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Nestmate Recognition and the Role of Cuticular Hydrocarbons in the African Termite Raiding Ant *Pachycondyla analis*

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Abstract Cuticular hydrocarbons (CHCs) are used for chemical communication among nestmates in many ant species, and they may play a role in the discrimination of nestmates and non-nestmates. Using the mandible opening response (MOR) bioassay, we tested the response of the African termite raiding ant, *Pachycondyla analis*, to CHC extracts of nestmates and non-nestmates. The ants were able to distinguish control chemical cues, from nestmate CHCs, and from non-nestmate CHCs, and, based on a CHC recognition threshold, aggression was demonstrated toward non-nestmates. Gas chromatography (GC) and GC-mass spectrometric analyses showed that CHC components of different ant colonies had chain lengths ranging from C_8 to C_{31}, comprising mainly *n*-alkanes, alkenes, and methyl branched alkanes, with the *n*-alkanes occurring in the same proportions among all colonies. The ants were grouped successfully according to their colonies of origin by using discriminant analysis of CHCs. We demonstrate that nestmate recognition occurs in *P. analis*, and that some of the cues involved are evidently alkenes and methyl-branched alkanes.

Key Words Nestmate recognition · Mandible opening response bioassay · *Pachycondyla analis* · Cuticular hydrocarbons · Ponerine ant · Formicidae · Ponerinae

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Introduction

Communication is a common and important phenomenon in all biological organisms. Among social insects, nestmate recognition enables integration within a colony and prevents non-colony members (both conspecifics and heterospecifics) from exploiting the colony's resources (Crozier and Pamilo 1996). The presence of non-nestmates (intruders) usually elicits active defensive behaviors (Hölldobler and Wilson 1990; Vander Meer and Morel 1998). Nestmate recognition in social insects can be adaptive because workers obtain benefits from aiding nestmates and discriminating against non-nestmates provided that the nestmates are more closely related to one another than to members of other conspecific colonies (Hölldobler 1995). The cues involved can be of genetic or environmental origin, and can differ among populations and seasons (Pirk et al. 2001), even in species that form super-colonies like *Formica exsecta* (Katzerke et al. 2006). The primary cues of communication in most insects are chemical in nature (Wyatt 2003) and are perceived by olfaction or contact chemoreception (Breed 1998).

Ants are among the dominant social insects in the world, and they employ complex forms of chemical communication. Over 100 exocrine glands have been described in social insects with more than half of these found in ants (Billen 2004). An array of signals and information on an individual's species, sex, age, caste, status, and relatedness, as well as alarm and trail pheromones are encoded in the secretions from these glands (Howard and Blomquist 2005).

Fielde (1901) advocated that nestmate discrimination signals are encoded in cuticular lipids, particularly those hydrocarbons that coat all insects. Since then, the role of cuticular hydrocarbons (CHCs) has been a subject of much debate, and various studies have attempted to determine

their functions in insect chemical communication. Examples of these roles include recognition at various levels, such as the individual (e.g., D'Ettore and Heinze 2005), nestmate (e.g., Wagner et al. 2000; Akino et al. 2004, Martin et al. 2008a,b), species (e.g., Neems and Butlin 1995; Dapporto 2007), kin (Arnold et al. 1996), and as cues for reproduction and division of labor (e.g., Dietemann et al. 2003; Martin and Drijfhout 2009). Most recently CHCs have been found to be responsible for enforcing altruism in ants (Smith et al. 2009). In adult insects, CHCs are synthesized internally in the oenocytes (Blomquist and Dilwith 1985) and, hence, are under a strong genetic influence reflecting an insect's genetic makeup (Lockey 1991). After synthesis, they are transferred to the cuticle by lipophorin (Schal et al. 2001). CHCs are made up of a homologous series of long, straight-chained saturated alkanes that can be modified by addition of methyl groups or the introduction of double bonds (Jackson and Morgan 1993).

The ant *Pachycondyla analis* (Formicidae: Ponerinae) is a specialized termite predator, which is widely distributed in sub-Saharan Africa (Lévieux 1966). This species, commonly referred to as 'Matabele ants', organizes group raids on termite species that mainly belong to the sub-family Macrotermitinae (Longhurst et al. 1978). There is no information on CHCs of *P. analis*, nor the role they play in nestmate recognition. We recently initiated a comprehensive investigation of the chemical ecology of this ant species, and in the present paper we report the results from a study of CHCs of different colonies of *P. analis* and the role they play in nestmate recognition.

