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A Chemotaxonomic Study of Cuticular Hydrocarbons on *Epilachna indica* (Family: Coccinellidae) from Sarawak

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ABSTRACT

The chemical composition of cuticular hydrocarbons of adult *Epilacnha indica* (ladybird beetles), collected from Kota Samarahan, Kota Padawan and Lanjak-Entimau, Sarawak were analyzed by using a capillary gas chromatography-mass spectrometer (GC-MS). Cuticular hydrocarbons extracted from 18 samples of adult *E. indica* (comprise of 90 individuals). Over 95% of the hydrocarbon peak areas consist of chain lengths from C_{18} to C_{38}. The proportions of n-alkanes between three different localities are significantly difference, except for n-dotriacontane and n-tetratriacontane. Comparison between Kota Samarahan and Kota Padawan samples revealed the significant different in hydrocarbon composition for even-numbered carbon n-alkanes ranging from *n*-C_{18} to *n*-C_{38} except for *n*-C_{32} and *n*-C_{34}. Several odd-numbered carbon n-alkanes such as *n*-C_{25}, *n*-C_{27}, *n*-C_{33} and *n*-C_{35} also showed significant difference in the composition between Kota Samarahan and Kota Padawan. Examination on components contributing to the differentiation of localities showed that *n*-C_{29}, *n*-C_{33} and *n*-C_{36} were important in discriminating three different localities. Discriminant function analysis (DFA) successfully classified all samples into three correct groups in 100% of cases, with cross-validation resulted in an error of 7.7%. Individuals from each locality were grouped in the range of 2.10 - 9.16% differences, with average of 43% different reflected between localities. *E. indica* samples collected from the forests containing simpler hydrocarbon pattern than samples collected around housing or industrial areas. Result showed that differences in microenvironment have influenced the composition and proportion of insect cuticular hydrocarbon. The finding reveals the potential of cuticular hydrocarbons profile to separate subpopulations of species.

Keywords: *Epilachna indica*, cuticular hydrocarbon, gas chromatography-mass spectrometer

INTRODUCTION

The chemotaxonomic significance of cuticular alkanes was demonstrated on several orders of insects including Isoptera (Haverty *et al*., 1991; Takematsu & Yamaoka, 1997), Lepidoptera (Arsene *et al*., 2002), Hymenoptera (Abdalla *et al*., 2003; Martin & Drijfhout, 2009; Nunes *et al*., 2010), Dictyoptera (Chapman *et al*., 1995), Diptera (Anyanwu *et al*., 2000), Orthoptera (Bounecheda *et al*., 2011; Chapman *et al*., 1995), Hemiptera (Juarez & Fernandez, 2007) and others. Species-specificity of cuticular hydrocarbons also had been studied for coleopteran families including Tenerbrionidae (Lockey, 1988), Chrysomelidae (Nelson & Charlet, 2003), Curculionidae (Lapointe *et al*., 2004), Silphidae (Whitlow, 2003) and the Scolytidae (Page *et al*., 1990). Several studies discovered that majority of hydrocarbons found in insects cuticular were aliphatic hydrocarbon range from C_{23} to C_{47} with odd-numbered compounds predominated the composition (Arsene *et al*., 2002; Chapman *et al*., 2000; Haverty *et al*., 1996; Juarez & Fernandez, 2007; Page *et al*., 1990). Three main hydrocarbon classes that have been identified in hydrocarbon mixtures namely, *n*-alkanes, alkenes and methylalkanes. *n*-alkane was considered as the most prevalent in the cuticular hydrocarbons mixture comprising more than 54% (Whitlow, 2003), where C_{22}

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and C_{29} are the usual principal compound of *n*-alkane in insects (Soliday *et al*., 1974).

