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# Multiple evolutionary origins lead to diversity in the metabolic profiles of ambrosia fungi

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## ABSTRACT

Ambrosia fungi are an ecological assemblage cultivated by ambrosia beetles as required nutrient sources. This mutualism evolved in multiple beetle and fungus lineages. Whether convergence in ecology led to convergent metabolism in ambrosia fungi is unknown. We compared the assimilation of 190 carbon sources in five independent pairs of ambrosia fungi and closely related, non-ambrosial species. Ecological convergence versus phylogenetic divergence in carbon source use was tested using variation partitioning. We found no convergence in carbon utilization capacities. Instead, metabolic variation was mostly explained by phylogenetic relationships. In addition, carbon usage in ambrosia fungi was equally diverse as that in non-ambrosial species. Thus, carbon metabolism of each ambrosia fungus is determined by its inherited metabolism, not the transition towards symbiosis. In contrast to other fungus-farming systems of termites and attine ants, the fungal symbionts of ambrosia beetles are functionally diverse, reflecting their independent evolutionary origins.

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## 1. Introduction

Fungus-farming by ambrosia beetles is a widespread, diverse, and ancient insect agricultural system that first arose over 100 million years ago (Vanderpool et al., 2017). Ambrosia beetles (Coleoptera: Platypodinae and Scolytinae) are an ecological assemblage of wood-boring weevils which typically colonize recently dead trees and cultivate fungal symbionts in breeding tunnels (galleries). Ambrosia beetles typically do not consume wood as a food source, but instead rely on the symbiotic fungi for nutrition (Batra, 1966). All ambrosia beetles have evolved a specialized sac-like structure (mycangium) for the transmission of the fungal symbiont among host trees after dispersal (Francke-Grossmann, 1956; Batra, 1963). Within Scolytinae and Platypodinae, ambrosia farming has more than eleven independent evolutionary origins (Jordal and Cognato, 2012; Hulcr and Stelinski, 2017). Analogous to the polyphyletic origins of ambrosia beetles, the ambrosia fungi have repeatedly evolved in the Dikarya (Ascomycota and Basidiomycota), in which at least seven families include lineages adapted to the ambrosia symbiosis (Ophiostomataceae, Ceratocystidaceae, Nectriaceae,

Bionectriaceae, Saccharomycetaceae, Peniophoraceae, and Meruliaceae) (Whitney et al., 1987; Endoh et al., 2008; Kolařík and Kirkendall, 2010; De Beer et al., 2013; Li et al., 2015; O'Donnell et al., 2015). Consequently, ambrosial fungi are not monophyletic, but phylogenetically diverse, arising from multiple, distantly related non-ambrosial lineages.

The repeated evolution of the ambrosial habit presents a unique opportunity to study patterns and processes in organisms transitioning from free-living, independent lifestyles to mutualistic symbioses. In other farming societies, such as fungus farming ants and fungus farming termites, the association has evolved only once within their respective groups, and the phylogenetic diversity of the fungal symbiont diversity is considerably smaller than that of the ambrosia fungi (Mueller et al., 2005; Hulcr and Stelinski, 2017). In contrast to these other fungus farming systems, ambrosia fungi evolved from lineages that are highly variable in their respective ecologies and metabolic requirements. One of the largest radiations is that of *Raffaelea*, a polyphyletic group of closely related clades within Ophiostomatales (Vanderpool et al., 2017), which specialize on large wood segments, typically tree trunks, that retain moisture longer than other tree parts (Romón et al., 2007; Foit, 2010). While most *Raffaelea* species are not specific to any particular tree taxon, some show preference for certain plant host families (Carrillo et al., 2014; Simmons et al., 2016). Similar ecology is found in

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*Ambrosiozyma*, a clade of hyphae-forming yeasts that are strictly associated with beetles colonizing large and humid tree trunks (Endoh et al., 2008; Yun et al., 2015). The ambrosia habit has also evolved multiple times in the Ceratocystidaceae, and gave rise to the related (but independent) genera *Ambrosiella*, *Meredithiella*, and *Phialophoropsis* (Mayers et al., 2015). The ancestral Ceratocystidaceae are often plant pathogens capable of early colonization of dying trees. The three ambrosia genera appear to have evolved a somewhat different ecology, ranging from preferences for thinner and drier material (*Ambrosiella*) to preference for large trunks (*Phialophoropsis*) and even living trees (*Meredithiella*) (Jaramillo et al., 2011). Most *Ambrosiella* species are not specific to any plant family, but the other two genera show some evidence of host specificity (Brady and Sutton, 1979; Mayers et al., 2015). In yet another independent origin of the ambrosia habit in the genus *Geosmithia*, the fungi are largely associated with phloem-feeding bark beetles, and typically occupy the drier parts of the phloem than those preferred by Ophiostomatales, such as branches and twigs (Kolařík and Jankowiak, 2013). The two ambrosial species, *Geosmithia microcorthyli* and *Geosmithia eupagioceri*, however, appear to live in larger and more humid trunks (Kolařík and Kirkendall, 2010). There are many additional fungal taxa that gave rise to the ambrosia habit, and each displays a certain degree of convergence as well as unique ecology (Hulcr and Stelinski, 2017). In short, the repeated evolution of a mutualistic nutritional association with a beetle vector among distantly related fungal lineages makes the ambrosia fungi a perfect model system for studying the evolution of fungus-animal mutualism in a phylogenetically and statistically robust, replicated framework.

