

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 as a means of 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 between chemical cues from control, nestmate and non-nestmate, and based on a CHC recognition threshold, aggression was demonstrated between non-nestmate. Gas chromatography (GC) and GC-mass spectrometric analyses showed that the CHC components of the different ant colonies had chain lengths ranging from C<sub>8</sub> to C<sub>31</sub>, comprising mainly *n*-alkanes, alkenes and methyl branched alkanes, with the *n*-alkanes occurring in the same proportions among all colonies. The ants were successfully grouped according to their colonies of origin using discriminant analysis. We demonstrate that nestmate recognition occurs in *P. analis* and that some of the cues involved could be encoded in the alkenes and methyl-branched alkanes.

**Key Words-** Nestmate recognition, mandible opening response bioassay, *Pachycondyla analis*, cuticular hydrocarbons, ponerine ant.

## 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 behaviours (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 between populations and season (Pirk et al. 2001) even in species which form super-colonies like *Formica exsecta* (Kutzerke et al. 2006). The primary cues of communication in most insects are chemical in nature (Wyatt, 2003), which 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 chemical communication of insects. Examples of these roles include; as cues for 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 and Martin et al. 2008b), species (e.g. Neems and Butlin, 1995; Dapporto, 2007), kin (Arnold et al. 1996), and as cues for reproduction and division of labour (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 synthesised 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 which could be modified by addition of methyl groups or the introduction of double bonds (Jackson and Morgan, 1993).

The ant *Pachycondyla analis* is a specialised termite predator, which is widely distributed in sub-Saharan Africa (Lévieux, 1966). This ant species, commonly referred to as ‘Matabele ants’, organises 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* and 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.

## MATERIALS AND METHODS

*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 ant colonies were kept in artificial nests boxes (20 cm × 20 cm × 20 cm) made of aluminium which were connected to a foraging arena (1.5 × 1.0 m) which was 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 on live termites (mainly from the subfamily Macrotermitinae) twice daily collected around the Duduville campus of *icipe* in Nairobi, Kenya.

*Extraction of CHCs for mandible opening response (MOR) bioassay.* Cuticular hydrocarbons from five ants per colony were extracted for use as sources of chemical stimuli in the mandible opening response (MOR) bioassay (Guerrieri and d’Ettorre, 2008). Ants previously in contact with their own colony odour were selected for extraction of CHCs. The ants were first killed by placing them on ice for 15 min and CHC extracted by washing them in 500 µl of pentane for 10 min. The solvent was evaporated under a gentle stream of nitrogen, and the residue dissolved in 50 µl of pentane and stored at -20°C until required for analysis. Twenty four extracts were prepared from each of the three colonies making a total of 72 extracts. A solvent control (pentane) was also subjected to the same extraction procedure. An average quantity corresponding to the extract of one ant (10µl) was poured unto the tip of a Pasteur pipette using a Hamilton syringe. The pipette tip was held downwards until the solvent

evaporated from the tip, 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 into 20 ml glass vials and were then immobilized by placing them on ice. The ants were then harnessed using methods previously described (Guerrieri and d’Ettorre, 2008). The ants were kept undisturbed in a room for 2 hours to recover and to habituate to the harness.

Aggression responses were quantified by presenting four different types of stimuli, viz., a) solvent extract only (CTRL); b) extract from colony 1 (C1); c) extract from colony 2 (C2); d) extract from colony 3 (C3) to the test ants from colonies 1, 2 and 3. 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 the extracts and the control.

In each trial, one stimulus was presented to a previously 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 gently touched for 5 sec with the tip of the stimulating pipette. When it opened its mandibles continuously i.e. displacing them from the resting position, it was recorded as aggression (score = 1). If the individual did not open its mandibles and instead antennate 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 the 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 four stimuli, thus a total of 72 ants were tested.

*Extraction of Cuticular Hydrocarbons (CHC) for Chemical Analyses.* Cuticular hydrocarbons were extracted in a similar way to those used for MOR, but this time each ant was extracted in 1 ml of solvent (pentane). The solvent was evaporated under a gentle stream of nitrogen, and re-suspended in 100  $\mu$ l of pentane and stored at -20 °C until required for analysis. Twenty four extracts were prepared from each of the three colonies making a total of 72 extracts. A solvent control (pentane) was also subjected to the same extraction procedure.

