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

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Abstract

In recent decades, multipartite mutualisms involving microorganisms such as fungi have been discovered in associations traditionally thought of as bipartite. Ant-plant mutualisms were long thought to be bipartite despite fungi being noticed in an epiphytic ant-plant over 100 years ago. We sequenced fungal DNA from the three distinct domatium chambers of the epiphytic ant-plant Myrmecodia beccarii Hook.f. to establish if fungal communities differ by chamber type across five locations spanning 675 km. The three chamber types serve different ant-associated functions including: 'waste' chambers, where ant workers deposit waste; 'nursery' chambers, where the brood are kept; and 'ventilation' chambers, that allow air into the domatium. Overall, fungi from the order Chaetothyriales dominated the chambers in terms of the proportion of 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. We identified 13 fungal OTUs as '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 were also significantly different, but variation between these chambers was less pronounced. Differences in dominance of the common OTUs drive the observed patterns in the fungal communities for each of the chamber types. This suggests a multipartite mutualism involving fungi exists in this ant-plant and that the role of fungi differs among chamber types.

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In recent decades, multipartite mutualisms involving microorganisms such as fungi have been discovered in associations traditionally thought of as bipartite. Ant-plant mutualisms were long thought to be bipartite despite fungi being noticed in an epiphytic ant-plant over 100 years ago. We sequenced fungal DNA from the three distinct domatium chambers of the epiphytic ant-plant *Myrmecodia beccarii* Hook.f. to establish if fungal communities differ by chamber type across five locations spanning 675 km. The three chamber types serve different ant-associated functions including: 'waste' chambers, where ant workers deposit waste; 'nursery' chambers, where the brood are kept; and 'ventilation' chambers, that allow air into the domatium. Overall, fungi from the order Chaetothyriales dominated the chambers in terms of the proportion of 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. We identified 13 fungal OTUs as '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 were also significantly different, but variation between these chambers was less pronounced. Differences in dominance of the common OTUs drive the observed

patterns in the fungal communities for each of the chamber types. This suggests a multipartite mutualism involving fungi exists in this ant-plant and that the role of fungi differs among chamber types.

KEYWORDS: Ant-plant, myrmecophyte, mutualism, symbiosis, fungi, *Myrmecodia beccarii* , *Philidris cordata* , metabarcoding, PacBio

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 & Goodrich-Blair, 2013). Examples of multipartite mutualisms include the coral-algae association that also involves bacteria, archaea and viruses (Rosenberg, Koren, Reshef, Efrony, & Zilber-Rosenberg, 2007); the fungus-farming attine ants and antibiotic-producing bacteria that control fungal garden parasites (Currie, Scott, Summerbell, & Malloch, 1999); and streptomyces bacteria that protects pollinating honeybees and strawberry plants from pathogenic fungi and bacteria (Kim, Cho, Jeon, Weller, & Thomashow, 2019). 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 (Mayer, Frederickson, McKey, & Blatrix, 2014; Voglmayr et al., 2011).

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 & Renner, 2015). In some ant-plants, the resident ants obtain food rewards from their host (e.g., extrafloral nectar or food bodies) (Hölldobler & 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 (Defossez, Djieto-Lordon, McKey, Selosse, & Blatrix, 2011; Gay, 1993; Huxley, 1978; Rickson, 1979; Rico-Gray, Barber, Thien, Ellgaard, & Toney, 1989; Treseder, Davidson, & Ehleringer, 1995).

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 in three independently evolved and geographically distinct ant-plant (tree) associations (Blatrix et al., 2013; Blatrix et al., 2012; Defossez et al., 2011). 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, Solano, Ayroles, Corbara, & Orivel, 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; C. Leroy et al., 2017; Céline Leroy et al., 2011; Mayer & Voglmayr, 2009; Nepel, Voglmayr, Schönenberger, & Mayer, 2014). The dominant fungi isolated from ant-plant systems studied so far are "black yeasts" from the orders Chaetothyriales and Capnodiales of phylum Ascomycota (Voglmayr et al., 2011).

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 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 & Renner, 2015). Fungi were first noticed in the epiphytic ant-plant *Myrmecodia tuberosa* Jack (Rubiaceae) (Miehe, 1911), but have since been mentioned rarely in the literature (Bailey, 1920; Huxley, 1978; Janzen, 1974) or dismissed as opportunistic (Bailey, 1920; Miehe, 1911).

