7927	<0.0001	waste vs. ventilation			

OTUID	Fungal taxon	DESeq2 results					mvabund results		
		Base Mean	log2 Fold Change	LFC SE	adjusted p-value	contrasts between chamber types	chamber type	location	inter-action
OTU0518†	Chaetothyriales sp	7.9028	6.1675	0.9531	**<0.0001**	waste vs. nursery	**<0.001**	ns	ns
			6.5854	0.9638	**<0.0001**	waste vs. ventilation			
OTU1026†	Tremellomycetes sp	10.6870	6.4035	0.9057	**<0.0001**	waste vs. nursery	**<0.001**	ns	ns
			6.2846	0.9152	**<0.0001**	waste vs. ventilation			
OTU1028	Tremellomycetes sp	0.5729	3.1547	0.8772	0.0008	waste vs. ventilation	**<0.001**	ns	ns
OTU1029†	Tremellomycetes sp	19.2890	6.5169	0.6642	**<0.0001**	waste vs. nursery	**<0.001**	**<0.001**	<0.05
			6.9120	0.6723	**<0.0001**	waste vs. ventilation			
OTU0281	Talaromyces sp	2.7017	3.8785	0.8275	**<0.0001**	nursery vs. ventilation	<0.01	ns	ns
OTU0300	Talaromyces sp	7.3471	3.0174	0.7015	**<0.0001**	waste vs. ventilation	**<0.001**	ns	ns
			4.5797	0.6925	**<0.0001**	nursery vs. ventilation			
OTU0347†	Chaetothyriales sp	41.1045	5.3899	0.6169	**<0.0001**	nursery vs. waste	**<0.001**	**<0.001**	ns
			2.5572	0.6309	0.0002	ventilation vs. waste			
			2.8327	0.6077	**<0.0001**	nursery vs. ventilation			
OTU0438	Eurotiomycetes sp	5.0590	3.0895	0.7077	**<0.0001**	nursery vs. waste	**<0.001**	ns	ns
			3.9125	0.7094	**<0.0001**	ventilation vs. waste			
OTU0544†	Fusarium sp	11.7027	3.2445	0.5006	**<0.0001**	nursery vs. waste	**<0.001**	ns	ns
			4.8572	0.4999	**<0.0001**	ventilation vs. waste			
			1.6127	0.4550	0.0027	ventilation vs. nursery			
OTU0563†	Hysteriales sp	13.3790	4.4893	0.7920	**<0.0001**	nursery vs. waste	<0.05	ns	ns
			2.3656	0.8049	0.0074	ventilation vs. waste			
OTU0648†	Exobasidiales sp	7.1766	3.9134	0.6214	**<0.0001**	nursery vs. waste	**<0.001**	ns	ns
			6.0686	0.6151	**<0.0001**	ventilation vs. waste			
			2.1552	0.5580	0.0011	ventilation vs. nursery			
OTU0670	Exobasidiales sp	1.0327	3.5540	0.7402	**<0.0001**	ventilation vs. waste	**<0.001**	ns	ns
OTU0677	Exobasidiales sp	3.6334	3.2921	0.6091	**<0.0001**	nursery vs. waste	**<0.001**	ns	ns
			5.4640	0.5907	**<0.0001**	ventilation vs. waste			
			2.1719	0.5443	0.0007	ventilation vs. nursery			
OTU0780	Capnodiales sp	2.0527	2.7730	0.7840	0.0016	nursery vs. waste	<0.05	ns	ns
			3.3875	0.7823	**<0.0001**	ventilation vs. waste			
OTU0782	Capnodiales sp	1.2627	4.1842	0.9293	**<0.0001**	ventilation vs. waste	**<0.001**	ns	ns
			3.3189	0.9241	0.0025	ventilation vs. nursery			
OTU0783	Capnodiales sp	1.0831	2.7067	0.7309	0.0006	ventilation vs. waste	<0.01	ns	ns

OTUID	Fungal taxon	DESeq2 results					mvabund results		
		Base Mean	log2 Fold Change	LFC SE	adjusted p-value	contrasts between chamber types	chamber type	location	inter-action
OTU0898	Cuniculitremaceae sp	4.2562	3.4739	0.7263	**<0.0001**	nursery vs. waste	<0.01	<0.01	ns
			3.6656	0.7291	**<0.0001**	ventilation vs. waste			
OTU0938	Fellomyces sp	1.6562	2.9681	0.5802	**<0.0001**	nursery vs. waste	**<0.001**	ns	ns
			3.8377	0.5676	**<0.0001**	ventilation vs. waste			
OTU0221	Sporothrix eucalyptigena	2.3369	2.7588	0.9430	0.0075	waste vs. ventilation	<0.01	<0.01	ns
			2.9882	0.9409	0.0092	nursery vs. ventilation			
OTU0291	Ustilaginomycotina sp	4.3914	2.5056	0.8336	0.0089	nursery vs. waste	**<0.001**	**<0.001**	ns
			4.1387	0.8282	**<0.0001**	ventilation vs. waste			
OTU0372	Chaetothyriales sp	3.0937	3.7334	1.14302	0.0040	nursery vs. waste	ns	ns	ns
			4.2716	1.15231	0.0018	nursery vs. ventilation			
OTU0373	Chaetothyriales sp	5.8739	4.5343	0.7829	**<0.0001**	nursery vs. waste	**<0.001**	ns	ns
			4.7105	0.7912	**<0.0001**	nursery vs. ventilation			
OTU0457	Talaromyces sp	3.8679	3.0144	0.7004	**<0.0001**	nursery vs. waste	ns	<0.01	ns
			3.1380	0.7073	**<0.0001**	nursery vs. ventilation			
OTU0561	Hysteriales sp	6.2862	3.5635	0.9930	0.0014	nursery vs. waste	ns	<0.01	ns
OTU0567	Hysteriales sp	5.3501	3.9853	0.9187	**<0.0001**	nursery vs. waste	ns	<0.01	ns
OTU0623	Cryptodiscus sp	3.7826	3.4673	0.9948	0.0019	nursery vs. waste	**<0.001**	ns	ns
			4.5019	0.9956	**<0.0001**	ventilation vs. waste			
OTU0667	Pezicula radicicola	6.2013	5.3410	1.2609	**<0.0001**	nursery vs. waste	ns	<0.01	ns
			4.8684	1.2750	0.0004	ventilation vs. waste			
OTU0674	Capnodiales sp	1.2467	2.2966	0.7384	0.0066	nursery vs. waste	<0.01	ns	ns
			2.6744	0.7361	0.0008	ventilation vs. waste			
OTU0746	Capnodiales sp	1.3859	3.3808	0.9349	0.0008	ventilation vs. waste	**<0.001**	<0.01	ns
OTU0815	Capnodiales sp	2.1288	2.5703	0.7953	0.0029	ventilation vs. waste	ns	<0.01	ns
OTU0821	Candida sp	1.0624	4.0572	1.3734	0.0073	waste vs. ventilation	**<0.001**	ns	ns
OTU0981	Kockovaella sp	2.1113	2.1745	0.7486	0.0078	ventilation vs. waste	ns	**<0.001**	ns

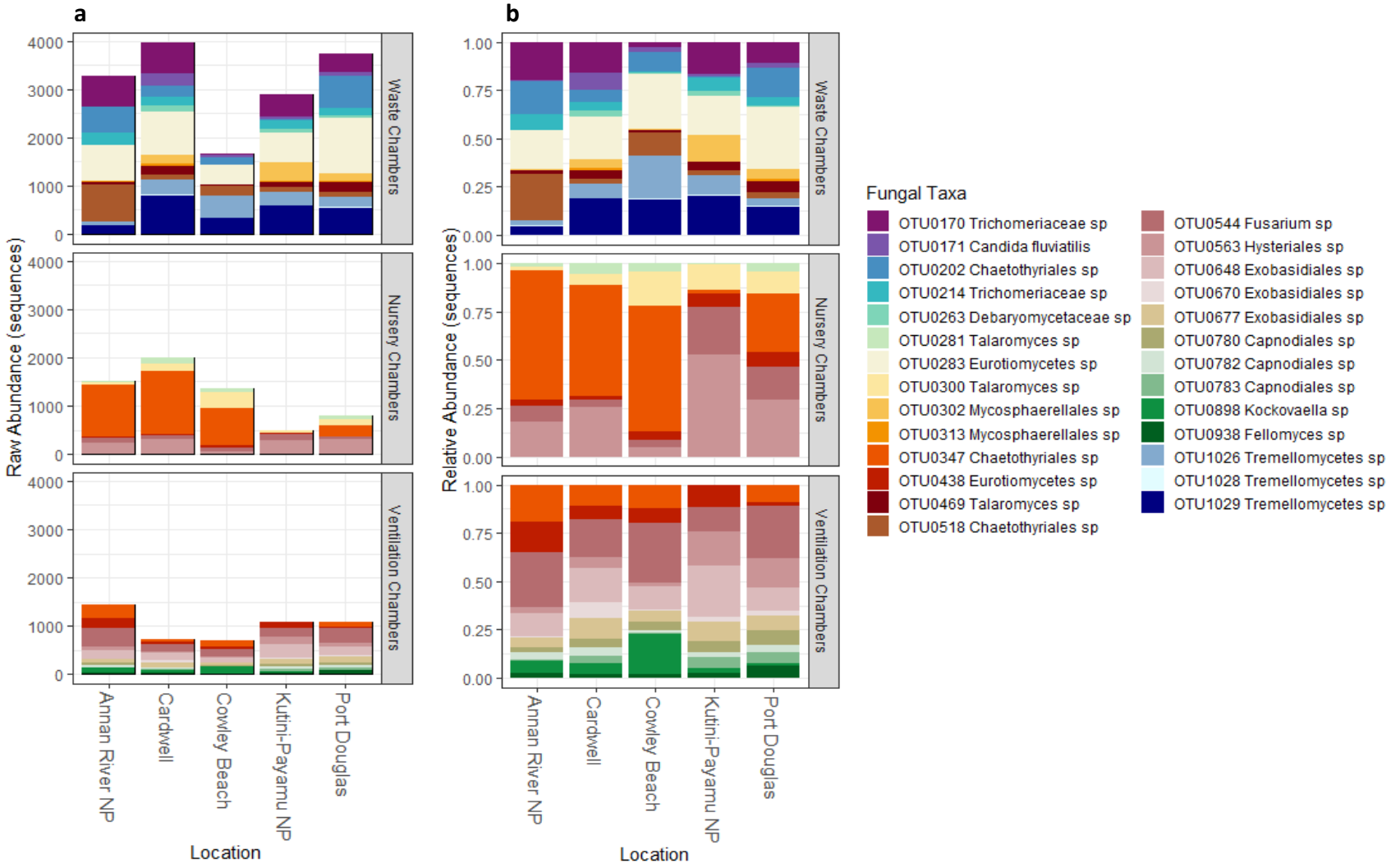

a
b
Raw Abundance (sequences)
Relative Abundance (sequences)
Waste Chambers
Nursery Chambers
Ventilation Chambers
Annan River NP
Cardwell
Cowley Beach
Kutini-Payamu NP
Port Douglas
Location
Fungal Taxa
OTU0170 Trichomeriaceae sp
OTU0171 Candida fluviatilis
OTU0202 Chaetothyriales sp
OTU0214 Trichomeriaceae sp
OTU0263 Debaryomycetaceae sp
OTU0281 Talaromyces sp
OTU0283 Eurotiomycetes sp
OTU0300 Talaromyces sp
OTU0302 Mycosphaerellales sp
OTU0313 Mycosphaerellales sp
OTU0347 Chaetothyriales sp
OTU0438 Eurotiomycetes sp
OTU0469 Talaromyces sp
OTU0518 Chaetothyriales sp
OTU0544 Fusarium sp
OTU0563 Hysteriales sp
OTU0648 Exobasidiales sp
OTU0670 Exobasidiales sp
OTU0677 Exobasidiales sp
OTU0780 Capnodiales sp
OTU0782 Capnodiales sp
OTU0783 Capnodiales sp
OTU0898 Kockovaella sp
OTU0938 Fellomyces sp
OTU1026 Tremellomycetes sp
OTU1028 Tremellomycetes sp
OTU1029 Tremellomycetes sp

Figure 2.6 (a) Raw sequence abundances and (b) Relative sequence abundances of the most common fungal operational taxonomic units (OTUs) in the three different chambers of *M. beccarii* (27 fungal OTUs in total) across the five locations surveyed. Fungal Taxa are the best match found in available databases (GenBank and UNITE). To be selected as one of the most common 27 OTUs, the OTU had to occur in at least 50% of at least one of the chamber types. Under this definition of 'most common', there were 6 OTUs in the nursery chambers, 12 OTUs in the ventilation chambers, and 13 OTUs in the waste chambers. Note that each of the most common OTUs for a chamber type may also occur in one, or both, of the other chamber types.

2.4 Discussion

This study is among the first to identify and compare the long overlooked fungal communities in the domatium chambers of an epiphytic ant-plant. I found strong associations between chamber type and fungal community composition across my five surveyed geographic locations. The fungal community in the waste chambers of *M. beccarii* was consistently distinct from the nursery and ventilation chambers across all five locations surveyed which spanned 675 km. The fungal communities within the nursery and ventilation chambers overlapped more than either did with the waste chambers but were also distinct from each other. The fungal OTUs found in each of the chamber types were generally not unique to each chamber type, however differences in OTU abundances drove the patterns I found in the fungal communities for each of the chamber types. As with other ant-plant systems studied to-date, Chaetothyriales fungi dominated in terms of the numbers and abundances of fungal OTUs in the chambers of *M. beccarii.*

2.4.1 Dominant fungal orders in the domatium chambers

Fungi from the order Chaetothyriales were dominant in the domatium chambers of *M. beccarii* in terms of the number of fungal OTUs and the abundances of OTUs. The dominance of Chaetothyriales fungi in *M. beccarii* is consistent with other studies of non-epiphytic ant-plant domatia in Cameroon, Malaysia and French Guiana (Defossez et al. 2009, Voglmayr et al. 2011, Blatrix et al. 2012, Blatrix et al. 2013, Nepel et al. 2016, Moreno et al. 2019). There were also high numbers of fungal OTUs from the order Capnodiales in the chambers of *M. beccarii* which, together with Chaetothyriales fungi, have also been found associated with ant-carton (Voglmayr et al. 2011) and ant nests (Schlick-Steiner et al. 2008). The presence of Eurotiales fungi in *M. beccarii* is not surprising given this order of fungi are ubiquitous in nature and include saprotrophic species as well as animal associated genera (Chen et al. 2015). Forty-six OTUs were unclassifiable at the order level suggesting there are species of fungi in this ant-plant that have never been sequenced before

according to the online databases available for comparison (GenBank and UNITE). My results suggest that epiphytic ant-plants may provide habitats for multiple novel fungal families and potentially order-level taxa, however it may be at least partly related to our poor understanding about the fungi inhabiting tree canopies in Australia.

2.4.2 The fungal communities in the domatium chambers

The waste, nursery, and ventilation chambers harboured different fungal communities that varied somewhat across locations. Between chambers, differences in fungal communities were driven primarily by variation in the relative sequence abundances of specific OTUs, rather than by unique differences in the identity or number of fungal OTUs in the different chambers. This is clear from the high number of fungal OTUs (94 of the 164 OTUs) that were shared among the three chambers that also collectively made up most (88.8%) of the total abundances. The high number of shared OTUs is not unexpected given the interconnectedness of the domatium chambers. The movement of ant workers among chambers potentially spreads fungal particles across other chamber types. However, despite many OTUs being found across chambers, some fungal OTUs occurred significantly more often in one chamber or another.

The waste chambers of *M. beccarii* contained 13 fungal OTUs that were significantly abundant and common across the five locations surveyed. The high abundances of these 13 common waste chamber OTUs (and low abundances in, or absence from, the other chambers) suggest that ant workers are maintaining and/or transporting fungi to the waste chambers (e.g. in faeces or other waste), and/or creating an environment suitable for specific fungi. Maintenance of fungi by ant workers in an ant-plant was first noticed by Miehe (1911) who observed fungal mats in the waste chambers of the epiphytic ant-plant *Myrmecodia tuberosa* (in Java) that had been cut neatly, and the only possible explanation was that the ant workers were trimming fungal hyphae. I also observed dense brown to black thick mats on most of the waste chamber surfaces of all dissected ant-plants. The waste chambers contain the colony's waste deposits and represent sources for plant nutrient acquisition (Huxley 1978, 1982). Therefore, it is reasonable to expect that at least some of these fungi are involved in the breakdown and releasing of nutrients from waste. Alternatively, fungi in the waste chambers may be cultivated as food or used for their secondary metabolites such as antimicrobial compounds that could be used by the ant colony as defence compounds against pathogens.

Common fungal OTUs in the waste chambers included four fungi from the order Chaetothyriales**.** One of these (OTU0202) matched at 99% similarity to a sequence found in the domatium of the ant-plant *Keetia hispida* (Rubiaceae) in Cameroon (accession number HQ634649) (Voglmayr et al. 2011). Although these two ant-plant species are from the family Rubiaceae, the ant species are from different sub-families (*Philidris cordata* (Dolichoderinae) in *M. beccarii* and *Crematogaster* sp. (Myrmicinae) in *K. hispida*) and these ant-plant systems have evolved separately on different continents. A Chaetothyriales fungal OTU found in *Azteca* sp. nests on *Cecropia* trees in Costa Rica was also isolated from domatia of *K. hispida* occupied by *Crematogaster margaritae* in Cameroon (Nepel et al. 2016, Vasse et al. 2017). My research supports the recent phylogenetic study reporting that some ant-associated Chaetothyriales fungi do not cluster according to the ant species, host ant-plant, or geographic origin (Vasse et al. 2017). The other three Chaetothyriales fungal OTUs found in the waste chambers across the five locations had sequence similarities that allowed identification of two of these OTUs to the family Trichomeriaceae (both as putative symbiotrophs according to FUNGuild) and the other to the order Chaetothyriales. Future research could investigate the chambers of other epiphytic ant-plants in the Australasian region to determine if these Chaetothyriales fungi are widespread in other epiphytic ant-plant systems, or whether they show any host plant and/or ant specificity.

Other (non-Chaetothyriales) fungi were also common and abundant in the waste chambers of *M. beccarii* and consistently found across the five locations surveyed. Three common OTUs were identified to the class Tremellomycetes which contains mostly yeasts that are mycoparasites or animal pathogens (Weiss et al. 2014). Their role (if any) in the waste chambers is yet to be determined, but it is possible that these yeasts act as mycoparasites on the mycelium of Chaetothyriales species. It has been suggested that the occurrence of fungi from orders such as Eurotiales, Hypocreales, Pleosporales, and Saccharomycetales are most likely contaminants (Vasse et al. 2017), opportunistic, or non-symbiotic competitors in ant-plant domatia (Blatrix et al. 2013). However, I found these non-Chaetothyriales fungal OTUs in more than 50% of the waste chambers (some with high abundance) but with very little occurrence in the other chamber types, suggesting a yet-to-be-established functional role.

The differences in the fungal communities between nursery and ventilation chambers were not so pronounced compared with the waste chambers. However, the abundances of most of the common fungal OTUs differed between the former two chamber types. The ventilation chambers lead into

the nursery chambers in the lower/middle part of the domatium of *M. beccarii*, whereas the waste chambers tend to be concentrated more towards the upper/middle portion of the domatium beneath the stem. This might explain why the nursery and ventilation chambers shared some fungal taxa that are relatively uncommon (or absent) in the waste chambers. Also, I often found brood in both the nursery and ventilation chambers, but rarely in the waste chambers (and then only pupae) and I observed ant workers moving brood between the nursery and ventilation chambers. This may be in response to temperature/humidity changes in this ant-plant and the movement of brood may further explain why there was overlap between these two chamber types as some fungi may be associated with the larvae.

Three fungi from the order Chaetothyriales were found in high abundances in the nursery chambers. Chaetothyriales OTU0347 was common at four of my locations and had a 97% match to a sequence isolated from domatia of the ant-plant *Leonardoxa africana letouzeyi* (ant species: *Aphomomyrmex afer*) in Cameroon (accession number KC951221) (Blatrix et al. 2013). The nursery chambers also contained two other Chaetothyriales fungi (OTUs 372 and 373) with high abundances but low frequency. These OTUs were matched with >98% identity similarity to sequences isolated from the ant-plant *Keetia hispida* (Rubiaceae) in Cameroon and *Saraca thaipingensis* (Fabaceae) in Malaysia respectively (Voglmayr et al. 2011). All domatium symbiont fungi isolated and sequenced previously are closely related to each other (Nepel et al. 2014), and the four Chaetothyriales fungal OTUs from this study support these findings. However, I also found other Chaetothyriales fungi that have not been recorded in other ant-plants.

The ventilation chambers were dominated by OTUs from the order Exobasidiales and Capnodiales. Fungi from Exobasidiales are known to be plant pathogens and are divided into four groups based on their morphology and the plant host range they parasitize, including plants from Ericanae, Lauraceae, monocots and palms (Begerow 2002). The Exobasidiales sequences found in this study could only be identified to the order level and have never been recorded before. Capnodiales fungi have been found in ant-carton in Cameroon and Malaysia (Voglmayr et al. 2011) and I often observed ant-carton in the ventilation chambers during this study, which may explain the occurrence of Capnodiales fungi. The greater exposure of ventilation chambers to the outside environment increases the likelihood of harbouring opportunistic fungi such as *Fusarium* OTU0544 which was abundant in all ventilation chambers.

2.4.3 Consistency in fungal OTU communities across the five locations

The significant geographic variation in abundances of 18 of the fungal OTUs and interaction between chamber and location for two of these OTUs indicates large variation in abundances across the five locations surveyed. The abundances and occurrences of any fungus in the domatium chambers of *M. beccarri* is likely to be influenced by interactions with other fungi and possibly other microorganisms such as bacteria and this could vary across locations due to, for example, different micro-climates outside domatia. Different numbers of ant workers in different ant-plants are also likely to alter the abundances of fungi in the domatium chambers they occupy. Seven of the 13 common waste chamber fungal OTUs were significantly more abundant at some locations and this may be due to the ant workers transporting/depositing different types (and amounts) of waste into the waste chambers at different locations. The fungal communities in the ventilation chambers were different across all locations and were the only chamber type to have a significant difference in fungal OTU richness at a location, being higher at Port Douglas compared to Cardwell. It is not surprising that the ventilation chambers had the most variation, given they are the most exposed of all chambers to the outside environment and therefore to a range of different fungi. I also observed ant workers entering domatia via large pores that are sometimes present on domatium and it is possible ant workers transport fungi from the outside environment into the ventilation chambers that could differ across locations. Only three of the pairwise comparisons of geographic locations for the nursery chambers were significant, suggesting the fungal communities in the nursery chambers are the most stable of the three chamber types. This could be because the brood of the colony are tended by ant workers in these chambers and it is likely the workers keep these chambers free of unwanted fungi. Despite these differences across locations, patterns in the occurrences and abundances of the common fungal OTUs discussed here were found in the domatium chambers of *M. beccarii*.

2.4.4 Conclusion

The consistent patterns in fungal communities among ant-plant chambers is extraordinary given their fragmented distribution across a broad range and the inclusion of specimens of *M. beccarii* from both national parks and suburban populations. The different chambers of this epiphytic ant-plant serve different purposes for the ant colony and the plant. It is in the waste chambers where the three potential players in this mutualism intersect: the ants deposit waste in the waste chambers, the fungal community is distinct in the waste chambers, and the plant absorbs nutrients

from the waste chambers. While I have not yet unequivocally determined what role/s fungi play in this ant-plant, I have achieved the first step in determining whether a multipartite mutualism exists by showing that the waste chambers contain a specific fungal community that is constant over a large portion of the distribution of this ant-plant. The role of fungi in this mutualism is likely to include the breakdown of organic waste in the waste chambers. However, fungi are involved in diverse interactions with other organisms. In this ant-plant, these interactions could include the production of antibiotic compounds that protect the brood in the nursery chambers from bacterial or fungal pathogens, nourishment of the ant colony, and other roles. It is also probable that some fungi are parasitic or opportunistic. Whether fungi perform any, or all, of these functions in ant-plants should be the focus of future research. Sampling of fungi in the chambers of other epiphytic ant-plants, as well as their resident ant workers, and the host trees and habitat in which epiphytic ant-plants live, could help explain how widespread and common (or not) fungi are in these ant-plants and in the environment generally. Answering these questions could ultimately unravel whether fungi are important in the evolution, maintenance, and stability of epiphytic ant-plant mutualisms.

2.5 Supplementary Information

Supplementary Table S2.1 Primers and molecular identifiers used in the study

Primers for high-throughput sequencing			
ITS1Fngs	GGTCATTTAGAGGAAGTAA		
ITS4ngs	TCCTSCGCTTATTGATATGC		
Multiplex Identifiers (MID)			
ACGAGTGCGT	ACTAGCAGTA	AGTGTATGTC	AGTCTGACTGA
ACGCTCGACA	AGTATACATA	ATAGATAGAC	AGTGAGCTCGA
AGACGCACTC	AGTCGAGAGA	ATATAGTCGC	ATAGCTCTCGA
ACTCGCGTGTC	AGTGCTACGA	ATCTACTGAC	ATCACGTGCGA
ATGATACGTCT	ACGATCGTATA	ACACGTAGATC	ATCGTAGCAGA
ACGAGAGATAC	ACGCAGTACGA	ACACGTGTCGC	ATCGTCTGTGA
ATACGACGTA	ACGCGTATACA	ACATACTCTAC	ATGTGTCTAGA
ATCACGTACTA	ACGTACAGTCA	ACGACACTATC	
ACGTCTAGTAC	ACGTACTCAGA	ACGAGACGCGC	
ATGTACTACTC	ACTATAGCGTA	ACGTATGCGAC	
ACATACGCGT	ATACGTCATCA	ACGTCGATCTC	
ACTACTATGT	ATAGTCGCATA	ACTAGTCACTC	
ACTGTACAGT	ATATATATACA	ACTCTACGCTC	
AGACTATACT	ATATGCTAGTA	ACTGTACATAC	
AGTACGCTAT	ATCACGCGAGA	ATAGACTGCAC	
ATAGAGTACT	ATCGATAGTGA	ATAGCTCTATC	
ACACGCTACGT	ATCGCTGCGTA	ATATAGACATC	
ACAGTAGACGT	ATCTGACGTCA	ATATGATACGC	
ACGACGTGACT	ATGAGTCAGTA	ATCACTCATAC	
ATACACACACT	ATGTAGTGTGA	ATCATCGAGTC	
ATACACGTGAT	ATGTCACACGA	ATCGAGCTCTC	
ATACAGATCGT	ATGTCGTCGCA	ATCGCAGACAC	
ATACGCTGTCT	ACACATACGC	ATCTGTCTCGC	
ATAGTGTAGAT	ACAGTCGTGC	ATGAGTGACGC	
ATCGATCACGT	ACGACAGCTC	ATGATGTGTAC	
ATCGCACTAGT	ACGTCTCATC	ATGCTATAGAC	
ATCTAGCGACT	ACTCATCTAC	ATGCTCGCTAC	
ATCTATACTAT	ACTCGCGCAC	ACGTGCAGCGA	
ATGACGTATGT	AGAGCGTCAC	ACTCACAGAGA	
ATGTGAGTAGT	AGCGACTAGC	AGACTCAGCGA	
AACAGTATATA	AGTAGTGATC	AGAGAGTGTGA	
ACGCGATCGA	AGTGACACAC	AGCTATCGCGA	

Supplementary Table S2.2 Taxonomic assignments of the 164 fungal operational taxonomic units (OTUs) collected from *Myrmecodia beccarii* waste, nursery and ventilation chambers (minimum read abundance >10) including: OTU identifier, Phylum, Order and Fungal Taxon (which represents the closest taxonomic match using the online available databases GenBank and UNITE), accession number (GenBank), species hypothesis number (UNITE) for the closest matched read from the databases, percentage sequence coverage to closest match, percentage sequence similarity to closest match, and total sequence abundances across samples.

OTUID	Phylum	Order	Fungal Taxon	Genbank Accession number	UNITE SH number	sequence coverage (%)	sequence similarity (%)	sequence abundance
OTU0463	Ascomycota	Botryosphaeriales	Endomelanconiopsis sp	FJ527864	SH195467.07FU	99.8	100	23
OTU0502	Ascomycota	Calosphaeriales	Jattaea aurea	KT716462	SH206445.07FU	100.0	99	59
OTU0287	Ascomycota	Capnodiales	Capnodiales sp	AY970168	SH012378.07FU	97.4	90	23
OTU0290	Ascomycota	Capnodiales	Capnodiales sp	AY970168	SH012378.07FU	95.8	91	34
OTU0674	Ascomycota	Capnodiales	Capnodiales sp	KC222753	SH025821.07FU	100.0	94	198
OTU0722	Ascomycota	Capnodiales	Capnodiales sp	JN890104	SH019572.07FU	97.2	91	12
OTU0746	Ascomycota	Capnodiales	Capnodiales sp	KC222753	SH025821.07FU	100.0	98	279
OTU0761	Ascomycota	Capnodiales	Capnodiales sp	KX908745	SH642194.07FU	100.0	95	301
OTU0780	Ascomycota	Capnodiales	Capnodiales sp	KC222753	SH025821.07FU	100.0	95	355
OTU0782	Ascomycota	Capnodiales	Capnodiales sp	KC222753	SH025821.07FU	100.0	99	205
OTU0783	Ascomycota	Capnodiales	Capnodiales sp	KC222753	SH025821.07FU	96.3	96	176
OTU0798	Ascomycota	Capnodiales	Capnodiales sp	JN890104	SH019572.07FU	96.5	93	35
OTU0805	Ascomycota	Capnodiales	Capnodiales sp	JN890104	SH019572.07FU	97.8	92	10
OTU0815	Ascomycota	Capnodiales	Capnodiales sp	JQ760724	SH025821.07FU	100.0	95	263
OTU0788	Ascomycota	Capnodiales	Cladosporium cladosporioides	KU314943		100.0	100	12
OTU0693	Ascomycota	Capnodiales	Devriesia sp	GU214635	SH202791.07FU	100.0	95	15
OTU0707	Ascomycota	Capnodiales	Devriesia sp	JQ905788	SH019575.07FU	97.8	99	82
OTU0763	Ascomycota	Capnodiales	Devriesia sp	JQ905788	SH019575.07FU	97.8	99	68

OTUID	Phylum	Order	Fungal Taxon	Genbank Accession number	UNITE SH number	sequence coverage (%)	sequence similarity (%)	sequence abundance
OTU0684	Ascomycota	Capnodiales	Devriesia strelitziicola	GU214635	SH202791.07FU	100.0	97	12
OTU0703	Ascomycota	Capnodiales	Toxicocladosporium rubrigenum	FJ790285	SH202299.07FU	100.0	99	15
OTU0289	Ascomycota	Chaetothyriales	Arthrocladium sp	KT337442	SH208969.07FU	100.0	98	13
OTU0135	Ascomycota	Chaetothyriales	Chaetothyriales sp	EU552107	SH198014.07FU	92.8	85	10
OTU0189	Ascomycota	Chaetothyriales	Chaetothyriales sp	KC222765	SH009575.07FU	99.6	91	19
OTU0191	Ascomycota	Chaetothyriales	Chaetothyriales sp	HQ634649	SH196444.07FU	100.0	99	22
OTU0365	Ascomycota	Chaetothyriales	Chaetothyriales sp	HQ634653	SH025817.07FU	99.0	98	32
OTU0372	Ascomycota	Chaetothyriales	Chaetothyriales sp	HQ634648	SH212029.07FU	100.0	100	424
OTU0384	Ascomycota	Chaetothyriales	Chaetothyriales sp	KC951221	SH212029.07FU	99.6	96	23
OTU0468	Ascomycota	Chaetothyriales	Chaetothyriales sp	JX014388	SH029055.07FU	100.0	96	101
OTU0504	Ascomycota	Chaetothyriales	Chaetothyriales sp	JX014388	SH029055.07FU	100.0	96	12
OTU0516	Ascomycota	Chaetothyriales	Chaetothyriales sp	KF675595	SH014029.07FU	98.4	85	51
OTU0518	Ascomycota	Chaetothyriales	Chaetothyriales sp	HM239979	SH212163.07FU	99.2	93	1283
OTU0536	Ascomycota	Chaetothyriales	Chaetothyriales sp	KF675595	SH014029.07FU	89.0	87	12
OTU0202	Ascomycota	Chaetothyriales	Chaetothyriales sp	HQ634649	SH196444.07FU	100.0	99	1678
OTU0347	Ascomycota	Chaetothyriales	Chaetothyriales sp	KC951221	SH212029.07FU	99.6	97	4070
OTU0373	Ascomycota	Chaetothyriales	Chaetothyriales sp	HQ634653	SH025817.07FU	99.0	98	767
OTU0187	Ascomycota	Chaetothyriales	Exophiala oligosperma	KP938216	SH193070.07FU	100.0	100	29
OTU0195	Ascomycota	Chaetothyriales	Fonsecaea brasiliensis	JN173796	SH188917.07FU	100.0	99	10
OTU0155	Ascomycota	Chaetothyriales	Fonsecaea sp	KF928456	SH188921.07FU	100.0	100	58
OTU0201	Ascomycota	Chaetothyriales	Fonsecaea sp	JF267657	SH009592.07FU	100.0	95	76
OTU0467	Ascomycota	Chaetothyriales	Phaeomoniella sp	JN225891	SH015548.07FU	100.0	96	127
OTU0170	Ascomycota	Chaetothyriales	Trichomeriaceae sp	KU195499	SH491217.07FU	100.0	95	2308

OTUID	Phylum	Order	Fungal Taxon	Genbank Accession number	UNITE SH number	sequence coverage (%)	sequence similarity (%)	sequence abundance
OTU0214	Ascomycota	Chaetothyriales	Trichomeriaceae sp	KU195499	SH491217.07FU	100.0	94	829
OTU0038	Ascomycota	Diaporthales	Diaporthales sp	KM199768	SH190605.07FU	100.0	86	11
OTU0267	Ascomycota	Eurotiales	Eurotiales sp	AB986366	SH523442.07FU	99.0	96	152
OTU0272	Ascomycota	Eurotiales	Eurotiales sp	AB986366	SH523442.07FU	99.4	94	185
OTU0293	Ascomycota	Eurotiales	Eurotiales sp	AB986366	SH523442.07FU	99.0	96	170
OTU0306	Ascomycota	Eurotiales	Eurotiales sp	AB986366	SH523442.07FU	99.2	92	99
OTU0314	Ascomycota	Eurotiales	Eurotiales sp	AB986366	SH523442.07FU	99.0	93	57
OTU0335	Ascomycota	Eurotiales	Eurotiales sp	AB986366	SH523442.07FU	98.8	94	118
OTU0402	Ascomycota	Eurotiales	Penicillium citreonigrum	KP329595	SH182483.07FU	100.0	99	116
OTU0458	Ascomycota	Eurotiales	Penicillium citreosulfuratum	KP016814	SH182483.07FU	100.0	100	59
OTU0739	Ascomycota	Eurotiales	Penicillium citrinum	LT558884		100.0	100	261
OTU0471	Ascomycota	Eurotiales	Penicillium multicolor	JN799647	SH182484.07FU	100.0	99	97
OTU0432	Ascomycota	Eurotiales	Penicillium singorense	LT558940		100.0	100	50
OTU0417	Ascomycota	Eurotiales	Talaromyces aculeatus	KP143766	SH209380.07FU	100.0	98	17
OTU0281	Ascomycota	Eurotiales	Talaromyces sp	KU141384	SH194198.07FU	100.0	100	375
OTU0300	Ascomycota	Eurotiales	Talaromyces sp	KJ608116	SH194198.07FU	100.0	99	859
OTU0457	Ascomycota	Eurotiales	Talaromyces sp	KF366489	SH209380.07FU	100.0	100	512
OTU0469	Ascomycota	Eurotiales	Talaromyces sp	KP143766	SH209380.07FU	100.0	99	602
OTU0486	Ascomycota	Eurotiales	Trichocomaceae sp	KP235558	SH209380.07FU	100.0	99	21
OTU0658	Ascomycota	Helotiales	Hyaloscyphaceae sp	KT270007	SH488999.07FU	99.4	97	53
OTU0667	Ascomycota	Helotiales	Pezicula radicicola	HQ889715	SH201622.07FU	100.0	100	619
OTU0654	Ascomycota	Helotiales	Proliferodiscus sp	JN033427	SH196496.07FU	100.0	97	538
OTU0632	Ascomycota	Helotiales	Scytalidium lignicola	HM214453	SH025355.07FU	100.0	97	24

OTUID	Phylum	Order	Fungal Taxon	Genbank Accession number	UNITE SH number	sequence coverage (%)	sequence similarity (%)	sequence abundance
OTU0336	Ascomycota	Hypocreales	Acremonium polychromum	KP184325	SH213543.07FU	100.0	100	16
OTU0876	Ascomycota	Hypocreales	Bionectriaceae sp	JQ905678	SH204399.07FU	100.0	99	21
OTU0619	Ascomycota	Hypocreales	Fusarium solani	AM412643	SH205225.07FU	100.0	98	23
OTU0544	Ascomycota	Hypocreales	Fusarium sp	JQ905732	SH025137.07FU	95.0	98	1606
OTU0638	Ascomycota	Hypocreales	Fusarium sp	GU595029	SH203010.07FU	100.0	100	81
OTU0651	Ascomycota	Hypocreales	Fusarium sp	KF675604	SH219676.07FU	100.0	100	10
OTU0338	Ascomycota	Hypocreales	Hypocreaceae sp	KU534625		100.0	100	41
OTU0233	Ascomycota	Hypocreales	Trichoderma harzianum	KU681407	SH190868.07FU	100.0	100	10
OTU0280	Ascomycota	Hypocreales	Trichoderma virens	LC123599	SH187757.07FU	100.0	100	19
OTU0561	Ascomycota	Hysteriales	Hysteriales sp	KF675741	SH021234.07FU	100.0	98	728
OTU0563	Ascomycota	Hysteriales	Hysteriales sp	KF675741	SH205606.07FU	100.0	97	1637
OTU0570	Ascomycota	Hysteriales	Hysteriales sp	KF675741	SH205606.07FU	100.0	96	32
OTU0302	Ascomycota	Mycosphaerellales	Mycosphaerellales sp	GU117898	SH1541673.08FU	80.9	84	731
OTU0313	Ascomycota	Mycosphaerellales	Mycosphaerellales sp	GU117898	SH1541673.08FU	81.1	83	97
OTU0221	Ascomycota	Ophiostomatales	Sporothrix eucalyptigena	KU865592		99.6	99	309
OTU0623	Ascomycota	Ostropales	Cryptodiscus sp	AJ877182	SH210980.07FU	100.0	99	536
OTU0519	Ascomycota	Ostropales	Ostropales sp	KF617267	SH025039.07FU	97.9	90	402
OTU0550	Ascomycota	Ostropales	Ostropales sp	KF617267	SH025039.07FU	97.9	90	73
OTU0719	Ascomycota	Ostropales	Ostropales sp	AJ877182	SH210980.07FU	95.3	88	45
OTU0737	Ascomycota	Ostropales	Ostropales sp	KF617267	SH025039.07FU	98.5	91	50
OTU0902	Ascomycota	Ostropales	Ostropales sp	AJ877182	SH210980.07FU	99.1	87	15
OTU0831	Ascomycota	Pleosporales	Pleosporales sp	DQ914714	SH016308.07FU	98.0	92	67
OTU0171	Ascomycota	Saccharomycetales	Candida fluviatilis	HQ652068	SH200664.07FU	100.0	98	472

OTUID	Phylum	Order	Fungal Taxon	Genbank Accession number	UNITE SH number	sequence coverage (%)	sequence similarity (%)	sequence abundance
OTU1048	Ascomycota	Saccharomycetales	Candida melibiosica	KJ939352		100.0	97	86
OTU0821	Ascomycota	Saccharomycetales	Candida sp	JQ683772	SH203686.07FU	98.3	96	157
OTU0977	Ascomycota	Saccharomycetales	Candida sp	KM504131	SH495679.07FU	100.0	99	193
OTU1001	Ascomycota	Saccharomycetales	Candida sp	KM504131	SH495679.07FU	100.0	94	78
OTU0263	Ascomycota	Saccharomycetales	Debaryomycetaceae sp	KP109748	SH192552.07FU	100.0	94	255
OTU0304	Ascomycota	Saccharomycetales	Debaryomycetaceae sp	AY553844	SH192555.07FU	100.0	92	153
OTU0308	Ascomycota	Saccharomycetales	Debaryomycetaceae sp	KP235619	SH192552.07FU	100.0	93	20
OTU1035	Ascomycota	Saccharomycetales	Hyphopichia sp	KP691036		98.9	95	10
OTU1036	Ascomycota	Saccharomycetales	Hyphopichia sp	KP691036		98.9	95	29
OTU0644	Ascomycota	Saccharomycetales	Sugiyamaella smithiae	KU883304		100.0	100	33
OTU0713	Ascomycota	Trichosphaeriales	Nigrospora oryzae	KC771457	SH177637.07FU	100.0	99	10
OTU0754	Ascomycota	Trichosphaeriales	Nigrospora oryzae	KT224807	SH177636.07FU	100.0	100	17
OTU0709	Ascomycota	Trichosphaeriales	Trichosphaeriales sp	KP975475	SH177638.07FU	100.0	100	14
OTU0331	Ascomycota	Xylariales	Pestalotiopsis colombiensis	KU715149		100.0	100	42
OTU0770	Ascomycota	Xylariales	Pestalotiopsis sinensis	KU715146		100.0	99	75
OTU0487	Ascomycota	unid. Dothideomycetes	Dothideomycetes sp	EU977184	SH021234.07FU	100.0	97	12
OTU0510	Ascomycota	unid. Dothideomycetes	Dothideomycetes sp	EU977184	SH021234.07FU	100.0	97	49
OTU0528	Ascomycota	unid. Dothideomycetes	Dothideomycetes sp	JQ760450	SH216757.07FU	100.0	99	39
OTU0583	Ascomycota	unid. Dothideomycetes	Dothideomycetes sp	EU977184	SH021234.07FU	100.0	95	21
OTU0599	Ascomycota	unid. Dothideomycetes	Dothideomycetes sp	GU595039	SH027549.07FU	100.0	98	95
OTU0802	Ascomycota	unid. Dothideomycetes	Dothideomycetes sp	KM519295	SH200715.07FU	97.4	90	26
OTU0860	Ascomycota	unid. Dothideomycetes	Dothideomycetes sp	KX908744		96.7	91	22
OTU0884	Ascomycota	unid. Dothideomycetes	Dothideomycetes sp	GQ999265	SH200708.07FU	99.6	81	10

