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SPECIES RECOGNITION FROM POSTPHARYNGEAL GLAND CONTENTS OF ANTS OF THE *Cataglyphis bicolor* GROUP

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Abstract—The *Cataglyphis bicolor* group of species of desert-dwelling ants, difficult to identify from morphological features alone, can be readily recognized by the contents of their postpharyngeal glands. Analysis by linked gas chromatography–mass spectrometry of glands from colonies identified only by code numbers showed in all samples straight and branched-chain alkanes and linear alkenes. *C. viaticus*, *C. bicolor*, and *C. savignyi*, the three species most difficult to distinguish morphologically, each contained distinctly different patterns of hydrocarbons, as illustrated by cluster analysis. The 16 most abundant hydrocarbons in the whole group of samples were selected and plotted as windrose diagrams. The differences in the windroses have more visual impact than gas chromatograms of the same data. The only case where there was any similarity was that between *C. bicolor* and *C. diehlii*, and even there the resemblance was not close. *C. bombycinus* is a sympatric species but is recognized as not belonging to the *bicolor* group by its different mandibular gland substances. It also was easily distinguished by its postpharyngeal gland contents from the other species.

Key Words—Formicinae, ant, exocrine secretion, hydrocarbons, species specificity, species diagnosis, postpharyngeal gland.

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INTRODUCTION

The work of Wehner (1994) on navigation in desert-dwelling *Cataglyphis* ants kindled our interest in this group and the possible use of semiochemicals to aid in distinguishing the species. There are over 100 described taxa of *Cataglyphis*, almost all living in desert or semidesert habitats, but it is very difficult to identify species from morphological characters alone. Santschi (1929) left a tangle of species and subspecific names for *Cataglyphis* from his years of work in Tunisia. Much of Wehner's work has been carried out on "*Cataglyphis bicolor*," but during the course of the work it became clear that either this species varied in appearance from place to place or it was really a group of species. The work of Agosti (1990) clarified the picture considerably, and our preliminary chemotaxonomic studies showed that identification of exocrine secretions could aid the process (Keegans et al., 1992). We have further shown that the *Cataglyphis bicolor* group shares a mandibular gland secretion that consists essentially of one compound, (*S*)-2-methyl-1-hexanol (Agosti et al., 1996), but as yet of unknown function.

The function of the postpharyngeal gland of ants also remains unknown in spite of various speculations. Some of us have shown that the postpharyngeal glands contain the same mixture of hydrocarbons as is found on the cuticle of at least a number of ant species selected at random (Bagnères and Morgan, 1991). Subsequently Hefetz's group has, through several publications, studied the relation between the cuticular and postpharyngeal hydrocarbons (Hefetz et al., 1996). There is much accumulated evidence indicating that the cuticular hydrocarbons are used in nestmate or species recognition in many insect classes (Singer and Espelie, 1996; Bonavita-Cougourdan et al., 1997; Doi et al., 1997; Heifetz et al., 1997) and it is currently thought that the hydrocarbons of the postpharyngeal gland play some part in this recognition.

In the present study, we have taken a chemotaxonomic approach to see if the postpharyngeal gland substances were helpful in defining species within the *C. bicolor* group. At the same time we have explored the use of diagrammatic representation to make differences and similarities in multicomponent mixtures readily apparent. Cluster analysis shows the distinct separation of species. We find that windrose diagrams are very helpful for this group. They can, at a glance, distinguish species. Windrose diagrams, first used to show the relative strength and frequency of winds at a given locality, are compact and visually striking.

METHODS AND MATERIALS

The ants were collected at sites in Tunisia and Egypt by D. Agosti during 1992 [for location see Wehner et al. (1994)]. The nests used for this study were also used in a study of Dufour glands (Gokçen et al., unpublished). The nests

were brought live to Zürich where the postpharngeal glands were dissected out as described by Morgan (1990) and sealed in glass capillaries for transport to Keele. There they were analyzed by linked gas chromatography–mass spectrometry (GC-MS), avoiding the use of solvent and injecting the glands directly into the gas chromatograph by the method of Morgan and Wadhams (1972). Ten individuals from each of 12 nests were analyzed but only five and seven individuals were available from the two nests identified as **gbom** and **kbom**, respectively.