*Chemical analyses.* Gas chromatography (GC) was carried out on a HP 5890 series II gas chromatograph equipped with a flame ionisation detector (FID) and a HP-5 column (30 m × 0.25mm ID × 0.25µm film thickness). Nitrogen was used as a 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 and at 10°C/min to 280°C, and held at this temperature for 13 min. GC-MS analysis was carried on an Agilent Technologies 7890A gas chromatograph equipped with a capillary column HP-5 MS (30 m × 0.25mm ID × 0.25µm film thickness) and coupled to 5795C mass spectrometer. One µl of each sample was injected in the splitless mode, and helium was used as the carrier gas at 1.0 ml min<sup>-1</sup>. The oven temperature was 35°C held for 5 min, increased to 280°C at 10°C min<sup>-1</sup>, and then held at this temperature for 15 min. The analysis was carried out at 70eV in the electron impact 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 mass spectral data with those of authentic standards. The other methyl-branched alkanes and alkenes were tentatively identified 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, 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 aggressive response of ants. We tested the differences in aggression response of ants to the 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 standardised to 100%. The standardised peak areas were then transformed following the method proposed by Aitchinson (1986):

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

where  $Z_{ij}$  is the standardised 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 generalised square distances between colonies and classification error rates were also calculated. All statistical analyses were carried out using SAS 9.1.3 statistical software.

## RESULTS

*Mandible opening response bioassay (MOR).* The number of ants that opened their mandibles when presented with the control (solvent) was significantly lower compared to an extract (*Wald's*  $\chi^2= 58.34$ ,  $df=1$ ,  $P < 0.0001$ ). There was significantly less mandible opening when ants were presented with a nestmate extract compared to an extract from a non-nestmate (*Wald's*  $\chi^2= 101.24$ ,  $df=6$ ,  $P < 0.0001$ ). In general, the levels of aggression (MOR) increased when an ant was presented with a non-nestmate stimulus compared to the nestmate extract and a control (*Wald's*  $\chi^2= 132.19$ ,  $df=2$ ,  $P < 0.0001$ , Fig. 1). Ants from colony 1 were slightly more aggressive than those from colonies 2 and 3 (Fig 1), although aggression between colonies were not significantly different (*Kruskal Wallis ANOVA*,  $\chi^2= 5.08$ ,  $df =2$ ,  $P =0.0815$ ).

*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<sub>8</sub> to C<sub>31</sub> (Fig. 2, Table 1). The n-alkanes, 2-methylheptadecane, 1-heptadecene, (Z)-9-tricosene and squalene had retention times and mass spectra which matched those of authentic standards. The identities of these compounds in the extracts were further confirmed by GC-MS co-injection studies. Other components in the extracts including methylbranched alkanes and alkenes were tentatively identified 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 between the colonies (Fig 3).

*CHC differentiation among colonies* Ants from the different colonies could be distinguished using the transformed peak areas of the 35 identified compounds (Fig 2) that differ among the

colonies. Using the stepwise DA, 17 variables clustered the ants according to their colonies of origin (*Wilk's*  $\lambda=0.0007$ ,  $df=34, 10$ ,  $P<0.0001$ ). Discriminating compounds selected by the stepwise DA were: 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-methyl nonadecane, squalene and hentriacontane.

Using the 17 compounds selected by the stepwise DA, ants were grouped into their colony of origin (*Wilk's*  $\lambda=0.0000$ ,  $df=34, 10$ ,  $P<0.0001$ ) with function 1 explaining 88.07 % of the variation separating colony 3 from both colonies 1 and 2, and function 2 explaining 11.93 % 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 using mandible opening as a measure of aggression between different colonies of *P. analis*. The result shows that *P. analis* discriminate between nestmates and non-nestmates since they were significantly more aggressive to extracts from non-nestmates (Fig. 1). These results are in agreement with those previously reported for *L. 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 set a recognition or an aggression threshold in ants. Recognition thresholds are usually based on a template odour 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 profile could be due to errors arising while reacting to recognition cues as shown by Vásquez et al. (2008) in the invasive Argentine ant (*Linepithema humile*) or due to lower threshold to avoid false-positive identification. In the present study, the MOR bioassay was successfully used to measure inter-colony aggression at the individual and colony level in *P. analis*.



