A comparative study of exocrine gland chemistry in *Trachymyrmex* and *Sericomyrmex* fungus-growing ants

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ABSTRACT

Ants possess many exocrine glands that produce a variety of compounds important for chemical communication. Fungus-growing ants, a tribe of over 230 species within the subfamily Myrmicinae, are unique among ants because they cultivate fungus gardens inside their nests as food. Here the chemistry of the exocrine glands of the two genera most closely related to the conspicuous leaf-cutting ants are examined. Based on a recent phylogeny of the fungus-growing ants, these genera comprise three clades that together link the more basal species to the most derived, leaf-cutting species. The leaf-cutting ants possess many derived characteristics such as extensive leaf-cutting behavior and massive colony sizes, effectively making them major herbivores in many Neotropical habitats. This is the first comparison of the chemistry of eight *Trachymyrmex* and one *Sericomyrmex* species in a phylogenetic context. Most of the compounds found in the *Trachymyrmex* species examined were terpenes. In one species, the major component was α,α-acariolide, the first example of this compound, which was only previously reported in mites, from an insect. Additionally, 3-octanol, 3-octanone, and 4-methyl-3-heptanone were detected, well-known mandibular gland compounds from a number of ant genera, together with high levels of undecane, likely from the Dufour's gland, all generally thought to be used as alarm pheromones. Overall the combination of compounds discovered was unique for each species but biosynthetic similarities corroborate, at a very basic level, the phylogenetic relationships.

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1. Introduction

Unlike most insects that rely mainly on vision, ants, with around 75 epithelial and secretory glands (Billen, 2009, 2011) use olfaction as their main sensory system (Morgan, 2008). However and perhaps not surprisingly, the function of the chemicals produced in most ant species is unknown (Morgan, 2008). Ants are able to use either single or a mixture of compounds at varying concentrations to alter the function of their glandular substances. Pheromone parsimony across species, in which the same compound can be produced by different glands yet have diverse functions, is practiced widely by ants (Morgan, 2008).

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Thus, function in one ant species may not always translate to the same function in another, and careful behavioral studies are needed to draw definite conclusions (Jackson and Morgan, 1993). This is just one reason that the remarkable convergence in ant exocrine gland products remains an exciting yet challenging field of research. Several review articles highlight the natural products of insects (Blum, 1969) and ants in particular (Attygalle and Morgan, 1984; Morgan, 2008). In some cases work on ant chemical ecology has been explicitly comparative, enhancing our understanding of the complexities of ant exocrine chemistry within genera (Blum et al., 1968; Cammaerts et al., 1983; Crewe and Blum, 1972) and even across a phylum (Blum and Edgar, 1971).

Within the fungus-growing ants (tribe Attini), the conspicuous leaf-cutting ants of the genera *Atta* and *Acromyrmex* have been subjects of a number of chemical ecology and natural product studies, and trail pheromones (Cross et al., 1979, 1982; Evershed and Morgan, 1983; Jaffé and Howse, 1979), alarm pheromones (Crewe and Blum, 1972; Crewe et al., 1972; Riley et al., 1974), and territorial markers (Jaffé et al., 1979) have been identified in several species. A few representative species within the tribe Attini have also been the subjects of a broader comparative analysis of alarm pheromones conducted by Crew and Blum (1972). This study used the phylogenetic relationships proposed by Weber (1958), but recent developments in our biological and especially phylogenetic understanding of the attine ants (Mehdiabadi and Schultz, 2010; Schultz and Brady, 2008) provide an opportunity to better understand evolutionary transitions of specific chemical compounds.

The tribe Attini has been divided into five agricultural groups, which can be simplified to two major groups: the 'higher' or most derived attines, including the leaf-cutting ants and the genera *Trachymyrmex* and *Sericomyrmex*, and the 'lower' attines, a diverse group of attine species belonging to clades that in most cases diverged earlier in attine evolution (Schultz and Brady, 2008; Fig. 1). *Trachymyrmex,* an ant genus of 47 recognized species (Agosti and Johnson, 2011) and *Sericomyrmex*, a genus of 19 recognized species (Agosti and Johnson, 2011), together occupy an intermediate phylogenetic position between the 'lower' attine ants and the leaf-cutting ants and possess many morphological and behavioral traits that are clearly intermediate between those two groups (Schultz and Brady, 2008; Fig. 1).