Ambrosia fungi all depend on beetle vectors for transmission between trees, and the beetle appears to provide nutrition to the fungal inoculum within the mycangium during its dispersal phase (Schneider and Rudinsky, 1969). Inside the wood, however, the fungi grow mostly around the beetle galleries, and in certain cases, they can also be detected many centimeters from the inoculation points, presumably as a result of rapid growth through the vascular system (Inch and Ploetz, 2012). Therefore, while the ambrosia fungus life cycle is dependent on beetle dispersal, the fungi are metabolically independent of their vector when growing in the xylem.

Beyond this general pattern, adaptations in the fungi to the symbiotic lifestyle remain poorly known. For instance, we do not know which labile and recalcitrant sources of carbon can be utilized by ambrosia fungi, whether fungi evolved metabolic modification to adapt to the ambrosia niche, or to what extent such metabolic modifications are shared across diverse lineages of ambrosia fungi. A few studies have provided evidence that ambrosia fungi have limited metabolic capacities. A minimal nutrient assay of a fungal symbiont of *Xyleborus ferrugineus* showed utilization of dextrose, but not cellulose as a carbon source to sustain the progeny of the beetle population (Norris and Baker, 1968). De Fine Licht and Biedermann (2012) revealed that the enzymatic activities in the gallery of *Xyleborinus saxesenii* targeted hemicellulose and simple sugars, rather than decomposing the complex compounds in plant cells. The amino acid composition of *X. ferrugineus* was largely shared by its fungal symbionts suggesting a mutual chemical interdependence (Abrahamson and Norris, 1970; Kok and Norris, 1972). While the above-mentioned studies have provided insights to the nutritional ecology of ophiostomatoid ambrosia fungi, very little is known about the metabolism of ambrosia fungi in groups other than Ophiostomatales, and no comparison between ambrosia fungus clades has been published. Here we utilize the multiple independent evolutionary origins of the ambrosia symbiosis as a natural experiment to explicitly test for convergent metabolic adaptations that transcend phylogenetic relationships. We conducted

the first comparison of the carbon source metabolic profiles of ambrosia fungi and their most closely related non-ambrosial species. We addressed four questions: (1) Have ambrosia fungi convergently evolved similar metabolic profiles? (2) Do ambrosia fungi, on average, use fewer or more specific compounds than their non-ambrosial relatives? (3) Are there specific nutrients that are utilized significantly better or worse in ambrosia than non-ambrosia fungi? (4) What are the relative contributions of phylogenetic history and life-styles (ambrosia versus non-ambrosia) to the metabolic profiles of fungi? We focus on carbon metabolism, because the goal of our study is the ecology of the fungi, and carbohydrates are likely the most commonly metabolized compounds in the environment of each fungus and may play a role in partitioning their niches (Hanson et al., 2008). Carbohydrates are also probably the most traded metabolic commodity between the fungus and the beetles, and thus carbohydrate metabolism has a high probability of showing adaptation to the mutualistic lifestyle.

## 2. Materials and methods

### 2.1. Fungal isolates

To compare the metabolic profiles between ambrosia fungi and their nearest available non-symbiotic relatives, five pairs, including four filamentous fungal pairs and a yeast fungal pair, were selected: *Raffaelea lauricola* and *Leptographium* sp. (Ophiostomataceae), *Ambrosiella roeperi* and *Huntiaella moniliformis* (Ceratocystidaceae), *Fusarium* sp. AF3 and *Fusarium solani* (FSSC “clade 3” sensu O’Donnell (2000)) (Nectriaceae), *G. microcorthyli* and *Geosmithia* sp. 8 (Bionectriaceae), *Ambrosiozyma platypodis* and *Ogataea minuta* (Saccharomycetaceae). The sources of each isolate are provided in Table 1.

### 2.2. Phenotype microarrays (PM) study

The carbon metabolic profiles of fungal isolates were assayed using PM1 and PM2A Biolog Phenotype MicroArrays™ (Biolog, Inc. Hayward, CA). Each 96-well microplate contains 95 different sole carbon sources with typical concentration for fungal growth (2–20 mM) and 1 well of negative control without substrate. Therefore, 190 different carbon sources were assayed for each fungal isolate.

Inoculum preparation followed the manufacturer’s instruction and Blumenstein et al. (2015b) adapted for filamentous fungi and yeasts. Agar chunks were collected from a 2-week old fungal culture into a 2 mL Eppendorf vial and moderately vortexed to obtain cell suspension. Fungal cell suspension was spread onto sterile semi-permeable cellophane which was appressed to Petri dishes of 2% MEA to prevent carry-over of agar residue when harvesting fungal tissues. Inoculated Petri dishes were cultivated in the dark at 25 °C for 14–28 d. Incubation time was dependent on the growth rate of each fungal isolate. Fungal cells were removed from the cellophane membrane with a sterile cotton swab and transferred into a 2 mL Eppendorf vial containing 1 mL of Biolog FF inoculating fluid (FF-IF). Isolates with thick hyphal mats (*A. roeperi* and *H. moniliformis*) were gently ground with a sterile pestle and then moderately stirred to obtain a homogeneous tissue mixture. The tissue mixture was added to the FF-IF tubes (18 mL) and adjusted to 60–62% of transmittance. A 37.5 µL of adjusted aliquot was added to another FF-IF tube (18 mL) to obtain the final inoculum. The final inoculum was inoculated into the microplate at 100 µL per well. Inoculated microplates were cultivated at 25 °C in darkness. Three technical replicate subcultures were analyzed for each fungal isolate on PM1 and PM2A respectively to account for measurement error.

**Table 1**

List of the ten fungal species used in the Phenotypic Microarray study, their corresponding acronyms, isolate sources, and 28S rRNA accession numbers.

