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The Florida Entomologist, Vol. 71, No. 3 (Sep., 1988), pp. 333-345
Published by Florida Entomological Society

HYDROCARBONS FOR IDENTIFICATION AND PHENETIC COMPARISONS: COCKROACHES, HONEY BEES AND TSETSE FLIES

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ABSTRACT

The hydrocarbon components of Asian and German cockroaches showed consistent differences by gas chromatography (GC) that did not depend on geographic origin, sex or age, and that did reliably identify individuals of these otherwise morphologically similar species. European honey bee workers and drones showed consistent GC patterns. Race-specific similarities in GC patterns were present in Africanized workers and drones from Central and South America. Principal components analysis separated data from different races. Comb waxes reflected the genetic ancestry of the workers that produced that wax. GC data was used to construct phenetic comparisons of 26 species and subspecies of tsetse flies using dried museum and fresh specimens.

RESUMEN

Cuando se usó la cromatografía de gas (CG), los componentes de hidrocarbón de las cucarachas Asiáticas y Alemanas demostraron consistentemente diferencias que no dependían de origen geográfico, sexo o edad, y que con seguridad identificaban a individuos de esas especies que son además morfológicamente idénticas. Obreras y drones de la abeja Europea consistentemente demostraron patrones por CG. Similaridades en patrones por CG específicos de las razas, estuvieron presente en obreras Africanizadas y drones de Centro y Sur America. Análisis de componentes principales separó datos de diferentes razas. Cera del panal reflejó el antecedente genético de las obreras que producieron esa cera. Se usaron datos del CG para construir comparaciones fenéticas de 26 especies y subespecies de moscas tsetse usando muestras secas y frescas obtenidas del museo.

There are often life forms difficult to identify in the comparison of insects species that are reproductively isolated, but closely related, or of subspecies, of sibling or incipient species, or even of races and morphs. The following is a description of the use of readily obtained hydrocarbon compounds from the cuticle of closely related insects as markers for insect taxa (genera and species) and populations (races), with an excursion into systematics. It appears that synthesis of these materials is often genetically determined, and is not affected by geographic origin or host of the specimens. Thus, it is hypothesized that the hydrocarbons may reliably identify individuals of otherwise morphologically similar species. Insects possess cuticular compounds of many chemical classes for waterproofing purposes, and most of the external materials may be removed by a brief rinse of a fresh or dried specimen in a nonpolar solvent such as hexane. After isolation of the hydrocarbons, gas chromatography (GC) is employed for rapid quantitative and qualitative analysis of the hydrocarbons. The specimen is retrieved undamaged.

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MATERIALS AND METHODS

German cockroaches, *Blattella germanica* (L.) were collected in the field in Alaska, and at several locations in Florida, Alabama and Louisiana, killed by freezing, and then dried. They also were obtained from colonies in Gainesville, Florida, as were Asian cockroaches, *B. asahinai* (Mizukubo), the latter from collections made in Florida. *B. vaga* (Hebard) were obtained from a colony at Virginia Polytechnic University, Blacksburg, Virginia.

European honey bees *Apis mellifera* (L.), were collected randomly in 1983 from commercial type colonies in Florida (FL) and Georgia (GA). African materials were collected in Zimbabwe. Drones from Argentina (ARG) were collected in Argentina. Comb wax samples were empty, non-sex brood cells that were freshly constructed without an artificial foundation. Wax was obtained from suspected Africanized colonies near Bakersfield, California. Africanized comb was collected in Panama and Venezuela.

Tsetse flies were obtained from colonies located in Europe, from several museums, or collected in Africa (unpublished data).

Extraction, column chromatography and GC analyses were carried out for individual insects as previously described (Carlson & Bolten 1984). The hexane extract was passed through a short column of silica gel to separate polar lipids from the nonpolar hydrocarbon fraction. GC analysis then separated the hydrocarbons by vapor pressure, or essentially by their molecular weight. A modern 15 to 30 meter by 0.3 mm id fused silica capillary column with a nonpolar bonded phase was used that may temperature programmed up to 325° to complete each analysis in 10 to 30 min. A 1.8 m x 2 mm. packed column of 3% OV-1 was used for tsetse data. The data were best recorded using a micro computer based system for rapid data analysis. Only a small portion (1/10 to 1/50) of an insect equivalent was usually a sufficient quantity for analysis.

The relative retention time is presented as a four digit number without a decimal (Kovats Index, KI). For example, the sequence of elution for hydrocarbons of 31 carbons chain length was: alkadiene, KI 3043; alkene, KI 3065 to 3075; n-alkane, KI 3100; internally branched methyl alkane, KI 3135, 5-methyl isomer, KI 3152; 3-methyl isomer, KI 3175. All hydrocarbons were identified by GC mass spectrometry and were consistent for the range of compounds seen in insects described here. Peak ratios were calculated from GC data by dividing the integrated area of one consistently appearing peak by another. In honey bees, the intensity of the KI 2900 peak was divided by the intensity of KI 2935 to produce R0. Other ratios were R2 = 3075/3265, R3 = 3265/3743, R4 = 3265/3765. Also, the totals of three methyl-branched alkanes in honey bee drones were summed in Table 1. For waxes, R2, R3 and R4, plus the total compounds eluting after KI 3300 were summed in Table 2.

