# Variation in Cuticular Hydrocarbons among Strains of the *Anopheles gambiae sensu stricto* by Analysis of Cuticular Hydrocarbons Using Gas Liquid Chromatography of Larvae

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*Cuticular hydrocarbons of larvae of individual strains of the* Anopheles gambiae sensu stricto *were investigated using gas liquid chromatography. Biomedical discriminant analysis involving multivariate statistics suggests that there was clear hydrocarbon difference between the Gambian(G3), the Nigerian (16CSS and, its malathion resistant substrain, REFMA) and the Tanzanian (KWA) strains. The high degree of segregation (95%) in hydrocarbons among the four strains investigated indicates that further analysis is needed to enable understanding of hydrocarbon variation in samples of* An. gambiae *especially from areas where these populations co-exist.*

Key words: *Anopheles gambiae sensu stricto* strains - cuticular hydrocarbon analysis

The morphological similarity of the members of the *Anopheles gambiae* complex has posed a problem to identification. Species A, B, and C of the complex were originally identified by their mating incompatibility by cross-mating with known species (Davidson 1964). Since then other techniques have been applied in the differentiation of the cryptic species including (a) detection of differential banding patterns of the polytene chromosomes from the ovarian nurse cells of the semi-gravid adult females (Coluzzi & Sabatini 1967); (b) examination of the fluorescent banding sequences of mitotic sex chromosomes (Gatti et al. 1977); (c) evaluation of electrophoretic isoenzyme frequencies (Mahon et al. 1976, Miles 1978); (d) analysis of differences in cuticular hydrocarbons by chromatography (Carlson & Service 1979, 1980, Hamilton & Service 1983); and (e) using DNA characteristics (dot hybridisation technique) for distinguishing adults of *An. gambiae sensu stricto* (s.s.) (Gale & Crampton 1987) as well as (g) using ribosomal RNA gene probe to differentiate members of the *An. gambiae* complex (Collins et al. 1987).

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Differentiation of larvae and adult females of both salt and mineral water species of most individuals of the complex have made use of anatomical features of pecten, setal branching, antennal coeloconica, palpal ratios, tarsal, palpal and wing markings as well as thoracic scales (White 1974). Apart from distinct egg morphology (Ramsdale & Leport 1967), all these morphological and meristic features showed much variation both in dimension and ratios. For the freshwater species, the only reliable means of identification until recently was almost entirely on chromosome analysis. This study is the first to use hydrocarbon analysis to discriminate larval mosquitoes.

The present investigation was prompted by the need to provide further information on the differentiation of members of the *gambiae* complex and the need to ascertain if the cuticular hydrocarbon technique which had been successfully applied for the identification of *An. gambiae* s.s. (Carlson & Service 1980), chromosomal forms of *An. gambiae* s.s. and *An. arabiensis* (Phillips et al. 1987), adults of *An. culicifacies* (Milligan et al. 1986) and *An. maculipennis* (Phillips et al. 1990), strains of *An. stephensi* (Anyanwu et al. 1993), would be appropriate for separating the strains of *An. gambiae* s.s.

## MATERIALS AND METHODS

*Experimental mosquitoes* - Samples of larvae were taken from adult colonies established from collections of *An. gambiae* s.s. at the London School of Tropical Medicine. They consist of four strains viz G3- originating from The Gambia, West Africa and colonized in London since 1975; 16CSS, derived in 1974 from wild caught adults

originating from Lagos, Nigeria (West Africa); KWA, strain from Kwale, Tanzania (East Africa) colonized in London since 1975; REFMA, colony formed in London since 1979 thought to be a substrain of 16CSS resistant to malathion.