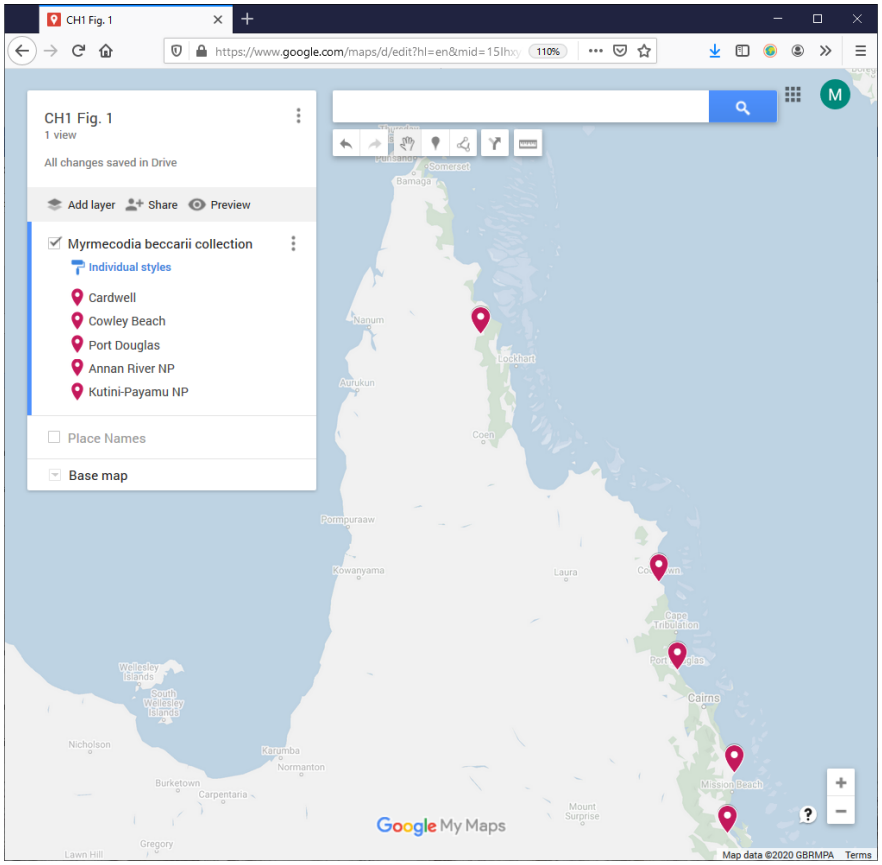
Our study investigated fungi in the epiphytic ant-plant *Myrmecodia beccarii* Hook.f. (Gentianales: Rubiaceae), endemic to northern Queensland, Australia. *Myrmecodia beccarii* is listed as vulnerable under the Environment Protection and Biodiversity Conservation Act 1999 (Commonwealth of Australia) 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, Lovatt, Bahr, Kahler, & Appelman, 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). The chambers include smooth-walled ('nursery') chambers where*P. cordata* keeps its brood (eggs, larvae, and pupae), warted ('waste') chambers where the ant workers deposit waste and defecate, 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. To our knowledge, there are no detailed studies focussing on fungi in epiphytic ant-plants, leaving questions about the identity and functioning of the fungi.

We hypothesized that there is a multipartite mutualism involving this ant-plant, its resident symbiont ants, and fungi. To test this, we sampled the fungal communities in the three domatium chambers of*M. beccarii* across five 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 chambers, and if so, is this consistent across geographical sites? If fungal communities are distinct in each of the chambers, it would suggest fungi play different roles in the different chambers. If there are distinct fungal communities across the five locations, it would indicate fungi have had a long association with this ant-plant mutualism. If these predictions are both correct, it will indicate a high likelihood that fungi have a mutualistic role in the ant-plant *M. beccarii.*

1. **MATERIALS AND METHODS**
2. **Study sites and sampling**

We collected 46 whole *Myrmecodia beccarii* plants from*Melaleuca* trees at five locations spanning 675 km in northern Queensland, Australia (Fig. 1) from March 2016 until March 2017. We 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). We 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. 1).



We 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 (approximately 1 to 21 days after collection). During this time, we 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 1 (A) Map of northern Queensland, Australia, showing the five locations where *Myrmecodia beccarii* ant-plants were collected during the study. (B) Known distribution of *M. beccarii*– red dots indicate where *M. beccarii* has been found.