Woodrow *et al*. (2000) reported that cuticular hydrocarbons of *Chyptotermes brevis* (Isoptera) comprised of 29% *n*-alkanes, while Chapman *et al*. (2000) found that *n*-alkanes occupied 40% to 60% of the total hydrocarbon extracted from *Schistocerca* spp. (Orthoptera). On the other hand, Harverty *et al*. (1996) reported that methylalkanes dominated the cuticular hydrocarbons composition in *Coptotermes formosanus* (Isoptera). These studies have shown that the types and abundance of cuticular hydrocarbons varied in different species of insects. Meanwhile, variation between two different geographically isolated populations is much higher ranged between 30-60% (Brown *et al*., 2000). The high variation in population through ecological isolation may reflect to the process of speciation (Brown *et al*., 2000; Wilgenburg *et al*., 2011). It was also suggested that the variation in cuticular hydrocarbons composition among population of the same species reflected the genetic differences rather than environment factors (Brown *et al*., 1997).

If a cuticular wax is to be used as a basis of systematic studies of phytophagus insects, it is clearly essential to have an understanding of the extent to which cuticular hydrocarbon varies within a well-recognized species at subpopulation level. Furthermore, examination of cuticular hydrocarbon variation is a necessary step for further taxonomic, genetic and ecological studies of phytophagous insects, particularly ladybird beetle in these regions. This study examine the variation of cuticular hydrocarbon of *E. indica* for three different localities based on group of *n*-alkanes compound, in which could serve as basis for developing optimal sampling regimes for further examination of this beetle.

MATERIALS AND METHODS

Species collection and Identification

A total of 90 adult Ladybird beetles *E. indica* from Kota Padawan, Kota Samarahan and Lanjak-Entimau were collected using beating trays, comprise 30 individuals for each sampling location. The specimens were frozen at -20°C before hydrocarbons extraction (Page *et al.*, 1990), and were identified by comparing them with voucher specimens in the Universiti Malaysia Sarawak Zoology Museum and also by using identification keys of Tung (1983).

Cuticular waxes extraction and analysis

Cuticular hydrocarbon of 18 samples, comprising five (5) individuals of adult *E. indica* for each sample, were extracted by immersing specimens in 10 mL chloroform for 1 min (Chapman *et al*., 1995). The extract was then allowed to evaporate at room temperature until dry and then dissolved with 10 mL of *n*-hexane. Following this, the extract was placed on top of a glass column chromatography packed with 4 cm of activated Biosil A (silica gel, 100-200 mesh) following that of Page *et al*. (1990) and eluted with 20 mL of *n*-hexane. The eluent was collected in a 50 mL pear-shaped flask, which then concentrated via a rotary evaporator, before evaporated to dryness under a stream of nitrogen, and finally dissolved in 50 µL *n*-hexane for Gas Chromatographic (GC) analysis. GC analysis was performed with a Shimadzu QP5000 PLUS.

Compound Identification and Analysis

Cuticular hydrocarbons extracted from 18 samples of adult *E. indica* were identified by comparing their GC retention times and mass fragmentation patterns of standard reference materials, the standard mixture of *n*-alkanes ranging from C_9 to C_{34}. The identified compounds were further confirmed by comparing their mass spectra with mass spectra standard library installed in the data system (computer), which provided by National Institute of Standards and Technology (NIST). Twenty one quantitative characters (Table 1), representing 21 types of *n*-alkanes compounds, were performed in SPSS 17.0 to estimate the similarity and dissimilarity of hydrocarbons compositions. Cluster analysis was carried out with the Squared Euclidean as the distance measure and the unweighted pair group average as the linkage rule.

