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Identification of the *Anopheles* Mosquitoes (Diptera: Culicidae) of Southern Iran Using Analysis of Cuticular Hydrocarbons

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Abstract: Cuticular hydrocarbons (CHCs) of the epicuticle wax layer are so far used to differentiate the insects in species and subspecies levels. In this study, four species of malaria vectors (genus *Anopheles*) were collected from various localities in southern Iran. Twenty specimens of each species were randomly selected and one epicuticular extract was prepared of every five specimens. FID-GC profiles of the extracts did not show any qualitative difference. Using significant difference of CHC mass at retention time (RT) 39.6, the two species of *An. sacharovi* and *An. fluviatilis* could be distinguished. Similarly, the two species of *An. superpictus* & *An. sacharovi* and *An. dthali* & *An. sacharovi* were differentiated by their CHC level at RT 28.5. *An. sacharovi* was distinguished by integratable peaks at RTs 29.7, 30.6, 30.7, 31 and 32.6 while the other three species just indicated trace peaks at the same RTs. Similarly, *An. dthali* could be known by an integratable peak at RT 26.2 while *An. fluviatilis* and *An. superpictus* indicated trace peaks at the same RT. Integratable peaks and traces at RTs 27.4 and 28.5 were respectively used to differentiate *An. superpictus* from *An. fluviatilis*. Lastly, CHC trace amount of *An. superpictus* at RT 39.6 is another indicator to distinguish it from *An. fluviatilis* with an integratable peak at the same RT. In harmony with other studies worldwide we hereby report that quantitative analysis of CHCs was successfully applied to differentiate the four *Anopheles* species of southern Iran.

Key words: *Anopheles*, mosquito, quantitative GC, cuticular hydrocarbon, Iran

Malaria is still an important endemic disease in southern and southeastern Iran (Edrissian, 2002), which transmitted by genus *Anopheles* Meigen, 1818 (Diptera: Culicidae). Based on different taxonomic methods, 22 to 28 *Anopheles* species have been so far reported in Iran (Dow, 1953; Sedaghat et al. 2003; Doosti et al. 2006), out of which 8 species are proved to be malaria vectors (Zaim et al. 1993; Sedaghat et al. 2003; Doosti et al. 2006).

One of the first steps in the control of malaria is precise identification of the disease vectors, their biology and ecology (Manoucheri et al. 1976). Using a morphological key (Deane, 1946) is the current method to identify the larva and adult of *Anopheles* mosquitoes which is based on the chaetotaxi, wing morphology, palpal and thoracic indices (White, 1978). The insect must be intact in order to be checked by a morphologic key, so old specimens can be hardly identified due to changes in morphological characters. In addition, identification of subspecies and siblings is not possible by morphological method (White, 1977).

Morphological characters and surface patterns of eggs (Ramsdale and Leport, 1967), hybridization and species mating (Davidson, 1964), cytogenetic and chromosomal investigations (Coluzzi and Sabatini, 1967), isoenzyme evaluation by electrophoresis (Miles, 1978), specific DNA features (Gale and Crampton, 1987) and analysis of cuticular hydrocarbons (CHCs) (Carlson and Service, 1979) are methods by which species, siblings, subspecies and allopatric populations of mosquitoes can be differentiated.

Application of the CHCs can be useful for identification of the species, siblings and allopatric populations (Anyanwu et al. 2000; Caputo et al. 2005; Caputo et al. 2007). Epicuticular wax layer of the insect integument contains long chain hydrocarbons including methyl-alkanes, n-alkenes and n-alkanes (Gibbs, 1998). Analysis of insect CHCs using gas chromatography has shown that there are qualitative or quantitative differences between different species and between male and females of the same species

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(Howard, 1993). CHC analysis of mosquitoes was first performed on *Anopheles gambiae* sensu stricto Giles, 1902 (Carlson and Service, 1979) and soon followed by other workers on *An. culicifacies* Giles, 1901 (Milligan et al. 1986), *An. arabiensis* Patton, 1905 (Phillips et al. 1987), *An. maculipennis* Meigen, 1818 siblings (Phillips et al. 1990), *An. stephensi* Liston, 1901 siblings (Anyanwu et al. 2000) and allopatric and sympatric populations of *Anopheles gambiae* s.s. (Caputo et al. 2007). Analysing CHCs in the current research, we would like to evaluate the technique potency for differentiation of malaria vectors of southern Iran. It is in particular an efficient tool to identify the old museum specimens whose chaetotoxy, wing scales or other morphological characters are lost over time.

