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Context is everything: mapping *Cyphomyrmex*-derived compounds to the fungus-growing ant phylogeny

Natalie Hamilton[1] · Tappey H. Jones[2] · Jonathan Z. Shik[3] · Bonnie Wall[4] · Ted R. Schultz[5] · Haley A. Blair[6] · Rachelle M. M. Adams[1]

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Abstract

Ants communicate using a suite of chemicals with a level of sophistication that is inextricably linked to their ecological dominance. The fungus-growing ants have been the focus of chemical ecology studies for decades, especially the leaf-cutting ants. Some create long, conspicuous foraging trails to harvest fresh vegetation used to sustain large farming systems that feed millions of workers. However, of the ca. 250 fungus-growing ant species, most feed detritus rather than fresh material to their gardens, and colony sizes are tens to hundreds of workers. Colonies within the attine genus *Cyphomyrmex* use distinct methods of agriculture (i.e., yeast and lower fungus agriculture). We compared compounds found in five species from the yeast-growing *Cyphomyrmex rimosus* group (*C. rimosus* and *C. salvini*) and the lower agriculture *Cyphomyrmex wheeleri* group (*C. costatus, C. longiscapus, C. muelleri*). Compounds identified were compared with those reported in the literature and mapped onto the attine-ant phylogeny, and glands of origin suggested. There were 10 compounds across five species and two are known alarm compounds, 1-octen-3-o1 and 3-octanol. Of the six farnesenes detected, the most notable was the diversity of gaster-derived compounds in *C. salvini* and the high abundance of (3*Z*, 6*E*)-α-7-ethylhomofarnesene and α-6-bishomofarnesene in the three *C. wheeleri* group species. We also found 2,5-dimethyl-3-isoamylpyrazine in the heads of yeast-growing species, an unexpected result because pyrazines are known trail substances in other attines. Our results expand our understanding of semiochemicals found in fungus-growing ants and provides a starting point to generate hypotheses for more extensive comparative studies.

Keywords *Cyphomyrmex* · Fungus-farming ants · Alarm pheromone · Trail pheromone · Chemical communication · Formicidae

Introduction

Ants (Hymenoptera: Formicidae) have over 75 glands producing chemicals that guide their social organization, contributing to their ecological dominance around the world (Jackson and Morgan 1993; Billen 2009). Chemicals provide

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✉ Rachelle M. M. Adams
Adams.1970@osu.edu

[1] Department of Evolution, Ecology, and Organismal Biology, The Ohio State University, 318 W. 12th Ave. 300 Aronoff Lab, Columbus, OH 43210, USA

[2] Department of Chemistry, Virginia Military Institute, Lexington, VA 24450, USA

[3] Department of Biology, Centre for Social Evolution, University of Copenhagen, Universitetsparken 15, 2100 Copenhagen, Denmark

[4] Department of Biology, University of Konstanz, Universitätsstraße 10, 78464 Konstanz, Germany

[5] Department of Entomology and Laboratories of Analytical Biology, National Museum of Natural History, Smithsonian Institution, 10th St. and Constitution Ave. NW, Washington, DC 20560, USA

[6] Department of Chemistry, University of Virginia, Charlotesville, VA 22904, USA

advantages over visual signals, enabling information transfer in dark nest cavities that can linger in the environment or dissipate quickly depending on their volatility (Morgan 2008). Semiochemicals (i.e., allomones and pheromones) are used in complex communication systems that influence territorial interactions (Salzemann et al. 1992; Cammaerts and Cammaerts 1998), nestmate recognition systems (Sainz-Borgo et al. 2013; Norman et al. 2014), foraging recruitment (Vander Meer et al. 1988) and alarm signaling (Jackson and Morgan 1993; Morgan 2008). The importance of these diverse functions illustrate the complexity of social organization and it is therefore not surprising that ants have evolved diverse glands to produce such compounds (reviewed in: Billen 2011).

