Cuticular Hydrocarbons Discriminate Distinct Colonies of *Melipona marginata* (Hymenoptera, Apinae, Meliponini)

by

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ABSTRACT

The aim of the present study was to characterize the variation of the chemical profiles among workers in different colonies of the stingless bee *Melipona marginata*. We used gas chromatography and mass spectrometry (CG-MS) and multivariate analysis of the bees' chemical from three colonies of two localities in southeast Brazil. The results showed that cuticular hydrocarbon profiles clearly separated distinct colonies. We show here the importance of using the chemical analyses for characterization of colony membership, in addition of the traditional techniques of diversity analyses.

Keywords: stingless bees, cuticular hydrocarbons, colonial signature, gas chromatography-mass spectrometry.

INTRODUCTION

Stingless bees are widely distributed in different Neotropical biomes (Sakagami 1982). Currently, morphometric and genetic analysis are used to study population structure and geographical variation between races, or even between populations of *Apis mellifera* (Francoy *et al.* 2006; Mendes *et al.* 2007). In addition, studies on mitochondrial DNA polymorphism (Torres *et al.* 2009) and cuticular hydrocarbons (Francisco *et al.* 2008) have been used to identify differences between species.

The epicuticle of all insects consists of a thin layer of lipids (Hadley 1985). These compounds are basically hydrocarbons whose function is to create a protective barrier against water loss, microorganisms and chemicals (Blomquist & Dillwith 1985; Lockey 1988; Nelson & Blonquist 1995). Hydrocarbons are always present in the cuticle of insects and can comprise up to 90 per cent of the surface lipids (Blomquist & Dillwith 1985; Hadley

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1985; Lockey 1988).

Cuticular hydrocarbons have been evolved as communication cues and play an exceptionally important role in the life of social insects (Howard & Blomquist 1982; Breed & Bennett 1987; Blomquist *et al.* 1998; Singer *et al.* 1998; Sledge *et al.* 2001; Howard & Blomquist 2005; Le Conte & Hefetz 2008; Provoust *et al.* 2008). Howard and Blomquist (2005) considered this secondary function of hydrocarbons as a reason for cuticular compound diversity found among insect groups.

Gamboa *et al.* (1996) classified the scents present in the recognition of body surface of social insects according to their origin in environmental and hereditary odors. The environmental odors would be those obtained by individuals from ambient conditions such as food and supplies brought to the nest, and the hereditary odors would be related to inherited genetic factors and maternal non-genetic factors. Thus, cuticular chemical profiles of individuals in colonies in different conditions can diverge under the influence of the environment, or remain distinct when the endogenous odors prevail.

Some bioassays have shown that surface hydrocarbons act as semiochemical cues and they are important tools for recognition systems for social insects (Provost *et al.* 2008). These compounds have been shown as the main compounds involved in the process of colony communication in several social insects as colony specificity, age, caste or sex of individuals (Blomquist *et al.* 1998; Monnin & Peters 1999; Sledge *et al.* 2001; Abdalla *et al.* 2003; Provost *et al.* 2008; Nunes *et al.* 2009; Tannure-Nascimento *et al.* 2009; Ferreira-Caliman *et al.* 2010).

Cuticular hydrocarbons have been used increasingly as a chemotaxonomic character for species recognition and also, in conjunction with discriminate analysis, for population differentiation (revised by Bagneres & Wicker-Thomas 2010). The technique was most widely used for species recognition in Diptera (Carlson 1988; Nelson *et al.* 1988; Kruger & Pappas 1989; Hoppe *et al.* 1990; Kruger *et al.* 1991; Anyanwu *et al.* 1994; Byrne *et al.* 1995; Bernier et at. 1998), in Hymenoptera (Fröhlich *et al.* 2000; Dall'Aglio-Holvorcem *et al.* 2009; Francisco *et al.* 2008; Sunamura *et al.* 2009) and in Isoptera (Haverty *et al* 1991; Klochkov *et al.* 2005).

Dapporto *et al.* (2004) showed that cuticular hydrocarbon patterns of *Polistes dominulus* are consistent with similarities among northern Tyrrhenian islands, as reported in previous biogeographic studies. In stingless bees, there are relatively few studies that investigate the chemical intra and inter-specific diversity. In this study we showed that is possible to differentiate bees according to their colonial profile.