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

At time of dissection, we 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). We 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). We 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 (A) *Myrmecodia beccarii* ant-plant on*Melaleuca* tree at Cardwell, Queensland. (B) *M. beccarii*(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 are 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. Photos: M. Greenfield.

DNA Extraction and Sequencing

We 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 we eluted 50 µL of purified DNA instead of 100 µL. We 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 S1). 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 their relative quantity 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. 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.

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, Bahram, Hiiesalu, & Tedersoo, 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, Flouri, Nichols, Quince, & Mahé, 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 & Godzik, 2006) at a threshold of 97% similarity, as commonly set in fungal molecular ecology (Kõljalg et al., 2013; Taylor et al., 2014). We 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).

We 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. We 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 ([dataset] Greenfield et al., 2020) which we further filtered to remove any OTUs with total read abundances <10, leaving a dataset with a total of 164 fungal OTUs (Table S2). This final step was performed because the focus of this study is on the dominant fungal taxa inside the domatium chambers of *M. beccarii* .

We 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.

We 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. We collapsed 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 the interconnectedness of the chambers in this ant-plant mean multiple samples (for each chamber type) per plant are not independent. This left a total of 135 samples comprising 46 nursery chambers, 44 ventilation chambers, and 45 waste chambers from the 46 ant-plants collected ([dataset] Greenfield et al., 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) 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.

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. Unique and shared fungal OTUs were investigated by creating a Venn diagram using the R package 'VennDiagram' (v1.6.20) (H. Chen, 2018). We used the multivariate abundance analysis package 'mvabund' (v4.0.1) (Wang, Naumann, Eddelbuettel, Wilshire, & Warton, 2020; Wang, Naumann, Wright, & Warton, 2012) 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". We 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, we used the "p.uni" argument to calculate univariate test statistics and their p-values (adjusted for multiple testing using a step-down resampling procedure). We 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.

We used the packages 'phyloseq' (v1.28.0) (McMurdie & 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, we standardized our 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 'DESeq2' package (v1.24.0) (Love, Huber, & Anders, 2014) and 'phyloseq' package was 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'. We used FUNGuild (v1.0) (Nguyen et al., 2016) to assign trophic modes to the significantly abundant fungal OTUs.

We used the dataset containing the 374 OTUs (minimum read abundances of 2) to compare fungal OTU richness using a generalized linear mixed effects model (glmer function) in the lme4 package (v1.1.21) (Bates, Maechler, Bolker, & Walker, 2015) with family = poisson and bobyqa optimization. Fixed effects included location and chamber type and an interaction term for location and chamber type. We also included an observation level random effect to account for overdispersion. We used the emmeans package (v1.4.5) (Lenth, 2020) for pairwise comparisons between locations for each of the chamber types.

1. **RESULTS**
2. **Dominant fungi in the domatium chambers**

We 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). 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. 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. 3B).

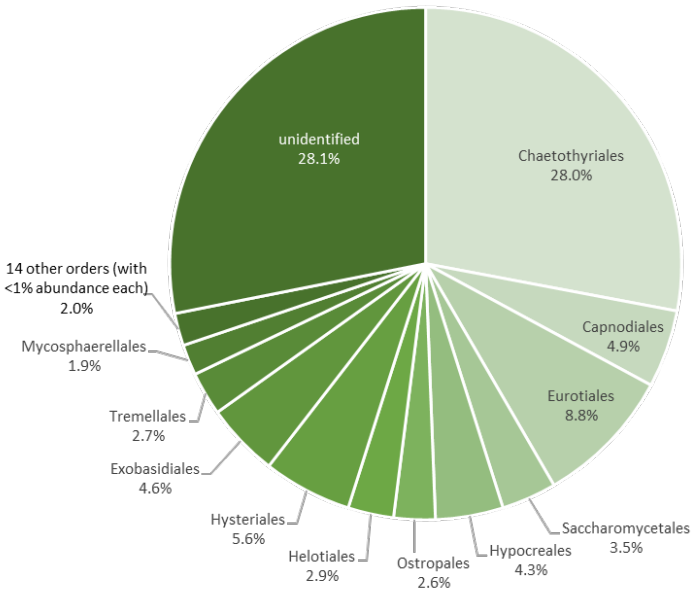


FIGURE 3 Dominant fungal taxonomic orders in the chambers of the ant-plant *Myrmecodia beccarii.* (A) Relative proportion of fungal operational taxonomic units (OTUs) found in the chambers of*M. beccarii* and assigned to fungal orders, showing Chaetothyriales, Capnodiales, and Eurotiales are dominant. The 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.