Methods and Materials

Study Species Three colonies of *P. analis* were excavated from Mpala Research Centre (0°17′N, 37°52′E) central Kenya, 250 km north of Nairobi. The colonies were kept in artificial nest boxes (20×20×20 cm) made of aluminium, which were connected to a foraging arena (1.5×1.0 m) made of Perspex. The nests were maintained at 25±1°C, with about 50–60% relative humidity and a 12L: 12D photoperiod. Ants were fed twice daily on live termites (mainly from the subfamily Macrotermitinae) collected around the Duduville campus of *ICIPE* in Nairobi, Kenya.

Extraction of CHCs for Bioassay CHCs from 5 ants per colony were extracted for use as sources of chemical stimuli in bioassays (Guerrieri and d'Ettorre, 2008). Ants previously in contact with their own colony odor were selected for extraction of CHCs. The ants were first killed by placing them on ice for 15 min, and CHCs were extracted by washing them in 500 µl of pentane for 10 min. Solvent was evaporated under a gentle stream of nitrogen, the residue was dissolved in 50 µl of pentane, and stored at −20°C until analysis. Twentyfour extracts were prepared from each of the 3 colonies making a total of 72 extracts. A solvent control (pentane) also was subjected to the same evaporation procedure. An average quantity corresponding to the extract of one ant (10 µl) was applied to the tip of a Pasteur pipette by using a Hamilton syringe. The pipette tip was held downwards until the solvent evaporated from the tip, thus leaving the residue of the extract around the lower and outer part of the pipette.

Mandible Opening Response (MOR) Bioassay Ants were removed from their colonies and transferred to 20 ml glass vials, and immobilized by placing them on ice. They then were harnessed using methods previously described (Guerrieri and d'Ettorre 2008), and were kept undisturbed for 2 hr to recover and habituate to the harness.

Aggression responses were quantified by presenting four different types of stimuli to the test ants from colonies 1, 2, and 3: a) solvent extract only (CTRL); b) extract from colony 1 (C1); c) extract from colony 2 (C2); d) extract from colony 3 (C3). For a test ant, extracts from individuals of its own colony served as nestmate stimuli, while extracts from individuals of other colonies served as non-nestmate stimuli. All ants were tested with all extracts and the control.

In each trial, one stimulus was presented to a harnessed individual ant. A test individual was removed from its resting place and allowed to habituate for 2 min prior to presenting it with the test stimulus. After habituation, its antennae were touched gently for 5 sec with the tip of the stimulating pipette. When the test ant opened its mandibles continuously (i.e., displacing them from the resting position), the behavior was recorded as aggression (score=1). If the individual did not open its mandibles, and instead antennated continuously, the response was recorded as non-aggressive (score=0) following the protocol of Guerrieri and d'Ettorre (2008). After presenting a stimulus, the test ant was returned to its resting place. Stimuli were presented at random to individual ants after an interval of 20 min to allow for the recovery of antennal receptors. From each of the three colonies studied, 24 ants chosen at random were tested with each of the 4 stimuli, thus a total of 72 ants were tested.

Extraction of Cuticular Hydrocarbons (CHCs) for Chemical Analyses Cuticular hydrocarbons were extracted in a way similar to those used for the MOR bioassay, but in this case each ant was extracted in 1 ml of pentane. The solvent was evaporated under a gentle stream of nitrogen, dissolved in 100 µl of pentane, and stored at −20°C until analysis. Twentyfour extracts were prepared from each of the three colonies, making a total of 72 extracts. A pentane control also was subjected to the same evaporation procedure.

Chemical Analyses Gas chromatography (GC) was carried out on an HP 5890 series II GC equipped with a flame ionization detector (FID) and an HP-5 column (30× 0.25 mm ID×0.25 μm film thickness). Nitrogen was used as carrier gas, with a column pressure of 46 psi and injection temperature of 250°C. One μl of sample was injected in the splitless mode, with the oven temperature programmed at 60°C for 5 min to 280°C at 10°C/min, and held at this temperature for 13 min. GC-mass spectrometry (MS) analysis was carried on an Agilent Technologies 7890A GC equipped with an HP-5 MS capillary column (30×0.25 mm ID×0.25 μm film thickness) coupled to 5795C MS. One microliter of each sample was injected in the splitless mode, and helium was used as carrier gas at 1.0 ml min^{-1}. The oven temperature was 35°C for 5 min, increased to 280°C at 10°C min^{-1} and then held at this temperature for 15 min. Spectra were recorded at 70 eV in the electron impact (EI) ionization mode. All the n-alkanes, 2-methylheptadecane, 1-heptadecene, (*Z*)-9-tricosene, and squalene were identified by GC-MS co-injection, and comparison of MS data with those of authentic standards. Other methyl-branched alkanes and alkenes were tentatively identified by using EI diagnostic ions (El-Sayed 2009).