We identified 35 different compounds in the CHCs of different colonies of *P. analis*. These were mainly alkanes, alkenes, and methyl-branched alkanes, as previously reported for other ant species (Dietemann et al. 2003; 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 (Fig. 3) unlike in *Formica japonica* where *n*-alkanes and (Z)-9-alkenes varied between colonies (Akino et al. 2004); and *F. exsecta* where (Z)-9-alkenes are colony specific (Martin et al. 2008b). In the genus *Pachycondyla*, species like *P. villosa* (Lucas et al. 2004) and *P. apicalis* (Soroker et al. 1998) have been shown to produce varying amounts of *n*-alkanes and alkenes. These differential amounts 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).

Nestmate recognition cues in *P. analis* could be encoded in the alkenes and methyl-branched alkanes in the CHCs. (Z)-9-Alkenes have been reported as nestmate recognition and as aggression cues in *Formica* ants (Akino et al. 2004; Martin et al. 2008a and b). They also serve as recognition cues in the desert ant *Cataglyphis niger* (Lahav et al. 2001). Nestmate recognition cues for *P. analis* identified in this study might serve two purposes, colony defence which is the traditional role of nestmate recognition and group raiding behaviour which may require 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 and using them in bioassays to see whether ants respond differently to the manipulated nestmate or non-nestmate CHCs.

The results from the discriminant function analysis clearly showed that clear cut differences existed in the CHC profiles between colonies of *P. analis*. The chosen 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 effectively be used to group the ants into their respective colonies correctly. The colony specific nature of CHCs in *P. analis* confirmed 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 groupings, based on CHC profiles, can explain the degree of aggression between different colonies. Colony 1 was further away from colonies 2 and 3, hence the high aggression, likewise colony 2 and 3 were closer together than colony one. These clear differences could explain the differential acceptance of workers from different colonies competing for the same resources. Intruders or encroachers are usually killed upon encounter (AAY personal observation). By contrast the invasive Argentine ant (*L. humile*) displays minimal nestmate discrimination and individual non-nestmates are often integrated into an alien colony (Vásquez et al. 2008).

Colony odour 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) and the relative importance can vary between populations (Pirk et al. 2001). Whatever the source is, it is predicted that each colony will display a uniform odour 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 which could 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 different groups of workers in a colony based on the roles they play and their body sizes (major and minor) might reveal differences in the CHCs as in other ant species.

In summary, the MOR bioassay was successfully used to measure responses of *P. analis* workers that show differences based on colony of origin. Aggression was found to be associated with colony odour, 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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## Figure Legends

FIG. 1. Mandible opening response (MOR)  $\pm$ se for, (A) ants from colony 1, (B) ants from colony 2 and (C) ants from colony 3 to the presented extracts.  $\square$  = Control solvent (pentane),  $\blacksquare$  = CHC extract from colony 1,  $\blacksquare$  = CHC extract from colony 2 and  $\blacksquare$  = CHC extract from colony 3. Ants responded significantly different to test stimulus and control (*Wald's*  $\chi^2=58.34$ ,  $P < 0.0001$ ). Response of ants to the extract of nestmate and those of non-nestmate was also differed significantly (*Wald's*  $\chi^2=101.24$ ,  $df=6$ ,  $P < 0.0001$ ). Same letters on bars represent means that are not significantly different.

Fig. 2. Total ion chromatograms for the cuticular hydrocarbons of 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 list of identified compounds).

FIG. 3 Proportions  $\pm$  SE of the different groups of hydrocarbons ( $\square$  = *n*-Alkanes,  $\blacksquare$  = Alkenes and  $\blacksquare$  = Methyl branched alkanes) in the cuticular hydrocarbon profiles of *P. analis* ants from colony 1, 2 and colony 3, error bars represent mean proportions  $\pm$ SE.