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. 4). The read abundances of these 94 shared OTUs made up 88.8% of the total. The nursery and ventilation chambers shared 28 OTUs which made up 3.7% 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.4).

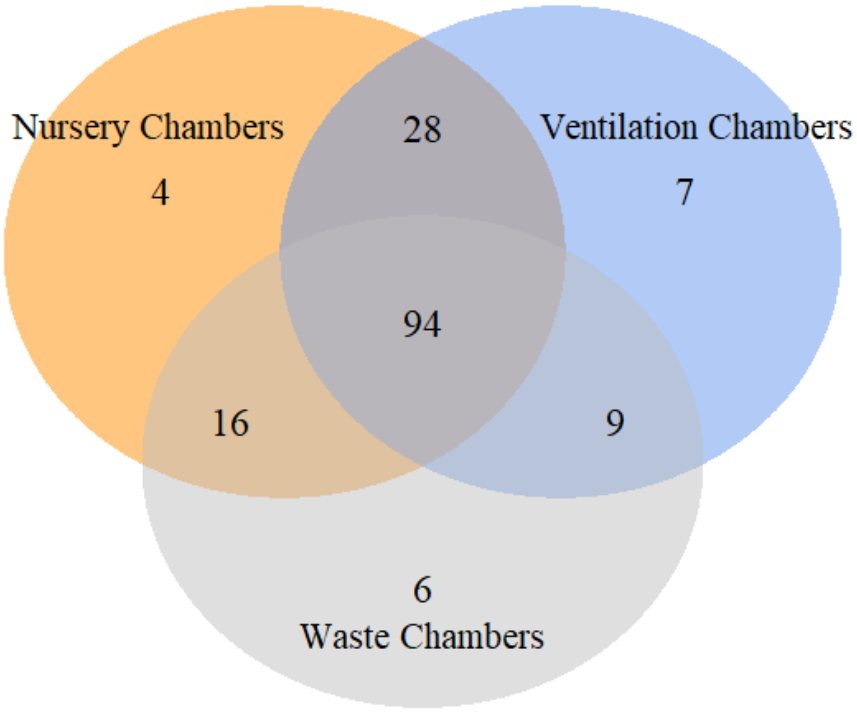


FIGURE 4 Venn diagram of unique and shared fungal operational taxonomic units (OTUs) among the three different chambers of the ant-plant *Myrmecodia beccarii*. The numbers displayed represent the number of fungal OTUs shared between and among chambers (overlapping shaded areas) and unique (not overlapping) to each chamber type (total 164 fungal OTUs). The total number of OTUs associated with each of the chambers is: waste chambers 125 OTUs; nursery chambers 142 OTUs; and ventilation chambers 138 OTUs.

Fungal communities among domatium chambers

Our 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. 5A). Significantly different fungal communities were also found between the nursery and ventilation chambers, but with less pronounced variation (LRT = 609, $p<0.001$) (Fig. 5A).

Fungal community differences across the geographic distribution of *Myrmecodia beccarii*

We 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. 5B-D). Our 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 1). The fungal OTU communities in the ventilation chambers showed fungal OTU community composition differed across all locations (Table 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=0.0018) and Cowley Beach (mean 24.0 ± 2.6 SE, p<0.0078) (Tables S3 and S4, Figure S2). All other pairwise comparisons of fungal OTU richness between the five locations for each of the three chamber types were not significant (Table S4).