Many compounds used for communication and defense have predicable source glands among seemingly distantly related ant species, but the conservation of semiochemicals within genera is less known. Functional roles present an additional layer, as some signals depend on compounds from multiple glands (Billen 2006; Morgan 2008). For instance, the secondary alcohols and corresponding ketones of ant mandibular glands are primarily responsible for the production of alarm pheromones, whereas long-chain hydrocarbons stored in the postpharyngeal gland (PPG) and produced in the Dufour's gland tend to facilitate nestmate recognition (Cammaerts et al. 1981; Jaffe and Marcuse 1983; Morgan 2008). Trail-marking chemicals and several alarm pheromones are also derived from abdominal glands (e.g., postpygidial, Dufour's, and venom glands) and are frequently made of linear and terpenoid hydrocarbons also known as farnesenes (Blum et al. 1964; Attygalle and Morgan 1984; Vander Meer et al. 1988). Furthermore, metapleural glands produce acids with antimicrobial properties that can protect the ants from entomopathogens but may also have a communicative function (Morgan 2008; Yek and Mueller 2011). To summarize, all ant species rely on chemical communication and most biosynthesize a remarkable diversity of compounds.

The fungus-growing ants (Formicidae: Myrmicinae: Attini: subtribe Attina; hereafter "attine" ants) comprise a monophyletic lineage that farms fungus for food (Mehdiabadi and Schultz 2009; Ward et al. 2015). Five key farming practices can be mapped onto the attine phylogeny: (1) lower agriculture, (2) yeast agriculture, (3) coral fungus agriculture, (4) higher agriculture, and (5) leaf-cutter agriculture (Schultz and Brady 2008). The genus *Cyphomyrmex* occupies a phylogenetically intermediate position between other lower fungus-growing ant genera and the "higher" attines (including the leaf-cutting ants). This is particularly interesting because *Cyphomyrmex* species from the *C. wheeleri* group (four species) practice lower agriculture and species from the *C. rimosus* group (19 species) practice yeast agriculture (Mehdiabadi et al. 2012; Albuquerque 2014). *Cyphomyrmex* species also exhibit defense behaviors ranging from crypsis (i.e., playing dead when disturbed) to aggressive attack (Kweskin 2004; Adams and Longino 2007; Adams et al. 2015). Thus, one might expect these species to produce correspondingly diverse semiochemicals to mediate these varied collective farming and defensive behaviors.

The chemical ecology of fungus-growing ants has been traditionally focused on the most derived leaf-cutter species due to their pest status and large-scale farming systems. More recently, several compounds have been found to be conserved across the attine phylogeny, in genera that diverged tens of millions of years ago (e.g., *Atta, Apterostigma, Trachymyrmex*, and *Sericomyrmex* (Crewe and Blum 1972; Adams et al. 2012; Hogan et al. 2017; Norman et al. 2017). Like other ant species, attines use semiochemicals in nestmate recognition (e.g., cuticular hydrocarbons) (Martin and Drijfhout 2009; Neupert et al. 2018), trail following (e.g., farnesenes, pyrazines) (David Morgan 2009 and references therein), nest marking (e.g., long-chain hydrocarbons) (Salzemann et al. 1992), and alarm behavior (e.g., 3-octanol) (Crewe and Blum 1972; Norman et al. 2017). There is a clear gap in knowledge surrounding the majority of attine-derived semiochemicals and comparative work is greatly needed. It is likely that *Cyphomyrmex*-derived semiochemicals are shared among various attines despite their divergent farming practices, but this has yet to be tested.

We described the chemical compounds found in the head and abdomen (i.e., gaster) of five *Cyphomyrmex* species and propose their glandular origins. To our knowledge, of the 23 extant *Cyphomyrmex* species (Antweb 2018), the glandular secretions of only *C. rimosus* have so far been studied (Crewe and Blum 1972). We place our findings in an evolutionary context to highlight the rarity and convergence of some compounds and discuss how farnesenes—likely used as trail pheromones—separate the *C. rimosus* group (yeast agriculture) and the *C. wheeleri* group (lower "mycelium" agriculture). *Cyphomyrmex* have great potential for highlighting possible mechanisms enabling transitions from yeast agriculture and lower agriculture to larger scale farming systems that arose over millions of years of attine evolution.

