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The Laboratory Environment Affects the Volatiles of Fungus Gardens in the Colonies of Fungus-farming Ants

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Abstract

The ability to recognize nestmates is critical to the ecological success of social insects. Fungus-farming "attine" ants (Formicidae: Myrmicinae: Attini: Attina) can recognize their nestmates and symbiotic fungi via chemoreception. Although it has been shown that mutualistic fungi release volatile compounds that elicit responses in fungus-farming ants, the compounds and the sensory mechanisms involved remain little studied. Here, we characterize compounds found in attine fungus gardens and explore the correlations between those compounds, fungal substrates, and the laboratory environment. We also characterize ant cuticular hydrocarbons from *Atta cephalotes* colonies of the same species maintained in the same laboratory conditions for two or more years. Using gas chromatography associated with mass spectrometry, we verified that both substrate (i.e., the food on which fungus gardens grow) and environmental origin may influence the volatiles the fungus releases. We found compounds related to the environment, including naphthalene. We show that the volatile profiles of fungal strains grown by *Atta cephalotes* are most similar to each other, whereas the profile of the fungus grown by ants in the genus *Cyphomyrmex* is more similar to that of their substrate than to the profiles of other cultivated fungi. Regarding cuticular hydrocarbons, we found that ants collected in the same location have more similar hydrocarbon profiles than ants of the same species collected in a different location, even if all the colonies had been maintained under the same conditions (temperature, substrate) for extended periods. Our results provide strong evidence that a combination of species genetics and environmental factors shape variations in the volatile chemical profiles of cultivated fungi. After long homogenization, ants still demonstrate a solid difference among the cuticular profiles.

Introduction

The symbiosis between "attine" ants (Formicidae: Myrmicinae: Attini: Attina) and their symbiotic fungi arose ≈ 60 million years ago in the ancestor of both groups (Mueller et al., 2001; Schultz & Brady, 2008; Branstetter et al., 2017). Attine ants comprise 245 species (Ferguson-Gow et al., 2014; Li et al., 2018) and are a monophyletic group (Schultz & Brady, 2008). They cultivate their food (Weber, 1958) by growing fungal species in five agricultural systems (Mehdiabadi & Schultz, 2009). The fungi are multiple species in Agaricaceae and Pterulaceae (Mueller et al., 2001). The ants forage provides substrates for fungal growth and feed on fungal mycelium (Mueller et al., 2001).

The co-evolutionary history of the association between fungus-farming ants (e.g., in lower attinas *Mycocepurus*, *Mycetophylax*, *Apterostigma*, and higher attinas *Atta* and *Acromyrmex* species) and their cultivated fungi includes elements of both cooperation and potential conflict (Richard et al., 2007; Shik et al., 2016). For instance, in "lower-attine"

(e.g., *Mycocepurus smithii*) agriculture, ants limit the quantities of proteins in garden substrates to maximize the nutritional content of their garden fungus (Shik et al., 2016). However, Branstetter and colleagues (2017) provided evidence that "higher-attine" fungus-farming ants (including leaf-cutting ants) and their fungal cultivars have been mutually dependent since the origin of higher-attine agriculture around 30 million years ago. The fungus communicates with the ants through chemical compounds, sharing information about the suitability of the substrate (North et al., 1999; Herz et al., 2008); this information is transmitted by contact (North et al., 1999; Richard et al., 2007a) and possibly by odor as well. Richard and colleagues (2007b) showed that the chemical profiles in three species of attine ants and their symbiotic fungi can be differentiated, and the ants can recognize other individuals from the same colony. This is the case even though different species of Attina may, in some cases, share the same fungal cultivar species (Jesovnik et al., 2017).