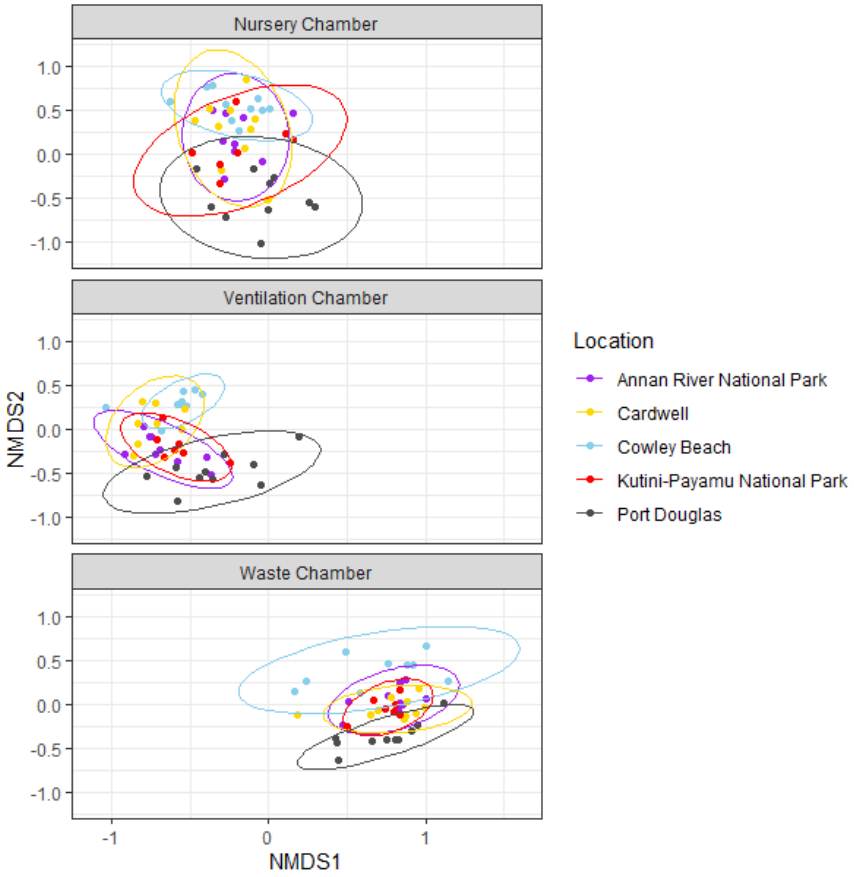


FIGURE 5 Non-metric multidimensional scaling (NMDS) ordinations displaying fungal OTU community compositions in the waste, nursery and ventilation chambers of *Myrmecodia beccarii* across five locations. Plot A is for the three chamber types for all locations combined, showing the fungal communities 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 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**	**Chamber Type**	**Chamber Type**	**Chamber Type**	**C**
	Waste LRT	**Waste p-value**	**Nursery LRT**	**Nursery p-value**	**V L**
Annan River NP vs Cardwell	207.6	0.034 *	195.3	0.177	2
Annan River NP vs Cowley Beach	195.3	0.049 *	251.5	0.046 *	2
Annan River NP vs Kutini-Payamu NP	199.7	0.043 *	186.7	0.177	2
Annan River NP vs Port Douglas	235.2	0.023 *	198.6	0.177	2
Cardwell vs Cowley Beach	241.9	0.023 *	216.5	0.112	1
Cardwell vs Kutini-Payamu NP	142.1	0.075	165.3	0.177	2
Cardwell vs Port Douglas	231.8	0.023 *	250.6	0.046 *	3
Cowley Beach vs Kutini-Payamu NP	237.5	0.023 *	237.2	0.061	2
Cowley Beach vs Port Douglas	280.7	0.006 **	360.3	0.002 **	3
Kutini-Payamu NP vs Port Douglas	191.4	0.049 *	191.9	0.177	2

To better understand why there were differences in the fungal community compositions across locations, we identified fungal OTUs individually that were significantly abundant in one or more of the three different chamber types. Our 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).

TABLE 2 Pairwise contrasts and multivariate abundance analysis to identify significantly differentially abundant fungal operational taxonomic units (OTUs) in the chambers of *Myrmecodia 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 mvabund p-values were calculated using 999 resampling iterations via PIT-trap resampling (to account for correlation in testing). The abbreviation "ns" refers to non-significance.

OTUID	**Fungal taxon**	**DESeq2 results**	**DESeq2 results**	**DESeq2 results**	**DESeq2 results**	**DESeq2 results**	**mvabund results**	**mvabund results**	**mv re su**
		Base Mean	**log2 Fold Change**	**LFC SE**	**adjusted p-value**	**contrasts between chamber types**	**chamber type**	**location**	**int ac**
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			

OTUID	Fungal taxon	DESeq2 results	DESeq2 results	DESeq2 results	DESeq2 results	DESeq2 results	mvabund results	mvabund results	mv results
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	5.0390	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
			7.1054	0.5974	<0.0001	waste vs. ventilation			
OTU0302	Mycosphaerellales sp	7.3570	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	DESeq2 results	DESeq2 results	DESeq2 results	DESeq2 results	mvabund results	mvabund results	mv results
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	10.2890	6.5169	0.6642	<0.0001	waste vs. nursery	<0.001	<0.001	<0
			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	11.1045	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