OTUID	Phylum	Order	Fungal Taxon	Genbank Accession number	UNITE SH number	sequence coverage (%)	sequence similarity (%)	sequence abundance
OTU0887	Ascomycota	unid. Dothideomycetes	Dothideomycetes sp	JN890113	SH459884.07FU	99.8	82	27
OTU0896	Ascomycota	unid. Dothideomycetes	Dothideomycetes sp	EU661873	SH001790.07FU	94.2	84	34
OTU0928	Ascomycota	unid. Dothideomycetes	Dothideomycetes sp	GU017512	SH001790.07FU	97.8	83	14
OTU0567	Ascomycota	unid. Dothideomycetes	Hysteriales sp	KF675741	SH021234.07FU	100.0	98	600
OTU0505	Ascomycota	unid. Eurotiomycetes	Eurotiales sp	KP202994	SH488776.07FU	88.6	88	143
OTU0266	Ascomycota	unid. Eurotiomycetes	Eurotiomycetes sp	DQ914677	SH206547.07FU	93.0	82	19
OTU0283	Ascomycota	unid. Eurotiomycetes	Eurotiomycetes sp	DQ914677	SH206547.07FU	93.0	83	3977
OTU0288	Ascomycota	unid. Eurotiomycetes	Eurotiomycetes sp	AM902038	SH468872.07FU	91.4	81	19
OTU0319	Ascomycota	unid. Eurotiomycetes	Eurotiomycetes sp	AM902038	SH468872.07FU	92.1	81	31
OTU0438	Ascomycota	unid. Eurotiomycetes	Eurotiomycetes sp	KX908623		95.8	86	679
OTU0478	Ascomycota	unid. Eurotiomycetes	Eurotiomycetes sp	KF675595	SH014029.07FU	98.3	84	11
OTU0501	Ascomycota	unid. Eurotiomycetes	Eurotiomycetes sp	KF675595	SH014029.07FU	98.4	84	262
OTU0600	Ascomycota	unid. Eurotiomycetes	Eurotiomycetes sp	DQ309129	SH469901.07FU	94.6	84	10
OTU0943	Ascomycota	unid. Lecanoromycetes	Lecanoromycetes	KF617293	SH200320.07FU	87.4	84	183
OTU1011	Ascomycota	unid. Sordariomycetes	Sordariomycetes sp	JQ905789	SH456958.07FU	88.9	89	94
OTU0485	Basidiomycota	Auriculariales	Auriculariales sp	JQ272342	SH027409.07FU	96.5	93	75
OTU0083	Basidiomycota	Cantharellales	Cantharellales sp	FM866336	SH007381.07FU	100.0	98	22
OTU0143	Basidiomycota	Corticiales	Marchandiomyces sp	AY583326	SH208841.07FU	100.0	96	36
OTU0628	Basidiomycota	Exobasidiales	Exobasidiales sp	KP730059		92.4	87	23
OTU0648	Basidiomycota	Exobasidiales	Exobasidiales sp	KP730059		92.4	88	1190
OTU0670	Basidiomycota	Exobasidiales	Exobasidiales sp	KP730059		92.4	87	167
OTU0677	Basidiomycota	Exobasidiales	Exobasidiales sp	KP730059		92.4	88	605
OTU0585	Basidiomycota	Septobasidiales	Septobasidiales sp	AB043972	SH010567.07FU	100.0	86	10

OTUID	Phylum	Order	Fungal Taxon	Genbank Accession number	UNITE SH number	sequence coverage (%)	sequence similarity (%)	sequence abundance
OTU0264	Basidiomycota	Sporidiobolales	Rhodotorula mucilaginosa	KU298469		100.0	100	38
OTU0145	Basidiomycota	Trechisporales	Trechispora sp	JF691276	SH009017.07FU	100.0	98	26
OTU0938	Basidiomycota	Tremellales	Fellomyces sp	AJ608646	SH204460.07FU	100.0	100	270
OTU0898	Basidiomycota	Tremellales	Kockovaella sp	KY103846	SH1523569.08FU	100.0	91	579
OTU0981	Basidiomycota	Tremellales	Kockovaella sp	KY103848	SH176359.07FU	100.0	95	285
OTU0633	Basidiomycota	Tremellales	Tremellales sp	AY518273	SH020544.07FU	97.7	94	20
OTU0929	Basidiomycota	Tremellales	Tremellales sp	KM246201	SH535758.07FU	100.0	92	20
OTU0531	Basidiomycota	unid. Basidiomycota	Basidiomycota sp	JX999053	SH029161.07FU	97.6	90	104
OTU0461	Basidiomycota	unid. Exobasidiomycetes	Exobasidiomycetes sp	JX575187	SH198249.07FU	94.0	81	59
OTU0665	Basidiomycota	unid. Pucciniomycetes	Pucciniomycetes sp	KJ832014	SH472715.07FU	81.5	82	11
OTU0652	Basidiomycota	unid. Tremellomycetes	Tremellomycetes sp	KF617649	SH025808.07FU	80.2	92	80
OTU1026	Basidiomycota	unid. Tremellomycetes	Tremellomycetes sp	JX999048	SH477174.07FU	90.4	87	1397
OTU1028	Basidiomycota	unid. Tremellomycetes	Tremellomycetes sp	JX999048	SH477174.07FU	90.4	86	81
OTU1029	Basidiomycota	unid. Tremellomycetes	Tremellomycetes sp	JX999048	SH477174.07FU	90.4	87	2650
OTU0291	Basidiomycota	unid. Ustilaginomycotina	unid. Ustilaginomycotina	AB180368	SH025674.07FU	41.3	91	580
OTU0040	Mortierellomycota	Mortierellales	Mortierellales sp	JX975889	SH007250.07FU	98.2	84	41
OTU0279	unid. Fungi	unid. Fungi	Fungi sp	KM104119	SH491823.07FU	100.0	99	48
OTU0309	unid. Fungi	unid. Fungi	Fungi sp	DQ309127		93.1	81	16
OTU0315	unid. Fungi	unid. Fungi	Fungi sp	KP889575	SH193251.07FU	98.5	78	21
OTU0332	unid. Fungi	unid. Fungi	Fungi sp	AJ877193	SH479386.07FU	100.0	93	16
OTU0602	unid. Fungi	unid. Fungi	Fungi sp	KP889786	SH208682.07FU	97.1	83	10
OTU0610	unid. Fungi	unid. Fungi	Fungi sp	KJ023736	SH218992.07FU	100.0	99	41
OTU0700	unid. Fungi	unid. Fungi	Fungi sp	KF675628	SH204253.07FU	100.0	86	55

OTUID	Phylum	Order	Fungal Taxon	Genbank Accession number	UNITE SH number	sequence coverage (%)	sequence similarity (%)	sequence abundance
OTU0716	unid. Fungi	unid. Fungi	Fungi sp	KU580767	SH521400.07FU	98.3	84	32
OTU0740	unid. Fungi	unid. Fungi	Fungi sp	AM999674	SH205434.07FU	99.6	84	22
OTU0744	unid. Fungi	unid. Fungi	Fungi sp	KC222753	SH025821.07FU	100.0	97	11
OTU0752	unid. Fungi	unid. Fungi	Fungi sp	KT722342		100.0	100	151
OTU0767	unid. Fungi	unid. Fungi	Fungi sp	KM104100	SH495907.07FU	96.5	86	15
OTU0837	unid. Fungi	unid. Fungi	Fungi sp	KC222753	SH025821.07FU	100.0	94	66
OTU0838	unid. Fungi	unid. Fungi	Fungi sp	KM104100	SH495907.07FU	99.6	86	17
OTU0853	unid. Fungi	unid. Fungi	Fungi sp	KF675509	SH210983.07FU	99.3	94	161

Supplementary Table S2.3 Output of a simple linear regression of fungal OTU richness and sequencing depth to test if OTU richness in the chambers of the ant-plant *Myrmecodia beccarii* is related to the number of sequences in the samples.

	Estimate	**Std. Error**	**t-value**	**p-value**
Intercept	16.40	1.7527	9.358	$2.65e^{-16}$
Sequencing depth	0.028	0.0048	5.745	$5.96e^{-08}$

Residual standard error: 9.084 on 133 degrees of freedom
Multiple R-squared: 0.1988, Adjusted R-squared: 0.1928
F-statistic: 33.01 on 1 and 133 DF, p-value: 5.963e-08

Supplementary Table S2.4 Fungal OTU richness for the nursery, ventilation, and waste chambers
of *Myrmecodia beccarii* across five locations: Annan River National Park, Cowley Beach, Cardwell, Kutini-Payamu National Park, and Port Douglas. Sample numbers (N), total OTU richness (all samples for a chamber type/location) and mean ± standard error (SE) OTU richness per sample for a chamber type/location combination.

Location	**Nursery chamber OTUs**			**Ventilation chamber OTUs**			**Waste chamber OTUs**		
	N	**Total**	**Mean ± SE**	**N**	**Total**	**Mean ± SE**	**N**	**Total**	**Mean ± SE**
Annan River NP	9	180	20.0 ± 2.65	9	263	29.2 ± 2.84	9	180	20.0 ± 2.26
Cowley Beach	10	214	21.4 ± 2.32	9	216	24.0 ± 2.60	9	194	21.6 ± 4.03
Cardwell	10	228	22.8 ± 3.13	9	201	22.3 ± 2.13	10	252	25.2 ± 2.51
Kutini-Payamu NP	7	162	23.1 ± 3.96	7	197	28.1 ± 2.37	7	178	25.4 ± 2.65
Port Douglas	10	268	26.8 ± 4.07	10	400	40.0 ± 3.27	10	299	29.9 ± 3.49

Supplementary Table S2.5 Fungal OTU richness. Output of linear mixed model to test the influence of chamber type (nursery, ventilation, waste) and geographic location (Annan River National Park, Cardwell, Cowley Beach, Kutini-Payamu National Park and Port Douglas) on fungal OTU richness (residuals) in the domatium chambers of *Myrmecodia beccarii*.

Type III Analysis of Variance Table with Satterthwaite's method

	Sum Sq	Mean Sq	NumDF	DenDF	F value	p-value	
location	516.26	129.07	4	40.83	2.706	0.0434	*
chamber	1624.35	812.17	2	79.95	17.029	6.91E-07	***
location : chamber	332.06	41.51	8	79.97	0.870	0.545	

Fixed effects and interactions	Estimate	SE	df	t-value	p-value	
(Intercept)	-4.106	2.690	105	-1.526	0.130	
location Cardwell	-0.145	3.708	105	-0.039	0.970	
location Cowley Beach	3.383	3.708	105	0.912	0.364	
location Kutini-Payamu NP	2.817	4.067	105	0.693	0.490	
location Port Douglas	5.813	3.708	105	1.568	0.120	
chamber ventilation	9.142	3.256	79	2.808	**0.006**	**
chamber waste	-3.745	3.256	79	-1.15	0.253	
location Cardwell : chamber ventilation	-4.226	4.558	80	-0.927	0.357	
location Cowley Beach : chamber ventilation	-5.381	4.559	80	-1.18	0.241	
location Kutini-Payamu NP : chamber ventilation	-4.760	4.922	79	-0.967	0.336	
location Port Douglas : chamber ventilation	1.307	4.488	79	0.291	0.772	
location Cardwell : chamber waste	4.187	4.488	79	0.933	0.354	
location Cowley Beach : chamber waste	3.013	4.559	80	0.661	0.511	
location Kutini-Payamu NP : chamber waste	0.257	4.922	79	0.052	0.958	
location Port Douglas : chamber waste	3.047	4.488	79	0.679	0.499	

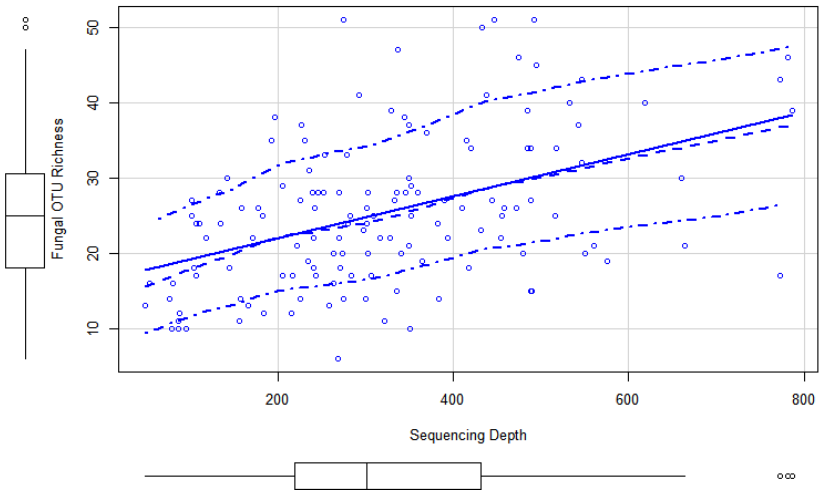
Significance codes: ***<0.001, **<0.01, *<0.05

Supplementary Table S2.6 Pairwise contrasts of Fungal OTU richness from a linear mixed model (see Supplementary Table S2.5).
Contrasts are between geographic locations (Annan River National Park, Cardwell, Cowley Beach, Kutini-Payamu Natonal Park) for each of the three chamber types (nursery, ventilation, waste), and were computed using estimated marginal means. Adjusted p-values using Tukey method.

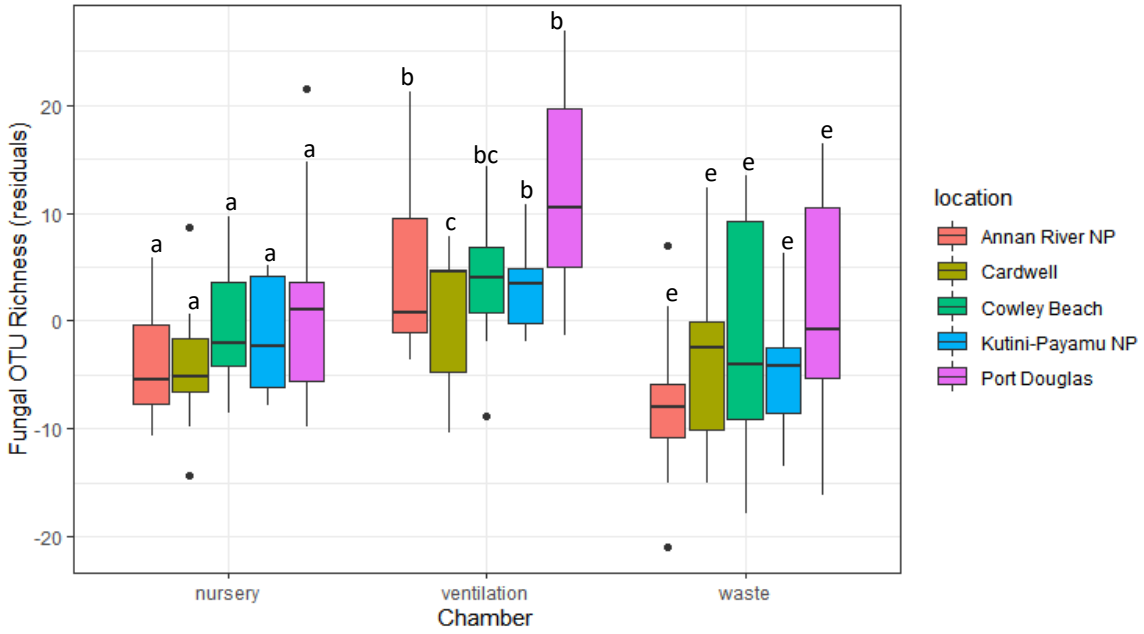
Pairwise contrasts between locations for each chamber type					
Chamber = Nursery	**Estimate**	**SE**	**df**	**t-ratio**	**p-value**
Annan River NP - Cardwell	0.1447	3.71	105	0.039	1.000
Annan River NP - Cowley Beach	-3.3826	3.71	105	-0.912	0.892
Annan River NP – Kutini-Payamu NP	-2.8174	4.07	105	-0.693	0.958
Annan River NP - Port Douglas	-5.8134	3.71	105	-1.568	0.521
Cardwell - Cowley Beach	-3.5273	3.61	105	-0.977	0.865
Cardwell – Kutini-Payamu NP	-2.9621	3.98	105	-0.745	0.945
Cardwell - Port Douglas	-5.9581	3.61	105	-1.651	0.469
Cowley Beach – Kutini-Payamu NP	0.5652	3.98	105	0.142	1.000
Cowley Beach - Port Douglas	-2.4308	3.61	105	-0.674	0.962
Kutini Payamu NP - Port Douglas	-2.9959	3.98	105	-0.753	0.943
Chamber = Ventilation					
Annan River NP - Cardwell	4.3709	3.8	107	1.152	0.779
Annan River NP - Cowley Beach	1.9985	3.8	107	0.527	0.985
Annan River NP - Kutini Payamu NP	1.9426	4.07	105	0.478	0.989
Annan River NP - Port Douglas	-7.1205	3.71	105	-1.92	0.313
Cardwell - Cowley Beach	-2.3724	3.79	109	-0.626	0.972
Cardwell - Kutini Payamu NP	-2.4283	4.06	107	-0.598	0.975
Cardwell - Port Douglas	-11.4914	3.7	107	-3.107	**0.020**
Cowley Beach - Kutini Payamu NP	-0.0559	4.06	107	-0.014	1.000
Cowley Beach - Port Douglas	-9.119	3.7	107	-2.465	0.106
Kutini Payamu NP - Port Douglas	-9.0631	3.98	105	-2.279	0.160
Chamber = Waste					
Annan River NP - Cardwell	-4.0427	3.71	105	-1.09	0.811
Annan River NP - Cowley Beach	-6.3952	3.8	107	-1.685	0.448
Annan River NP - Kutini Payamu NP	-3.0745	4.07	105	-0.756	0.942
Annan River NP - Port Douglas	-8.86	3.71	105	-2.389	0.126
Cardwell - Cowley Beach	-2.3525	3.7	107	-0.636	0.969
Cardwell - Kutini Payamu NP	0.9681	3.98	105	0.243	0.999
Cardwell - Port Douglas	-4.8173	3.61	105	-1.335	0.670
Cowley Beach - Kutini Payamu NP	3.3206	4.06	107	0.818	0.925
Cowley Beach - Port Douglas	-2.4648	3.7	107	-0.666	0.963
Kutini Payamu NP - Port Douglas	-5.7855	3.98	105	-1.455	0.590

Supplementary Table S2.6 (continued)

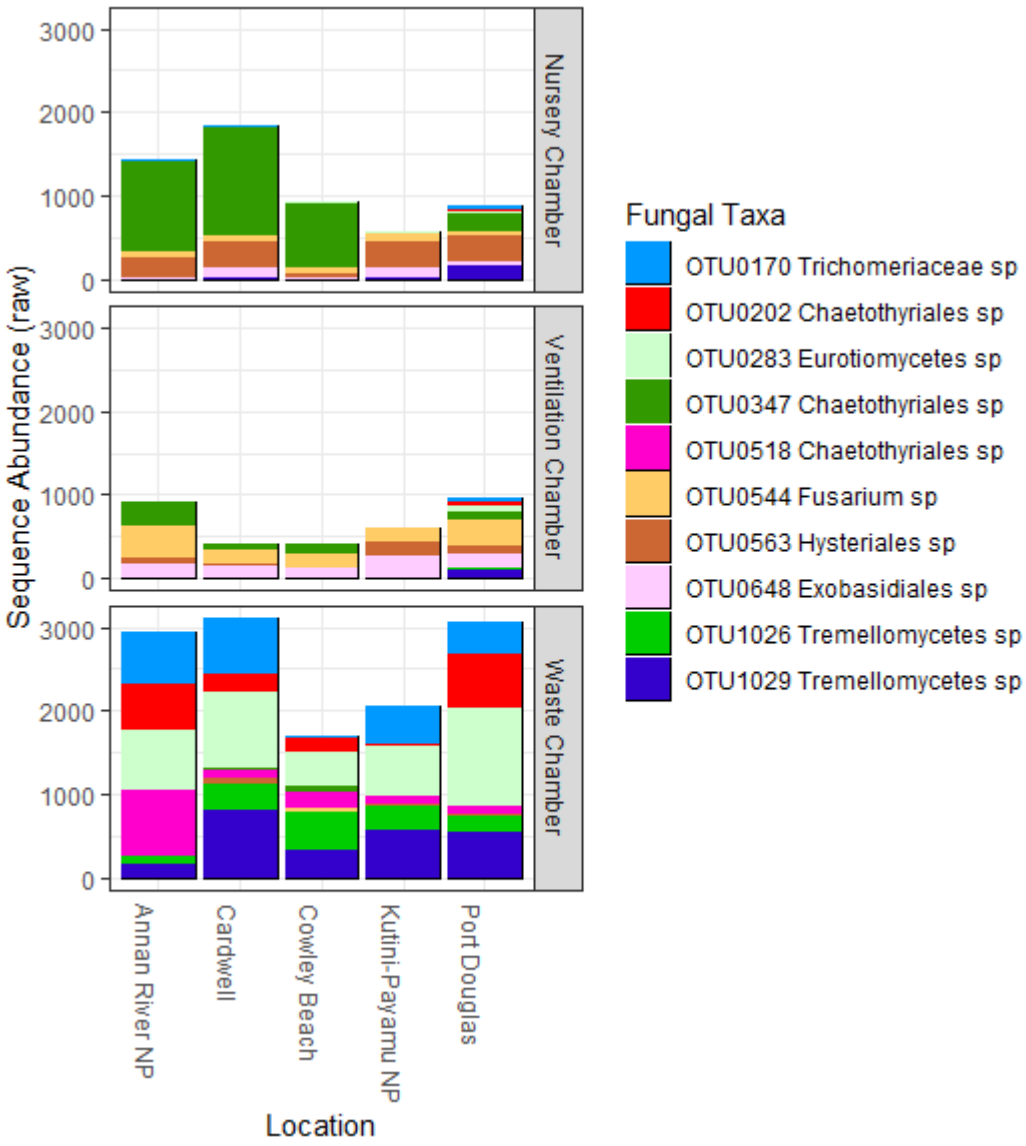
Estimated marginal means for each geographical location for each chamber type					
Chamber = Nursery	**EM mean**	**SE**	**df**	**lower CL**	**upper CL**
Annan River NP	-4.106	2.69	105	-9.44	1.23
Cardwell	-4.25	2.55	105	-9.31	0.81
Cowley Beach	-0.723	2.55	105	-5.78	4.34
Kutini Payamu NP	-1.288	3.05	105	-7.34	4.76
Port Douglas	1.708	2.55	105	-3.35	6.77
Chamber = Ventilation					
Annan River NP	5.036	2.69	105	-0.30	10.37
Cardwell	0.665	2.68	109	-4.64	5.97
Cowley Beach	3.037	2.68	109	-2.27	8.34
Kutini Payamu NP	3.093	3.05	105	-2.96	9.14
Port Douglas	12.156	2.55	105	7.10	17.22
Chamber = Waste					
Annan River NP	-7.851	2.69	105	-13.19	-2.52
Cardwell	-3.808	2.55	105	-8.87	1.25
Cowley Beach	-1.456	2.68	109	-6.76	3.85
Kutini Payamu NP	-4.777	3.05	105	-10.82	1.27
Port Douglas	1.009	2.55	105	-4.05	6.07



Supplementary Figure S2.1 Scatterplot showing the relationship between fungal OTU richness (observed values) in the chambers of the ant-plant *Myrmecodia beccarii* and sequencing depth (number of sequences per sample).



Supplementary Figure S2.2 Fungal OTU richness compared within each chamber type of *M. beccarii* (nursery, ventilation, and waste chambers) using a linear mixed model and pairwise contrasts across locations. Different letters above bars represent significant differences in fungal OTU richness. The residuals for fungal OTU richness from a simple linear regression were used instead of the observed values for richness to take account for the positive relationship between fungal OTU richness and sequencing depth in the samples.



Supplementary Figure S2.3 Top 10 fungal operational taxonomic units (OTUs) with the highest number of sequences for each of the three different chamber types: nursery, ventilation, and waste chambers across the five locations surveyed: Annan River National Park, Cardwell, Cowley Beach, Kutini-Payamu National Park, and Port Douglas.

Chapter 3 - Fungi inhabiting the chambers of an epiphytic ant-plant are transported by resident ant workers

Philidris cordata ant workers
Photo: Melinda Greenfield

Chapter 3 - Fungi inhabiting the chambers of an epiphytic ant-plant are transported by resident ant workers

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Statement of contribution of others:

M.J.G. designed the experiment, conducted the field work, grew ant-plants from seed, ran the experiment, carried out the dissections, collected and collated the data, performed the bioinformatics, analysed the data, created the figures and tables, and wrote this chapter. S.E.A. and L.L. provided advice on the design of the study, interpretation of results, and edited the chapter. B.C.C. provided advice on interpreting the data. P.Y. provided input and advice on data analyses. Y.I. and P.L. assisted with data collection. S.A. provided input and advice on bioinformatics. L.T. provided laboratory space, primers and reagents, and advice on high-throughput sequencing.

3.1 Introduction

Mutualisms are interactions between species, usually from different kingdoms, where there is a net benefit to the partners involved in the association (Bronstein 1998, Herre et al. 1999, Bronstein 2015). The categorisation of mutualisms is often based on the services and rewards exchanged between the partners that benefit, and broadly include nutrition, protection, and transportation (Bronstein 1998, 2001, Biedermann and Vega 2020). For example, the stingless bee *Scaptotrigona depilis* (Hymenoptera: Apidae) and a yeast fungus from the genus *Zygosaccharomyces* (Saccharomycetaceae) are involved in a nutritional exchange where the larvae of the bee is dependent on the fungus which it consumes for an essential steroid precursor to complete its development, and the fungus feeds on the sugar-rich brood cells (Hartfelder and Engels 1989, Paludo et al. 2018). Protective exchanges include the pea aphid *Acyrthosiphon pisum* (Hemiptera: Aphididae) which harbours bacterial symbionts that defend it against a parasitoid wasp (Oliver et al. 2003, Scarborough et al. 2005, Gauthier et al. 2015). Transportation services occur in most flowering plants – e.g. pollination and seed dispersal by insects, birds or other animals in exchange for nectar or fruit rewards (Thompson 1982). Transportation services are common in mutualisms involving fungi because fungi are typically sessile and require transport in order to disperse.

Fungi disperse in a variety of ways including abiotic forms of transport such as spores dispersed by wind (Golan and Pringle 2017) and fungi dispersed via water on driftwood that floats across oceans (Rama et al. 2014). In mutualistic interactions, biotic forms of transport often occur, for example the northern bettong *Bettongia tropica* eats ectomycorrhizal truffle fungi and disperses truffle spores through faeces (Nuske et al. 2018). In fungus-insect mutualisms, transportation services to fungi are usually provided by the insect hosts and often include nutrition and protection as well (Biedermann and Vega 2020). For example, ambrosia beetles (Curculionidae: Platypodinae) farm fungi for food and receive antimicrobial defence from fungi, while the fungi benefit from transport in specialised pocket-like structures called mycangia on female beetles (vertical transmission), as well as nutrition from substrate, and protection against natural enemies (Skelton et al. 2019, Biedermann and Vega 2020). Transport of fungi are also found in fungus-farming termites (Blattodea: Isoptera, sub-family Macrotermitinae) that cultivate fungus gardens. The termite colony benefits nutritionally from the nitrogen rich fungal biomass, while the fungi benefit from growing in a favourable environment, nutrition via pre-digested plant material, protection from competitive fungi and microbial infections (Korb and Aanen 2003) and transport. After a new colony is founded by alates, transportation of fungi in most termite

fungus-farming mutualisms is by the first workers (horizontal transmission) who collect spores of fruiting fungi during foraging expeditions (Korb and Aanen 2003, Aanen and Boomsma 2006, Aanen et al. 2009).

Ants are among the most renowned transporters of their mutualist fungi. The fungus-farming leaf-cutter ants (tribe Attini: Formicidae) are obligately dependent on their fungus gardens for food so transport of fungal cultivars is essential for the establishment of new colonies (Hölldobler and Wilson 1990, Schultz and Brady 2008). Transportation of the fungal cultivar is carried out by mated foundress queens (vertical transmission) that carry pellets of the fungus in their infrabuccal pockets (located in the mouth cavity) (Mueller et al. 1998, Mueller et al. 2001, Fernandez-Marin et al. 2004). Horizontal cultivar transmission also occurs during colony founding by queens that actively search for replacement cultivars if required (Green et al. 2002, O'Fallon 2008, Poulsen et al. 2009, Howe et al. 2018). The fungus is also obligately dependent on the leaf-cutter ant workers for food who collect fresh vegetation to feed their fungal garden and mechanically remove fungal competitors (Mueller et al. 2001). Other benefits include the accumulation of microbial biofilms on the integument of the ants including actinomycetes bacteria that produce antibiotics believed to protect against ant disease, and a different biofilm in the fungal gardens that protects against garden diseases (Barke et al. 2011, Mueller 2012). Mutualisms have also been found in other ant-fungi interactions including in myrmecophytes ("ant-plants") that involve the plant, the resident ants, and fungi.

Myrmecophytic ant-plant mutualisms are where the plant provides nesting space to ants in specialised hollow structures called domatia (singular: domatium) (Chomicki and Renner 2015). Many terrestrial ant-plants also provide food rewards to the ants (e.g. extrafloral nectar or food bodies) (Hölldobler and Wilson 1990) and some ants rear hemipterans in domatia to obtain honeydew (Beattie et al. 1985). Ant-plants often benefit from protection provided by their resident ants including defence against enemies including herbivores (Janzen 1972, Rosumek et al. 2009), pathogens (Letourneau 1998) and encroaching vegetation (Frederickson et al. 2005) and often provide nutrients to the plant by depositing waste inside domatia (Huxley 1978, Rico-Gray et al. 1989, Gay 1993, Treseder et al. 1995, Defossez et al. 2011, Mayer et al. 2014, Baker et al. 2017, Volp et al. 2022).

Over the last decade, multipartite interactions involving plants, ants and fungi have been found in terrestrial ant-plant mutualisms. For example, fungal patches in domatia are used as food by ants in several ant-plant/plant-ant mutualisms including: *Barteria fistulosa* Mast. (Passifloraceae)/ *Tetraponera aethiops* Smith (Pseudomyrmecinae), *Tachigali* sp. Aubl.

(Fabaceae: Caesalpinioideae) /*Pseudomyrmex penetrator* Smith (Pseudomyrmecinae) and *Leonardoxa africana* (Baill.) Aubrév. subsp. *africana* (Fabaceae: Caesalpinioideae)/ *Petalomyrmex phylax* Snelling (Formicinae) (Defossez et al. 2009, Defossez et al. 2011, Blatrix et al. 2012, Blatrix et al. 2013). In *L. a. africana,* the *P. phylax* ant workers were filmed depositing detritus and defecating on a fungal patch, as well as chewing hyphae and transporting fragments of the fungus (Defossez et al. 2009). Vertical transmission of cultivated fungi has been identified in the Costa Rican terrestrial ant-plant *Cecropia* (Urticaceae), which is inhabited by *Azteca* ants (Dolichoderinae) (Mayer et al. 2018). In that ant-plant system, fungal OTU diversity does not differ between fungal patches produced by foundress queens and the fungal patches of the established natal colonies from which the foundress queens originated (Mayer et al. 2018). In an ant-exclusion experiment with the epiphytic ant-plant *Myrmecodia tuberosa* (Rubiaceae), Huxley (1978) noticed a fungus was introduced by the ant workers to the accessible seedlings, but fungi were not the focus of that study and were not investigated further. Despite the recent studies, little is known about fungal transmission in most ant-plant mutualisms and whether horizontal or vertical transmission of fungi occurs.

Myrmecodia beccarii Hook.f. is an epiphytic ant-plant endemic to tropical north Queensland, Australia. The domatium of *M. beccarii* has a network of chambers that are utilised by its resident *Philidris cordata* (Dolichoderinae) ant colonies for different purposes. I have described the waste, nursery, and ventilation chambers in chapter 2 of this thesis. A survey of fungi within the chambers of *M. beccarii* showed that fungal communities in the waste chambers were consistently different from fungal communities in the nursery and ventilation chambers at five locations across 675 km of coastline (chapter 2; Greenfield et al. 2021), which suggests fungi play diverse and important roles in this ant-plant mutualism. It is not yet known how fungi become established in those chambers.

The spatial distribution of *M. beccarii* ant-plants is clustered within and between trees, and also at the scale of habitat and *M. beccarii* is considered to be dispersal limited (Tsen 2011). Individuals of *M. beccarii* are usually spaced short distances from each other, or on top of each other within trees (pers. obs.) (Figs. 3.1 and 3.2) and this clustering occurs in other epiphytic ant-plants from the family Rubiaceae (Janzen 1974, Chomicki and Renner 2016). This clustered distribution is believed to be because *P. cordata* ant workers disperse seeds of *Myrmecodia* ant-plants by collecting, carrying, and placing seeds in nutrient-rich carton runways which become future domatia for the ant colony (Janzen 1974, Huxley 1980, Sommer 1990). Seedlings of *M. beccarii* have also been observed growing in amongst the layers of paperbark of *Melaleuca* trees

near *M. beccarii* ant-plants (pers. obs) and this may also be a result of *P. cordata* ant workers planting seeds of *M. beccarii* or seeds falling from *M. beccarii* ant-plants located above. Seed dispersal by ant workers suggests *P. cordata* colonies in *Myrmecodia* spp. inhabit multiple domatia (are polydomous). Recently, it was reported that *P. nagasau* inhabiting *Squamellaria* spp. (Rubiaceae) in Fiji are polydomous and the ant workers plant their host's seeds in cracks in tree bark rather than in ant carton (Chomicki and Renner 2016). Longer-distance dispersal of *M. beccarii* seeds is believed to be by the mistletoe bird *Dicaeum hirundinaceum* (Dicaeidae).

I hypothesised that *P. cordata* ant workers transport chamber fungi between mature *M. beccarii* ant-plants and nearby plantlets. To test this hypothesis, I manipulated ant access to *M. beccarii* ant-plantlets (approximately 18 months old) placed in cages with a mature ant-plant colonised with *P. cordata*. I predicted fungal OTU sequence abundance and richness in chambers of the ant-accessible plantlets would be greater than in the ant-excluded plantlets at the end of the three-month experiment. I also predicted the ant-accessible plantlets and mature ant-plants would share fungal OTUs and have similar fungal OTU communities. Further, I predicted I would find fungi on the exoskeleton surfaces and/or in the heads or abdomens of *P. cordata* ant workers that match the fungal OTUs found in the ant-accessible plantlets.

Figure 3.1 Cluster of *M. beccarii* ant-plants.

Figure 3.2 *Melaleuca* tree with seven *M. beccarii* ant-plants (indicated by orange arrows).

3.2 Methods

3.2.1 Collection of mature *Myrmecodia beccarii* ant-plants

I collected 15 mature *M. beccarii* ant-plants (with their resident ant colonies) from *Melaleuca viridiflora* trees (Fig. 3.3) in Girringun National Park at Rungoo in north Queensland, Australia (18°29'58.8" S, 146°11'04.00" E, elevation 8-9 m) in late August 2017. Each ant-plant had an approximate domatium base circumference of 400-550 mm. At time of collection, I confirmed the resident ant workers were *P. cordata.* Upon removal from its host *Melaleuca* tree, I placed each ant-plant into a plastic box container (27 L). To prevent ant workers from escaping during transport, I prepared these containers as described in the methods section 2.3.1 of chapter 2 (Greenfield et al. 2021). The *M. beccarii* ant-plants were kept overnight in a greenhouse prior to the start of the experiment.

Figure 3.3 Mature *M. beccarii* ant-plant on a *Melaleuca viridiflora* tree at Girringun National Park, Rungoo, Queensland, Australia

3.2.2 Cultivation of ant-plantlets

I collected fruits from stock mature *M. beccarii* ant-plants held in a greenhouse at James Cook University Cairns Campus (16°48'58.83"S, 145°41'16.73"E) in early 2016. I collected 4-5 seeds from each fruit and placed the seeds on paper towel for 1-2 days to allow them to dry (a pilot study showed this helped germination). Seedling trays (300 mm wide x 350 mm length cm x 60 mm height) were filled with a 30-40 mm layer of coarse orchid potting mix (Scotts Osmocote®; Evergreen Garden Care Australia, Bella Vista, NSW, Australia) and topped with a 15-20 mm thick

layer of moist sphagnum moss (Brunnings Garden Products Pty Ltd, Oakleigh South, VIC, Australia). I sowed the *M. beccarii* seeds directly on top of the sphagnum moss (approximately 60 seeds/tray) and lightly watered them approximately 3-4 times per week for the first six months to keep the sphagnum moss slightly moist. When the plantlets were approximately 6 months old, they were transplanted into 30-cell seedling trays (REKO®, HomeLeisure, Mulgrave, VIC, 3070) which contained a mixture of two parts orchid potting mix to one part perlite (Brunnings Garden Products Pty Ltd). A 10 mm layer of sphagnum moss was placed on the surface around each of the *M. beccarii* plantlets to protect the roots. The plantlets were grown in the greenhouse for a further 12 months prior to the start of the experiment during which I watered the growing plantlets every two days in the dry season (May – October) and every 3-4 days in the wet season (November – April) by an overhead automatic watering system (Fig. 3.4). The legs of the greenhouse benches were placed into buckets containing water to exclude any ants from gaining access to the plantlets (Fig. 3.4). I fertilised the plantlets with Charlie Carp® Premium Organic Fertiliser (Charlie Carp Ltd, Deniliquin, NSW, Australia) at one-quarter strength (2.5 ml/L) of the manufacturer's recommended application rate by direct overhead application with a watering can to the point of run-off every 4 weeks during the dry season and every 1-2 weeks during the wet season. The last application of fertiliser was 14 days before the start of the experiment in early September 2017. No fertiliser was applied to the plantlets during the experiment.

Figure 3.4 *M. beccarii* plantlets (approximately 18 months old) growing in greenhouse at James Cook University prior to the start of the experiment

3.2.3 Preparation of cages

I prepared 15 plastic box containers (109 L, 740 mm in length, 535 mm in width, 440 mm in height) as cages for each mature *M. beccarii* ant-plant (Fig. 3.5). I cut a panel out of the lid which was replaced with a rectangle of mesh (400mm x 180 mm) to allow airflow while preventing any organisms from entering or leaving the cages. I applied Fluon® to the inside walls of the cages and a 25 mm strip of Tangle-Trap® around the upper inside edge of the cages to prevent the ant workers from escaping. I also applied Tangle-Trap® to the entire surface of the undersides of the lids to prevent any alates from escaping if they emerged during the experiment.

Figure 3.5 Cage used in ant-exclusion experiment.

3.2.4 Experimental design

I conducted the experiment in a greenhouse at James Cook University, Cairns Campus (16°48'58.83"S, 145°41'16.73"E) for three months from September to December 2017 using a 2 x 2 factorial design with ant-exclusion and fungicide application as treatments (Fig. 3.6). The results from the ant-excluded versus ant-accessible treatments (treatments 1 and 2 in Fig. 3.6) are the subject of this chapter. The results of the fungicide treatment are discussed in the next chapter of this thesis. I placed each of the 15 collected mature *M. beccarii* ant-plants in the centre of a cage with four *M. beccarii* plantlets placed on small upturned pots (diameter 55 mm, height 50 mm), one in each corner (Fig. 3.6) and randomly assigned treatments to the plantlets. The two plantlets that were ant-excluded were placed in moats containing mineral oil (Coles Supermarkets Australia Pty Ltd, Hawthorn, Victoria, Australia) and I applied TangleTrap® to the rim of the moats to prevent the ant colony from accessing them. I provided food to the ant colonies by placing two meal worms in the cage (one on each side of mature ant-plants) 2-3 times per week and 3-4 mL of 50% sucrose solution in two plastic vials (7 mL capacity each) plugged with a small ball of cotton wool (Fig. 3.6) 3-4 times per week (one vial on each side of mature ant-plants). I lightly watered the mature *M. beccarii* ant-plants and plantlets 3-4 times

per week with tap water using a pressurised hand-sprayer. The average temperature in the greenhouse was 27.3°C (with a range of 19.0°C to 43.3°C) and relative humidity averaged 75.5% (with a range of 32.3 - 97.5%) during the three months of the experiment.

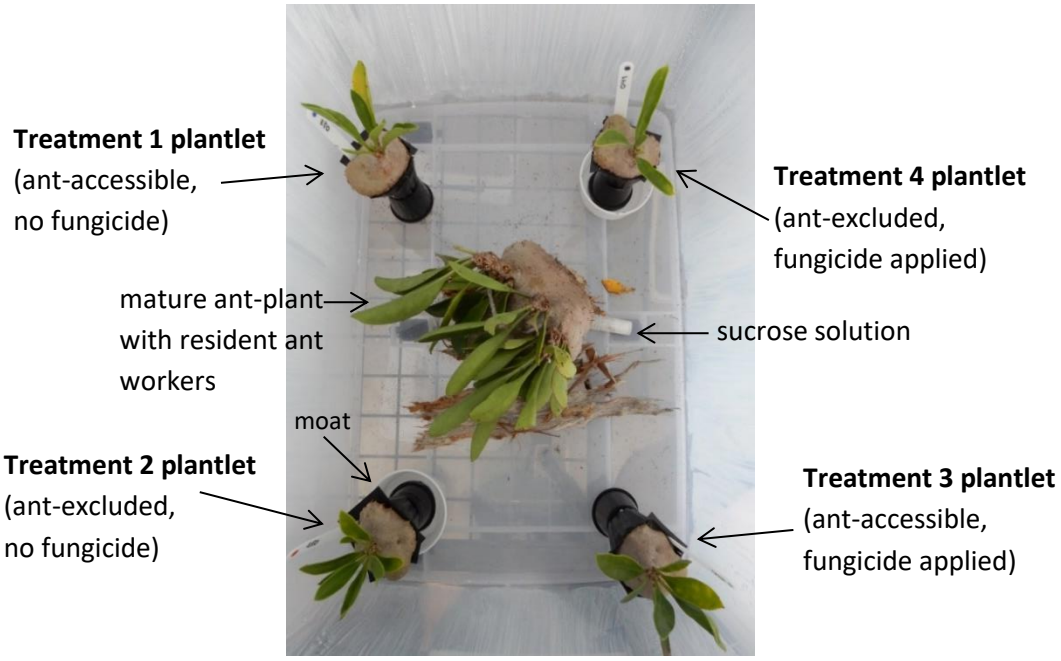

Figure 3.6 Experimental set up with *M. beccarii* plantlets using a 2 x 2 factorial design with ant-exclusion and fungicide application as treatments. A mature ant-plant with its resident *P. cordata* ant colony was placed in each of the cages and was the source of ant workers. Ant workers were allowed access to two plantlets (treatments 1 and 3) and excluded from accessing two plantlets using moats (treatments 2 and 4). Treatments 1 and 2 relate to this chapter 3. Fungicide was applied to treatments 3 and 4. Treatments 1 and 3 were accessible to the ant workers and are analysed in chapter 4.