RESULTS

Overall, hydrocarbons are made up over 95% of the analyze peak areas detected by GC/MS

in all 18 samples of cuticular wax *E. indica*. The cuticular hydrocarbons of *E. indica* were represented by normal alkanes, alkenes and methyl-branched alkanes. Group of *n*-alkanes and methyl alkanes dominated the hydrocarbon profile of *E. indica*, while double bond *n*-alkenes were detected in minute amount in all the samples examined. GC/MS analysis showed that each sample contained between 100–120 peaks in the chromatograms. However, only 70 compounds were considered as cuticular hydrocarbon profile of *E. indica* as those compounds were detected in all samples examined, while the other minor components were detected in few samples and represent in trace. Furthermore, only peak above 0.05% of the total area were considered for data analysis. However, the hydrocarbon ranged from C_{18} to C_{38} was constantly detected among individuals within the species (Figure 1).

Although differences in hydrocarbon composition within populations are relatively small, differences are greater between geographical populations. The magnitude of these differences is also clearly illustrated by GC/MC analysis presented in Figure 1. Cuticular hydrocarbons pattern of samples from Kota Samarahan showed a more complex with a wider range compared to samples from Kota Padawan and Lanjak-Entimau. However, both Kota Samarahan and Kota Padawan population showed similar hydrocarbon chain series ranging from C_{18} to C_{38} although *n*-alkanes from C_{18} to C_{26} were found in small quantity in Kota Padawan. Cuticular hydrocarbon from Lanjak-Entimau samples contained a homologous series from C_{18} to C_{37} which is shorter from those two other samples.

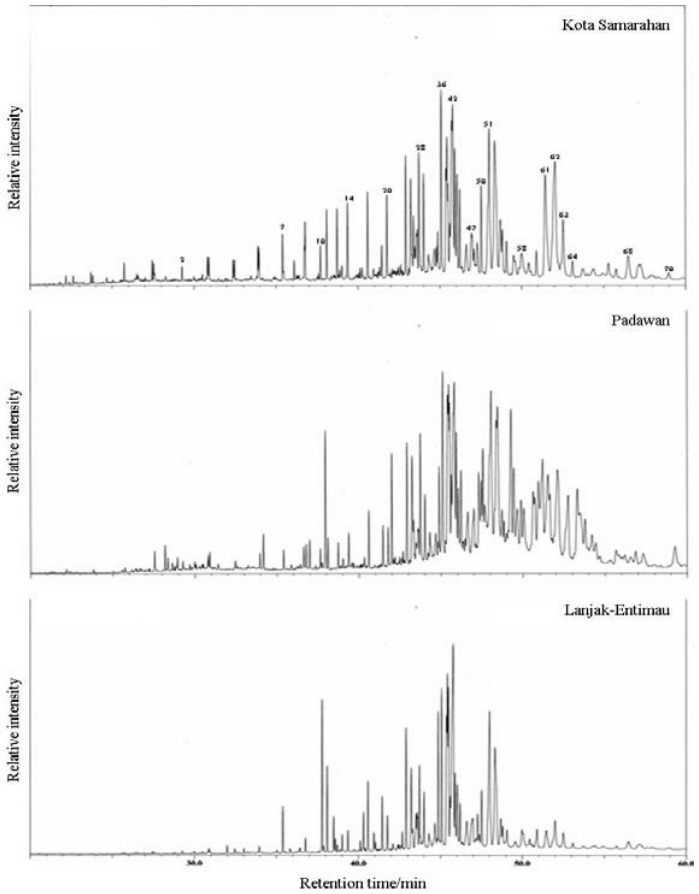

Figure 1. Total chromatogram of GC analysis for cuticular surface lipids of the *Epilacnha indica* from different localities.

Table 1. *n*-alkanes identified in the cuticle of matured *Epilacnha indica*. The data listed the common hydrocarbons detected in all samples examined using GC/MS analysis.