Fungi	Taxonomy	Acronym <sup>a</sup>	Ambrosia <sup>b</sup>	Isolate no. <sup>c</sup>	Isolate information	Isolation sources
<i>Raffaella lauricola</i>	Ophiostomataceae	Rla	Y	10980	mycelium of <i>Xyleborus glabratus</i>	Forest Pathology Lab, UF
<i>Leptographium</i> sp.	Ophiostomataceae	Lsp	N	11032	crushed head sample of <i>Xyleborus festinus</i> (phoretic symbiotic fungus)	Forest Entomology Lab, UF
<i>Ambrosiella roeperi</i>	Ceratocystidaceae	Aro	Y	14766	mycelium of <i>Xylosandrus crassiusculus</i>	Forest Entomology Lab, UF
<i>Hunttiella moniliformis</i>	Ceratocystidaceae	Hmo	N	14053	<i>Quercus ellipsoidalis</i> (sapwood of a 2–3 weeks cut stump)	PPRI Culture Collection; source no. CMW9989
<i>Fusarium</i> sp. AF3	Nectriaceae	Faf	Y	6459	mycelium of <i>Euwallacea interjectus</i>	Forest Entomology Lab, UF
<i>Fusarium solani</i>	Nectriaceae	Fso	N	14852	surface wash of <i>Xylosandrus compactus</i> (phoretic symbiotic fungus)	Forest Entomology Lab, UF
<i>Geosmithia microcorthyli</i>	Bionectriaceae	Gmi	Y	11071	ambrosial layer on the gallery wall of <i>Microcorthylus</i> sp.	CCF Culture Collection of Fungi; source no. CCF3861
<i>Geosmithia</i> sp.8	Bionectriaceae	Gsp	N	11072	surface wash of <i>Scolytus intricatus</i> (phoretic symbiotic fungus)	CCF Culture Collection of Fungi; source no. CCF3350
<i>Ambrosiozyma platypodis</i>	Saccharomycetaceae	Apl	Y	14853	whole beetle ground-up <i>Platypus koryoensis</i>	College of Life Sciences and Biotechnology, Korea University; source no. PKGY10-1
<i>Ogataea minuta</i>	Saccharomycetaceae	Omi	N	14259	fermenting mushrooms ( <i>Mycena pura</i> )	Phaff Yeast Culture Collection, UC Davis; source no. UCDFST 50-341

<sup>a</sup> Acronyms used in the plots.<sup>b</sup> Ambrosia ecology of fungal species were determined based on reported references or isolation information.<sup>c</sup> Isolation number in the database of Hulcr's lab in University of Florida.

Biolog plates were read using an Emax microplate reader (software: Softmax<sup>®</sup> Pro 3) with wavelengths at 750 nm. The OD<sub>750</sub> measures the biomass, i.e. mycelia and conidia production, of each well. The first read was performed approximately 30 min after inoculation as a blank value specifically for each microplate (T=0). Readings were then carried out at intervals of 24 h for 8 d.

### 2.3. Phylogenetic distances among fungal species

To assess the contribution of phylogenetic signal to the metabolic variance among fungal isolates, all pairwise phylogenetic distances of assayed fungi were calculated based on partial 28S ribosomal DNA sequences. Given the distant relatedness of our assayed fungi, the relatively conserved 28S region was chosen to circumvent the potential issue that may have resulted from using a fast-evolving region such as the ITS region. Partial 28S rDNA was amplified using the primer pair LROR/LR5 (Vilgalys and Hester, 1990; Rehner and Samuels, 1994). DNA extraction of fungal isolates was carried out using Extract-N-Amplify Plant PCR kit (Sigma-Aldrich) from fungal tissues cultured on 2% MEA (malt extract agar, BD Difco). PCR reaction mixture consisted of 50–100 ng template DNA, 1.25 U Taq polymerase (Takara Bio Inc), 200 μM dNTP, 0.5 μM of each primer, and 5% DMSO (V/V). The PCR conditions were as follows: 95 °C for 4 min, followed by 35 cycles of 94 °C for 30 s, 40 s at 50–55 °C, and 1 min at 72 °C. The final extension step was 10 min at 72 °C. Purification of PCR products and DNA sequencing were carried out at Genewiz, Inc (South Plainfield, NJ, USA). Sequences were deposited on NCBI GenBank (accession no. MG954239–MG954248). Sequences were aligned using the online version of MAFFT (Katoh and Standley, 2013). The aligned sequences were trimmed to equal lengths (853 bp including gaps). Tree topology was inferred with Maximum Likelihood analysis using RAxML 8.2.2 (Stamatakis, 2014) with default setting and 1000 pseudoreplicate search for bootstrap value estimation. Dendrogram tree was visualized and edited using TreeGraph2 (Stöver and Müller, 2010). The NEXUS file containing partial 28S sequences and a RAxML inferred phylogeny was deposited in TreeBASE (study no. S22300).

### 2.4. Statistical analyses

All statistical analyses were performed using R v.3.4.1 (R Core Team, 2013). Graphs were generated using the package ggplot2 (Wickham, 2016). The OD<sub>750</sub> absorbance values were normalized following Garland and Mills (1991). The blank value of each plate (T=0) was first subtracted from raw absorbance values; any negative values were forced to zero for analytic convenience as recommended by Garland and Mills (1991). An average well color development (AWCD) of each plate was calculated as the sum of absorbance values obtained from 95 wells (excluding the control well) divided by 95. The subtracted values were then divided by the AWCD to obtain the normalized values. All statistical analyses were conducted using the normalized values unless otherwise stated.