In the evolution of fungus-farming ants and their fungi, "higher-attine" fungi possibly evolved from a facultative, free-living symbiont to an obligate, tightly mutualistic symbiont due to the transport of the ancestral higher-attine fungus by ants to a dry environment (Branstetter et al., 2017). Unable to escape and interbreed with free-living conspecifics, the ancestral higher-attine fungus coevolved with its ant farmer and became domesticated, i.e., no longer capable of living independently without its ant hosts (Chapela et al., 1994; Schultz & Brady, 2008). Fungus-farming ants and their fungal cultivars provide a model system for the study of symbiotic evolution (Chapela et al., 1994), in which both partners have mutually overlapping interests and conflicts of interest (Shik et al., 2016). In this paper, we aim to investigate how colonies reared under laboratory conditions with distinct substrates can affect the volatiles released by symbiotic fungi in six fungus-farming ant species (*Cyphomyrmex muellerii, Sericomyrmex mayri, Mycetosoritis hartmanni, Trachymyrmex* sp., and *Atta cephalotes*). Additionally, we examine whether cuticular hydrocarbons (CHCs) from *Atta cephalotes* colonies collected from multiple countries remain stable after more than two years in the laboratory.

Material and Methods

Volatiles from fungus, food source, and ant room

We sampled small pieces (~ 1 g) of the fungus gardens of five ant species (Table 1). The samples were maintained in glass headspace vials of 20 ml (22x75) sterilized under UV light for 30 min. In all cases, we used polydimethylsiloxane (PDMS) fiber 57300 from Supelco (100 µm [nonbonded phase], needle size 24 ga). Before exposing the fiber to the volatile, we heated the sample for two minutes at 35 ºC and maintained this temperature throughout the extraction for colonies. Volatile extraction times varied depending on colony species (Table 1). We used ten replicates for the species Atta sexdens and Sericomyrmex mayri due to the quantity of fungus available.

We also sampled the substrates offered to the ants as a carbon source for the fungus (leaves, oak saplings, insect frass, orange peels) and an empty cleaned box as a control group. The methodology for extracting these volatiles involved heating at 35 °C for 8 minutes, followed by an 8-minute for adsorption of volatile compounds. To quantify the volatiles in the air where the colonies were located, the SPME fiber was cleaned in the equipment and exposed in the room where the colonies were located and in the corridors leading to them for 30 minutes.

All analyses were performed with a 6890-5975 GCMS (Agilent, Santa Clara, CA) with electron ionization. We used a temperature ramp proposed by Mendez-Bravo and colleagues (2018). After a preliminary test, this proved to fit our purpose best (running time/good chromatogram). The temperature ramp proceeded as follows: initial oven temperature of 40 ˚C standing for 3 min, increased to 160 ˚C at a constant rate of 15 ˚C/minute and increased to 250 ˚C at a rate of 10 ˚C/min. We used helium carrier gas (1.0 ml min-1, constant flow) with an HP 5MS + GP column.

Volatiles from fungi and leaves (the substrate for leaf-cutter fungal cultivars) were identified using the GCMS library Nist08, Nist08s, and FFSC13, Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry (Adams, 2017). We also compared the results with the NIST Webbook spectra (National Institute of Standards and Technology 2017), when available (https://webbook.nist.gov/chemistry).

Table 1. Time of fiber exposition to each fungus sample.

Ant Species[1]	Fiber exposition Time
Atta cephalotes	8 min
Sericomyrmex mayri	8 min
Trachymyrmex sp.	8 min
Cyphomyrmex mulleri	48 h
Mycetosoritis hartmanni	68 h

Symbiotic ant species. Volatiles were collected from fungus garden samples cultivated by laboratory colonies of these species.

Statistical Analysis

In order to visualize the data from the volatiles, we used an NMDS ordering (non-metric dimensional scaling) (Clarke, 1993). This type of analysis was chosen because it maintains a relationship between data points in which similar points are more closely clustered (Gotelli & Ellison, 2004; Legendre & Legendre, 2012; Buttigieg & Ramette, 2014). We used Bray-Curtis distances because they can be used for raw count data and, in addition, the measure of dissimilarity treats both high and low values as variables with the same weight (Bray & Curtis, 1957; Gotelli & Ellison, 2004; Legendre &

Legendre, 2012). To verify the goodness of fit of the data, we used two different techniques: stress and R^2, as suggested by Buttigieg and Ramette (2014). Moreover, to measure the fit of the NMDS, we used the 'rule of thumb": stress > 0.2, the ordination is arbitrary; stress approximately 0.2 ordination is suspect; stress ≤ 0.1, the ordination is fair; and stress ≤ 0.05 is a good ordination (Legendre & Legendre, 2012).