No.	Retention time[b]	Formula Compound[c]	Name of Compound[d,e]	Kota Padawan[a]	Lanjak-Entimau[a]	Kota Samarahan[a]
				[Mean ± Std.]	[Mean ± Std.]	[Mean ± Std.]
1	27.458	$C_{18}H_{38}$	*n*-octadecane	0.70 ± 0.22	0.16 ± 0.02	1.41 ± 0.39
2	29.267	$C_{19}H_{40}$	*n*-pristane	0.59 ± 0.27	0.25 ± 0.02	0.92 ± 0.25
3	30.905	$C_{20}H_{42}$	*n*-eicosane	0.87 ± 0.34	0.34 ± 0.17	1.67 ± 0.75
4	32.470	$C_{21}H_{44}$	*n*-heneicosane	0.59 ± 0.43	0.38 ± 0.22	1.44 ± 0.25
5	33.965	$C_{22}H_{46}$	*n*-decosane	0.65 ± 0.25	0.33 ± 0.18	2.31 ± 0.38
6	35.401	$C_{23}H_{48}$	*n*-tricosane	1.23 ± 0.75	5.17 ± 0.72	1.63 ± 0.84
7	36.778	$C_{24}H_{50}$	*n*-tetracosane	1.11 ± 0.67	0.95 ± 0.29	3.58 ± 0.87
8	38.098	$C_{25}H_{52}$	*n*-pentacosane	2.20 ± 0.61	10.62 ± 1.19	4.91 ± 0.75
9	39.368	$C_{26}H_{54}$	*n*-hexacosane	1.15 ± 0.59	1.92 ± 0.64	5.49 ± 0.76
10	40.592	$C_{27}H_{56}$	*n*-heptacosane	3.14 ± 0.95	8.36 ± 1.14	6.83 ± 0.47
11	41.770	$C_{28}H_{58}$	*n*-octacosane	2.42 ± 0.76	3.39 ± 0.30	6.61 ± 0.37
12	42.910	$C_{29}H_{60}$	*n*-nonacosane	8.56 ± 0.52	15.88 ± 0.76	9.27 ± 0.57
13	44.011	$C_{30}H_{62}$	*n*-triacontane	4.00 ± 1.12	6.29 ± 0.69	7.57 ± 0.69
14	45.076	$C_{31}H_{64}$	*n*-hentriacontane	16.86 ± 0.53	19.86 ± 2.44	15.47 ± 1.71
15	46.213	$C_{32}H_{66}$	*n*-dotriacontane	7.65 ± 1.55	7.08 ± 0.43	7.25 ± 0.51
16	47.527	$C_{33}H_{68}$	*n*-tritriacontane	11.93 ± 1.10	9.04 ± 0.72	8.03 ± 0.67
17	49.103	$C_{34}H_{70}$	*n*-tetratriacontane	4.40 ± 1.76	3.57 ± 1.13	4.74 ± 0.56
18	50.991	$C_{35}H_{72}$	*n*-pentatriacontane	14.43 ± 1.79	4.35 ± 1.29	4.85 ± 0.90
19	53.153	$C_{36}H_{74}$	*n*-hexatriacontane	10.75 ± 1.27	1.50 ± 0.32	2.90 ± 0.56
20	55.768	$C_{37}H_{76}$	*n*-heptatriacontane	2.45 ± 0.84	0.62 ± 0.17	1.78 ± 0.18
21	59.001	$C_{38}H_{78}$	*n*-octatriacontane	4.24 ± 0.81	0.00 ± 0.00	1.52 ± 0.35

[a] Values are the averages for 18 samples of *E. indica* collected from three different localities.

[b] Retention time correspond to the value in the range of -0.015 to +0.020, which was used as a measure of acceptance on the samples data

[c] Formula given based on straight-chained alkanes, C_nH_{2n+2}.

[d] Compounds were determined from GC/MS analysis. The peaks that represent mean less than 0.05% were not included in the data table.

[e] The compounds' name were based on The Pherobase: Database of Insect Pheromones and Semiochemicals by El-Sayed (2013).