Materials and methods

Sample collections

In May 2015 and 2017, *Cyphomyrmex* colonies were collected from four sites in Parque Nacional Soberanía Forest, Panamá. Three were along Pipeline Road (Rio La Seda: N 09.16330, W 79.39605; 16E creek: N 9.16379, W 79.74244; 8W creek: N 9.16093, W 79.75181) and one on Plantation Road (N 09.15277, W 79.73680), the latter located southeast across the Río Chagres. Compounds were extracted from freshly collected colonies of five *Cyphomyrmex* species [i.e., *C. rimosus* (2 colonies), *C. salvini* (6 colonies), *C. costatus* (3 colonies), *C. muelleri* (9 colonies), and *C. longiscapus* (5 colonies)] in HPLC grade methanol solvent (1–11 individuals per vial). Species were identified using keys of (Kempf 1965; Snelling and Longino 1992) and vouchers were deposited at the Smithsonian Institution National Museum of Natural History, Washington DC. Whole ants or trisected ants, dissected into head, mesosoma (thorax), and gaster (abdomen), were placed into separate glass vials with 50–200 µL of solvent (Table 1). The mesosoma region did not yield volatile compounds and was therefore not considered further in the study. To minimize contamination between the ant body parts, the forceps were washed in ethanol, methanol, and pentane after each cut. Trisections of 1–3 individuals per colony were used to identify the body regions where compounds were most abundant. Whole-body extracts confirmed the ubiquity of trace compounds detected. Extractions of one species with ants from the same or different colonies are described as "replicate samples" hereafter. Reported compounds were found in at least trace amounts in two or more extracts of workers of the same species.

Analysis of *Cyphomyrmex*-derived compounds

Samples of extracts were analyzed by THJ and HAB at the Virginia Military Institute with gas chromatography–mass spectrometry (GC–MS) using a Shimadzu QP-2010 GC–MS equipped with an RTX-5, 30 m × 0.25 mm i.d. column. The carrier gas was helium with a constant flow of 1 ml/min. The temperature program was from 60 to 250 °C changing 10 °C/min and held at the upper temperature for 20 min. The mass spectrometer was operated in EI mode at 70 eV, and scanning was set to 40 to 450 AMU at 1.5 scans/s. Peaks on chromatograms were identified by database search (NIST Mass Spectral Data base, V.2, US Department of Commerce, Gaithersburg, MD), published literature spectra, and by direct comparison with commercially available authentic samples. Single ion searches were necessary in the cases where the signals were barely above the baseline in the chromatogram (electronic supplementary material 1; Fig. 1).

Table 1 Collection information

Species	Collection code	Collection location	Sample type
C. rimosus	RMMA150504-04	Gamboa Forest	W, H, G
	RMMA170506-02	16E	W
C. salvini	JS150509-05	Rio La Seda	W, H, G
	JZS150525-01	16E	W, H, G
	JS150525-01	16E	W, H, G
	RMMA170520-01	Rio La Seda	W
	RMMA170222-01	8W	W
	RMMA170527-02	8W	W
C. costatus	JS150509-01A	Rio La Seda	W, H, G
	RMMA150517-05W	Plantation Rd.	W, H, G
	RMMA170513-02	16E	W
C. muelleri	CTH170511-02	16E	W
	NMH170515-03	16E	W
	NMH170520-01	Rio La Seda	H, G
	NMH170520-02	Rio La Seda	W
	RMMA170515-03	Rio La Seda	W
	RMMA170527-01	8W	W
	RMMA170527-04	8W	W
	RMMA170527-05	8W	W
C. longiscapus	RMMA170527-07	8W	W
	BMW150517-05	Plantation Rd.	W, H, G
	NMH170515-01	16E	W, H, G
	NMH170515-04	Rio La Seda	W
	RMMA170515-01	Rio La Seda	W
	SS150523-02	16E	W, H, G

Collection codes contain the collector's initials, date of collection (year/month/day), and colony number. Collector's initials: Rachelle M.M. Adams (RMMA), Natalie M. Hamilton (NMH), Conor T. Hogan (CTH), Jonathan Z. Shik (JZS), Jacob Spile (JS), and Bonnie M. Wall (BMW). Sample type: Whole-body sample (W), Head (H), and Gaster (G)

Results

Among the five *Cyphomyrmex* species studied, we detected and identified four head-derived compounds (Table 2), six putative trail-following pheromones (Table 3), and various alkenes (C11–C17) (Fig. 1). The "mushroom alcohol" 1-octen-3-ol (octenol) was present in all *Cyphomyrmex* species, whereas 3-octanol and nonanal were found in all species except *C. rimosus*. *Cyphomyrmex salvini* samples contained 2,5-dimethyl-3-isoamylpyrazine (**4**) in the whole-body and head samples. This compound was also detected in a whole ant sample of *C. rimosus* (RMMA150504-04). 1-octen-3-ol, 3-octanol, and nonanal were identified using authentic samples from Sigma-Aldrich (SA CAS# 3391-86-4, 589-98-0, and 124-19-6 respectively). 2,5-Dimethyl-3-isoamylpyrazine was determined by comparison to the