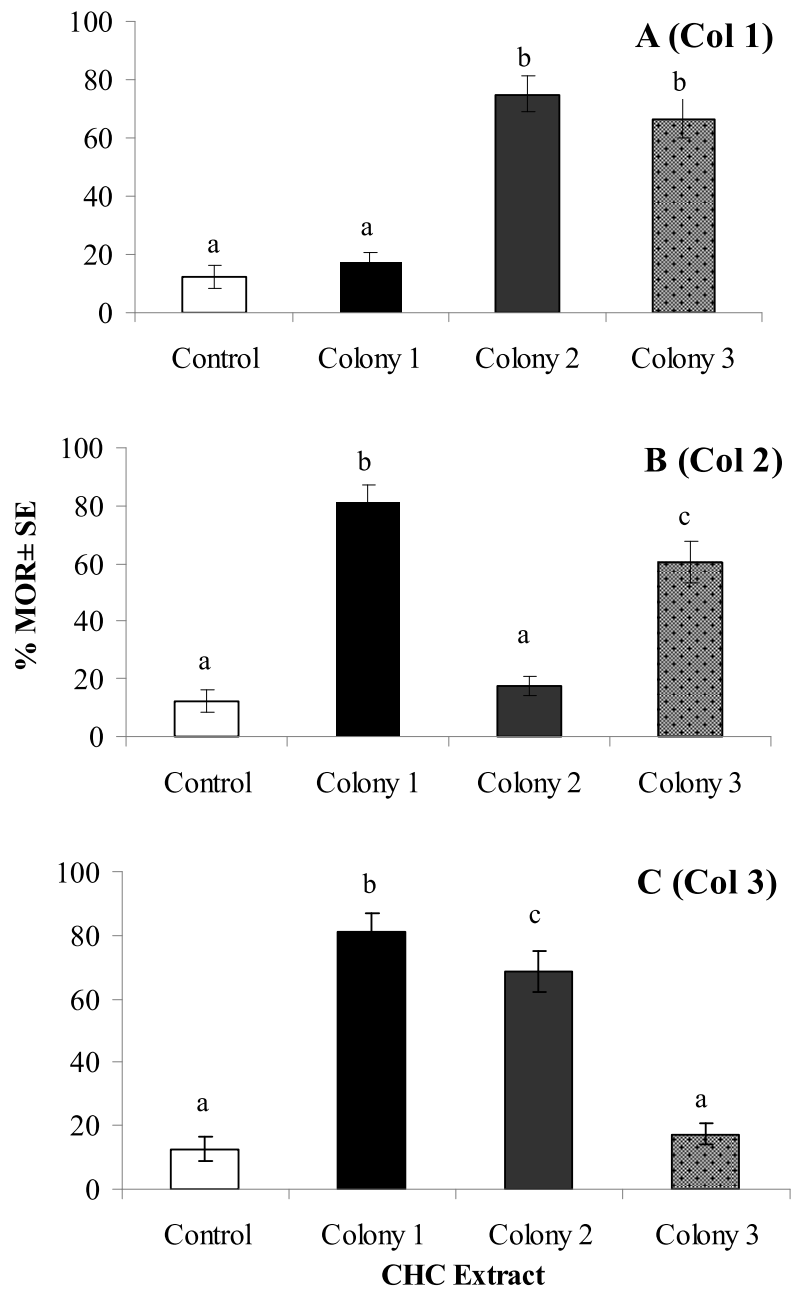
FIG. 4 Discriminant function analysis of ants from the three colonies of *P. analis* based on relative proportions of 17 cuticular hydrocarbons determined in stepwise fashion. Colony 1 (filled triangles), Colony 2 (circles) and Colony 3 (squares). Circles around individual points are arbitrarily drawn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

TABLE 1 Table 1. Compounds identified from the cuticular hydrocarbon profiles of *Pachycondyla analis*, along with retention indices and diagnostic ions. Peak numbers corresponds to labelled peaks in Fig. 2.