Approximately six weeks into the experiment, I observed female and male alates (winged reproductive ants) emerging from the mature ant-plants in some of the cages. To avoid contamination of the ant-plantlets on moats by alates, I placed mesh covers made of fine tulle fabric (1 mm^2 holes) over the ant-excluded plantlets on moats. I used elastic bands to keep the tulle in place around the base of the pots and 15 mm fold-back clips to hold the tulle in place at the top (Figure 3.7A), the latter of which allowed easy removal for access to the plantlets for watering. I also placed tulle covers over the ant-accessible plantlets to remove any effect of the tulle on the experimental plantlets, but I did not fix them around the base of the pots which allowed the ant workers continued access (Figure 3.7B). However, for one night there were no tulle covers on the experimental ant-plantlets and two ant-excluded plantlets (cages 16 and 18) had one dealate queen each and one ant-excluded plantlet (cage 32) had an alate male at the end of the experiment (Supplementary Table S3.1).

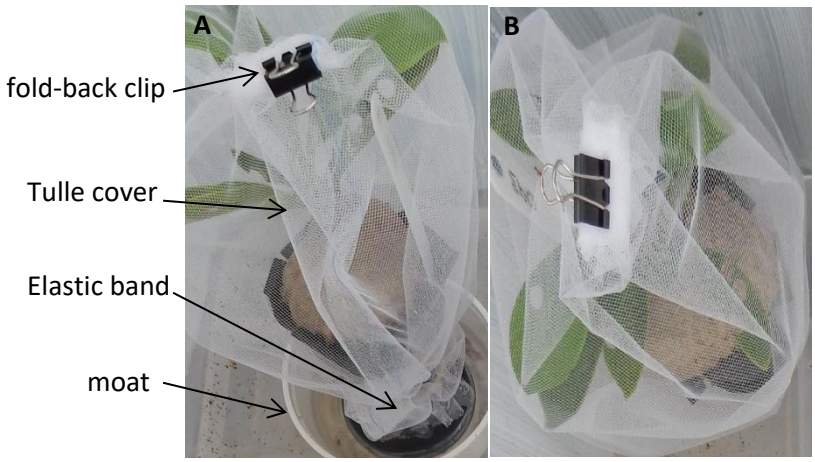

Figure 3.7 Tulle coverings: (A) ant-excluded plantlets on moats and (B) ant-accessible plantlets. Tulle coverings were held in place by elastic bands at the base of ant-excluded plantlets to prevent alates (winged reproductive adults) from accessing ant-excluded plantlets. No elastic bands were used to fix tulle around the bases of the ant-accessible plantlets to allow continued access by the ant workers.

3.2.5 Collection of fungi from plant chambers (including DNA extraction)

Prior to the dissections, I euthanised the ant colonies in each of the 15 mature *M. beccarii* ant-plants and plantlets using chloroform. Each mature ant-plant was placed in a sealed plastic container (20 L capacity) with 5 cotton balls, each soaked in 1 mL chloroform. I dissected the mature ant-plants following the methodology outlined in chapter 2 (Greenfield et al. 2021). Briefly, I cut each mature ant-plant domatium vertically into approximately 10 mm cross-sections ("slices") with a knife which was flame-sterilised using 99.5% ethanol between slices. I aimed to collect nine chamber surface samples for each mature ant-plant (three samples of each chamber type). To do this, I selected three slices: one slice from each end of the domatium and one slice from the middle of the domatium. For each slice, I collected 4-5 chamber surfaces per sample of each chamber type (waste, nursery, and ventilation) using a scalpel and placed these samples into individual 1.5 mL tubes (total sample weight of 25 mg ± 5 mg for each chamber type for each slice). I placed each of the 30 ant-accessible plantlets in a sealed plastic container (5 L capacity) with 2 cotton balls each soaked in 1 mL of chloroform. For the 30 ant-excluded plantlets, there was no need to do this because there were no ant workers in the ant-excluded plantlets. I collected a total of 3 chamber surface samples (1 of each chamber type) where possible for each plantlet (total sample weight 25 mg ± 5 mg for each chamber type). I followed the methodology outlined above for the mature ant-plants, but each of the three chamber samples consisted of 4-5 chamber surfaces collected from all three cross-sections because there were fewer chambers in the plantlets due to their small size. I flame-sterilised all equipment

between each sample collected from the mature ant-plants and plantlets during dissections including the knife, scalpel and forceps using 99.5% ethanol.

Controls included dissection controls and extraction controls. For the dissection controls, a 1.5 mL control tube was left open during ant-plant dissections to account for potential contaminants in the laboratory during sample processing, the contents of which were then included in subsequent sample processing (DNA extraction, PCR, and sequencing). I extracted DNA from the plant chamber surface samples to determine the identity of fungi in the different chamber samples, using the DNeasy Plant Mini Kit (Qiagen Pty Ltd, Victoria, Australia) and the method outlined in chapter 2 (Greenfield et al. 2021). Extraction controls were used to control for contamination during DNA extractions, such as contamination of reagents.

3.2.6 Ant colony collection

I collected the ant workers alive in each of the cages at the end of the experiment, including those on the surface of the plants and inside the chambers of the mature ant-plants and ant-accessible plantlets, as well as from the floors of the cages. All ant worker samples were stored in 99% ethanol until they were dried for 24 hours overnight in an oven set at 40°C. I weighed a sub-sample of 50 ant workers from each cage and divided that value by 50 to reach an average weight of one ant worker for each cage. To calculate the approximate number of workers for each cage, I weighed all of the ant workers from that cage and divided that by the average ant worker weight to obtain an approximate abundance of ant workers per cage (Supplementary Table S3.1). I also recorded the presence of alate and dealate queens, males, eggs, larvae, and pupae from any of the ant-plants including the mature ant-plants, ant-accessible plantlets, and ant-excluded plantlets in each cage (Supplementary Table S3.1).

3.2.7 Collection of fungi from ant workers (including DNA extraction)

To sample fungi from the ant workers, I collected 10 *P. cordata* ant workers from each of the cages at the end of the experiment and stored them in 2 mL tubes in a freezer at -20°C until processing. To sample the exoskeleton surfaces of the ant workers, I used forceps to place each batch of 10 ant workers into 2 mL tubes and added 500 µL of buffer solution from the PowerBead tubes in the DNeasy® PowerSoil® Kit (Qiagen Pty Ltd, Victoria) and 60 µL of C1 solution. I inverted the 2 mL tubes five times to combine the contents and placed them into a tissuelyser on low speed to gently mix the contents (frequency 10/second for 1 minute duration). I then transferred the solution in the 2 mL tubes back to the PowerBead tubes, leaving the ant workers behind in the original 2 mL tubes. I did not surface sterilise the ant worker

samples after the exoskeleton wash because it may have destroyed the fungi in the infrabuccal pockets. I dissected each batch of 10 ant worker samples (after the exoskeleton wash) using flame sterilised forceps and a scalpel to remove the heads, which were placed in their batches of 10 into the lids of PowerBead tubes and crushed with the rounded end of a disposable inoculating loop. The lid was secured and the PowerBead tubes were inverted five times to mix the buffer with the contents of the ant workers' heads. I placed the 2 mL tubes containing the samples into a tissuelyser to mix the contents (frequency 20/second for 1 minute duration). The same process was used for batches of the abdomens of the ant workers. The scalpel and forceps used throughout were flame-sterilised between batches using 99.5% ethanol. For dissection controls, the forceps and scalpel were dipped in 1.5 mL tubes containing sterile water in between the processing of batches of samples to control for cross contamination of the tools. The contents of the dissection controls were included in subsequent sample processing (DNA extraction, PCR, and sequencing). I placed the PowerBead tubes in a hot water bath at 65°C for 10 minutes and inverted the tubes 2-3 times during the 10 minutes. I then followed the recommended protocol for DNA extraction outlined in the DNeasy® PowerSoil® Kit from step 5 to the end. Extraction controls were used to control for contamination during DNA extractions, such as contamination of solutions. I had a total of 44 ant worker samples comprising 13 exoskeleton washes, 13 ant worker heads, 13 ant worker abdomens, 3 dissection controls, and 2 extraction controls.

3.2.8 PCR and Sequencing - all samples

I performed polymerase chain reaction (PCR) using the forward primer ITS1Fngs (GGTCATTTAGAGGAAGTAA) (Tedersoo et al, 2015a,b) and reverse primer ITS4ngsUni (CCTSCSCTTANTDATATGC) (Tedersoo & Lindahl, 2016) to target the full internal transcribed spacer (ITS) region (ITS1-5.8S-ITS2), the formal barcode for identification of fungi in molecular studies (Schoch et al. 2012). The primers were tagged with 10-11 base pair unique identifiers for multiplexing (Supplementary Table S3.2). The same PCR cocktail and amplification procedure was used as that outlined in chapter 2 (Greenfield et al. 2021) including negative and positive controls, purification of amplicons and quantification of DNA. Purified PCR products for the samples were arranged in five libraries and subjected to SMRTbell library preparation following Pacific Biosciences Amplicon library preparation protocol (Greenfield et al. 2021).

3.2.9 Bioinformatics and data collation

The 10 SMRT cells yielded CCS reads totalling 276293 (library 1), 289635 (library 2), 350792 (library 3), 324229 (library 4), and 376987 (library 6) [1]. Libraries 1, 2, 3 and two-thirds of library 4 were for the plant chamber samples and library 6 was for the ant samples. I performed the Bioinformatics analyses of the PacBio sequencing data (for full ITS region) using PipeCraft (v1.0) (Anslan et al. 2017) using the same technique as that outlined in chapter 2 (Greenfield et al. 2021). I created a database of the 374 fungal OTUs (minimum abundance of 2) found in *M. beccarii* ant-plants collected during the survey (chapter 2; Greenfield et al. 2021) and called it "APSurvey database". I used BLASTn search for the most abundant sequence of each non-singleton cluster (i.e., OTU) against UNITE (v7.2) reference databases and our APSurvey database for taxonomic assignment of OTUs (e-value = 0.001, word size=7, reward = 1, penalty = -1, gap opening cost = 1, gap extension cost = 2). OTUs with e-values[2] of $<e^{-50}$ were used to assign sequences as reliable to the fungal kingdom and e-values $>e^{-20}$ were considered unknown and removed from the dataset. Five OTUs had no BLAST hit and were removed. This left a dataset with a total 1279 fungal OTUs which was split to create: (1) a dataset with 786 fungal OTUs collected from waste, nursery, and ventilation chambers of mature ant-plants and all plantlets, and (2) a dataset with 176 fungal OTUs collected from the exoskeletons, heads, and abdomens of the ant workers. The lower number of OTUs in these datasets is because OTUs with abundances of zero after the split were removed.

I removed some samples from the plant dataset with 786 fungal OTUs because they were either missing samples, or not relevant to this chapter. I removed the sequencing data for the plantlets that received fungicide treatment because that data relates to chapter 4 of this thesis. I removed sequencing data for two cages (AE12 and AE23) that were missing samples from the dataset to ensure a balanced design for analyses. One ant-excluded plantlet in cage AE23 died during the experiment and the mature ant-plant and one ant-excluded plantlet in cage AE12 had insufficient chambers to collect adequate amounts of material. This left 117 samples from mature ant-plants (13 cages x 9 samples) which I then pooled to reduce the nine chamber samples per plant to 3 samples per plant – resulting in there being one nursery chamber sample,

[1] There was a fifth library with 274,366 CCS reads that, together with the one-third of library 4, is not included here because it relates to a field experiment which has not been included in this thesis.

[2] The e-value (also known as expect value) is a parameter that describes the number of hits one can expect to see by chance when searching a database of a particular size. Essentially it describes the random background noise. For example, an e-value of 1 assigned to a hit can be interpreted as meaning that in the database, one might expect to see 1 match with a similar score simply by chance. The lower the e-value (closer to zero) the more significant the match.

one ventilation chamber sample and one waste chamber sample for each mature ant-plant. This was necessary because the interconnectedness of the chambers in this ant-plant mean multiple samples (for each chamber type) per plant are not independent. The final dataset contained a total of 117 plant chamber samples including 39 for the mature ant-plants, 39 for the ant-accessible plantlets, and 39 for the ant-excluded plantlets (each comprising 13 nursery chambers, 13 ventilation chambers, and 13 waste chambers). I had a total of 199 samples for the plants, ants, and laboratory controls. There were 153 samples for the plants including the 117 plant chamber samples and their associated dissection (13) and extraction (15) controls, plus four positive and four negative PCR controls. For the ant workers, there were 46 samples which included the 39 ant worker samples and their associated dissection (3) and extraction (2) controls, plus one positive and one negative control.

After the above sample data collation, a dataset with 657 fungal OTUs remained for the plant chamber samples (mature ant-plants, ant-accessible plantlets (no fungicide), and ant-excluded plantlets (no fungicide)), and a dataset with 176 fungal OTUs for the ant worker samples (exoskeletons, heads, and abdomens).

3.2.9.1 Plant chamber samples

The plant chamber samples dataset with 657 fungal OTUs was filtered further before splitting it into two final datasets: one which contained 484 fungal OTUs with minimum read abundances of 2 (used for OTU richness); and another which contained 218 fungal OTUs with minimum read abundances of 10 (for all other analyses). The further filtering included several steps. I checked OTUs with e-values between e^{-20} and e^{-50} against the ten best matches for assignment to kingdom fungi or removal (resulting in 30 OTUs being detected as chimeric sequences and removed). I also removed 34 OTUs that had low sequence coverage (<70%). The single positive PCR control OTU was removed along with three OTUs that were found only in positive controls and one OTU found only in a negative control. I removed 12 OTUs that were in chamber samples and also in positive controls (10 OTUs), negative controls (1 OTU) or both positive and negative controls (1). I removed global OTU singletons (92 OTUs in total, each with only one occurrence in the dataset) to avoid potentially erroneous sequences which left the dataset containing 484 fungal OTUs with minimum read abundances of 2. I then removed a further 257 OTUs with sequence abundances <10 across all plant samples and another 9 OTUs that did not have a minimum of 10 abundances in at least one of the plant types (ant-accessible plantlets or ant-excluded plantlets or the mature ant-plants) which left the dataset of 218 fungal OTUs (Supplementary Table S3.3). This final step was performed because the focus of this study is on

the dominant fungal taxa inside the domatium chambers of *M. beccarii* and, according to previous studies, excluding rare species makes the community matrix more coherent and less noisy and hence strengthens the statistical power (Tedersoo et al. 2015). I used sequence similarity thresholds of >97%, >90%, >85%, >80%, >75% and >70% to match roughly species, genus, family, order, class, and phylum levels respectively (Nilsson et al., 2019). Of the 218 OTUs, 162 OTUs (74.3%) matched the taxonomic identity of >97% to pre-existing fungal ITS sequences in existing databases (UNITE and my APSurvey database). A further 36 OTUs (16.5%) matched at 90-97% and the remaining 20 OTUs (9.2%) matched to closest taxa at <90% sequence similarity.

3.2.9.2 Ant samples

The ant chamber samples dataset with 176 fungal OTUs was filtered further to create a single dataset containing 127 fungal OTUs for analyses. The further filtering included several steps. I checked OTUs with e-values between e^{-20} and e^{-50} against the ten best matches for assignment to kingdom fungi or removal (resulting in 5 OTUs being detected as chimeric sequences and removed). I also removed 13 OTUs that had low sequence coverage (<70%). The single positive PCR control OTU was removed along with three OTUs that were found only in positive controls. I removed 13 OTUs that were in chamber samples and also in positive controls. I then removed global OTU singletons (14 OTUs in total) to avoid potentially erroneous sequences. This left a dataset of 127 OTUs with a minimum abundance of 2 across the whole dataset of 39 ant samples (Supplementary Table S3.3). This lower threshold for minimum abundance was used for the ant samples because the abundances of OTUs in the ant samples were much lower overall in comparison to the plant samples. Of the 127 OTUs, 104 OTUs (81.3%) matched the taxonomic identity of >97% to pre-existing fungal ITS sequences in existing databases (UNITE and my APSurvey database). A further 15 OTUs (11.7%) matched at 90-97% and the remaining 9 OTUs (7.0%) matched to closest taxa at <90% sequence similarity.

3.2.10 Statistical analyses

There were two datasets for the 117 plant chamber samples: one contained data for the 484 fungal OTUs with a minimum read abundance of 2 and the other contained data for the 218 fungal OTUs with a minimum read abundance of 10 (supplementary Table S3.3). I used the dataset with 484 OTUs to test whether ant exclusion altered fungal OTU richness in the waste chambers (because richness relies on the rarer OTUs). I used the dataset with 218 OTUs for all other plant chamber analyses. The dataset for the 39 ant worker samples contained 127 fungal

OTUs with a minimum read abundance of 2 and was used to test whether fungal OTU abundances varied across the ant worker samples (heads, abdomens, exoskeletons).

Statistical analyses were conducted in R version 4.0.5 (R Core Team 2021). I used the dataset containing sequence abundance data for the 484 fungal OTUs (minimum read abundances of 2) from 117 samples to compare fungal OTU richness among chambers of the treatment plantlets (ant-accessible plantlets and ant-excluded plantlets). This dataset was used for the richness analysis because richness relies on the rarer OTUs. First, I tested if OTU richness is dependent on sample sequencing depth using the package 'car' (v3.0.11) (Fox and Weisberg 2018). I used the package 'ggfortify' (v0.4.12) (Horikoshi and Tang 2016, Tang et al. 2016) to visualize the data from which an outlier was identified (using Cook's Distance) and removed (Supplementary Fig. S3.1). I found OTU richness was significantly positively correlated to sequencing depth (Supplementary Table S3.4, Fig. S3.2), so I calculated the residuals for richness for each sample from the regression to account for the relationship in our subsequent analysis. I used the packages 'lme4' (v1.1.27.1) (Bates et al. 2015) and 'lmerTest' (v3.1.3) (Kuznetsova et al. 2017) to determine whether fungal OTU richness (residuals) varies with treatment and chamber type. Fixed effects included treatment (ant-accessible plantlets and ant-excluded plantlets) and chamber type (waste, nursery, ventilation) with an interaction term, and cage ID was a random effect. I used the package 'emmeans' (v1.6.2.1) (Lenth 2021) for pairwise comparisons between the treatment plantlets for each chamber type. I used the package 'ggplot2' (v3.3.5) (Wickham 2016) to create the box plot.

I used the dataset containing sequence abundance data for the 218 fungal OTUs (minimum read abundances of 10) from 117 samples for all other plant chamber analyses (see Supplementary Table S3.3 plant data). I used the package 'lme4' (v1.1.27.1) (Bates et al. 2015) to perform a generalized linear mixed model (glmer function) with a Poisson distribution to compare fungal OTU abundances in the chambers of the ant-accessible and ant-excluded plantlets. Fixed effects included treatment (ant-accessible plantlets and ant-excluded plantlets) and chamber type (waste, nursery, ventilation), with an interaction term, and cage ID was a random effect. An observation level random effect was also included to account for overdispersion (Harrison 2014) and the 'emmeans' package (v1.6.2.1) (Lenth 2021) was used to make pairwise comparisons between the treatment plantlets for each chamber type. I used the package 'ggplot2' (v3.3.5) (Wickham 2016) to create the box plot.

I used the packages 'phyloseq' (v1.34.0) (McMurdie and Holmes 2013), 'vegan' (v2.5.7) (Oksanen et al. 2020) and 'ggplot2' (v3.3.5) (Wickham 2016) to create ordination plots in order

to visualise differences in the fungal communities in the three different chambers of the two treatment plantlets (ant-accessible and ant-excluded) and included the mature ant-plants as a reference fungal community. First, I standardized the OTU matrix with a Hellinger-transformation (to account for varying sampling and sequencing depth) and performed non-metric multidimensional scaling (NMDS) with Bray-Curtis distance measure faceted by chamber type. I compared the fungal OTU community composition in the chambers of the two treatment plantlets and mature ant-plants using PERMANOVA on the Hellinger-transformed OTU matrix using the package 'vegan' (v2.5.7) (Oksanen et al. 2020). Treatment, chamber, and ant abundance were included as fixed factors and CageID as a random factor. I did not compare each chamber type for all treatments combined because the results are likely to be driven by the greater abundances of fungal OTUs found in the mature ant-plants, which would obscure any differences between the two treatment plantlets. Instead, I performed separate PERMANOVA analyses for each chamber type and used the package 'pairwiseAdonis' (v0.0.4) (Martinez Arbizu 2020) for pairwise comparisons between the mature ant-plants and the two ant-plantlets.

The packages 'DESeq2' (v1.24.0) (Love et al. 2014) and 'phyloseq' (v1.34.0) (McMurdie and Holmes 2013) were used to investigate whether any fungal OTUs were significantly abundant in contrasts of the different chambers of the ant-accessible plantlets and ant-excluded plantlets and in contrasts of the different samples from the *P. cordata* ant workers (exoskeletons, heads, and abdomens). Abundance OTU data was first loaded into Phyloseq and imported into DESEq2 using the phyloseq_to_deseq2 function. The DESeq2 model for the plants included both chamber type and treatment and the model for the ant worker samples included sample type. For both models, the significance test was set to "Wald", fitType set to "local" and multiple inference correction set to "Benjamini-Hochberg". Pairwise contrasts on chamber type (for plants) and sample type (for ants) were then carried out with DESeq2 to identify significantly abundant OTUs.

I used the dataset containing 218 fungal OTUs collected from mature ant-plants, ant-accessible plantlets and ant-excluded plantlets to create plots displaying the dominant fungal OTUs across the 13 cages. The package 'phyloseq' (v1.34.0) (McMurdie and Holmes 2013) was used to identify OTUs that occurred with a minimum abundance of 10 in at least 50% of the mature ant-plants, and/or 20% of the ant-accessible plantlets, and/or 20% of the ant-excluded plantlets. I used 'ggplot2' (v3.3.5) (Wickham 2016) to create the plots displaying the dominant fungal OTUs.

I used presence/absence datasets to create two venn diagrams to investigate shared and unique fungal OTUs in the *M. becarrii* ant-plants and *P. cordata* ant workers using the R package 'VennDiagram' (v1.6.20) (Chen 2018). The first figure with venn diagrams is for the shared and unique fungal OTUs in the mature ant-plants, ant-accessible plantlets, and ant-excluded plantlets (218 OTUs). The second venn diagram is for fungal OTUs shared and unique in/on the *P. cordata* ant workers and the ant-accessible and ant-excluded plantlets (238 OTUs).

To test for differences in fungal OTU abundances across ant worker samples, I used the dataset containing 127 fungal OTUs (minimum read abundances of 2) from 39 samples collected from *P. cordata* ant workers (see Supplementary Table S3.3 ant data). I used the package 'lme4' (v1.1.27.1) (Bates et al. 2015) to perform a generalized linear-mixed model (glmer) function with a Poisson distribution. Fixed effects included sample type (exoskeleton washes, heads and abdomens) and cage ID was a random effect. An observation level random effect was also included to account for overdispersion (Harrison 2014) and the 'emmeans' package (v1.6.2.1) (Lenth 2021) was used to make pairwise comparisons between the three ant sample types. I used the package 'ggplot2' (v3.3.5) (Wickham 2016) to create the box plot.

3.3 Results

3.3.1 *Philidris cordata* ant colonies

The number of *P. cordata* ant workers alive in each of the 13 cages at the end of the experiment varied (Fig. 3.8, Supplementary Table S3.1). Four of the 13 mature ant-plants each had a dealate queen and an additional seven mature ant-plants contained brood (eggs, larvae, and/or pupae) but no dealate queen (Table S3.1). Eleven of the 13 mature ant-plants had alate queens and 10 mature ant-plants had males. Three of the ant-accessible plantlets had one alate queen each (cages AE16, AE24 and AE32) and one ant-accessible plantlet (in cage AE25) contained pupae and larvae but no dealate queen. Two ant-excluded plantlets (in cages AE16 and AE18) each contained a dealate queen and one ant-excluded plantlet (in cage AE32) contained a male.

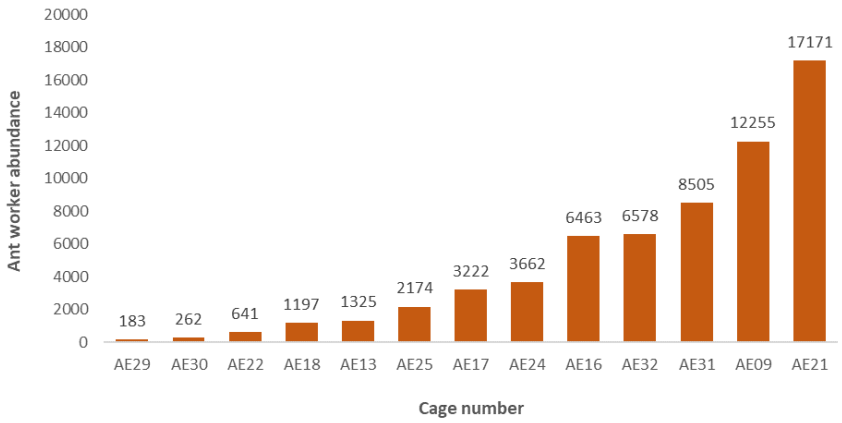

Figure 3.8 Approximate abundance of *Philidris cordata* ant workers in each of the 13 cages in the ant-exclusion experiment. All living ant workers were collected from each cage including on and inside the mature ant-plants and plantlets as well as the floor of the cages.

3.3.2 Fungal operational taxonomic units (OTUs)

I detected a total of 236,311 sequences from 287 distinct fungal OTUs from samples collected from the chambers of *M. beccarii* ant-plants (218 OTUs from 117 samples) and *Philidris cordata* ant workers (127 OTUs from 39 samples) at the end of the experiment (Supplementary Table S3.3). The plant chamber samples included 229,964 sequences from 218 OTUs (minimum abundances >10) from the waste, nursery and ventilation chambers of the mature ant-plants, ant-accessible plantlets, and ant-excluded plantlets. The ant worker samples included 6263 sequences from 127 OTUs (minimum abundances >2) from the ant worker exoskeletons, heads, and abdomens.

3.3.3 Fungal OTU abundances in the chambers of *M. beccarii* plantlets

Fungal OTU sequence abundances in the ant-accessible plantlets were 2.6 times higher for waste chambers and 1.9 times higher for nursery chambers compared to the ant-excluded plantlets (Figure 3.9, Table 3.1, Supplementary Table S3.5). In the ventilation chambers, fungal OTU sequence abundances did not differ significantly between the two treatment plantlets (Figure 3.9, Table 3.1).

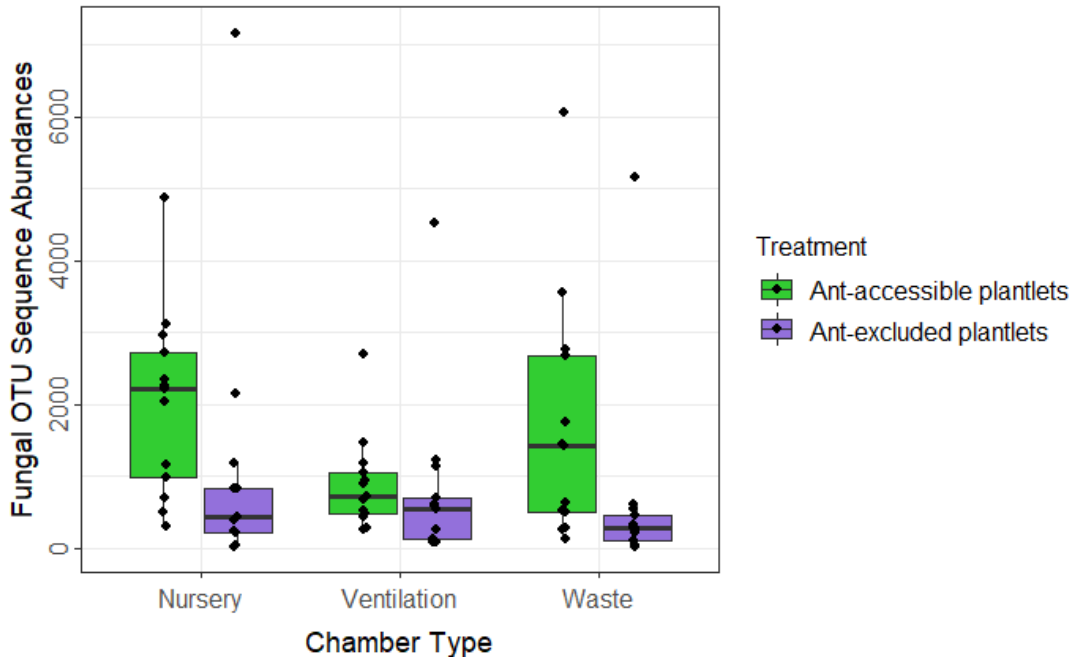

Figure 3.9 Fungal OTU sequence abundances in the domatium chambers of *M. beccarii* treatment plantlets (ant-accessible plantlets = green and ant-excluded plantlets = purple) grouped by chamber type (nursery, ventilation, and waste). The solid line represents the median, the black dots are the data points (n=13).

Table 3.1 Summary of generalised linear mixed effects model results testing the effects of ant-exclusion and chamber type on fungal OTU sequence abundances in the domatium chambers of *M. beccarii* plantlets including main effects and interactions (upper section of table). Lower section of table shows the pairwise contrasts between treatments for each chamber type.

Explanatory variables	**Estimate**	**SE**	**z value**	**p-value**
(Intercept)	7.36	0.33	22.62	<0.0001
ants excluded	-1.52	0.43	-3.55	**0.0004**
chamber ventilation	-0.78	0.43	-1.81	0.07
chamber waste	-0.47	0.43	-1.10	0.27
treatment ants excluded: chamber ventilation	0.91	0.61	1.50	0.13
treatment ants excluded: chamber waste	0.08	0.61	0.13	0.89
Pairwise contrasts between ant-accessible and ant-excluded plantlets for each chamber type				
nursery chamber	1.53	0.43	3.55	**0.0004**
ventilation chamber	0.61	0.43	1.43	0.71
waste chamber	1.45	0.43	3.37	**0.0008**

3.3.4 Fungal OTU richness in the chambers of *M. beccarii* plantlets

Both treatment and chamber type influenced fungal OTU richness but pairwise contrasts between the ant-accessible and ant-excluded plantlets for the three chamber types showed no significance.

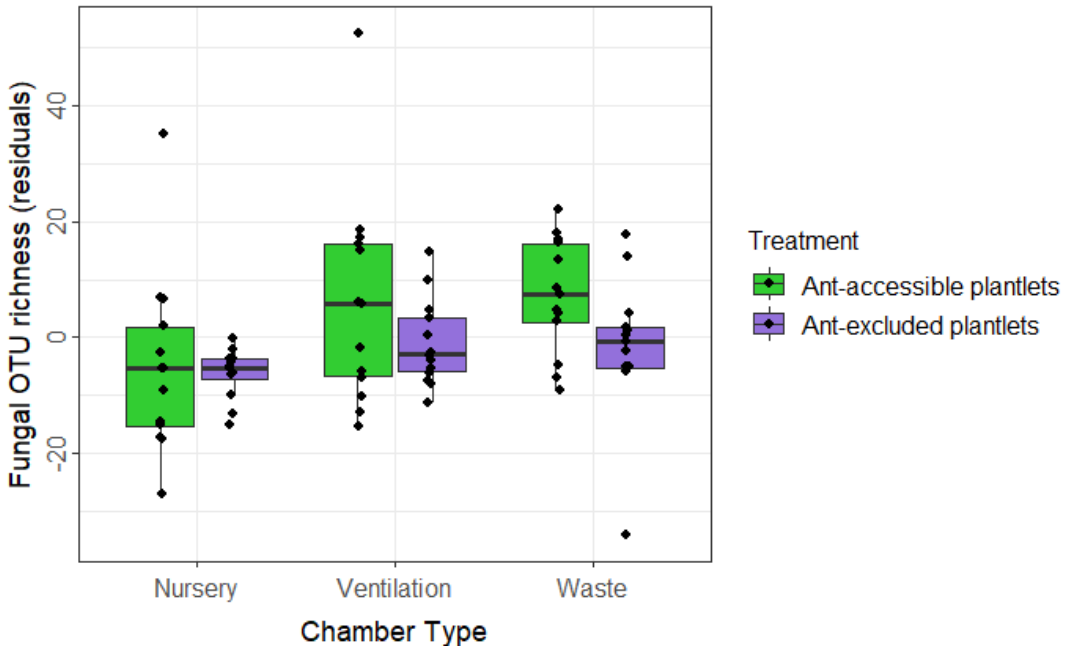

Figure 3.10 Fungal OTU richness in the domatium chambers of *M. beccarii* treatment plantlets (ant-accessible plantlets = green and ant-excluded plantlets = purple) grouped by chamber type (nursery, ventilation, and waste)**.** The solid line represents the median, the black dots are the data points (n=13).

Table 3.2 Summary of linear mixed effects model results testing the effects of ant-exclusion and chamber type on fungal OTU richness in the domatium chambers of *M. beccarii* plantlets including main effects and interactions (upper section of table). Lower section of table shows the pairwise contrasts between treatments for each chamber type.

Explanatory variables	Sum Sq	Mean Sq	NumDF	DenDF	F value	p-value
treatment	647.91	647.91	1	58.7	4.41	**0.0399**
chamber	1172.67	586.33	2	58.7	3.99	**0.0237**
treatment: chamber	185.19	92.6	2	58.7	0.63	0.54

Pairwise contrasts between ant- accessible and ant-excluded plantlets for each chamber type					
	Estimate	**SE**	**df**	**t-ratio**	**p-value**
nursery chamber	1.49	4.86	59.7	0.306	0.76
ventilation chamber	7.17	4.75	59	1.508	0.14
waste chamber	8.76	4.75	59	1.844	0.07

3.3.5 Fungal OTU community composition in the plant chambers

The fungal OTU community composition in the domatium chambers of *M. beccarii* ant-plants varied with treatment, and the effect of treatment varied with chamber type (Fig. 3.11, Table 3.3). There was a weak (R^2=1.7%) but significant effect of ant abundance on the fungal OTU community composition, and the effect of treatment also varied with ant abundance (Table 3.3). For the separate analyses for each chamber type, treatment influenced fungal OTU communities in the nursery, ventilation, and waste chambers for all pairwise comparisons, but the amount of variation explained between the OTU communities for each comparison differed (Table 3.3). For example, contrasts of mature ant-plants versus ant-excluded plantlets displayed greater amounts of explained variation for the nursery (R^2=34%) and waste (R^2=47%) chambers compared to contrasts of mature ant-plants versus ant-accessible plantlets for nursery (R^2=27%) and waste (R^2=28%) chambers (Fig. 3.11A&C, Table 3.3). A similar amount of variation was explained in the ventilation chambers for contrasts between mature ant-plants versus ant-accessible plantlets (R^2=37%) and mature ant-plants versus ant-excluded plantlets (R^2=38%) (Fig. 3.11B, Table 3.3). Contrasts of ant-accessible plantlets versus ant-excluded plantlets explained the least amount of variation: nursery (R^2=9%), ventilation (R^2=8%), and waste (R^2=13%). For all pairwise comparisons, there were large amounts of unexplained residual variation (Table 3.3).

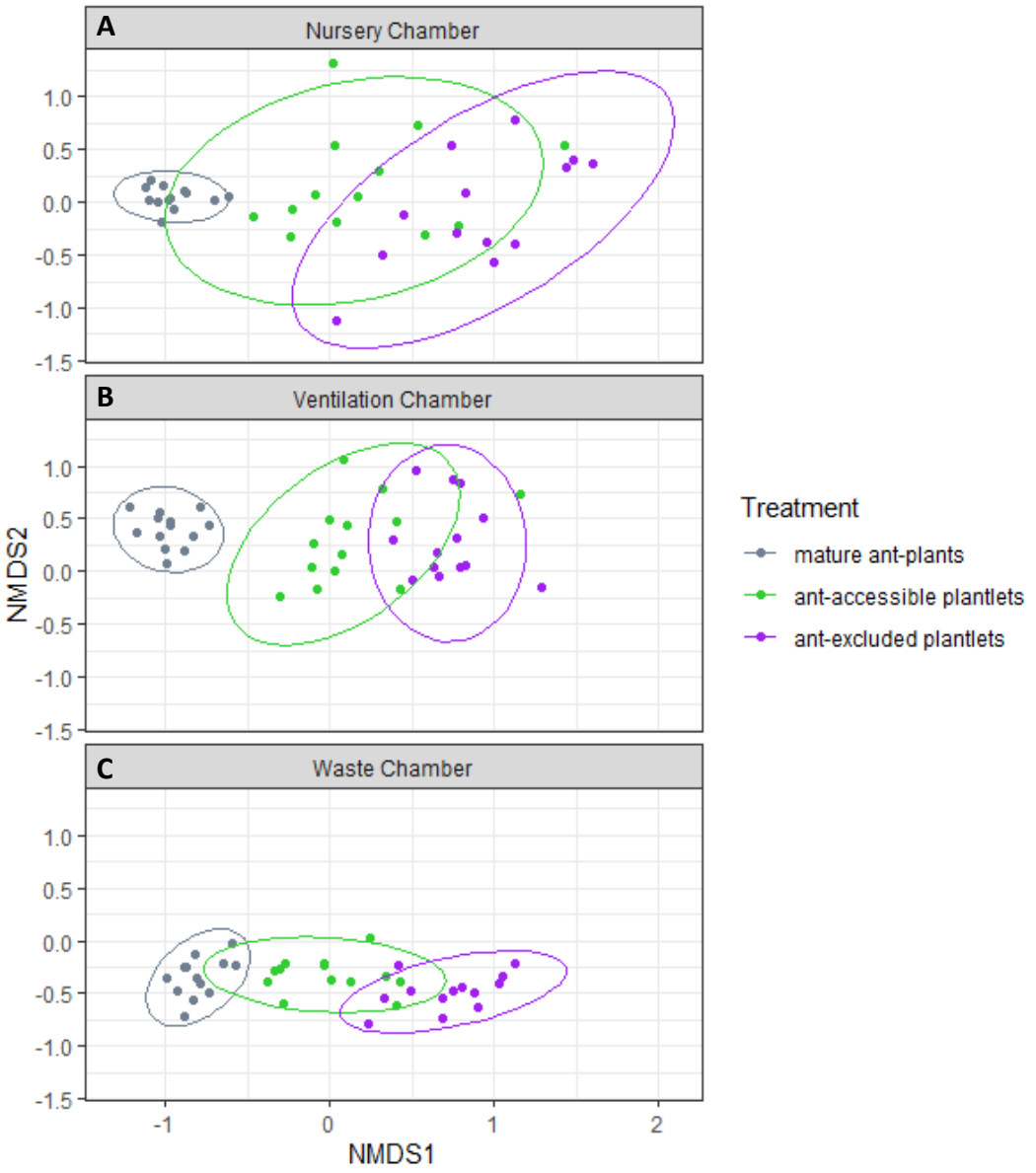

Figure 3.11 Non-metric multidimensional scaling (NMDS) ordinations showing fungal OTU community compositions in (A) nursery, (B) ventilation, and (C) waste chambers of *M. beccarii* mature ant-plants (grey), ant-accessible plantlets (green), and ant-excluded plantlets (purple). Each point on a plot is a sample of a fungal community collected from one of the three chambers from the mature ant-plants or the treatment plantlets (ant-accessible or ant-excluded). These ordination plots include 218 fungal operational taxonomic units (OTUs). Stress = 0.146.

Table 3.3 Fungal OTU community composition - PERMANOVA results of Hellinger-transformed OTU matrix to test influence of treatment (ant-accessible plantlets, ant-excluded plantlets, and mature ant-plants) and chamber type (nursery, ventilation, waste) on fungal OTU community composition in the chambers of *M. beccarii* plantlets. Upper section shows main effects and interactions, and lower section shows the pairwise contrasts for each chamber type.

PERMANOVA Summary	Df	Sum Sq	Pseudo-F	R^2	p-value
Main effects and interactions					
treatment	2	8.997	18.0771	0.19767	**0.0001**
chamber	2	4.672	9.3869	0.10264	**0.0001**
ant abundance	1	0.762	3.062	0.01674	**0.0001**
treatment:chamber	4	4.458	4.4783	0.09794	**0.0001**
treatment:ant abundance	2	1.049	2.1418	0.02305	**0.0013**
chamber:ant abundance	2	0.556	1.1354	0.01222	0.219
treatment:chamber:ant abundance	4	0.774	0.7897	0.017	0.789
Residual	99	26.626		0.53274	
Total	116	45.514		1	
Nursery chambers					
treatment	2	4.1951	7.2436	0.28471	**0.0001**
ant abundance	1	0.4047	1.3975	0.02746	**0.0001**
Residuals	35	10.1349		0.68783	
Total	38	14.7346		1	
mature ant-plants vs ant-accessible plantlets					
treatment	1	2.2554	8.7509	0.2672	**0.002**
Residuals	24	6.1857		0.7328	
Total	25	8.4412		1	
mature ant-plants vs ant-excluded plantlets					
treatment	1	3.1338	12.292	0.33869	**0.001**
Residuals	24	6.1188		0.66131	
Total	25	9.2526		1	
ant-accessible vs ant-excluded plantlets					
treatment	1	0.9034	2.4708	0.09334	**0.018**
Residuals	24	8.7747		0.90666	
Total	25	9.678		1	
Ventilation chambers					
treatment	2	4.7443	9.6271	0.34535	**0.0001**
ant abundance	1	0.3693	1.4986	0.02688	**0.0001**
Residuals	35	8.6242		0.62777	
Total	38	13.7378		1	
mature ant-plants vs ant-accessible plantlets					
treatment	1	3.0287	13.955	0.36767	**0.001**
Residuals	24	5.2087		0.63233	
Total	25	8.2374		1	
mature ant-plants vs ant-excluded plantlets					
treatment	1	3.4638	14.816	0.3817	**0.002**
Residuals	24	5.6108		0.6183	
Total	25	9.0746		1	
ant-accessible vs ant-excluded plantlets					
treatment	1	0.6241	2.0897	0.0801	**0.011**
Residuals	24	7.1673		0.9199	
Total	25	7.7914		1	

PERMANOVA Summary (cont'd)	Df	Sum Sq	Pseudo-F	R^2	p-value
Waste chambers					
treatment	2	4.5148	10.9556	0.36499	**0.0001**
ant abundance	1	0.6432	3.1214	0.052	**0.0001**
Residuals	35	7.2117		0.58302	
Total	38	12.3696		1	
mature ant-plants vs ant-accessible plantlets					
Treatment	1	1.8167	9.3931	0.28129	**0.001**
Residuals	24	4.6417		0.71871	
Total	25	6.4583		1	
mature ant-plants vs ant-excluded plantlets					
treatment	1	3.9486	21.287	0.47005	**0.001**
Residuals	24	4.4518		0.52995	
Total	25	8.4004		1	
ant-accessible vs ant-excluded plantlets					
treatment	1	1.0069	3.6526	0.13209	**0.004**
Residuals	24	6.6163		0.86791	
Total	25	7.6232		1	

3.3.6 Fungal OTUs shared among the plants

The ant-accessible plantlets and mature ant-plants shared the greatest number of fungal OTUs (60) for all chambers combined (27.5% of the 218 OTUs; Fig. 3.12A). The ant-accessible and ant-excluded plantlets shared 38 OTUs (17.4%) while the mature ant-plants and ant-excluded plantlets shared only 6 of the 218 OTUS (2.75%, Fig. 3.12A). The ant-accessible plantlets and mature ant-plants shared 26.1% of OTUs in the waste chambers, 23.6% in the nursery chambers, and 20.9% in the ventilation chambers (Fig. 3.12B-D). In contrast, the ant-excluded plantlets and mature ant-plants shared 0.62% of OTUs in the waste chambers, 2.2% in the nursery chambers and 2.0% in the ventilation chambers (Fig. 3.12B-D). The greatest number of OTUs shared between ant-accessible and ant-excluded plantlets was in the ventilation chambers, which is also where the greatest number of unique fungal OTUs were found for the mature ant-plants.