The GC-MS was performed with a Hewlett-Packard 5890 gas chromatograph linked to a 5970B Mass Selective Detector, a quadrupole mass spectrometer using 70 eV ionization, and set to monitor m/z 35–450. The system was controlled by an HP Series 300 computer with HP 59970C ChemStation software. A fused silica capillary column [12 m × 0.32 m × 0.25 mm film thickness of 5% phenyl–95% methylsiloxane (non-polar) phase; SGE, Milton Keynes, UK] was used for chromatography. A surface-deactivated, fused silica retention gap (1 m × 0.2 mm ID) at the injection end was used to protect the column, with a similar piece of deactivated fused silica capillary (5 m) between the column and the mass spectrometer to ensure the column was operating under a positive pressure of helium, which was used as carrier gas at 1 ml/min. The samples were heated in the injector at 250°C for 3 min before crushing the glass capillary.

The column was initially at 100°C for 2 min, then heated at 8°C/min to 280°C, and finally held at that temperature to give a total run time of 40 min. The injector was operated splitless for the injection and 30 sec after.

Identification of the compounds was made chiefly by comparison of retention time and fragmentation pattern with a series of straight-chain hydrocarbons from C_{10} to C_{30} and with the aid of several mass spectral libraries. The presence and position of methyl branching in hydrocarbons was identified from characteristic mass spectral fragmentation. The positions of double bonds in alkenes were not determined. For quantitative analysis, peak areas for each component in the chromatogram were determined by computer integration, and the percentage of each substance in the gland was calculated. The average percentage composition was calculated for each colony together with the sample standard deviation (SD) for each of the compounds, and then the same was done for each group of colonies identified by the same code.

For cluster analysis the program Systat 6.0 (SPSS Inc., Chicago Illinois, USA) was used. Mean hydrocarbon composition for each species was standardized and the standardized data submitted to hierarchical cluster analysis using Euclidian distances.

The 16 most abundant compounds in all colonies and species were selected from the average values of these compounds for each species, and windrose diagrams were constructed for these 16 as they occurred in each colony. The mean amount of each substance in each colony was plotted as the area of the segment of the circle for Figures 1–3.