Although this study did not included branched chain alkanes in quantitative analysis, qualitative examination on branched chain alkanes in samples collected from three different localities indicated that several compounds showed significant difference in their relative abundance. 3-MeC_{24}, 5-MeC_{25}, 12/14-MeC_{34}, 11-MeC_{25} 13, 17-diMeC_{35} and 11, 15, 23-triMeC_{35} are among branched chain alkanes detected abundantly at least at one of the localities. Furthermore, several unsaturated hydrocarbons including 1-C_{19}ene, 1-C_{20}ene, 1-C_{21}ene, 1-C_{22}ene, 9-C_{23}ene, 1-C_{24}ene, and a few branched chain hydrocarbons such as 7-mehtyalkane and 11-methylalkane were not detected at least in one of the localities.

n-alkanes from C_{18} to C_{38} were detected in 18 samples analyzed, with the exceptions of *n*-C_{37} compounds for Lanjak-Entimau. However, hydrocarbon compounds showed different concentration at different locality. Further examination on gas chromatogram of GC/MS revealed that *n*-alkanes were common for four replicates samples in each locality, but the mean relative proportions of several *n*-alkanes varied significantly. The shorter chain *n*-alkanes between *n*-C_{23} and *n*-C_{31} were the most abundant hydrocarbons in Lanjak- Entimau samples, while longer chain *n*-alkanes ranging from *n*-C_{33} to *n*-C_{38} were abundant in Kota Padawan samples (Figure 2).

Analysis of variance signified that there was a significant difference of proportions of *n*-alkanes between three different localities, except *n*-dotriacontane (*n*-C_{32}) (F=0.352, p-value=0.713>0.05) and *n*-tetratriacontane (*n*-C_{34}) (F=0.930, p-value=0.430>0.05). However, *n*-hentriacontane (*n*-C_{31}) compound were not significant at P>0.01, while other *n*-alkanes show significant different with p value <0.001. The highest variance of *n*-alkanes between the three different localities was *n*-C_{29}, followed by *n*-C_{36} at 95% confident interval with F values were 160.638 and 147.196, respectively.

Significant different of certain *n*-alkanes between Lanjak-Entimau and Kota Samarahan revealed with Tukey's HSD test (Honestly Significantly Different), that is hydrocarbon composition at lower chain length *n*-alkanes ranging from *n*-C_{18} to *n*-C_{26} with p-value <0.01. In contrast, longer chain length *n*-alkanes from *n*-C_{35} to *n*-C_{38} showed a significant different between Kota Padawan and Lanjak-Entimau, with exception of *n*-C_{23} and *n*-C_{25}. Comparison between Kota Samarahan and Kota Padawan samples revealed the significant different in hydrocarbon composition for even-numbered carbon *n*-alkanes ranging from *n*-C_{18} to *n*-C_{38} except *n*-C_{32} and *n*-C_{34}. Several odd-numbered carbon *n*-alkanes such as *n*-C_{25}, *n*-C_{27}, *n*- C_{33}