Chemicals *n*-Undecane, *n*-Dodecane, *n*-tridecane, *n*-tetradecane, *n*-pentadecane, *n*-hexadecane, and *n*-heptadecane with the purity of >99% were obtained from Aldrich Chemical Company (Gillingham, Dorset, UK). *n*-Octadecane, *n*-nonadecane, *n*-eicosane, *n*-heneicosane, *n*-docosane, *n*-tricosane, *n*-tetracosane, *n*-pentacosane, *n*-hexacosane, *n*-heptacosane, *n*-nonacosane, and *n*-hentriacontane were provided by Dr. Peter Teal, USDA/ARS-CMAVE, Florida, USA. 1-Heptadecene, (*Z*)-9-tricosene, squalene, and 2-methylheptadecane were provided by Dr. Antony Hooper, Rothamsted Research, Harpenden, UK.

Statistical Analyses Logistic regression was performed on the dichotomous data (1 vs. 0) on the aggressive responses of ants. We tested the differences in aggression response of ants to the solvent control, nestmate and non-nestmate extracts. The levels of aggression between colonies were tested using *Kruskal Wallis* ANOVA. The relative areas of the peaks of the individual compounds in the CHC profile for each ant were standardized to 100%. The standardized peak areas were then transformed following the method proposed by Aitchinson (1986):

$$Z_{ij} = \ln\left[Y_{ij}/g\left(Y_j\right)\right]$$

where Z_{ij} is the standardized peak area *i* for individual ant *j*, Y*ij* is the observed peak area *i* for individual ant *j*, and g (Y*j*) is the geometric mean of all peak areas for ant *j* included in the analyses. We performed a stepwise discriminant function analysis (stepwise DA) on the transformed variables followed by canonical discriminant analysis on the selected peaks to determine whether the colonies could be separated on the basis of their CHC profiles. Pairwise generalized square distances between colonies and classification error rates were also calculated. All statistical analyses were carried out using SAS 9.1.2 statistical software.

Results

MOR Bioassay The number of ants that opened their mandibles when presented with the solvent control was lower compared to an extract (*Wald's* χ^2=58.34, *df*=1, *P*<0.001). There was less mandible opening when ants were presented with a nestmate extract compared to an extract from a non-nestmate (*Wald's* χ^2=101.24, *df*=6, *P*<0.001). In general, the levels of aggression (i.e., MOR) increased

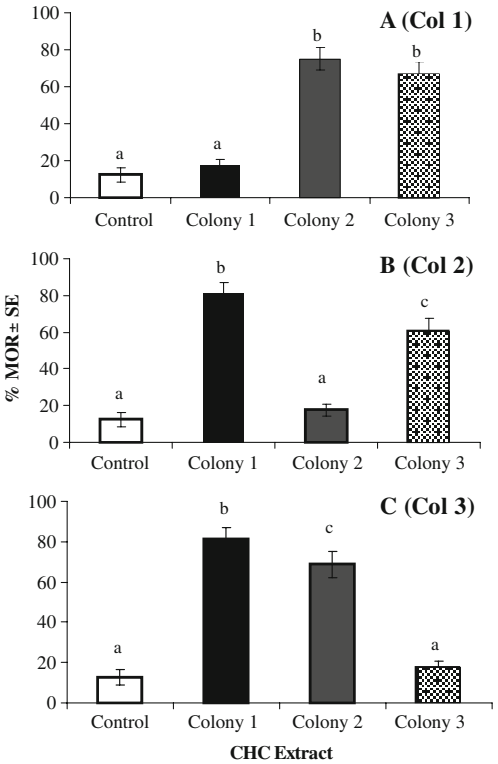

Fig. 1 Mandible opening response (MOR)±SE for, Pachycondyla ants (**A**) from colony 1, (**B**) from colony 2, and (**C**) from colony 3 to the presented extracts. □=Control solvent (pentane), ■=CHC extract from colony 1, ▩=CHC extract from colony 2 and ✚=CHC extract from colony 3. Ants responded significantly different to test stimulus and control (*Wald's* χ^2=58.34, *P*<0.001). Response of ants to the extract of nestmate and those of non-nestmate also differed significantly (*Wald's* χ^2=101.24, *df*=6, *P*<0.001). The same letters on bars represent means that are not significantly different