### 2.5. Convergence of carbon assimilation test

We hypothesized that ambrosia fungi converged on an overall similar metabolic profile as a result of having similar life histories. To test this hypothesis, we conducted a distance-based test of homogeneity in multivariate dispersion between ambrosia and non-ambrosial isolates (Anderson, 2006). Specifically, this test determines if groups differ in how variable they are among observations within each group. From our hypothesis that ambrosial fungi have converged on similar metabolic profiles, we predicted that ambrosia fungi would show reduced variability in metabolic profiles when compared to non-ambrosial fungi. The test was implemented on a Bray-Curtis distance matrix using the betadisper () function of the vegan package for R (Oksanen et al., 2013).

### 2.6. Metabolic niche breadth test

Our objective was to determine if ambrosial species have diminished or expanded abilities to utilize diverse carbon resources when compared to phylogenetically related, non-ambrosial species. We used OD<sub>750</sub> absorption values as an indicator of fungal growth for each isolate on each carbon source at 8 d. These values were used to calculate the effective number of carbon sources equally utilized by each isolate. This was done by first calculating Simpson's

index using the diversity () function in Vegan. Simpson's index was then converted to Hill's numbers (Hill, 1973) which is the number of equivalent values required to produce the observed Simpson diversity index (Jost, 2006). This value thus reflects the number of carbon sources that can be used, as well as the degree to which each was used.

A mixed-modeling approach was used to account for variation due to fungal clade while determining if there was a significant effect of ambrosia life style on the diversity of carbon sources that each fungal isolate could metabolize. Models were fitted using the lme () function in the R package nlme (Pinheiro et al., 2017). We considered ambrosial lifestyle (yes or no) as a fixed effect. To find the best model structure, we examined a model with no random terms (model 1), a model with a random intercept for fungal clade (model 2), and a third model with a random intercept and slope (model 3). The best model structure was chosen according to lowest Akaike information criterion (AIC). The best model was validated by visual inspection of residuals, plots of residuals versus fitted values, and residuals by main effect (Zuur et al., 2009).

### 2.7. Do ambrosia fungi share one or a few preferred carbon sources?

Even if ambrosia fungi do not share overall metabolic profiles, they may still have converged on the utilization of a few key compounds. To test for this possibility, we conducted a linear model-based analysis of the multivariate Biolog profiles to determine if there were significant differences in the utilization of individual carbon compounds in ambrosial versus non-ambrosial fungi. We used the manylm () function of the mvabund package for R (Wang et al., 2012) to fit individual linear models for each carbon source with an alpha correction for multiple comparisons, as well as an overall multivariate model. The OD<sub>750</sub> absorption values at 8 d were used as an indicator of fungal growth for each isolate. For each model, the response variable was the average normalized OD<sub>750</sub> values of all 3 replicates for each isolate, and life style (ambrosia versus non-ambrosia) was the predictor. In a separate analysis to determine if isolates within each clade differed in utilization of each carbon source, a one-tailed *t*-test on the OD<sub>750</sub> values of three replicates of each carbon source between ambrosia and non-ambrosia in a designated species pair (i.e. Ophiostomataceae, Ceratocystidaceae, Nectriaceae, Bionectriaceae, and Saccharomycetaceae) to investigate whether the ambrosia fungi significantly used better ( $P < 0.05$ ) of a specific compound. Because this analysis was exploratory (i.e. not testing a specific hypothesis), we did not apply alpha corrections for *t*-tests.

### 2.8. How much is carbon assimilation explained by phylogenetic origin versus symbiotic habit?

We conducted a variation partitioning analysis to separate the effects of lifestyle from those of phylogenetic relationships on the metabolic profiles of fungi. Pairwise phylogenetic distances were obtained from the trimmed sequences using a dist.ml () function in the phangorn package for R with default settings (Schliep, 2011). The phylogenetic distance matrix was then decomposed to phylogenetic eigenvectors by principal coordinates analysis, using the pcoa () function in the ape package for R (Paradis et al., 2004) following the methods of Dray et al. (2006) and Tedersoo et al. (2013). Phylogenetic eigenvectors are orthogonal vectors that describe relationships among taxa at various phylogenetic scales (approximately different taxonomic levels) (Diniz-Filho et al., 2012) and can be used as independent predictor variables in univariate and multivariate modeling. The response variable was the OD<sub>750</sub> absorption value averaged for all three replicates per isolate

measured at day 8. These values were Wisconsin double-standardized. To identify significant phylogenetic eigenvectors and to avoid over fitting our final model, we conducted forward multivariate model selection (Blanchet et al., 2008) using the forward.sel function of the packfor package for R (Dray et al., 2009). We then used variation partitioning to partial out the amount of variation explained by all phylogenetic eigenvectors retained by model selection from the variation explained by lifestyle (ambrosia versus non-ambrosia). Variation partitioning was conducted using the varpart () function in the vegan package for R (Oksanen et al., 2013). Significance of each fraction was determined by redundancy analysis, using the rda () function in the vegan package (Oksanen et al., 2013).

## 3. Results

All of the assayed fungi grew in most simple sugars (monosaccharides to polysaccharides) and polymers. Some substrates, such as L-fucose, D-psicose, N-acetyl-D-galactosamine, and 2,3-butanediol yielded a low OD<sub>750</sub> value in all fungal species. Most of the amino acids and amides provided in this version of the Biolog assay were able to sustain the growth of each species, except methionine which was not utilized by Ophiostomataceae and Saccharomycetaceae species, and supported only limited growth of other fungi (Supplementary Table 1).