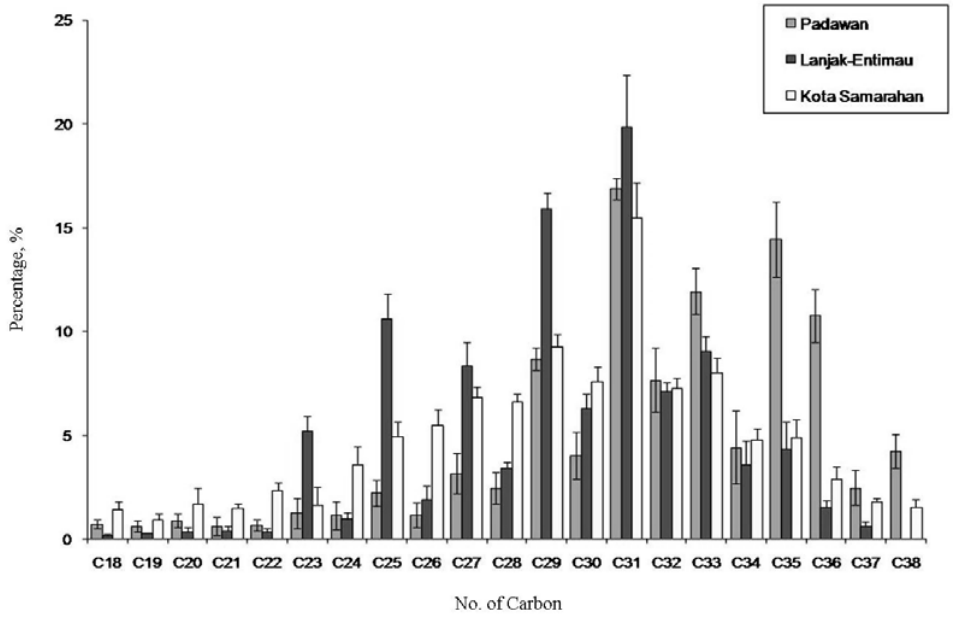

Figure 2. Amounts (%) of *n*-alkanes extracted from *Epilacnha indica* collected from three different localities (n=18). Error bars represent standard deviations.

and *n*-C_{35} also showed significant difference in composition between Kota Samarahan and Kota Padawan.

A discriminant function analysis (DFA) evaluates the pattern of *n*-alkanes abundance between individuals. All variance in the data set was explained by the first two discriminant functions. More than 65% was contributed by the function 1, while 35.2% of variance explained by function 2. Examination on components contributing to the differentiation of localities showed that *n*-C_{29}, *n*-C_{33} and *n*-C_{36} were important in discriminating three different localities although other compounds were found to be significantly different through analysis of variance. DFA successfully classified all samples into three correct groups in 100% of cases, with cross-validation resulted in an error of 7.7%. The pattern of segregation of the populations is graphically represented in Figure 3. This result indicated that insects from a single population are generally similar to each other. Quantitative data from cuticular hydrocarbons of *E. indica* can discriminate samples into their respective group even from small numbers of samples.

Similar to that of DFA, four (4) samples out of six (6) samples from each locality were also used in cluster analysis. Cluster analysis successfully separated the samples into 3 centroid groups. Each centroid group consists of all samples from the same sampling sites (locality). Group 1 showed the samples collected from Kota Samarahan, while group 2 and 3 represent samples from Lanjak-Entimau and Kota Padawan, respectively (see Figure 4). The dendrogram had been successfully constructed using *n*-alkanes proportions. The result implied that the samples examined were placed in respected group. Individuals from each location were grouped in the range of 2.10 - 9.16 % differences, with average of 43% different reflected between localities. Comparison between Kota Samarahan-Lanjak-Entimau samples and Kota Samarahan-Kota Padawan samples showed similarity up to 71.8% and 55.5%, while Kota Padawan-Lanjak-Entimau had only 39.9% similarity. The finding reveals the potential of cuticular hydrocarbons profile to separate subpopulations of species.

DISCUSSION

Distributions of cuticular hydrocarbons in *E. indica* are typical of those commonly found in insects, and there was no unusual compounds detected. No dominant hydrocarbons exceeding 10% of the total hydrocarbons concentration were detected. This profile complimented the report by Kosaki and Yamaoka (1996). However, in their study, they found hydrocarbon ranges from C_{23} to C_{35} in *E. indica*. While in this study, the distribution of cuticular hydrocarbon is in the range of C_{18} to C_{38}. It was probably due to the population from tropical region producing longer carbon chain length to prevent desiccation compare to those in temperate region.

The occurrence of variation in cuticular hydrocarbon compositions of insects was pronounced from various levels. Several studies were also reported that the variation in distribution patterns of cuticular hydrocarbon have shown among individuals of the species (Brown *et al*., 1998; Chapman *et al*., 1995). This study found that *E. indica* samples collected from the forests containing simpler hydrocarbon pattern than samples collected in housing or industrial areas. This shows that differences in micro-environment have influenced the composition and proportion of insect cuticular hydrocarbon. It was suggested that the degree of direct exposure to sunlight and level of humidity could be part of the factor that contribute to the variation between subpopulation.