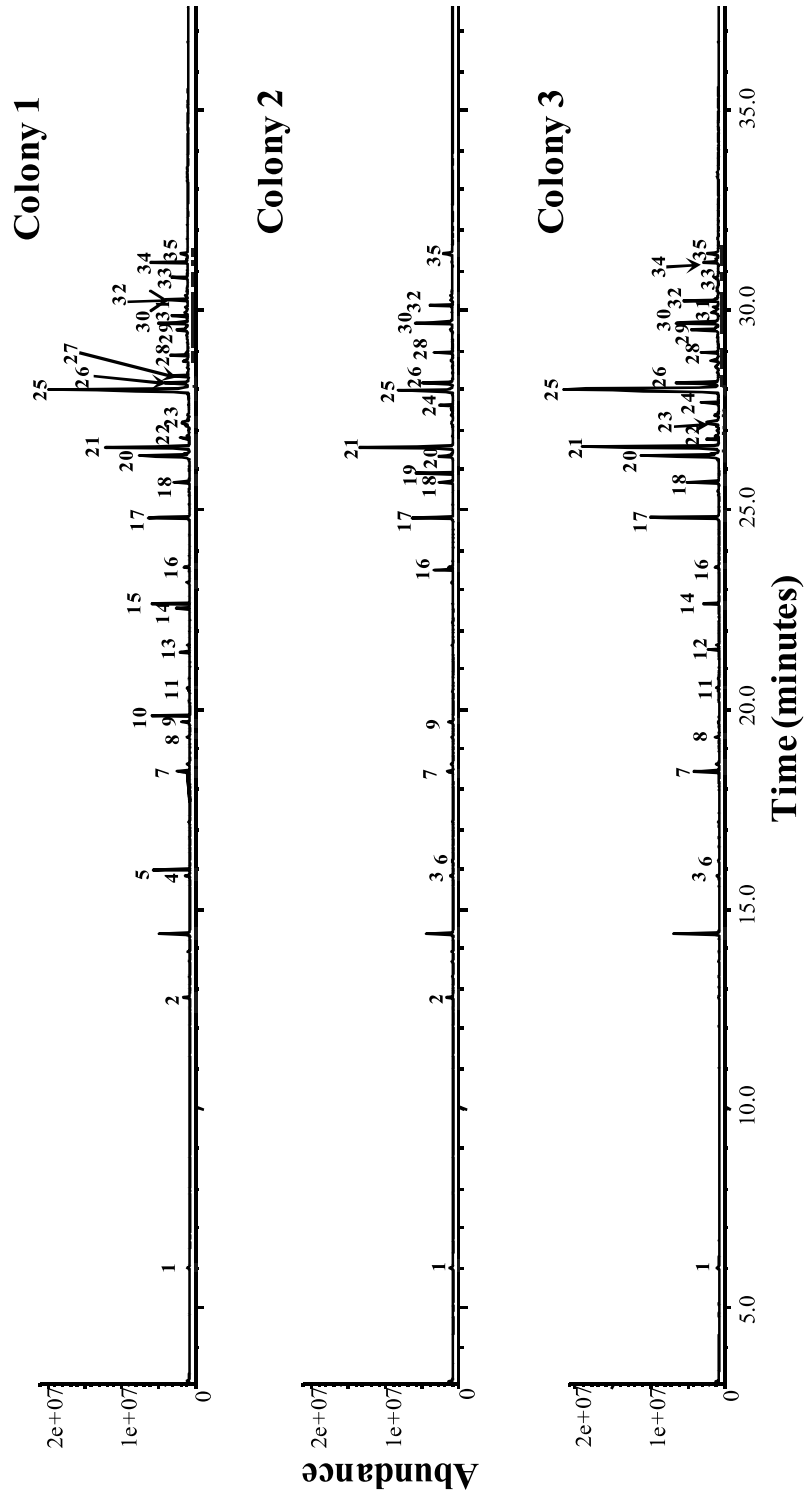
<b>Peak No</b>	<b>Compound</b>	<b>Retention index</b>	<b>Diagnostic EI-MS ions(m/z)</b>
1	<i>n</i> -Octane	800	114
2	<i>n</i> -Undecane	1100	156
3	5-Methylundecane	1154	43, 57, 71, 85, 99, 112
4	3-Methylundecane	1169	43, 57, 71, 85, 99, 112, 141, 170
5	3,8-Dimethyldecane	1063	57, 71, 85, 99, 113, 141, 155, 170
6	<i>n</i> -Tridecane	1300	184
7	<i>n</i> -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	<i>n</i> -Octadecane	1800	254
14	9-Nonadecene	1875	43, 55, 69, 83, 97, 111, 125, 139, 153, 167, 238, 266
15	<i>n</i> -Nonadecane	1900	268
16	<i>n</i> -Eicosane	2000	282
17	<i>n</i> -Heneicosane	2100	296
18	1-Docosene	2195	43, 57, 69, 83, 97, 111, 125, 280, 308
19	<i>n</i> -Docosane	2200	310
20	( <i>Z</i> )-9-Tricosene	2270	43, 55, 69, 83, 97, 111, 125, 139, 153, 223, 237, 294, 322
21	<i>n</i> -Tricosane	2300	324
22	Unidentified		
23	1-Tetracosene	2396	43, 57, 69, 85, 97, 113, 309, 338
24	<i>n</i> -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	<i>n</i> -Pentacosane	2500	352
28	( <i>Z</i> )-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	<i>n</i> -Hexacosane	2600	366
31	<i>n</i> -Heptacosane	2700	380
32	<i>n</i> -Octacosane	2800	394
33	Squalene	2663	41, 55, 69, 81, 95, 109, 121, 136, 148, 341, 367, 410
34	<i>n</i> -Nonacosane	2900	408
35	<i>n</i> -Hentriacontane	3100	436