MATERIALS AND METHODS

Collection of mosquito specimens

Anopheles mosquitoes were collected either as adult by aspiration or larval rearing. Site of collection, dates and number of collected mosquitoes are indicated in Table 1. Larvae were reared on aquarium fish food at 25°C, 75-80% RH and 12L: 12D photoperiodism. Female mosquitoes were identified using the key to the Iranian species of *Anopheles* (Shahgudian, 1960) and its illustrations right after collection and stored in −30°C until the experiment time.

Chemical extraction

Twenty specimens of each species were randomly chosen and groups of five specimens were extracted in 400 mL n-hexane for five minutes to get enough cuticular wax without extra contamination from the internal lipids according to Phillips et al. (1987).

Quantitative gas chromatography

CHCs of the identical extract of each species was four time (n=4) monitored by quantitative gas chromatography. One mL of each extract was injected to a GC Varian 3800 gas chromatograph in split mode (split ratio 50%) equipped with a CP-Sil 8cb (5% Phenyl and 95% dimethyl polysiloxane, non-polar) bounded phase fused silica capillary column (Varian: 0.12 mm, ID 0.25 mm, length: 50 m) connected to a flame ionization detector (FID). Helium was used as a carrier gas at 1 mL/min velocity. Injector and detector temperature were set at 300°C. The temperature programme was started at 32°C and ramped at 2°C/min to 52°C, then maintained for 3 min. Thereafter, the temperature was raised at 5°C/min to 72°C, maintained for 2 min, followed by a ramp at 5°C/min to 230°C and another one at 2°C/min to 285°C, ultimately cooled to the starting temperature. CHC peak areas were integrated by Saturn® Workstation package, Saturn view ™ version 5.2.1, 1989-1998, Varian Associates, Inc.

Mass calculation and carbon chain estimation

Using mixture of a given value of the three external standards of n-pentadecane ($C_{15}H_{32}$), n-pentacosane ($C_{25}H_{52}$) and n-dotriacontane ($C_{32}H_{66}$) and their relevant integrated peak areas, mass of each hydrocarbon in the cuticle extracts was accordingly calculated. Using the eluting time of the external standards, it is also possible to estimate the chain length of CHCs in a chromatogram.

Statsitical analysis

Kolmogorov-Smirnov test was applied in order to verify the normal distribution of the data. Hydrocarbon mass of the four *Anopheles* species at retention time (RT) 6.6-8.7 and also CHC mass of the species *An. dthali* Patton 1905,

Table 1. Collecting locations of *Anopheles* mosquitoes in Southern Iran, 2007.

Anopheles Species	*An. dthali*	*An. fluviatilis*	*An. superpictus*	*An. sacharovi*
Geographical location (Reference grid)	Kazeroon County Islam-abad **(29°47'N, 51°33'E)**	Kazeroon County Pirsabz **(29°48'N, 51°38'E)**	Darab County Arabe chegini **(28°47'N, 54°22'E)**	Marvdasht County Garm-abad **(30°3'N, 52°47'E)**
	Kazeroon County Ali-abad Ghuri **(29°48'N, 51°34'E)**			
	Kazeroon County Ghaemieh **(29°50'N, 51°35'E)**			Marvdasht County *Fath-abad* **(29°56'N, 52°48'E)**
	Kazeroon County Pirsabz **(29°48'N, 51°38'E)**			
Collecting date	September 2006 & June 2007	June 2007	May & June 2oo6	September 2006
Method of collection & type of habitat	Hand Catch: 1. Indoor (Islam-abad) 2. Outdoor (all above localities)	Larval collection	Larval collection	Outdoor hand catch
Number of specimens	360	75	110	94
Number of females	175	40	48	45

An. sacharovi (Favre, 1903) and *An. superpictus* Grassi, 1899 at RT 28.5 were compared using ANOVA and the Tukey *HSD* post hoc test. If an integratable peak was only appeared in two species at a specific RT, the non-parametric test of Mann-Whitney *U*-test was used. Reproducibility of the experiments at RT 39.6 for the two species of *An. fluviatilis* James, 1902 and *An. sacharovi* was tested upon four replications at 99.9% ($P<0.001$). The confidence level for differentiation of *An. dthali* v.s. *An. superpictus* and *An. superpictus* v.s. *An. sacharovi* at RT 28.5 was 95% ($P<0.05$) while for *An. dthali* v.s. *An. sacharovi* was 99% ($P<0.01$). All statistical analyses were fulfilled with SPSS statistical package ver.11.5.0 (GS-35F-5899H, USA).

