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CUTICULAR COMPONENT ANALYSIS FOR DISCRIMINATION OF *Aedes Aegypti* (DIPTERA: CULICIDAE) FROM SEVEN LOCALITIES IN SOUTH KALIMANTAN

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ABSTRACT

Cuticular components of female ***Aedes aegypti*** *from seven localities (populations) in South Kalimantan were compared. Mosquitoes from four populations of Banjarmasin, and one population each of Pelaihari, Barabai, and Kotabaru were sampled. Cuticular hydrocarbons were removed from adult female and were separated and quantified by gas chromatography. Stepwise discriminant analysis determined the degree of differences between populations. Using the concentration of the hydrocarbons in a linear discriminant function and the estimator obtained from cross validation, it was demostrated that specimens could be correctly allocated the population to which they belong with an average success rate of 80.6%. All (100%) specimens of populations outside Banjarmasin were correctly identified. There was reduced segregation of the four Banjarmasin populations, suggesting greater similarity in the hydrocarbons of* ***Aedes aegypti*** *from these populations. It was suggested that the greater similarity correlated with increased contact between populations in the region.*

Key words: *Aedes aegypti, cuticular hydrocarbons, gas chromatography, discriminant analysis, intraspecific variation*

INTRODUCTION

Aedes aegypti is one of the most widely distributed mosquitoes in Southeast Asia. In Indonesia, this species can be found in all province (Djakaria, 1988), and most probably it has developed intraspecific, geographic variations. This species is the most potent vector for dengue virus and is the key factor in dengue hemorrhagic fever (DHF) eradication program. Consequently, knowledge of the genetic diversity of this species, particularly that governs response to control measures and relates to capacity to transmit the virus, is essential. In view of the advantages and disadvantages of various techniques so far developed, it is necessary to use different taxonomic techniques in studying this variation.

Gas chromatographic analysis of cuticular components has been proven useful in distinguishing and identifying members of species complexes and strains of mosquitoes. Examples include *Anopheles* (Milligan *et al.*, 1986; Kittayapong *et al.*, 1990; Phillips *et al.*, 1990; Anyanwu *et al.*, 1993; Kittayapong *et al.*, 1993; Milligan *et al.*, 1993); *Culex quinquefasciatus* (Chen *et al.*, 1990); and *Aedes albopictus* (Kruger *et al.*, 1991; Kruger and Pappas, 1993). The method has the sensitivity needed for reliable determination of individual specimen with the advantage that it can be applied in cases where the condition of specimen unsuitable for the application of morphological, genetic, or other biochemical identification techniques.

Gafur (2004) applied the technique to *Aedes aegypti* from Banjarmasin, the capital of the province of South Kalimantan. The study revealed that two populations of this species, that were 7 km separated, showed differences in their cuticular component profiles of adult females. However, cuticular components were extracted from pooled batches of specimens, so it was not clear how reliably individual specimens could be identified. In the present study, cuticular components were extracted from individual specimens of *Aedes aegypti* from several sites in South Kalimantan. The objective of the study was to investigate further the intraspecific variations of adult female *Aedes aegypti* in the region. The question is whether there are differences in cuticular components between more distantly separated populations in South Kalimantan.

MATERIALS AND METHODS

Mosquitoes

Collections were made in four towns: Banjarmasin, Barabai, Pelaihari, and Kotabaru (Figure 1). One village in each town was chosen as sampling site, except for Banjarmasin in which four villages were selected as sampling sites. Adult mosquitoes were obtained by collecting larvae during daytime from indoor and outdoor man-made water-holding containers, e.g. those of drinking, washing, and bathing water in concrete tanks, drums, clay jars, buckets, and aquariums. The collected larvae were placed in 200 ml glass jars half-filled with tap water. Every two days the water was replaced and the larvae were fed with goldfish pellet. Emerging adults were collected using aspirator and were subsequently kept in papercups until death. In accordance with the study objective, only females were used in gas chromatographic analysis. These were kept in petri dishes until extraction.