### 3.1. Convergence of carbon assimilation test

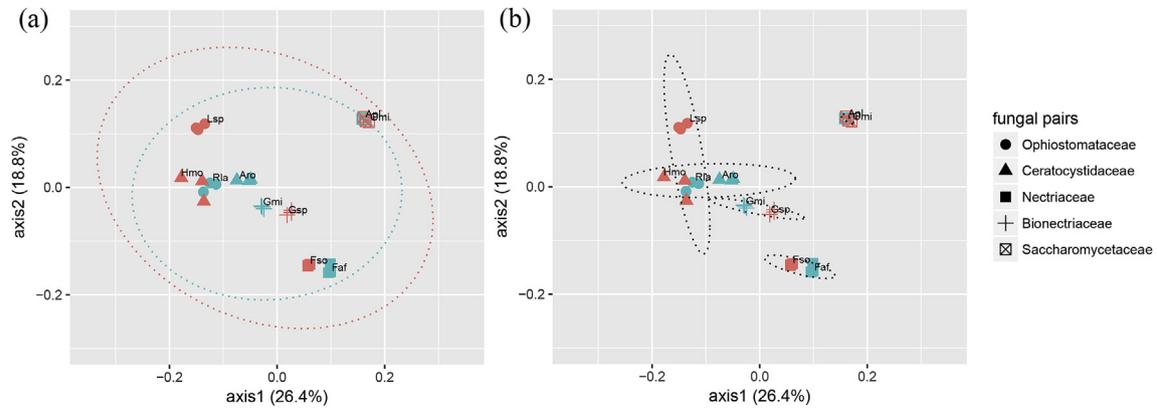
Test of homogeneity in multivariate dispersion revealed no significant difference in variability among isolates between ambrosia fungi and non-ambrosial fungi (betadisper,  $F = 0.988$ ,  $P = 0.33$ ). It indicated that ambrosia fungi have not converged on a similar metabolic profile. Instead, ordination revealed a strong phylogenetic signal in metabolic profile, demonstrating that metabolic profiles are conserved within lineages throughout the evolutionary transition to an ambrosia life-style (Fig. 1). The first two ordination axes of multivariate scaling plot explained 45.2% of the variations. Visually, the three technical replicates of each assayed fungus were strongly clustered except for those of *H. moniliformis* which showed slight separation (Fig. 1). The ambrosia fungi and non-ambrosial fungi fully overlapped in their distribution on the plot (Fig. 1A). The Saccharomycetaceae pair (*O. minuta* and *A. platypodis*) was the most strongly clustered group, followed by the Nectriaceae pair (*F. sp. AF3* and *F. solani*), the Bionectriaceae pair (*G. microcorthyli* and *G. sp.8*), and then the Ceratocystidaceae pair (*A. roeperi* and *H. moniliformis*). The metabolic profiles of the Ophiostomataceae (*R. lauricola* and *Leptographium sp.*) were relatively distant from each other (Fig. 1B).

### 3.2. Metabolic niche breadth test

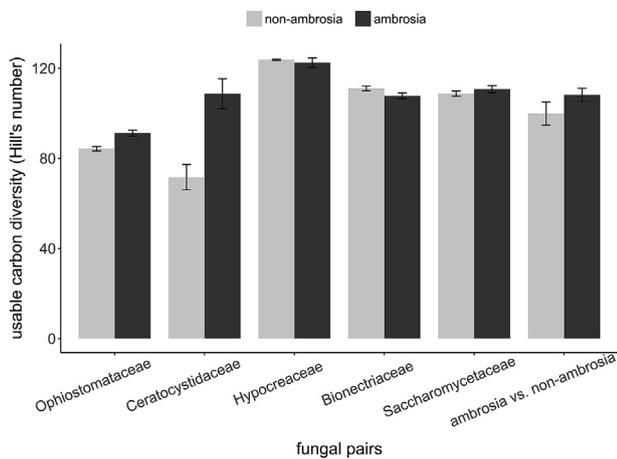
We found no evidence that evolution of the ambrosial habit resulted in decreased carbon metabolic capacity. The ambrosia fungi utilized slightly more carbon sources than did non-ambrosial fungi (out of the 190 tested, ambrosia: mean 108.17, SD 11.45, non-ambrosia: mean 99.89, SD 20.11; Fig. 2) but the difference was not statistically significant (linear mixed-effects model,  $t = 1.21$ ,  $P = 0.24$ ). The best model structure according to the AIC was the fixed effect of ambrosia lifestyle, with random intercept and slope for phylogenetic pairings in the mixed model (model 3) (Table 2).

### 3.3. Do ambrosia fungi share one or a few preferred carbon sources?

We found no evidence that ambrosia fungi have converged on



**Fig. 1.** Plots of multivariate analysis showing the metabolic dissimilarities of carbon sources among fungal species. (A) Similar profiles were observed among ambrosial and non-ambrosial fungi. Ellipses illustrate 1 standard deviation around the centroids for ambrosia fungi (green) versus non-ambrosial fungi (red). (B) Fungal lineages varied in metabolic profile. Ellipses are drawn for phylogenetic pairs [symbol codes: ● Ophiostomataceae pair, *Raffaella lauricola* (Rla) and *Leptographium* sp. (Lsp); ▲, Ceratocystidaceae pair, *Ambrosiella roeperi* (Aro) and *Huntia moniliformis* (Hmo); ■, Nectriaceae pair, *Fusarium* sp. AF3 (Faf) and *F. solani* (Fso); +, Bionectriaceae pair, *Geosmithia microcorthyli* (Gmi) and *G. sp.8* (Gsp); ☒, Saccharomycetaceae pair, *Ambrosiomyces platypodis* (Apl) and *Ogataea minuta* (Omi)]. Points represent individual samples and ellipses drawn with 95% confidence intervals fitted onto the spatial ordination.