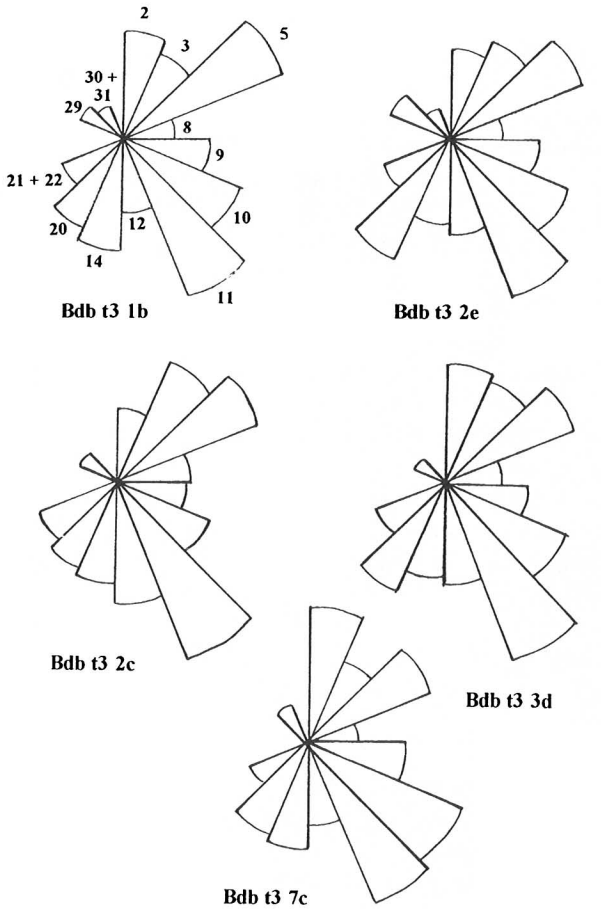

FIG. 1. Windrose diagrams of the mean amounts of the principal hydrocarbon constituents of the postpharyngeal glands of five colonies of ants identified as **bdb** (*Cataglyphis bicolor*). The names of the numbered components are given in bold in Table 1. The designation t3 represents transect 3 of Tunisia, and 1b, 1c, etc., indicate the place of collection and the colony. For locations, see map in Agosti et al. (1995).

RESULTS

The study was carried out blind. Colonies of the *C. bicolor* group were collected at various sites in Tunisia, identified only by a code [e.g., t3 indicates the transect (see Wehner et al., 1994; Agosti et al., 1996); 1–10, the collection point on the transect, and a–d, the colony collected at that point, and three letters

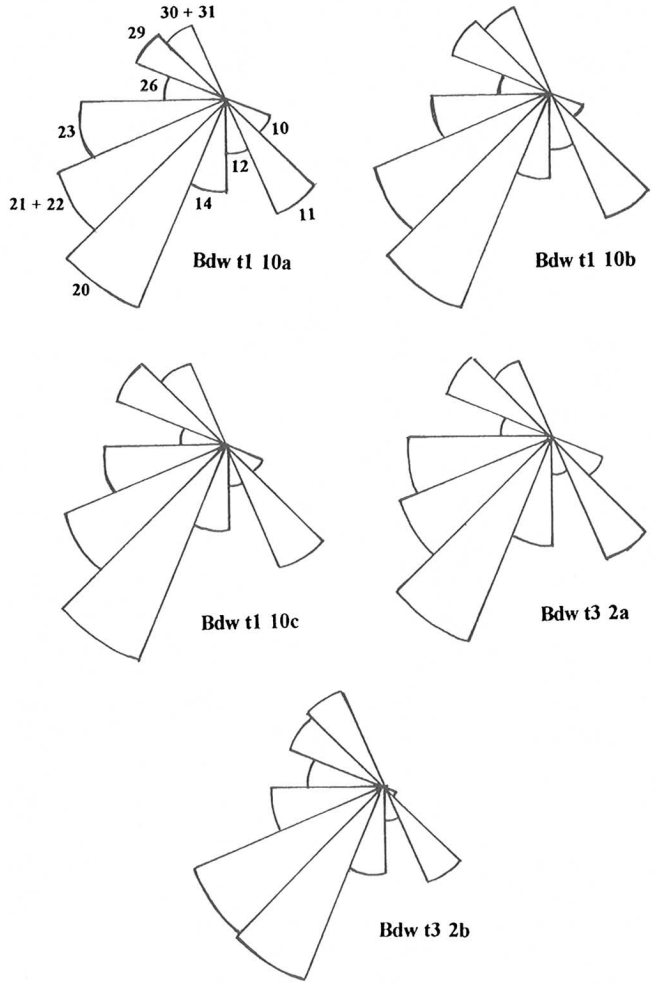

FIG. 2. Windroses for the mean amounts of the principal hydrocarbons in the postpharyngeal glands of five colonies of Tunisian ants identified as **bdw** (*Cataglyphis savignyi*). The location description is as in Figure 1.

which referred to some morphological character, Table 1]. The code names were only united with the new species names (*bicolor*, *diehlii*, *savignyi*, *viaticus*, and *bombycinus*) derived from morphological examination of the male genitalia, after the analyses were finished, the colonies grouped by type and reported. A total of 14 colonies were included in the study.