Sample Preparation

Cuticular components were extracted from dead dry mosquitoes by immersing individual specimen in 100 μl hexane for 10 minutes in a reacti vial. The extract was then transferred to a new clean glass vial. The reacti vial was washed with 100 μl hexane for 3 minutes and the wash solution was transferred to the glass vial. The extract was evaporated to near dryness under nitrogen and resuspended in 2 μl of hexane containing 100 ppm pentadecane (Merck) as an internal standard for GC analysis.

Gas Chromatography

Cuticular components were quantified with a Shimadzu 17A gas chromatograph using a 30 m DB1 capillary column of 0.25 mm internal diameter with 0.25 μm film thickness (J.W. Scientific). The chromatograph was connected to a Shimadzu QP5000 mass spectrometer. The analysis was programmed to begin at 50° C, which remained constant for 8 minutes. Thereafter, the oven temperature increased by 20° C/minute until 260° C where it was maintained for 22 minutes. The injector and interface temperatures were 250° C and 280° C, respectively. The carrier gas (helium) was flowing 10 ml/min. Peaks identity and quantity were determined using Class 5000 software (Shimadzu Corp.).

Data Analysis

The chromatograms (profiles) from each specimen illustrate, by a series of peaks, the kind and quantity of compounds in the cuticular extract. The area under each peak of a chromatogram is proportional to the concentration of a compound in the extract. To compensate for differences in injection volume and machine response, the area under each peak was divided by the area for pentadecane; these ratios formed the data for statistical analysis after logarithmic transformation to improve marginal normality. Transformation $\log(X + 1)$ was applied to avoid negative infinity when the area under a peak was zero.

The objective of data analysis was to compare the cuticular component profiles of the populations under study and to determine whether differences in the profiles could be used for identification. Discriminant analysis was used as it has the capacity to determine which component(s) are most useful in distinguishing populations by finding linear combinations of variables. The stepwise discriminant analysis applied was that of SPSS v.11.5 which employs the 'leave-one-out classification'. In this approach, each individual was excluded from the analysis and was then identified using discriminant functions derived from all the data except the data for that individual. Differences in the cuticular components between populations were quantified by the proportion of 'correct classification' as determined by the discriminant analysis. The 'F to enter' criterion was set to 8, which is a conservative value, to avoid overoptimistic succesful result, so the results were probably pessimistic but unbiased (Phillips *et al.*, 1990).

RESULTS

Thirty six specimens from seven populations were analyzed. Up to 25 hydrocarbon peaks were obtained from each specimen. These were numbered 1–25 in order of retention time. Of these, four peaks (2, 10, 11, and 12) were not significantly different among populations studied and were then excluded from further analysis.

Stepwise discriminant analysis selected four peaks (peak 3, 13, 18, and 22) that gave best separation between populations. The use of all peaks in the analysis did not improve the separation. Table 1 gives summary of the discriminant analysis.

Using the chromatographic characteristics, each specimen was identified by assigning to the population to which its probability of membership was greatest. All Pelaihari, Barabai, and Kotabaru specimens were correctly identified, indicating greater difference in hydrocarbon compositions. Among Banjarmasin specimens, all Pelambuan specimens were also correctly identified, whereas among those of Kuin Cerucuk, Karang Mekar, and Sungai Jingah the success rate were 40%, 50%, and 80%, respectively. Cross validation gave an average of 80.6% of correct identification. Table 2 gives details of identification success, and shows the degree and direction of misclassification.