This study observed that the *n*-alkanes were identical in particular location for each individual population, but the relative concentrations of several *n*-alkanes varied significantly. The differences in *n*-alkanes proportion within population are relatively small where greater differences were observed among different geographical population. The minor quantitative differences among same compound were also shown in other insects (Brown *et al*., 2000). It showed that the differences are from quantitative variation among the same components, although there are qualitative differences among branched alkanes (excluded in the statistical analysis).

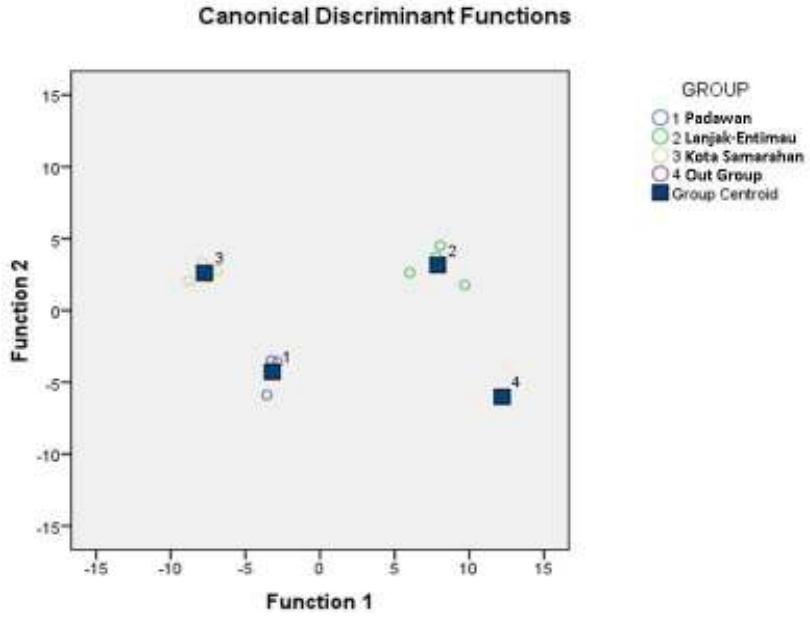

Figure 3. DFA displaying the relative position of 12 samples from three different sampling locations with an adult sample used as out group in the analysis.

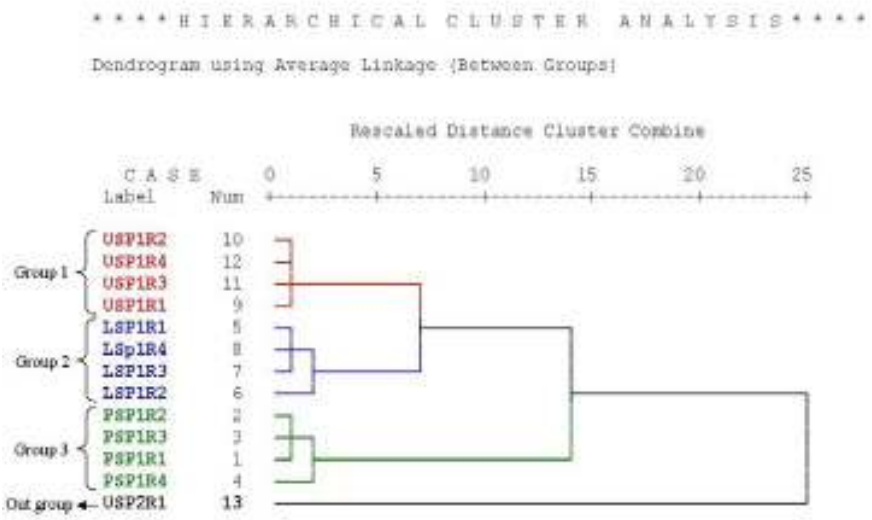

Figure 4. A tree diagram (dendrograms) from a cluster analysis using the maximum 21 *n*-alkanes detected in each sample. The samples were collected from three different sampling locations. USP1R1-USP1R4, LSP1R1-LSP1R4 and PSP1R1-PSP1R4 represent samples of *E. Indica* from Kota Samarahan, Lanjak-Entimau and Kota Padawan, respectively.