MATERIAL AND METHODS

Study species and colonies

Melipona marginata is a Brazilian species of stingless bees and is distributed throughout the states of São Paulo, Paraná, Santa Catarina and Bahia (Silveira *et al.* 2002). The species is also known locally as manduri, manduri-menos, minduri, gurupu do miúdo and taipeira (Nogueira-Neto 1970).

Colonies of *Melipona marginata* Peletiere obtained from Cunha (two colonies) and Itanhaém (one colony) (fig. 1) were placed in wooden boxes and kept at the meliponary of Universidade de São Paulo, Ribeirão Preto. Because cuticular hydrocarbon composition can vary with tasks perfomred (Ferreira-Caliman *et al.* 2010) we observed the activity of individuals and in order to collect individuals belonging to different functional roles, we collected 10 newly emerged workers, 10 nursing workers and 10 foragers of

Fig.1. São Paulo State and the cities of Cunha and Itanhaém.

each colony.

Chemical analyses

Individual bees were subjected to a superficial extraction with hexane (1ml per individual, 1 minute). After eliminating the solvent under N_2 flow, the apolar extract was suspended in 50 µl of hexane and 1µl injected in a combined gas chromatography-mass spectrometer (GC-MS: SHIMADZU, model QP2010). Separation was achieved on a DB-5MS column 30m and the gas carrier was helium at 1.0 ml min^{-1}. The oven temperature was initially set to 150°C, and ramped up 3°C min^{-1} until it reached 280°C. Analyses were performed in splitless mode. The mass spectra were obtained by 70 eV ionization.

The data were analyzed with GCMS solutions for Windows (Shimadzu Corporation) and the chemical compounds were identified based on their mass spectra by comparison with Wiley Library data and with a standard solution of different synthetic hydrocarbons.

Statistical analyses

Principal component analysis (PCA) was used to define the main compound peaks to be compared. Following this, a stepwise discriminant function analysis was used to observe if combinations of variables could be useful in the predicting group. In this method, variables are successively added to the model based on the higher *F* to enter values, adding no more variables when the *F*-ratio is no longer significant. Wilks' λ values were used to verify the individual contribution of each variable to the model. The Wilks' λ statistic for the overall discrimination is computed as the ratio of the determinant of the within-groups variance/covariance matrix to the determinant of the total variance/covariance matrix. When this value is close to 1.0, then the residual is high and the variable is not a good discriminator, while a value closer to 0 means that the residual is low and the variable is a good discriminator (Rao 1973).

To avoid errors in compositional sample data, the area under each peak was transformed according to the following formula: $Z = \ln[A_p/g(A_p)]$, where A_p is the area of the peak and $g(A_p)$ is the geometric mean peak area (Aitchison

1986). The statistical analyses were performed using the software Statistic 7.0 for Windows (Statsoft, inc).

RESULTS

Table 1: Mean (%) and standard deviation of the relative concentrations of cuticular hydrocarbons in the workers of *Melipona marginata*. RT= Retention Time (min). N=30 for each colony.

RT	Compound	Cunha 1	Cunha 2	Itanhaém
18.193	Heneicosane	0.97 ± 0.28	0.71 ± 0.50	-
23.500	Tricosene	2.26 ± 0.98	-	-
23.908	Tricosane	15.19 ± 5.91	18.31 ± 6.03	10.97 ± 3.42
26.577	Tetracosane	0.66 ± 0.31	0.61 ± 0.34	0.57 ± 0.24
28.329	Pentacosene – 1	1.27 ± 0.48	1.27 ± 0.58	0.70 ± 0.50
28.459	Pentacosene – 2	4.25 ± 2.72	3.04 ± 1.99	2.02 ± 0.48
29.207	Pentacosane	20.26 ± 6.19	22.10 ± 6.46	19.16 ± 6.86
31.017	Hexacosene	-	0.46 ± 0.12	0.30 ± 0.08
31.711	Hexacosane	0.37 ± 0.07	0.40 ± 0.09	0.63 ± 0.20
33.377	Heptacosene – 1	0.87 ± 0.56	4.36 ± 3.69	1.16 ± 0.68
33.524	Heptacosene – 2	10.23 ± 13.67	12.66 ± 12.48	18.33 ± 10.54
33.720	Heptacosene – 3	2.60 ± 1.47	1.46 ± 1.05	5.98 ± 6.59
34.163	Heptacosane	7.17 ± 2.51	9.50 ± 2.48	8.83 ± 6.46
36.465	Octacosane	0.59 ± 0.18	0.64 ± 0.40	0.78 ± 0.46
37.597	Nonacosadiene – 1	0.61 ± 0.44	4.75 ± 2.04	1.48 ± 0.97
37.734	Nonacosadiene – 2	0.85 ± 0.64	5.70 ± 1.76	0.99 ± 0.48
38.110	Nonacosene – 1	6.44 ± 7.78	7.58 ± 7.21	11.07 ± 6.62
38.243	Nonacosene – 2	0.65 ± 0.49	2.59 ± 1.79	6.97 ± 2.16
38.380	Nonacosene – 3	11.35 ± 3.22	0.66 ± 0.16	-
38.500	Nonacosene – 4	-	0.69 ± 0.22	-
38.815	Nonacosane	2.05 ± 1.01	3.25 ± 1.93	2.02 ± 0.78
41.943	Hentriacontadiene – 1	1.88 ± 2.25	4.61 ± 4.40	2.84 ± 1.11
42.091	Hentriacontadiene – 2	0.75 ± 0.27	4.00 ± 2.96	2.20 ± 0.62
42.239	Hentriacontene – 1	4.30 ± 2.84	2.77 ± 1.98	0.90 ± 0.66
42.442	Hentriacontene – 2	1.61 ± 1.21	-	2.62 ± 2.47
42.547	Hentriacontene – 3	0.75 ± 0.39	-	5.30 ± 2.03
42.675	Hentriacontene – 4	3.77 ± 1.17	2.40 ± 0.69	2.14 ± 1.19
43.179	Hentriacontane	1.49 ± 0.89	1.43 ± 0.34	0.81 ± 0.29