when an ant was presented with a non-nestmate stimulus compared to a nestmate extract or a solvent control (*Wald's* χ^2=132.19, *df*=2, *P*<0.001, Fig. 1). Ants from colony 1 were slightly more aggressive than those from colonies 2 and 3 (Fig. 1), although aggression between colonies was not significantly different (*Kruskal Wallis ANOVA*, χ^2= 5.08, *df*=2, *P*=0.082).

CHC Profiles of P. analis GC-MS analysis revealed that the CHCs of *P. analis* were a complex mixture of alkanes, alkenes, and methyl-branched alkanes ranging from C_8 to C_{31} (Fig. 2, Table 1). The *n*-alkanes, 2-methylheptadecane, 1-heptadecene, (*Z*)-9-tricosene, and squalene had retention times and mass spectra that matched those of authentic standards. The identities of these compounds in the extracts were confirmed further by GC-MS co-injection. Other components in the extracts, including methyl-branched alkanes and alkenes, were identified tentatively from their characteristic mass spectral diagnostic ions (El-Sayed 2009). The major components varied between colonies, with (*Z*)-9-tricosene being present in varying proportions in all the colonies. The proportions of alkanes in the extracts remained constant, while there was variation in the proportions of the alkenes and the methyl-branched alkanes between colonies (Fig. 3).

CHC Differentiation Among Colonies Ants from the different colonies could be distinguished by using the transformed peak areas of the 35 identified compounds (Fig. 2) that differed among the colonies. Using the stepwise DA, 17 variables clustered the ants according to their colonies of origin (*Wilk's* λ=0.0007, *df*=34, 10, *P*< 0.001). Discriminating compounds selected by the stepwise DA were: undecane, 3-methylundecane, 3,6-dimethylundecane, 3,8-dimethyl decane, pentadecane, heptadecane, 3-methylheptadecane, 2,6,10,14-tetramethylpentadecane, octadecane, nonadecane, heneicosane, tricosane, 1-nonadecene, 9-nonadecene, 9-methylnonadecane, squalene, and hentriacontane.

Using the 17 compounds selected by the stepwise DA, ants were grouped into their colony of origin (*Wilk's* λ= 0.0000, *df*=34, 10, *P*<0.001), with function 1 explaining 88.07 % of the variation separating colony 3 from both colonies 1 and 2, and function 2 explaining 11.93 % of the variation further separating colony 3 from 2 and 1 (Fig. 4). All the ants were grouped into their colonies correctly based on their CHC profiles.

Discussion

In this study, we demonstrated the use of a 'yes or no' aggression bioassay by using mandible opening as a measure of aggression between different colonies of *P. analis*. The results show that *P. analis* discriminate between nestmates and non-nestmates, since they were significantly more aggressive toward extracts of non-nestmates. These results