Atta cephalotes *cuticular hydrocarbons*

Unlike volatiles, cuticular hydrocarbons (CHCs) are molecularly heavier and have different conformations. For this reason, we used different methodologies to maximize the data sample; all methods are described below.

We used ten randomly selected ants from the colonies, stored them in tubes, and transferred them to a storage freezer at -20 ºC to anesthetize the ants and preserve the cuticular compounds (Martin and Drijfhout 2009b). The individuals were washed for four minutes in an amount of solvent sufficient to immerse the entire ant; the cuticular hydrocarbons were extracted using n-Hexane (95%, Sigma-Aldrich). Hydrocarbons were subsequently removed and stored inside Glass tubes containing 95% ethanol for future studies. The extract (solvent + CHC) was placed in a flow chamber at room temperature for 24 hours to evaporate the solvent. We added 5 µL of solvent afterward, put the sample into a sonic bath for 10 seconds, and injected two µL into the GCMS. The GC was operated in splitless mode, with an injection temperature of 250 ˚C and sampling time of 2.6 min. The GC temperature ramp was 150 ºC, increasing to a 5 °C/min to 280 °C, remaining at this temperature for 10 minutes, and then 280 °C to 310 °C at a rate of 5 °C/min, remaining for 10 minutes at 310 °C. Each sample spent 52 minutes in the GCMS. We used helium carrier gas and an HP5MS + GP 0.25 mm column (Agilent). The results were compared with standard solutions (C21-C40 Linear Alkanes, Sigma-Aldrich), and the unsaturated ones were identified by molecular ions and retention time.

Atta cephalotes *cuticular hydrocarbons analyses*

We used the NMDS with a Bray-Curtis similarity matrix to compare cuticular hydrocarbons. We tested the data heterogeneity using the PerMaDisp analysis (Anderson 2006). This test measures the distance between the centroid points (spatial median) in a multivariate space (Anderson 2006; Oksanen et al. 2017). In addition, to analyze the chemical compounds of individuals from each colony of *Atta cephalotes*, we did a Permutational Multivariate ANOVA (PerMAnova) (Anderson 2001, 2017). We used the dissimilarity matrix as the response variable and the colony as an explanatory variable. For that analysis, we used 999 free permutations. We used the 'adonis2' function of the vegan statistical package v.2.4.4 (Oksanen et al. 2017) for R software v.3.4.3 (R Foundation for Statistical Computing, 2016). We used the 'Pillai-trace' test for peer-to-peer comparison of the results because it is more robust for multivariate use (Hand & Taylor, 1987). The *p* values were corrected using the 'false discovery rate' (fdr) method (Bretz et al., 2016).

A discriminant percentage similarity (Simper) analysis was performed (Clarke, 1993). This analysis shows how much each variable, in our case, compound chemical, influenced the difference between the groups (Gotelli & Ellison, 2004). This analysis resulted in peer-to-peer comparisons; we selected the compounds with a discriminant percentage of up to 70% (ranging up to 1% due to the percentage of explanation of the compounds). We also used 999 permutations for this analysis. We also used a hierarchical cluster analysis to ordinate the data from *Atta cephalotes*. We used the UPGMA (unweighted pair-group method using arithmetic averages) or Average-linkage (Gotelli & Ellison, 2004) method for these analyses. The UPGMA uses the average of dissimilarity (or measure distance) between the members in each group (Gotelli & Ellison, 2004; Legendre & Legendre, 2012). In addition, we could use both multivariate methods for each sampled species, but with no repetition. We performed a permutational multivariate analysis of variance to test the differences between the colony profiles (PerMAnova) (Anderson, 2001, 2017). All statistical analyses were performed in R v3.5.1 (R Core Team, 2018).

Results

Volatiles from fungi, food sources, and ant room

We identified several volatile compounds in the Supplementary Material (SMT Table 1). Volatiles obtained from fungi colonies varied between six (*Mycetosoritis hartmanni*) to sixteen compounds (*Atta* colonies from Peru). All samples had a high abundance of naphthalene ($C_{10}H_8$), although there was no trace of that compound in the symbiotic fungi from *Atta* colonies. We also found naphthalene in the ants room (~5.5%), and food and nest boxes (Fig 1).