Cockroaches

Analysis by GC gave excellent results in identification problems with the three *Blattella* species found in North America. An undescribed species of flying cockroach was recently reported in Lakeland, Florida that evaded ready identification by experts. GC analysis showed immediately (Fig. 1a) that all males, females and nymphs possessed very similar patterns of hydrocarbons, with a group of large GC peaks at 29 to 30 carbons in chain length and a group of smaller peaks at 31 to 32 carbons in chain length, but with little other material. This species, later identified from males as *B. asahinai* were subsequently found in Pinellas and Hillsborough Counties. Very different patterns were found in *B. germanica* males, females and nymphs (Fig. 1b), in which a group of small GC peaks appeared at 27 to 28 carbons together with a group of large peaks at 29 to 30 carbons, with very little material at 31 to 32 carbons. Retention indeces (KI)

TABLE 1. PEAK RATIOS (R) OBTAINED FROM GC ANALYSIS OF AFRICAN, AFRICANIZED AND EUROPEAN HONEY BEE DRONES: (MEANS ±S.D.).[a]

R Values	Zimbabwe (n=9)	Argentina (n=29)	Florida (n=21)	Georgia (n=18)
Ro	0.6 (0.1)b	0.2 (±0.1)a	1.7 (±1.3)a	0.8 (±0.5)b
R2	0.2 (±0.01)a	0.4 (±0.1)a	0.7 (±0.06)c	1.1 (±0.1)b
R3	5.2 (±1.0)a	6.3 (±1.1)a	19.2 (±5.3)b	8.0 (±1.6)a
R4	3.0 (±0.2)a	3.1 (±0.4)a	18.1 (±4.4)b	20.0 (±5.1)b
% of Methyl alkanes[b]				
	18.0 (±2.3)b	25.6 (±5.6)a	n.a.	12.6 (±3.8)b

[a]SD: standard deviation
[b]KI 2735, 2935, 3135

were assigned to all prominent peaks that were internally consistent with the mass spectra of selected specimens. Patterns of GC peaks were consistent in all specimens from the Continental U.S. and the Orient (Carlson & Brenner 1988). No changes could be ascribed to location, food, or degree of resistance to pesticides. An indication of the genetic basis of these components is shown in the pattern found in a hybrid nymph of *B. asahinai* and *B. germanica* (Fig. 1c), as this nymph appeared to contain a mixture of materials from both parents. All hybrids examined to date display this intermediate pattern regardless of age or direction of crossing (unpublished data).

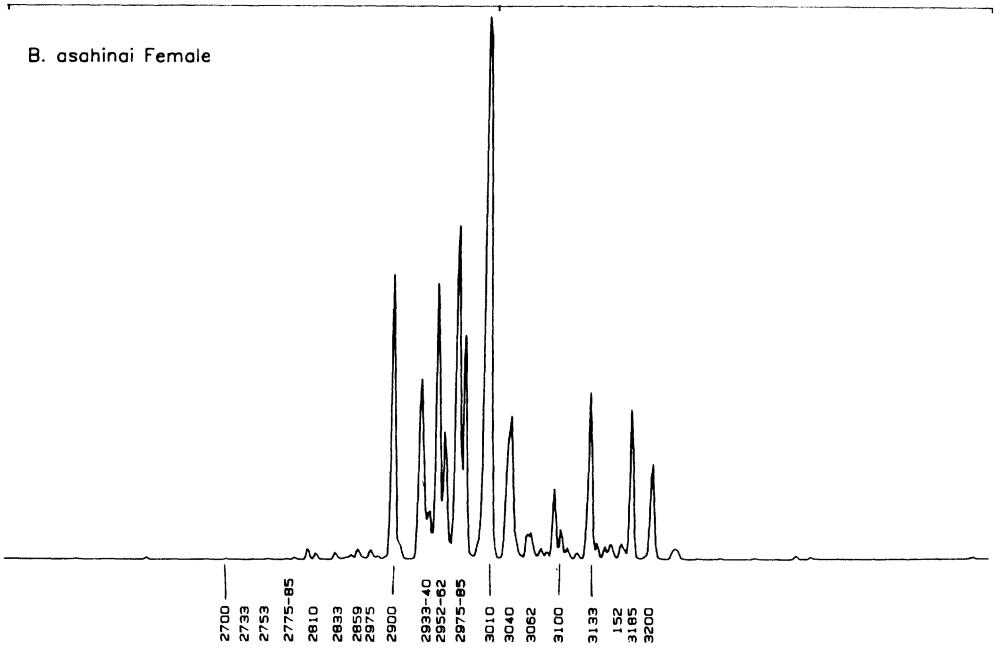
However, the hydrocarbon patterns of *B. vaga* were internally consistent, but quite unlike *B. asahinai* or *B. germanica* patterns, with methyl-branched components eluting in a wide range between standards of 32 carbons and 42 carbons, considerably after those mentioned above (Fig. 1d). This interesting variability within one genus has implications for the use of hydrocarbon data in phenetic comparison, as in the tsetse fly study mentioned below.