The reduced segregation among Banjarmasin populations were reflected in the classification output. A good proportion of Kuin Cerucuk was misclassified as Karang Mekar and vice versa. However, despite a high proportion of Kuin Cerucuk misclassified as Pelambuan, all specimens of the latter were correctly classified. The considerable difficulty in differentiating Karang Mekar and Kuin Cerucuk was consistent with a greater similarity (smaller Mahalanobis distance) between the two populations

Table 1. Summary of discriminant analysis of cuticular hydrocarbons of *Aedes aegypti* from seven localities in South Kalimantan

	Discriminant Function			
	1	2	3	4
% of variance	83.6	12.5	3.8	0.1
Standardized discriminant function coefficient:				
peak 3	–0.045	1.136	0.015	–0.368
peak 13	0.255	–0.070	0.982	0.136
peak 18	–0.001	0.922	0.056	0.789
peak 22	0.996	–0.060	–0.134	–0.205
Mean function score:				
Karang Mekar	–2.531	0.557	–0.874	0.239
Kuin Cerucuk	–1.970	–1.053	–1.601	1.483E-02
Pelambuan	–3.863	–1.132	–1.479	–0.155
Sungai Jingah	–2.522	5.622	0.839	–0.166
Pelaihari	–4.809	–0.814	2.342	0.279
Barabai	–0.201	–3.519	1.785	–0.213
Kotabaru	16.214	0.294	–7.28E-02	4.010E-02

Table 2. Cross-validated classification results for *Aedes aegypti* from seven localities in South Kalimantan

	n	KMR	KCK	PLB	SJG	PLH	BRB	KTB
KMR	6	50.0	33.3	0	0	16.7	0	0
KCK	5	20.0	40.0	40.0	0	0	0	0
PLB	6	0	0	100.0	0	0	0	0
SJG	5	20.0	0	0	80.0	0	0	0
PLH	4	0	0	0	0	100.0	0	0
BRB	5	0	0	0	0	0	100.0	0
KTB	5	0	0	0	0	0	0	100.0

80.6% of cross-validated grouped cases correctly classified.
KMR: Karang Mekar; KCK: Kuin Cerucuk; PLB: Pelambuan; SJG: Sungai Jingah; PLH: Pelaihari; BRB: Barabai; KTB: Kotabaru

Table 3. F-values, indicating matrix of the Mahalanobis distances between the populations of *Aedes aegypti* from seven localities in South Kalimantan

	KCK	PLB	SJG	PLH	BRB	KTB
KMR	2.132	3.460	17.577	9.368	17.922	215.254
KCK		2.219	28.497	11.819	11.612	187.608
PLB			32.268	8.490	18.192	248.855
SJG				24.465	50.343	213.126
PLH					14.492	223.678
BRB						161.093

See legends on table 2 for abbreviations

(see Table 3). When either one of the two populations was excluded, the proportion of correct allocation greatly improved (up to 90%).

In Figure 2 individuals are plotted according to their discriminant scores. Kotabaru, Barabai, and Pelaihari populations can be clearly distinguished from the others, while Banjarmasin populations are more or less overlap.

DISCUSSION

The high degree of correct allocation indicates that significant variation occurs among the populations of *Aedes aegypti*. Data on hydrocarbons presented in this paper shows that there are distinguishable populations of *Aedes aegypti* in South Kalimantan.

The degree of separation and the Mahalanobis distance between populations is consistent with the distance between the populations (Figure 1). Kotabaru is the farthest town from Banjarmasin. It is more than 300 km separated from the capital of the province, including large uninhabited areas. Moreover, it lies on an island separated by a small strait from the mainland. These must be sufficient to create barrier. The Mahalanobis distances of Kotabaru and all four Banjarmasin populations are far higher than those between populations of Banjarmasin and other towns. On the other hand, the four Banjarmasin populations are no more than 5 km separated. The Mahalanobis distances between these populations are unsurprisingly small. However, since *Aedes aegypti* are not strong fliers and, on the other hand, they are anthropophilic and tend to breed near houses, contact between the four Banjarmasin populations is also to certain extent reduced. Barabai is about 150 km from Banjarmasin, while Pelaihari is only about 50 km separated from the capital. The greater Mahalanobis distance between Banjarmasin and Barabai than that between Banjarmasin and Pelaihari is consistent with the distance of the two towns from Banjarmasin. There seems to be a high correlation between the reduced contact with a smaller similarity in cuticular hydrocarbon composition of *Aedes aegypti* populations.