**Fig. 2.** Boxplot of fitted values for linear mixed model showing the non-significant effects of the ambrosia lifestyle on the diversity of carbon sources utilized by the fungal isolates. The Y-axis represents the number of carbon sources that were utilized equally well by each isolate (explained in Methods).

similar metabolic capacities across lineage for any particular carbon source (Fig. 1). Linear model-based analysis found no significant difference in carbon utilization between ambrosia and non-ambrosia fungi in the multivariate model ( $F_{8,1} = 120.9$ ,  $P = 0.924$ ), and there were no significant differences in utilization of any individual carbon sources (in all cases  $P_{adj} > 0.05$ ).

Similarly, a heatmap of  $t$ -test comparisons of the normalized OD<sub>750</sub> values (8d) within each fungal pair showed that no single specific carbon compound was significantly used better in all ambrosia fungi than all non-ambrosial counterparts (Supplement Fig. 1). A more detailed, within-clade analysis suggested that there

**Table 2**

Mixed model test for metabolic breath of ambrosia fungi. Model 1 included fixed effect only, model 2 included fixed effect with random slope for phylogenetic pair, and model 3 included fixed effect with random intercept and slope for phylogenetic pair (see Methods).

Model	df	AIC	L.Ratio	P-value
1	3	247.3956	NA	NA
2	4	229.8695	19.52611	9.92E-06
3	6	213.199	20.67054	3.25E-05

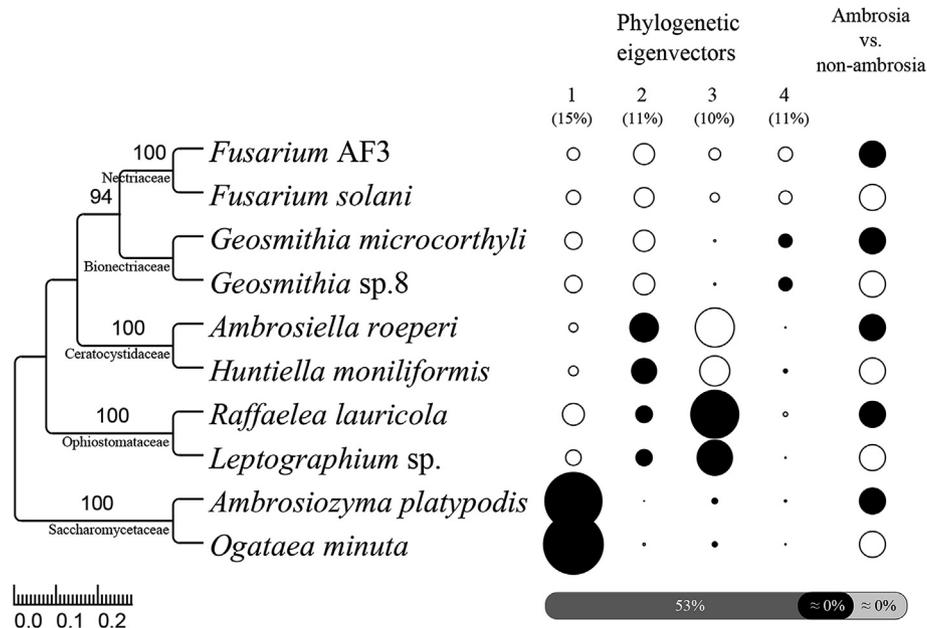
were two compounds, lactulose and N-acetyl- $\beta$ -D-mannosamine, which were significantly used better in four ambrosia fungi than their non-ambrosia relatives: *A. roeperi* [lactulose ( $t = 5.28$ ,  $P < 0.05$ ); N-acetyl- $\beta$ -D-mannosamine ( $t = 5.00$ ,  $P < 0.05$ )], *F. sp. AF3* ( $t = 4.14$ ,  $P < 0.05$ ;  $t = 4.60$ ,  $P < 0.05$ , respectively), *G. microcorthyli* ( $t = 2.82$ ,  $P < 0.05$ ;  $t = 4.36$ ,  $P < 0.05$ , respectively), and *A. platypodis* ( $t = 3.85$ ,  $P < 0.05$ ;  $t = 6.56$ ,  $P < 0.05$ , respectively) (Supplementary Fig. 1).

#### 3.4. How much is carbon assimilation explained by phylogenetic origin versus lifestyle?

The carbon metabolic profiles of fungal species were structured along phylogenetic relations among species rather than their lifestyle (ambrosia versus non-ambrosia) (Fig. 3). Model selection retained the first four phylogenetic eigenvectors to explain variation in metabolic profiles among isolates. These eigenvectors cumulatively explained 47.2% of the variation in metabolic profiles among all sampled fungi (Table 3). The first phylogenetic eigenvector (PE#1) described the phylogenetic separation between the Saccharomycetaceae group and other fungal groups and explained 15% of the variation observed in metabolic profiles among isolates. The second, PE#2, described the phylogenetic separation between Nectriaceae/Bionectriaceae (Hypocreales) and other fungal groups and explained 11% of the variation in metabolic profiles. PE#3 represented the separation between Nectriaceae/Bionectriaceae/Ceratocystidaceae (Hypocreomycetidae) and other fungal groups and explained 10%, and PE#4 represented the *Fusarium* spp./*Geosmithia* spp. split and explained 11%. A subsequent analysis partitioning the variables of phylogenetic eigenvectors and lifestyle indicated that 53% of the metabolic variations were independently explained by phylogenetic eigenvectors, -6% by lifestyle, and -0.5% by variation shared by both variables (Fig. 3). RDA indicated that only the phylogenetic fraction was significant ( $F = 3.28$ ,  $P = 0.002$ ). The unexplained residuals were 53% (Fig. 3).