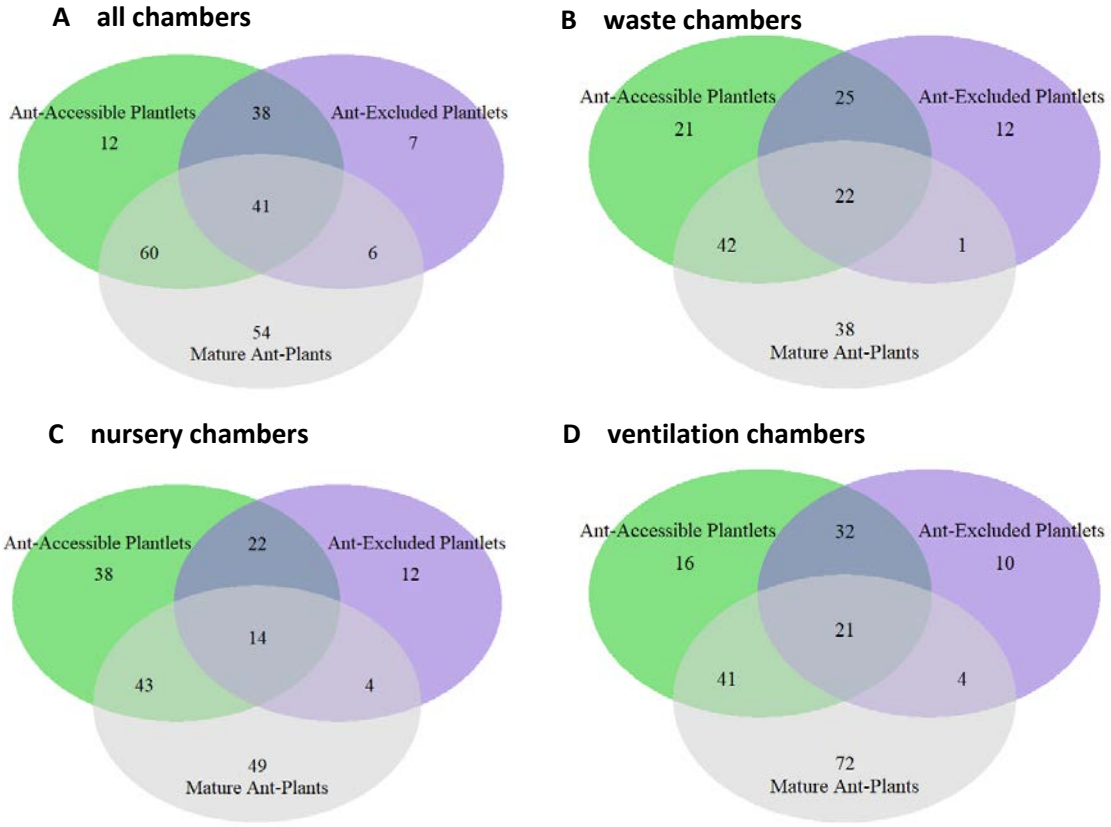

Figure 3.12 Venn diagrams of unique and shared fungal operational taxonomic units (OTUs) in: (A) all three chambers (218 OTUs), (B) waste chambers (161 OTUs), (C) nursery chambers (182 OTUs), and (D) ventilation chambers (196 OTUs) of *Myrmecodia beccarii* for the ant-accessible plantlets (green), ant-excluded plantlets (purple) and mature ant-plants (grey).
The numbers displayed represent the number of fungal OTUs shared between and among (overlapping circles) and unique to the ant-accessible plantlets, ant-excluded plantlets, and mature ant-plants. The mature ant-plants had 161 OTUs, ant-accessible plantlets had 151 OTUs, and ant-excluded plantlets had 92 OTUs.

3.3.7 Fungal OTU sequence abundances on or in the *P. cordata* ant workers

A total 127 fungal OTUs with a minimum sequence abundance of 2 were sequenced from *P. cordata* ant worker exoskeletons (81 OTUs), crushed heads (69 OTUs), and crushed abdomens (30 OTUs) (Supplementary Table S3.3). Fungal OTU sequence abundances were approximately three times greater in the exoskeleton wash samples compared to the crushed abdomens of the ant workers and approximately 2.5 times greater in the crushed heads compared to the crushed abdomens (Fig. 3.13, Supplementary Table S3.7, Table 3.4). Fungal OTU sequence abundances did not differ between the exoskeleton wash and the crushed head samples (Fig. 3.13, Table 3.4).

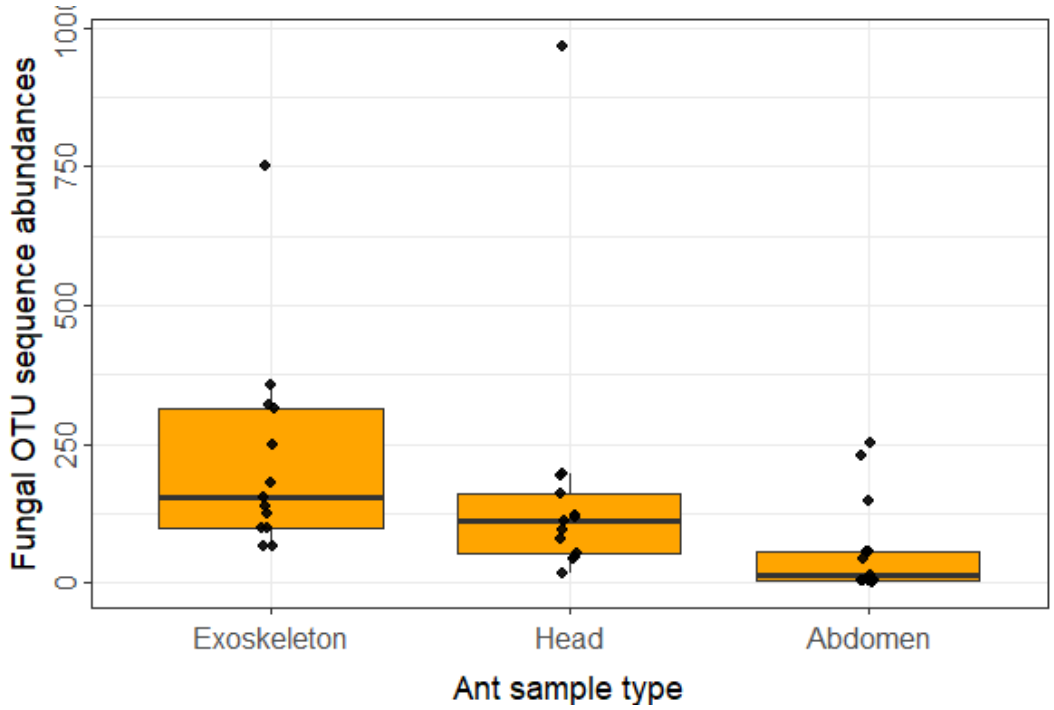

Figure 3.13 Fungal OTU sequence abundances on/in samples from *P. cordata* ant workers including exoskeletons, crushed heads, and crushed abdomens. The solid line represents the median, the black dots are the data points (n=13).

Table 3.4 Summary output of generalised linear mixed model to test the influence of ant sample type (exoskeleton wash, heads, abdomens) on fungal OTU sequence abundances in and on *P. cordata* ant workers inhabiting *M. beccarii* ant-plants including main effects (upper section of table) and pairwise contrasts between sample types (lower section).

Summary	Estimate	SE	z-value	p-value
(Intercept)	5.1635	0.3062	16.866	**<0.0001**
head	-0.5321	0.4097	-1.299	0.194
abdomen	-2.0322	0.4168	-4.876	**<0.0001**
Pairwise contrasts between sample types				
exoskeleton vs head	0.532	0.410	1.299	0.3958
exoskeleton vs abdomen	2.032	0.417	4.876	**0.0001**
head vs abdomen	1.50	0.417	3.594	**0.0009**

3.3.8 Fungal OTUs shared between plantlets and ant workers

Of the 127 fungal OTUs found associated with the ant workers, 31 OTUs (24.4%) were shared with the ant-accessible plantlets and no OTUs were shared solely with the ant-excluded plantlets (Fig 3.14). Twenty-two OTUs were shared among the ant workers, ant-accessible plantlets, and ant-excluded plantlets. There were 74 OTUs that were unique to the ant workers,

41 OTUs unique to the chambers of the ant-accessible plantlets and 13 OTUs that were unique to the chambers of the ant-excluded plantlets (Fig. 3.14).

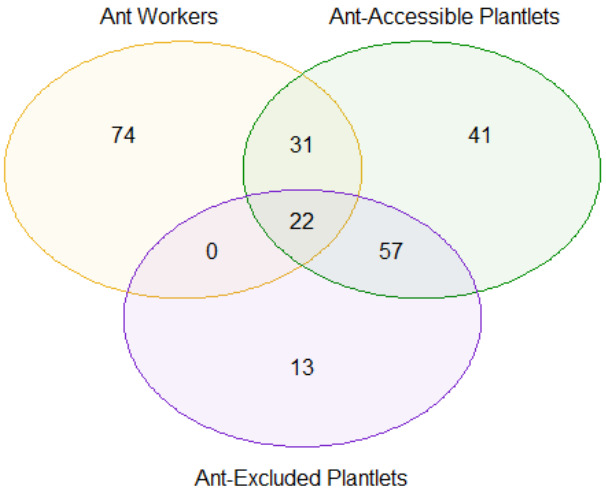

Figure 3.14 Venn diagram of unique and shared fungal OTUs collected from *Philidris cordata* ant workers (exoskeletons, heads, and abdomens combined) (orange), and from chambers of *Myrmecodia beccarii* ant-accessible plantlets (green) and ant-excluded plantlets (purple).
The numbers displayed represent the number of fungal OTUs shared between and among (overlapping circles) and unique to the ant workers (total 127 OTUs), the ant-accessible plantlets (total 151 OTUs) and ant-excluded plantlets (total 92 OTUs).

3.3.9 Dominant fungal OTUs

Forty-one of the 218 fungal OTUs collected from plant samples were 'dominant' (Table 3.5). To be considered dominant, the sample had to occur in a chamber type with a minimum abundance of 10 in at least 50% of mature ant-plant samples, and/or at least 20% of: ant-accessible plantlets, or ant-excluded plantlets. Thirteen of these 41 dominant fungal OTUs were significantly more abundant in ant-accessible plantlets compared to ant-excluded plantlets and one OTU (OTU0186) was more abundant in the ant-excluded plantlets (Table 3.6). Eight of the 41 dominant OTUs had a close match (>97% sequence similarity) to fungal OTUs found previously during the ant-plant survey (OTU0281, OTU0296, OTU1188, OTU0836, OTU0737, OTU0850, OTU0379, OTU0752) and two fungal OTUs had a 96% match (OTU0509 and OTU0436) (chapter 2; Greenfield et al. 2021) (Table 3.5). The ant workers were associated with 24 of the 41 dominant OTUs from the plant samples (Table 3.5). These 24 OTUs included 13 of the 21 dominant OTUs in the waste chambers, 9 of the 14 dominant OTUs in the nursery chambers and 12 of the 21 dominant OTUs in the ventilation chambers (Table 3.5).

Seven of the 41 dominant fungal OTUs in the plant chamber samples (namely OTU0296, OTU1188, OTU0422, OTU1266, OTU0089, OTU0509 and OTU0436) occurred in all chamber types of the mature ant-plants and almost all chamber types of the ant-accessible plantlets (Table 3.5, rows 1-7). In contrast, all seven of these fungal OTUs were absent or occurred rarely in chambers of the ant-excluded plantlets (Table 3.5, rows 1-7). Fungal OTU abundances for all seven of these OTUs were higher in the ant-accessible plantlets compared to the ant-excluded plantlets for the nursery and ventilation chambers for OTU0436, and in the waste chambers for the other six OTUs (Table 3.6). All seven of these fungal OTUs were in the ant worker samples (Table 3.7). Fungal OTU0296 is from the order Chaetothyriales and had a 99% match to fungal OTU0202 found previously in a survey of ant-plants (chapter 2; Greenfield et al. 2021) and has also been isolated from domatia of a terrestrial ant-plant in Cameroon, Africa (Voglmayr et al. 2011) and an epiphytic ant-plant in Thailand (Blatrix et al. 2021).

An additional 19 of the 41 fungal OTUs were dominant in one or more of the chambers of the mature ant-plants and 15 of these (79%) were also found in the chambers of the ant-accessible plantlets, but not dominant (Table 3.5, rows 8-26). For example, OTU0281 *Candida fluviatilis* with a 98% match to OTU0171 in the ant-plant survey (chapter 2; Greenfield et al. 2021) was found in 9 out of 13 waste chamber samples but the abundances of this OTU were less than 10 in 7 of those samples (Table 3.5). OTU0281 was the most abundant fungal OTU associated with the *P. cordata* ant worker samples (Table 3.7) and was significantly more abundant in samples collected from the exoskeletons and heads of the ant workers than in the abdomens of the workers (Table 3.8).

For the ant worker samples, 29 of the 127 fungal OTUs were dominant (occurred with minimum abundance of 2 in at least 20% of at least one of the sample types: exoskeletons, heads, or abdomens) (Table 3.7). Of the 29 dominant fungal OTUs associated with ant workers, 27 (93%) were collected from exoskeletons (22 of which dominant), 24 OTUs (83%) were collected from heads (15 of which were dominant), and 11 OTUs (38%) were collected from abdomens (2 of which were dominant).

Table 3.5 Dominant fungal operational taxonomic units (OTUs) in domatium chambers of *Myrmecodia beccarii* with minimum read abundance of 10 in at least: 50% of mature ant-plant samples (grey), and/or 20% of ant-accessible plantlets (green), and/or 20% of ant-excluded plantlets (purple).
WC = waste chamber, NC = nursery chamber, VC = ventilation chamber. Values on left side of table headed "Relative Abundance" are sequence abundances of OTUs across samples. Middle column entitled "total abundance" is the total sequence abundance across all samples for each OTU. Values on right side of table headed "Occurrence" are percentages of chamber samples that had the fungal OTU and colour-shaded values highlight where OTUs were dominant for a plant/chamber combination. OTU presence in *P. cordata* ant samples is indicated by E (exoskeleton), H (head), and A (abdomen) and those in bold indicate the OTU was dominant in that ant sample. OTU IDs marked with * are fungal OTUs that were significantly abundant in contrasts between ant-accessible and ant-excluded plantlets (Table 3.6). OTU IDs underlined were found previously during the ant-plant survey (chapter 2; Greenfield et al. 2021).

	Fungal OTU ID	Relative Abundance (% sequence abundance in chamber samples)									Total abundance	Relative Occurrence (% of chamber samples with OTU)									OTU presence in *P. cordata* ant samples
		mature ant-plants			ant-accessible plantlets			ant-excluded plantlets				mature ant-plants			ant-accessible plantlets			ant-excluded plantlets			
		WC	NC	VC	WC	NC	VC	WC	NC	VC		WC	NC	VC	WC	NC	VC	WC	NC	VC	
1	OTU0296*	26.33	0.37	4.79	67.71	0.68	0.12	0.00	0.00	0.00	8400	84.6	15.4	30.8	46.2	30.8	15.4	0.0	0.0	0.0	H
2	OTU1188*	60.15	4.02	0.76	18.09	16.56	0.42	0.00	0.00	0.00	1443	69.2	38.5	15.4	53.8	46.2	30.8	0.0	0.0	0.0	**E** H
3	OTU0422*	71.30	0.48	5.07	22.48	0.53	0.11	0.03	0.01	0.00	11971	100	76.9	38.5	61.5	38.5	38.5	23.1	7.7	0.0	E H A
4	OTU1266*	94.77	0.64	1.64	2.75	0.15	0.04	0.00	0.00	0.00	7152	100	69.2	53.8	61.5	38.5	15.4	0.0	0.0	0.0	E
5	OTU0089*	88.52	5.23	1.19	4.81	0.24	0.00	0.01	0.00	0.00	22772	100	84.6	53.8	76.9	15.4	0.0	15.4	0.0	0.0	E **H**
6	OTU0509*	0.87	66.29	32.27	0.41	0.11	0.03	0.01	0.00	0.00	21390	100	100	100	61.5	38.5	38.5	15.4	0.0	0.0	**E H**
7	OTU0436*	0.20	36.03	14.46	0.20	11.25	37.85	0.00	0.00	0.00	3955	23.1	84.6	100	46.2	38.5	38.5	0.0	0.0	0.0	H
8	OTU0281*	83.03	2.03	0.37	5.44	8.49	0.65	0.00	0.00	0.00	1084	100	30.8	15.4	69.2	30.8	15.4	0.0	0.0	0.0	**E H**
9	OTU0299*	87.79	5.23	0.83	5.51	0.55	0.09	0.00	0.00	0.00	1089	100	61.5	7.7	53.8	15.4	7.7	0.0	0.0	0.0	**H**
10	OTU0366	99.15	0.12	0.38	0.26	0.00	0.00	0.00	0.06	0.03	3428	100	15.4	15.4	30.8	0.0	0.0	0.0	7.7	7.7	E
11	OTU0391	99.94	0.00	0.06	0.00	0.00	0.00	0.00	0.00	0.00	1774	100	0.0	7.7	0.0	0.0	0.0	0.0	0.0	0.0	-
12	OTU0685	98.61	0.24	0.65	0.47	0.00	0.00	0.00	0.03	0.00	3386	69.2	15.4	15.4	15.4	0.0	0.0	0.0	7.7	0.0	-
13	OTU0450	99.47	0.05	0.24	0.14	0.05	0.00	0.00	0.05	0.00	2087	61.5	7.7	7.7	15.4	7.7	0.0	0.0	7.7	0.0	E
14	OTU0448	1.80	97.16	1.03	0.00	0.00	0.00	0.00	0.00	0.00	388	15.4	84.6	23.1	0.0	0.0	0.0	0.0	0.0	0.0	-
15	OTU0752	0.44	70.36	29.14	0.00	0.01	0.00	0.00	0.00	0.04	11245	53.8	100	84.6	0.0	7.7	0.0	0.0	0.0	7.7	E
16	OTU0550	0.12	33.44	66.23	0.18	0.02	0.00	0.00	0.00	0.00	4871	38.5	100	100	15.4	7.7	0.0	0.0	0.0	0.0	E

	Fungal OTU ID	Relative Abundance (% sequence abundance in chamber samples)									Total abundance	Relative Occurrence (% of chamber samples with OTU)									OTU presence in *P. cordata* ant samples
		mature ant-plants			ant-accessible plantlets			ant-excluded plantlets				mature ant-plants			ant-accessible plantlets			ant-excluded plantlets			
		WC	NC	VC	WC	NC	VC	WC	NC	VC		WC	NC	VC	WC	NC	VC	WC	NC	VC	
17	OTU0836	1.29	37.34	61.37	0.00	0.00	0.00	0.00	0.00	0.00	5276	53.8	84.6	76.9	0.0	0.0	0.0	0.0	0.0	0.0	-
18	OTU0737	2.47	20.64	76.11	0.07	0.27	0.44	0.00	0.00	0.00	2960	76.9	92.3	100	15.4	15.4	30.8	0.0	0.0	0.0	**E H**
19	OTU1190	1.74	29.24	68.49	0.00	0.00	0.53	0.00	0.00	0.00	749	23.1	92.3	100	0.0	0.0	23.1	0.0	0.0	0.0	E
20	OTU0634	0.32	4.93	94.54	0.00	0.00	0.11	0.00	0.00	0.11	1867	7.7	38.5	92.3	0.0	0.0	7.7	0.0	0.0	15.4	-
21	OTU0850	0.00	2.30	97.51	0.00	0.10	0.10	0.00	0.00	0.00	1044	0.0	15.4	61.5	0.0	7.7	7.7	0.0	0.0	0.0	-
22	OTU0951	0.26	10.55	88.96	0.08	0.00	0.15	0.00	0.00	0.00	2664	30.8	84.6	92.3	15.4	0.0	15.4	0.0	0.0	0.0	**E** H
23	OTU0974	0.35	3.77	95.53	0.09	0.09	0.18	0.00	0.00	0.00	1141	30.8	69.2	100	7.7	7.7	7.7	0.0	0.0	0.0	**E**
24	OTU0995	0.00	1.13	98.87	0.00	0.00	0.00	0.00	0.00	0.00	970	0.0	38.5	100	0.0	0.0	0.0	0.0	0.0	0.0	-
25	OTU1054	0.17	13.34	85.10	0.00	1.04	0.35	0.00	0.00	0.00	577	7.7	53.8	84.6	0.0	15.4	15.4	0.0	0.0	0.0	E
26	OTU1122	0.12	52.85	41.96	0.00	3.96	1.11	0.00	0.00	0.00	1616	7.7	76.9	76.9	0.0	7.7	23.1	0.0	0.0	0.0	E H
27	OTU0471*	2.54	12.68	2.98	10.85	20.11	4.93	18.86	27.03	0.03	28303	100	100	76.9	92.3	84.6	76.9	38.5	30.8	23.1	**E H** A
28	OTU0573	0.08	0.76	0.28	1.25	4.25	17.29	3.24	41.17	31.69	5033	30.8	15.4	15.4	69.2	84.6	69.2	84.6	76.9	92.3	-
29	OTU0417	0.21	0.15	0.15	42.39	45.89	1.84	7.30	1.72	0.34	5221	23.1	15.4	7.7	84.6	38.5	38.5	92.3	38.5	38.5	-
30	OTU0835	0.07	0.00	0.00	42.48	8.92	2.14	31.27	11.80	3.32	1356	7.7	0.0	0.0	92.3	30.8	30.8	92.3	23.1	30.8	-
31	OTU0338	0.47	0.00	0.35	48.82	0.12	0.12	49.88	0.00	0.24	850	7.7	0.0	7.7	30.8	7.7	7.7	69.2	0.0	7.7	E
32	OTU0169*	0.18	2.60	0.23	0.09	96.04	0.87	0.00	0.00	0.00	2195	23.1	23.1	23.1	7.7	23.1	15.4	0.0	0.0	0.0	E H
33	OTU0379*	1.39	2.29	2.02	91.80	2.18	0.32	0.00	0.00	0.00	1877	30.8	23.1	23.1	46.2	38.5	30.8	0.0	0.0	0.0	**-**
34	OTU1035	0.36	0.00	0.00	77.28	8.14	2.95	2.33	2.77	6.17	1118	7.7	0.0	0.0	53.8	38.5	38.5	23.1	15.4	30.8	-
35	OTU0038	3.15	0.00	0.00	2.36	8.66	22.83	0.00	0.00	62.99	127	15.4	0.0	0.0	15.4	7.7	38.5	0.0	0.0	38.5	H A
36	OTU0257	0.00	0.00	0.18	0.36	25.77	26.50	1.27	1.27	44.65	551	0.0	0.0	7.7	15.4	23.1	46.2	23.1	30.8	53.8	-
37	OTU0231	0.00	2.03	0.58	14.24	0.00	2.33	33.14	46.51	1.16	344	0.0	15.4	7.7	53.8	0.0	23.1	38.5	46.2	23.1	H
38	OTU1111	0.00	0.00	0.00	0.00	0.15	0.46	0.15	13.81	85.43	1970	0.0	0.0	0.0	0.0	23.1	38.5	7.7	23.1	23.1	-
39	OTU0057	0.00	0.00	0.00	14.08	8.90	4.97	51.55	13.87	6.63	483	0.0	0.0	0.0	61.5	38.5	46.2	61.5	38.5	46.2	-
40	OTU1021*	0.00	0.00	0.00	0.05	28.22	43.13	0.00	0.00	28.60	13388	0.0	0.0	0.0	23.1	30.8	69.2	0.0	0.0	53.8	**-**
41	OTU1010	0.00	0.00	0.00	0.00	55.10	7.65	0.00	13.02	24.23	4771	0.0	0.0	0.0	0.0	30.8	30.8	0.0	23.1	23.1	-

Table 3.6 DESeq2 analysis showing significantly abundant fungal operational taxonomic units (OTUs) in contrasts between samples collected from the nursery, ventilation, and waste chambers of *Myrmecodia beccarii* ant-accessible and ant-excluded plantlets at the end of the ant-exclusion experiment. Fungal Taxon represents the closest match to a sequence in the UNITE database or the APSurvey database created from fungal sequences obtained during the ant-plant survey (chapter 2; Greenfield et al. 2021) in which case "APSurvey OTU ID" is the identifier given to the fungal OTUs collected during the survey. OTUs with * were dominant (minimum abundance 10 in at least: 20% of the ant-accessible plantlets and/or 20% of the ant-excluded plantlets. Shading is for clarity when reading table. OTUs in bold were found in *P. cordata* ant worker samples.

OTU ID	Fungal Taxon	APSurvey OTU ID	Chamber Type	Contrasts between plants	Base Mean	log2 Fold Change	LFC SE	Adjusted p-value
OTU0089*	Fungi sp.	OTU0170 (94%)	waste	ant-accessible vs ant-excluded	448.614	8.076	1.033	1.45E-13
OTU0169*	Eurotiales sp.		nursery	ant-accessible vs ant-excluded	10.712	23.504	3.580	2.11E-09
OTU0186	Sebacinales sp.		nursery	ant-excluded vs ant-accessible	8.777	28.224	3.580	1.73E-13
OTU0281*	*Candida fluviatilis*	OTU0171 (98%)	waste	ant-accessible vs ant-excluded	15.090	4.825	0.915	2.42E-06
OTU0296*	Chaetothyriales sp.	OTU0202 (99%)	waste	ant-accessible vs ant-excluded	264.113	27.449	1.700	1.40E-56
OTU0299*	Fungi sp.	OTU0170 (94%)	waste	ant-accessible vs ant-excluded	21.323	4.404	1.059	3.87E-04
OTU0379*	*Arthrocladium* sp.	OTU0289 (98%)	waste	ant-accessible vs ant-excluded	45.522	26.200	1.971	1.35E-38
OTU0422*	Eurotiomycetes sp.		waste	ant-accessible vs ant-excluded	214.738	9.581	1.182	1.91E-14
OTU0436*	Eurotiales sp.	OTU0267 (96%)	nursery	ant-accessible vs ant-excluded	35.494	23.631	1.766	1.31E-38
OTU0471*	Talaromyces sp.		ventilation	ant-accessible vs ant-excluded	17.607	5.149	1.253	3.55E-03
OTU0509*	Chaetothyriales sp.	OTU0347 (96%)	waste	ant-accessible vs ant-excluded	6.089	4.806	0.931	3.80E-06
			ventilation	ant-accessible vs ant-excluded	23.367	23.775	1.328	2.23E-69
OTU1021*	Fungi sp.		nursery	ant-accessible vs ant-excluded	29.259	38.670	3.579	2.69E-25
OTU1188*	*Candida* sp.	OTU0977 (99%)	waste	ant-accessible vs ant-excluded	18.086	6.540	1.410	4.77E-05
OTU1266*	Tremellomycetes sp.		waste	ant-accessible vs ant-excluded	136.847	6.715	1.019	9.70E-10

Table 3.7 Twenty-nine dominant fungal OTUs on or in samples from *P. cordata* ant workers including exoskeletons (exo), heads (hds), and abdomens (abs). Relative Abundance is the percentage sequence abundance for each fungal OTU for each ant sample type relative to the other samples, total sequence abundance is the total raw abundances across all ant samples, and Occurrence is the percentage of ant samples that had the fungal OTU for a sample type. OTU presence in the three different chambers of *M. beccarii* mature ant-plants, ant-accessible plantlets, and ant-excluded plantlets is indicated by ticks. Shaded values highlight where the OTUs were dominant for ant samples (minimum read abundance 2 in at least 20% of at least one of the ant worker sample types – all in orange) or plant samples (minimum abundance 10 in at least: 50% of mature ant-plant samples (grey) and/or 20% of ant-accessible plantlets (green) and/or 20% of ant-excluded plantlets (purple). WC = waste chamber, NC = nursery chamber, VC = ventilation chamber. OTU IDs marked with * are fungal OTUs that were significantly abundant in contrasts between samples collected from ant workers.

Fungal OTU ID	Relative abundance (% sequence abundance in ant samples)			Total sequence abundance	Occurrence (% of ant samples with OTU)			OTU presence in ant-plant chambers								
								mature ant-plants			ant-accessible plantlets			ant-excluded plantlets		
	exo	hds	abs		exo	hds	abs	WC	NC	VC	WC	NC	VC	WC	NC	VC
OTU0281*	52.3	47.7	0.0	644	57.1	64.3	0.0	✓	✓	✓	✓	✓	✓			
OTU0471	18.5	71.5	10.0	130	21.4	21.4	7.1	✓	✓	✓	✓	✓	✓	✓	✓	✓
OTU0509	53.8	46.2	0.0	225	35.7	50.0	0.0	✓	✓	✓	✓	✓	✓	✓		
OTU0587	62.3	23.2	14.5	69	50.0	21.4	7.1		✓	✓	✓					
OTU0737	68.9	31.1	0.0	45	28.6	28.6	0.0	✓	✓	✓	✓	✓	✓			
OTU0946	68.0	10.3	21.7	175	28.6	28.6	14.3	✓	✓	✓	✓	✓	✓	✓	✓	✓
OTU0957	63.0	6.5	30.4	46	42.9	21.4	14.3	✓	✓	✓	✓	✓	✓	✓	✓	✓
OTU0206	66.7	33.3	0.0	48	21.4	14.3	0.0		✓	✓		✓	✓			
OTU0235	85.0	15.0	0.0	60	28.6	7.1	0.0	✓	✓	✓	✓	✓	✓			
OTU0241	55.6	44.4	0.0	18	21.4	14.3	0.0	✓		✓	✓	✓	✓	✓		✓
OTU0513	100.0	0.0	0.0	34	35.7	0.0	0.0									
OTU0749	88.0	1.3	10.7	75	35.7	7.1	14.3	✓	✓				✓	✓		
OTU0759	84.4	2.2	13.3	45	21.4	7.1	7.1	✓	✓	✓	✓	✓	✓			
OTU0862	21.4	78.5	0.2	576	50.0	7.1	7.1	✓		✓		✓				
OTU0951	93.3	6.7	0.0	104	21.4	14.3	0.0	✓	✓	✓	✓		✓			
OTU0959	100.0	0.0	0.0	65	42.9	0.0	0.0									
OTU0974	100.0	0.0	0.0	22	21.4	0.0	0.0	✓	✓	✓	✓	✓	✓			
OTU1138	97.9	2.1	0.0	47	21.4	7.1	0.0	✓	✓	✓	✓	✓	✓		✓	✓

Fungal OTU ID	Relative abundance (% sequence abundance in ant samples)			Total sequence abundance	Occurrence (% of ant samples with OTU)			OTU presence in ant-plant chambers								
								mature ant-plants			ant-accessible plantlets			ant-excluded plantlets		
	exo	hds	abs		exo	hds	abs	WC	NC	VC	WC	NC	VC	WC	NC	VC
OTU1154	100.0	0.0	0.0	29	21.4	0.0	0.0	✓	✓	✓						
OTU1188	70.5	29.5	0.0	88	42.9	14.3	0.0	✓	✓	✓	✓	✓	✓			
OTU1238	100.0	0.0	0.0	153	71.4	0.0	0.0									
OTU0036	34.4	32.2	33.4	410	21.4	50.0	28.6									
OTU0924	12.8	55.1	32.1	78	14.3	42.9	21.4	✓	✓		✓	✓	✓	✓		✓
OTU0089	20.5	79.5	0.0	146	7.1	57.1	0.0	✓	✓	✓	✓	✓		✓		
OTU0299	0.0	100.0	0.0	16	0.0	42.9	0.0	✓	✓	✓	✓	✓	✓			
OTU0362	92.9	7.1	0.0	551	7.1	21.4	0.0	✓	✓		✓	✓				
OTU0400	75.5	24.5	0.0	102	7.1	21.4	0.0	✓	✓	✓			✓			
OTU0898	0.0	87.2	12.8	117	0.0	50.0	7.1				✓	✓	✓			
OTU0923	17.9	76.8	5.4	56	14.3	28.6	7.1									

Table 3.8 DESeq2 analysis showing significantly abundant fungal OTUs in contrasts between different samples collected from *Philidris cordata* ant workers including exoskeletons, heads, and abdomens. Fungal Taxon represents the closest match to a sequence in the UNITE database or the APSurvey database created from fungal sequences obtained during the ant-plant survey (chapter 2; Greenfield et al. 2021) in which case "APSurvey OTU ID" is the identifier given to the fungal OTU in that survey. OTUs in bold were significantly abundant in plant chamber samples.

OTU ID	Fungal Taxon	APSurvey OTU ID	Contrasts between samples	Base Mean	log2 Fold Change	LFC SE	Adjusted p-value
OTU0281	*Candida fluviatilis*	OTU0171 (98%)	exoskeleton vs abdomen	13.19481	6.526085	1.463525	0.00091
			head vs abdomen	13.19481	6.636342	1.464025	0.00064

3.4 Discussion

This study is the first to investigate whether resident ant workers transport fungi between epiphytic ant-plants and my results strongly suggest that they do. The ant-accessible plantlets had higher fungal OTU sequence abundances in the nursery and waste chambers compared to the ant-excluded plantlets but similar abundances in the ventilation chambers. The nursery and waste chambers are where the ant workers keep their brood and deposit their waste and are the innermost chambers where fungi are least likely to disperse on their own. The ventilation chambers are the most exposed to the outside environment where fungi could disperse by other means. The ant-accessible plantlets and mature ant-plants had the highest number of shared fungal OTUs (60) and overlapped in their fungal community compositions for nursery and waste chambers, whereas the ant-excluded plantlets shared few OTUs (6) with the mature ant-plants. Seven fungal OTUs were dominant in the waste and nursery chambers of the ant-accessible plantlets and mature ant-plants that were absent (4) or occurred rarely (3) in the ant-excluded plantlets, and all of these dominant OTUs were found on or in *P. cordata* ant workers. A further 15 OTUs were dominant in chambers of mature ant-plants that also occurred in the ant-accessible plantlets, 12 of which were found on or in the *P. cordata* ant workers. The *P. cordata* ant workers shared 31 fungal OTUs solely with the ant-accessible plantlets but no OTUs were shared solely with the ant-excluded plantlets.

3.4.1 Fungi and the *P. cordata* ant workers

The transfer of fungi by ant workers was most likely via the exoskeleton and heads of the ant workers, not the abdomens. Of the 29 dominant fungal OTUs collected from the three types of ant samples, a high

proportion (76%) were dominant in exoskeletons suggesting ant workers are indirectly transporting fungi to the chambers of *M. beccarii* on the surface of their bodies. Approximately half of the 29 OTUs were dominant in the heads which suggests the *P. cordata* ant workers may be carrying fungal fragments or spores in their infrabuccal pockets as found in other ant species (Little et al. 2003), the contents of which form a pellet that is expelled as waste (Little et al. 2003) or fed to larvae (Wheeler and Bailey 1920, Blatrix et al. 2012). In other ant species, the infrabuccal pocket is used by founding queens to transport fungi vertically when they leave their natal nest to establish a new colony, e.g. in the *Azteca/Cecropia* ant-plant system (Mayer et al. 2018) and in leaf-cutter ants when they transport their fungal cultivar (Mueller et al. 1998, Mueller et al. 2001, Fernandez-Marin et al. 2004). Future research could examine whether the infrabuccal pockets of *P. cordata* ant workers and alate queens contain the dominant fungi that are found in the chambers of *M. beccarii*. Only two of the 29 OTUs (7%) were dominant in the abdomens of the *P. cordata* ant workers. The low number of fungi obtained from the crushed abdomens (compared to that found on the exoskeletons and in the heads) suggests the washing of the workers did remove fungi from the surface of the abdomens. Whether the remaining fungal OTUs from the crushed abdomens came from the gut contents or the outer surface cannot be confirmed but it does suggest that ant workers were not eating fungi and also that no or very few fungi were being deposited by ant workers in ant faeces in the waste chambers.

3.4.2 Fungi in the chambers of *M. beccarii*

The six fungal OTUs that were dominant in the waste chambers of ant-accessible plantlets and mature ant-plants and also on or in the *P. cordata* ant workers (but absent or rare in the ant-excluded plantlets) provides the most support for the hypothesis that ants transfer fungi between *M. beccarii* ant-plants. These six OTUs were also found previously in the ant-plant survey (chapter 2; Greenfield et al. 2021) which suggests these fungi may be playing roles in the waste chambers. In addition, the waste chambers of ant-accessible plantlets and mature ant-plants shared 40 times the number of fungal OTUs shared between the waste chambers of mature ant-plants and ant-excluded plantlets. The waste chambers are where the ant workers deposit waste (e.g. faeces) and the surfaces of waste chambers have wart-like structures that can absorb nutrients for the plant (Huxley 1978, Volp et al. 2022). It is possible the ant workers transferred fungi (e.g. on their bodies or in infrabuccal pellets) at the same time as depositing waste (e.g. faeces) in the waste chambers of the ant-accessible plantlets. It may be that the waste deposited by ant workers provides an ideal environment suitable for the transferred fungi to thrive (e.g. saprotrophic fungi that feed on waste). Alternatively, the waste chambers themselves may create conditions favourable for the

fungi to survive including plant-produced compounds that help fungi grow, or the location of the waste chambers in the domatium of *M. beccarii* may create environmental conditions (e.g. humidity) that assist certain fungi to grow. Some of the dominant fungi found in the waste chambers in this experiment may serve other purposes, e.g. food for the ant colony (Blatrix et al. 2012), or antibiotics to protect the colony from microbial pathogens (Moreno et al. 2019), or fungi may act as natural biofilters that clean the air in the domatium (Mayer et al. 2021).

For the nursery chambers, the sequence abundances of fungal OTUs were higher in the ant-accessible plantlets compared to the ant-excluded plantlets suggesting the ant workers did move fungi to the nursery chambers of the ant-accessible plantlets. Also, the number of fungal OTUs shared between the mature ant-plants and ant-accessible plantlets was ten times that shared with the ant-excluded plantlets. It was expected the fungal OTU community composition in the nursery chambers of the ant-accessible plantlets would be similar to that found in the nursery chambers of the mature ant-plants, but this was not the case. This may be due to the low abundances and occurrences of fungal OTUs in the ant-accessible plantlets that were shared with mature ant-plants; only one fungal OTU was dominant in the nursery chambers of both the ant-accessible plantlets and mature ant-plants. An explanation is that the nursery chambers of the ant-accessible plantlets were not yet being used to house brood and therefore the fungal communities associated with mature ant-plant nurseries had not developed in the nursery chambers of the ant-accessible plantlets. Only one ant-accessible plantlet had brood (larvae and pupae) at the end of the experiment. The presence of particular fungi may be connected to brood, e.g. fungi may be food for larvae or used in antimicrobial defence for the larvae, and it may be that only in the presence of brood the fungal community becomes established.

The ventilation chambers of the ant-accessible plantlets and ant-excluded plantlets had similar fungal OTU sequence abundances. This is not surprising given the function of the ventilation chambers is to allow airflow into the chambers (Janzen 1974, Huxley 1980), and they are the most exposed of all chambers to the outside environment and to air-borne fungal spores. It is likely that the ant-accessible and ant-excluded plantlets harboured similar amounts of fungi in the ventilation chambers because they were exposed to the same environment during (and prior to) the experiment. I observed *P. cordata* ant workers travelling through the ventilation chambers so there is potential for fungi to be transported to the ventilation chambers directly (e.g. infrabuccal pellets) or indirectly (e.g. from the body surfaces of the ant workers).

3.4.3 Dominant fungal OTUs

Some of the fungal OTUs isolated from the chambers and ant workers' exoskeletons, heads, and abdomens in this experiment could be playing a role in this mutualism. A Chaetothyriales species OTU0509 with a 96% match to OTU0347 (chapter 2; Greenfield et al. 2021) was dominant in all chambers of the mature ant-plants but most abundant in the nursery and ventilation chambers consistent with what was found in mature wild ant-plants during the survey (chapter 2; Greenfield et al. 2021). Chaetothyriales species OTU0509 was also found on the exoskeletons and in heads of *P. cordata* ant workers and was transferred to the ant-accessible plantlets. It may be that OTU0509 plays some role for the larvae of *P. cordata*. Chaetothyriales species OTU0509 had a 99% match to a Chaetothyriales fungus found in 93% of domatia from the terrestrial ant-plant *Leonardoxa africana letouzeyi* (Fabaceae: Caesalpinioideae) occupied by the mutualist ant *Aphomomyrmex afer* (Formicinae) (Defossez et al. 2009, Blatrix et al. 2013). Another Chaetothyriales species OTU0296 was dominant in the waste chambers of the mature ant-plants and ant-accessible plants and found in the heads of *P. cordata* ant workers. This Chaetothyriales OTU0296 had a 99% match to OTU0202 (chapter 2; Greenfield et al. 2021) which had a 99% match to Chaetothyriales isolate Kh-Nk3-2 found in the ant-plant *Keetia hispida* (Rubiaceae) in Cameroon (Voglmayr et al. 2011). The occurrence of closely related Chaetothyriales fungi in ant-plants from Australia and Africa suggests these fungi have a long association with ants and ant-plants. The occurrence of *Candida fluviatilis* OTU0281 with a 98% match to OTU0171 (chapter 2; Greenfield et al. 2021) occurred in the waste chambers of both the mature ant-plants (dominant) and ant-accessible plantlets (but not dominant) as well as on the exoskeletons and heads of the *P. cordata* ant workers suggests a possible role in the *P. cordata/M. beccarii* mutualism. *Candida fluviatilis* is a saprophyte considered important in bioremediation processes for its ability to biodegrade hydrocarbons such as oil-contaminated soils (Vetrova et al. 2022) and also has potential to neutralise acidic environments (Mitsuya et al. 2017). The presence of *C. fluviatilis* in the waste chambers of the mature ant-plants and ant-accessible plantlets suggests it may play a role in the breakdown of organic waste in the waste chambers.