We detected traces of DEET (N,N'-Diethyl-3-methylbenzamide) in the fungus gardens of *M. hartmanni*, *S. mayri,* and *Trachymyrmex* sp. (SMT 2). In the samples of the fungus of *M. hartmanni*, we found 34.25 % of DEET (97% confidence); in the fungus of *S. mayri,* we found 27.64 % of the volatiles of DEET (97% confidence), and in the fungus of *Trachymyrmex* sp. we found 14.99% (97% confidence) (SMT 1). DEET did not occur in any non-fungus-garden sample.

In *Leucogaricus gongylophorus,* we did not find any trace of naphthalene nor DEET. We did, however, find several compounds that are naphthalene derivatives. In both *Atta* colonies, the most abundant volatile was Cedrene <-beta> ($C_{15}H_{24}$), a derivative of cedar oil. Moreover, almost all compounds found in the *L. gongylophorus* volatiles have 15 carbon atoms (Fig 1).

The cluster analysis identified five main clusters: 1) *Atta* fungus; 2) *Mycetosoritis*, *Sericomyrmex,* and *Trachymyrmex* fungi; 3) *Cyphomyrmex* fungus and orange; 4) ant colony

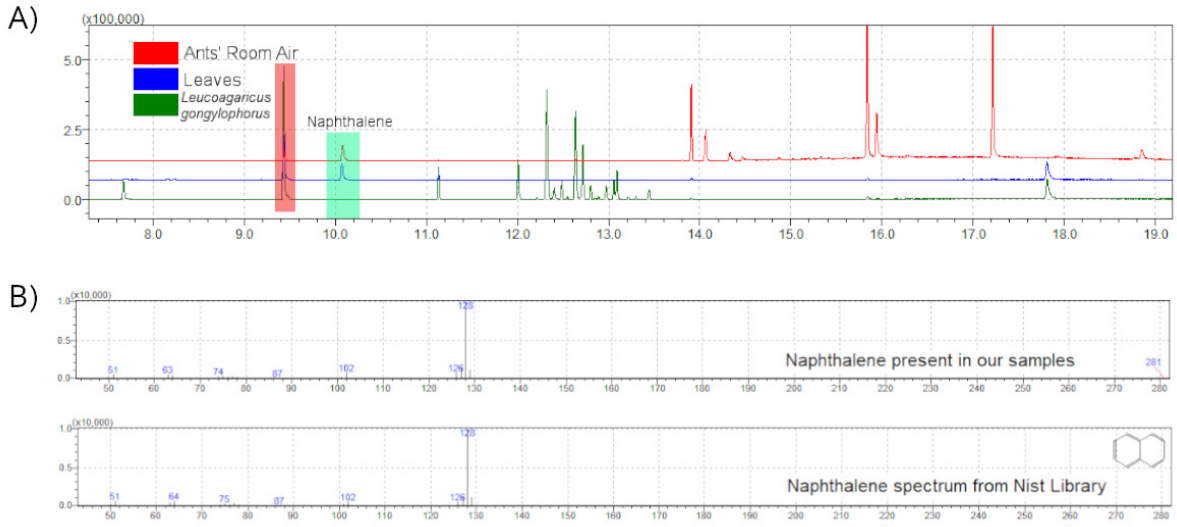

Fig 1. Presence of Naphthalene in samples of *Leucogaricus gongylophorus*. A) Chromatograms from ambient air taken from the ant-room laboratory, Leaves (food) and *L. gongylophorus* (symbiotic fungus from Atta colonies). The red square represents a contamination from the fiber (Phenol). In the green square the Naphthalene peak present in the samples. B) Mass Spectrum (MS) found in our samples from fungi species and Ants Room versus the spectrum from Nist Webbook Library (www.webbook.nist.gov). The ion 281 is a contamination from the column.

rearing Lab (Ant's room); 5) leaves, caterpillar frass (i.e., feces, also used as a garden substrate), and oak catkins (another garden substrate) (SMT 3). Volatiles in the two strains of the fungus cultivated by the *Atta* species, *Leucogaricus gongylophorus*, shared a high similarity (~ 70% similarity). However, the *Atta* fungus had the most dissimilar profile of all the fungal cultivars analyzed (~ 0% similarity with others). In contrast, the *Cyphomyrmex* fungus was more similar to the orange (the primary *Cyphomyrmex* fungus-garden substrate) than to any fungi from other ant species.