There were 33 hydrocarbon peaks recognized in the study, not all of them

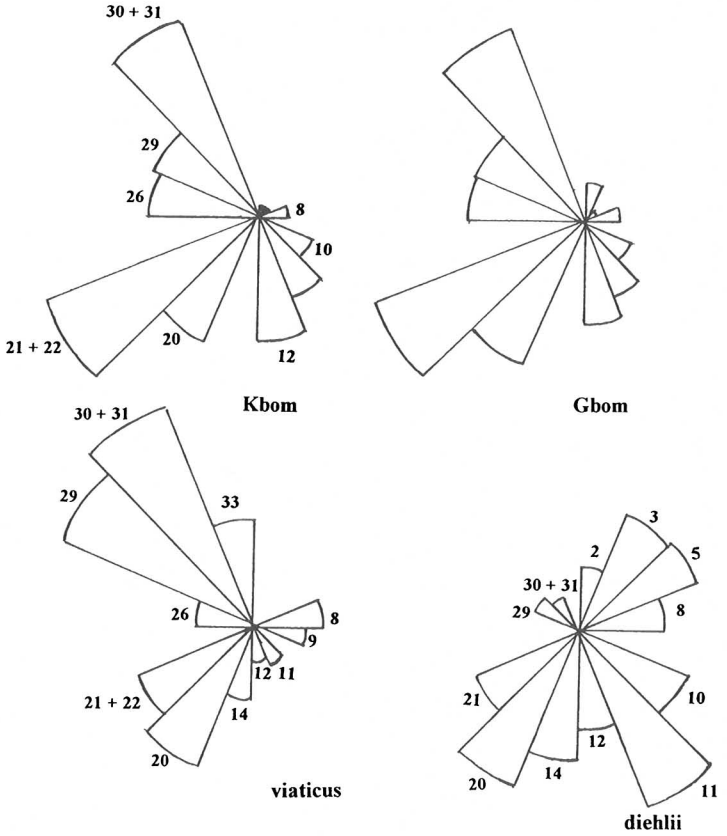

FIG. 3. Windroses for the mean amounts of the principal hydrocarbons of the postpharyngeal glands of two colonies of *Cataglyphis bombycinus* collected at Kebili (**kbom**) and Gafsa (**gbom**) in Tunisia, one colony of *Cataglyphis viaticus* (**bb**) from northern Tunisia and one of *Cataglyphis diehlii* (**diehl**) from southern Tunisia.

detected in any one sample. Some peaks consisted of more than one component (e.g., 9-methylheptacosane, 11-methylheptacosane, and 13-methylheptacosane eluted together as a single peak). Where there is a methyl branch near the middle of an alkyl chain the chromatographic column used is unable to separate the isomers. In the case of 7- and 9-methylnonacosane and 11,17-dimethylnonacosane, the peaks were sufficiently separated to integrate separately in some samples, but in others they were not, so these compounds have all been put together for representation on the windroses. In a few cases of branched alkanes that gave very weak mass spectra, it was not possible to determine the position of the methyl branch, so they are referred to as x-methylalkane. The closest resemblance in

postpharyngeal gland contents encountered was between **bdb** (*C. bicolor*) and **diehl**, but even here the differences were quite clear. *C. diehlii* lacked pentacosene (peak 1) and heptacosene (peak 9) and had much more of peak 20 and 21. The latter was absent from *C. bicolor*. It will be shown later that these two are also clearly separated by their Dufour gland contents (Gokçen et al., unpublished).

When all compounds had been identified, the 16 most abundant peaks from all the species were selected and the amounts of each of those peaks for a colony were plotted as windroses. The 16 compounds selected are shown in bold in Table 1 and are plotted on the windroses starting at 12 o'clock and moving clockwise around the diagram. The mean percentage composition of the secretion for each species is given in Table 1, while the mean percentage of the 16 selected compounds for each colony is plotted in Figures 1–3. The five colonies labelled **bdb** all fitted together with the same pattern (Figure 1) with a mixture of 9-, 11-, and 13-methylheptacosane (peak 11) as the largest component. The five samples of **bdw** (Figure 2) also had the same appearance when plotted as a windrose, with the mixture of 11- and 13-methylnonacosane (peak 20) as the largest component. The two samples called **kbom** and **gbom** were very similar to each other with components 21 + 22 and 30 + 31 clearly dominant. The remaining samples called **bb**, with peaks 29 and 30 + 31 very large, and **diehl**, with 11 and 20 largest, were all different (Figure 3).