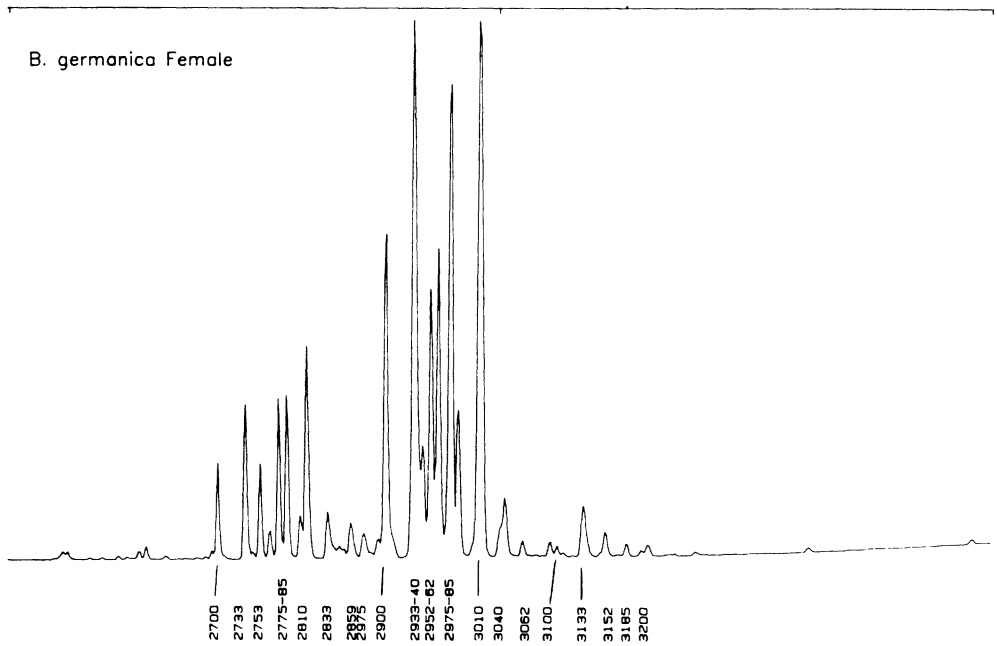
Principal components analysis was utilized to study the data from *B. germanica* and *B. asahinai*, and 12 GC peaks were selected for cluster analysis using the percent composition of each peak. The clusters were well separated. A scoring system was devised that used the 95% confidence intervals of the quantity of each of the 12 peaks for 120 specimens. All instars and adults of *B. asahinai* scored +7 to +12 with a mean of +10.5, whereas the *B. germanica* scored –7 to –12, with a mean of –10.5 (Fig. 2). Blind samples were successfully identified (40/40 = 100%) as were individual oothecae, legs, wings and cast skins by the same scoring system (25/25 = 100%, Fig. 3).

TABLE 2. PEAK RATIOS (R) OBTAINED FROM GC ANALYSIS OF AFRICAN, AFRICANIZED AND EUROPEAN/FLORIDA HONEY BEE COMB WAX: (MEANS).

R Values	Zimbabwe	Panama	Florida 1[a]	Florida 2
R2 3075/3265	0.18	0.19	0.33	0.44
R3 3265/3443	18.30	24.98	56.23	48.60
R4 3265/3465	3.65	3.30	17.04	24.30
% of total, HC larger than KI 3300				
	5.4	6.4	1.2	1.2

[a]FLA 1 and FLA 2 are from same colony.


B. asahinai Female
2700
2733
2753
2775-85
2810
2833
2859
2975
2900
2933-40
2952-62
2975-85
3010
3040
3062
3100
3133
152
3185
3200


B. germanica Female
2700
2733
2753
2775-85
2810
2833
2859
2975
2900
2933-40
2952-82
2975-85
3010
3040
3062
3100
3133
3152
3185
3200