OTUID	Fungal taxon	DESeq2 results	DESeq2 results	DESeq2 results	DESeq2 results	DESeq2 results	mvabund results	mvabund results	mv results
			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	DESeq2 results	DESeq2 results	DESeq2 results	DESeq2 results	mvabund results	mvabund results	mv results
OTU0898	Cuniculitrema 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	Ustilaginomycetes sp	4.8914	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

OTUID	Fungal taxon	DESeq2 results	DESeq2 results	DESeq2 results	DESeq2 results	DESeq2 results	mvabund results	mvabund results	mv results
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

Dominant fungal taxa

We identified 27 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. 6, Table 3, grey shaded values). These 27 OTUs were a subset of the 41 fungal OTUs previously identified as significantly differentially abundant (Table 2) and 10 of these 27 fungal OTUs recorded the highest sequence abundances (Fig. S1). Each of the 27 OTUs occurred in more than one chamber type but with significantly different abundances (Fig. 6, Tables 2 and 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 3).

In the waste chambers, we found 13 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 3). The 13 common OTUs were either very low in abundance or absent in the nursery and ventilation chambers (Table 3, Figure 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 3). 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 3). 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 Hypocrealean OTU0544 (*Fusarium*sp.) occurred in low abundances in the nursery chambers (<27%) compared to the ventilation chambers (>70% of the total abundances) (Table 3). 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 3).

The 13 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). 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, Fig. 6).

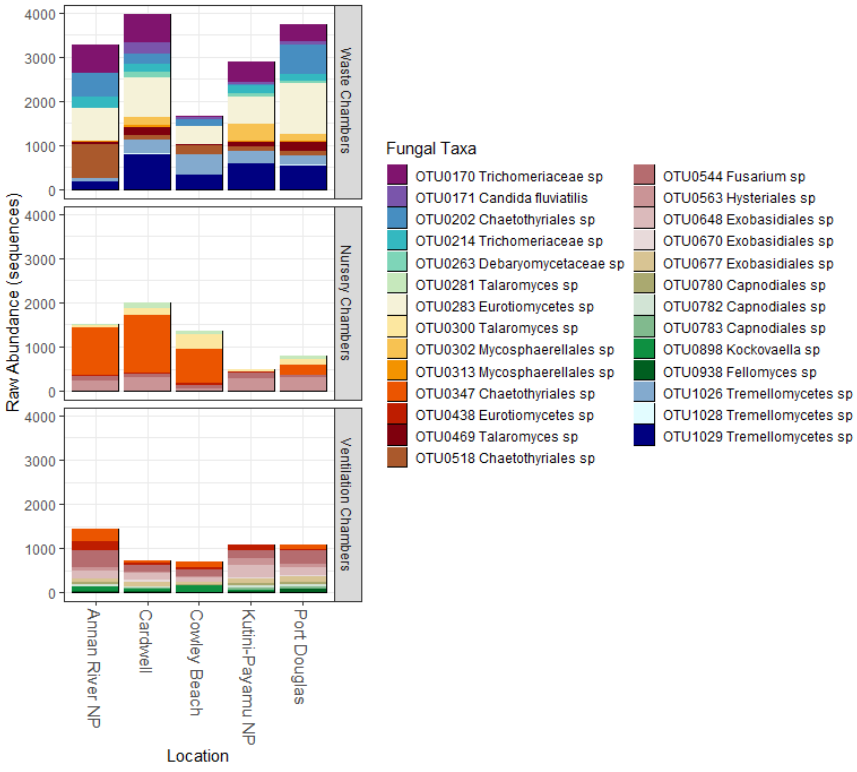


FIGURE 6 (A) Raw (sequence) abundances (A) and Relative (sequence) abundances (B) of the most common fungal operational taxonomic units (OTUs) in the three different chambers of*Myrmecodia beccarii* (27 fungal OTUs in total) across the five locations surveyed. Fungal Taxa are the best match found in 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.

