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Comparison of cuticular hydrocarbon profiles of fire ants *Solenopsis richteri* from the same colony, using capillary column gas chromatography with pattern recognition

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ABSTRACT

The cuticular hydrocarbon profiles of workers of similar age and sub-caste of the black fire ant, *Solenopsis richteri* Forel, were examined by capillary gas chromatography and computer-based pattern recognition procedures. The data show statistically significant and reproducible differences in the hydrocarbon profiles of workers of similar age and sub-caste from the same colony.

INTRODUCTION

It is well known that cuticular hydrocarbons in insects play a role in chemical communication[1–4]. The literature on cuticular hydrocarbons is extensive. It includes the chemical characteristics of materials extracted from the genus *Solenopsis* (fire ants). The cuticular hydrocarbons of these ants are chemically characterized by the presence of alkanes, primarily homologous series of *n*-alkanes and internally branched monomethyl- and dimethylalkanes[3,4]. Studies have also been conducted to correlate gas chromatographic (GC) patterns of the cuticular hydrocarbons to variables such as colony, caste, etc.[5–8]. The primary tools for such investigations are embodied in the science of pattern recognition. Pattern recognition is a general analysis method where data sets obtained from samples are treated by appropriate algorithms for correlation to elements of individuality or to functional group properties[9–11]. In the study of cuticular hydrocarbons, gas chromatograms from different groups of insects are compared. Similarities or differences that are not easily observable by the human eye are pointed out. This process is most efficiently done by computer. Dozens of chromatograms, each consisting of a hundred components or more, can readily be processed. After suitable data pre-treatment, the pattern recognition system looks for natural groupings or clusters. The end result of such manip-

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ulations is the observance of specific chromatographic peaks or groups of peaks that are indicative of the individual sample sets.

In a series of previous papers, we have examined cuticular hydrocarbon patterns for two of the four species of the genus *Solenopsis*[5]. Further studies on the black fire ant (*Solenopsis richteri*) showed that statistically significant differences appeared between the GC profiles of insects from different castes and those originating from separate colonies[6–8]. The variations between different colonies are generally small. Careful examination, however, shows that somewhat larger differences can be observed between neighboring colonies[7]. It has been reported recently that harvester ants (*Pogonomyrmex barbatus*) can distinguish between neighbors and strangers[12].

In classical sample preparation methods, a large number of insects are usually extracted. The nature of this batch process tends to equalize differences that may exist between smaller sub-sets or individual insects. We have introduced a sensitive procedure that permits establishment of profiles for single insects[13]. During our studies, we have noted that individual workers from the same colony produced slight but reproducible differences in their hydrocarbon profiles.

In this last of four communications, we present evidence for statistically significant variations that cannot be ascribed to experimental error. We do not know if these observations have biological significance.

EXPERIMENTAL

Sample preparation

Individual foragers from the same colony were homogenized in a 250-μl borosilicate glass Potter-Elvehjem tissue grinder (Fisher Scientific, Norcross, GA, U.S.A.) with approximately 200 μl of redistilled *n*-hexane. After the ant was thoroughly ground up, 1 μl of internal standard (containing 250 ng/μl each of docosane, *n*-C_{22}, and dotriacontane, *n*-C_{32}, in *n*-hexane) was added to the liquid. The supernatant was transferred to a 1-ml vial. The residue was washed several times with small quantities of fresh *n*-hexane, and the washings added to the vial. Finally, the extract was made up to 200 μl with *n*-hexane (due to evaporation during the workup). A total of eight foragers of approximately the same size were treated in this manner. The experiments were repeated using foragers from five different colonies of the black fire ant.

Control

The sampling and workup procedure was also performed without the use of a forager: approximately 200 μl of *n*-hexane were placed in the tissue grinder and the homogenation step was performed by grinding the pestle in the solvent for a few minutes. The internal standard was added and the solution transferred, together with the washings, to a sample vial.