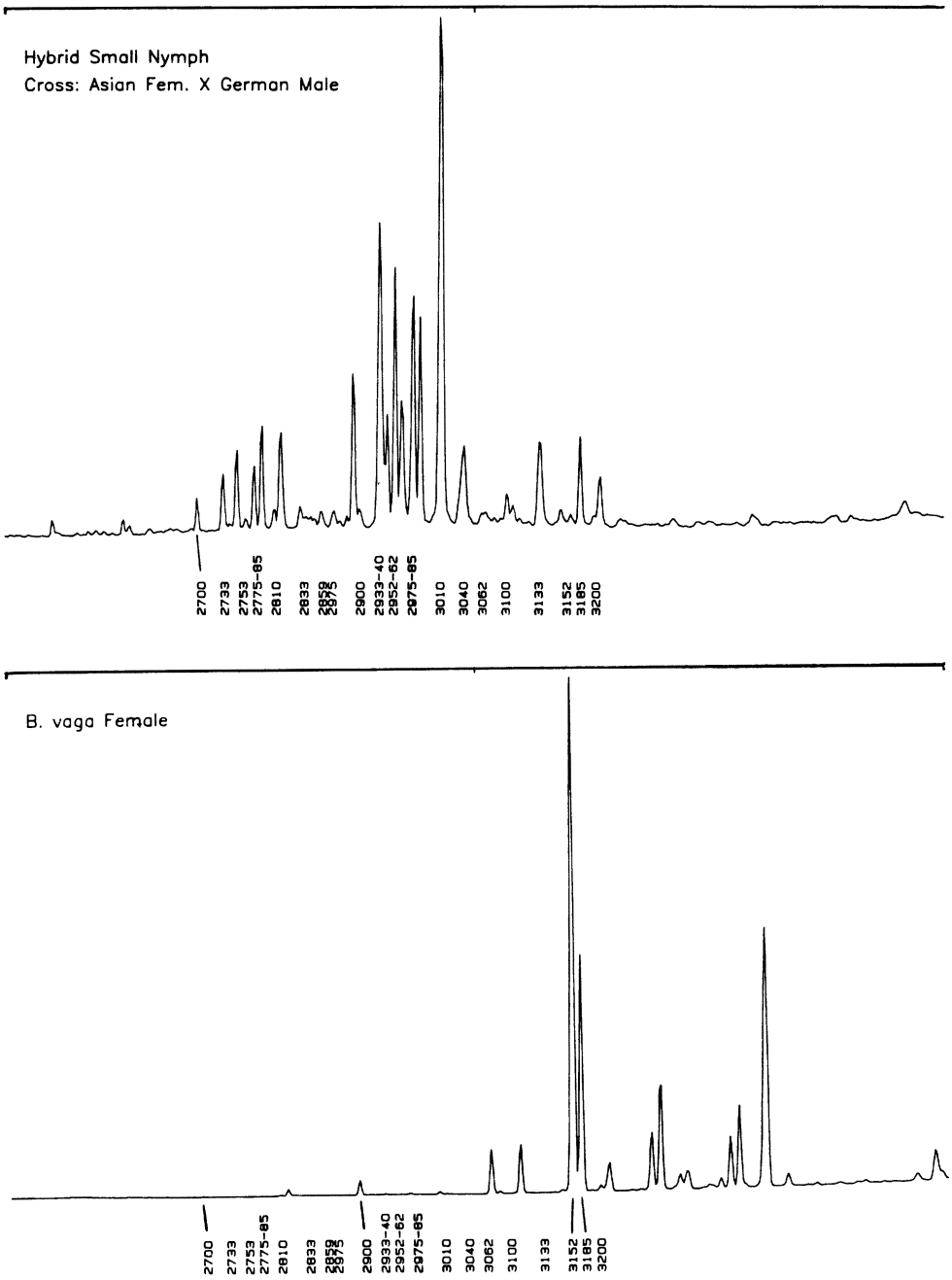


Fig. 1. GC of hydrocarbons from: a. female *Blattella asahinai*. b. female *B. germanica*. c. hybrid of *B. asahinai* and *B. germanica*. d. *B. vaga*.

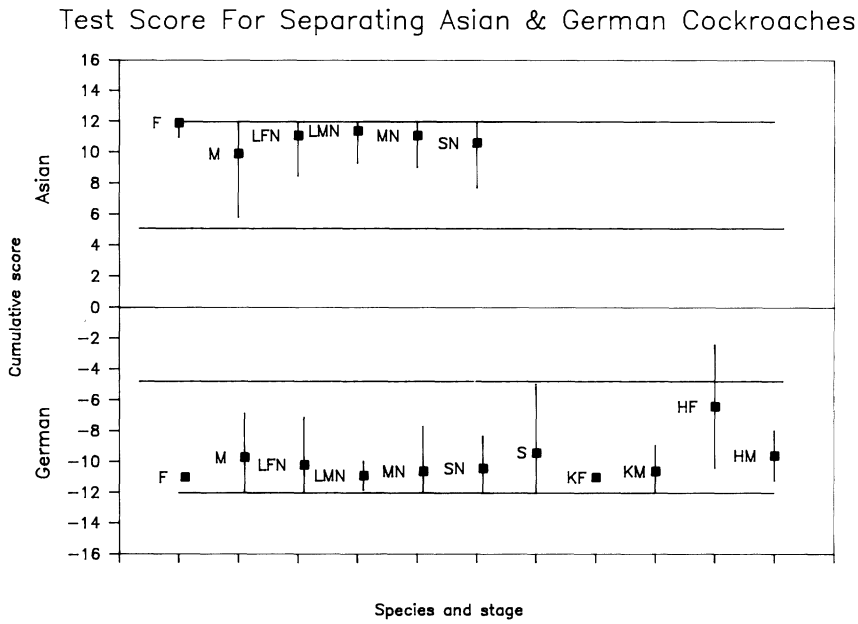


Fig. 2. Mean Cumulative Scores (x) and 99% confidence intervals of observations for various stages or strains of German (– values) and Asian cockroaches (+ values): F,f = female, M,m = male, N = nymph, LMN = large male nymph, MN = medium nymph, SN = small nymph, K = Alaska, S = wild German, H = Hydroprene treated.

Honey Bees

The synthesis of hydrocarbon compounds by the honey bee, *Apis mellifera*, is apparently under genetic control. Several series of lipids are always present within every individual of each species regardless of race. Honey bees and waxes of several races, and species including giant honey bees contained four major classes of long chain hydrocarbons of 21 to 43 carbons, alkadienes, alkenes, n-alkanes and methyl-branched alkanes (unpublished data).