Atta cephalotes *cuticular hydrocarbons*

In cuticular hydrocarbon profiles from colonies of *Atta cephalotes*, we found a significant difference in the number of compounds ($F = 6.374$, $df = 2$, $p = 0.005$). Tukey's pairwise analysis did not indicate a difference between the colonies from Peru ($p = 0.995$); however, it did indicate a difference between the colony from Panama and both Peru colonies (Panama X Peru1: $p = 0.013$; Panama X Peru2: $p = 0.010$).

The compositions of the profiles from the *Atta* colonies were different (pseudo-$F = 126.5$, number of permutations = 9999, $p < 0.001$; Pairwise test: $p < 0.001$ to all comparisons) (SMT 4). The ordination analysis (NMDS) shows stress of 0.118, considered a good ordination (Fig 2). The cluster analysis shows the same results as the NMDS. However, the cluster analysis indicates a substantial similarity among all the *Atta* colonies from Peru and a lower similarity between those with Panama one.

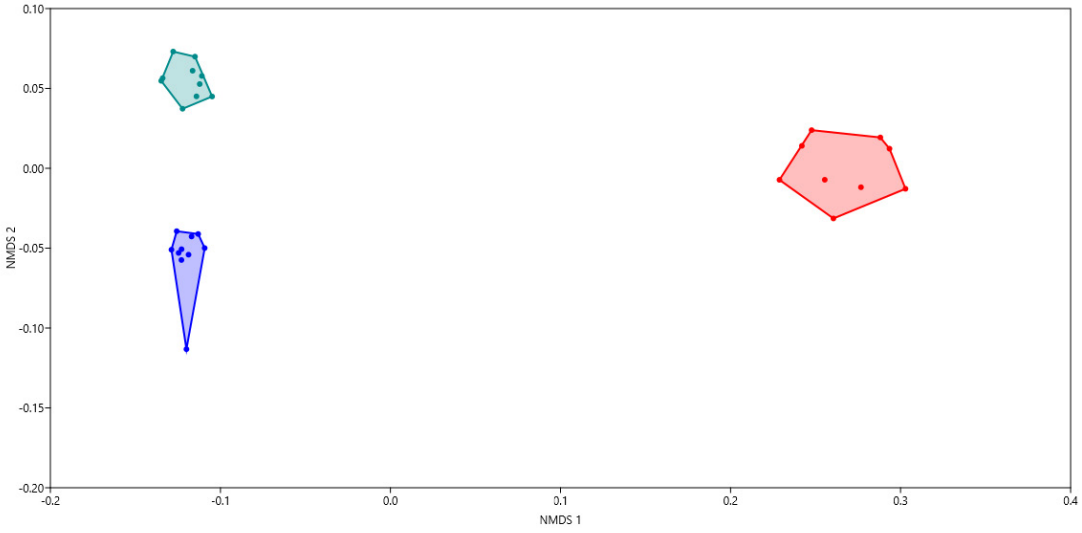

Fig 2. NMDS ordination to composition of CHC in *Atta cephalotes*. Both colonies from Peru were collected in 2004 (blue and cyan), and the Panama colony was collected in 2014 (red). Stress = 0.118.

Discussion

We found complex blends of volatile compounds in the mutualistic fungi cultivated by attine ants. The fungi, in general, can emit many types of volatiles (Dickschat, 2017). As each fungus-growing ant group has a specific kind of fungus (Schultz & Brady, 2008), we expected to find a high diversity of volatiles related to those fungi. The substrates that the ants use as food for their fungal cultivars can affect the type and number of released volatiles (Pasanen et al., 1997; Morath et al., 2012). Our samples had several food sources, which provided a high substrate diversity. We believe that the fungal volatile diversity correlates with fungal phylogeny and abiotic factors such as food (Pasanen et al., 1997; Morath et al., 2012) and environmental odor (Németh et al., 1993).