Gas chromatography

The extracts were examined by capillary GC using the splitless injection mode. The gas chromatograph was a Hewlett-Packard 5830A instrument, equipped with a Grob-type split/splitless injection port and a flame ionization detector. The column was a 10 m × 0.25 mm I.D. glass capillary, coated with a 0.25-μm film of SE-30. This column was produced in-house, following standard procedures[8]. Initial oven temper-

ature was 40°C. Thirty seconds after injection, the injector purge was activated and the oven heated rapidly to 85°C. Thereafter, the oven was temperature-programmed to 300°C at a rate of 8°C/min. The helium carrier gas was set at a flow-rate of approximately 1 ml/min. Five replicate injections, of 1.8 μl each, were made from each sample.

The samples were also examined by GC–mass spectrometry (MS), using a Hewlett-packard 5895A GC/MS system operated in the electron impact mode (70 eV). A 15 m × 0.32 mm I.D. fused-silica capillary column, coated with a 0.10-μm film of 5% phenylmethylpolysiloxane (DB-5, J&W Scientific, Folsum, CA, U.S.A.) was used.

Data analysis

The retention times and peak areas from the integrated GC reports were submitted to a mainframe computer (UNIVAC 1100). The ARTHUR chemometrics system package that contains several pattern recognition subroutines was used to analyze the data[14]. To avoid bias, unsupervised learning procedures were used; that is, the computer was not supplied with the category (colony source of individual foragers) to which each of the gas chromatograms belonged. Seven marker peaks (consisting of the two internal standards and five peaks from the cuticular hydrocarbons) were encoded with the data set, which was then transduced into multivariate form[5,15]. The marker peaks are necessary in order to normalize chromatograms and to correct for small shifts in retention time. The raw data consisted of 41 data vectors, each comprised of 26 features. The data were then autoscaled, to prevent bias from features having greatly differing magnitudes[14], and then submitted to two graphical display routines contained in the ARTHUR package. Autoscaled data were displayed in two-dimensional space using the non-linear mapping (NLM) procedure[9]. The autoscaled data were also transformed using the Karhunen–Loeve (KL) projection, a method based on principal components analysis[9], and then plotted using combinations of the first three principal components.

RESULTS AND DISCUSSION

It has been shown in the black fire ant that hydrocarbon profiles obtained by homogenation in solvent such as *n*-hexane are nearly identical with those obtained by topical washing with the same solvent, despite the fact that one method probes primarily the surface of the insect, whereas the other extracts both the surface and the internal body parts[8,16]. The methods differ only in the total amount of hydrocarbons that can be recovered in a given period of time. Replicate analyses of small numbers of insects produce virtually identical results. We have previously described a sensitive method for examining the profiles of single insects, using a pyroprobe to generate headspace samples[13] However, in the application described herein, the headspace procedure proved unsatisfactory, since it was not possible to obtain replicate samples from a single fire ant which were suitable to run on the pyroprobe. The most satisfactory method, therefore, was solvent extraction.

To minimize or exclude extraneous effects, care was taken to eliminate extraneous variables. It has been reported that cuticular hydrocarbons of adult insects change with age[17]. Since hydrocarbons remain relatively unchanged in older adults[18], it was decided to use foragers in this study. Foragers constitute the oldest workers in the colony.

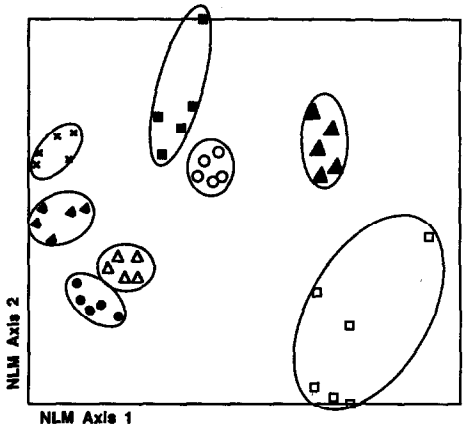

Fig. 1. Non–linear map (NLM) of the autoscaled data set obtained from hydrocarbon profiles of eight foragers, each from the same colony.