Cuticular hydrocarbons of honey bee workers of Africanized (AV) and European ancestry (EU) were found to be different by GC (Carlson & Bolten 1984). The hydrocarbons included a homologous series of C35 to C43 alkenes and alkadienes that totalled 22% in AV and 1 to 3% in EU bees. Ratios between peaks allowed identification without measuring every peak (Carlson & Bolten 1984). Principal components analysis was used to map data from ten GC peaks of AF and EU bees (Fig. 4), (Lavine & Carlson 1987).

Sting shaft hydrocarbons of EU bees were analyzed and evaluated for their chemotaxonomic suitability (McDaniel et al. 1984). Examination of F1 hybrids from artificially inseminated EU queens with feral Venezuelan drone sperm resulted in workers with GC patterns that appeared to be Africanized by R2 and R4 values (unpublished data).

Honey Bee Drones

The hydrocarbon patterns determined for all drones were grossly similar as all drones had about as much methyl-branched alkanes as n-alkanes. However, both R2

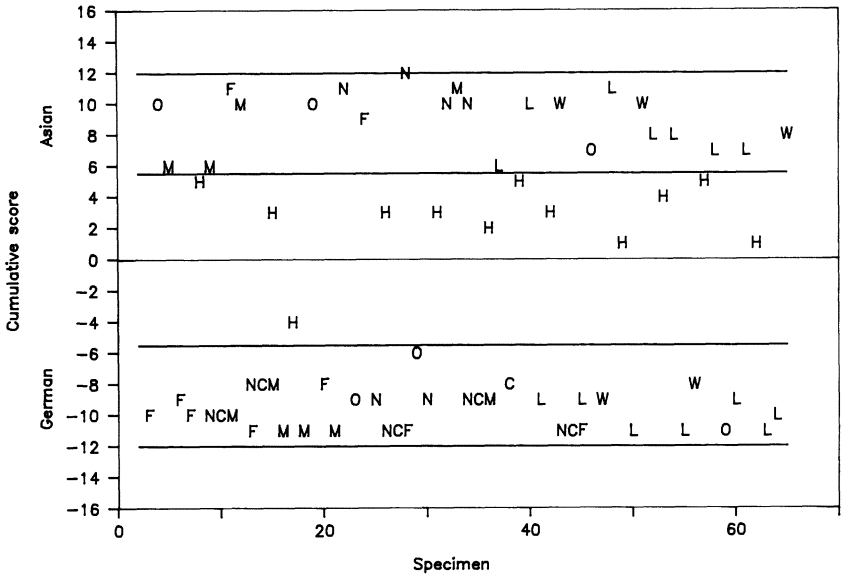


Fig 3. Plot of Cumulative Scores for 65 German (– values) and Asian (+ values) cockroach specens submitted blind. M = male, F = female, N = nymph, L = leg, C = cast skin, W = wing, H = hybrid, NC = New Caledonia, O = oothecae.

and R4 of FL (Fig. 5a) and GA European drones were significantly different from both ZIM and ARG (Fig. 5b) drone values (Table 1) ($P < 0.01$, student's t-test). No overlapping values were observed.

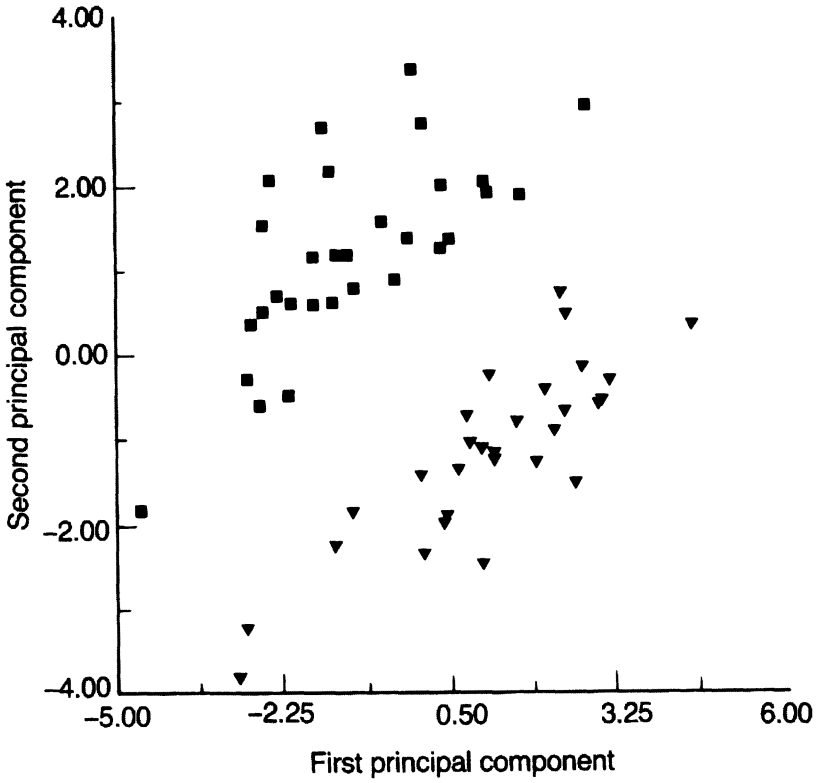
Comb waxes

The hydrocarbon (HC) composition of honey bee waxes resembled that of the bees that are presumed to have secreted that wax.