This study, using individual specimens in chromatographic analysis, confirmed Gafur (2004), who used pooled specimens, that there is close similarity in

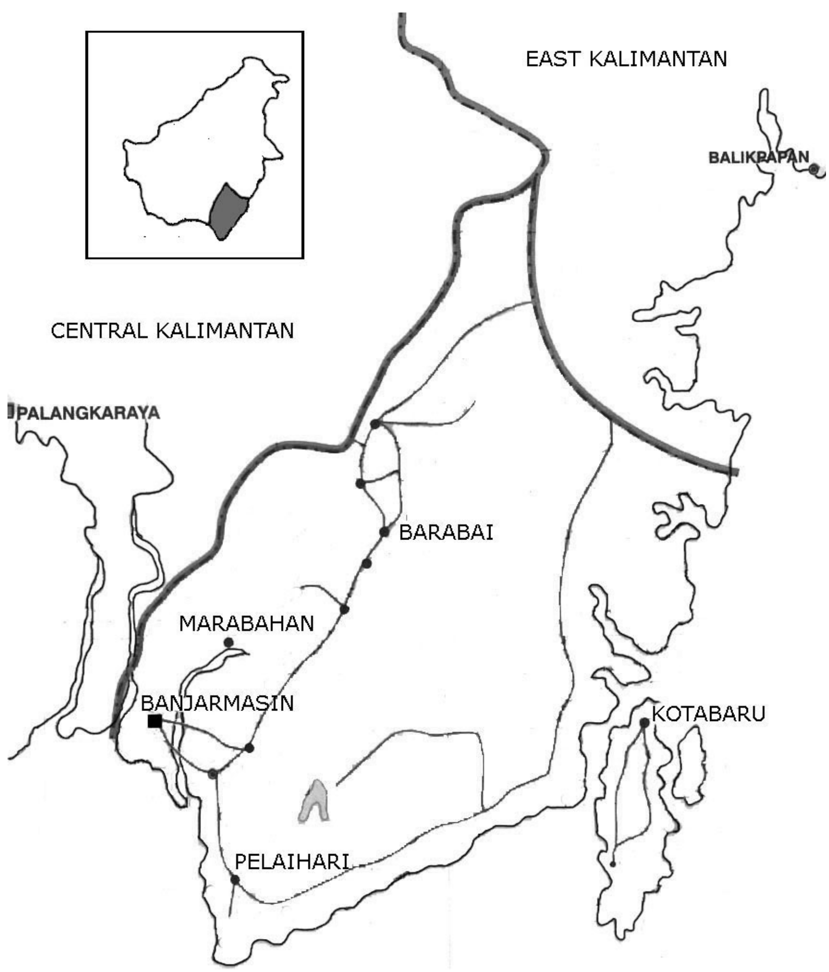

Figure 1. Map of South Kalimantan showing four towns at which sampling sites were located.

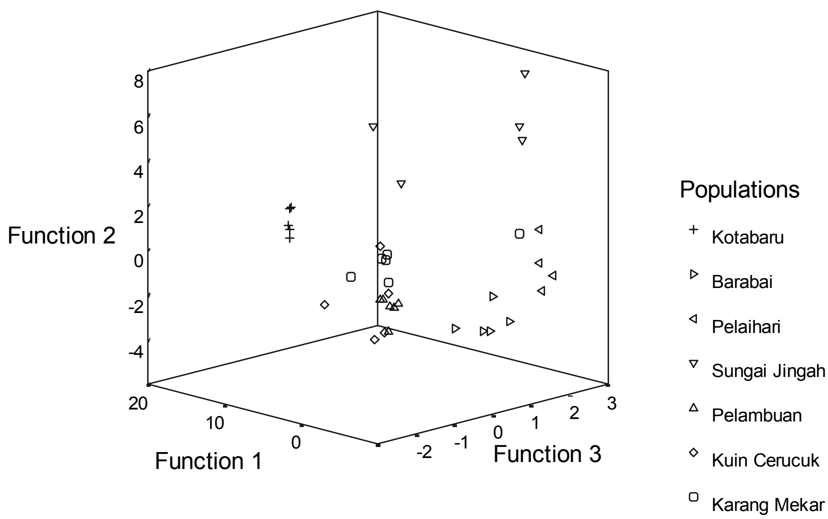

Figure 2. Discriminant function plot showing distribution of individuals of *Aedes aegypti* from seven localities in South Kalimantan in the space of the first three discriminant functions.