For this reason we decided to carry out the first comparison of the chemistry of a set of *Trachymyrmex* and one *Sericomyrmex* species in a phylogenetic context.

2. Materials and methods

2.1. Material and extraction

Specimens analyzed in this study were mainly collected in central Panamá near Gamboa, Colon Province, from 2007 to 2011. The *Trachymyrmex septentrionalis* sample was collected in the United States, Virginia, in 2004 (Table 1). Species identities were determined by TRS, and specimens have been deposited at the Smithsonian Institution National Museum of Natural History, Washington, DC, and/or in the dry collections of the Smithsonian Tropical Research Institute, Balboa, Panamá.

Five to 20 worker ants were taken from live colonies in the field or laboratory and placed directly into a small amount (c. 100 μl) of 100% methanol solvent in glass vials with Teflon-lined stoppers, either while alive or after being freeze killed. Pure methanol was used as a solvent since it extracts a wide range of both hydrophobic and hydrophilic organic compounds except heavy (C30+) hydrocarbons. For most species, samples were analyzed using sets of whole and trisected workers
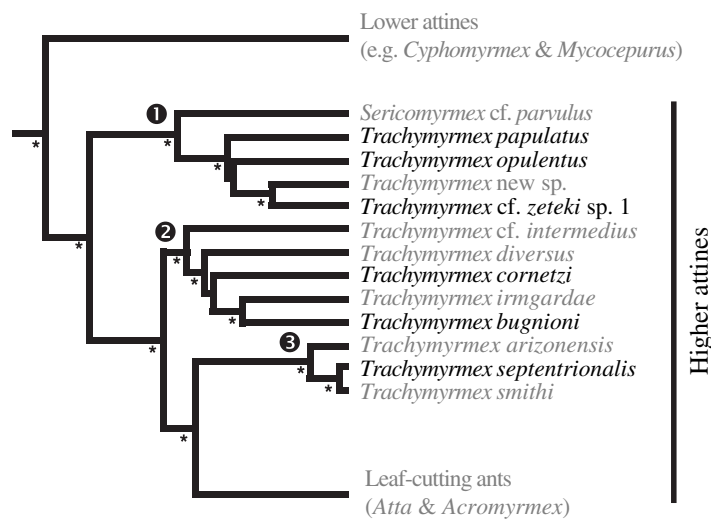
Fig. 1. A detail from the recent fungus-growing ant phylogeny of Schultz and Brady (2008). Asterisks at the nodes are posterior probabilities >0.98, as determined by Bayesian nucleotide-model and Bayesian codon-model analyses. Species names in black indicate taxa that are the same or similar to species in this study. Note that *Sericomyrmex* cf. *amabilis*, *T.* cf. *ithmicus*, and *T.* cf. *zeteki* sp. 2 were not included in the phylogeny of Schultz and Brady (2008) but are placed within *Trachymyrmex* clade 1 (Fig. 3). Numbers refer to clades discussed in the text. The lower attine species, belonging to all genera diverging earlier in attine evolution than the clades in the figure, have been reduced to a single branch, as have the leaf-cutter clades.

Table 1

Sample and collection information. Collectors were Rachelle M. M. Adams (RMMA), Henrik H. de Fine Licht (HHdFL), Steve A. Rehner (SAR), and Hermógenes Fernández-Marín (HF-M).