The volatiles from food sources were more like each other than those of the volatiles originating in the fungi. This may be partly because the foods directly interact with the ambient environment (Németh et al., 1993). Some food sources are stored in plastic boxes and are in direct contact with the environment. However, other foods, such as leaves, caterpillar frass, and orange rinds, are stored in the refrigerator and frequently withdrawn to feed the colonies. Even brief contact between fungal substrates and the environment may be sufficient to acquire environmental volatiles (Németh et al., 1993).

Naphthalene traces were the most abundant fungal volatiles, reinforcing our previous argument about transferring environmental volatiles to the fungus gardens. We removed ant colonies from the ambient laboratory environment at NMNH in Washington, DC, to a naphthalene-free environment at the Smithsonian Museum Support Center in Suitland, MD, for 48 hours before the fiber extraction. It is possible that 48 hours was insufficient to eliminate naphthalene from the gardens. Importantly, we did not find naphthalene in colonies of the ant species in the higher-attine genera *Atta*. A 2014 Smithsonian Institution health and safety report about the floor of the NMNH in which the ant colonies are maintained indicates that the quantity of naphthalene in the air is insignificant. As previously mentioned, it was one of the more abundant compounds in our samples and in the ambient air control we conducted using SPME in 2018.

Naphthalene has many functions, including as an insecticidal and antibiotic agent (Daisy et al., 2002; Buckpitt et al., 2010). Some fungi species can produce naphthalene (Daisy et al., 2002; Gond et al., 2013), an insecticide (Daisy et al., 2002). However, in the case of fungus-farming ants, it is unlikely that the production of naphthalene by the mutualistic fungi would prove beneficial.

In addition to naphthalene, we identified another insecticidal compound, DEET (N, N-diethyl-3-methylbenzamide). DEET blocks the electrophysiological pathway of olfactory neurons in insects (Ditzen et al., 2008). Unlike naphthalene, we did not detect any trace of DEET in the air, food, or laboratory equipment (boxes, tubes, plates, etc.). This compound is one of the most used insecticides worldwide (Tavares et al., 2018). DEET may play a role in colony defense against parasitic insects. Microbial symbionts that defend their hosts are not rare (Little et al., 2006; Adams et al., 2013; Cremer et al., 2018). In some fungus-growing ants, for example, there is a tripartite interaction among ants, fungal cultivars, and bacteria (Currie, 2001) in which the bacteria produce compounds that protect the fungus and the ants against pathogens (Currie, 2001). Our results may be the first case of a defensive volatile compound originating in the cultivated fungus. Further studies are necessary to understand how ant colonies can metabolize and incorporate DEET in their colonies

Our results indicate colony origin can influence cuticular hydrocarbon richness in fungus-farming ants. In analyses of cuticular compounds from *Atta cephalotes*, individuals sampled from a colony collected in Panama had a smaller number of cuticular hydrocarbons than did the two colonies collected in Peru. Several studies show that the synergism between environment and genetics shapes the cuticular hydrocarbon profiles (Leonhardt et al., 2013; Menzel et al., 2017a, b). Colonies sampled for this study had been maintained in the laboratory for a long time after being collected, 14 years for the Peru and four years for the Panama colonies. That would seem enough time to homogenize cuticular hydrocarbons in laboratory conditions (Soroker et al., 2003). Homogenization might also be expected because all three colonies were fed with the same diet of leaves and otherwise maintained under identical conditions (e.g., temperature and humidity), leading to the expectation that any observed differences are likely due to genetics.

While the number of hydrocarbons differed between the Panamanian and Peruvian colonies of *Atta cephalotes*, cuticular hydrocarbon profiles differed among all three colonies, suggesting colonial variation (van Wilgenburg et al. 2011). This result corroborates the hypothesis of colony-specific odors (Haverty et al. 1988; Liu et al. 2001). Our results contribute to understanding several aspects of maintaining ant colonies in artificial environments.