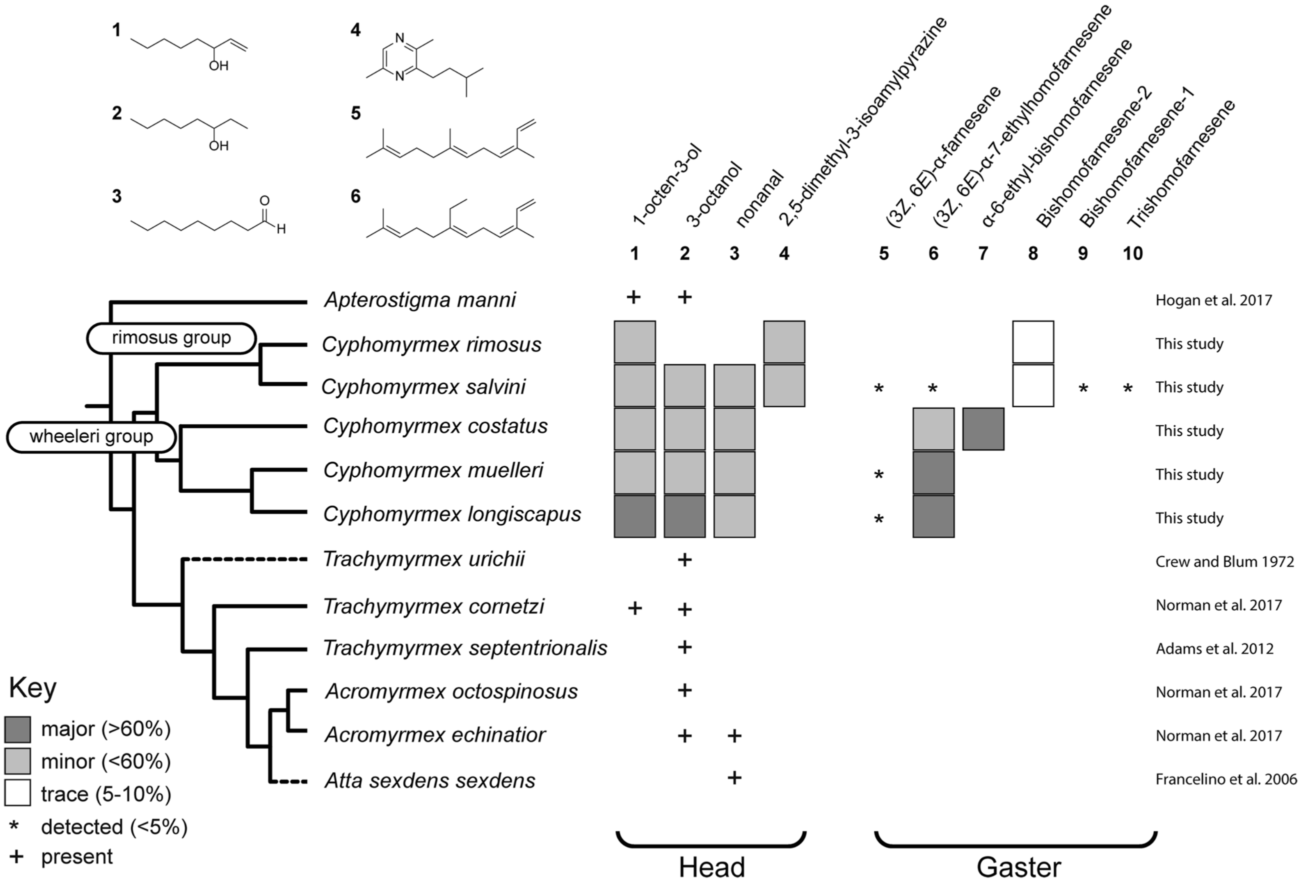

Fig. 1 *Cyphomyrmex* chemicals compared across a sampling of the attine phylogeny. Compounds marked with numbers correspond to the structures shown at top. Shading of each square represents the relative quantities of compound found in the head or gaster: dark gray (major component > 60%), light gray (minor component < 60%), white (5–10%), and "asterisk" (detected). Data from the literature were added for comparison and are not an exhaustive list of compounds found in these species. If a compound was found in other studies this is indicated by a "plus". Species relationships inferred from the phylogeny of Branstetter et al. (2017). Positons of species not included in the Branstetter et al. (2017) study are approximated based on morphological similarities. The *C. rimosus* group (yeast agriculture) and the *C. wheeleri* group (lower agriculture) are labeled at the appropriate nodes