#### 4. Discussion

This study took advantage of the multiple independent origins of ambrosia beetle-fungus symbioses as a natural evolutionary experiment to understand how insect cultivation of nutritional symbionts affects the evolution of the symbionts' metabolic capacities. Our results revealed the importance of evolutionary



**Fig. 3.** An ultrametric phylogeny of fungal isolates and their relation to significant phylogenetic eigenvectors and lifestyles (circles). A Venn diagram below the eigenvector circles represents variation in the carbon metabolic profiles among fungal species explained by the phylogenetic eigenvectors (medium gray), the lifestyles (light gray), and the shared variation explained by both variables (black). Values above the phylogeny branches indicate bootstrap support >70 of clades. Fungal families were indicated below the phylogeny branches. The size of the circles corresponding to each species represents their coordinates on an eigenvector ranging from  $-1$  to  $1$  (the sizes corresponding to the absolute value of each eigenvector). Circles of the lifestyles are binary (yes or no). Filled circles: positive values, ambrosia fungi; open circles: negative values, non-ambrosia.

**Table 3**  
Determination coefficients, F values and P values of phylogenetic eigenvectors in forward model selection.

	R <sup>2</sup> adjusted	F value	P value
PE#1	0.1506102	2.45687854	0.005
PE#2	0.1095265	2.17662138	0.003
PE#3	0.0988725	2.11190103	0.012
PE#4	0.1131826	2.39768954	0.021

origins rather than the ecological strategies (ambrosia lifestyle in our case) in shaping the metabolic capacities of the symbiotic fungi tested. Carbon metabolism does not show any convergence among ambrosia fungi and there is no significant change in the diversity of carbon sources that fungi can utilize. Instead, metabolic capacities were different between fungal clades and were conserved within each clade. Consequently, each origin of the ambrosial crop features a functionally distinct symbiont, and together ambrosia symbioses represent an ecologically diverse suite of mutualistic associations. This conclusion is supported by several findings: (1) Carbon metabolism profiles were equally variable among ambrosial and non-ambrosial fungi. (2) There were no significant differences between ambrosia and non-ambrosia fungi in aggregate composition of carbon sources that were utilized or specific types of carbon that could be utilized. (3) The majority of variation among isolates in carbon metabolism profiles (53%) was explained by phylogenetic relationships, whereas lifestyle (ambrosia versus non-ambrosia) did not explain a significant fraction of variation.

#### 4.1. Suitability of the method

The three replicates of each assayed fungus on the plot of multivariate analysis were strongly clustered, suggesting the high reproducibility and feasibility of the Biolog microarray in profiling metabolic capacities of filamentous fungi (Pinzari et al., 2016). The slight variation among replicates of *H. moniliformis* on the

multivariate analysis plot was likely due to the melanized mycelium, which may cause biased readings (Morris and Nicholls, 1978; Pinzari et al., 2016).

It was not our focus to investigate the utilization of each specific substrate by each fungus. Solid interpretation of the usage of each individual substrate may not be warranted due to the limitations within the PM system, e.g. the production of non-active biomass, extracellular pigments of fungi, or cross-well effects caused by volatiles (Blumenstein et al., 2015b; Aylward et al., 2017). Instead we focused on broader compound use patterns for a comparative evolutionary analysis, for which the Biolog PM system is excellent.

#### 4.2. Carbon assimilation in relation to fungal phylogeny and lifestyle

The phylogenetic relatedness of the assayed fungi was a strong predictor of the metabolic variation. One of the most notorious difficulties in the inference of ecological patterns among organisms is the phylogenetic non-independence of the analyzed taxa (Harvey and Pagel, 1991). The eigenvector-based variation partitioning allowed us to separate the effects of fungal phylogeny from that of the ambrosia lifestyle on their metabolism. The practice of using eigenvector-based variation partitioning was demonstrated in another ecological study (Tedesoo et al., 2013). Here, this approach enabled us to compare the independent and shared contribution of the phylogenetic and ecological variables to the variation in fungal metabolism (Desdevises et al., 2003).

The class-level split between the Saccharomycetaceae pair (Saccharomycetes) and the rest of the fungi (Sordariomycetes) contributed the highest phylogeny effect (PE#1, 15%). The other three phylogenetic eigenvectors, the order-level split [Hypocreales (Nectriaceae and Bionectriaceae) vs. others, PE#2], subclass-level split [Hypocreomycetidae (Nectriaceae, Bionectriaceae, Ceratocystidaceae) vs. others, PE#3], and genus-level split (*Fusarium* spp./*Geosmithia* spp., PE#4) contributed similar effects on the variations

(ca. 10%). The similar explanatory power of all four phylogenetic eigenvectors suggests that there was no specific taxonomic-level effect on the metabolic differences of fungi. Even the well-studied ambrosia fungus clades that are millions of years old, i.e. the Ophiostomataceae and Ceratocystidaceae, have not undergone any major metabolic modification after their involvement in the ambrosia symbiosis.

Variation partitioning revealed that the fungus lifestyle has no discernible effect on the metabolic profiles, even after the effects of phylogeny had been removed. The conservation of the metabolic profile of each ambrosia fungus may also partly explain ecological differences between major clades of the beetle vectors, specifically their microhabitat preferences within a tree and along the tree decay time gradient (Kolařík and Kirkendall, 2010; Seifert et al., 2013; Mayers et al., 2015). In an extreme example, the fungal symbiont *Flavodon ambrosius*, a unique ambrosial fungus capable of aggressive white-rot, has resulted in a shift of the optimal stage of tree decay for the beetle vector (Kasson et al., 2016; Li et al., 2017). Our results support the previously espoused hypothesis that it is the fungal metabolism, not the beetle host choice, which places the more conservative constraint on the overall ecological niche of each symbiotic pair (Macedo-Reis et al., 2016; Hulcr and Stelinski, 2017).