3.4.4 Conclusion

Mutualisms involve an exchange of services and rewards between/among the partners involved so determining what is being exchanged and by whom is an important part of identifying the partners and the role(s) they play in a mutualism. While my study has confirmed that the *P. cordata* ant workers provide transportation services to some fungi, I have not determined what if any role these fungi play in the mutualism for the ant colony or for *M. beccarii* ant-plants. Some of the fungal OTUs found during this

study may be key players in the mutualism, some may be opportunistic and/or parasitic, and others may be 'weeds'. For example, fungi may play a role in protection of the ant colony (e.g. antibiotic) or fungi may be food for larvae. Some fungi may benefit in protecting the ant-plant against pathogenic microbes. Future research could sample fungi from the gut contents of *P. cordata* larvae, workers, and queens and from the infrabuccal pockets of the alate queens and ant workers. Sampling fungi from the environment around *M. beccarii* ant-plants (e.g. host tree bark and leaves, soil beneath the host) would provide insight into if/where domatium fungi live outside domatia. *Myrmecodia beccarii* lives in a nutrient poor environment so nutritional services provided by mutualists would be expected to occur. In chapter 4, I will investigate if fungi play a role in the transfer of nutrients in the waste chambers of *M. beccarii*. This will determine if fungi are a central player in nutrient provision between the *P. cordata* ant workers and the ant-plant.

3.5 Supplementary Information

Supplementary Table S3.1 Ant colony data for *Philidris cordata* collected from *Myrmecodia beccarii* mature ant-plants and plantlets and their cages. Each cage contained a mature ant-plant with its resident ant colony and ant-plantlets that were either accessible to the ant workers or excluded from ant workers (placed on a moat). The number of ant workers is the total number of ant workers alive collected from the mature ant-plants, ant-accessible plantlets and from the cage they were in at the end of the experiment. The abbreviation "na" under Treatment means not applicable as the mature ant-plants were not a treatment in this experiment. Ticks indicate presence at the end of the experiment. *cont. indicates possible contamination of chambers by dealate queens if they carried fungi from mature ant-plant to an ant-excluded plantlets.

Cage ID	Plant ID	Treatment	Number of ant workers	Dealate Queen	Male	Alate Queen	Pupae	Larvae	Eggs
AE09	mature09	na	12255	✓	✓	✓	✓	✓	✓
	063	ant-accessible plantlet		no	no	no	no	no	no
	040	ant-excluded plantlet		no	no	no	no	no	no
AE13	mature13	na	1325	no	no	✓	no	✓	✓
	024	ant-accessible plantlet		no	no	no	no	no	no
	025	ant-excluded plantlet		no	no	no	no	no	no
AE16	mature16	na	6463	no	✓	✓	no	no	✓
	010	ant-accessible plantlet		✓	no	✓	no	no	no
	060	ant-excluded plantlet		✓ *cont.	no	no	no	no	no
AE17	mature17	na	3222	no	✓	✓	✓	no	no
	029	ant-accessible plantlet		no	no	no	no	no	no
	044	ant-excluded plantlet		no	no	no	no	no	no
AE18	mature18	na	1197	✓	✓	✓	✓	✓	✓
	053	ant-accessible plantlet		no	no	no	no	no	no
	003	ant-excluded plantlet		✓ *cont.	no	no	no	no	✓*
AE21	mature21	na	17171	no	✓	no	✓	✓	✓
	023	ant-accessible plantlet		no	no	no	no	no	no
	062	ant-excluded plantlet		no	no	no	no	no	no

Cage ID	Plant ID	Treatment	Number of ant workers	Dealate Queen	Male	Alate Queen	Pupae	Larvae	Eggs
AE22	mature22	na	641	✓	✓	✓	no	✓	no
	004	ant-accessible plantlet		no	no	no	no	no	no
	045	ant-excluded plantlet		no	no	no	no	no	no
AE24	mature24	na	3662	no	✓	✓	no	✓	✓
	031	ant-accessible plantlet		no	no	✓	no	no	no
	011	ant-excluded plantlet		no	no	no	no	no	no
AE25	mature25	na	2174	no	no	✓	✓	✓	no
	035	ant-accessible plantlet		no	no	no	✓	✓	no
	059	ant-excluded plantlet		no	no	no	no	no	no
AE29	mature29	na	183	no	✓	✓	✓	✓	✓
	036	ant-accessible plantlet		no	no	no	no	no	no
	071	ant-excluded plantlet		no	no	no	no	no	no
AE30	mature30	na	262	no	no	no	no	no	no
	056	ant-accessible plantlet		no	no	no	no	no	no
	015	ant-excluded plantlet		no	no	no	no	no	no
AE31	mature31	na	8505	✓	✓	✓	no	no	no
	026	ant-accessible plantlet		no	no	no	no	no	no
	046	ant-excluded plantlet		no	no	no	no	no	no
AE32	mature32	na	6578	no	✓	✓	no	no	no
	047	ant-accessible plantlet		no	no	✓	no	no	no
	041	ant-excluded plantlet		no	✓*cont.	no	no	no	no

Supplementary Table S3.2 Primers and molecular identifiers used in the study

Primers for high-throughput sequencing	
ITS1Fngs	GGTCATTTAGAGGAAGTAA
ITS4ngsUni	CCTCCSCTTANTDATATGC

Multiplex Identifiers (MID)

ACGAGTGCGT	ACTAGCAGTA	AGTGTATGTC	AGTCTGACTGA
ACGCTCGACA	AGTATACATA	ATAGATAGAC	AGTGAGCTCGA
AGACGCACTC	AGTCGAGAGA	ATATAGTCGC	ATAGCTCTCGA
ACTCGCGTGTC	AGTGCTACGA	ATCTACTGAC	ATCACGTGCGA
ATGATACGTCT	ACGATCGTATA	ACACGTAGATC	ATCGTAGCAGA
ACGAGAGATAC	ACGCAGTACGA	ACACGTGTCGC	ATCGTCTGTGA
ATACGACGTA	ACGCGTATACA	ACATACTCTAC	ATGTGTCTAGA
ATCACGTACTA	ACGTACAGTCA	ACGACACTATC	
ACGTCTAGTAC	ACGTACTCAGA	ACGAGACGCGC	
ATGTACTACTC	ACTATAGCGTA	ACGTATGCGAC	
ACATACGCGT	ATACGTCATCA	ACGTCGATCTC	
ACTACTATGT	ATAGTCGCATA	ACTAGTCACTC	
ACTGTACAGT	ATATATATACA	ACTCTACGCTC	
AGACTATACT	ATATGCTAGTA	ACTGTACATAC	
AGTACGCTAT	ATCACGCGAGA	ATAGACTGCAC	
ATAGAGTACT	ATCGATAGTGA	ATAGCTCTATC	
ACACGCTACGT	ATCGCTGCGTA	ATATAGACATC	
ACAGTAGACGT	ATCTGACGTCA	ATATGATACGC	
ACGACGTGACT	ATGAGTCAGTA	ATCACTCATAC	
ATACACACACT	ATGTAGTGTGA	ATCATCGAGTC	
ATACACGTGAT	ATGTCACACGA	ATCGAGCTCTC	
ATACAGATCGT	ATGTCGTCGCA	ATCGCAGACAC	
ATACGCTGTCT	ACACATACGC	ATCTGTCTCGC	
ATAGTGTAGAT	ACAGTCGTGC	ATGAGTGACGC	
ATCGATCACGT	ACGACAGCTC	ATGATGTGTAC	
ATCGCACTAGT	ACGTCTCATC	ATGCTATAGAC	
ATCTAGCGACT	ACTCATCTAC	ATGCTCGCTAC	
ATCTATACTAT	ACTCGCGCAC	ACGTGCAGCGA	
ATGACGTATGT	AGAGCGTCAC	ACTCACAGAGA	
ATGTGAGTAGT	AGCGACTAGC	AGACTCAGCGA	
AACAGTATATA	AGTAGTGATC	AGAGAGTGTGA	
ACGCGATCGA	AGTGACACAC	AGCTATCGCGA	

Supplementary Table S3.3 Taxonomic assignment of 287 fungal operational taxonomic units (OTUs) in the chambers of *Myrmecodia beccarii* ant-plants (218 OTUs) and/or *Philidris. cordata* ant worker samples (127 OTUs) at the end of the experiment. The chamber sample OTUs have a minimum read abundance of 10 in at least one of the plant types (mature ant-plants, ant-accessible plantlets, ant-excluded plantlets) and include waste (WC), nursery (NC), and ventilation (VC) chambers. The samples collected from ant workers have a minimum read abundance of 2 and include exoskeletons (EXO), heads (HEAD), and abdomens (ABD). Fungal taxon and Order are closest taxonomic match using UNITE database and the APSurvey database from an ant-plant survey (chapter 2; Greenfield et al. 2021). If OTU had a match >96% to an OTU found during ant-plant survey, the OTU ID from that survey appears under the column headed "APSurvey OTU ID" together with the percentage sequence similarity. The UNITE SH number and Genbank Accession numbers are included as well as the percentage sequence similarity. Total sequence abundances are provided separately for plant and ant samples. OTU IDs marked with * represent 13 fungal OTUs found to be significantly abundant in contrasts between the ant-accessible and ant-excluded plantlets (Supplementary Table S3.5). Fungal OTU IDs and their raw abundances are in bold if they were dominant (i.e., occurring in at least 50% of mature ant-plants, or 20% in ant-accessible or ant-excluded plantlets (41 OTUs), or in 20% of ant samples (29 OTUs). If an OTU was dominant in both plant and ant samples, the OTU ID is also italicised. Shading of rows is for the purpose of clarity.

OTU ID	Fungal Taxon	Order	APSurvey OTU ID & sequence similarity	UNITE SH number	GenBank Accession number	seq similarity (%)	total abundance in plants	total abundance in ants	Raw abundances in samples from:											
									mature ant-plants			ant-accessible plantlets			ant-excluded plantlets			ant workers		
									WC	NC	VC	WC	NC	VC	WC	NC	VC	EXOSK	HEAD	ABD
OTU0035	*Malassezia globosa*	Malasseziales		SH1565715.08FU	KM454161	98	0	4											1	3
OTU0036	*Malassezia* sp.	Malasseziales		SH1515384.08FU	KT334699	99	0	410										**141**	**132**	**137**
OTU0037	*Malassezia globosa*	Malasseziales		SH1565714.08FU	JN882325	99	3	3		1				1	1				3	
OTU0038	*Moesziomyces aphidis*	Ustilaginales		SH1509407.08FU	KY037841	99	127	15	4			3	11	29			**80**		4	11
OTU0044	*Pseudozyma hubeiensis*	Ustilaginales		SH1145432.08FU	KY806117	99	15	0	2		1		1	10			1			
OTU0051	*Malassezia slooffiae*	Malasseziales		SH1519721.08FU	KY104167	99	0	43										43		
OTU0052	*Malassezia* sp.	Malasseziales		SH1565716.08FU	AM901732	99	0	6											6	
OTU0054	*Verruconis* sp.	Venturiales		SH1549842.08FU	LM644533	94	66	0				16	4	7	30	8	1			
OTU0056	*Hanseniaspora thailandica*	Saccharomycetales		SH1547319.08FU	KY580387	99	0	8										8		
OTU0057	*Verruconis gallopava*	Venturiales		SH1549842.08FU	LM644533	99	483	0				68	43	24	**249**	**67**	**32**			
OTU0059	Fungi sp.	unknown		SH211882.07FU	HQ611279	98	19	0		1	18									
OTU0066	Agaricales sp.	Agaricales		SH1155222.08FU	GU256961	95	29	0								25	4			

OTU ID	Fungal Taxon	Order	APSurvey OTU ID & sequence similarity	UNITE SH number	GenBank Accession number	seq similarity (%)	total abundance in plants	total abundance in ants	Raw abundances in samples from:											
									mature ant-plants			ant-accessible plantlets			ant-excluded plantlets			ant workers		
									WC	NC	VC	WC	NC	VC	WC	NC	VC	EXOSK	HEAD	ABD
OTU0089*	Fungi sp.	unknown		SH491217.07FU	KU195499	100	22772	146	**20157**	**1191**	272	**1095**	55		2			30	**116**	
OTU0099	*Ochroconis musae*	Venturiales		SH1549545.08FU	KT272078	96	0	6											6	
OTU0106	*Rhytidhysteron* sp.	Hysteriales		SH1569504.08FU	KX960807	99	33	0			25	4		2	1	1				
OTU0109	*Rhodotorula* sp.	Sporidiobolales		SH1528261.08FU	FM957563	98	30	0				1	1	15			13			
OTU0112	Agaricomycetes sp.	unknown		SH1185637.08FU	HM037681	92	1856	0				932	538	225	161					
OTU0116	Clavulinaceae sp.	Cantharellales		SH1517446.08FU	KT779284	97	48	0								48				
OTU0117	Agaricomycetes sp.	unknown		SH1185637.08FU	HM037681	94	179	0							138	8	33			
OTU0129	*Amplistroma* sp.	Hypocreales		SH1238250.08FU	KC907376	91	23	0		23										
OTU0130	Fungi sp.	unknown		SH193251.07FU	KP889575	99	21	0	1	1	19									
OTU0137	Basidiomycota	unknown		SH016338.07FU	AB907585	99	13	0	10		3									
OTU0141	*Gerhardtia* sp.	Agaricales		SH1186681.08FU	KP012736	90	0	6											6	
OTU0142	Sebacinaceae sp.	Sebacinales		SH1551358.08FU	JX317317	94	23	0				1	1	4		13	4			
OTU0145	*Sebacina* sp.	Sebacinales		SH1176700.08FU	AB831798	90	101	0					8	93						
OTU0152	*Hasegawazyma lactosa*	Erythrobasidiales		SH1514516.08FU	FJ515208	99	94	0				6	28	10	4		46			
OTU0163	*Ganoderma australe*	Polyporales		SH1555690.08FU	KJ654550	98	20	0	1			7	6		2	3	1			
OTU0166	*Ganoderma* sp.	Polyporales		SH1555638.08FU	KY471677	98	18	0				17	1							
OTU0168	Fungi sp.	unknown		SH1506973.08FU	KR015395	99	0	4											4	
OTU0169*	Eurotiales sp.	Eurotiales		SH523442.07FU	AB986366	98	2195	23	4	57	5	2	**2108**	19				22	1	
OTU0174	*Sterigmatomyces halophilus*	Agaricostilbales		SH1546026.08FU	KY792630	99	30	3	4		2	6	15	3				3		
OTU0175	*Colacogloea terpenoidalis*	Microbotryomycetes OIS		SH1522429.08FU	KY102579	99	86	0	11			34	21	15	5					
OTU0179	Serendipitaceae sp.	Sebacinales		SH1555098.08FU	JX317218	90	24	0				16	8							

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OTU0183	*Sterigmatomyces elviae*	Agaricostilbales		SH1522694.08FU	AB038054	84	0	10											10	
OTU0184	*Ganoderma fornicatum*	Polyporales		SH1555646.08FU	JX840347	99	13	0			2		11							
OTU0186	Sebacinales sp.	Sebacinales			JF691010	87	1812	0							97	1174	541			
OTU0188	*Malassezia sympodialis*	Malasseziales		SH1553528.08FU	AY743645	94	0	6											6	
OTU0193	Chaetothyriales sp.	Chaetothyriales		SH014776.07FU	HQ634633	99	63	0	7	5	5	46								
OTU0197	*Abundisporus pubertatis*	Polyporales		SH1565950.08FU	KC787566	88	3	3		1				2					3	
OTU0201	Dothideomycetes sp.	unknown		SH1523711.08FU	JQ760123	88	0	13											13	
OTU0202	*Rigidoporus vinctus*	Polyporales		SH1610876.08FU	MF169503	99	0	2												2
OTU0206	Exobasidiomycetidae sp.	Exobasidiomycetidae OIS		SH188444.07FU	KF435880	99	26	48		6	16		1	3				**32**	16	
OTU0222	Fungi sp.	unknown		SH1551238.08FU	KT328661	99	6	4		1				2		2	1	4		
OTU0231	*Fonsecaea* sp.	Chaetothyriales		SH1529604.08FU	KF928456	99	344	2		7	2	49		8	**114**	160	4		2	
OTU0235	*Schwanniomyces polymorphus*	Saccharomycetales		SH1516577.08FU	KY105394	99	110	60	19	23	2	59	6	1				**51**	9	
OTU0237	*Sistotremastrum guttuliferum*	Trechisporales		SH1172756.08FU	GQ999454	98	269	0					269							
OTU0240	Fungi sp.	unknown		SH1191583.08FU	KR016504	98	0	17											17	
OTU0241	*Meira* sp.	Exobasidiales		SH1649628.08FU	KF435880	96	75	18	1		9	5	12	37	2		9	**10**	8	
OTU0242	*Meira argovae*	Exobasidiales		SH1649628.08FU	KY437699	92	2	4			2							2	2	
OTU0247	Tremellales sp.	Tremellales		SH1245523.08FU	HG938309	81	29	0				27	1	1						
OTU0254	*Loweporus tephroporus*	Polyporales		SH1555634.08FU	MH634493	99	11	0			1	2	8							

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OTU0257	*Cyphellophora oxyspora*	Chaetothyriales		SH1573966.08FU	KY792629	99	551	0			1	2	142	146	7	7	**246**			
OTU0260	*Malassezia dermatis*	Malasseziales		SH1553524.08FU	KP825483	100	0	16										10	1	5
OTU0266	Malasseziales sp.	Malasseziales		SH1547563.08FU	KY322606	99	4	173		1			2	1				149	20	4
OTU0267	Peniophoraceae sp.	Russulales		SH1646404.08FU	EU593550	99	0	66											66	
OTU0276	*Fonsecaea brasiliensis*	Chaetothyriales		SH1529592.08FU	KP132190	99	40	0				17	5	4	8	1	5			
OTU0277	*Cladophialophora immunda*	Chaetothyriales		SH1529600.08FU	KC886406	100	94	0				36	9	13	26	9	1			
OTU0281*	*Candida fluviatilis*	Saccharomycetales	OTU0171 (98%)	SH200664.07FU	HQ652068	99	1084	644	**900**	22	4	59	92	7				**337**	**307**	
OTU0284	Fungi sp.	unknown		SH1561396.08FU	KF800665	100	0	17											17	
OTU0296*	Chaetothyriales sp.	Chaetothyriales	OTU0202 (99%)	SH196444.07FU	HQ634649	96	8400	10	**2212**	31	402	**5688**	57	10					10	
OTU0299*	Fungi sp.	unknown		SH491217.07FU	KU195499	97	1089	16	**956**	57	9	60	6	1					**16**	
OTU0302	*Peltigera canina*	Peltigerales		SH1569986.08FU	LT852809	99	0	19										19		
OTU0309	*Sporothrix eucalyptigena*	Ophiostomatales		SH1552347.08FU	KU865588	99	626	0	338	155	102	27	3	1						
OTU0312	Orbiliaceae sp.	Orbiliales		SH1142967.08FU	LC146730	85	17	0			17									
OTU0320	*Rhinocladiella similis*	Chaetothyriales		SH1575246.08FU	KY780281	97	122	2		1	1	44	5	9		56	6	2		
OTU0321	Fungi sp.	unknown		SH1161897.08FU	KT758121	99	0	14												14
OTU0324	Agaricomycetes sp.	unknown		SH1571979.08FU	UDB036216	78	91	0				1		87			3			
OTU0338	*Trichoderma harzianum*	Hypocreales		SH1567965.08FU	KX092003	99	850	2	4		3	**415**	1	1	**424**		2	2		
OTU0339	Chaetothyriales sp.	Chaetothyriales		SH1563587.08FU	EU624333	84	22	0	22											
OTU0341	Herpotrichiellaceae sp.	Chaetothyriales		SH497366.07FU	LC128795	99	20	0				4			4	11	1			

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OTU0345	Fungi sp.	unknown		SH1555632.08FU	KR015297	99	0	5											5	
OTU0349	*Exophiala spinifera*	Chaetothyriales		SH1575245.08FU	MH010935	99	18	0					8	7		2	1			
OTU0361	*Cyphellophora guyanensis*	Chaetothyriales		SH1636098.08FU	KC455240	97	83	0	1	10	72									
OTU0362	*Rhodotorula mucilaginosa*	Sporidiobolales		SH1558726.08FU	KY611842	99	37	551	26	1		8	2					512	**39**	
OTU0365	Fungi sp.	unknown		SH1567297.08FU	KU580407	88	42	0		42										
OTU0366	Debaryomycetaceae sp.	Saccharomycetales		SH192552.07FU	KP109748	96	3428	2	**3399**	4	13	9				2	1	2		
OTU0370	Tubulicrinaceae sp.	Hymenochaetales		SH1245737.08FU	KU659609	86	16	0		16										
OTU0374	*Phaeomoniella* sp.	Chaetothyriales	OTU0278 (96%)	SH197744.07FU	GQ999270	99	14	0			14									
OTU0376	Fungi sp	unknown		SH1173553.08FU	KF675434	89	0	3											3	
OTU0377	*Spencermartinsiella* sp.	Saccharomycetales		SH1550041.08FU	KY105461	92	0	9										9		
OTU0379*	*Arthrocladium* sp.	Pezizomycotina_OIS	OTU0289 (98%)	SH208969.07FU	KT337442	99	1877	0	26	43	38	**1723**	41	6						
OTU0380	Debaryomycetaceae sp.	Saccharomycetales		SH192552.07FU	KP109748	97	267	0	184		2	74	7							
OTU0386	Elsinoaceae sp.	Myriangiales		SH1214444.08FU	KX887231	85	44	0				3	36	1	1		3			
OTU0388	Capnodiales sp.	Capnodiales		SH012378.07FU	AY970168	98	155	0		4	150				1					
OTU0391	Saccharomycetales sp.	Saccharomycetales		SH192555.07FU	AY553844	97	1774	0	**1773**		1									
OTU0400	Fungi sp.	unknown	OTU0291 (99%)	SH491823.07FU	KM104119	99	1414	102	26	280	1106			2				77	**25**	
OTU0407	*Phaeoacremonium parasiticum*	Togniniales		SH1581104.08FU	LC203479	99	67	0				6	39	9	11	2				

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OTU0410	Fungi sp.	unknown		SH479386.07FU	AJ877193	99	40	0	39			1								
OTU0417	*Trichoderma atroviride*	Hypocreales		SH1567966.08FU	MF541418	99	5221	0	11	8	8	**2213**	2396	96	**381**	90	18			
OTU0419	Eurotiales sp.	Eurotiales		SH523442.07FU	AB986366	99	938	3	9	632	297								3	
OTU0421	Xylariales sp.	Xylariales		SH1149881.08FU	KX096659	88	14	0						10	3	1				
OTU0422*	Eurotiomycetes sp.	unknown		SH206547.07FU	DQ914677	98	11971	26	**8535**	57	607	**2691**	64	13	3	1		4	21	1
OTU0423	Talaromyces sp.	Eurotiales		SH1516169.08FU	FJ491803	95	54	0			54									
OTU0430	*Resinicium saccharicola*	Hymenochaetales		SH1509371.08FU	GU054177	99	2607	0					1992			615				
OTU0433	*Lecanorales* sp.	Lecanorales		SH1515811.08FU	AY969753	80	0	9										9		
OTU0435	*Meyerozyma caribbica*	Saccharomycetales		SH1516570.08FU	MG871185	100	5	72	2		1		2						72	
OTU0436*	Eurotiales sp.	Eurotiales	OTU0267 (96%)	SH523442.07FU	AB986366	99	3955	4	8	**1425**	**572**	8	**445**	**1497**					4	
OTU0438	Eurotiales sp.	Eurotiales	OTU0293 (96%)	SH523442.07FU	AB986366	95	30	0						1			29			
OTU0443	*Phaeoacremonium venezuelense*	Togniniales		SH1581109.08FU	KF764568	99	59	0				28	1	1	18	9	2			
OTU0446	*Acremonium polychromum*	Hypocreales		SH1555961.08FU	MH469511	99	108	31	11	2	9		86					1	30	
OTU0448	Eurotiales sp.	Eurotiales		SH523442.07FU	AB986366	99	388	0	7	**377**	4									
OTU0450	Fungi sp.	unknown		SH479386.07FU	AJ877193	99	2087	14	**2076**	1	5	3	1			1		14		
OTU0454	*Metacordyceps* sp.	Hypocreales		SH1644900.08FU	JX978429	94	43	0	26	6	1	3		7						

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OTU0462	Sporobolomyces sp.	Sporidiobolales		SH1575126.08FU	KY436087	99	0	2												2
OTU0465	*Stypella variabilis*	Auriculariales		SH1211934.08FU	UDB035272	91	0	25												25
OTU0468	*Talaromyces radicus*	Eurotiales		SH1516156.08FU	MH298756	99	35	0	18	7	7			3						
OTU0470	Fungi sp.	unknown		SH479386.07FU	AJ877193	99	68	0	67		1									
OTU0471*	Talaromyces sp.	Eurotiales		SH1552152.08FU	MH784402	99	28303	130	**719**	**3589**	**844**	**3070**	**5692**	**1394**	**5337**	7650	8	**24**	**93**	13
OTU0477	Fungi sp.	unknown		SH1182330.08FU	KT328742	87	0	8										8		
OTU0478	Auriculariales sp.	Auriculariales		SH1246437.08FU	KF800127	84	0	5											5	
OTU0482	Phaeomoniellales sp.	Phaeomoniellales		SH1538725.08FU	KF675294	89	36	0				14	6	11	3		2			
OTU0493	*Talaromyces diversus*	Eurotiales	OTU0376 (98%)	SH204611.07FU	KJ775712	99	48	0	4	10	1				1	32				
OTU0498	*Aspergillus* sp.	Eurotiales		SH1649119.08FU	EF652038	98	0	8										8		
OTU0507	*Aspergillus flavus*	Eurotiales		SH1161994.08FU	KY065346	99	499	20	229	173	25	27	41	3			1	20		
OTU0509*	Chaetothyriales sp.	Chaetothyriales	OTU0347 (96%)	SH212029.07FU	KC951221	99	21390	225	**187**	**14180**	**6903**	**88**	23	6	3			**121**	**104**	
OTU0512	*Resinicium friabile*	Hymenochaetales		SH1509370.08FU	KR015420	99	19	0					19							
OTU0513	*Dactylella oxyspora*	Orbiliales		SH1511060.08FU	DQ494356	94	0	34										**34**		
OTU0514	*Leptospora rubella*	Pleosporales		SH1595853.08FU	KX757835	99	0	15												15
OTU0515	Chaetothyriales sp.	Chaetothyriales		SH1648673.08FU	KX822513	98	49	0	4	33		12								
OTU0524	Eurotiomycetes sp.	unknown		SH1578170.08FU	KX908623	79	30	0			30									
OTU0533	*Penicillium daleae*	Eurotiales		SH1160518.08FU	KY315582	97	242	0	105	57	33	41	2	1	1	2				
OTU0545	*Occultifur* sp.	Cystobasidiales		SH1613466.08FU	KF675690	99	2	13	1								1	13		
OTU0548	Fungi sp.	unknown		SH1526400.08FU	MG016006	99	65	0	2		1	8	47	2	3		2			
OTU0550	Eurotiomycetes sp.	unknown			KX908623	98	4871	31	6	**1629**	**3226**	9	1					31		
OTU0563	Dothideales sp.	Dothideales		SH1515068.08FU	FJ037769	99	0	5												5
OTU0567	*Occultifur* sp.	Cystobasidiales		SH1613485.08FU	KM527117	98	5	33				1	1				3	19		14

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OTU0573	Fungi sp.	unknown		SH1516144.08FU	KU837575	99	5033	0	4	38	14	**63**	**214**	**870**	**163**	**2072**	**1595**			
OTU0577	Myriangiaceae sp.	Myriangiales		SH1214634.08FU	AY843169	86	47	0			47									
OTU0587	Exobasidiomycetes sp.	unknown		SH198249.07FU	JX575187	99	80	69		7	72	1						**43**	**16**	10
OTU0589	Conioscyphales sp.	Conioscyphales		SH1179227.08FU	GQ924027	93	162	0				5	20	33	100	2	2			
OTU0591	*Penicillium adametzii*	Eurotiales		SH1160531.08FU	JN714932	99	42	0		7	26	1	7	1						
OTU0603	Eurotiomycetes	unknown			KX908623	96	13	0			13									
OTU0604	*Metapochonia* sp.	Hypocreales		SH1244656.08FU	KX426574	92	21	0			21									
OTU0605	*Cystobasidium calyptogenae*	Cystobasidiales		SH1613462.08FU	KY103129	99	20	0				1		4	1	2	12			
OTU0608	Stachybotryaceae sp.	Hypocreales		SH1557966.08FU	KJ834351	99	146	0	11				135							
OTU0611	Eurotiomycetes sp.	unknown			KX908225	99	57	0	4	16	37									
OTU0620	*Penicillium consobrinum*	Eurotiales		SH1529989.08FU	MG490873	99	646	0	36	284	317	3	1	5						
OTU0623	Monocillium sp.	Hypocreales		SH1170580.08FU	MG826938	93	77	0	3	53	7	3	11							
OTU0624	*Phaeomoniella* sp.	Chaetothyriales	OTU0477 (96%)	SH015548.07FU	JN225891	100	130	0		2	128									
OTU0629	*Dendryphiella paravinosa*	Pleosporales		SH1573931.08FU	LT963357	92	0	15											13	2
OTU0631	Conioscyphales sp.	Conioscyphales		SH1179227.08FU	GQ924027	95	44	0			12	17	11	4						
OTU0634	Chaetothyriales sp.	Chaetothyriales	OTU0468 (96%)	SH029055.07FU	JX014388	99	1867	0	6	92	**1765**			2			2			
OTU0640	*Penicillium* sp.	Eurotiales		SH1163579.08FU	KY445779	99	69	0	6	3	54	6								
OTU0642	Talaromyces sp.	Eurotiales		SH1516144.08FU	KF366489	100	30	0		13	3	6	3		2	3				
OTU0647	Talaromyces sp.	Eurotiales		SH1516144.08FU	KF366489	97	4414	0	82	2953	1365	4	4	1	1	3	1			
OTU0652	*Rhodotorula* sp.	Sporidiobolales		SH1182329.08FU	KU057818	94	19	0	18		1									

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OTU0655	Talaromyces sp.	Eurotiales		SH1516149.08FU	KU866647	98	24	0					2	22						
OTU0658	Chaetothyriales sp.	Chaetothyriales		SH014029.07FU	KF675595	99	339	0	218	42	69	4	6							
OTU0675	Basidiomycota sp.	unidentified Basidiomycota		SH029161.07FU	JX999053	99	233	6		2	231							6		
OTU0682	Talaromyces sp.	Eurotiales		SH1516144.08FU	KY474345	99	28	0				15		13						
OTU0683	Capnodiales sp.	Capnodiales		SH1175713.08FU	KT329205	98	0	208												208
OTU0684	Fungi sp.	unknown		SH1233882.08FU	KY496842	100	20	0				1	19							
OTU0685	Chaetothyriales sp.	Chaetothyriales		SH212163.07FU	HM239979	98	3386	0	**3339**	8	22	16				1				
OTU0689	*Jattaea aurea*	Calosphaeriales	OTU0502 (99%)	SH206445.07FU	KT716462	99	133	0		36	97									
OTU0707	*Phaeophleospora hymenocallidicola*	Capnodiales		SH1606643.08FU	KR476739	99	11	5				2	1	6	1		1		5	
OTU0710	*Coniochaeta* sp.	Coniochaetales		SH1645099.08FU	KJ583241	99	108	0					1		104		3			
OTU0713	Xylariaceae sp	Xylariales		SH1237656.08FU	AB741616	94	0	5											5	
OTU0714	Hypocreales sp.	Hypocreales		SH492807.07FU	KF534505	99	20	0	2	2	16									
OTU0718	*Neodevriesia* sp.	Capnodiales		SH1549747.08FU	MG545073	98	17	0				3	3	5	6					
OTU0722	Eurotiomycetes sp.	unknown		SH464476.07FU	FJ475738	99	14	9	7	1	6							9		
OTU0723	*Neodevriesia* sp.	Capnodiales		SH1549728.08FU	KJ740813	95	12	0					6	4			2			
OTU0733	*Neodevriesia* sp.	Capnodiales		SH1549728.08FU	GQ999499	99	45	7	1			3	7	27	1	3	3		7	
OTU0737	*Fusarium* sp.	unknown	OTU0544 (99%)	SH1209625.08FU	KX247125	98	2960	45	73	**611**	**2253**	2	8	13				**31**	**14**	
OTU0738	*Symbiotaphrina* sp.	Symbiotaphrinales		SH1649916.08FU	KC215110	95	14	0	1	9	2	1	1							
OTU0749	*Wallemia mellicola*	Wallemiales		SH1605560.08FU	KJ494616	99	13	75	1	5				6	1			**66**	1	8

OTU ID	Fungal Taxon	Order	APSurvey OTU ID & sequence similarity	UNITE SH number	GenBank Accession number	seq similarity (%)	total abundance in plants	total abundance in ants	Raw abundances in samples from:											
									mature ant-plants			ant-accessible plantlets			ant-excluded plantlets			ant workers		
									WC	NC	VC	WC	NC	VC	WC	NC	VC	EXOSK	HEAD	ABD
OTU0750	Fungi sp.	unknown		SH208682.07FU	KP889786	99	24	0	1	13	10									
OTU0751	*Acremonium* sp.	Hypocreales		SH1209356.08FU	HQ608111	91	24	0		24										
OTU0752	Dothideomycetes sp.	unknown	OTU0567 (97%)	SH021234.07FU	EU977184	97	11245	2	50	**7912**	**3277**		1				5	2		
OTU0753	*Fusarium solani*	Hypocreales	OTU0619 (98%)	SH205225.07FU	AM412643	99	228	138	60	12	6	1	149					14	124	
OTU0754	Fungi sp.	unknown		SH204253.07FU	KF675628	85	24	0	24											
OTU0759	Fungi sp.	unknown	OTU0610 (99%)	SH218992.07FU	KJ023736	99	65	45	2	8	49	1	4	1				**38**	1	6
OTU0761	*Scytalidium* sp.	Helotiales		SH1185415.08FU	HM134143	99	209	0				134		1	74					
OTU0762	Fungi sp	unknown		SH1560628.08FU	KY038596	99	0	4										4		
OTU0766	Conioscyphales sp.	Conioscyphales		SH1179227.08FU	GQ924027	87	56	2	1		1	6	43	5				2		
OTU0767	*Cryptodiscus* sp.	Ostropales		SH1514193.08FU	AJ877197	99	1641	0		1541	99		1							
OTU0770	*Clonostachys* sp.	Hypocreales		SH1522825.08FU	KY413687	100	6	12	1				3	1	1				12	
OTU0772	Hypocreales sp.	Hypocreales		SH1560529.08FU	KY379587	99	0	7										7		
OTU0777	*Fusarium solani*	Hypocreales		SH1546322.08FU	KF918557	99	28	46	5	1			21	1					46	
OTU0779	*Scytalidium lignicola*	Helotiales		SH1185468.08FU	HM214453	99	68	0				66				2				
OTU0812	*Rhinocladiella indica*	Chaetothyriales		SH1552278.08FU	UDB035057	99	42	0						38	2		2			
OTU0815	Hyaloscyphaceae sp.	Helotiales	OTU0658 (97%)	SH488999.07FU	KT270007	99	123	0		15	94		1	13						
OTU0828	Tremellomycetes sp.	unknown		SH025808.07FU	KF617649	99	16	0		3	11			2						
OTU0834	Fungi sp.	unknown		SH204253.07FU	KF675628	99	100	0			7				93					
OTU0835	*Mariannaea* sp.	Hypocreales		SH1506691.08FU	MH734517	99	1356	0	1			**576**	121	29	**424**	160	45			

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									WC	NC	VC	WC	NC	VC	WC	NC	VC	EXOSK	HEAD	ABD
OTU0836	*Pezicula radicicola*	Helotiales	OTU0667 (97%)	SH201622.07FU	HQ889715	99	5276	0	68	**1970**	**3238**									
OTU0837	Tremellomycetes	unknown		SH203621.07FU	KF225912	99	322	0				270	1	11	15	24	1			
OTU0845	*Chloridium* sp.	Chaetosphaeriales		SH1517983.08FU	AB734790	97	2671	0				315	1022	20	135	710	469			
OTU0846	Helotiales sp.	Helotiales		SH1236101.08FU	GQ892249	95	23	0				3	7		5	6	2			
OTU0847	Ascomycota sp.	unknown		SH1150020.08FU	KU550122	87	25	0				1		23	1					
OTU0849	Pucciniomycetes sp.	unknown		SH472715.07FU	KJ832014	98	188	0		1	187									
OTU0850	*Proliferodiscus* sp.	Helotiales	OTU0654 (97%)	SH196496.07FU	JN033427	99	1044	0		24	**1018**		1	1						
OTU0851	Fungi sp.	unknown		SH204253.07FU	KF675628	95	34	0			29				5					
OTU0855	Ascomycota sp.	unknown		SH1231841.08FU	LC143823	89	11	0				1				1	9			
OTU0856	Tremellales sp.	Tremellales		SH525820.07FU	KM079158	99	0	16										16		
OTU0862	*Gliocladium* sp.	Hypocreales		SH1644389.08FU	DQ682585	99	146	576	37		1		108					**123**	452	1
OTU0868	*Toxicocladosporium rubrigenum*	Capnodiales		SH1572798.08FU	FJ790285	98	0	7										7		
OTU0869	Fungi sp.	unknown		SH1506424.08FU	MF971291	84	0	12											12	
OTU0873	Ascomycota sp.	unknown			KU687400	89	2327	0	214	7	3	1	2039	63						
OTU0883	Sordariales sp.	Sordariales		SH1563553.08FU	AB986406	89	23	0							16	1	6			
OTU0885	*Nigrospora* sp.	Trichosphaeriales		SH1549606.08FU	MG976425	99	167	0	8	1	2	72	61	6	4		13			
OTU0887	*Cryptococcus* sp.	Tremellales	OTU0708 (99%)	SH205048.07FU	JN581131	99	12	0		1	1						10			
OTU0893	*Crucellisporiopsis* sp.	Helotiales		SH1565365.08FU	KC978015	100	106	0			106									
OTU0894	Exobasidiales sp.	Exobasidiales		SH1239966.08FU	FJ357790	87	20	0				1	9	9	1					

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									WC	NC	VC	WC	NC	VC	WC	NC	VC	EXOSK	HEAD	ABD
OTU0898	*Toxicocladosporium strelitziae*	Capnodiales		SH1572794.08FU	KU255041	98	10	117				5	3	2					**102**	15
OTU0900	*Nigrospora oryzae*	Trichosphaeriales		SH1549605.08FU	MG832530	99	216	228	12	6	10	67	36	39		6	40		12	216
OTU0902	Fungi sp.	unknown		SH521400.07FU	KU580767	99	160	0		17	143									
OTU0903	*Sarocladium* sp.	Hypocreales		SH1569895.08FU	KT328719	99	0	28												28
OTU0905	*Nigrospora oryzae*	Trichosphaeriales		SH1549605.08FU	MG489868	98	6	3	1			3				1	1			3
OTU0906	Fungi sp.	unknown		SH204253.07FU	KF675628	95	19	0			19									
OTU0920	Pleosporales sp.	Pleosporales		SH1614491.08FU	KM516291	100	0	4										4		
OTU0923	*Cladosporium sphaerospermum*	Capnodiales		SH1572792.08FU	KF177679	99	39	56	8	1		16	11	2			1	10	**43**	3
OTU0924	*Cladosporium sphaerospermum*	Capnodiales		SH1572792.08FU	MF467891	99	30	78	4	1		5	9	6	2		3	10	**43**	**25**
OTU0926	*Aspergillus pseudoglaucus*	Eurotiales		SH1572953.08FU	LC317577	99	22	0	1				21							
OTU0930	*Penicillium* sp.	Eurotiales		SH1529986.08FU	KF367520	100	251	8	72	16	38	46	69	10				8		
OTU0934	Fungi sp.	unknown		SH495907.07FU	KM104100	99	27	0			27									
OTU0946	*Cladosporium aciculare*	Capnodiales		SH1572792.08FU	KT600411	99	198	175	26	3	24	22	57	39	16	2	9	**119**	**18**	38
OTU0948	*Phialemoniopsis* sp.	Xylariales		SH1541595.08FU	JQ070570	100	0	2										2		
OTU0951	Exobasidiales sp.	Exobasidiales			KP730059	99	2664	104	7	281	**2370**	2		4				**97**	7	
OTU0957	*Cladosporium oxysporum*	Capnodiales		SH1572792.08FU	MG063185	99	57	46	4	2	6	14	13	3	2	1	12	**29**	**3**	14
OTU0958	*Devriesia* sp.	Capnodiales	OTU0707 (99%)	SH019575.07FU	JQ905788	97	133	0		1	129						3			

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									WC	NC	VC	WC	NC	VC	WC	NC	VC	EXOSK	HEAD	ABD
OTU0959	*Fusarium oxysporum*	Hypocreales		SH1610159.08FU	MH333106	95	0	65										**65**		
OTU0965	Fungi sp.	unknown		SH521400.07FU	KU581110	99	14	0		2	12									
OTU0972	*Neopestalotiopsis* sp.	Xylariales		SH1552672.08FU	KY463476	100	32	0	4	3	1	7	1	4	6	3	3			
OTU0974	Fungi sp.	unknown	OTU0783 (96%)	SH025821.07FU	KC222753	99	1141	22	4	43	**1090**	1	1	2				**22**		
OTU0975	Dothideomycetes sp.	unknown		SH200715.07FU	KM519295	99	129	0			129									
OTU0976	Fungi sp.	unknown			KX908745	99	53	0			53									
OTU0978	*Corynelia uberata*	Coryneliales		SH1517323.08FU	KU204605	90	0	12										12		
OTU0986	*Devriesia* sp.	Capnodiales	OTU0763 (99%)	SH019575.07FU	JQ905788	99	14	0			13	1								
OTU0987	Fungi sp.	unknown		SH025821.07FU	KC222753	96	196	0	1		195									
OTU0993	*Zygosporium* sp.	Xylariales		SH1507866.08FU	MF621585	100	0	5											5	
OTU0995	Fungi sp.	unknown		SH1176541.08FU	KC222753	99	970	0		11	**959**									
OTU1006	Capnodiales sp.	Capnodiales		SH019572.07FU	JN890104	99	23	0		6	17									
OTU1010	Fungi sp.	unknown		SH1514197.08FU	KF675509	96	4771	0					2629	**365**		621	**1156**			
OTU1021*	Fungi sp.	unknown		SH210983.07FU	KF675509	98	13388	0				7	**3778**	**5774**			**3829**			
OTU1025	*Fusarium* sp.	Hypocreales		SH1560532.08FU	JQ905675	98	50	0	3	36	11									
OTU1026	*Candida* sp.	Saccharomycetales	OTU0821 (96%)	SH203686.07FU	JQ683772	98	2437	11	2218	13	4	3	199					11		
OTU1029	*Antennariella placitae*	Capnodiales		SH1549156.08FU	MH250008	99	3	172			3							172		