Table 2 Relative peak area average of head-derived compounds and retention data in minutes

Compound #		Peak area average ± SE			
Species	(n/rs)	1	2	3	4
C. rimosus	(1/5)	–	–	–	–
C. salvini	(3/30)	22.8 ± 0.91	5.75 ± 1.59	–	51.9 ± 10.7
C. costatus	(2/21)	15.5 ± 0.75	20.2 ± 5.75	14.1 ± 4.2	–
C. muelleri	(1/1)	25.2	19	14.2	–
C. longiscapus	(3/18)	32.9 ± 8.61	32.8 ± 4.52	13.9 ± 3.44	–
Retention time		10.2	10.6	12.86	16.36

Numbers correspond to compounds indicated in Fig. 1

n number of colonies sampled and *rs* number of replicate samples (rs)

NIST Library 367,291 and published spectra (Xu et al. 2018).

Extracts of the gasters of three *C. wheeleri* group species (*C. costatus, C. longiscapus*, and *C. muelleri*) contained sesquiterpenoid farnesenes and their homologs as major hydrocarbon components, while *C. rimosus* and *C. salvini* had these compounds in low amounts (≤ 10%). The gas chromatograms of the extracts of *C. longiscapus* and *C. muelleri* were indistinguishable, revealing the presence of two compounds in a 1:10 ratio. The first eluting compound

Table 3 Relative peak area average of gaster-derived farnesenes and retention data in minutes

Compound # (carbons)		Peak area average ± SE					
Species	(n/rs)	5 (C_{15})	6 (C_{16})	7 (C_{17})	8 (C_{17})	9 (C_{17})	10 (C_{18})
C. rimosus	(2/8)	–	–	–	26.4 ± 0	–	–
C. salvini	(6/53)	5.833 ± 0.68	6.6 ± 0.76	–	25.75 ± 3.28	9.33 ± 1.06	13.28 ± 1.82
C. costatus	(2/15)	–	16.2 ± 0.3	63.5 ± 2.8	–	–	–
C. muelleri	(9/35)	6.17 ± 0.67	72.2 ± 01.56	–	–	–	–
C. longiscapus	(5/34)	7.97 ± 1.33	68.2 ± 3.27	–	–	–	–
Retention time		18.738	19.575	19.654	19.769	20.015	20.698

Numbers correspond to compounds indicated in Fig. 1 and the number of carbons is in parentheses

n number of colonies sampled and *rs* number of replicate samples (rs)

had a parent ion at $m/z = 204$ and a mass spectrum that matched (3*Z*, 6*E*)-α-farnesene (**5**) in the NIST Library 141,112. The second compound had a mass spectrum with parent ions at $m/z = 218$, which suggested a homofarnesene. Hydrogen bubbled through a small portion of the extract containing < 1 mg of PtO_2 and as expected, the minor component had a mass spectrum that matched that reported for 2,6,10-trimethyldodecane, farnesene (NIST Library 62,132). After hydrogenation, the mass spectrum of the major component had $M^+ = 226$ ($C_{16}H_{34}$), and significant fragments at $m/z = 197$ (M-29) and 111, and it matched that reported for 2,10-dimethyl-6-ethyldodecane (Attygalle and Morgan 1982; Compound 2). Upon closer examination of the original mass spectrum, the ion at $m/z = 133$ (22) and the rest of the spectrum matched the mass spectrum reported for (3*Z*, 6*E*)-α-7-ethylhomofarnesene (**6**). We have provided the mass spectra of the peaks in electronic supplementary material 1; Fig. 2. Other possibilities for the double-bond geometries and positions were ruled out from mass spectra reported in the literature. Accordingly, the (E, E) isomer is reported to have a noticeable ion at $m/z = 137$, which was not observed, along with the ion at $m/z = 133$. Likewise, β-7-ethylhomofarnesene is reported to have noticeable ions at $m/z = 147$ and 134 which were not present in the spectrum of (**6**) (Jackson et al. 1990).