| Collection No. & date	Species	Location	Collector
RMMA110529-01 May 2011	*Sericomyrmex* cf. *amabilis*	Plantation Rd, Panamá N 9° 4.705′ W 79° 39.594	RMMA
C#001–070508 May 2008	*T. bugnioni*	Pipeline Rd ~1–2 km, Panamá N 9° 8.077′ W 79° 43.334′	HHdFL
C#005–270408 April 2008	*T. cornetzi*	Gamboa Blvd, Panamá N 9° 7.023′ W 79° 41.898′	HHdFL
C#010–010508 May 2008	*T. cornetzi*	Pipeline Rd ~1–2 km, Panamá N 9° 8.077′ W 79° 43.334′	HHdFL
RMMA100521–16 May 2010	*T.* cf. *isthmicus*	El Llano, Panamá N 9° 16.813′ W 78° 57.643′	RMMA
RMMA100512–04 May 2010	*T. opulentus*	Pipeline Rd 9.5 km, Panamá N 9° 9.652′ W 79° 45.169′	RMMA
Hdfl25052010–4 May 2010	*T. n. sp.* nr. *papulatus*	Gamboa Forest, Panamá N 9° 7.023′ W 79° 41.898′	HHdFL
C#003–130507 May 2007	*T. n. sp.* nr. *papulatus*	Gamboa Forest, Panamá N 9° 7.023′ W 79° 41.898′	HHdFL
SAR040627–01 May 2004	*T. septentrionalis*	Virginia, USA N 4° 5.301′ W 52° 40.631′	SAR
C#029–0509 May 2009	*T. zeteki*	Gamboa, Panamá N 9° 7.023′ W 79° 41.898′	HF-M
RMMA100629–16 June 2010	*T.* cf. *zeteki* 2	Pipeline Road ~6 km, Panamá N 09° 9.770′ W 79° 44.715′	RMMA

(Fig. 3). For trisection, live ants were frozen and then divided into three parts with a solvent-rinsed dissection blade: head, thorax (including legs), and abdomen (i.e. gaster). Care was taken to minimize contamination between ant body parts by washing the forceps and blade first in ethanol, then in methanol, after each division. The heads of the workers from one colony were then combined in a glass vial with methanol, the thoraxes in a second vial and the gasters in a third vial. Samples from whole ants, heads, thoraxes, and gasters were analyzed using the same GC–MS methods. Results for trisected ants are only reported as belonging to a particular body region if that compound was not found in samples from the other body regions. Following the analysis, ants were placed in vials containing ethanol for possible genetic analysis and vouchering.