OTU ID	Fungal Taxon	Order	APSurvey OTU ID & sequence similarity	UNITE SH number	GenBank Accession number	seq similarity (%)	total abundance in plants	total abundance in ants	Raw abundances in samples from:											
									mature ant-plants			ant-accessible plantlets			ant-excluded plantlets			ant workers		
									WC	NC	VC	WC	NC	VC	WC	NC	VC	EXOSK	HEAD	ABD
OTU1035	*Papiliotrema laurentii*	Tremellales		SH1576892.08FU	KY445944	99	1118	0	4			**864**	91	33	26	31	69			
OTU1036	Fungi sp.	unknown		SH025821.07FU	KC222753	99	371	0		28	342			1						
OTU1038	*Fellomyces* sp.	Tremellales		SH212023.07FU	NR_073234	99	14	13		5	8			1				13		
OTU1039	*Acaromyces ingoldii*	Exobasidiales		SH1562717.08FU	KR909167	100	44	29	3	2	30	2	1	5			1	29		
OTU1040	Fungi sp.	unknown		SH495907.07FU	KM104100	96	18	0		1	17									
OTU1041	Fungi sp.	unknown		SH025821.07FU	KC222753	99	24	0	1		23									
OTU1044	Capnodiales sp.	Capnodiales		SH025821.07FU	JQ760724	96	85	0			85									
OTU1051	Ascomycota sp.	unknown		SH1547057.08FU	MG571637	99	6	8				1		1		2	2	8		
OTU1054	Capnodiales sp.	Capnodiales		SH025821.07FU	JQ760724	99	577	0	1	77	**491**		6	2						
OTU1066	Dothideomycetes sp.	unknown			KX908744	99	43	4		5	38							4		
OTU1067	Fungi sp.	unknown		SH495907.07FU	KM104100	98	11	0	1	2	8									
OTU1073	*Lasiodiplodia theobromae*	Botryosphaeriales		SH1507365.08FU	KU296917	99	0	21										21		
OTU1076	Fungi sp.	unknown		SH025821.07FU	KC222753	99	45	0		1	42	1		1						
OTU1083	Dothideomycetes sp.	unknown		SH200708.07FU	GQ999265	98	83	4		2	78		1		2			4		
OTU1089	*Kockovaella* sp.	Tremellales		SH1155938.08FU	KT328747	97	62	0					4	4			54			
OTU1096	Tremellales sp.	Tremellales		SH212025.07FU	KR857010	100	20	13	1	6	12		1					13		

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									mature ant-plants			ant-accessible plantlets			ant-excluded plantlets			ant workers		
									WC	NC	VC	WC	NC	VC	WC	NC	VC	EXOSK	HEAD	ABD
OTU1105	*Phaeosphaeriopsis* sp.	Pleosporales		SH1646320.08FU	KU529841	96	86	0				1		31	48	5	1			
OTU1107	Teratosphaeriaceae sp.	Capnodiales		SH1521438.08FU	KT581771	94	0	35										35		
OTU1111	*Fellomyces* sp.	Tremellales		SH1155951.08FU	JQ861271	99	1970	0					3	9	3	272	**1683**			
OTU1118	*Cryptococcus* sp.	Tremellales		SH1576903.08FU	JN635412	99	50	0				4	14	5			27			
OTU1122	Fungi sp.	unknown		SH495340.07FU	KT328732	99	1616	11	2	854	**678**		64	18				1	10	
OTU1123	Fungi sp.	unknown		SH1552750.08FU	KY496833	99	26	0									26			
OTU1132	Fungi sp.	unknown		SH1183477.08FU	KF675720	96	58	0					10	48						
OTU1136	Dothideomycetes sp.	unknown		SH1237705.08FU	JN890113	95	21	0			1		1	2		5	12			
OTU1138	*Fellomyces penicillatus*	Tremellales		SH1539826.08FU	KY103409	98	527	47	30	161	307	1	4	9		5	10	**46**	1	
OTU1154	*Fellomyces polyborus*	Tremellales		SH1539825.08FU	AJ608672	97	25	29	7	5	13							**29**		
OTU1158	Fungi sp.	unknown		SH200320.07FU	KF617293	98	124	0	93	29	2									
OTU1165	*Penidiella* sp.	Capnodiales	OTU0970 (98%)	SH492567.07FU	KP269064	99	891	0		737	148			6						
OTU1178	*Apiotrichum mycotoxinivorans*	Trichosporonales		SH1616869.08FU	KY792632	99	79	0	10			42	4	15	4	1	3			
OTU1183	*Penidiella* sp.	Capnodiales	OTU0970 (98%)	SH492567.07FU	KP269064	94	42	0		42										
OTU1184	*Rhynchogastrema* sp.	Tremellales		SH1576898.08FU	AB915390	99	0	3												3
OTU1187	*Papiliotrema* sp.	Tremellales		SH1576891.08FU	LC191360	99	54	0	15		28	1		10						
OTU1188*	*Candida* sp.	Saccharomycetales	OTU0977 (99%)	SH495679.07FU	KM504131	99	1443	88	**868**	58	11	**261**	239	6				**62**	26	
OTU1190	*Fellomyces* sp.	Tremellales	OTU0981 (95%)	SH176359.07FU	NR_073258	97	749	7	13	**219**	**513**			4				7		

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									WC	NC	VC	WC	NC	VC	WC	NC	VC	EXOSK	HEAD	ABD
OTU1195	*Calocera* sp.	Dacrymycetales		SH1563450.08FU	KP013031	98	24	0							24					
OTU1198	*Candida tropicalis*	Saccharomycetales		SH1644517.08FU	KY065354	99	0	30											30	
OTU1202	Pucciniomycetes sp.	unknown		SH029175.07FU	HM209413	92	15	0	1	1	13									
OTU1205	Pucciniomycetes sp.	unknown		SH029175.07FU	HM209413	96	20	0	1		19									
OTU1209	*Candida parapsilosis*	Saccharomycetales		SH1570474.08FU	MG386882	100	3	18		2		1						10	8	
OTU1213	Saccharomycetales sp.	Saccharomycetales		SH495679.07FU	KM504131	99	1002	6	364	233	2		402	1					6	
OTU1220	Tremellales sp.	Tremellales		SH1177278.08FU	KP986516	84	14	0			14									
OTU1233	Ascomycota sp.	unknown		SH1648483.08FU	FJ999654	99	19	0				9	4	6						
OTU1238	Sordariomycetes sp.	unknown		SH456958.07FU	JQ905789	98	0	153										**153**		
OTU1263	Archaeorhizomycetes sp.	unknown		SH180885.07FU	JQ346855	100	15	0		15										
OTU1266*	Tremellomycetes sp.	unknown		SH477174.07FU	JX999048	99	7152	9	**6778**	46	117	**197**	11	3				9		
OTU1270	*Hyphopichia* sp.	Saccharomycetales			KP691036	100	4	23	3	1								8	15	
OTU1274	*Hyphopichia pseudoburtonii*	Saccharomycetales			KY103607	99	2	18	1	1								6	12	
OTU1282	*Clavispora lusitaniae*	Saccharomycetales			MG015987	99	0	6											5	1
OTU1283	*Clavispora lusitaniae*	Saccharomycetales		SH1569452.08FU	LC413209	100	0	7										7		
OTU1284	*Clavispora* sp.	Saccharomycetales			MG637448	99	0	6										6		

Supplementary Table S3.4 Output of a simple linear regression to test the relationship between fungal OTU richness (observed values) and sequencing depth (number of sequences per sample) in the chambers of the ant-plant *M. beccarii.*

	Estimate	**Std. Error**	**t-value**	**p-value**
Intercept	13.003	1.9735	6.59	5.42E-09
Sequencing depth	0.0074	0.0012	6.43	1.06E-08

Residual standard error: 13.11 on 75 degrees of freedom
Multiple R-squared: 0.3555, Adjusted R-squared: 0.3469
F-statistic: 41.37 on 1 and 75 DF, p-value: 1.056e-08

Supplementary Table S3.5 Fungal OTU abundance in chambers of *M. beccarii* ant-accessible plantlets, ant-excluded plantlets and mature ant-plants including all chambers together and each chamber type: nursery, ventilation, and waste. Included are the number of samples (N), the total OTU abundance (for all samples for a plant/chamber combination), and the mean ± standard error (SE).

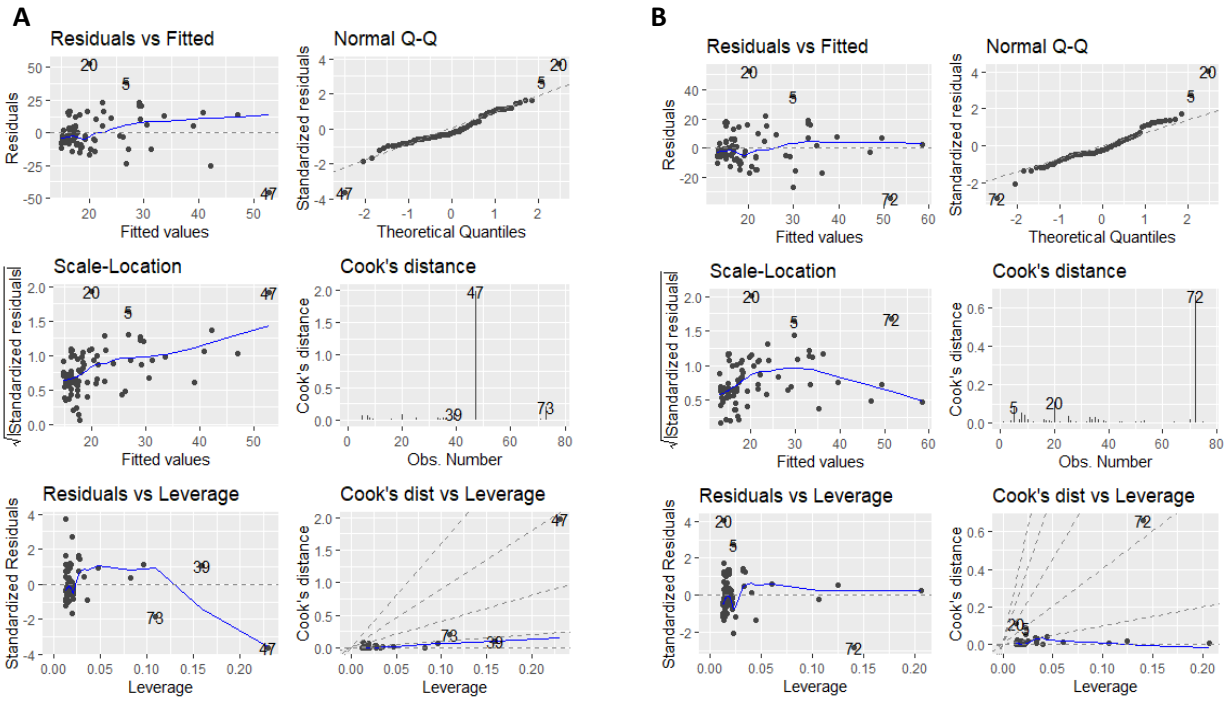
Treatment	Ant-accessible plantlets			Ant-excluded plantlets			Mature ant-plants		
	N	Total	Mean ± SE	N	Total	Mean ± SE	N	Total	Mean ± SE
All chambers	39	60054	1540 ± 216	39	32763	840 ± 240	39	138291	3546 ± 308
Nursery chambers	13	26276	2021 ± 357	13	13989	1076 ± 533	13	42929	3302 ± 591
Ventilation chambers	13	11667	898 ± 181	13	10349	796 ± 331	13	38264	2943 ± 526
Waste chambers	13	22111	1701 ± 477	13	8425	648 ± 380	13	57098	4392 ± 427

Supplementary Table S3.6 Fungal OTU richness in chambers of *M. beccarii* ant-accessible plantlets, ant-excluded plantlets and mature ant-plants including all chambers together and each chamber type: nursery, ventilation, and waste. Included are the number of samples (N), the total OTU richness (for all samples for a plant/chamber combination), and the mean ± standard error (SE).

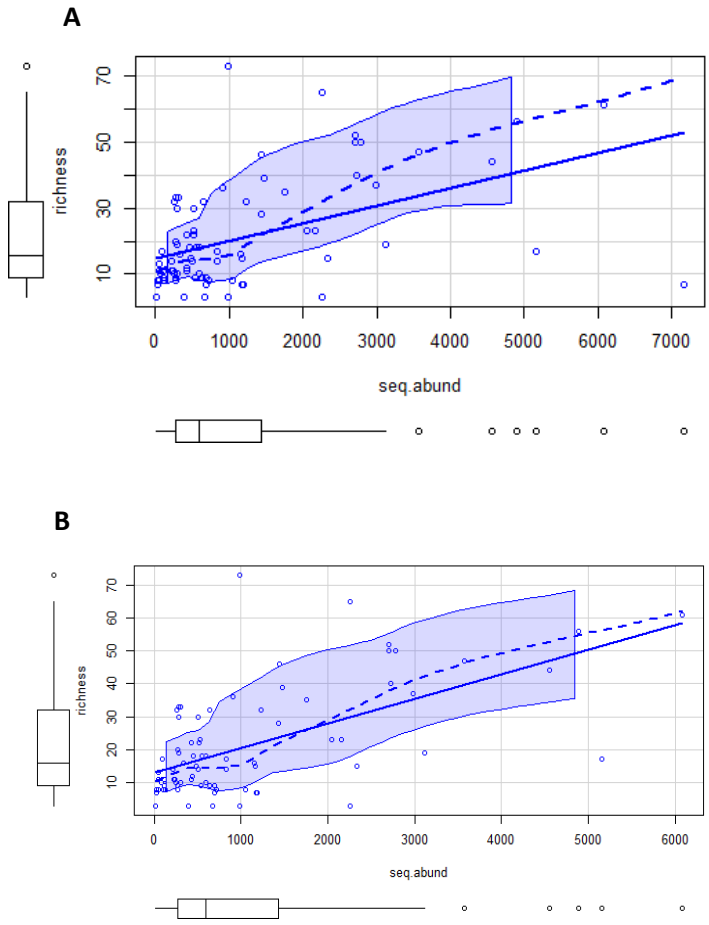
Treatment	Ant-accessible plantlets			Ant-excluded plantlets			Mature ant-plants		
	N	Total	Mean ± SE	N	Total	Mean ± SEz	N	Total	Mean ± SE
All chambers	39	1064	27.3 ± 3.1	39	583	14.9 ± 1.4	39	1741	44.6 ± 2.9
Nursery chambers	13	301	23.2 ± 5.6	13	139	10.7 ± 1.5	13	471	36.2 ± 3.2
Ventilation chambers	13	335	25.8 ± 5.6	13	232	17.8 ± 3.1	13	746	57.4 ± 6.2
Waste chambers	13	428	32.9 ± 4.8	13	212	16.3 ± 2.3	13	524	40.3 ± 2.8

Supplementary Table S3.7 Fungal OTU abundances in samples collected in/on *P. cordata* ant workers including exoskeletons, heads, and abdomens. Included are the number of samples (N), the total OTU abundance for a sample type, and the mean ± standard error (SE).

Treatment	N	Total	Mean ± SE
Exoskeletons	13	2934	225.7 ± 51.7
Heads	13	2205	169.6 ± 68.1
Abdomens	13	832	64.0 ± 24.4



Supplementary Figure S3.1 Diagnostic plots including outlier identification for the linear model testing the relationship between fungal OTU richness (observed values) and sequencing depth (number of sequences per sample) in the chambers of the treatment *M. beccarii* plantlets (ant-accessible plantlets and ant-excluded plantlets). Plot A displayed an outlier sample (number 47) (Cook's distance plot) which was removed prior to running the model, and Plot B shows the plots after the outlier was removed.



Supplementary Figure S3.2 Scatterplots showing the relationship between fungal OTU richness (observed values) and sequencing depth (number of sequences per sample) in the chambers of the treatment *M. beccarii* plantlets (ant-accessible plantlets and ant-excluded plantlets). Plot A is before the outlier sample number 47 was removed prior to running the model, and Plot B is after the outlier was removed.

Chapter 4 - Ant workers transfer nitrogen to their host ant-plant waste chambers, but the role of fungi remains a mystery

Chapter 4 - Ant workers transfer nitrogen to their host ant-plant waste chambers, but the role of fungi remains a mystery

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Statement of contribution of others:

M.J.G. designed the experiment, conducted the field work, grew ant-plants from seed, ran the experiment, carried out the dissections, collected and collated the data, performed the bioinformatics, analysed the data, created the figures and tables, and wrote this chapter. S.E.A. and L.L. provided advice on the design of the study, interpretation of results, and edited the chapter. B.C.C. provided advice on interpreting the data. P.Y. provided input and advice on data analyses. S.A. provided input and advice on bioinformatics. L.T. provided laboratory space, primers and reagents, and advice on high-throughput sequencing.

4.1 Introduction

Nitrogen is essential for plant growth but is a limiting nutrient in terrestrial ecosystems and consequently plants have developed ways of obtaining it through interactions with other organisms. Some plants have evolved specialised structures to capture nitrogen. For example, the pitcher plant *Nepenthes lowii* Hook.f. (Nepenthaceae) is visited by the tree shrew *Tupaia montana* Thomas (Scandentia) which feeds on nectary exudates under the lid of the pitcher plant and then defecates into the pitcher, providing nitrogen to the plant (Clarke et al. 2009). Rhizobia bacteria infect roots of legumes, inducing nodules within which the bacteria fix atmospheric nitrogen making it available as ammonium to the plant, and in return the bacteria receives carbon and other nutrients (Marx 2004). Other plants interact directly or indirectly with fungi to obtain nitrogen.

The most well-known plant-fungal interaction involving nutrients is mycorrhiza, an ancient mutualism between fungi and plants that allowed plants to make the transition from sea to land (Smith and Read 2008, Bidartondo et al. 2011). Approximately 90% of vascular plants form mutualistic associations with mycorrhizal fungi which mobilise nitrogen from soil organic matter and supply it to plants in exchange for carbon (Brundrett 2009, Lindahl and Tunlid 2015). In cold-stressed habitats like Antarctica, mycorrhizas are usually absent or low in abundance. Instead, root endophytic *Penicillium* species promote plant growth via nitrogen mineralisation and nutrient acquisition in two species of native Antarctic vascular plants (Oses-Pedraza et al. 2020). An indirect example of fungi providing nutrients to plants is saprotrophic fungi that break down dead organic matter, transforming it into smaller molecules which can lead to a release of soluble compounds making it available to plants and other organisms, e.g. free-living decomposers of leaf-litter (Lindahl and Tunlid 2015).

Some plants benefit from nutrients provided through the waste management practices of ants. Ants practice waste management of faecal and other refuse material including dead nestmates because waste accumulation can lead to harmful pathogens for the colony (Schmid-Hempel 1998, Farji-Brener et al. 2016). The accumulation of waste materials in and around ant nests elevates the level of nutrients which provide better sites in which plants can germinate and establish compared to the surrounding soils (Beattie 1985). For example, leaf-cutting *Atta* and *Acromyrmex* ants create waste chambers where they deposit nest refuse underground in the deepest part of the nest or in external piles depending on habitat, colony size, and the natural history of the species (Farji-Brener et al.

2016). In an experiment by Sternberg et al. (2007), leaves labelled with isotopic nitrogen (^{15}N) were fed to leaf-cutting ants (*Atta colombica* and *Atta laevigata*) and the ^{15}N tracer was later found in leaves of trees surrounding the nest sites, confirming the trees obtained nitrogen from the ant nests.

In terrestrial ant-plants, the ants benefit from a protective domatium, and sometimes nutritional rewards (e.g. extra-floral nectaries) (Hölldobler and Wilson 1990), while the ants often provide their host ant-plant with protection against enemies (e.g. herbivores), and some ants deposit faeces inside the domatium effectively fertilising their host terrestrial ant-plant (Cabrera and Jaffe 1994, Sagers et al. 2000, Fischer et al. 2003, Solano and Dejean 2004). For example, the ant-plant *Maita guianensis* Aublet (Melastomataceae) has protuberances within its domatium leaf pouches, and 80% of the host plant nitrogen was derived from its *Pheidole minutula* Mayr (Myrmicinae) ant workers (Solano and Dejean 2004). Fungi belonging to the order Chaetothyriales have been found in domatia of terrestrial ant-plants in Africa, South America (Blatrix et al. 2009, Defossez et al. 2009, Blatrix et al. 2013) and Central America (Voglmayr et al. 2011, Mayer et al. 2014, Nepel et al. 2014, Nepel et al. 2016). These studies have reported fungi as a source of food for the ant colony (Blatrix et al. 2012, Mayer et al. 2018) and the ant workers fertilise fungi by depositing their faeces on fungal patches inside domatium (Defossez et al. 2009, Defossez et al. 2011). In the ant-plant *Hirtella physophora* (Chrysobalanaceae), the resident *Allomerus decemarticulatus* (Myrmicinae) ants build galleries and traps to capture prey by combining host plant trichomes and fungal hyphae (as a structural component) and the fungi *Trimmatostroma* sp. (Chaetothyriales) also play a role in the transfer of nitrogen to the host plant (Leroy et al. 2011, Leroy et al. 2017, Orivel et al. 2017).

Epiphytic ant-plants are particularly nitrogen limited (Hietz et al. 2022) because they live in the nutrient-poor habitat of tree canopies. This means the provision of nutrients by ant workers to their host epiphytic ant-plant is believed to be an essential service (Huxley 1980). Some epiphytic ant-plants absorb nutrients from ant-deposited waste in specialised waste chambers inside the domatium, while other domatia are able to absorb the waste directly through the domatium surface (Janzen 1974, Huxley 1980, Rico-Gray et al. 1989, Gegenbauer et al. 2012). Some examples of studies where epiphytic ant-plants obtained nitrogen from ant-deposited waste include ant-plants from the families: (a) Rubiaceae with *Philidris* and *Pheidole* ants (Chomicki and Renner 2016, Chomicki and Renner 2019, Volp et al. 2022); (b) Orchidaceae with a variety of ants (Rico-Gray et al. 1989, Gegenbauer et al. 2012), (c) Asclepiadaceae with *Philidris* ants (Treseder et al. 1995), and (d)

the ferns Pteridaceae with *Pheidole* ants (Watkins et al. 2008) and Polypodiaceae with *Philidris* and *Crematogaster* ants (Gay 1993). Some studies have focussed on other nutrients such as sulphur, phosphate and carbon that were transferred from ant waste to host epiphytic ant-plants in ant-plants from the families Rubiaceae with *Philidris* ants, and Orchidaceae with various ants (Huxley 1978, Rickson 1979, Rico-Gray et al. 1989).

Myrmecodia beccarii Hook.f. (Gentianales: Rubiaceae) is an epiphytic ant-plant endemic to northern Queensland, Australia. *Myrmecodia beccarii* grows three types of specialised chambers: brood ('nursery') chambers where the resident *Philidris cordata* Smith F. (Hymenoptera: Formicidae) ant workers keep their brood, ventilation chambers that allow air flow into the domatium, and waste chambers with wart-like structures that absorb nutrients from waste deposited by the ant workers (Volp et al. 2022). These wart-like structures in the waste chambers of Rubiaceous epiphytic ant-plants were shown to absorb liquid substances including India ink and water and are believed to function as roots (Miehe 1911, Janzen 1974, Huxley 1980). A consistently specific fungal community has been found in the waste chambers of *M. beccarii* collected across 675 km of its distribution (chapter 2; Greenfield et al. 2021) and the resident ant workers transport fungi between *M. beccarii* ant-plants (chapter 3). We also know that the resident *P. cordata* ant workers feed *M. beccarii* (Volp et al. 2022). However, the possible role of fungi in nutrient transfer has never been investigated in epiphytic ant-plants.

I hypothesised that fungi are involved in the transfer of nitrogen from *P. cordata* ant workers to the ant-plant *M. beccarii*. To test this hypothesis, I manipulated ant access and fungal communities in *M. beccarii* ant-plantlets (greenhouse-grown, approximately 18 months old) that were placed in cages with mature ant-plants colonised with *P. cordata* ant colonies (collected from the wild). I traced nitrogen transfer from the ant workers to the leaves of the plantlets using the stable isotope ^{15}N which was fed to the ant workers. At the end of the experiment, I extracted and sequenced fungal DNA from the waste chambers of the plantlets and mature ant-plants to determine if the fungicide treatment worked by comparing: (a) fungal OTU richness, (b) fungal OTU sequence abundances, (c) fungal community compositions, (d) fungal functional guilds, and (e) dominant fungal OTUs, which could also be used to identify fungi involved in the transfer of nitrogen. I collected leaves from the plantlets to compare the ^{15}N values between treatments to test the hypothesis that fungi are involved in this ant-plant mutualism.

If my hypothesis is supported and the fungicide treatment is effective, I expect fungal OTU sequence abundances and richness will be lower, and fungal functional guilds, dominant fungal OTUs, and fungal community composition will be different in the plantlets that have received the fungicide treatment compared to the plantlets that have not. If nitrogen transfer to ant-plants requires both the ant workers and fungi, the $\delta^{15}N$ values should be higher in the ant-accessible/no fungicide treatment plantlets compared to the ant-accessible/fungicide treatment plantlets. If the ant workers are involved in the transfer of nitrogen and fungi are not involved, the $\delta^{15}N$ values will not differ between ant-accessible plantlets with or without fungicide treatment (assuming the fungicide treatment is effective in removing the fungal community).

4.2 Methods

4.2.1 Collection of mature *Myrmecodia beccarii* ant-plants and cultivation of plantlets

The methods used to collect 15 mature *M. beccarii* ant-plants (with their resident ant colonies) from a wild population and the cultivation of ant-plantlets for use in this experiment are outlined in chapter 3.

4.2.2 Experimental Design

I conducted the experiment in a greenhouse at James Cook University, Cairns Campus (16°48'58.83"S, 145°41'16.73"E) for three months from September to December 2017 using a 2 x 2 factorial design with ant-exclusion and fungicide application as treatments (Fig. 4.1). The preparation and set-up of the cages and maintenance of the experiment is explained in chapter 3. For ease of reference, I have included a photograph of the cage set-up here (Figure 4.1). The results of the ant-accessible/no fungicide treatment plantlets versus ant-excluded/no fungicide treatment plantlets (treatments 1 and 2 in Figure 4.1) were used to test the hypothesis that fungi are transferred by ant workers (chapter 3 of this thesis). The results of the ant-accessible/fungicide treatment plantlets versus ant-accessible/no fungicide treatment plantlets (treatments 1 and 3 in Figure 4.1) were used to test the hypothesis that fungi are involved in the transfer of nitrogen to *M. beccarii* plantlets and are analysed in this chapter.

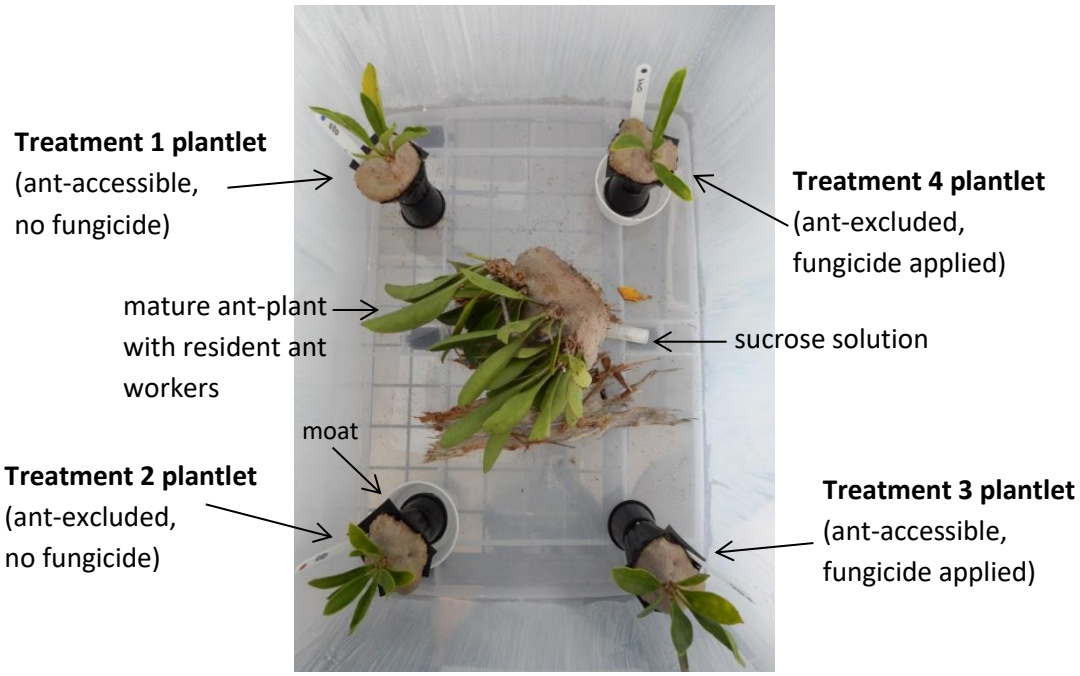


Figure 4.1 Experimental set up with *M. beccarii* plantlets using a 2 x 2 factorial design with ant-exclusion and fungicide application as treatments. A mature ant-plant with its resident *P. cordata* ant colony was placed in each of the cages and was the source of ant workers. The ant workers were allowed access to two plantlets (treatments 1 and 3) and excluded from accessing two plantlets using moats (treatments 2 and 4). Fungicide was applied to treatments 3 and 4. Treatments 1 and 3 were accessible to the ant workers and are analysed in this chapter. Treatments 1 and 2 relate to chapter 3.

4.2.3 Fungicide application

I applied fungicide once per week during the 12 weeks of the experiment using injection and spray applications to suppress the fungal communities in the domatium chambers of the two plantlets that received the fungicide treatment (treatments 3 and 4, Fig. 4.1). The injections consisted of 1.0 mL (per fungicide treatment plantlet) of the anti-mycotic drug Amphotericin B (Sigma-Aldrich Pty. Ltd, Castle Hill, NSW, Australia) at a concentration of 20 mg/L (in sterile water) which was injected into the domatium tissue around the base of the stem using sterile Terumo® hypodermic needles (23-gauge x 25 mm) and 1.0 mL Hapool® syringes (Livingstone International, Mascot, NSW, Australia). Amphotericin B has been used in a previous study to remove fungi from the surface of ant workers (Little and Currie 2008) and was therefore considered safe for use with *P. cordata* ant workers. The spray application consisted of 0.5 mL (per plantlet) of the fungicide Triforine® (Kendon

Chemical & MNFG. CO. Pty Ltd, Fairfield, Victoria, Australia) which I applied to the leaves using a 100 mL capacity atomiser at a concentration of 15 mL/L of water (the manufacturer's recommended application). Triforine® is a systemic fungicide that is stated to have low toxicity to bees and therefore unlikely to harm ants (Kendon Chemical & MNFG. Co. Pty. Ltd, Fairfield, Victoria, Australia). I conducted a pilot study over four weeks prior to the experiment to confirm that *P. cordata* ant workers and *M. beccarii* plantlets would not be adversely affected (or ant workers deterred) by the Triforine® spray application and/or the Amphotericin B injections. I used this pilot study to also test whether different concentrations of the Amphotericin B injections (2.5 mg/L, 5 mg/L, 10 mg/L and 20 mg/L) negatively affected the ant-plantlets. The pilot study plantlets did not show any detrimental effects of the Amphotericin B application and therefore, I chose 20 mg/L as optimal because it was the highest concentration. The other two plantlets (one accessible to workers and one that was inaccessible to workers – treatments 1 and 2, Fig. 4.1) were injected and sprayed with sterile water. I began the first application of fungicide five days prior to placing the plantlets in the cages with the mature ant-plants and their resident ant colonies.

4.2.4 Nitrogen tracing using stable isotopes

I traced nitrogen using the stable isotope ^{15}N to determine if fungi are involved in the transfer of nitrogen from worker ants to the *M. beccarii* plantlets. I fed the *P. cordata* ant workers in each of the cages with a 50% sucrose solution labelled with ^{15}N-enriched glycine (99 atom% ^{15}N, Sigma-Aldrich Pty Ltd, North Ryde, NSW, Australia) which can be used to trace the passage of nitrogen from worker ants to the plant without harming the ants or the plants. I provided each of the ant colonies in the 15 cages with a total 20 µL daily of the labelled ^{15}N solution for a period of 13 days (equivalent to 1 mg per day of ^{15}N-enriched glycine). The 20 µL droplet was placed onto a plastic disc (15 mm in diameter) that was fixed onto the domatium of each of the mature ant-plants using a non-toxic pliable adhesive (Blue Tac®, Bostik Australia Pty Ltd, Victoria, Australia) (Fig. 4.2). The discs were placed on the mature ant-plants because the mature ant-plants were at the centre of the cage, equally distant to the ant-accessible plantlets.

Figure 4.2 ^{15}N Feeding station consisting of a plastic disc attached to the surface of a *M. beccarii* domatium. The 20 µL droplet of ^{15}N is being consumed by *P. cordata* ant workers.

Prior to the start of feeding the ^{15}N-enriched glycine to the ant colonies, I collected five control (baseline) leaf disc samples (5 mm diameter) from five individual leaves of the mature ant-plants and four to five individual leaves (five wherever possible, but sometimes only four leaves were available) from all plantlets in the 15 cages using a standard hole punch. The batches of leaf discs for all plants were rinsed (to remove any fungicide residue from the fungicide treated plantlets) by placing them into a 1.5 ml Eppendorf tube with 1.0 mL of sterile water, shaking by hand for 10 seconds, and draining them on paper towels. I dehydrated the discs by placing them in permeable bags (tea bag material) on silica gel beads. The same method was used to collect leaf discs at four weeks, eight weeks and 12 weeks after the ^{15}N-enriched glycine was first provided to the ant workers. At the end of the experiment, each batch of leaf discs was freeze-dried for 24 hours and ground into a fine powder with a mortar and pestle, weighed to obtain 4 to 5 mg of powdered material, and transferred to tin capsules. I rinsed the mortar and pestle using 70% ethanol and tap water in between samples. The leaf samples were analysed using an isotope ratio mass spectrometer (Sercon HS20-22 fitted with a GSL inlet) at the Central Analytical Research Facility at Queensland University of Technology, Brisbane, Queensland, Australia. The two stable isotopes of nitrogen (^{15}N and ^{14}N) were used to calculate the stable isotope composition of a sample which is

expressed as a delta (δ) value per mil (‰). The $\delta^{15}N$ values for the nitrogen isotopic composition were calculated using the equation:

$$\boldsymbol{\delta^{15}N = \frac{(R_{sample} - R_{std})}{R_{std}} \times 1000}$$

Where R_{sample} is the molar ratio of the heavy isotope (^{15}N) to the light isotope (^{14}N), and R_{std} is the $^{15}N/^{14}N$ ratio of atmospheric N_2 (the standard used for nitrogen).

4.2.5 Collection of fungi from plant chambers (including DNA extraction, PCR, and sequencing)

I dissected the 15 mature *M. beccarii* ant-plants and 60 plantlets as outlined in the methods section of chapter 3. Briefly, I aimed to collect nine chamber surface samples for each mature ant-plant (three samples of each chamber type) and three chamber surface samples for each plantlet (one sample of each chamber type per plantlet). I extracted DNA from the chamber samples and amplified and sequenced fungal DNA as outlined in chapter 3 including controls for dissections, extractions, and PCR amplification.

4.2.6 Bioinformatics and data collation

The bioinformatics process for the samples collected during the experiment is explained in the methods section of chapter 3, up to the generation of the dataset for plant chamber samples with a total 786 fungal OTUs. This dataset of 786 fungal OTUs included samples collected from the waste, nursery, and ventilation chambers of the mature ant-plants and the four treatment plantlets. I removed some samples from the dataset of 786 fungal OTUs because they were either missing samples, or not relevant to this chapter. One ant-excluded plantlet in cage AE23 died during the experiment and the mature ant-plant and one ant-excluded plantlet in cage AE12 had insufficient chambers to collect adequate amounts of material. I removed sequencing data for these two cages to ensure a balanced design for analyses. I removed sequencing data for the nursery and ventilation chambers from the dataset for this chapter because the waste chambers are where the ants deposit waste and therefore the stable isotope (^{15}N). This left 39 waste chamber samples from mature ant-plants (13 cages x three samples per plant) which I then pooled to reduce the three waste chamber samples per plant to one waste chamber sample per plant. This was necessary because the interconnectedness of the chambers in this ant-plant mean multiple samples per plant are not independent. After removal of these samples, the final dataset contained 482 fungal OTUs across

93 samples including 65 waste chamber samples comprising 13 from mature ant-plants, and 52 from the four treatment plantlets (13 waste chamber samples x 4) plus 28 controls (13 dissection controls and 15 extraction controls).

The plant chamber samples dataset with 482 fungal OTUs was filtered further to create a final dataset with 123 fungal OTUs. The further filtering included several steps as follows. I checked OTUs with e-values between e^{-20} and e^{-50} against the ten best matches for assignment to kingdom fungi or removal (resulting in 30 OTUs being detected as chimeric sequences and removed). I also removed 34 OTUs with low sequence coverage (<70%). The single positive PCR control OTU was removed along with three OTUs that were found only in positive controls and one OTU found only in a negative control. I removed 13 OTUs that were in chamber samples and also in positive controls (11 OTUs), negative controls (1 OTU) or both positive and negative controls (1). I removed global OTU singletons (92 OTUs in total, each with only one occurrence in the dataset) to avoid potentially erroneous sequences which left a dataset containing 308 fungal OTUs with minimum read abundances of 2. I then removed a further 168 OTUs with sequence abundances <10 across all plant samples and another 17 OTUs that did not have a minimum of 10 abundances in at least one of the plant types (mature ant-plants or any of the four treatment plantlets) which left the dataset of 123 fungal OTUs (Supplementary Table S4.1). This final step was performed because the focus of this study is on the dominant fungal taxa inside the domatium chambers of *M. beccarii* and, according to previous studies, excluding rare species makes the community matrix more coherent and hence strengthens the statistical power (Tedersoo et al. 2015). I used sequence similarity thresholds of >97%, >90%, >85%, >80%, >75% and >70% to match approximate species, genus, family, order, class, and phylum levels respectively (Nilsson et al., 2019). Of the 123 OTUs, 91 OTUs (74.0%) matched the taxonomic identity of >97% to pre-existing fungal ITS sequences in existing databases (UNITE and my APSurvey database). A further 22 OTUs (18.0%) matched at 90-97% and the remaining 10 OTUs (8.0%) matched to closest taxa at <90% sequence similarity.

4.2.7 Statistical analyses

There were two datasets for the 65 waste chamber samples: one contained data for the 308 fungal OTUs with a minimum read abundance of 2 and the other contained data for the 123 fungal OTUs with a minimum read abundance of 10 (Supplementary Table S4.1). I used the dataset with 308 OTUs to test whether fungicide application altered fungal OTU richness in the waste chambers (because richness relies on the rarer OTUs). I used the dataset with 123 OTUs to test whether

fungicide application influenced fungal OTU abundances and community composition in the waste chambers, and to test whether $\delta^{15}N$ values differed between the leaves of the four treatment plantlets. All statistical analyses were conducted in R version 4.0.5 (R Core Team 2021). I used the package 'ggplot2' (v3.3.5) (Wickham 2016) to create the plots.

I tested whether fungicide application reduced fungal OTU richness in the waste chambers of the plantlets using the dataset with 308 OTUs. First, I performed a simple linear regression to test if OTU richness is dependent on sample sequencing depth using the package 'car' (v3.0.11) (Fox and Weisberg 2018). I used the package 'ggfortify' (v0.4.12) (Horikoshi and Tang 2016, Tang et al. 2016) to visualize the data from which two outliers were identified (using Cook's Distance) and removed (Supplementary Fig. S4.1). I found OTU richness was significantly positively correlated to sequencing depth (Supplementary Table S4.2), so I calculated the residuals for richness for each sample from the regression to account for the relationship in my subsequent analysis. I then used a linear mixed effects model to test whether fungal OTU richness (residuals) is reduced by fungicide treatment using the packages 'lme4' (v1.1.27.1) (Bates et al. 2015) and 'lmerTest' (v3.1.3) (Kuznetsova et al. 2017). Fixed effects included fungicide and ant exclusion with an interaction term for ant exclusion: fungicide. I included cage ID as a random effect. I used the package 'emmeans' (v1.6.2.1) (Lenth 2021) for pairwise comparisons between the treatment plantlets. This analysis was also performed on the data without removing the two outliers mentioned above, and the outcome was the same but when the outliers are included, the model results in a singularity.

I tested whether fungicide application reduced fungal OTU abundances in the waste chambers of the plantlets using the dataset containing 123 fungal OTUs. I used the package 'lme4' (v1.1.27.1) (Bates et al. 2015) to perform a generalized linear mixed model (glmer function) with a Poisson distribution. Fixed effects included fungicide and ant exclusion with an interaction term for ant exclusion: fungicide. I included cage ID as a random effect. An observation level random effect was also included to account for overdispersion (Harrison 2014).

I tested whether fungicide application altered the fungal OTU community composition in the waste chambers of the plantlets using the dataset containing 123 fungal OTUs. I used the packages 'phyloseq' (v1.34.0) (McMurdie and Holmes 2013), 'vegan' (v2.5.7) (Oksanen et al. 2020) and 'ggplot2' (v3.3.5) (Wickham 2016) to create non-metric multidimensional scaling (NMDS) ordination plots to visualise differences in the fungal communities in the waste chambers. The mature ant-

plants (no treatment) were included as a reference fungal community. First, I standardized the OTU matrix with a Hellinger-transformation (to account for varying sampling and sequencing depth) and performed the NMDS with Bray-Curtis distance measure and k=3 dimensions. Binary = TRUE was included to make the data binary (presence/absence of fungal OTUs) to remove the effect of abundance on the ordination because the mature ant-plants had greater abundances of fungal OTUs compared to the plantlets. I compared the fungal OTU community compositions in the chambers of the plantlets using PERMANOVA on the Hellinger-transformed OTU matrix using the package 'vegan' (v2.5.7) (Oksanen et al. 2020). Fungicide and ant exclusion were included as fixed factors with an interaction term for ant exclusion:fungicide and strata=cageID, method = "bray" and permutations=9999.

To test my hypothesis that fungi are required for the transfer of nitrogen to the plantlets, I used the package 'lme4' (v1.1.27.1) (Bates et al. 2015) to perform a generalized linear mixed model (glmer function) with a Gamma distribution and log link function. Fixed effects included ant exclusion and fungicide with an interaction term for ant exclusion:fungicide. I included cage ID as a random effect.