Fresh FL wax values were R2 = 0.33, R3 = 56 and R4 = 17, and the HC that eluted after KI 3300 totalled 1.2% in several samples (Table 2, Fig 6a). In contrast, fresh Panama wax (Fig. 6b) showed R2 = 0.19, R3 = 24.9, and R4 = 3.3, with the total HC that eluted after KI 3300 at 6%. Mexican and California wax patterns were similar to Florida patterns.

Differences in HCP of Africanized and European workers and drones can be evaluated by comparison of R2 and R4 values. The use of HCP for identifying drones include the same advantages as in identifying HCP of workers: dried specimens can be stored indefinitely, they are undamaged by extraction with solvent, and statistically useful numbers result from replicated GC analysis. Effects of location, genetic background, diet and age of drones are unknown, but it appears that HCP from at least two populations of European drones from the Southeastern US were alike enough to differentiated from Africanized and African drones.

Similarly, wax samples dissolved in hexane can be separated by chromatography for characterization by GC, and the collected fractions can be retained indefinitely for future reference.



A principal components representation of the pattern space defined by the 10 GC peaks for Venezuelan foragers.
The first two principal components account for 65% of the total cumulative variance. The squares represent Africanized foragers, and the inverted triangles are European foragers.

Fig. 4. Cluster analysis of data from Africanized and European honey bees.

Tsetse Flies

Unique patterns and quantities of cuticular alkanes were seen in the GC data of replicated samples of 26 species and subspecies of *Glossina* of both sexes. The distinctive patterns allowed visual assignment of most chromatograms as to sex and species in 10 species studied first (Carlson 1981). This effort was expanded to include specimens of all 26 species available, excluding only four rare forest species for which only type specimens were available (unpublished data). Early collections of flies from the British Museum (1899 to 1909) were used with results equally as good as for modern specimens, and all specimens were recovered undamaged. Patterns of hydrocarbons from old and recent specimens showed little change for individual *G. m. morsitans* males (1909 vs. 1983), *G. m. morsitans* females (1985 vs. 1907), and *G. p. palpalis females* (1977 vs.

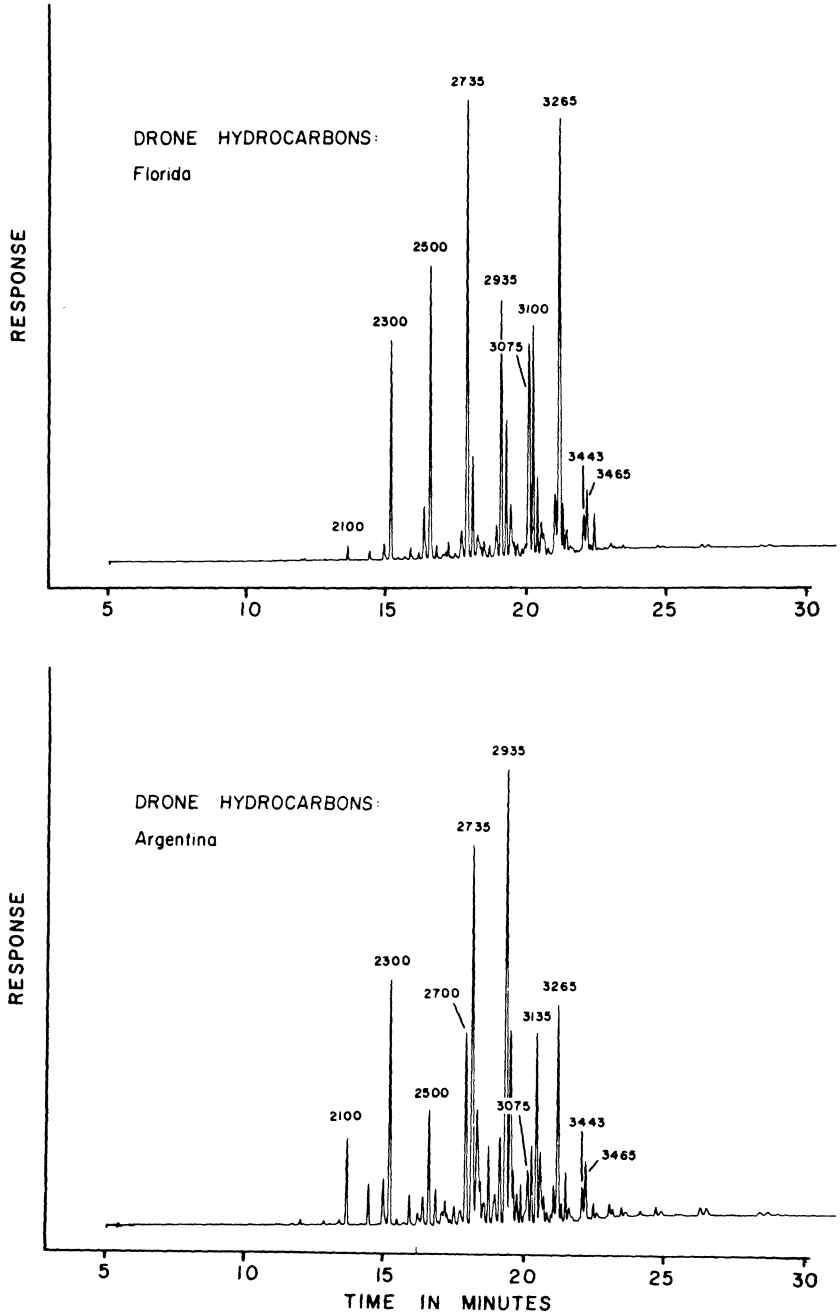


Fig. 5. GC of hydrocarbons from: a. Florida drones b. Argentina drones.