Conclusion

Our results suggest several questions about ecology and disease in social insects, including whether or not ants are affected by naphthalene in the symbiont fungus and whether fungal cultivars may produce compounds that contribute to pest and pathogen control in attine ant colonies. Among the many compounds we characterized among the volatiles of fungus gardens, two are clearly “compounds of interest”: naphthalene and DEET, both insecticides. Although naphthalene was a contaminant from the ambient environment, it remains important to understand why it was only found in some garden samples but not in those taken from

the higher-attine ants in the genera *Sericomyrmex* and *Atta*. In contrast, although DEET was never detected in any part of the laboratory environment, it consistently occurred in garden samples from *Mycetosoritis hartmanni*, *Sericomyrmex mayri, Trachymyrmex* sp. Further exploration of the occurrences of these compounds in the fungal cultivars of attine ants may contribute to understanding both evolutions of agriculture and social immunity in fungus-farming ants.

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Conflicts of interest/Competing interests

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Author's Contribution

All authors participate in all stages of this paper.

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Supplementary Material

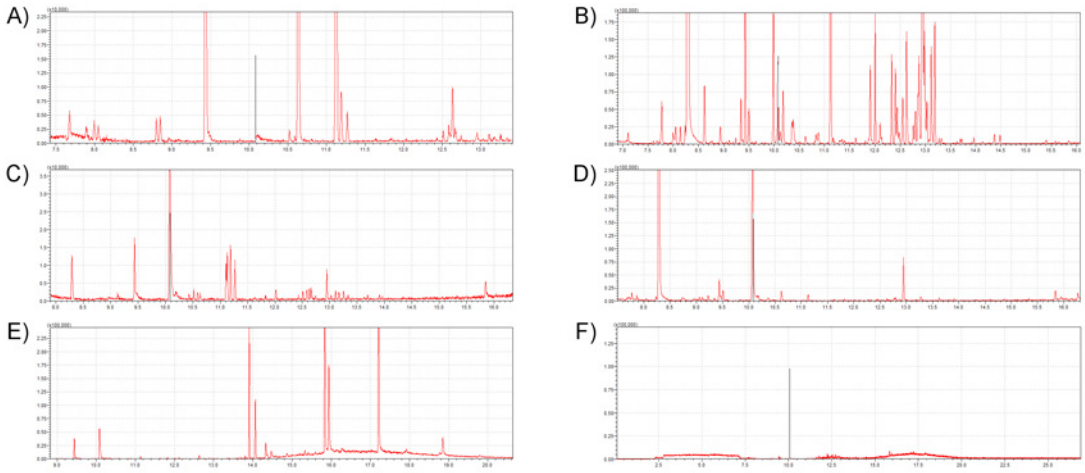

Fig S1. Chromatograms of volatiles from sources related to the colonies. A) Leaves; B) Orange; C) Oak; D) Insect frass; E) Ant room and F) Empty cleaned nest boxes.

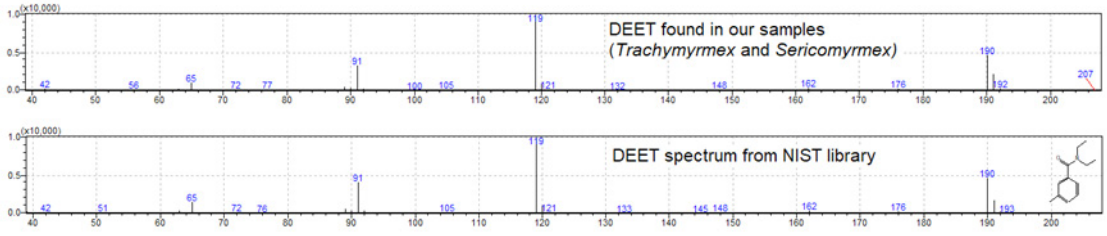

Fig S2. Mass Spectrum (MS) found in our samples from fungi samples of *Sericomyrmex mayri* and *Trachymyrmex* sp. versus the spectrum from Nist Webbook Library (www.webbook.nist.gov).