Analysis of the worker cuticular waxes identified 28 hydrocarbons (Table 1). These compounds consisted of a mixture of alkanes, alkenes and alkadienes from C21 to C31.

Generally, the alkanes were predominant at shorter chain lengths (C23, C25 and C27), while alkenes and alkadienes were progressively more abundant with longer chains (C29 and C31) (Table 1).

These worker bees showed a complex mixture of isomers differing in the position of the double bonds. Due to the difficulty of identifying the position of these double bonds, the compounds were ranked in order according to their retention time.

Few hydrocarbons were present only in one or two colonies. In general, bees from three colonies showed a similar profile of cuticular hydrocarbons, regardless of source population (Table 1). Nonacosene-4 was found in just one colony (Cunha-2). Compounds with most representative concentrations (above 1%) of the three colonies were: Tricosane, Pentacosene-2, Pentacosane, Heptacosene-2, Heptacosene-3, Heptacosane, Nonacosene-1, Nonacosane, Hentriacontadiene-2 and Hentriacontene-4. On the other hand, the individu-

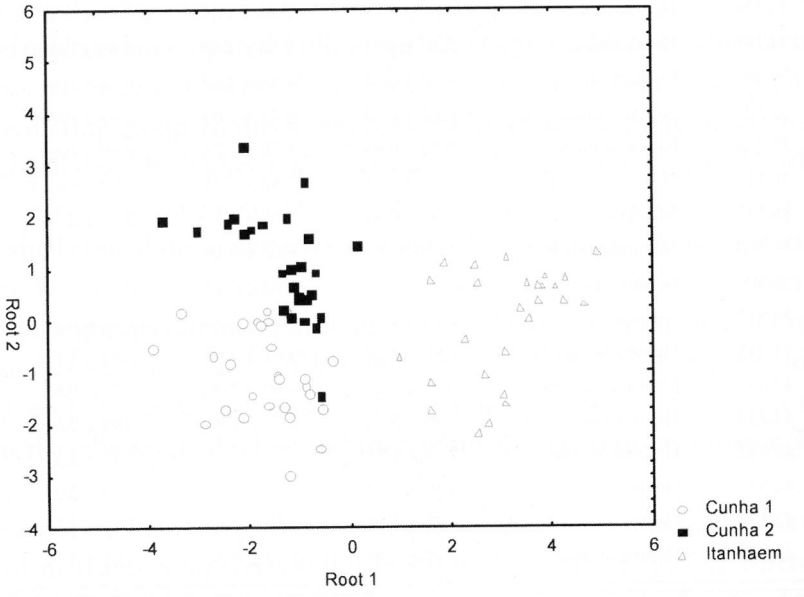

Fig. 2. Discriminant analysis using cuticular compounds of *M. marginata* workers from three different sites Cunha-SP (two colonies) and Itanhaém-SP (one colony). N= 30 individuals per colony.

als sampled from the colony from Itanhaém showed a certain similarity between those from the colonies of Cunha. Hentriacontene-2 and Hentriacontene-3 were shared by bees from the colony from Cunha-1 and Itanhaém, and Hexacosene was the only hydrocarbon shared by individuals from two colonies of Cunha-2 and Itanhaém.