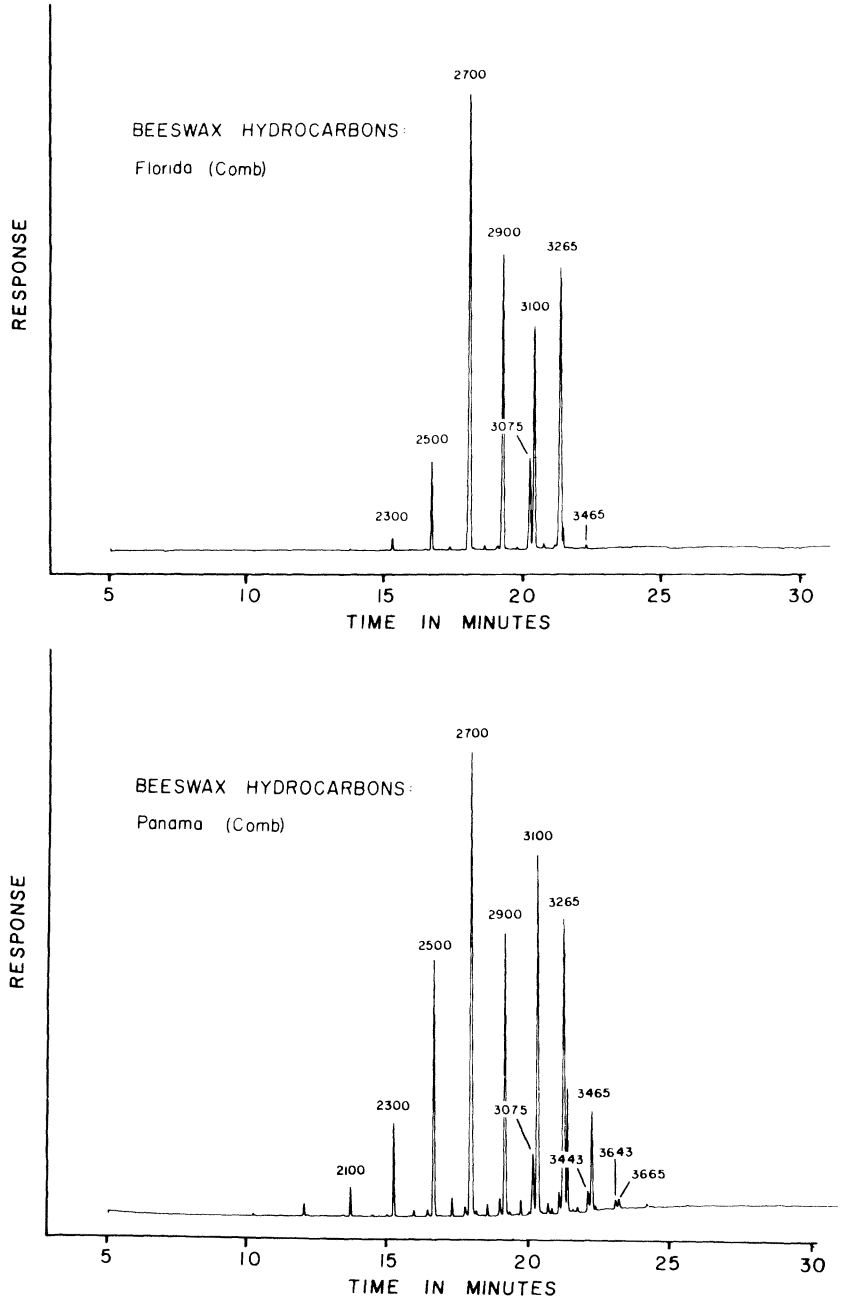


Fig. 6. GC of hydrocarbons from: a. Florida comb wax. b. Panama comb wax.