TABLE 3 Fungal Operational Taxonomic Units (OTUs) that are significantly differentially abundant in at least one of the chamber types of the ant-plant *Myrmecodia beccarii* with fungal taxon (closest match in online databases GenBank and UNITE), GenBank accession number, UNITE species hypothesis number, sequence similarity (%), sequence abundance, percentage sequence abundance in chamber samples, and percentage of chambers with the OTU. Grey shading indicates the OTU was 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 Simi (%)	Seq abund	% sequence abundance in chamber samples	% sequence abundance in chamber samples	% sequence abundance in chamber samples	% of chamber samples with OTU	% of chamber samples with OTU	% of chamber samples with OTU	F t r
						NC	VC	WC	NC	VC	WC	
OTU0170	Trichomeriaceae sp	KU195499	SH491217.07	97	2308	4.7	1.4	93.9	32.6	9.1	68.9	S
OTU0171	Candida fluviatilis	HQ652068	SH200664.08	97	472	7.2	0.4	92.4	19.6	4.5	66.7	S
OTU0202[a]	Chaetothyriales sp	HQ634649	SH196444.07	97	1678	1.8	3.2	94.9	17.4	9.1	80.0	-
OTU0214	Trichomeriaceae sp	KU195499	SH491217.07	97	829	4.3	2.2	93.5	21.7	9.1	68.9	S
OTU0263	Debaryomyces sp	KP109748	SH192552.07	97	255	0.8	0.0	99.2	2.2	0.0	62.2	S
OTU0283	Eurotiomycetes sp	DQ914677	SH206547.08	97	3977	1.4	2.3	96.4	34.8	9.1	100.0	-
OTU0302	Mycosphaerellales sp	GU117898	SH154167.08	98	731	0.5	0.3	99.2	4.3	2.3	57.8	-
OTU0313	Mycosphaerellales sp	GU117898	SH154167.08	98	97	1.0	0.0	99.0	2.2	0.0	60.0	-
OTU0469	Talaromyces sp	KP143766	SH209380.07	97	602	7.1	0.8	92.0	19.6	9.1	64.4	S
OTU0518	Chaetothyriales sp	HM239979	SH212163.07	97	1283	0.1	0.0	99.9	2.2	0.0	57.8	-
OTU1026	Tremellomycetes sp	JX999048	SH477174.07	97	1397	1.4	2.6	96.1	6.5	4.5	71.1	-
OTU1028	Tremellomycetes sp	JX999048	SH477174.07	97	81	4.9	0.0	95.1	4.3	0.0	60.0	-
OTU1029	Tremellomycetes sp	JX999048	SH477174.07	97	2650	5.9	3.4	90.7	13.0	11.4	97.8	-
OTU0281	Talaromyces sp	KU141384	SH194198.07	97	375	84.5	3.2	12.3	52.2	13.6	26.7	S

OTUID	Fungal taxon	GenBank Accession number	UNITE SH number	Seq Simi (%)	Seq abund	% sequence abundance in chamber samples	% sequence abundance in chamber samples	% sequence abundance in chamber samples	% of chamber samples with OTU	% of chamber samples with OTU	% of chamber samples with OTU	F t r
OTU0300	Talaromyces sp	KJ608116	SH194198.07FU		859	77.2	2.8	20.0	69.6	25.0	44.4	S
OTU0347[b]	Chaetothyriales sp	KC951221	SH212029.07FU		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	SH025137.08FU		1606	24.2	72.6	3.2	73.9	100.0	22.2	F S S
OTU0563	Hysteriales sp	KF675741	SH205606.07FU		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		355	44.5	52.1	3.4	28.3	61.4	13.3	-
OTU0782	Capnodiales sp	KC222753	SH025821.07FU		205	4.9	94.1	1.0	13.0	52.3	2.2	-
OTU0783	Capnodiales sp	KC222753	SH025821.07FU		176	26.1	69.3	4.5	21.7	56.8	13.3	-
OTU0898	Cuniculitrema sp	KY103846	SH152356.08FU		579	40.4	56.5	3.1	43.5	59.1	6.7	-
OTU0938	Fellomyces sp	AJ608646	SH204460.07FU		270	33.7	64.8	1.5	43.5	75.0	8.9	F S S
OTU0221	Sporothrix eucalyptigena	KU865592	-	99	309	61.5	0.3	38.2	23.9	2.3	44.4	F S S
OTU0291	Ustilaginomycotina sp	AB180368	SH025674.07FU		580	20.9	78.3	0.9	34.8	43.2	6.7	-
OTU0372[c]	Chaetothyriales sp	HQ634648	SH212029.07FU		424	89.9	4.5	5.7	30.4	11.4	15.6	-
OTU0373[b]	Chaetothyriales sp	HQ634653	SH025817.07FU		767	94.0	2.6	3.4	45.7	22.7	26.7	-
OTU0457	Talaromyces sp	KF366489	SH209380.07FU		512	70.5	14.3	15.2	47.8	34.1	40.0	S
OTU0561	Hysteriales sp	KF675741	SH021234.07FU		728	57.8	38.7	3.4	34.8	25.0	17.8	-