In *C. costatus*, a similar gas chromatogram was observed except that the farnesenes were one-carbon and two-carbon homologs of (3*Z*, 6*E*)-α-farnesene (**5**). The minor component had a mass spectrum and retention time identical to those of (3*Z*, 6*E*)-α-7-ethylhomofarnesene (**6**) in *C. longiscapus* and *C. muelleri*, while the major component α-6-bishomofarnesene (**7**) had a mass spectrum with a parent ion at $m/z = 232$, which suggested a bishomofarnesene. Hydrogen was bubbled through a small portion of the extract containing < 1 mg of PtO_2 and after hydrogenation, and the minor component had $M^+ = 226$ and significant fragments at $m/z = 197$ (M-29) and 111. This matched 2,10-dimethyl-6-ethyldodecane reported above from the hydrogenation of the *C. longiscapus* extract. The major component of this mixture had $M^+ = 240$ ($C_{17}H_{36}$) and significant fragments at $m/z = 211$(M-29) and 111. This suggested a saturated hydrocarbon with an internal ethyl group and the additional carbon on one end of the molecule, so that its precursor, α-6-bishomofarnesene (**7**) would be a one-carbon homolog of (3*Z*, 6*E*)-α-7-ethylhomofarnesene (**6**). We have provided the mass spectrum for compound (**7**) in electronic supplementary material 1; Fig. 3, as to the best of our knowledge, this compound has not been previously reported.

In *C. rimosus* and *C. salvini*, where the farnesenes appear in low amounts, two bishomofarnesenes (**8** and **9**) and one trishomofarnesene (**10**) had mass spectra that matched those published in Ali et al. (2007). There the mass spectrum of "bishomofarnesene-2" (Ali et al. 2007: Fig. 1, spectrum a) matched that of (**8**) exactly, while the mass spectrum of "bishomofarnesene-1" (Ali et al. 2007: Fig. 1, spectrum a) matched that of (**9**) exactly. Similarly, the mass spectrum of "trishomofarnesene-1" (Ali et al. 2007: Fig. 5, spectrum b) matched that of (**10**) exactly. Bishomofarnesene-2 (**8**) was the only terpenoid compound found in *C. rimosus*. In contrast, *C. salvini* was found to have small amounts of (**5, 6, 8, 9**, and **10**).

Discussion

Our results indicate that several chemicals are conserved within the genus *Cyphomyrmex*, but also occur in other terminal attine taxa (Fig. 1). All *Cyphomyrmex* species in our study contain 1-octen-3-ol (**1**) in minor (< 60%) or major (> 60%) amounts (Fig. 1). The function is yet to be determined in ants, but 1-octen-3-ol (**1**) is also found in the distantly related species, *Apterostigma manni* (Hogan et al. 2017) and *Trachymyrmex cornetzi* (Norman et al. 2017) function as a repellent in millipedes (Ômura et al. 2002). All species excluding *C. rimosus* have 3-octanol (**2**). This compound induces strong mandible-opening response in *Acromyrmex echinatior* and acts as an attractant in *Myrmica* ants (Cammaerts et al. 1981; Norman et al. 2017) and therefore may function as an alarm pheromone in *Cyphomyrmex* species. Nonanal (**3**) has a citrus odor and small amounts have been found in the mandibular glands of *Acromyrmex* and *Atta* species (Hughes et al. 2001; Francelino et al. 2006; Norman et al. 2017). *Trachymyrmex fuscus* also produces nonanal (**3**) along with an array of acids in its metaplural gland (Vieira et al. 2012). Behavioral studies are needed to determine pheromonal function of 1-octen-3-ol, 3-octanol, and nonanal in the *Cyphomyrmex* genus.