2.2. GC–MS

2–5 µl of methanol extract of whole ants or trisections was carefully drawn from each sample using a standard Hamilton syringe, and injected directly into the port of a Shimadzu QP-2010 GC–MS equipped with an RTX-5, 30 m × 0.25 mm i.d. column. The instrument was programmed from 60 °C to 250 °C at 10 °C min^{-1}. The mass spectrometer was operated in EI mode at 70 eV, and scanning was set to 40 to 450 AMU at 1.5 scans sec^{-1}.
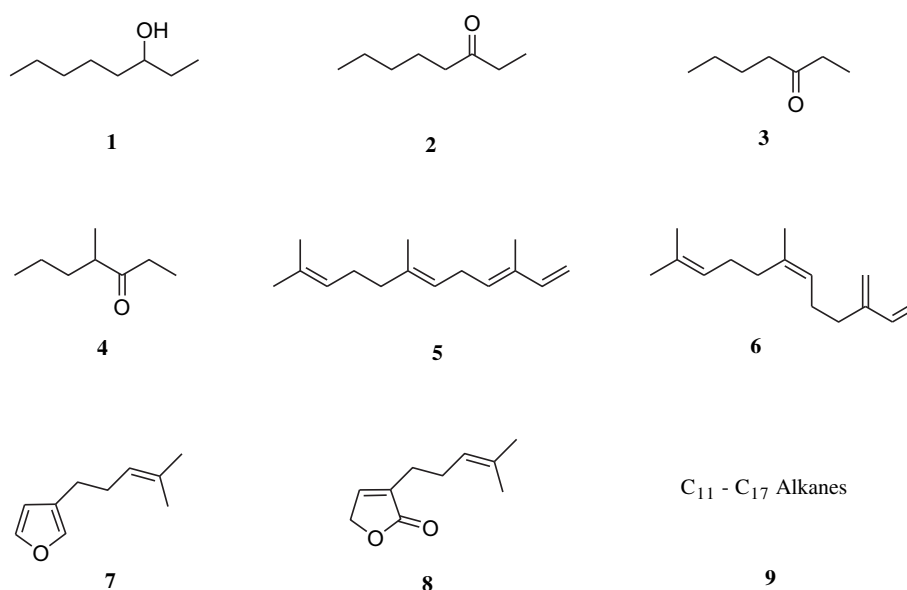
Fig. 2. Compounds detected in *Trachymyrmex* and *Sericomyrmex* species. (**1**) = 3-octanol, (**2**) = 3-octanone, (**3**) = 3-heptanone, (**4**) = 4-methyl-3-heptanone, (**5**) = E,E-α-farnesene, (**6**) = Z-β-farnesene, (7) = Perillene, (**8**) = α,α-acariolide, (**9**) = C$_{11}$–C$_{17}$ alkanes (predominantly undecane, but also C$_{13}$, C$_{15}$, and C$_{17}$).
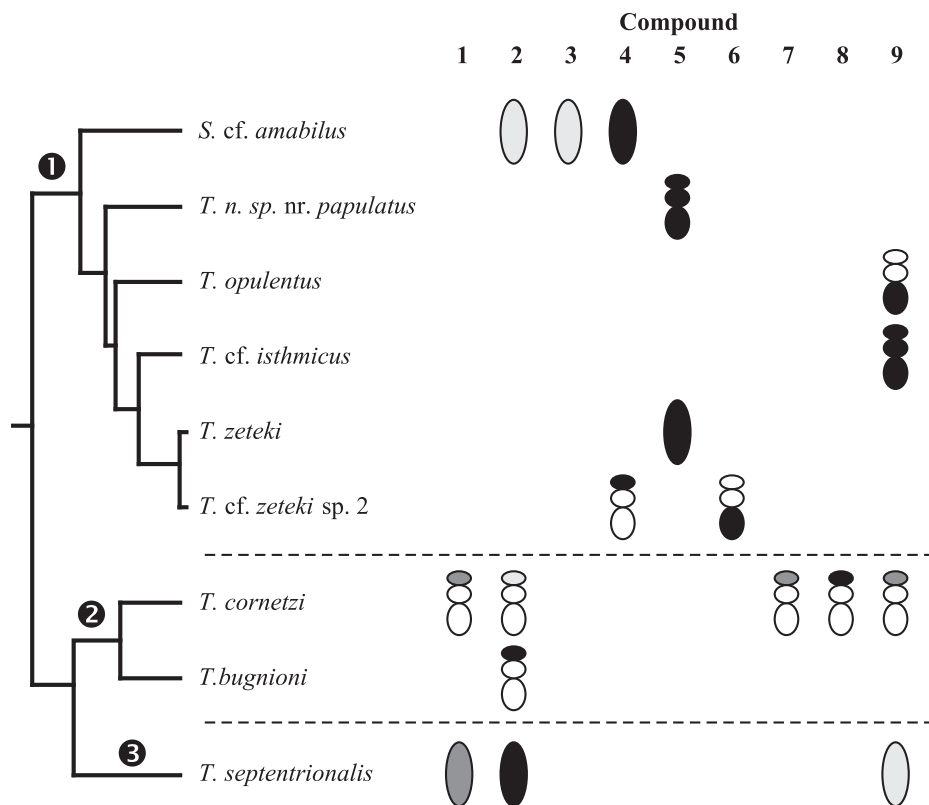
Fig. 3. Compounds identified in *Trachymyrmex* and *Sericomyrmex* species with numbers corresponding to structures in Fig. 2. Trisected samples are shown as three-part symbols, representing the head, thorax and abdomen from top to bottom. Samples that were not trisected are shown as ellipses. Shading of each symbol represents the quantities of compound found in each sample: black (major component, >20%), mid-grey (minor component, <20%) and light grey (trace amount). To the left of the table are species relationships inferred from the phylogeny of Schultz and Brady (2008) and morphological similarity (see discussion). 1, 2, and 3 refer to the clades discussed in the text. See Fig. 1 for a more detailed phylogeny.

3. Results and discussion

GC–MS identified a series of compounds in our samples that were unique to one or a few species (see below), plus typical insect fatty acids (mostly C16, C18 and C20), which appeared as their methyl esters. Since such fatty acids are present in all insect material, they were not analyzed further. For trisected ants, all compounds that were identified from particular body regions were also identified from whole ant extracts. No other compounds were detected in our GC-MS analyses.