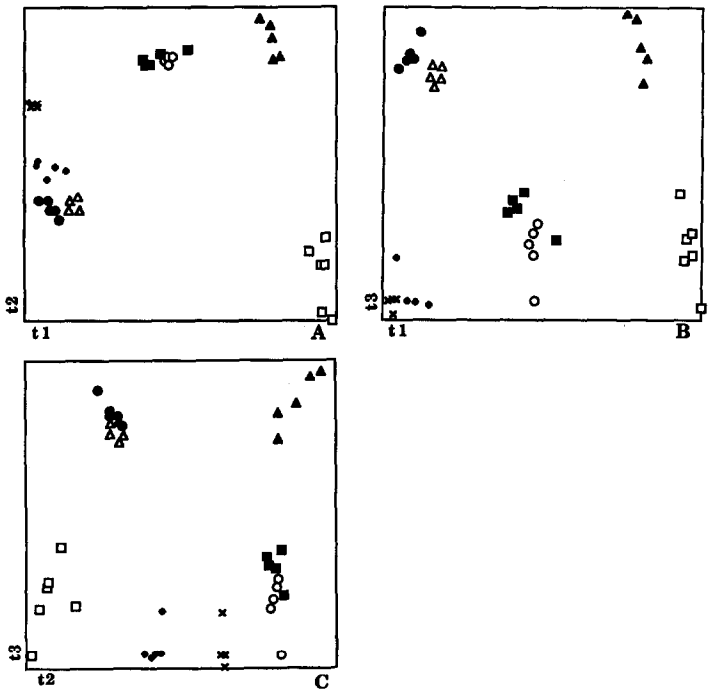

Fig. 2. Principal components (KL) plots (A–C) of the hydrocarbon profiles of eight foragers, each from the same colony. t1 = First principal component; t2 = second principal component; t3 = third principal component. The symbols are the same as in Fig. 1.

The results of these studies show statistically significant and reproducible differences in the hydrocarbon profiles of foragers from the same colony. Fig. 1 shows the NLM plot of the autoscaled data. The profiles for each of the foragers form distinct and well-defined clusters. The computer had no information concerning the different categories in the data set. The groupings observed in the NLM plot are therefore natural clusterings, which have not been enhanced by feature weighting, feature selection, or other mathematical manipulations that might lead to distortions. Only the investigator knew the categories (*i.e.*, individual foragers) to which the objects (chromatographic profiles) belonged. It is statistically significant, therefore, that these natural clusters also correspond to the various categories (individual foragers) in the data set. The cluster designated by the open squares shows a rather large spread. It is likely that the data point at the top right of the cluster is an outlier (due to an experimental error). When a sixth replicate was examined, it was located approximately at the center of the cluster, making it more likely that the aforementioned data point is an outlier.