Three populations namely Kota Samarahan, Kota Padawan and Lanjak-Entimau, were remarkably different from one another. It seems that the quantitative differences among population of *E. indica* derive from micro-environment factor since each sampling site representing different type of micro-environment. Thus, it affects the level of

evaporation, whereby evaporation of water through cuticle can be expected to be lower at higher humidity or at low temperature. Therefore, smaller amount of cuticular hydrocarbons may be needed to protect insects from desiccation. Noorman and Den Otter (2002) stated that relative humidity and temperature have a temporary effect on the composition of insects' cuticular hydrocarbons.

The result of discriminant analysis also suggests that the hydrocarbon differences in ladybirds' *E. indica* may be due to geographical isolation. Discriminant function analysis (DFA) revealed that cuticular hydrocarbons compounds can be used to identify and group individuals into their own population. Individual from same population would share similar hydrocarbon compounds, but insects collected from different locations posses different cuticular hydrocarbons composition. The variable in cuticular hydrocarbon profiles within a single species has raised questions regarding the ecological meaning of this variability (Howard, 1993). Anyanwu *et al*. (2000) proposed that the patterns of segregation or isolation in hydrocarbons adds to the distinct esterase enzyme characteristic and chromosomal differences, thus providing further information on the differences between the population of species studied.

It may be concluded that cuticular hydrocarbons are primarily genetic control, but the production are affected by environmental factors as a form of adaptation (Frentiu & Chenowelth, 2010). Chapman *et al*. (2000) stated that such variations among individuals occurred within the limit of the character or profile of cuticular hydrocarbon in the species. However, the variations in cuticular hydrocarbon profiles, either in type of compounds or relative concentrations, between subpopulation are still not fully understood. Several researchers such as Brown *et al*. (1998) and Anyanwu *et al.* (2000) suggested that these differences represent geographical variation, and could play as future speciation mechanisms (Phillips *et al*., 1990). For example, geographic population was the major determinant of variation in the melting points of cuticular lipids of grasshoppers (Gibbs *et al.*, 1991). While, Haverty *et al*. (2000) revealed that there is a seasonal variation in the proportion of several hydrocarbons which reflected the behavior of the species.

Though quantitative differences suggested that some genetic variation among geographical variation does exist, the present of sibling species or subspecies may not be sufficiently pronounced. The differences were also found in worm fly *Chrysoma* sp. (Brown *et al*., 1998), cockroach *Macropanesthia* sp. (Brown *et al*., 2000), grasshopper *Schistocerca* spp. (Chapman *et al*., 2000), wasps *Polistes* spp. (Dani, 2006) and others. According to Brown *et al*. (2000), differences in hydrocarbon composition within populations are relatively small, while the differences among different geographical population showed much greater variation. However, the status of quantitative differences, based on proportion of *n*-alkanes compounds between locations, which is up to 43% remains unclear.

CONCLUSION

Variation does occur in the relative abundance of the hydrocarbons in the cuticular lipids of a single species of ladybird beetle. The differences in micro-environment have influenced the composition and proportion of insect cuticular hydrocarbon. If cuticular hydrocarbons are used as a component of taxonomic studies on ladybird beetles, conclusions certainly cannot be based on single specimens, or even on a number of specimens from a single habitat. Therefore, studies on specimens of known taxa from a variety of habitats could draw meaningful conclusions about the usefulness of cuticular hydrocarbons in insect taxonomy, particularly on species complex.

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