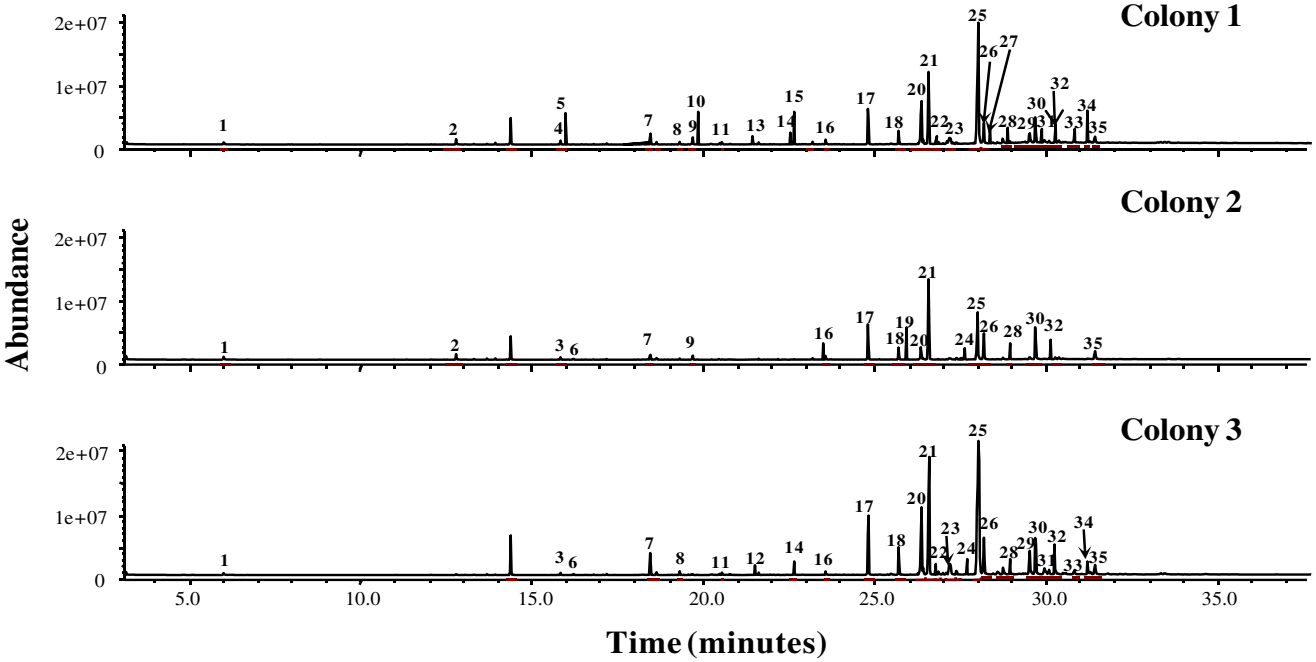

Fig. 2 Total ion chromatograms for the cuticular hydrocarbons of Pachycondyla ants from the colonies studied. Colony 1=CHC extracts from colony 1 ants, Colony 2=CHC extracts from colony 2 ants, Colony 3=CHC extracts from colony 3 ants (see Table 1 for the list of identified compounds)

Table 1 Compounds identified from the cuticular hydrocarbon profiles of *Pachycondyla analis,* along with retention indices and diagnostic ions

No[a]	Compound	RI[b]	Diagnostic EI-MS ions(m/z)
1	*n*-Octane	800	114
2	*n*-Undecane	1100	156
3	5-Methylundecane	1154	43, 57, 71, 85, 99, 112
4	3-Methylundecane	1169	43, 57, 71, 85, 99, 112, 141, 170
5	3,8-Dimethyldecane	1063	57, 71, 85, 99, 113, 141, 155, 170
6	*n*-Tridecane	1300	184
7	*n*-Pentadecane	1500	212
8	3-Methylpentadecane	1572	43, 57, 71, 85, 99, 113, 127, 141, 155, 168, 197, 226
9	2-Methylheptadecane	1765	43, 57, 71,85, 99, 113, 127, 141, 155, 169, 183, 195, 211, 239, 254
10	1-Heptadecene	1679	83,97,111,125,196, 210,239
11	8-Heptadecene	1679	41, 55, 69, 83, 97, 111, 125, 140, 238
12	5-Octadecene	1789	43, 55, 69, 83, 97, 111, 125, 139, 166, 180, 195, 224, 252
13	*n*-Octadecane	1800	254
14	9-Nonadecene	1875	43, 55, 69, 83, 97, 111, 125, 139, 153, 167, 238, 266
15	*n*-Nonadecane	1900	268
16	*n*-Eicosane	2000	282
17	*n*-Heneicosane	2100	296
18	1-Docosene	2195	43, 57, 69, 83, 97, 111, 125, 280,308
19	*n*-Docosane	2200	310
20	*(Z)*-9-Tricosene	2270	43, 55, 69, 83, 97, 111, 125, 139, 153, 223, 237, 294, 322
21	*n*-Tricosane	2300	324
22	Unidentified		
23	1-Tetracosene	2396	43, 57, 69, 85, 97, 113, 309, 338
24	*n*-Tetracosane	2400	338
25	Cyclotetracosane	2445	43, 57, 69, 83, 97, 111, 125, 139, 153, 207, 392
26	9-Pentacosene	2465	43, 57, 69, 85, 97, 113, 141, 169, 197, 326, 350
27	*n*-Pentacosane	2500	352
28	*(Z)*-12-Pentacosene	2496	43, 57, 69, 83, 97, 125, 236, 257, 290, 322, 350
29	1-Hexacosene	2593	43, 57, 69, 83, 97, 111, 125, 139, 336, 364
30	*n*-Hexacosane	2600	366
31	*n*-Heptacosane	2700	380
32	*n*-Octacosane	2800	394
33	Squalene	2663	41, 55, 69, 81, 95, 109, 121, 136, 148, 341, 367, 410
34	*n*-Nonacosane	2900	408
35	*n*-Hentriacontane	3100	436