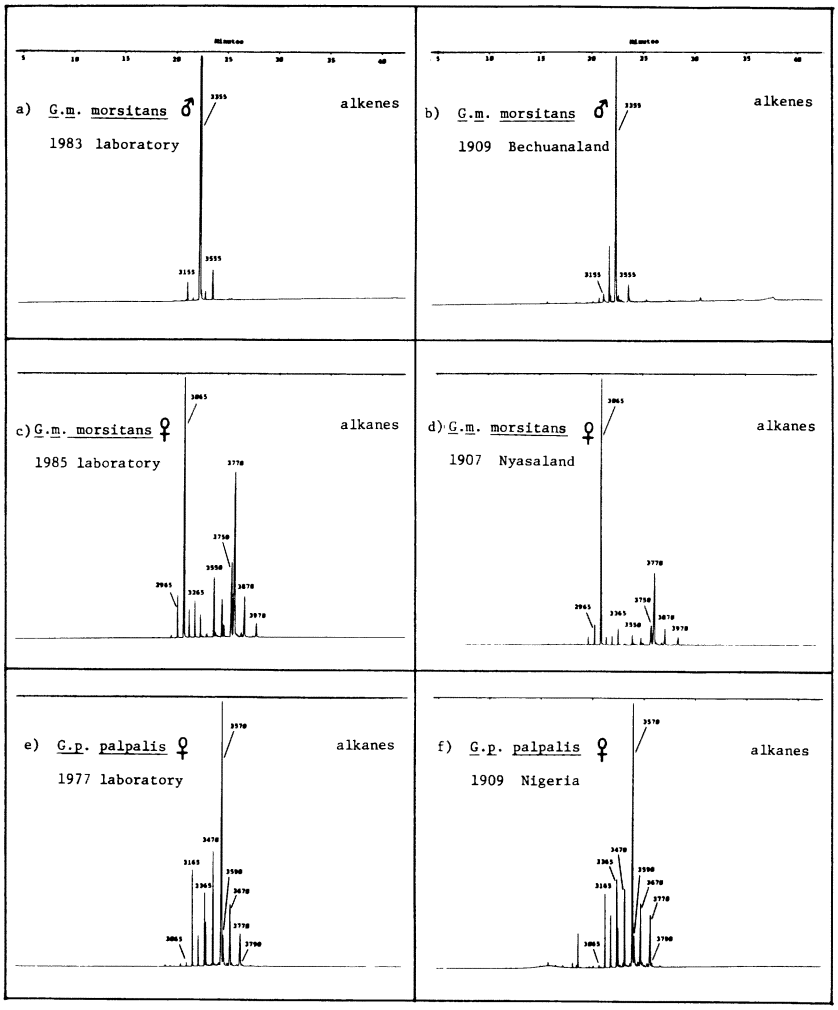


Fig. 7. GC of hydrocarbons from old museum and recently collected tsetse flies.

1909) (Fig. 7). Thus it appears that hydrocarbon patterns are highly conserved in tsetse flies and these materials, the endpoint of genetically determined biosynthetic pathways in the insect, may be utilized for phenetic comparisons by some scheme. Percent composition data was accumulated for two to 10 specimens or groups of each species using packed column GC. While recognizing that use of percent composition data for multivariate statistical treatment can introduce bias, an effort was made to get useful indicators for phenetic relationships by selection of common peaks within each group. These peaks represented most of the materials found, and only a few small peaks were not represented. Each GC peak was used as a "character"; five arbitrarily set "character states" were possible depending upon peak area; a = major (>20%), b = large (10-20%), c = intermediate (5-10%), d = small (2-5%), and e = very small, trace or no detectable peak (0-2%). In the example given here, 8 of the 11 GC peaks were the same

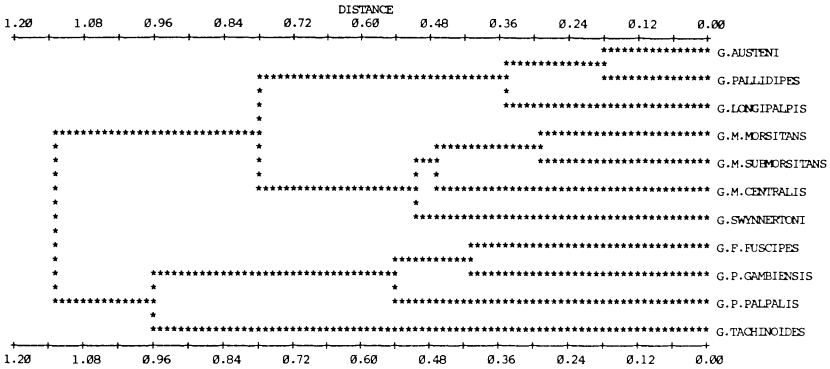


Fig. 8. Phenogram of *Glossina morsitans* and *G. palpalis* male tsetse based on UPGMA Cluster Analysis of 11 GC peaks via Nei (1978) Unbiased Genetic Distance.

for the *G. morsitans* and the *G. palpalis* group males. Phenograms for males of 11 species of these two groups based on UPGMA cluster analysis of GC peaks only were constructed using BIOSYS-1. Complete phenetic separations were found between the two groups (Fig. 8), that agreed quite well with recognized group classifications based on traditional morphological and genetic information. The oth[illegible]ies have also been evaluated by this method for a study to be published elsewh[illegible]

Conclusion

Hydrocarbon patterns obtained by GC appear to be very useful in identification in species complexes or within populations of morphologically indistinguishable insects. This is helpful particularly where traditional taxonomic methods are difficult or highly technical and time consuming as with nuclear or mitochondrial DNA. Hydrocarbon analysis can be readily performed with modern GC equipment particularly when long-lasting bonded phase capillary columns are used, and an answer obtained in less than an hour. Also, the specimens are not damaged by extraction with hexane. If further chemical separation is desirable, as to isolate and identify specific alkene and alkadiene isomers, this can be done by well established chromatographic methods for characterization by GC and GC mass spectrometry. We have found that badly damaged specimens, usually cockroaches, have been submitted for identification after a forceful collection experience. Fortunately, the cuticular components are unaffected by such crushing, and good results from GC are still to be expected.

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