DISCUSSION

In the northern part of Tunisia are found the *C. bicolor* group ants described here as **bb**, later defined as *C. viaticus*; then as one moves southward, there is a mixture of **bb** and **bdb**, now recognized as *C. bicolor*. Further south again, as the climate becomes drier, one finds only **bdb** or *C. bicolor*, and then a little further on a mixture of that and **bdw**, now defined as *C. savignyi*, which then continues southward to the Schotts (see Figure 7 in Wehner et al., 1994). Samples of **bdb** from each of the three areas in which it occurs (i.e., with **bb**, alone, and with **bdw**) have been analyzed. Chemically, **bdb** is still the same species in all these regions (see Figure 1). Included in the study were two colonies of the smaller species, *C. bombycinus*, not belonging to the *bicolor* group. These samples collected near Gafsa and Kebili and labeled **gbom** and **kbom** were included for comparison. They have already been eliminated from the *C. bicolor* group by their different mandibular gland secretion (Agosti et al., 1996; Gokçen et al., unpublished). Nevertheless they give clearly distinct patterns from the others, with peaks 21 + 22 and 30 + 31 most prominent (see Figure 3). Another species, *C. diehlii* belongs to the *bicolor* group but is significantly smaller than those labeled **bb**, **bdb** and **bdw**. *C. bicolor* and *C. savignyi*, which are more difficult

TABLE 1. AVERAGE PERCENTAGE COMPOSITION AND SAMPLE STANDARD DEVIATION (SD) FROM ALL SPECIMENS OF POSTPHARYNGEAL GLANDS FOR EACH SPECIES OF *Cataglyphis* ANT IN THIS STUDY

Peak number	Compound	*bicolor* **bdb** (N = 50)		*savignyi* **bdw** (N = 50)		*bombycinus* **bom** (N = 12)		*viaticus* **bb** (N = 10)		*diehlii* **diehl** (N = 10)	
		%	SD	%	SD	%	SD	%	SD	%	SD
1	Pentacosene	2.70	0.95								
2	**Pentacosane**	6.14	3.14			0.49	0.4			2.3	1.8
3	**11-Methylpentacosane**	6.21	1.87			T				8.4	3.7
	13-Methylpentacosane										
4	5-Methylpentacosane	2.30	0.67			T				1.2	0.7
5	**3-Methylpentacosane**	11.76	2.50			T		2.6	1.9	9.0	3.0
6	Hexacosane	3.88	0.80							2.4	1.4
7	x-Methylhexacosane	3.45	0.89							3.5	1.5
8	**4-Methylhexacosane**	1.86	0.64			0.51	0.4	1.4	1.0	4.3	1.3
9	**Heptacosene**	4.25	1.13								
10	**Heptacosane**	8.61	4.13	0.97	0.85	1.62	1.2			7.7	6.5
11	**9-Methylheptacosane**										
	11-Methylheptacosane	18.39	2.99	9.03	1.85	3.72	0.7	0.9	0.4	18.6	4.3
	13-Methylheptacosane										
12	**11,15-Dimethylheptacosane**	5.12	2.17	1.18	0.77	7.21	1.6	0.6	0.3	5.4	1.4
13	9,13-Dimethylheptacosane			1.23	0.40						
14	**3-Methylheptacosane**	5.57	1.57	4.57	1.35			2.9	1.2	8.7	3.0