[a] No=Peak numbers referring to Fig. 2

[b] *RI* Retention Index

are in agreement with those previously reported for the invasive Argentine ant (*Linepithema humile*) workers by Vásquez et al. (2008), where they showed evidence that CHCs are used in queen adoption, confirming that the MOR is a sensitive assay that can be used effectively to determine recognition or aggression thresholds in ants. Recognition thresholds usually are based on a template odor that is characteristic of a given colony, with ants deciding to accept or reject an individual when it smells greater than a minimum similarity threshold or below a dissimilarity threshold (Reeve 1989).

Aggression towards nestmates of similar CHC profiles could be due to errors arising while reacting to recognition cues, as shown by Vásquez et al. (2008) in *L. humile,* or due to a lower threshold to avoid false-positive identification. In the present study, the MOR bioassay was used successfully to measure inter-colony aggression at the individual and colony levels in *P. analis*.

We identified 35 different compounds in the CHCs of different *P. analis* colonies. These compounds were mainly alkanes, alkenes, and methyl-branched alkanes, as previously reported for other ant species (Dietemann et al. 2003;

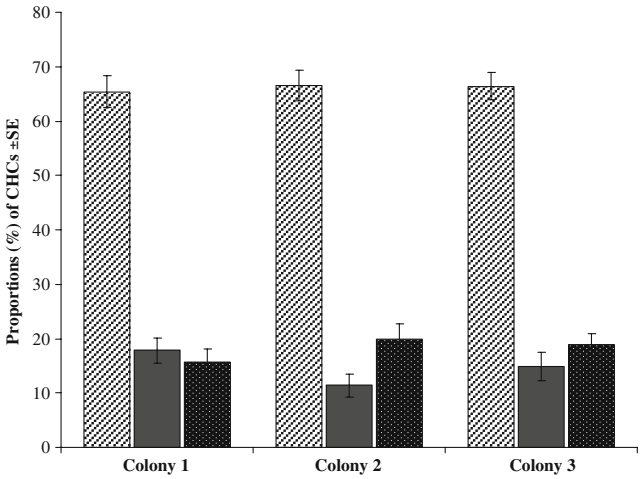

Fig. 3 Proportions±SE of the different groups of hydrocarbons (▨=*n*-Alkanes, ■=Alkenes and ▩=Methyl-branched alkanes) in the cuticular hydrocarbon profiles of *Pachycondyla analis* ants from colony 1, 2 and colony 3 (*error bars* represent mean proportions±SE)

Lucas et al. 2005; Martin et al. 2008b), with (*Z*)-9-tricosene occurring in variable proportions between colonies. In *P. analis, n*-alkanes occurred roughly in the same proportion in all colonies, with the alkenes and methyl-branched alkanes present in different proportions between the colonies, unlike in *Formica japonica* where *n*-alkanes and (*Z*)-9-alkenes varied between colonies (Akino et al. 2004) and in *F. exsecta* where (*Z*)-9-alkenes are colony specific (Martin et al. 2008b). In the genus *Pachycondyla,* species

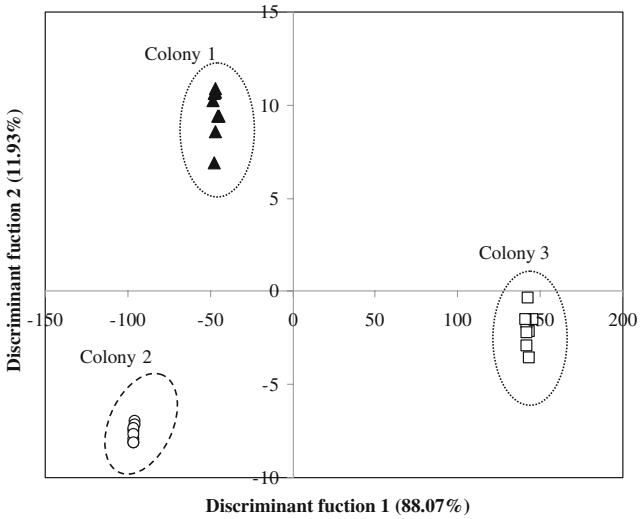

Fig. 4 Discriminant function analysis of ants from the three colonies of *Pachycondyla analis* based on relative proportions of 17 cuticular hydrocarbons determined in stepwise fashion. Colony 1 (*filled triangles*), Colony 2 (*circles*) and Colony 3 (*squares*). Circles around individual points are arbitrarily drawn to denote each colony. All individuals were clearly grouped into their respective colonies based on their CHC profiles