RESULTS

Quantitative differences of CHCs

Seventeen hydrocarbons were totally recognized at RT 6.6 to 39.6 in the chromatogram profiles of the four species of *An. dthali*, *An. superpictus*, *An. fluviatilis* and *An. sacharovi* which mean±SEM of their calculated masses are summarized in Table 2. *Anopheles sacharovi* and *An. fluviatilis* respectively showed the highest and the lowest number of integratable peaks (Table 3). All quantitative differences of the four *Anopheles* species were observed at RTs 26.2 to 39.6 (representing long chain hydrocarbons) with no quantitative difference at RTs 6.6- 26.2 (Fig. 1).

Carbon chain estimation

Comparing these hydrocarbon peaks and that of the external standards the approximate length of the carbon chain could be estimated. Peaks, eluted earlier than 27.51 min (RT of $C_{15}H_{32}$) have shorter chain than pentadecane (10 compounds); those eluted between retention time of $C_{15}H_{32}$ (27.51 min) and $C_{25}H_{52}$ (38.64 min) have a carbon chain of 15-25 (6 compounds) and just one hydrocarbon appeared between 38.64 (RT of $C_{25}H_{52}$) and 39.64 min (RT of $C_{32}H_{66}$) and thus a C chain between 25- 32.

DISCUSSION

Our study indicated the efficiency of the quantitative analysis of cuticular hydrocarbons to differentiate *Anopheles* species of southern Iran; however no qualitative difference was detectable. Statistical analyses on the mass quantity of CHCs of the four species indicated the significant differences among species.

Two species of *An. sacharovi* and *An. fluviatilis* could be differentiated via significant difference ($P<0.001$) of CHC mass at RT 39.6 (Fig. 2), while *An. dthali* v.s. *An. superpictus* and *An. superpictus* v.s. *An. sacharovi* ($P<0.05$) and *An. dthali* v.s. *An. sacharovi* ($P<0.01$) were

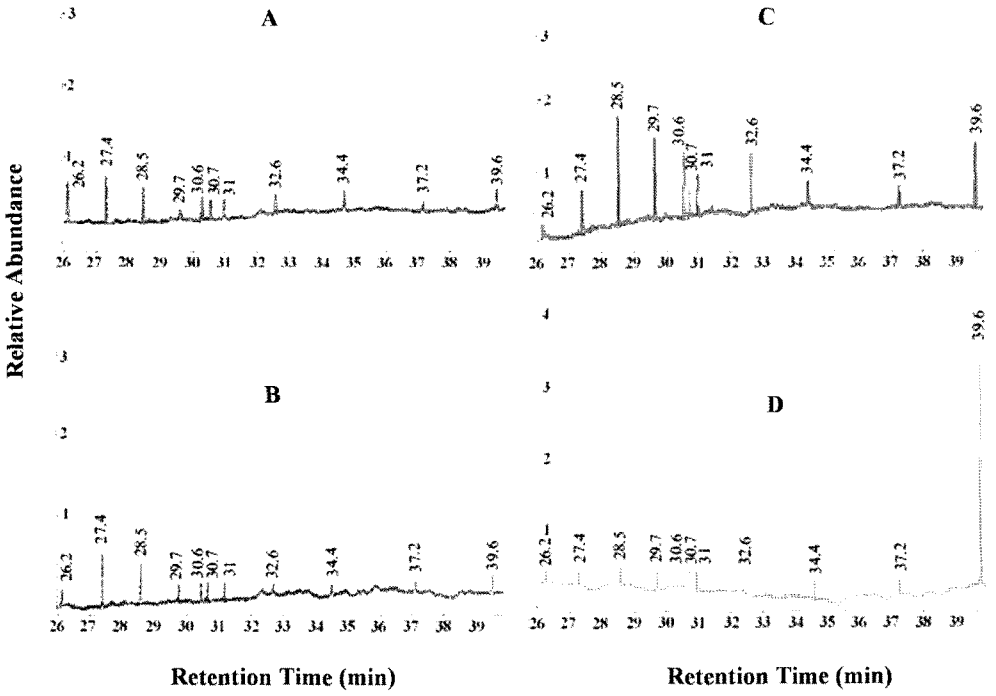

Fig. 1 FID-GC profile, indicating CHC relative abundance against Retention Time (minute) for the *Anopheles* species of Southern Iran, (2008). (A): *Anopheles dthali*, (B): *Anopheles superpictus*, (C): *Anopheles sacharovi*, (D): *Anopheles fluviatilis*. The precise retention time of each CHC is shown on the relevant peak. RTs 6.3- 26.2 have not been showed because of no qualitative or quantitative difference. Solvent (hexane) RT: 6.3.