15	Octacosane	2.69	1.16	1.28	0.63	T				1.0	0.8
16	13-Methyloctacosane	2.63	0.78	2.68	0.45	2.22	0.4	T		3.0	1.5
	16-Methyloctacosane										
17	4-Methyloctacosane	1.99	0.64	1.74	0.33	3.93	0.6	0.8	0.4	0.7	0.6
18	Nonacosene	0.93	0.45	2.59	0.52						
19	Nonacosane	0.94	0.64	2.29	1.92	2.09	1.5	1.2	1.0	0.7	0.6
20	**11-Methylnonacosane**										
	13-Methylnonacosane	6.20	1.84	26.39	3.33	11.38	2.1	12.5	2.6	15.0	5.6
	15-Methylnonacosane										
21	**7-Methylnonacosane**			18.25	3.84	5.50	0.9	8.7	1.9	6.8	4.7
	9-Methylnonacosane										
22	**11,17-Dimethylnonacosane**	2.62	0.78			21.76	1.7				
23	**3,9-Dimethylnonacosane**			8.87	3.05						
24	Triacontane			1.93	0.66						
25	Unknown hydrocarbon	1.13	0.54	2.09	0.44	2.83	0.6	3.0	0.3	0.9	0.7
26	**12-Methyltriacontane**			1.89	0.75	6.49	1.5	1.9	1.0		
27	**Hentriacontene**			2.47	1.30						
28	Unknown hydrocarbon			1.07	0.70						
29	**11-Methylhentriacontane**										
	13-Methylhentriacontane	1.27	0.60	5.87	1.30	7.04	0.8	24.4	1.1	1.3	1.1
30	**13,17-Dimethylhentriacontane**					23.60	2.8	30.2	6.8		
31	**5-Methylhentriacontane**	0.56	0.28	4.36	1.22					0.8	0.8
32	9-Methyltritriacontane							3.7	1.4		
	11-Methyltritriacontane										
33	**11,17-Dimethyltritriacontane**							6.5	1.7		

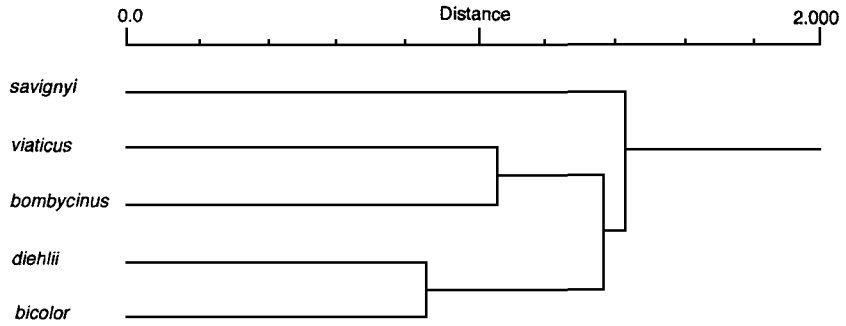

FIG. 4. Cluster analysis by the single-linkage method, using all the hydrocarbons of the postpharyngeal glands of the species of *Cataglyphis* studied here. Distance is the Euclidean distance.

to separate by their Dufour glands (Gokçen et al., unpublished), have distinctly different postpharyngeal gland contents (see Figures 1 and 2).

Cluster analysis was also performed on the total hydrocarbons of each species using the single linkage method (nearest neighbor), and separation of the species was clear (Figure 4). For example, *C. bicolor* and *C. diehlii* (which are morphologically distinct) are the most similar pair, with a separation distance of 0.863, *C. viaticus* and *C. bombycinus* form another pair of separation 1.096, but *C. bombycinus* is the one not belonging to the *bicolor* group. These pairs join at 1.370 and *C. savignyi* joins at 1.417. It is therefore very satisfying to find that the diagnosis of species based upon the difficult morphological criteria is supported completely by the chemical differences, and the results presented here strengthen the hypothesis that species can be defined by their chemical secretions.

Windrose diagrams, as used here, make a visual impact in pointing out these differences, in a much clearer way than a gas chromatogram does and more immediately than a table of statistical data. Twelve, 16, or 20 compounds can be conveniently plotted. We used the 16 most abundant compounds chosen from all the species, based on the mean values of these compounds for the species. We considered a statistical analysis of the data collected but found the differences between species was so clear that statistical treatment was not necessary. Similar diagrams have been used by Kaib et al. (1993) with equal success to illustrate differences in cuticular hydrocarbons of *Harpagoxenus sublaevis* and their slaves *Leptothorax* ants.

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