cuticular hydrocarbons between *Aedes aegypti* from Karang Mekar and from Kuin Cerucuk, two adjacent populations in Banjarmasin.

There are several explanations for the differences in the cuticular hydrocarbons. It may be argued that differences in environmental factors could be responsible. In many arthropods, the quantity of cuticular hydrocarbons can be affected by environmental factors: habitat, temperature, relative humidity, and season (Hadley, 1977; Toolson and Hadley, 1979; Toolson, 1982). In some species, dietary hydrocarbons can be incorporated directly into the cuticular hydrocarbons, altering the overall composition (Blomquist and Dillwith, 1985). Alternatively, the differences might be due to differences in the age of individuals in the samples as age-dependent changes in the cuticular hydrocarbon have been observed in houseflies (Silhacek *et al.*, 1972). Finally, the differences are genetic.

In the present study, adult female mosquitoes were used, which were always in papercups without meals since emergence until death. The larvae collected from the three populations were reared in relatively similar condition. These treatments could minimize the possibility that differences in cuticular hydrocarbon profiles were due to differences in condition of specimens. Adult females used died naturally after 3–4 days in papercups as a result of starvation. Since female mosquitoes reach sexual maturity before first bloodmeal, it could be reasonably assumed that all specimens had reached sexual maturity and, thus, definitive cuticular hydrocarbon composition. The effect of age differences was, if any, also kept to minimum as the specimens had reached definitive cuticular hydrocarbon composition. Besides, cuticular hydrocarbons are chemically very stable that specimens of more that 70 years old collection showed only minor differences from much younger ones (Carlson *et al.*, 1993). Therefore, by using only hydrocarbon peaks, the effect of different waiting time for each specimen before GC analysis could be eliminated.

The author then considers that the differences among the populations are genetic. This is in line with Anyanwu *et al.* (1993) that cuticular hydrocarbon are associated to reproductive isolation, i.e. genetic divergence, in insects. Gafur (2004) arrived at similar conclusion that the differences in cuticular hydrocarbon composition are most probably genetic. Further investigation, however, is needed to evaluate whether the technique of collection is obligatory to reveal the differences or the use of wild-caught adults, which is more practical, is also possible.

The discriminant functions can be used to allocate new individuals of unknown identity, provided that they are from the populations under study. Cuticular hydrocarbon variation is the first intraspecific variation detected in *Aedes aegypti* from South Kalimantan. Moreover, cuticular hydrocarbon analysis is so far the only method for identification/ discrimination of *Aedes aegypti* from this region. This is particularly important in case where the populations (a) are bioecologically different, (b) have different vectorial capacity, or (c) show different response to insecticides and other vector control measures, as cuticular lipids play role in controlling the entry of insecticides and other chemicals from environment (Brooks, 1976) and provide physical and chemical protection from microorganisms (Blomquist and Dillwith, 1985). In addition, cuticular hydrocarbons have great potential for use in tracing geographic origins of populations as has been utilized for the Formosan Subterranean termite (Haverty *et al.*, 1990).

In conclusion, preliminary analysis of cuticular components of female *Aedes aegypti* from seven localities in South Kalimantan revealed the biodiversity of this species in the province. Chromatograms showed a few GC peaks that might be of value in identification of populations. The present technique appears to be powerful for differentiating populations of *Aedes aegypti* in the province. In view of these promising results, more specimens as well as more populations should be tested to determine the intraspecific variability of cuticular components of *Aedes aegypti.*

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