The compounds identified from our samples of two genera within the higher attines showed considerable biological diversity. The chemical structure of the compounds reported is presented in Fig. 2, with numbers in bold referring to specific compounds or sets of compounds. We detected α,α-acariolide (**8**) and perillene (**7**) and the farnesene isomers (**5**) and (**6**). Additionally 3-octanol (**1**), 3-octanone (**2**), 3-heptanone (**3**) and 4-methyl-3-heptanone (**4**) were detected, which are well-known mandibular gland compounds from several ant genera, generally thought to be used as alarm pheromones (Morgan, 2008; Morgan et al., 2008). The identities of (**1**), (**2**), (**3**), (**4**), (**5**), (**6**) and the normal alkanes were established by comparison with pure stock samples. Results are presented in a table beside a phylogeny adapted from Schultz and Brady (2008) and based on morphological similarity in Fig. 3.

3.1. Trisections and possible glandular sources

3.1.1. Head

Of all species trisected, three head extractions yielded results unique to that body part and a total of five known mandibular gland substances were detected (Fig. 3).

Firstly, 3-octanone (compound **2**), is commonly found in ant mandibular glands (Attygalle and Morgan, 1984; Morgan, 2008) including those of *Cyphomyrmex*, *Trachymyrmex*, *Acromyrmex* (Crewe and Blum, 1972), and *Atta* species (Hughes et al., 2001). We found (**2**) in the head or whole body extracts of four of the nine species analyzed. *Trachymyrmex cornetzi* and *Trachymyrmex bugnioni* head extracts yielded this compound in trace (<1%) and major (>20%) levels, respectively. *Sericomyrmex* cf. *amabilis* and *T. septentrionalis* whole body extracts (neither of these species was trisected) also contained this volatile secretion. Previous work suggests that (**2**) alone or in combination with 3-octanol (compound **1**) serves as a mandibular gland derived alarm substance in *T. septentrionalis* (Crewe and Blum, 1972) and other ant species (Crewe and Blum, 1970; Crewe et al., 1972). Here we report (**1**) and (**2**) together, in *T. cornetzi* and *T. septentrionalis* (Fig. 3).

Secondly, *Sericomyrmex* cf. *amabilis* extracts contained 3-heptanone (compound **3**), a mandibular gland substance of two *Myrmica* species (Cammaerts et al., 1983) and *Atta* (Hernandez et al., 2006).

Thirdly, 4-methyl-3-heptanone (**4**), thought to be a vital releaser of alarm response in *Atta* (Blum et al., 1968), is a known mandibular gland product in a number of ant species and was detected in the head sample of *T.* cf. *zeteki* sp. 2 (Morgan, 2008; Morgan et al., 2008 and references therein). It was also detected in whole body extract of *S.* cf. *amabilis*. This non-terpenoid ketone has been found in several leaf-cutter species (Blum et al., 1968; Crewe and Blum, 1972; Moser et al., 1968) and interestingly, has not yet, to our knowledge, been found in the lower attines. It has however, been found in arachnids and may function similarly as an alarm substance (Blum and Edgar, 1971).

Finally, the monoterpenes, perillene (**7**) and α,α-acariolide (**8**) were found. The former was detected only in *T. cornetzi* head extracts, and is a unique furan terpene whose distinctive mass spectrum matched that reported in the literature (Stein et al., 2008). It has also been identified from the mandibular glands of *Tetramorium* and *Lasius* ants (Kern et al., 1997; Longhurst et al., 1980). The monoterpene lactone, α,α-acariolide (**8**), was found as a major component in the head of *T. cornetzi* as well, and only previously in astigmatid mites (Tarui et al., 2002). The mass spectrum and gas chromatographic retention time of (**8**) were identical to those of a synthetic sample prepared by the method of Tarui et al. (2002).

Although not dissected and analyzed, our results suggest that the extracted compounds may originate from the mandibular glands, which often contain volatile alcohols, ketones, and monoterpenes in ants, and are thought to be used mainly in defense and alarm communication (Hölldobler and Wilson, 1990). Six of the nine compounds identified belong to these groups of substances, one, α,α-acariolide (**8**), being identified in ants for the first time.