*Solvent extraction of cuticular lipid* - Cuticular lipid extraction essentially followed the technique of Phillips et al. (1988) which involved dipping each dead dry/desicated mosquito larva in 10 µl n-hexane (spectrophotometric grade) at room temperature for 1 min to give enough cuticular wax for hydrocarbon analysis without undue contamination by the insect's internal lipids; evaporating the extract to dryness, adding 2 µl of 10 ppm pentadecane($C_{15}H_{32}$) to the concentrated sample extract as a reference standard to aid quantification of the hydrocarbon components in each extract as well as ensure reproducibility from sample to sample and, after 1 min of resuspension, injecting the entire extract unto the capillary column.

*Chromatography* - Chromatographic analysis used a Perkin Elmer 8420 machine fitted with a flame ionisation detector (FID). Oven temperatures were programmed to start at 75$^{o}$C, rise at a ramp rate of 25$^{o}$C/min to 120$^{o}$C for 2 min and then rise again at 15$^{o}$C/min to 310$^{o}$C for 15 min. Helium, flowing at 6.8 ml/min through the column, was used to convey the hydrocarbon constituents to the Flame Ionisation Detector where the deflections they cause is recorded in the integrator and are reflected as peaks in the resulting chromatogram. GLC analysis was initiated when each resuspended extract was loaded directly onto the column injector using a microsyringe.

*Discriminant analysis* - The hydrocarbon peaks emerging from each sample extract were numbered by comparison to known hydrocarbon standards for case of recognition. The area under each peak on the chromatogram represented the concentration of a hydrocarbon component in the extract. The concentrations of each component were calculated by comparing the area under each peak with that of the internal standard, and these formed the data for statistical analysis.

Multivariate analysis of variance using the BMDP 7M package of Dixon (1988) was then applied to test for significant differences in the hydrocarbon quantity between the samples (Phillips et al. 1988, Anyanwu et al. 1993). To aid this hydrocarbon characterisation, the F-to-enter ratio was set at 6.0, a conservative value, which best enhances differences between groups in addition to reducing to reasonable level the number of variables needed to separate groups. This discriminant package further employs a jack-knifed ("leave-one-out-and then classify") estimator to gauge the proportion of correct classifications made by the discriminant analysis. Here each individual involved in the classification is left out each time the classification function used to assign it to a group is being computed. This serves as an empirical measure of the assessment of successful classification.

## RESULTS

Overall, 185 mosquito larvae were analyzed (52 each of 3 strains, G3, 16CSS and KWA and 29 of the REFMA strain) each giving a GC profile (Fig. 1) for further analysis. Discriminant analysis indicated that, of a profile of 80-100 peaks, variability in hydrocarbon composition between individuals of *An. gambiae* s.s. strains is dependent on peaks 7, 17, 22, 33, 34, 35, 44, 45 and 47. Visually systematic differences between individual peaks may be difficult to estimate. Nonetheless, it can be seen, for instance, that peak 7 is more pronounced in KWA and REFMA but reduced in G3 and 16CSS. Again, whereas peaks 17 and 45 are more prominent in KWA than in the others, peak 22 is smaller in REFMA than in the rest. Peak 47 is conspicuous in 16CSS and REFMA, peak 44 is diminished in all the cases.

For segregation among the four strains, three classification functions have been created from the data analysis (Table I). How well these groups separate from one another depends on the impact made by the different variables on the discriminant functions. Of the peaks selected in this analysis, four of them: 22, 17, 47 and 33 in that order, showed the highest loading on the first discriminant function (Table I). These four peaks account for the separation of G3 and KWA strains. This impact is reflected on their rating on the canonical variables evaluated at group means (Table II). On the first discriminant function KWA group is some distance from the rest, while 16CSS and REFMA are nearly inseparable.

On the other hand, the second discriminant function is affected largely by values on peaks 47 and 33. On this function, REFMA is separated from the other groups. Similarly the highest contribution to the third discriminant function was made by peaks 44, 5 and 17. This function is responsible for the separation of G3 and 16CSS. Thus on the 1st d.f, the KWA group is some distance from the rest but 16CSS and REFMA are close together (Table II). On the second discriminant function REFMA is far removed from G3 and 16CSS. It takes both discriminant functions to separate KWA, REFMA from G3 and 16CSS.