I used the database FungalTraits (Põlme et al. 2020) to investigate fungal guilds across the 123 fungal OTUs in the waste chambers of the four treatment plantlets of the factorial experiment and the mature ant-plants (Supplementary Table S4.1).

4.3 Results

4.3.1 Effect of fungicide application on fungal OTU richness, abundance, and community composition

I detected a total of 108,003 sequences from 123 fungal OTUs from 65 samples collected from the waste chambers of the mature ant-plants and all four treatments of the factorial experiment (Supplementary Table S4.1).

Fungicide application did not significantly reduce fungal OTU richness or abundances in the waste chambers of the ant-accessible plantlets (Fig. 4.3, Table 4.1(a)-(b)). There was a weak (R^2=3%) but significant effect of fungicide application on fungal OTU community composition in the waste chambers of the ant-accessible plantlets (Fig. 4.4, Table 4.2) and there was a large amount (R^2=65%) of unexplained variation (Table 4.1).

Ant exclusion lowered fungal OTU sequence abundances (Table 4.1(b)) and significantly altered the fungal OTU community composition in the waste chambers (Table 4.2, R^2=31%)). The effect of ant exclusion on fungi in the waste chambers of the treatment plantlets is the subject of chapter 3 but full model results (including ant exclusion) are presented here in the tables. Figures 4.3 and 4.4 do not include the ant-excluded plantlets because the results presented here relate to whether the fungicide application reduced fungi in the waste chambers of the ant-accessible plantlets and ant exclusion is not relevant.

Table 4.1 Summary of linear and generalised linear mixed effects model results testing the effects of fungicide application and ant exclusion on: (a) fungal OTU richness in the waste chambers, (b) fungal OTU abundances in the waste chambers, and (c) δ15N values in the leaves of the plantlets in the factorial experiment.

Response and Explanatory variables	Estimate	SE	t/z- value	p-value
(a) Fungal OTU richness in waste chambers				
(Intercept)	2.83	2.33	1.218	0.23
ant exclusion	-2.51	2.96	-0.848	0.40
fungicide	-2.51	2.96	-0.849	0.40
ant exclusion: fungicide	-1.37	4.18	-0.327	0.75
(b) Fungal OTU abundance in waste chambers				
(Intercept)	6.88	0.31	22.141	**<0.001**
ant exclusion	-1.47	0.44	-3.37	**<0.001**
fungicide	-0.34	0.44	-0.783	0.43
ant exclusion: fungicide	0.85	0.61	1.387	0.16
(c) δ^{15}N values in the leaves of plantlets				
(Intercept)	4.95	0.17	28.663	**<0.0001**
ant exclusion	-2.31	0.18	-12.645	**<0.0001**
fungicide	0.19	0.18	1.06	0.29
ant exclusion: fungicide	-0.19	0.26	-0.726	0.47

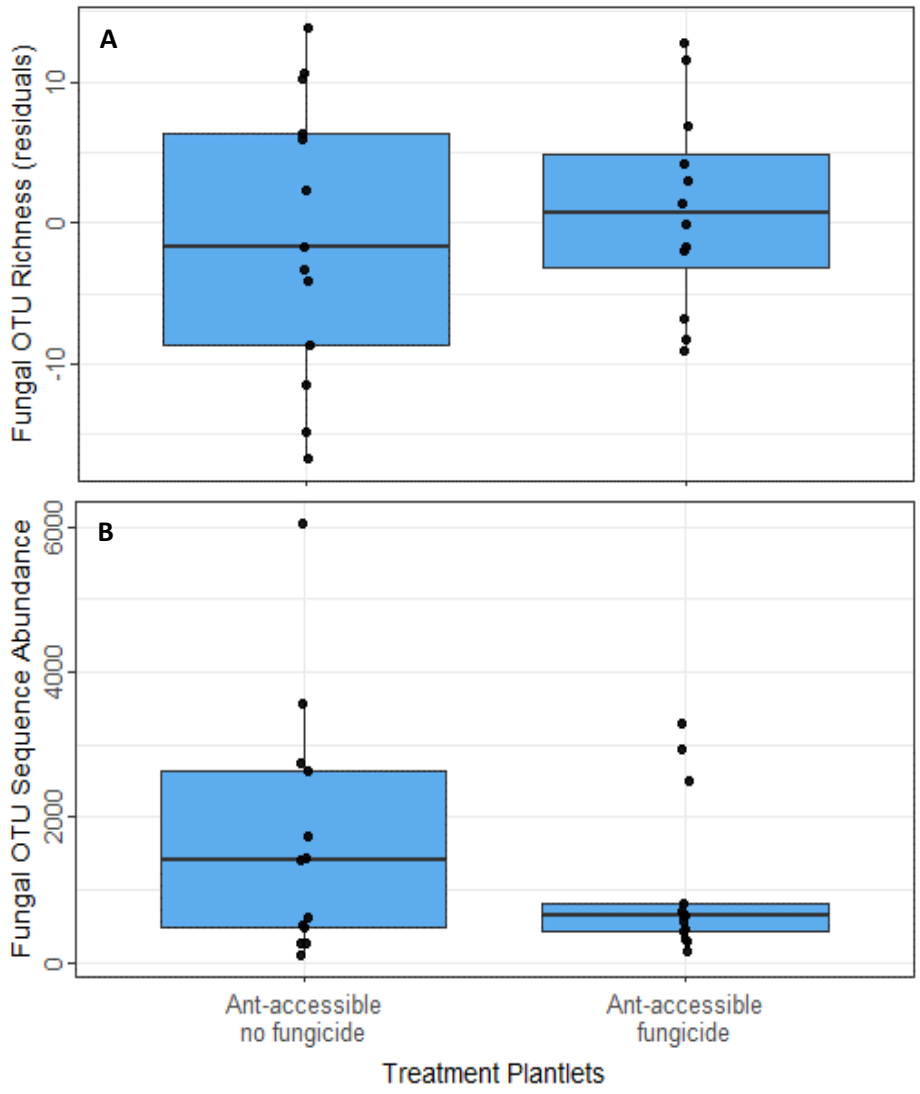


Figure 4.3 (A) Fungal OTU richness and (B) Fungal OTU sequence abundances in the waste chambers of ant-accessible *M. beccarii* plantlets treated with or without fungicide application. The solid line represents the median, the black dots are the data points. Table 4.1(a-b) shows the linear and generalised linear model results.

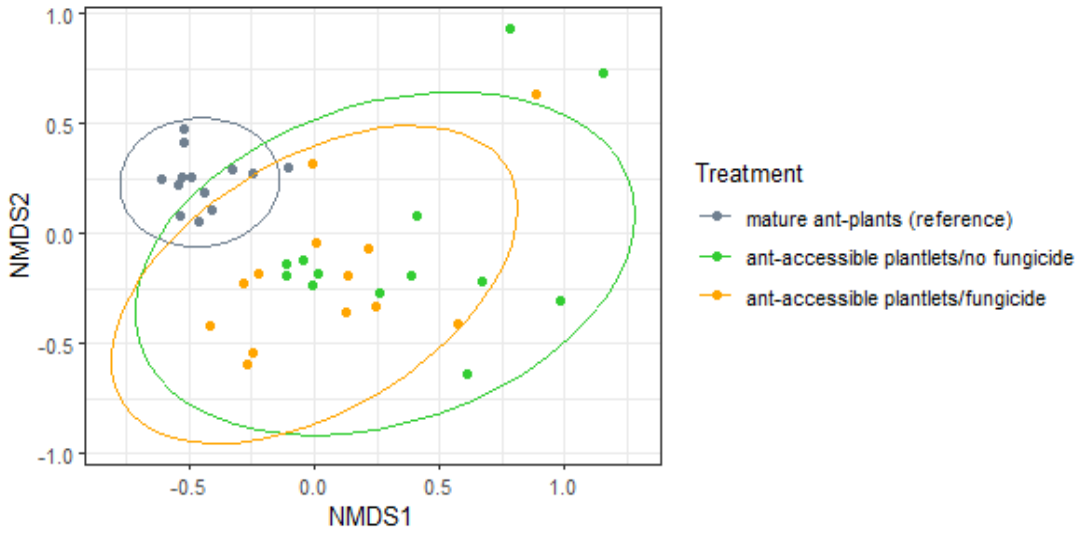


Figure 4.4 NMDS ordination displaying fungal OTU (operational taxonomic units) community compositions in the waste chambers of *Myrmecodia beccarii* mature ant-plants (grey), ant-accessible plantlets/no fungicide (green), ant-accessible plantlets/fungicide (orange).
This ordination plot includes 123 fungal OTUs from 39 waste chamber samples. A Hellinger transformation was used to account for varying sampling and sequencing depth and Bray Curtis distance was used with k=3 dimensions, binary=TRUE. Stress 0.124. Table 4.2 shows the PERMANOVA results.

Table 4.2 Fungal OTU community composition in the plantlets. Results of PERMANOVA to test the effect of ant exclusion and fungicide application on fungal OTU community composition in the waste chambers of *M. beccarii* plantlets.

Summary	Df	SumSq	R2	F	Pr(>F)
ant exclusion	2	6.5041	0.31263	14.4454	**<0.001**
fungicide	1	0.6043	0.02905	2.6842	**0.02**
ant exclusion: fungicide	1	0.1887	0.00907	0.838	0.49
Residual	60	13.5078	0.64926		
Total	64	20.8049	1		

4.3.2 $\delta^{15}N$ values in the leaves of treatment plantlets

The $\delta^{15}N$ values did not differ between the ant-accessible plantlets with and without fungicide (Fig. 4.5, Table 4.1(c)). Ant exclusion influenced the $\delta^{15}N$ values but the ant workers were unable to access the ant-excluded plantlets so it was expected that the ^{15}N values would be very low for the ant-excluded plantlets (Fig. 4.5., Table 4.1(c)).

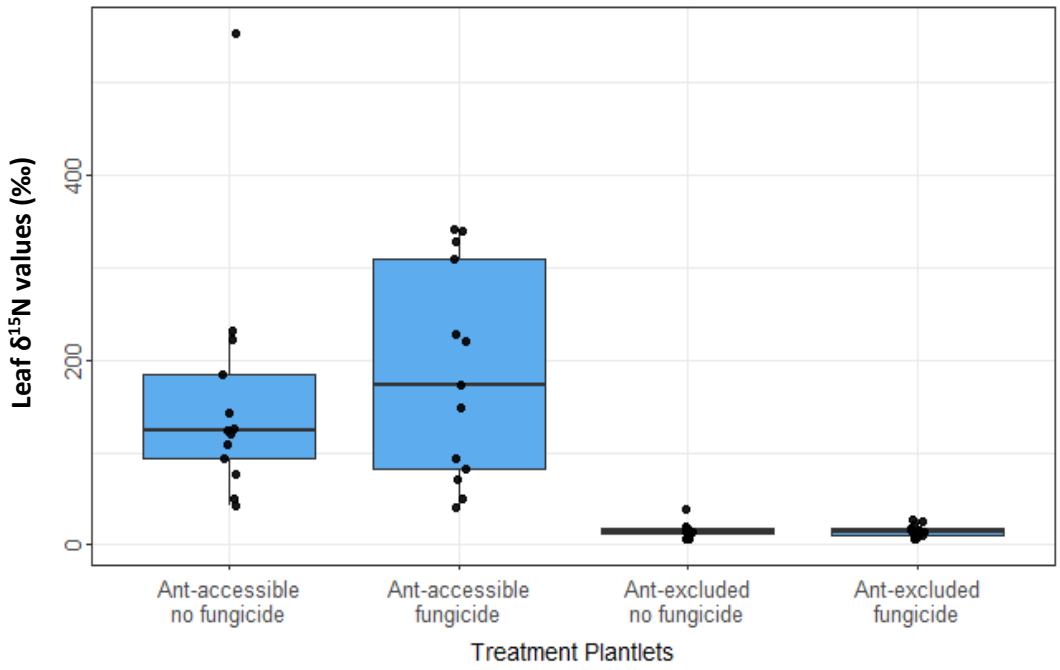


Figure 4.5 δ15N values in leaves of *Myrmecodia beccarii* treatment plantlets that were accessible to or excluded from *Philidris cordata* ant-workers and treated with or without fungicide. The solid line represents the median, the black dots are the data points (n=13 for each plantlet). Table 4.1(c) shows the generalised linear model results.

4.3.3 Fungal Guilds

The 123 fungal OTUs from the waste chambers of the mature ant-plants and the four treatment plantlets were assigned to fungal guilds. In terms of fungal OTU richness for all samples combined, a total 53 of the 123 OTUs (43%) were not assigned to a guild, and their sequence abundances made up a large proportion (67%) of the total abundances (Fig. 4.6(A)&(B), Supplementary Tables S4.4 & S4.5). Of the 123 OTUs, 35.0% (43 OTUs) were saprotrophs including litter, wood, soil, nectar/tap, and unspecified saprotrophs which together comprised 25% of the total OTU sequence abundances (Fig. 4.6(A)&(B), Tables S4.4 & S4.5). Twelve OTUs (9.8%) were assigned as parasites which comprised 6.9% of abundances and 14 OTUs (11.4%) were assigned as plant pathogens but comprised only 1% of the total abundances.

In the mature ant-plants, a large proportion of fungal OTUs (43.5%) could not be assigned to a guild and these comprised 89% of the total OTU sequence abundances (Fig. 4.6(C)&(D), Tables S4.4 & S4.5). Saprotrophs comprised 39% of fungal OTU richness in the mature ant-plants including litter, wood, soil, nectar/tap, and unspecified saprotrophs. Collectively, these saprotrophs accounted for

9.6% of the total sequence abundances in the mature ant-plants, 7% of which were nectar/tap saprotrophs (Fig. 4.6(C)&(D), Tables S4.4 & S4.5). The mature ant-plants had relatively small abundances of fungal OTUs assigned as plant pathogens and parasites (collectively 0.9%) comprising 16% of the total fungal OTU richness in the mature ant-plants (Fig. 4.6(C)&(D), Tables S4.4 & S4.5).

For the two ant-accessible plantlets (with and without fungicide), the relative proportions of fungal OTUs assigned to the different guilds were similar in terms of OTU richness (Fig. 4.6(C)) but the relative abundances show some differences and similarities (Fig. 4.6(D), Tables S4.4 & S4.5). For example, saprotrophs (including litter, wood, soil, nectar/tap, and unspecified saprotrophs) comprised 41-42% of fungal OTU richness in both ant-accessible plantlets (with and without fungicide) but the relative sequence abundances of those saprotrophic OTUs in the ant-accessible plantlets were 31% (no fungicide) and 42% (fungicide). The relative proportion of OTU abundances that were unable to be assigned to a guild was approximately 52% for both of the ant-accessible plantlets (with and without fungicide).

4.3.4 Dominant Fungal OTUs in the waste chambers

Of the 123 fungal OTUs in the waste chambers, 23 were dominant (Table S4.3, Fig. S4.2), i.e., they had a minimum read abundance of 10 in at least 50% of the mature ant-plant samples, and/or 20% of one of the ant-accessible or ant-excluded plantlets (both with and without fungicide). The total sequence abundances of the 23 dominant fungal OTUs (94203) comprised 87% of the total abundances (108003). Twenty-two of the dominant fungal OTUs were found in both ant-accessible plantlets with and without fungicide.

Three of the 23 dominant OTUs (OTU0296, OTU0281, OTU1188) were found in mature ant-plants and the ant-accessible plantlets regardless of fungicide treatment and all three had close matches to fungal OTUs isolated from the waste chambers of *M. beccarii* previously (chapter 2; Greenfield et al. 2021). OTU0296 (*Chaetothyriales* sp.) had a 99% match to OTU0202 from the previous survey, OTU0281 (*Candida fluviatilis*) had a 98% match to OTU0171, and OTU1188 had a 99% match to OTU0977 from the previous survey (chapter 2; Greenfield et al. 2021).

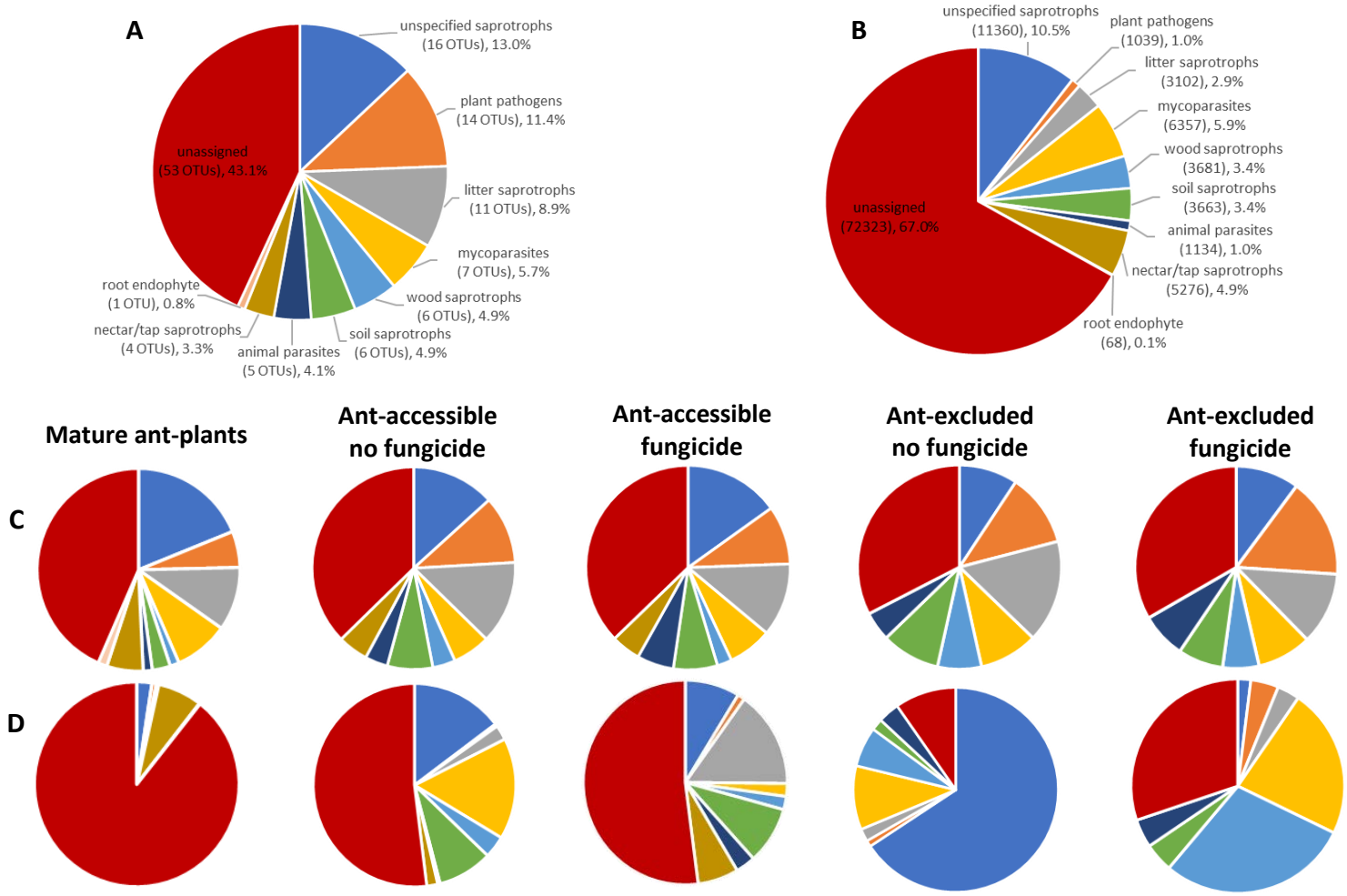


Figure 4.6 Relative proportion of fungal OTUs assigned to fungal guilds including for all samples combined: (A) OTU richness (total 123), (B) OTU sequence abundances (total 108003), and for each of the plants separately: (C) OTU (richness), and (D) OTU sequence abundances. Samples were sequenced from waste chambers of mature ant-plants and four treatment plantlets in the factorial experiment. (C) & (D) are colour-coded to correspond to guilds displayed in (A) and (B).

4.4 Discussion

Myrmecodia beccarii has specialised waste chambers where specific fungi have consistently been found (chapter 2; Greenfield et al. 2021) and nutrients are absorbed from ant-deposited waste (Volp et al. 2022), suggesting fungi might play a nutritional role in this ant-plant mutualism. In the present study, the ant workers successfully transferred the ^{15}N nitrogen tracer to the ant-accessible plantlets confirming the ant workers were feeding nitrogen to the *M. beccarii* plantlets. The fungicide treatment had a weak effect on fungal OTU community compositions in the waste chambers and ^{15}N values did not differ between leaves of ant-accessible plantlets despite fungicide application. However, neither fungal OTU abundances nor fungal OTU richness differed between the ant-accessible plantlets. Also, the fungal guilds and dominant OTUs were also alike in the ant-accessible plantlets, making it difficult to conclude whether or not fungi played a role in the transfer of nitrogen from ant workers to the *M. beccarii* plantlets.

4.4.1 The effect of the fungicide application on the fungal community

However, while there was a significant effect of the fungicide application on fungal OTU community compositions, the effect was weak (R^2=3%) suggesting the fungicide only partially worked. The lack of difference in OTU richness and abundance indicates the fungicide treatment failed to eliminate or adequately reduce fungi in the waste chambers of ant-accessible plantlets that received fungicide. The proportion of fungal OTUs assigned to each of the fungal guilds was remarkably similar for the ant-accessible plantlets (regardless of fungicide treatment) which suggests the compositions of the fungal communities, from a functional perspective, were alike. Finally, 22 of 23 dominant fungal OTUs were found in waste chambers of ant-accessible plantlets (with and without fungicide) which means they were also taxonomically similar.

4.4.2 The transfer of nitrogen in the waste chambers

The $\delta^{15}N$ values in the leaves of the ant-accessible plantlets did not differ despite the fungicide treatment, suggesting fungi did not play a role in the transfer of nitrogen to the ant-accessible plantlets. If the waste chamber fungi were not involved in the transfer of the ^{15}N tracer from ant faeces to the ant-accessible plantlets, it would indicate that *M. beccarii* can absorb nutrients directly via the wart-like protuberances on the surfaces of the waste chambers. This

would support earlier studies of other epiphytic ant-plants that found or suggested nutrients can be absorbed from ant-deposited waste directly through the walls of the domatium (Janzen 1974, Huxley 1978, Rico-Gray et al. 1989, Gay 1993, Watkins et al. 2008, Gegenbauer et al. 2012). In another study, fungi were investigated in the terrestrial ant-plant *L. a. africana* inhabited by *P. phylax* ants, where it was shown that the inner domatium surfaces absorb simple compounds (e.g. glucose and glycine) directly and transfer them to other parts of the plant without a fungal intermediary (Defossez et al. 2011). It is therefore possible that fungi were not required for the $\delta^{15}N$ tracer in the ant faeces to be absorbed via the waste chambers of the ant-accessible plantlets in the present study.

The lack of differences in $\delta^{15}N$ values in leaves of ant-accessible plantlets (with/without fungicide), no differences in fungal OTU richness and sequence abundances, and similarity in fungal guilds and dominant OTUs means I cannot conclude that fungi were not involved in transferring nitrogen. Evidence of nitrogen being transferred to plants via fungi has been confirmed previously in the terrestrial ant-plant *H. physophora*, where a species of *Trimmatostroma* fungi (order Chaetothyriales) improved nitrogen uptake (Leroy et al. 2011, Leroy et al. 2017). Further, in the *L. a. Africana/P. phylax* ant-plant mutualism, Defossez et al. (2011) found that nitrogen was distributed homogenously among the ants, plant, and fungus up to 660 days after being introduced, indicating nitrogen was being recycled within the system, however the role of the fungus remains unclear. As the fungicide application failed in the present study, it is not possible to conclude whether or not any of the fungal OTUs in the waste chambers were involved in the transfer of nitrogen from the ant-deposited faeces to the ant-plantlets.

It is possible that certain waste chamber fungi may not be required for the direct absorption of nutrients from ant faeces but are required to breakdown other detritus such as dead insects, thereby making nutrients from more complex molecules available to *M. beccarii* as has been suggested previously (Huxley 1978, Gay 1993, Defossez et al. 2011, Blatrix et al. 2021). It has been shown that nutrients from dead insects brought into waste chambers by ant workers can be absorbed by ant-plants and translocated to the upper portions of the stem (Rickson 1979). However, fungi have never been investigated as a potential player in nutrient transfer from insect remains to an epiphytic ant-plant. I did not find any dead insects in any of the ant-accessible plantlets at the end of the experiment, but this was expected as the

experiment was conducted in cages with lids and the only insects that were available to the ant workers were mealworms that I fed to the workers. I did find parts of dismembered mealworms in the waste chambers of two of the mature ant-plants at the end of the experiment. If fungi are involved in the breakdown of dead insects and other waste, it would seem likely those fungi are saprotrophs.

4.4.3 Saprotrophic fungi

Fungal OTUs assigned as saprophytic fungi were dominant in terms of richness, accounting for 40% of OTUs in the mature ant-plants and ant-accessible plantlets. However, the sequence abundances of these saprotrophic OTUs totalled only 9.6% for the mature ant-plants, comprising 7% nectar/tap saprotrophs and 2.6% litter, wood, soil, and unspecified saprotrophs. The nectar/tap saprotrophs consisted of four fungal OTUs all from the order Saccharomycetales, two of which were species of *Candida* (OTU1188 and OTU0281). These two OTUs were found previously in the ant-plant survey (chapter 2; Greenfield et al. 2021) and both were transported by *P. cordata* ant workers between individual *M. beccarii* ant-plants (chapter 3) suggesting a possible role in the waste chambers. *Candida fluviatilis* OTU0281 was the most abundant OTU isolated from *P. cordata* ant workers (chapter 3) and had a 98% match to OTU0171 which was found previously (chapter 2; Greenfield et al. 2021). *Candida fluviatilis* is known to biodegrade hydrocarbons (Vetrova et al. 2022) suggesting it may have a role in the breakdown of organic waste in the waste chambers. The small proportional abundances of saprotrophs for litter, wood, soil, and unspecified saprotrophs in the mature ant-plants suggests they were probably not important in the waste chambers of *M. beccarii* and instead are opportunistic. It is also possible these litter, wood, soil, and unspecified saprotrophs were transferred from the accessible plantlets to the mature ant-plants by the ant workers.

4.4.4 Fungal OTUs not assigned to fungal guilds

A large proportion of fungal OTU sequence abundances were unable to be assigned to any guilds. The three-month duration of the experiment may have been insufficient for the fungal community in the mature ant-plants to be established in the ant-accessible plantlets. This is evident in the assignment of fungal OTUs to functional guilds, particularly with regard to the OTU sequence abundances. For example, some OTUs could not be assigned to any guild, and

these comprised the majority of relative OTU sequence abundances in mature ant-plants (89%) and approximately half of OTU sequence abundances in the ant-accessible plantlets. These unassigned OTUs have not been recorded before with respect to their functional roles in ecosystems so it remains to be discovered what (if any) roles they play in the waste chambers of *M. beccarii*. One of these unassigned OTUs (OTU0296, Chaetothyriales sp.) was dominant in mature ant-plants and ant-accessible plantlets despite fungicide treatment and was found on heads of ant workers (chapter 3) and had a 99% match to OTU0202 which was isolated from the waste chambers of *M. beccarii* previously (chapter 2; Greenfield et al. 2021). This OTU0296/0202 is of interest because it had a close taxonomic match to a Chaetothyriales fungus (KhNk3-2) found in domatium of the ant-plant *Keetia hispida* in Cameroon occupied by *Crematogaster* ants (Voglmayr et al. 2011) and was more recently isolated from pitcher leaves of the ant-plant *Dischidia major* occupied by *Philidris* ants in Thailand (Blatrix et al. 2021). The role of this fungus in these different ant-plant systems on different continents remains to be determined.

4.4.5 The fungicide application technique

As far as I am aware, the fungicide application technique used in this study to remove fungi from domatium of an epiphytic ant-plant has not been attempted before, and several scenarios could explain why it failed to eliminate/suppress the fungi: (a) the fungicides used were not targeted specifically for the fungi located in the waste chambers, some or all of which may have been resistant to the chosen fungicides; (b) the application frequency and/or concentration of the fungicides were not sufficient, given the ant workers may have been continuously bringing fungi from the mature ant-plants into the waste chambers of the ant-accessible plantlets (chapter 3); (c) the application method for injection of the amphotericin B fungicide was ineffective; and (d) all or some of these scenarios combined. Injection sites for the amphotericin B were chosen based on our observations from prior dissections that the waste chambers tend to be located in the domatium beneath the stem around the upper part of the domatium. However, as I could not see the waste chambers, it is possible some waste chambers did not receive the injections of fungicide or did not receive enough fungicide over the duration of the experiment.

4.4.6 Conclusion

The role of fungi in this ant-plant mutualism remains unknown, but there are interesting outcomes from this study and avenues for future research. Saprotrophic fungi were dominant in the waste chambers of mature ant-plants in terms of richness but unassigned OTUs dominated in terms of abundance. Saprotrophs are known to breakdown organic matter, and it is possible that saprophytic fungi found in the waste chambers play a role in the breakdown of non-faecal waste such as dead insects and other detritus. Two fungal OTUs assigned as saprotrophs, both *Candida* species (OTU0281 and OTU1188), stood out in the waste chambers. Future research could isolate these OTUs in order to study them more closely – for instance, enzymatic assays to investigate the ability of these OTUs to breakdown various substances such as chitin, cellulose, and lignin (Baldrian et al. 2011). Alternatively, fungi may not be involved in nutrient transfer and instead be used as a source of food for the ant colony. Future studies could sample the gut contents of workers, larvae, alate queens of *P. cordata* ant colonies to establish whether or not they are consuming any fungi from the waste chambers. Fungal OTUs suspected of being food for the colony could be isolated onto media and a cafeteria experiment could be run to offer the workers different fungi and observe their behaviour. Fungal OTUs that have potential as antibiotics could be isolated and experimental assays conducted to determine if there is potential of any fungal OTUs against bacteria in the chambers or on members of the colony. Bacterial DNA would need to be sequenced prior to such assays. We still have much to learn about the fungal community in the waste chambers of *M. beccarii.* A large portion of the OTUs in the mature ant-plants could not be assigned to any functional guild. Unassigned OTU0296 (Chaetothyriales sp.) warrants further investigation because it was dominant in mature ant-plants and ant-accessible plantlets, was found on heads of ant workers (chapter 3) and has previously been found in two other ant-plants in Cameroon and Thailand.

4.5 Supplementary Information

Supplementary Table S4.1 Taxonomic assignment of 123 fungal operational taxonomic units (OTUs) in the waste chambers of *M. beccarii* ant-plants. The OTUs have a minimum read abundance of 10 in at least one of the plant types including mature ant-plants and the plantlets: ant-accessible/no fungicide, ant-accessible/fungicide, ant-excluded/no fungicide), and ant-excluded/fungicide. Fungal taxon and Order are closest taxonomic match using UNITE database and the APSurvey database from an ant-plant survey (Greenfield et al. 2021). If OTU had a match >97% to an OTU found during ant-plant survey, the OTU ID from that survey appears under the column headed "APSurvey OTU ID" together with the percentage sequence similarity. The UNITE SH number and Genbank Accession numbers are included as well as the percentage sequence similarity of the OTU sequence. Fungal guild is the primary lifestyle of the fungal OTU at the level of genera using the online database FungalTraits and if blank, the OTU was unassigned. Fungal OTU IDs in bold (23 in total) were dominant (i.e., occurring with a minimum sequence abundance of 10 in at least 50% of mature ant-plants, or 20% of ant-accessible or ant-excluded plantlets).

OTU ID	Fungal Taxon	Order	APSurvey OTU ID & sequence similarity	UNITE SH number	GenBank Accession number	Sequence similarity	Total abundance	Fungal Guild
OTU0038	*Moesziomyces aphidis*	Ustilaginales		SH1509407.08FU	KY037841	99%	27	plant pathogen
OTU0054	*Verruconis* sp.	Venturiales		SH1549842.08FU	LM644533	94%	90	animal parasite
OTU0057	*Verruconis gallopava*	Venturiales		SH1549842.08FU	LM644533	99%	825	animal parasite
OTU0089	Fungi sp.	unknown		SH491217.07FU	KU195499	100%	23756	
OTU0112	Agaricomycetes sp.	unknown		SH1185637.08FU	HM037681	92%	1093	
OTU0116	Clavulinaceae sp.	Cantharellales		SH1517446.08FU	KT779284	97%	15	
OTU0117	Agaricomycetes sp.	unknown		SH1185637.08FU	HM037681	94%	185	
OTU0129	*Amplistroma* sp.	Hypocreales		SH1238250.08FU	KC907376	91%	321	wood saprotroph
OTU0137	Basidiomycota sp.	unknown		SH016338.07FU	AB907585	99%	10	
OTU0166	*Ganoderma* sp.	Polyporales		SH1555638.08FU	KY471677	98%	25	plant pathogen
OTU0169	Eurotiales sp.	Eurotiales		SH523442.07FU	AB986366	98%	23	
OTU0174	*Sterigmatomyces halophilus*	Agaricostilbales		SH1546026.08FU	KY792630	99%	22	unspecified saprotroph

OTU ID	Fungal Taxon	Order	APSurvey OTU ID & sequence similarity	UNITE SH number	GenBank Accession number	Sequence similarity	Total abundance	Fungal Guild
OTU0175	*Colacogloea terpenoidalis*	Microbotryomycetes (order Incertae sedis)		SH1522429.08FU	KY102579	99%	304	myco-parasite
OTU0176	Elsinoaceae sp.	Myriangiales		SH1613720.08FU	KX887199	82%	10	
OTU0179	Serendipitaceae sp.	Sebacinales		SH1555098.08FU	JX317218	90%	16	
OTU0186	Sebacinales sp.	Sebacinales			JF691010	87%	97	
OTU0193	Chaetothyriales sp.	Chaetothyriales		SH014776.07FU	HQ634633	99%	69	
OTU0204	Sebacinales sp.	Sebacinales		SH1544495.08FU	KF359622	85%	77	
OTU0207	*Exophiala* sp.	Chaetothyriales		SH1529655.08FU	KF614882	96%	15	animal parasite
OTU0219	Chaetothyriales sp.	Chaetothyriales		SH1529656.08FU	KF614875	98%	81	
OTU0231	*Fonsecaea* sp.	Chaetothyriales		SH1529604.08FU	KF928456	99%	682	soil saprotroph
OTU0235	*Schwanniomyces polymorphus*	Saccharomycetales		SH1516577.08FU	KY105394	99%	298	nectar/tap saprotroph
OTU0241	*Meira* sp.	Exobasidiales		SH1649628.08FU	KF435880	96%	33	plant pathogen
OTU0247	Tremellales sp.	Tremellales		SH1245523.08FU	HG938309	81%	27	
OTU0257	*Cyphellophora oxyspora*	Chaetothyriales		SH1573966.08FU	KY792629	99%	66	litter saprotroph
OTU0276	*Fonsecaea brasiliensis*	Chaetothyriales		SH1529592.08FU	KP132190	99%	53	soil saprotroph
OTU0277	*Cladophialophora immunda*	Chaetothyriales		SH1529600.08FU	KC886406	100%	149	soil saprotroph
OTU0281	*Candida fluviatilis*	Saccharomycetales	OTU0171 (98%)	SH200664.07FU	HQ652068	99%	1246	nectar/tap saprotroph
OTU0296	Chaetothyriales sp.	Chaetothyriales	OTU0202 (99%)	SH196444.07FU	HQ634649	96%	8849	
OTU0299	Fungi sp.	unknown		SH491217.07FU	KU195499	97%	1177	

OTU ID	Fungal Taxon	Order	APSurvey OTU ID & sequence similarity	UNITE SH number	GenBank Accession number	Sequence similarity	Total abundance	Fungal Guild
OTU0309	*Sporothrix eucalyptigena*	Ophiostomatales		SH1552347.08FU	KU865588	99%	442	plant pathogen
OTU0320	*Rhinocladiella similis*	Chaetothyriales		SH1575246.08FU	KY780281	97%	75	soil saprotroph
OTU0324	Agaricomycetes sp.	unknown		SH1571979.08FU	UDB036216	78%	43	
OTU0338	*Trichoderma harzianum*	Hypocreales		SH1567965.08FU	KX092003	99%	1364	myco-parasite
OTU0339	Chaetothyriales sp.	Chaetothyriales		SH1563587.08FU	EU624333	84%	22	
OTU0340	*Lepteutypa fuckelii*	Xylariales		SH1563692.08FU	KT949902	92%	31	plant pathogen
OTU0349	*Exophiala spinifera*	Chaetothyriales		SH1575245.08FU	MH010935	99%	62	animal parasite
OTU0362	*Rhodotorula mucilaginosa*	Sporidiobolales		SH1558726.08FU	KY611842	99%	54	unspecified saprotroph
OTU0366	Debaryomycetaceae sp.	Saccharomycetales		SH192552.07FU	KP109748	96%	3415	
OTU0379	*Arthrocladium* sp.	Pezizomycotina (order Incertae sedis)	OTU0289 (98%)	SH208969.07FU	KT337442	99%	2576	soil saprotroph
OTU0380	Debaryomycetaceae sp.	Saccharomycetales		SH192552.07FU	KP109748	97%	389	
OTU0391	Saccharomycetales sp.	Saccharomycetales		SH192555.07FU	AY553844	97%	1774	
OTU0400	Fungi sp.	unknown	OTU0291 (99%)	SH491823.07FU	KM104119	99%	28	
OTU0407	*Phaeoacremonium parasiticum*	Togniniales		SH1581104.08FU	LC203479	99%	28	plant pathogen
OTU0410	Fungi sp.	unknown		SH479386.07FU	AJ877193	99%	41	
OTU0417	*Trichoderma atroviride*	Hypocreales		SH1567966.08FU	MF541418	99%	3658	myco-parasite
OTU0422	Eurotiomycetes sp.	unknown		SH206547.07FU	DQ914677	98%	13042	

OTU ID	Fungal Taxon	Order	APSurvey OTU ID & sequence similarity	UNITE SH number	GenBank Accession number	Sequence similarity	Total abundance	Fungal Guild
OTU0436	Eurotiales sp.	Eurotiales		SH523442.07FU	AB986366	99%	30	
OTU0443	*Phaeoacremonium venezuelense*	Togniniales		SH1581109.08FU	KF764568	99%	86	plant pathogen
OTU0446	*Acremonium polychromum*	Hypocreales		SH1555961.08FU	MH469511	99%	58	unspecified saprotroph
OTU0450	Fungi sp.	unknown		SH479386.07FU	AJ877193	99%	2081	
OTU0454	*Metacordyceps* sp.	Hypocreales		SH1644900.08FU	JX978429	94%	142	animal parasite
OTU0468	*Talaromyces radicus*	Eurotiales		SH1516156.08FU	MH298756	99%	18	unspecified saprotroph
OTU0470	Fungi sp.	unknown		SH479386.07FU	AJ877193	99%	68	
OTU0471	*Talaromyces* sp.	Eurotiales		SH1552152.08FU	MH784402	99%	10004	unspecified saprotroph
OTU0482	Phaeomoniellales sp.	Phaeomoniellales		SH1538725.08FU	KF675294	89%	30	
OTU0507	*Aspergillus flavus*	Eurotiales		SH1161994.08FU	KY065346	99%	411	unspecified saprotroph
OTU0509	Chaetothyriales sp.	Chaetothyriales		SH212029.07FU	KC951221	99%	494	
OTU0510	*Paraconiothyrium estuarinum*	Pleosporales		SH1525446.08FU	KX611652	99%	51	wood saprotroph
OTU0515	Chaetothyriales sp.	Chaetothyriales		SH1648673.08FU	KX822513	98%	71	
OTU0533	*Penicillium daleae*	Eurotiales		SH1160518.08FU	KY315582	97%	173	unspecified saprotroph
OTU0573	Fungi sp.	unknown		SH1516144.08FU	KU837575	99%	2478	
OTU0581	*Diaporthe* sp.	Diaporthales		SH1540624.08FU	KF159970	98%	13	plant pathogen
OTU0589	Conioscyphales sp.	Conioscyphales		SH1179227.08FU	GQ924027	93%	115	
OTU0608	Stachybotryaceae sp.	Hypocreales		SH1557966.08FU	KJ834351	99%	12	

OTU ID	Fungal Taxon	Order	APSurvey OTU ID & sequence similarity	UNITE SH number	GenBank Accession number	Sequence similarity	Total abundance	Fungal Guild
OTU0616	*Phaeoacremonium krajdenii*	Togniniales		SH1581115.08FU	KU060814	94%	40	plant pathogen
OTU0620	*Penicillium consobrinum*	Eurotiales		SH1529989.08FU	MG490873	99%	58	unspecified saprotroph
OTU0631	Conioscyphales sp.	Conioscyphales		SH1179227.08FU	GQ924027	95%	20	
OTU0647	Talaromyces sp.	Eurotiales		SH1516144.08FU	KF366489	97%	95	unspecified saprotroph
OTU0652	*Rhodotorula* sp.	Sporidiobolales		SH1182329.08FU	KU057818	94%	18	unspecified saprotroph
OTU0658	Chaetothyriales sp.	Chaetothyriales		SH014029.07FU	KF675595	99%	228	
OTU0672	*Diaporthe* sp.	Diaporthales		SH1540607.08FU	EU054414	99%	51	plant pathogen
OTU0682	Talaromyces sp.	Eurotiales		SH1516144.08FU	KY474345	99%	18	unspecified saprotroph
OTU0685	Chaetothyriales sp.	Chaetothyriales		SH212163.07FU	HM239979	98%	3378	
OTU0710	Coniochaeta sp.	Coniochaetales		SH1645099.08FU	KJ583241	99%	108	unspecified saprotroph
OTU0737	Fungi sp.	unknown	OTU0544 (99%)		KX247125	98%	135	
OTU0752	Dothideomycetes sp.	unknown	OTU0567 (97%)	SH021234.07FU	EU977184	97%	50	
OTU0753	*Fusarium solani*	Hypocreales	OTU0619 (98%)	SH205225.07FU	AM412643	99%	71	plant pathogen
OTU0754	Fungi sp.	unknown		SH204253.07FU	KF675628	85%	24	
OTU0761	*Scytalidium* sp.	Helotiales		SH1185415.08FU	HM134143	99%	226	wood saprotroph
OTU0773	*Chaetomium* sp.	Sordariales		SH1615600.08FU	GQ922579	99%	164	litter saprotroph
OTU0779	*Scytalidium lignicola*	Helotiales		SH1185468.08FU	HM214453	99%	66	wood saprotroph

OTU ID	Fungal Taxon	Order	APSurvey OTU ID & sequence similarity	UNITE SH number	GenBank Accession number	Sequence similarity	Total abundance	Fungal Guild
OTU0834	Fungi sp.	unknown		SH204253.07FU	KF675628	99%	93	
OTU0835	*Mariannaea* sp.	Hypocreales		SH1506691.08FU	MH734517	99%	2993	wood saprotroph
OTU0836	*Pezicula radicicola*	Helotiales	OTU0667 (97%)	SH201622.07FU	HQ889715	99%	68	root endophyte
OTU0837	Tremellomycetes	unknown		SH203621.07FU	KF225912	99%	362	
OTU0845	*Chloridium* sp.	Chaetosphaeriales		SH1517983.08FU	AB734790	97%	2162	litter saprotroph
OTU0846	Helotiales sp.	Helotiales		SH1236101.08FU	GQ892249	95%	77	
OTU0858	Xylariales sp.	Xylariales		SH1513186.08FU	MG649300	99%	26	
OTU0862	*Gliocladium* sp.	Hypocreales		SH1644389.08FU	DQ682585	99%	45	myco-parasite
OTU0870	*Corynespora* sp.	Pleosporales		SH1591179.08FU	JQ686921	100%	14	plant pathogen
OTU0873	Ascomycota sp.	unknown			KU687400	89%	232	
OTU0883	Sordariales sp.	Sordariales		SH1563553.08FU	AB986406	89%	16	
OTU0884	Hypocreales sp.	Hypocreales		SH1178082.08FU	JQ905794	92%	10	
OTU0885	*Nigrospora* sp.	Trichosphaeriales		SH1549606.08FU	MG976425	99%	174	litter saprotroph
OTU0900	*Nigrospora oryzae*	Trichosphaeriales		SH1549605.08FU	MG832530	99%	256	litter saprotroph
OTU0905	*Nigrospora oryzae*	Trichosphaeriales		SH1549605.08FU	MG489868	98%	15	litter saprotroph
OTU0923	*Cladosporium sphaerospermum*	Capnodiales		SH1572792.08FU	KF177679	99%	40	litter saprotroph
OTU0924	*Cladosporium sphaerospermum*	Capnodiales		SH1572792.08FU	MF467891	99%	39	litter saprotroph
OTU0930	*Penicillium* sp.	Eurotiales		SH1529986.08FU	KF367520	100%	225	unspecified saprotroph
OTU0943	*Lecythophora* sp.	Coniochaetales	OTU0731 (98%)		KT265801	100%	48	unspecified saprotroph

OTU ID	Fungal Taxon	Order	APSurvey OTU ID & sequence similarity	UNITE SH number	GenBank Accession number	Sequence similarity	Total abundance	Fungal Guild
OTU0944	Xylariales sp.	Xylariales		SH1149880.08FU	HM595556	94%	10	
OTU0946	*Cladosporium aciculare*	Capnodiales		SH1572792.08FU	KT600411	99%	125	litter saprotroph
OTU0954	*Nigrospora oryzae*	Trichosphaeriales		SH1549605.08FU	MG322174	100%	15	litter saprotroph
OTU0957	*Cladosporium oxysporum*	Capnodiales		SH1572792.08FU	MG063185	99%	46	litter saprotroph
OTU0959	*Fusarium oxysporum*	Hypocreales		SH1610159.08FU	MH333106	95%	123	plant pathogen
OTU0961	Fungi sp.	unknown		SH1190232.08FU	MF976652	90%	80	
OTU0974	Fungi sp.	unknown		SH025821.07FU	KC222753	99%	15	
OTU1010	Fungi sp.	unknown		SH1514197.08FU	KF675509	96%	161	
OTU1021	Fungi sp.	unknown		SH210983.07FU	KF675509	98%	100	
OTU1026	*Candida* sp.	Saccharomycetales		SH203686.07FU	JQ683772	98%	2230	nectar/tap saprotroph
OTU1035	*Papiliotrema laurentii*	Tremellales		SH1576892.08FU	KY445944	99%	933	myco parasite
OTU1105	*Phaeosphaeriopsis* sp.	Pleosporales		SH1646320.08FU	KU529841	96%	55	plant pathogen
OTU1138	*Fellomyces penicillatus*	Tremellales		SH1539826.08FU	KY103409	98%	35	unspecified saprotroph
OTU1158	Fungi sp.	unknown		SH200320.07FU	KF617293	98%	93	
OTU1178	*Apiotrichum mycotoxinivorans*	Trichosporonales		SH1616869.08FU	KY792632	99%	128	soil saprotroph
OTU1187	*Papiliotrema* sp.	Tremellales		SH1576891.08FU	LC191360	99%	23	myco-parasite
OTU1188	*Candida* sp.	Saccharomycetales	OTU0977 (99%)	SH495679.07FU	KM504131	99%	1502	nectar/tap saprotroph

OTU ID	Fungal Taxon	Order	APSurvey OTU ID & sequence similarity	UNITE SH number	GenBank Accession number	Sequence similarity	Total abundance	Fungal Guild
OTU1190	*Fellomyces* sp.	Tremellales		SH176359.07FU	NR 073258	97%	15	unspecified saprotroph
OTU1195	*Calocera* sp.	Dacrymycetales		SH1563450.08FU	KP013031	98%	24	wood saprotroph
OTU1213	Saccharomycetales sp.	Saccharomycetales		SH495679.07FU	KM504131	99%	442	
OTU1221	*Tremella* sp.	Tremellales		SH1523540.08FU	FN428949	99%	30	myco-parasite
OTU1266	Tremellomycetes sp.	unknown		SH477174.07FU	JX999048	99%	7153	

Supplementary Table S4.2 Output of a simple linear regression to test relationship between fungal OTU richness (observed values) and sequencing depth (number of sequences per sample) in the waste chambers of the treatment *M. beccarii* plantlets in the ant exclusion/fungicide application factorial experiment.