3.1.2. Thorax

No compounds unique to the thorax were found. The thorax of ants houses the large, paired metapleural glands (Yek and Mueller, 2011), which are unique to the subfamily, and a variety of functions have been proposed for them including the production of recognition compounds, territorial marking, sanitation, and chemical defense (Yek and Mueller, 2011). The metapleural glands have been particularly well studied in the leaf-cutting ants, where they have been shown to produce large quantities of fatty acids (Nascimento et al., 1996; Ortius-Lechner et al., 2000) that are effective against a range of pathogens and parasites of the ants and the fungus garden (Bot et al., 2002; Mendonca et al., 2009). Unfortunately these fatty acids cannot be distinguished from those derived from other body tissues in our analysis, so further studies are required to investigate their presence in non-leafcutting attines.

3.1.3. Gaster

The Dufour's gland of ants in the subfamily Myrmicinae produces an array of exclusively aliphatic hydrocarbons (Hölldobler and Wilson, 1990), which is consistent with the alkanes and farnesenes we detected. Terpenoid hydrocarbons, used as pheromones in some ant species, have previously been found in the Dufour's gland and pygidial glands located in the gaster of ants (Attygalle et al., 1983; Attygalle and Morgan, 1984; Jackson and Morgan, 1993 and references therein).

In the case of *T.* cf. *isthmicus* whole ant extractions, the mixture of alkanes (**9**) was composed mainly of undecane (60%) with lesser amounts of tri-, penta-, and heptadecane. These alkanes were found predominantly in the gaster extracts of the trisected sample of *Trachymyrmex opulentus*, which suggests that the gaster is their likely source. Undecane has been identified from the Dufour's gland and is known to be an aggregation (Jackson and Morgan, 1993) and alarm pheromone (Hölldobler and Wilson, 1990).

The sesquiterpenes *E,E*-α-farnesene (**5**) and *Z*-β-farnesene (**6**) were found in three species. The more common (**5**) was found in large amounts in *Trachymyrmex n. sp.* nr. *papulatus* and *Trachymyrmex zeteki,* while (**6**) was found in the gaster of *T.* cf. *zeteki* sp. 2. The large amounts of (**5**) in *Trachymyrmex n. sp.* nr. *papulatus* and *T. zeteki,* were not unique to the gaster extractions in these collections, but this could be due to contamination before or during trisections. *E,E*-α-farnesene (**5**) is found in plants (Bungert et al., 2002) and rodents (Brennan and Keverne, 2004) and is a known trail pheromone component of *Solenopsis invicta.* Farnesenes in general have been identified from the Dufour's gland in ants (Bergstrom and Lofqvist, 1968; Cavill et al., 1967; Vander Meer, 1983), which may well be the source gland in our samples.

3.2. Phylogenetic context

Comparisons of exocrine gland chemistry can be a powerful systematic tool (Jackson and Morgan, 1993). Although alarm pheromones may not be species- or even caste-specific (Hughes et al., 2001; Nascimento et al., 1993, 1996), mate attraction and trail pheromones can be. Similarly, hydrocarbon profiles and other chemical signatures can be helpful in the discovery of sister or cryptic species (Bagnères and Wicker-Thomas, 2010), whereas the study of biosynthetic pathways can elucidate distant levels of phylogenetic relationship at the genus or tribe level (Jackson and Morgan, 1993). Thus, in systems where the phylogeny is known, discussion of the chemical properties in a phylogenetic context is warranted. Here we discuss our results in the context of the most complete molecular based phylogeny for the genera *Trachymyrmex* and *Sericomyrmex* by Schultz and Brady (2008). In that phylogeny, the genera *Trachymyrmex* and *Sericomyrmex* form three distinct clades (Fig. 1): clade 1 is most distantly related to the leaf-cutting ants and contains both *Trachymyrmex* species and *Sericomyrmex* species, whereas the remaining two clades (2 and 3) are entirely made up by subsets of species within *Trachymyrmex* (Fig. 1).

3.2.1. Clade 1

This clade contains *Sericomyrmex* species as well as *Trachymyrmex* species classified in the *T. opulentus*, *Trachymyrmex urichi*, and *Trachymyrmex jamaicensis* species groups (Mayhe-Nunes and Brandão, 2007; Schultz and Brady, 2008) and is represented in the present study by *S.* cf. *amabilis*, *T. n. sp.* nr. *papulatus*, *T. opulentus*, *T. zeteki*, and *T.* cf. *zeteki* sp. 2. These five species have the ketones (**2**), (**3**) and (**4**), along with the sesquiterpenes (**5**) and (**6**), and C_{11}–C_{17} alkanes (**9**), suggesting that these compounds, with the exception of (**2**) and (**9**), are clade-specific. 4-methyl-3-heptanone (**5**), shared between *S. amabilis* and *T.* cf. *zeteki* sp. 2, is a known alarm pheromone and has convergently evolved in a variety of taxa across phyla (Blum and Edgar, 1971; Cammaerts-Tricot et al., 1976).