Table 2. Mean ± SEM of CHC masses based on integratable peaks (RTs 6.6- 39.6) of the four *Anopheles* species of Southern Iran.

Retention Time (min)	*Dthali*	*Superpictus*	*Fluviatilis*	*Sacharovi*
6.6	0.532769±0.111569	0.452945±0.02759	0.476821±0.041207	0.473932±0.039788
6.7	117.689±27.10233	104.6112±9.2705	106.9748±6.292474	104.256±4.555323
7.3	5.609779±1.145782	4.988285±0.515373	5.056554±0.302906	5.028963±0.154837
7.4	0.498516±0.111942	0.434256±0.125741	0.52593±0.049299	0.491363±0.027454
7.5	0.513136±0.130154	0.460255±0.022462	0.478236±0.033066	0.507065±0.054564
7.8	3.579812±0.811787	3.217421±0.28693	3.370996±0.232274	3.360915±0.263919
8.1	3.743174±0.790008	3.301961±0.301144	2.681351±1.318438	3.382315±0.192317
8.7	0.6956±0.146436	0.584235±0.025135	0.512076±0.118555	0.617544±0.062876
26.2	0.249848±0.019866	Trace	Trace	Trace
27.4	0.319649±0.049581	0.313223±0.054279	Trace	0.454242±0.232649
28.5	0.265571±0.006818	0.280872±0.010705	Trace	1.187962±0.141204
29.7	Trace	Trace	Trace	1.033937±0.152574
30.6	Trace	Trace	Trace	0.989014±0.245982
30.7	Trace	Trace	Trace	0.414389±0.052293
31	Trace	Trace	Trace	0.668894±0.127561
32.6	Trace	Trace	Trace	0.965669±0.262363
39.6	Trace	Trace	2.029908 0.209234	1.026332±0.40069

Table 3. Number of integratable peaks in the four *Anopheles* species of Southern Iran. Total peaks (integratables and non-integratables): 17.

Number of integratable peaks	Species
16	Anopheles sacharovi
11	Anopheles dthali
10	Anopheles superpictus
9	Anopheles fluviatilis

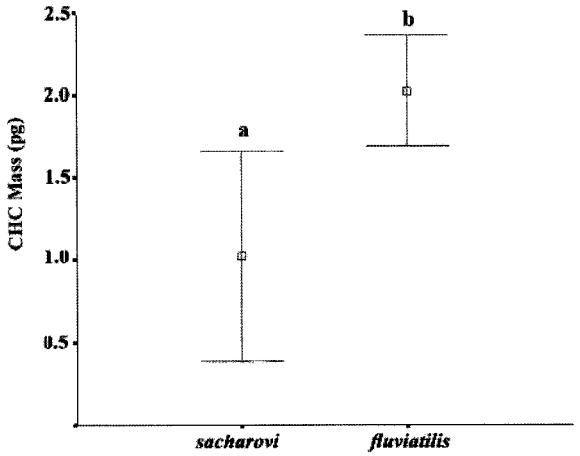

Fig. 2 CHC mass quantity of *An. sacharovi* and *An. fluviatilis* at RT 39.6. Central rectangles and the bars respectively indicate mean and confidence interval. Mann-Whitney *U*-test, n= 4, P< 0.001, Bars with different letters indicate statistically significant difference. Pg: Picogram.

distinguished by their CHC mass at RT 28.5 (Fig. 3).