such as *P. villosa* (Lucas et al. 2004) and *P. apicalis* (Soroker et al. 1998) produce varying amounts of *n*-alkanes and alkenes. Differential amounts of these compounds may be influenced by environmental conditions, including temperature and relative humidity, as reported in a previous study on the desert harvester ant, *Pogonomyrmex barbatus* (Wagner et al. 2001).

The alkenes and methyl-branched alkanes in the CHCs could constitute nestmate recognition cues in *P. analis*. (*Z*)-9-Alkene has been reported as a nestmate recognition and aggression cue in *Formica* ants (Akino et al. 2004; Martin et al. 2008a,b), and as a recognition cue in the desert ant, *Cataglyphis niger* (Lahav et al. 2001). Nestmate recognition hydrocarbons identified in this study for *P. analis* might serve two purposes, colony defense (the traditional role of nestmate recognition), and recognition of nestmates when foraging. The roles played by the alkenes and methyl-branched alkanes in nestmate recognition and aggression in *P. analis* need to be further investigated by manipulating the CHC profiles of ants using synthetic compounds to see whether ants respond differently to the manipulated nestmate or non-nestmate CHCs.

The results from the discriminant function analysis show clearly that differences exist in the CHC profiles between colonies of *P. analis*. The implicated compounds (undecane, 3-methylundecane, 3,6-dimethylundecane, 3,8-dimethyldecane, pentadecane, heptadecane, 3-methylheptadecane, 2,6,10,14-tetramethylpentadecane, octadecane, nonadecane, heneicosane, tricosane, 1-nonadecene, 9-nonadecene, 9-methylnonadecane, squalene, and tetratriacontane) can be used effectively to correctly group the ants into their respective colonies. The colony-specific nature of CHCs in *P. analis* is consistent with findings in other ant species (e.g., Lahav et al. 2001; Akino et al. 2004; Lucas et al. 2004; Denis et al. 2006; Martin et al. 2008a,b). We demonstrated that these clear-cut groupings, based on CHC profiles, can explain the degree of aggression between different colonies. Colony 1 was further away from colonies 2 and 3, and exhibited higher aggression toward the latter colonies; likewise, colony 2 and 3 were closer together than colony 1, and were less aggressive toward each other's workers. Thus, these CHC differences could explain the differential acceptance of workers from other colonies competing for the same resources. Intruders or encroachers are usually killed upon encounter (AAY personal observation). By contrast the invasive Argentine ant displays minimal nestmate discrimination and individual non-nestmates are often integrated into an alien colony (Vásquez et al. 2008).

Colony odor recognition cues in ants are phenotypic and are derived either from the environment (diet, nesting sources) or produced endogenously (genetically determined or both) (Vander Meer and Morel 1998); the relative

importance of environmental and genetic differences can vary between populations (Pirk et al. 2001). Whatever the source of CHCs is, it is predicted that each colony will display a uniform odor that constitutes a gestalt. However, in some studies, it has been shown that this is not always the case because different castes within a colony may possess different CHC profiles that code different information within the colony (Dietemann et al. 2003; Martin and Drijfhout, 2009). In the present study, we showed that CHCs in *P. analis* are colony specific. However, a further investigation of workers in a colony based on the roles they play and their body sizes (major vs. minor workers) might reveal differences in the CHCs as is the case in some other ant species.

In summary, the MOR bioassay was used successfully to measure differential responses of *P. analis* workers based on colony of origin. Aggression was found to be associated with colony odor, mainly in the CHCs. As in other ant species, CHCs in *P. analis* comprise three main groups; *n*-alkanes, alkenes, and methyl-branched alkanes. The *n*-alkanes were consistent between colonies, with the alkenes and methyl alkanes serving as possible nestmate recognition cues.

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