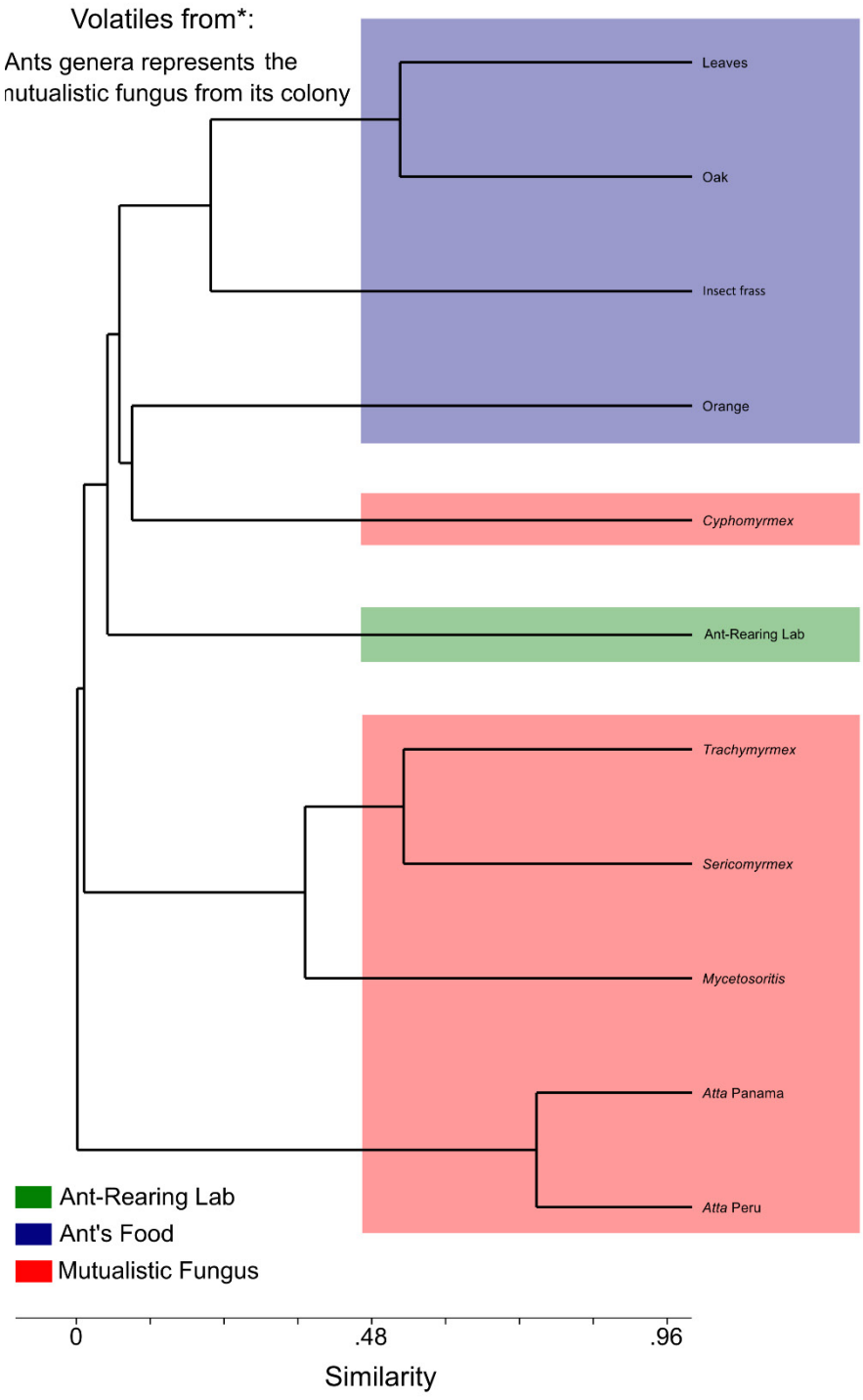

Fig S3. Cluster analysis based on volatiles from garden fungi and their substrates.

Fig S4. Cuticular hydrocarbon profile clustering (UPMGA) using the (dis)similarity method of Bray-Curtis. Individuals from colonies of *Atta cephalotes* from the same country are more similar to each other than to individuals of *Atta cephalotes* from Panama.