By plotting individuals according to their scores on the first two discriminant functions which act as rectangular coordinates, a scatter plot of the type shown in Fig. 2 gives a graphic illustration of the spacial disposition of the four groups relative to one another and hence a picture of the extent of

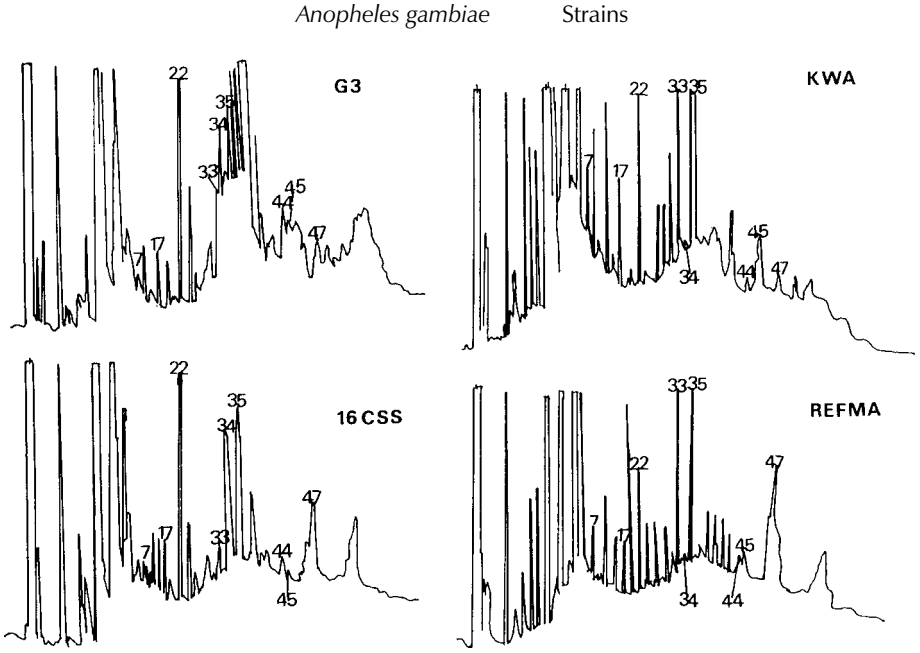


Fig. 1: gas chromatography profile of four strains of *Anopheles gambiae sensu stricto* hydrocarbons, showing the subset of peaks which accounted for the segregation larvae.

TABLE I

Discriminant function coefficients for distinguising the four strains of *Anopheles gambiae*

| Component number | Standardised (by pooled within variance) coefficients for canonical variables | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| 5 | -0.07652 | 0.01292 | -0.58149 |
| 17 | -0.54594 | 0.36139 | 0.39629 |
| 22 | 0.70051 | 0.09396 | -0.08855 |
| 33 | -0.17367 | -0.64507 | 0.28374 |
| 34 | 0.15892 | -0.03698 | -0.033580 |
| 44 | 0.14880 | 0.23765 | 1.34963 |
| 45 | -0.14880 | -0.007 | -0.32718 |
| 47 | 0.21383 | -0.00907 | 0.34302 |

TABLE II

Canonical variables (evaluated at group means) for the four strains of *Anopheles gambiae*

| Group | Discriminant function | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| G3 | 1.68340 | 0.90735 | 1.20754 |
| 16CSS | 0.88845 | 0.67331 | -1.49503 |
| KWA | -3.03764 | 0.20797 | 0.24391 |
| REFMA | 0.83521 | -3.20720 | 0.07814 |

hydrocarbon discrimination between the strains investigated.

Jack-knifed estimators of the proportion of correct identifications was used to test the accuracy of the classifications made by the discriminant analysis; 95% correct segregation among the four strains of *An. gambiae* s.s. larvae was achieved (Table IV).