Anopheles sacharovi was distinguished by integratable

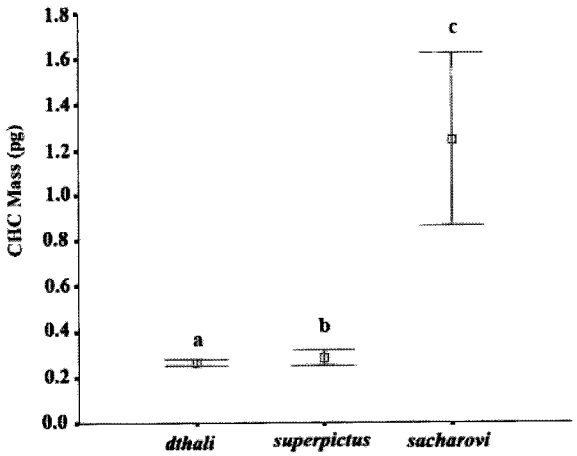

Fig. 3 CHC mass quantity of *An. sacharovi, An. dthali* and *An. superpictus* at RT 28.5. Central rectangles and the bars respectively indicate mean and confidence interval. ANOVA followed by Tukey post *HSD* hoc test, n= 4, Comparison of *An. dthali* v.s. *An. superpictus* and *An. superpictus* v.s. *An. sacharovi* (P< 0.05), Comparison of *An. dthali* v.s. *An. sacharovi* (P< 0.01); Bars with different letters indicate statistically significant difference. Pg: Picogram.

peaks at RTs 29.7, 30.6, 30.7, 31 and 32.6; whereas the other species had only trace peaks for the same RTs (Fig. 4). A trace is a considerably low peak area and its relevant mass is far below the sensitivity and measuring capability of the detector; hence numerically could not be validated.

Anopheles dthali showed an integratable peak at RT 26.2 which was identical, comparing the trace peak of *An. fluviatilis* and *An. superpictus* at the above retention time (Fig. 4).

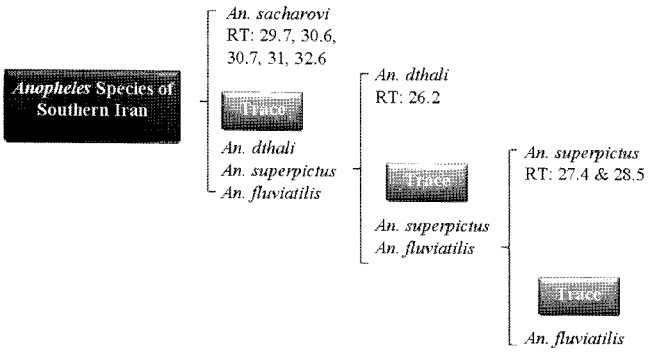

Fig. 4 *Anopheles* species of southern Iran and their systemic differentiation using CHC integratable and non-integratable trace peaks. RT of integratable peaks is indicated.

Anopheles superpictus and *An. fluviatilis* could be differentiated by integratable peaks of *superpictus* at RTs 27.4 and 28.5 (trace for *fluviatilis*) and additionally, the integratable peak of *fluviatilis* at RT 39.6 (trace for *supersticus*) (Fig. 4). Similar results were obtained on *An. gambiae* s.s. (Carlson and Service, 1979; Caputo et al. 2007), *An. culicifacies* (Milligan et al. 1986), *An. arabiensis* (Phillips et al. 1987) and *An. arabiensis* (Caputo et al. 2007). All other workers similarly reported quantitative differences without any qualitative diversity (Hamilton and Service, 1983; Kittayapong et al. 1993).

Anyanwu et al. (2000) indicated that CHC differences between *An. gambiae* strains can be due to geographical difference of diverse populations. It is of course not clear to what extent these differences are affected by genetic and ecological factors.

CHCs have protective role and also prevent loss of water via integument. Besides, they behave as semiochemical and have a role in mating and forming populations. An insect identifies its mate using CHCs and hence siblings derive over the time from their origin by geographical distribution (Marchand, 1984; Howard and Blomquist, 2005). We have recently found that the CHC quantity of malaria vectors and non-vector species of genus *Anopheles*, e.g. *An. Claviger*, bears a remarkable difference even though they were collected in a unique locality (Nikbakhtzadeh et al. 2008). This can be a considerable hint and imply to another prerequisite for an *Anopheles* mosquito in order to gain vectorial capacity. This study is the first part of a comprehensive project on CHCs and their application in differentiating of *Anopheles* mosquitoes in Iran. We are currently working on vector and non-vector *Anopheles* species, siblings of malaria vectors, the effect of geographical isolation on the CHCs of metapopulations and age estimation of vectors using this technique. Because of some controversies in literature, further studies are needed to verify the stability and reliability of CHC relative abundance within sexes and different life stages.

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