OTUID	Fungal taxon	GenBank Accession number	UNITE SH number	Seq Simi (%)	Seq abund	% sequence abundance in chamber samples	% sequence abundance in chamber samples	% sequence abundance in chamber samples	% of chamber samples with OTU	% of chamber samples with OTU	% of chamber samples with OTU	F t r
OTU0567	Hysteriales sp	KF675741	SH021234.08FU	97	600	60.0	37.0	3.0	47.8	27.3	8.9	-
OTU0623	Cryptodiscus sp	AJ877182	SH210980.08FU	97	536	48.3	51.5	0.2	26.1	47.7	2.2	S
OTU0667	Pezicula radicicola	HQ889715	SH201622.08FU	100	619	50.2	48.5	1.3	19.6	20.5	8.9	P S
OTU0674	Capnodiales sp	KC222753	SH025821.08FU	94	198	37.4	59.1	3.5	37.0	45.5	15.6	-
OTU0746	Capnodiales sp	KC222753	SH025821.08FU	98	279	2.5	96.8	0.7	8.7	47.7	2.2	-
OTU0815	Capnodiales sp	JQ760724	SH025821.08FU	97	263	46.8	48.7	4.6	39.1	38.6	11.1	-
OTU0821	Candida sp	JQ683772	SH203686.08FU	96	157	0.6	0.0	99.4	2.2	0.0	37.8	S
OTU0981	Kockovaella sp	KY103848	SH176359.08FU	97	285	48.8	46.0	5.3	34.8	45.5	11.1	P S S

[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)

DISCUSSION

This study is the first to identify and compare the long overlooked fungal communities in the domatium chambers of an epiphytic ant-plant. The fungal community in the waste chambers of *M. beccarii* was consistently distinct from the other two chambers across all five locations surveyed which spanned 675 km. The nursery and ventilation chambers also exhibited differences, but with less pronounced variation. The type of fungal OTUs found in each of the chambers were generally not unique to each chamber type however differences in OTU abundances are driving the patterns we found in the fungal communities for each of the chamber types. As with other ant-plant systems studied to-date, Chaetothyrialean fungi dominated in terms of the numbers and abundances of fungal OTUs in the chambers of

M. beccarii.

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 (Blatrix et al., 2013; Blatrix et al., 2012; Defossez et al., 2009; Nepel et al., 2016; Voglmayr et al., 2011). There were also high numbers of fungal OTUs from the order Capnodiales in the chambers of *M. beccarri*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 general (K. 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).

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 and 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. We 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). Our 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 Chaetothyrieales. 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, Bauer, Sampaio, & Oberwinkler, 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, we 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, we often found brood in both the nursery and ventilation chambers, but rarely in the waste chambers (and then only pupae) and we 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 our locations and had a 97% match to a sequence isolated from domatia of the ant-plant*Leonardoxa africana letouzeyi* (ant species: *Aphomomyrmex after*) 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, we 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 we often observed ant-carton in the ventilation chambers during this study, which may explain the occurrence of Capnodiales fungi in the ventilation chambers. 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.

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 significant differences in fungal

OTU richness across locations, being higher at Port Douglas compared to Cowley Beach and 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. We 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 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* .

Conclusions

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 we have not yet unequivocally determined what role/s fungi play in this ant-plant, we 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 so many different interactions with other organisms, and their role in this ant-plant may include various other functions such as the production of antibiotic compounds that keep the brood in the nursery chambers safe from bacterial or fungal pathogens, or fungi may be used as a source of food for the ant colony. 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.

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AUTHOR CONTRIBUTION

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.

DATA ACCESSIBILITY

The sequencing data has been deposited in Sequence Read Archive (SRA) under BioProject PRJNA661078. Supplementary Table S2 contains a list of the 164 fungal operational taxonomic units (OTUs) with minimum read abundance of 10, including taxonomic assignment and (closest match in GenBank and UNITE databases), Accession number (GenBank), Species Hypothesis number (UNITE), e-value, % sequence coverage, % sequence similarity. The full dataset of 374 fungal OTUs (minimum read abundance of 2) has been deposited in the James Cook University Tropical Data Hub and will be made publicly available at time of publication (doi to be advised) ([dataset] Greenfield et al., 2020). This 374 OTU dataset includes taxonomic information for each of the fungal OTUs, and additional data including OTU sequence abundances across each of the samples, and sample data including metadata relating to each sample.

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