## DISCUSSION

Using discriminant analysis contrasts in hydrocarbons have been demonstrated among the larvae of four strains of *An. gambiae* s.s. from the 95% success rate in their discrimination. The results indicate, for example, that the hydrocarbons of the Gambian (G3), Nigerian (16CSS and its malathion resistant sub-strain, REFMA) and the Tanzanian (KWA) strains of *An. gambiae* s.s. are different.

The low levels of misclassification of populations of *An. gambiae* s.s. indicates that some individuals from different strains share identical hydrocarbons or this may be due to the correlated nature of the variables responsible for their separation or a reflection of interrelatedness of the strains (notice overlap in distribution between G3 and 16CSS strains: Fig. 2). Lockey (1976) had demonstrated that marked similarities in hydrocarbon profiles was common even among distantly related species.

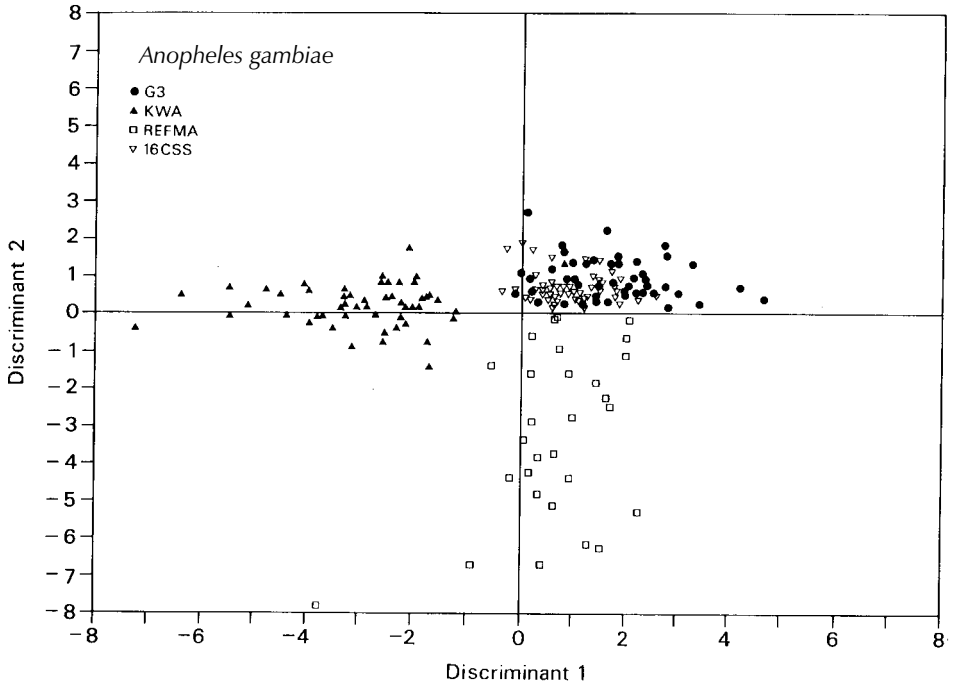


Fig. 2: discriminant function plot showing the distribution of individuals of four *Anopheles gambiae* s.s. strains.

Hydrocarbon differences amongst strains of the *gambiae* species analyzed here could be considered as reflecting geographical variation. For instance, G3 strain (from The Gambia) could be distinguished from 16CSS (from Nigeria) with a 97% confidence rate. There was 100% segregation between the Nigerian (16CSS) and the Tanzanian (KWA) strains. From pairwise comparisons (Table IV), it has been possible to separate any two strains within the *An. gambiae* species with an average success rate of 95%.

The matrix of distance (Mahalanobis') between the groups lends further support to the hydrocarbon relationship among the strains of *An. gambiae* s.s. studied (Table V). G3 strain appears to show greater resemblance to the Nigerian strain (16CSS), being separated from it by 22.06 whereas its distance from KWA is as much as 65.46.