**FIG. 1**



**FIG. 2**



**Fig. 3**

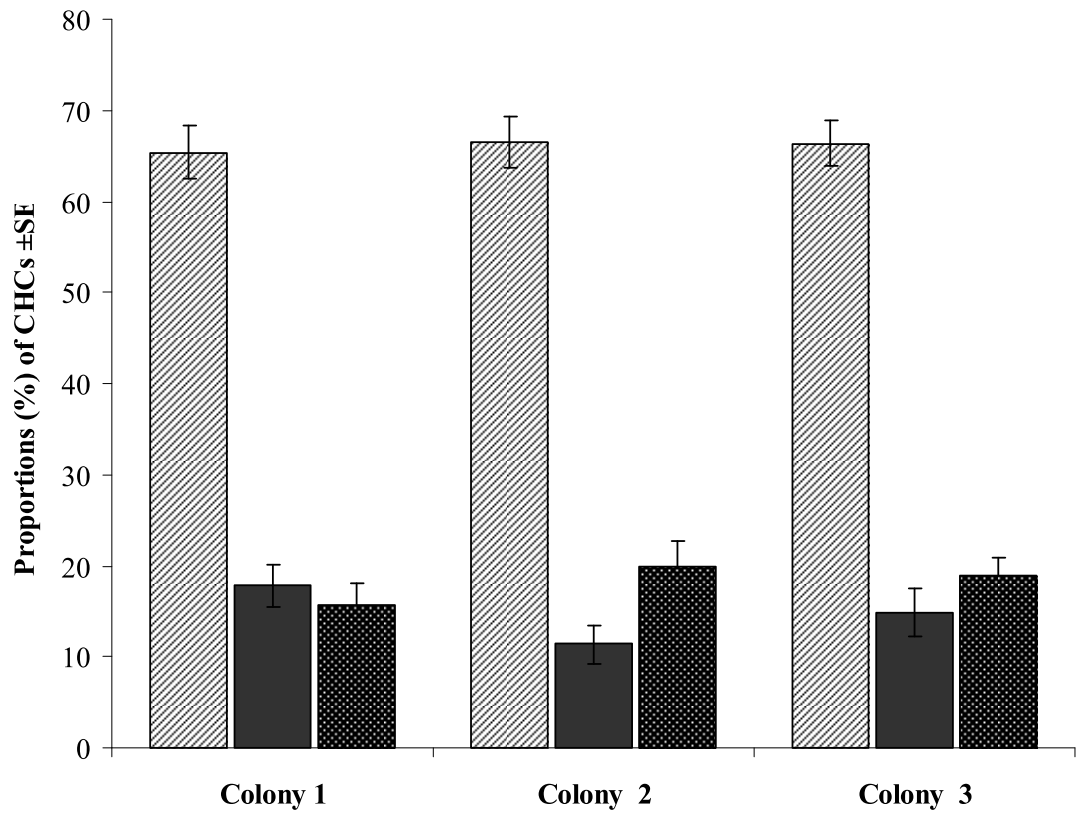


FIG. 4