The principal components (PC) analysis of the data set (using the KL projec-

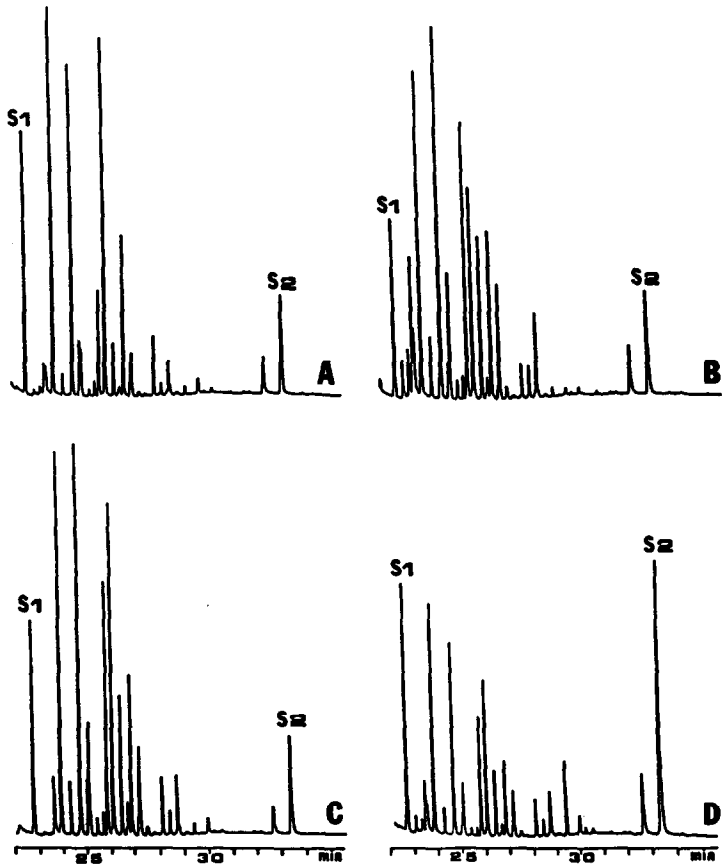

Fig. 3. Sample chromatograms of the hydrocarbon profiles of four foragers from the same colony of the black fire ant. S1 and S2 are internal standards (docosane and dotriacontane, respectively). A, B, C and D indicate the profiles of four different forgers from the same colony.

tion) also shows good correlation for each of the ant profiles, although the groupings were not as well focussed as in the NLM method. This is in accordance with previous observations[7]. Fig. 2 shows the KL plots of the autoscaled data set. Together, the first three principal components accounted for 83.7% of the explained variance in the data set. Some of the clusters in Fig. 2 show less than five objects. This is because two or more objects were "overlapping" in the two-dimensional data space, a phenomenon that sometimes occurs with linear transformation methods when projecting multi-dimensional data onto a two-dimensional space.

Sample hydrocarbon profiles of four foragers from the same colony are shown in Fig. 3. GC–MS analyses indicated that the components eluting between the two internal standards (S1 and S2) were hydrocarbons. The unusually large peak for the second internal standards (S2) relative to S1 in Fig. 3D was due to the forager possessing the same compound in its own hydrocarbon profile. Visual inspection reveals differences among the profiles of the foragers. The relative standard deviation among replicates from the same sample was less than 0.08, indicating that the reproducibility among the sample replicates was good and within expected experimented error. The observed differences in the profiles are therefore due to the samples themselves and not to sampling or instrumental errors. The control experiment was devoid of any extraneous material. When the GC profiles of foragers from several other colonies were examined, reproducible differences among the hydrocarbon profiles of foragers from the same colony were also evident.

The results of all these experiments are summarized in Table I. The chromatograms from each experiment were first normalized, to eliminate any bias caused by differences in sample size. Then the variation (relative standard deviation, R.S.D.) in the normalized peak area for each of the 26 features was compared for each sample. The resulting 26 R.S.D. values were then averaged to obtain an average relative standard deviation for each sample, which was then compared to that for other samples, as indicated in Table I. The results for category 1 of Table I indicate that the sampling procedure is highly reproducible. In category 2, replicate analyses from the homogenate extracts of individual foragers are compared. Category 3 compares the homogenate extracts of different foragers.

These studies show detectable differences in the hydrocarbon patterns of different foragers from the same nest. It is not known whether these differences have a functional significance. Individual recognition of nestmates has been reported in some ant species where workers are few in number and maintain dominance hierarchies[19–21].

TABLE I

VARIATIONS WITHIN AND BETWEEN REPLICATES FOR DIFFERENT SAMPLE CATEGORIES

Category		*Average R.S.D.*
(1)	Internal standard mixture (control) (7 replicates)	0.13
(2)	Extract of homogenate from single fire ant workers (35 replicates)	0.12
(3)	Extracts of homogenates from different fire ant workers (35 replicates)	0.42

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