The compounds with a significant presence in the three colonies of *M. marginata* were Tricosane, Pentacosane, Heptacosene – 2, Heptacosane and Nonacosene – 1. The compounds that appears only in Cunha colonies were Heneicosane, Tricosene (Cunha 1) Nonacosene – 3 and Nonacosene – 4 (Cunha 2).

For the discriminant analyses we used nine of the 29 original compounds: Tricosane, Pentacosene-2, Pentacosane, Heptacosene-2, Heptacosene-3, Heptacosane, Nonacosene-2, Nonacosane and Hentriacontene-1. The results showed that cuticular hydrocarbon profiles of individuals of colonies from Cunha were more similar to each other than profiles found in the colony from Itanhaém. Stepwise discriminant analyses of the main compounds significantly separated the individuals according to their origin (Global Model: Wilks'$\lambda = 0.078$; $F_{16.142} = 22.88$; $p < 0.001$) and the main important hydrocarbons for this separation were tricosane, pentacosene - 2, pentacosane, heptacosene - 1, heptacosene – 2, heptacosane, nonacosene - 2, nonacosane and hentriacontene - 1.

Although four eigenvalues of the correlation matrix were higher than 1 (cumulative variation = 91.37%), two factors explained the variation between the predicted groups. Factor 1 of the principal component analysis described 39.94% of the variation among groups, while factor 2 described 22.03% of total variance. The classification matrix based on the discriminant analysis separated 100% of workers from Itanhaém, 88.89% and 96.29% of workers from Cunha 1 and Cunha 2, respectively. The plot for discriminant analysis showed that the cuticular profile of Itanhaém workers was completely segregated from the other two worker groups (Fig. 2). This result was supported by the Mahalanobis distance, which confirmed a higher chemical similarity

Table 2: Result of discriminant analyses. N=30 individuals for each colony.

Worker Groups	Mahalanobis Distance	F-values (df = 9.7)	p- level
Cunha 1 x Cunha 2	4.98	6.7	< 0.001
Cunha 1 x Itanhaém	30.75	41.40	< 0.001
Cunha 2 x Itanhaém	24.97	33.62	< 0.001

between workers from Cunha colonies than these individuals to Itanhaém workers (Table 2).

DISCUSSION

Our analyses of worker surface hydrocarbons of *Melipona marginata* from two colonies from Cunha and one from Itanhaém has shown that all workers from the one area are distinguishable from those from the other area. The hydrocarbons of workers analyzed consisted of a mixture of alkanes, alkenes and alkadienes from C21 to C31. Most of the same hydrocarbons were found in the cuticles of the workers from both regions, but the respective proportions of several components differed greatly and a few hydrocarbons found in bees from one region were absent in those isolated from the other region. These data are consistent with studies by Espelie *et al.* (1990) and Francisco *et al.* (2008) for the species *Rhopalicus pulchripennis* and *Plebeia remota*, respectively.

The workers from Itanhaém produced a well defined cluster (100% correct allocation) while 11.11% (colony 1) and 3.71% (colony 2) of individuals from Cunha were not allocated within their predicted groups (Figure 2). These differences may be the basis for olfactory recognition of the population origin and can be a result of different genetic lineages, as reported in stingless bees (Francisco *et al.* 2008), ants (Sunamura *et al.* 2009; Dall'Aglio-Holvorcem *et al.* 2009) and in honey bees (Arnold *et al.* 1996).

Unlike the studies made by Francisco *et al.* (2008), our study used stingless bees collected in different localities, but kept in a common place. Thus, the environment cannot be considered as the main factor responsible for the differentiation of cuticular chemical profiles of the distinct populations. It is possible that the cuticular hydrocarbon composition of bees reflects genetic differences within the two geographic groups.

Several studies have shown that cuticular hydrocarbon mixtures can be used to discriminate insect taxa. Previous studies suggest that cuticular hydrocarbons can be use as an additional tool for taxonomic classification of species of hymenoptera (Espelie *et al.* 1990; Dapporto *et al.* 2004; Francisco *et al.* 2008). Our study also showed that hydrocarbon profiles appear to be very useful to identify colonies from distinct populations in stingless bees.

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