Additionally, 2,5-dimethyl-3-isoamylpyrazine (**4**) was detected in whole ant and head samples of *C. salvini* and in the heads of *C. rimosus*, but never in the gaster-only samples, suggesting this compound could originate in the mandibular glands. 2,5-dimethyl-3-isoamylpyrazine (**4**) has also been found in a bacterial associate of *Atta sexdens rubropilosa* (Silva-Junior et al. 2018) and in the mandibular glands of the distantly related *Anochetus kempfi* and *A. mayri* (Formicidae: Ponerinae: Ponerini) (Jones et al. 1999). Similar pyrazines have been reported from the mandibular glands of *Iridomyrmex* (Formicidae: Dolichoderinae) (Cavill et al. 1984; Morgan et al. 1999 and references therein). However, our result was unexpected because pyrazines are known to serve as trail substances in attine ants (Cross et al. 1979; Evershed et al. 1982; Vander Meer et al. 1988; Morgan 2008). To our knowledge, farnesenes (**5–10**), found in the *Cyphomyrmex* species sampled, have not been found in other fungus-growing ant species (Fig. 1; Table 2). However, (E,E)-α-farnesene was reported in whole-body samples of two *Trachymyrmex* species (i.e., *T. n. sp.* nr. *papulatus* and *T.* cf. *zeteki*) and (*Z*-β)-farnesene was found in gaster samples of *T.* cf. *zeteki* sp 2 (Adams et al. 2012). Farnesenes were also found in the Dufour's gland of other Myrmicinae ant genera (e.g., *Solenopsis, Pheidole*) suggesting this may be the source gland (Vander Meer et al. 1988; Ali et al. 2007). Although surveyed by Adams et al. (2012), no farnesenes were detected in *Trachymyrmex opulentus, Trachymyrmex* cf. *isthmicus, Trachymyrmex cornetzi, Trachymyrmex bugnioni* and *Trachymyrmex septentrionalis* suggesting there is variability in the presence of farnesenes in a single genus, although this is not true for *Cyphomyrmex* according to our study.

The occurrence and abundance of farnesenes distinguish the *C. rimosus* group from the *C. wheeleri* group. The gaster extracts of the two yeast-growing species (i.e., *C. rimosus* and *C. salvini*) contain compounds in very small quantities, while farnesenes are major components in the three *C. wheeleri* group species. Additionally, the farnesenes in the *C. wheeleri* group are comprised of (3*Z*, 6*E*)-α-farnesene (**5**) and what appear to be its homologs, (3*Z*, 6*E*)-α-7-ethylhomofarnesene (**6**) and the bishomofarnesene (**7**). On the other hand, the two species in the *C. rimosus* group have small amounts of farnesene homologs reported in abundance in *Pheidole* species (Ali et al. 2007), compound (**8**) in the case of *C. rimosus*, and (**8, 9**, and **10**) from *C. salvini*, which also has small amounts of (**5** and **6**). While the farnesenes detected in the two species groups examined here are different, we cannot conclude that the overall pattern of *Cyphomyrmex*-derived farnesenes is taxonomically informative, but it is possible that a pattern may emerge with more sampling.

While a diversity of farnesenes is present throughout the *Cyphomyrmex* clade, *C. salvini* has the widest range of compounds—albeit in low quantities. Larger colonies call for more caste specialization (Ferguson-Gow et al. 2014), therefore complex signals may be useful for refined communication between nestmates (Beckers et al. 1989; Jaffe et al. 2012). The mycelium-growing species have small colonies (ca. 20–100 individuals) and are often solitary foragers (Adams unpublished; Ferguson-Gow et al. 2014). In contrast, some yeast-growing species can have colonies with over 100 (e.g., *C. salvini*) to over 4000 individuals (e.g., *C. cornutus*) and have long foraging lines (Adams unpublished; Adams and Longino 2007; Ferguson-Gow et al. 2014). *Cyphomyrmex salvini* is thought to be a complex of sibling species with 6–7 morphospecies in Costa Rica alone (AntWeb). Future work focused on farnesenes in the *C. salvini* complex may reveal species-specific patterns that correlate with life history traits (e.g., colony size and foraging strategy) and function.

Overall, our results indicate a remarkable diversity of compounds among the *Cyphomyrmex* species. Many compounds are conserved within the genus *Cyphomyrmex* and found in a diversity of attine species. Due to pheromone parsimony, where chemical function may not be preserved across species, careful behavioral analyses are necessary to determine a chemical's function (Jackson and Morgan 1993). Because ants spread compounds all over their bodies when grooming, glandular sources can be difficult to detect (Richard et al. 2007). The evolution of exocrine chemicals within fungus-growing ants has the potential to be specific to the type of agriculture utilized by the ant species (Mehdiabadi et al. 2012), but this remains to be broadly tested. Each year, new fungus-growing species are being discovered and their natural history described. Focused work integrating behavior, semiochemicals, and phylogenetic relationships will provide a clearer understanding of the complexities of these fascinating ants.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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