Identification of cryptic species has capitalised on intraspecific differences in the hydrocarbons of the insect cuticle. This has been found useful in particular for the differentiation of sibling species of *Anopheles* mosquitoes (Carlson & Service 1979, 1980, Hamilton & Service 1983, Milligan et al. 1986), *Simulium* blackflies (Carlson & Walsh 1981) and in the sandflies (Ryan et al. 1986).

Although the biological implications of variations in cuticular hydrocarbons between very closely related species have not been fully studied. Phillips and Milligan (1986), Phillips et al. (1987) and Milligan et al. (unpubl. data) suggest that within such relationships species-specific differences do exist which tend to highlight the effect of geographical variation and possible incipient speciation mechanisms.

The species-specific function performed by insect cuticular hydrocarbons in various environments have been outlined by Howard and Blomquist (1982) and Phillips et al. (1988). They serve mainly to prevent dessication and to assist in "chemical communication" (Lockey 1980, Howard & Blomquist 1982).

Discrimination between the strains of *An. gambiae*, here analysed, was based on the quantities of various hydrocarbons (Table III). Milligan et al. (1990 unpubl. data) argued that the hydrocarbon discrimination is based on the relative concentrations of the component chemicals, rather than their mere presence or absence. Similarly, Hamilton and Service (1983) observed that, although the fourth instars of *An. gambiae* and *An. arabiensis* had similar cuticular hydrocarbons, there was still differences in the relative levels of some of these chemicals.

It is thought that hydrocarbons are not just passive protectors against desication, they may additionally play a leading role in mate selection/recognition and population divergence. Hydrocarbon

TABLE III

Jack-knifed classification scores showing the number of individuals of *Anopheles gambiae* correctly allocated to their strain

| | | No. of cases classified into | | | |
|---|---|---|---|---|---|
| Group | No. | G3 | 16CSS | KWA | REFMA |
| G3 | 52 | 49 (94.23%) | 3 (5.77%) | 0 (0.0%) | 0 (0.05) |
| 16CSS | 52 | 0 (0.0%) | 52 (100.0%) | 0 (0.0%) | 0 (0.0%) |
| KWA | 52 | 0 (0.0%) | 0 (0.0%) | 52 (100.0%) | 0 (0.0%) |
| REFMA | 29 | 2 (6.90%) | 4 (13.7%) | 0 (0.0%) | 23 (79.31%) |
| Total | 185 | | | | |

% correct (classification) = 95.1%

TABLE IV

Result of pairwise discrimination among strains of *Anopheles gambiae* based on their hydrocarbon characters

| | G3(A) | 16CSS(B) | KWA(C) |
|---|---|---|---|
| 16CSS(B) | 97.1 | | |
| KWA (C) | 99.0 | 100 | |
| REFMA(D) | 95.1 | 97.5 | 98.8 |

TABLE V

F-matrix indicating the Mahalanobis' measure of distance between the strains of *Anopheles gambiae* studied

| | G3 | 16CSS | KWA |
|---|---|---|---|
| 16CSS | 22.06 | | |
| KWA | 65.46 | 51.51 | |
| REFMA | 37.42 | 34.67 | 32.77 |

differences among sympatric populations of *An. gambiae* s.s. (Phillips et al. 1987, Milligan et al. 1990 unpubl.) might reflect a semiological function of the compounds enabling the insect to recognise potential mates in those locations where sibling species coexist. In some insects, part of the mate recognition mechanism has been linked with the detection of specific hydrocarbon compounds and other components of the cuticular lipid layer e.g fatty acids, alcohols, sterols, aldehydes etc. (Jallon 1984, Bonavita-Courgourdan et al. 1987, Pescke 1987).

Although our limited sample analysis here using laboratory colonies of *An. gambiae* s.s. provides evidence for intraspecific hydrocarbon differences, this variation could have wider implications for populations in the wild. It is not certain the extent to which hydrocarbons may be influenced by genetic, ecological and other geographical differences particularly in the wild. However further analysis of samples of *An. gambiae* drawn from different populations especially in areas where members of the sibling species coexist, will help provide a clearer picture.

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