3.2.2. Clade 2

Clade two is represented here by *T. cornetzi* and *T. bugnioni*. These samples contained the straight chain compounds 3-octanol (**1**) and 3-octanone (**2**), along with the terpenes perillene (**7**) and α,α-acariolide (**8**), plus C_{11}–C_{17} alkanes (**9**). Compound (**1**) is shared with *T. septentrionalis* while (**7**) and (**8**) are unique to *T. cornetzi* and, not surprisingly, C_{11}–C_{17} alkanes span the *Trachymyrmex/Sericomyrmex* phylogeny. The compounds in clade two are common in ants (Attygalle and Morgan, 1984; Morgan, 2008) or have convergently evolved across phyla.

The monoterpenes perillene (**7**) and α,α-acariolide (**8**), found in clade 2, and the sesquiterpenes *E,E*-α-farnesene (**5**) and *Z*-β-farnesene (**6**), found in clade 1, are likely derived from the same biosynthetic pathway, with (**5**) and (**6**) having one more isoprene unit. *E,E*-α-farnesene (**5**) is shared by *T. n. sp.* nr. *papulatus* and *T. zeteki,* supporting the hypothesis of recent common ancestry of the two species. However, *E,E*-α-farnesene is widespread and this terpenoid hydrocarbon is also found in other distantly related ant species (Vander Meer, 1983) thus convergence is equally probable. The form of farnesene found in *T.* cf. *zeteki* sp. 2 differs in geometry and double bond position from that of *T. zeteki* corroborating the hypothesis that they are separate species (T. R. Schultz and R. M. M. Adams, unpublished). At the same time, the additional isoprene unit shared by (**5**) and (**6**), and similarity in the biosynthetic pathway, suggest these taxa are close relatives and thus supports the hypothesis that *T. zeteki* and *T.* cf. *zeteki* sp. 2 may be sister species, as their morphology and natural history suggest (T. R. Schultz and R. M. M. Adams, unpublished).

3.2.3. Clade 3

Finally, the third clade, sister to the leaf-cutters (Schultz and Brady, 2008; Fig. 1), is represented by the North American species *T. septentrionalis,* which contained 3-octanol (**1**), 3-octanone (**2**) and C_{11}–C_{17} alkanes (**9**), none of which are unique to this species. 3-octanone (**2**) has previously been found in the head of *T. septentrionalis* and leaf-cutting species (Crewe and Blum, 1972; Riley et al., 1974). In this study, *Sericomyrmex* cf. *amabilis* and *T. septentrionalis* whole-body extracts also contained 3-octanone (**2**) in trace (<1%) and major (>20%) levels, respectively, thus spanning the entire range of *Sericomyrmex/Trachymyrmex* groups in the attine phylogeny (Fig. 3). Interestingly, previous work examining the alarm pheromones and mandibular gland secretions of two lower attine species, *Cyphomyrmex rimosus* and *Mycoceperus goeldii* (see Fig. 1), did not find 3-octanone (Blum et al., 1981; Crewe and Blum, 1972), which, barring the limited taxonomic sampling, suggest that this compound might be unique to the higher attine clades. This is supported by the fact that this compound is a large constituent of the samples from all species in clades 2 and 3, which are more closely related to the leaf-cutting ants, but is only found in trace quantities in one member (*S.* cf. *amabilis*) of the more distantly related clade 1.

3.3. Conclusion

Here we have presented the exocrine chemistry of eight *Trachymyrmex* and one *Sericomyrmex* species and discussed it at the glandular level and in a phylogenetic context. Research on these transitional genera will lead to a deeper understanding of the compounds identified in the fungus-growing ants. In the future, a comparison of species across the attine tribe will provide valuable insight into the evolution of the biosynthetic pathways, elucidating formation and function of the exocrine compounds found in these ants.

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