#### 4.3. Metabolic breadth of ambrosia and non-ambrosia fungi

Literature on insect symbioses is focused on a few well-studied models, most of which are intracellular prokaryotic mutualists. In most of these, metabolic capacity has universally degraded over evolutionary time, compared with free-living relatives (McCutcheon and Moran, 2012). In obligate internal eukaryotic symbionts, such as insect intracellular fungi or mycorrhizal fungi, a more complex pattern is emerging where the genome size and complexity overall does not change dramatically, but the metabolic pathway repertoire is distinctly adapted towards the symbiotic lifestyle (Tisserant et al., 2013; Vogel and Moran, 2013). In other fungus farming systems, such as the leaf cutter ants, the fungal cultivar has also undergone selection for symbiosis-related metabolic traits, rather than an overall reduction (Nygaard et al., 2016). Ambrosia fungi, however, are not internal symbionts, and they do not depend on the insect “farmer” for substrate delivery. For most of their lifecycle, ambrosia fungi are nutritionally independent organisms inside tree tissues, and only depend on the vector's provisions during a short dispersal when they are transported inside a mycangium (Hulcr and Stelinski, 2017). The evolution of their metabolism therefore does not face the same constraints as that of intracellular mutualists, internal gut symbionts, or farmed crops. As a result, their metabolic repertoire is not expected to diminish compared to non-symbiotic fungi. This is the pattern that we confirm here: even after millions of years of involvement in a symbiotic relationship with the beetles (Jordal and Cognato, 2012; Vanderpool et al., 2017), most ambrosia fungi still function as non-ambrosial relatives in terms of their carbon metabolism.

Most ambrosia fungi are not known to decompose lignocellulose, the main component of tree tissues (Kasson et al., 2016). The carbon source panel used here did not include lignocellulosic polymers, therefore we could not directly test the capacity of ambrosia fungi to utilize complex carbon chains such as cellulose, xylan, and lignin. Instead, our assay included many simpler molecules and confirms that most ambrosia fungi grow well on more labile sources from dying or freshly dead trees such as sugars, which is consistent with a previous report (De Fine Licht and Biedermann, 2012) and similar to closely-related non-ambrosial species in Ophiostomataceae (Norris and Baker, 1968; Kirisits, 2004; Blumenstein et al., 2015a). The report of efficient lignin digestion of an ambrosial *Fusarium* by Norris (1980) is questionable

since the studied vector beetles in the genus *Xyleborus*, but this beetle genus is not known to culture *Fusarium*.

We did not recover any particular compound that was utilized significantly better by the ambrosia species than their non-ambrosia counterparts (Supplement Fig. 1). The better utilization of lactulose and N-acetyl- $\beta$ -D-mannosamine in four ambrosia fungi stands unresolved at this point. The lack of capacity to use this biosynthetic precursor of sialic acid, was linked to the over-production of chitinase in some *Trichoderma harzianum* strains (Nagy et al., 2007), but this correlation was not universally found in other fungi. Lactulose is a lactose derived compound that is commonly used in medical treatments (Lederle et al., 1990), but its metabolic significance in fungi is unknown. The ecological interpretation of the utilization of these two compounds by ambrosia fungi is unclear.

The wide breadth of the carbon metabolism in ambrosia fungi echoes their capacity to grow well on artificial media. It is, however, important to note an aspect of circularity in this reasoning, which is that we were only able to study fungi that grow well *in vitro*. Several ambrosia systems include fastidious fungi that do not grow on standard media, such as fungi associated with beetles in the tribes of Corthylini, Hyorrhynchini, and Xyloterini (Batra, 1963; Beaver, 1984; Mayers et al., 2015). These fungi may show different patterns of metabolism evolution. The historical absence of studies on the recalcitrant ambrosia fungi is a caveat not only for our work, but for the field of ambrosia symbiosis research as a whole.

Another important caveat is that the metabolic profiles of the fungi represented here should not be taken as representative of the entire families, because only two representatives per family were tested. This analysis is strictly comparative, designed to determine the effect of the ambrosial ecology, rather than to characterize broader groups of fungi.

Our assay was restricted to the utilization of carbon sources, but ignored other chemical components of fungal metabolism, such as nitrogenous and phosphorus-rich compounds, fats, and sterols (Ayes et al., 2000) and did not address compound synthesis. To truly understand the mechanisms behind the symbiosis, it will be important to test not only the compounds that the fungus consumes, but mostly the compounds that the fungus produces in abundance and provides to the beetle through swollen conidia inside the gallery. Whether there is any evolutionary convergence in the production of compounds among ambrosia fungi should be the focus of future studies.

## 5. Conclusion

Ambrosia fungi showed no detectable convergence in carbon metabolism. Instead, they largely utilize the same compounds and in a similar degree as their closest non-ambrosial relatives. Therefore, evolutionary origin is a much better explanatory variable for our understanding of ambrosia fungus metabolism than its association with the beetle vector. It further stresses the notion that ambrosia fungi, rather than beetles, drive the metabolism and ecology of this particular farming mutualism, and that there is not one, but many different ambrosia symbioses. The present phenotypic study of ambrosia fungi calls for further research on the genomics of ambrosia fungi as well as the examination of paralleled genomic adaptation in beetle vector and tree host.

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## Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.funeco.2018.03.006>.

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