	Estimate	Std. Error	t-value	p-value
Intercept	16.76276	1.485811	11.282	<0.0001
Sequencing depth	0.008997	0.001057	8.515	<0.0001

Residual standard error: 8.274 on 48 degrees of freedom
Multiple R-squared: 0.6017, Adjusted R-squared: 0.5934
F-statistic: 72.51 on 1 and 48 DF, p-value: 3.750e-07

Supplementary Table S4.3 Dominant fungal operational taxonomic units (OTUs) in waste chambers of *M. beccarii* with minimum read abundance of 10 in at least 50% of mature ant-plant samples (grey), and/or at least 20% of one of the ant plantlets, i.e., ant-accessible/no fungicide (AANF, dark green), ant-accessible plantlets/fungicide (AAF, light green), ant-excluded plantlets/no fungicide (AENF, dark purple), ant-excluded plantlets/fungicide (AEF, light purple). Fungal Taxon represents the closest match to a sequence in the UNITE database or the APSurvey database created from fungal sequences obtained during the ant-plant survey (chapter 2, Greenfield et al. 2021) in which case the OTU ID is underlined (for more details see Supplementary Table S4.1). Column entitled "total abundance" is the total sequence abundances across all samples for each OTU. Values headed "Relative Abundance" are percentage sequence abundances of OTUs across the waste chamber samples. Values under "Occurrence" are percentages of chamber samples (for each plant type) that had the fungal OTU and colour-shaded values highlight where OTUs were dominant as defined above. Under "Fungal Guild" the abbreviation SAP = saprotroph and if a cell is left blank, the OTU unassigned.

Fungal OTU ID	Fungal Taxon	total abundance	Relative Abundance (% sequence abundances in waste chamber samples)					Occurrence (% of waste chamber samples with OTU)					Fungal Guild
			MAT	AANF	AAF	AENF	AEF	MAT	AANF	AAF	AENF	AEF	
OTU0391	Saccharomycetales sp.	1774	99.9	0	0	0	0.1	100	0	0	0	7.7	
OTU0450	Fungi sp.	2081	99.8	0.1	0.1	0	0	61.5	15.4	7.7	0	0	
OTU0366	Debaryomycetaceae sp.	3415	99.5	0.3	0.2	0	0	100	30.8	30.8	0	0	
OTU0685	Chaetothyriales sp.	3378	98.8	0.5	0.7	0	0	69.2	15.4	23.1	0	0	
OTU1266	Tremellomycetes sp.	7153	94.8	2.8	2.5	0	0.01	100	61.5	53.8	0	7.7	
OTU0089	Fungi sp.	23756	84.9	4.6	10.5	0.01	0.03	100	76.9	92.3	15.4	23.1	
OTU0422	Eurotiomycetes sp.	13042	65.4	20.6	13.9	0.02	0.04	100	61.5	84.6	23.1	23.1	
OTU0296[a]	Chaetothyriales sp.	8849	25.0	64.3	10.7	0	0.02	84.6	46.2	61.5	0	7.7	
OTU1188	*Candida* sp.	1502	57.8	17.4	24.8	0	0	69.2	53.8	61.5	0	0	nectar/tap SAP
OTU0509	Chaetothyriales sp.	494	37.9	17.8	43.7	0.6	0	100	61.5	61.5	15.4	0	
OTU0471	*Talaromyces* sp.	10004	7.2	30.7	7.9	53.3	0.8	100	92.3	84.6	38.5	38.5	unspecified SAP
OTU0281	*Candida fluviatilis*	1246	72.2	4.7	23.0	0	0	100	69.2	84.6	0	0	nectar/tap SAP
OTU0299	Fungi sp.	1177	81.2	5.1	13.7	0	0	100	53.8	69.2	0	0	
OTU0930	*Penicillium* sp.	225	32.0	20.4	43.1	0	4.4	53.8	46.2	76.9	0	7.7	unspecified SAP

Fungal OTU ID	Fungal Taxon	total abundance	Relative Abundance (% sequence abundances in waste chamber samples)					Occurrence (% of waste chamber samples with OTU)					Fungal Guild
			MAT	AANF	AAF	AENF	AEF	MAT	AANF	AAF	AENF	AEF	
OTU0379	*Arthrocladium* sp.	2576	1.0	66.9	31.8	0	0.3	30.8	46.2	76.9	0	15.4	soil SAP
OTU1035	*Papiliotrema laurentii*	933	0.4	92.6	2.8	2.8	1.4	7.7	53.8	30.8	23.1	15.4	mycoparasite
OTU0573	Fungi sp.	2478	0.2	2.5	21.3	6.6	69.4	30.8	69.2	61.5	84.6	100	
OTU0057	*Verruconis gallopava*	825	0	8.2	36.7	30.2	24.8	0	61.5	53.8	61.5	61.5	animal parasite
OTU0231	*Fonsecaea* sp.	682	0	7.2	43.3	16.7	32.8	0	53.8	46.2	38.5	61.5	soil SAP
OTU0417	*Trichoderma atroviride*	3658	0.3	60.5	6.0	10.4	22.8	23.1	84.6	61.5	92.3	84.6	mycoparasite
OTU0338	*Trichoderma harzianum*	1364	0.3	30.4	1.0	31.1	37.2	7.7	30.8	23.1	69.2	69.2	mycoparasite
OTU0835	*Mariannaea* sp.	2993	0.03	19.2	9.4	14.2	57.2	7.7	92.3	30.8	92.3	76.9	wood SAP
OTU0175	*Colacogloea terpenoidalis*	598	1.8	5.7	1.2	0.8	41.3	23.1	46.2	23.1	23.1	38.5	mycoparasite

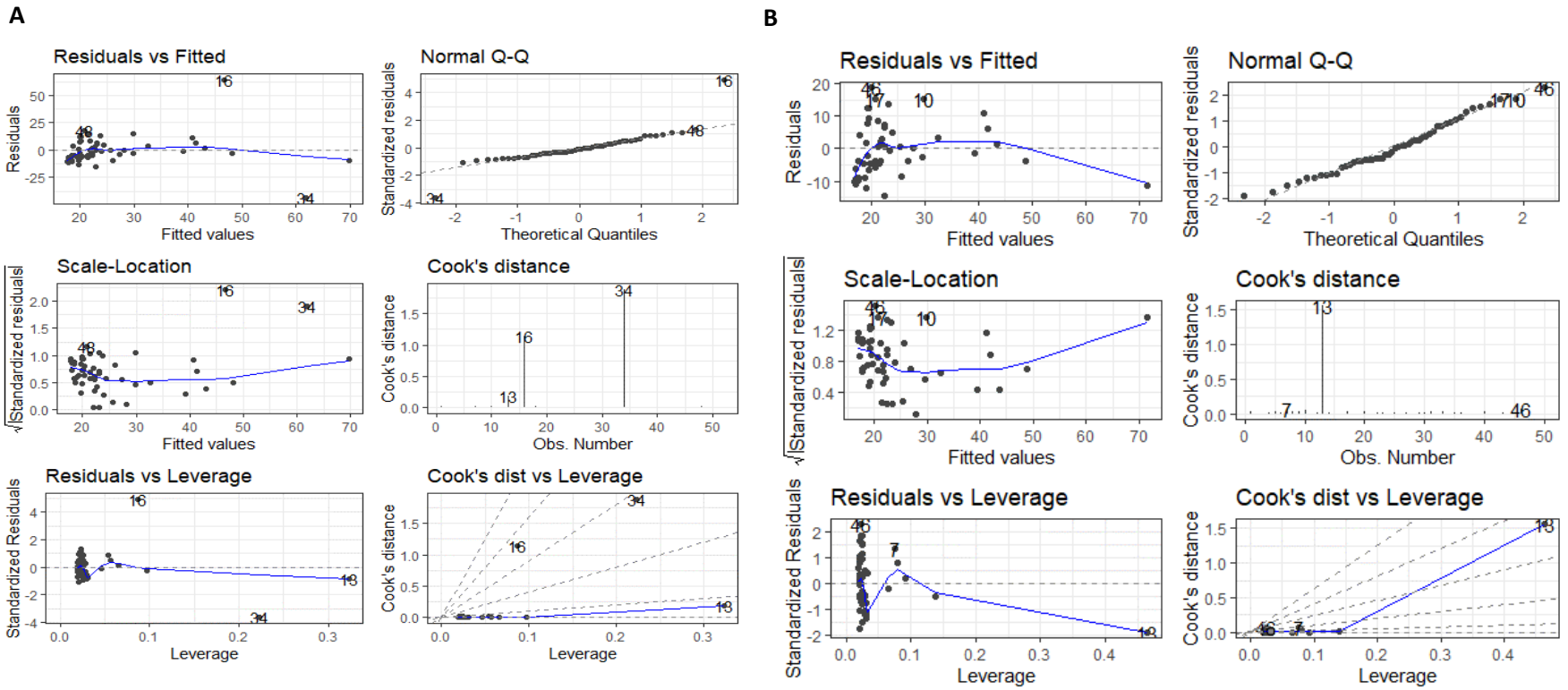
[a] Fungal OTU0296 – closest match 99% to OTU0202 from ant-plant survey (Greenfield et al. 2021) which had a closest match to KhNk3-2 from domatia of terrestrial ant-plant *Keetia hispida* (Rubiaceae) in Cameroon (ant species: *Crematogaster* sp. (Myrmicinae)) (Voglmayr et al. 2011).

Supplementary Table S4.4 Assignment of fungal OTUs to fungal guilds including the number of OTUs assigned to each of the guilds (OTU richness) and relative OTU richness across guilds for each plant including the mature ant-plants and the four plantlets in the factorial experiment: ant-accessible/no fungicide, ant-accessible/fungicide, ant-excluded/no fungicide, and ant-excluded/fungicide. There were 123 fungal OTUs from 65 waste chamber samples.

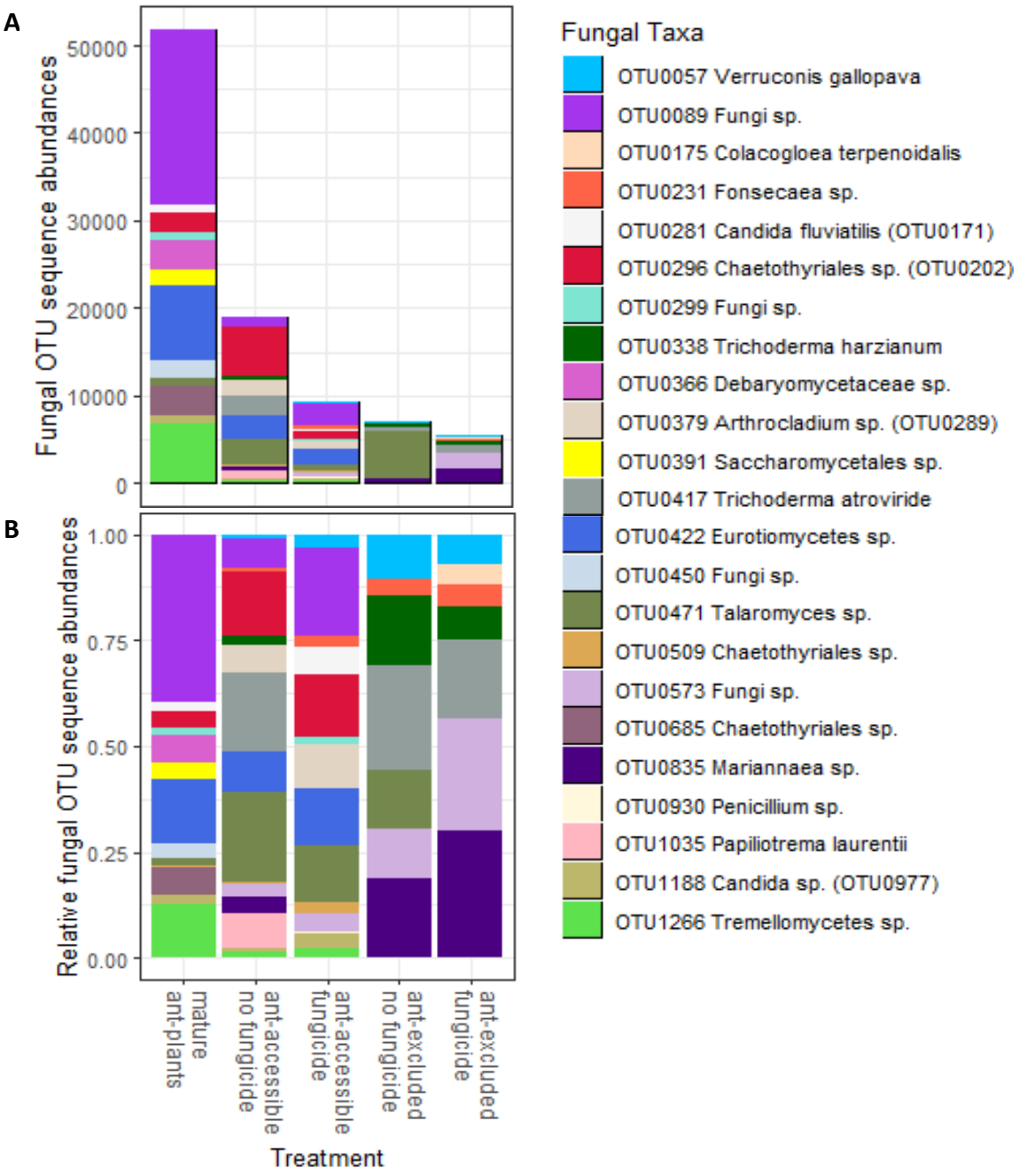
	OTU richness						Relative OTU richness (%)					
			ant-accessible		ant-excluded				ant-accessible		ant-excluded	
Fungal Guild	Total for all samples	mature ant-plants	no fungicide	fungicide	mature ant-plants	fungicide	Total for all samples	mature ant-plants	no fungicide	fungicide	no fungicide	fungicide
unspecified saprotrophs	16	13	11	13	4	7	13.0	18.8	13.3	15.1	9.3	10.1
litter saprotrophs	11	7	11	10	7	8	8.9	10.1	13.3	11.6	16.3	11.6
wood saprotrophs	6	1	3	2	3	4	4.9	1.4	3.6	2.3	7.0	5.8
soil saprotrophs	6	2	6	6	4	5	4.9	2.9	7.2	7.0	9.3	7.2
nectar/tap saprotrophs	4	4	4	4	0	0	3.3	5.8	4.8	4.7	0	0
plant pathogens	14	4	9	8	5	11	11.4	5.8	10.8	9.3	11.6	15.9
mycoparasites	7	6	5	6	4	6	5.7	8.7	6.0	7.0	9.3	8.7
animal parasites	5	1	3	5	2	5	4.1	1.4	3.6	5.8	4.7	7.2
root endophytes	1	1	0	0	0	0	0.8	1.4	0	0	0	0
unassigned	53	30	31	32	14	23	43.1	43.5	37.3	37.2	32.6	33.3
Total	123	69	83	86	43	69	100	100	100	100	100	100

Supplementary Table S4.5 Assignment of fungal OTU sequence abundances to fungal guilds including the sequence abundances of OTUs assigned to each of the guilds and relative OTU sequence abundances across guilds for each plant including the mature ant-plants and the four plantlets in the factorial experiment: ant-accessible/no fungicide, ant-accessible/fungicide, ant-excluded/no fungicide, and ant-excluded/fungicide. There were a total 108003 sequence abundances from 123 fungal OTUs collected from 65 waste chamber samples.

	OTU sequence abundances						**Relative OTU sequence abundances (%)**					
			ant-accessible		ant-excluded				ant-accessible		ant-excluded	
Fungal Guild	Total for all samples	mature ant-plants	no fungicide	mature ant-plants	mature ant-plants	mature ant-plants	Total for all samples	mature ant-plants	no fungicide	fungicide	no fungicide	fungicide
unspecified saprotrophs	11360	1363	3222	1190	5443	142	10.5	2.4	14.7	8.7	65.7	2.0
litter saprotrophs	3102	63	520	2106	167	246	2.9	0.1	2.4	15.3	2.0	3.4
wood saprotrophs	3681	1	776	286	522	2096	3.4	0.002	3.5	2.1	6.3	29.0
soil saprotrophs	3663	36	1911	1247	152	317	3.4	0.1	8.7	9.1	1.8	4.4
nectar/tap saprotrophs	5276	4005	382	889	0	0	4.9	7.0	1.7	6.5	0	0
plant pathogens	1039	403	90	160	81	305	1.0	0.7	0.4	1.2	1.0	4.2
mycoparasites	6357	82	3527	278	836	1634	5.9	0.1	16.1	2.0	10.1	22.6
animal parasites	1134	26	87	431	279	311	1.0	0.05	0.4	3.1	3.4	4.3
root endophytes	68	68	0	0	0	0	0.1	0.1	0	0	0	0
unassigned	72323	50838	11359	7143	803	2180	67.0	89.4	51.9	52.0	9.7	30.1
Total	108003	56885	21874	13730	8283	7231	100	100	100	100	100	100



Supplementary Figure S4.1 Diagnostic plots including outlier identification for the linear model testing the relationship between fungal OTU richness (observed values) and sequencing depth (number of sequences per sample) in the waste chambers of the treatment *Myrmecodia beccarii* plantlets in the ant exclusion/fungicide application factorial experiment. Plot A displayed two outliers (samples numbered 16 and 34 on the Cook's distance plot) which were removed prior to running the model, and Plot B shows the diagnostic plots after the outlier was removed.



Supplementary Figure S4.2 Dominant fungal operational taxonomic units (OTUs) in the waste chambers of *M. beccarii* including the mature ant-plants and four treatment plantlets including (A) fungal OTU sequence abundances and (B) relative fungal OTU sequence abundances.
If a fungal OTU had a close match to an OTU previously found in the ant-plant survey (chapter 2; Greenfield et al. 2021) the OTU ID for that close match is included at the end in parentheses.

Chapter 5 - Synthesis and conclusions

5.1 Thesis overview

I have endeavoured throughout this thesis to establish whether fungi are involved in the *Philidris cordata-Myrmecodia beccarii* ant-plant mutualism. I have achieved this, in part, by finding evidence that supports fungi being involved in this mutualism. I found a pattern in the fungal communities in each of the chambers of *M. beccarii* that was consistent over the 675 km of fragmented distribution of *M. beccarii* which suggests fungi have a long association with this ant-plant (chapter 2; Greenfield et al. 2021). I also found that the *P. cordata* ant workers transport fungi between *M. beccarii* ant-plants indirectly on their exoskeletons, and possibly more directly from their heads, which would ensure dispersal for the chamber fungi (chapter 3). However, I was unable to conclude that fungi are involved in the transfer of nitrogen to *M. beccarii* from waste deposited by the ant workers (chapter 4). I did find that saprotrophic fungi dominate the waste chambers of *M. beccarii* in terms of richness, suggesting fungi could be playing a role in nutrient transfer from organic waste deposited by the ant workers (chapter 4). In terms of abundance, fungal OTUs that could not be assigned to trophic guilds were dominant, including Chaetothyriales fungi, indicating that we have much more to learn about the functional roles of fungi in this ant-plant (chapter 4).

In conducting this study, I have taken the first steps to determine what role(s) fungi may be playing in the *P. cordata/M. beccarii* ant-plant mutualism by: (1) identifying the fungi/fungal community, (2) establishing how fungi are transferred to (or with) other partners in the mutualism over generations, and (c) investigating what services or rewards are being exchanged between fungi and the other partners in the mutualism. The survey and experiments I conducted to collect fungal samples, which were identified using high throughput sequencing of fungal DNA, allowed me to answer the following questions:

1. What fungi are in the domatium chambers of *M. beccarii*?
2. Does the fungal community differ with chamber type?
3. Does the fungal community differ across locations within the distribution of *M. beccarii*?
4. Are fungi transported by *P. cordata* workers between *M. beccarii* ant-plants and if so, how?
5. Do fungi play a nutritional role in the *P. cordata/M. beccarii* mutualism?

In this final chapter, I summarise and synthesise my key findings, address the limitations of my research project, and provide some future directions.

5.2 Summary and synthesis of key findings

I have documented the fungi that are in the domatium chambers of *M. beccarii* and found that the fungal community differs with chamber type across locations within the distribution of *M. beccarii.* There appears to be a long association between *M. beccarii*, the resident *P. cordata* ants, and the dominant fungi in these fungal communities. I investigated the entire fungal community in the domatium chambers of *M. beccarii* because the three types of chambers serve different functions for the ant colony. I hypothesised that the fungal communities would differ by chamber type. I found consistent and specific patterns in the fungal communities within the chambers of *M. beccarii* across the five sites surveyed which is remarkable given that the distribution of *M. beccarii* is fragmented and my sites included national parks and state forests as well as suburban residential streets (chapter 2). In particular, the waste chambers had a fungal community distinct from that found in the nursery and ventilation chambers (which were also distinct but had some overlap in their fungal communities) (chapter 2). It is in the waste chambers where the three potential players in this mutualism most likely interact: the ants deposit waste, the plant absorbs nutrients from waste, and I found that the fungal community is distinct from the other chambers.

Fungi are transported by *P. cordata* ant workers between *M. beccarii* ant-plants via the exoskeleton, and also the head/infrabuccal pockets. There was horizontal transmission of fungi by *P. cordata* ant workers that transferred fungi from the mature *M. beccarii* ant-plants to the ant-accessible *M. beccarii* plantlets (chapter 3). In the ant exclusion experiment, I found that the ant-accessible plantlets had higher fungal OTU sequence abundances in the nursery and waste chambers compared to the ant-excluded plantlets but similar abundances in the ventilation chambers (chapter 3). Also, a greater number of fungal OTUs (60) were shared between the mature ant-plants and ant-accessible plantlets compared to the ant-excluded plantlets (6 OTUs) (chapter 3). The *P. cordata* ant workers shared 31 fungal OTUs solely with the ant-accessible plantlets but none were shared solely with the ant-excluded plantlets (chapter 3). Fungi were mostly transferred via the exoskeletons and heads of the ant workers and much less fungi were found in the abdomens.

The transport of fungi on the exoskeletons of the ants suggests indirect transmission of fungi which raises questions about how the fungal communities that I found in chapter 2 eventually became so distinct. The three-month duration of the experiment was likely insufficient time for the entire fungal communities to become established in the three chambers. However, I believe that once

established, it is possible the *P. cordata* ant workers have a role in making the fungal communities distinct by pruning fungi in order to control them. This type of ant behaviour in an epiphytic ant-plants was first noticed over 100 years ago by Miehe (1911) in the closely related ant-plant *M. tuberosa*. In addition, the plant chambers may play a role in where fungi can grow, e.g. once there are ant worker faeces and other organic matter in the waste chambers, the growth conditions for certain fungi (e.g. saprotrophs) are likely to be ideal. In the nursery chambers, the presence of brood may also influence the fungal community if certain fungi are food for larvae or have antibiotic functions.

The fungal OTU abundances were similar in the ventilation chambers of the ant-accessible and ant-excluded plantlets which suggests either fungi do not grow well in those chambers or that the ant workers are managing fungi (chapter 3). Relatively few fungi were found in the abdomens of the ant workers suggesting *P. cordata* workers do not eat the fungi they are transporting (chapter 3). Fungi were also isolated from the ant worker heads which means fungi may have been transferred directly, i.e. from the infrabuccal pockets of the workers (chapter 3). However, there is also the possibility that fungi were isolated from the surface of the heads if the exoskeleton wash did not sufficiently remove them.

There is very little known about how fungi are transmitted between either terrestrial or epiphytic ant-plants. I sampled fungi from the *P. cordata* ant workers because we know that *M. beccarii* ant-plants are often clustered within trees and that they are polydomous, i.e. one *P. cordata* ant colony occupies multiple domatia on a host tree. It therefore seemed reasonable to expect the ant workers could be transferring fungi between *M. beccarii* ant-plants. To my knowledge, the only other study investigating the transport of fungi in an ant-plant is in three *Cecropia* species inhabited by four different *Azteca* ant species. In that study, Mayer et al. (2018) found vertical transmission of fungi by founding queens that carry fungi in their infrabuccal pockets when they leave their natal nest to start a new colony and that the new queens feed these fungi to their larvae. Mayer et al. (2018) did not sample ant workers and I was not able to sample the queens in this study. Therefore, it is difficult to compare with this study. The *Cecropia/Azteca* ant-plant system is also different in many ways to the *P. cordata/M. beccarii* system in that *Cecropia* is a neotropical pioneer tree with multiple hollow stem internodes for domatia and is occupied by multiple species of *Azteca.* However, both of these ant-plants have the challenge of starting new ant colonies in ant-plants that might be some distance

from their natal colony. This could mean that queens are also responsible for transferring fungi to new nests in the *P. cordata/M. beccarii* mutualism.

I was unable to confirm whether or not fungi play a nutritional role in the *P. cordata/M. beccarii* mutualism because my results were inconclusive. I investigated whether fungi are involved in the transfer of nitrogen from the *P. cordata* ant workers to *M. beccarii* via the waste chambers, which is where the workers deposit their faeces and other waste. After feeding a stable isotope of nitrogen (^{15}N) to the ant workers at the beginning of the experiment, I was able to trace nitrogen from the ant workers to the leaves of the *M. beccarii* plantlets that had been treated with or without fungicide. I confirmed that the *P. cordata* ant workers feed their host *M. beccarii* ant-plant by depositing their faeces in the waste chambers (chapter 4). However, after sampling the leaves at the end of the experiment, I found there was no difference in the $\delta^{15}N$ values of the plantlets despite the fungicide treatment (chapter 4). This was explained by the finding that the fungal communities did not differ in terms of abundance, richness, and functional guilds which indicated that the fungicide application did not effectively reduce or remove the fungal communities in the waste chambers of the plantlets (chapter 4).

Although I could not conclude whether or not fungi are involved in the transfer of nitrogen from the ant workers to the ant-plantlets, my findings provide some insights. For example, the fungal community in the waste chambers of the ant-accessible plantlets was similar to the waste chambers of the mature ant-plants but it was not the same, suggesting more time was required to allow the fungal community to become established in newly occupied *M. beccarii* ant-plants. Also, a large proportion of the fungal community in the mature ant-plants could not be assigned to any functional guild. This highlighted how little we know about fungi in general and how epiphytic ant-plants are an unexplored niche for fungi. I also found that particular fungal OTUs that were encountered throughout my thesis, were transferred by the ant workers (chapter 4). This included a fungal OTU from the order Chaethothyriales (chapter 4).

Across my three sets of data, I found certain fungal OTUs that repeatedly were dominant in the chambers, and some were also found on the exoskeletons and/or heads of the ant workers. For example, a Chaetothyriales fungal OTU (labelled OTU0202 in chapter 2 and with a 99% match to OTU0296 in chapters 3 & 4) was consistently found in the waste chambers throughout my project and was also isolated from the heads of the ant workers (chapter 3). This Chaetothyriales

OTU0202/296 fungus had a very close match (99%) to a sequence named KhNk3-2 isolated from the domatium of *K. hispida,* occupied by *Crematogaster* sp. ants in Cameroon (Voglmayr et al. 2011) and recently, this fungal species was also isolated from the epiphytic ant-plant *D. major* occupied by *Philidris* ants in Thailand (Blatrix et al. 2021). To my knowledge, the role of this Chaetothyriales fungus is not yet known. Another Chaetothyriales fungal OTU (labelled OTU0347 in chapter 2 with a 96% match to OTU0509 in chapters 3 & 4) was found in the nursery chambers during my project. A 96% match is not considered sufficient to call this OTU the same species. However, these OTUs are closely related and may serve the same function, possibly as food for larvae.

Most studies of fungi in terrestrial ant-plants have focussed on Chaetothyriales fungi because fungi from that order are dominant and consistently isolated from fungal patches inside domatium of terrestrial ant-plants or in ant-carton walls of nests and galleries (Mayer and Voglmayr 2009). A recent phylogenetic study found sufficient support for a clade of Chaetothyriales fungi obtained from ant domatia to be recognised as a separate family (Quan et al. 2021). These domatium and ant-carton fungi are closely related, but their morphology and ecology differ, which indicates the roles they play are different in these interactions (Voglmayr et al. 2011). For example in domatia, Chaetothyriales fungi have been a source of food for ant colonies (Defossez et al. 2011, Blatrix et al. 2012) whereas in ant-carton, the ant workers combine Chaetothyriales fungi with plant trichomes to build traps to capture prey so the role is structural (Dejean et al. 2005, Mayer and Voglmayr 2009, Nepel et al. 2014). The ant carton Chaetothyriales fungi in those traps are fed by the ant workers and the fungi transfers nitrogen to the plant (Leroy et al. 2011, Leroy et al. 2017).

I was also interested in other (non-Chaetothyriales) fungi because I thought there might be patterns in the fungal communities in the chambers of *M. beccarii* that included other fungi. This proved to be the case as there were several fungal OTUs that I consistently found in the waste chambers during this research project. For example, OTU0171 *Candida fluviatilis* was the most abundant fungus isolated from the exoskeletons and heads of the *P. cordata* ant workers and was dominant in the waste chambers throughout my project. *Candida fluviatilis* is a saprotrophic fungus with the ability to biodegrade hydrocarbons (Vetrova et al. 2022) and has potential for neutralising acidic environments (Mitsuya et al. 2017). *Candida fluviatilis* may also be playing a saprotrophic role and/or contributes to improving the chamber environment in the *P. cordata/M. beccarii* ant-plant mutualism.

5.3 Limitations and questions for future research

Research projects usually end in more questions than answers, and here I discuss some limitations of my research and propose questions for future research. One limitation of my survey data was that I did not compare the fungal communities I found inside *M. beccarii* domatia with communities in the surrounding environment. A future research question could be: are the dominant fungi I found during my project restricted to the chambers of *M. beccarii* ant-plants? To answer this, samples could be taken for fungal DNA analysis from *M. beccarii's* host *Melaleuca* trees (on/under bark, on leaves, etc.) and in soil under host trees. The surfaces of *M. beccarii* could also be sampled. This could determine if any dominant fungi are exclusive to *M. beccarii*, which would suggest those fungi are dependent on this ant-plant mutualism for dispersal and survival and also suggest a possible role in the mutualism. Alternatively, if a dominant fungus is common in the environment outside the domatium, it might indicate that they are opportunistic or non-symbiont fungal competitors, e.g. fungi from the orders Eurotiales, Hypocreales, Pleosporales, and Saccharomycetales (Blatrix et al. 2013, Vasse et al. 2017). Those species could then be excluded as mutualists, providing more focus for future research.

There were some limitations to the methodology used to sample fungi directly from ants. This would need to be addressed to answer future questions about whether fungi are carried in the infrabuccal pockets or in the gut of *P. cordata*. When I collected the fungal samples, I washed the workers in a solution from the DNeasy® PowerSoil® Kit (Qiagen Pty Ltd, Victoria) to obtain fungi from their exoskeletons, and then extracted DNA from the exoskeleton wash. I used the same ant workers which I dissected to separate the heads and abdomens, both of which I crushed before extracting DNA. However, I do not know for sure if the surface of the heads and abdomens were completely free of fungi. Future research should remove the infrabuccal pellets from the ant worker heads to examine the contents of the infrabuccal pocket using microscopy and also sequence the fungi directly from the infrabuccal pocket. If fungi were found in the infrabuccal pellets, it would indicate a more direct transfer of fungi to the chambers of *M. beccarii*. To confirm whether or not ant workers are consuming fungi, the abdomens of *P. cordata* could be dissected to obtain the gut contents which could then be used to extract fungal DNA directly.

The above methodology could also be used to determine if *P. cordata* founding queens carry fungi in their infrabuccal pockets, in their guts, or on their bodies to establish the fungal community in an

unoccupied ant-plant. The answer to this question would help explain how a fungal community becomes established in *M. beccarii* if a seed of *M. beccarii* is dispersed some distance from where it first originated, e.g. by a mistletoebird. It would also be possible to sample the surface and gut contents of the larvae of *P. cordata.* If fungi were found on the surface of the larvae, it would suggest a possible antibiotic function of the fungus and if fungi were found in their gut contents, it would suggest larvae are feeding on those fungi.

The duration of the experiment outlined in chapters 3 and 4 was three months and during that time, some of the fungi in the mature ant-plants was transported to the ant-accessible plantlets by the ant workers. I found that the fungal communities were distinctly different between the mature and the younger plantlets (chapter 3). This likely means that the experiment was too short to allow the full establishment of the fungal community. A longer experiment may have enabled me to see if fungal communities in the plantlets converged to resemble more closely those in the chambers of the mature ant-plants. Another way to explore this would be using an experiment conducted in the field where greenhouse-grown ant-plants are placed around mature wild ant-plants and then the greenhouse-grown ant-plants are harvested over a longer time (e.g. a year) to see how long it takes for the fungal communities to converge. This could also be attempted by planting seeds of *M. beccarii* around mature *M. beccarii* ant-plants.

During my project, I attempted to culture the fungi I found in the chambers of *M. beccarii* onto agar plates. Unfortunately, time and resources did not allow me to continue with this effort. Fungi from the order Chaetothyriales are poorly understood and it would be beneficial to isolate and culture Chaetothyriales fungi that were found in *M. beccarii*. A cafeteria experiment could then be carried out to test whether the ant-workers are selective about Chaetothyriales fungi. Assays could also be conducted with Chaetothyriales fungi to test whether they are capable of degrading chitin, cellulose, and lignin. Similar culturing and experiments could be conducted on other non-Chaetothyriales fungi, e.g. the *Candida* species to determine what role(s) they may be playing. The results of such work could reveal fungal species that are useful, for example in bioremediation (Mitsuya et al. 2017, Vetrova et al. 2022).

I also attempted an experiment using fungicide treatment on ant-accessible plantlets to try and eliminate fungi in order to ascertain if fungi are required for the transfer of nitrogen (chapter 4). The fungicide was ineffective and the results inconclusive. I believe my inability to see precisely

where the waste chambers were located made it difficult to eliminate the fungi in the waste chambers and it is also possible that the fungicide application type and/or rate was insufficient to target the fungi present in the waste chambers.

Several studies have shown that ant-plants are capable of absorbing nutrients directly via domatium surfaces (Janzen 1974, Huxley 1978, Rico-Gray et al. 1989, Gay 1993, Watkins et al. 2008, Gegenbauer et al. 2012). A different approach to take to see whether fungi are involved in the transfer of nitrogen in *M. beccarii* could be to use a direct method of applying the nitrogen tracer to the waste chambers (Defossez et al. 2011). Carcasses of insects that have been enriched in ^{15}N could be directly introduced into the waste chambers. This would mean accessing the chambers directly which could be done by cutting slices of the domatia. When I was in the field, I occasionally saw *M. beccarii* ant-plants with damaged domatia that exposed the chambers, but the plants otherwise appeared to be healthy. This suggests it would be possible to cut a small slice off of the upper side of the domatium of *M. beccarii* in order to directly access waste chamber surfaces without harming the plant. The cut off section of domatium could be replaced back over the exposed cut surface to reduce desiccation and protect the waste chambers from contamination by other organisms.

Using this direct access methodology, the experiment could be conducted with *M. beccarii* ant-plants from: (a) the wild with *P. cordata* ant workers removed and fungi presumed to be present; and (b) greenhouse-grown *M. beccarii* ant-plants, that are ant-free and free of fungi that would be found in a wild ant-plant. First, I would test whether nitrogen can be absorbed directly by the wart-like structures in the waste chambers of *M. beccarii* by applying the nitrogen isotope ^{15}N directly onto the surfaces of the waste chambers of greenhouse grown *M. beccarii* ant-plants. Secondly, I would test whether fungi are involved in the breakdown of waste from insect carcasses by placing dead mealworms (or other insect larvae) enriched with ^{15}N directly onto the waste chamber surfaces of both the wild and greenhouse-grown ant-plants.

DNA could be sampled from the waste chambers and sequenced to identify fungi in all of the waste chambers of the ant-plants at the beginning and end of the experiment and DNA could also be sampled from the mealworms together with microscopy to investigate if any fungi are consuming the mealworms. This experiment would confirm whether or not fungi play a role in this ant-plant mutualism.

5.4 Summary of findings

Together the findings from my three data chapters have advanced our knowledge about the *P. cordata/M. beccarii* ant-plant mutualism despite the role of fungi remaining a mystery. Fungal communities inhabiting the domatium chambers of *M. beccarii* differed among the chamber types and this pattern was consistent across a large portion of the distribution of *M. beccarii.* Certain Chaetothyriales fungi found in *M. beccarii* also occur in ant-plants on other continents suggesting a long association between ant-plants and their resident ants. We also now know more about how the resident *P. cordata* ant workers interact with fungi by the way they transport fungi between *M. beccarii* ant-plants. The exoskeletons were where most of the fungi were found on the ants indicating indirect transport of fungi. However, the heads also contained fungi suggesting there may be more direct transfer of fungi via the infrabuccal pocket which remains to be tested. Although the fungicide treatment was ineffective at removing fungi during the experiment in chapter 4, some useful information was gained including knowledge of the large proportion of fungi that were unable to be assigned to functional guilds, highlighting how little we know about fungi in ant-plants and in general. There may be fungi inside *M. beccarii* that are useful in industries such as bioremediation. The discovery of interactions involving microorganisms such as fungi in ant-plants could lead to a reinterpretation of mutualistic associations among partners and may influence our understanding of how these relationships evolved and how they are maintained. Mutualisms may be particularly vulnerable to a changing climate so learning about how microorganisms interact with other players within mutualisms could also be useful in conservation efforts for the species involved.

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