Chemical and Genetic Markers for Identification of Honey Origin from Its Bee Speciation

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***Abstract*—The need for accurate and reliable methods for identification of honey origin is important for reducing honey fraud. This study has identified suitable chemical and genetic markers to determine the origin of honey from its bee source of *Apis* honey bees or *Trigona* stingless bee. In the chemical analysis, moisture, fructose, glucose, sucrose, free acidity, and colour intensity were chemical markers identified for differentiating honey by its bee origin. The use of principal component analysis (PCA) and hierarchical cluster analysis (HCA) on honey composition have successfully classified honey into groups of *Apis* and *Trigona*. In the DNA-based method, mitochondrial cytochrome *c* oxidase subunit I (COI) gene was used as a genetic marker to identify honey origin by its bee species accurately from the clear groupings and distinct clusters in phylogenetic trees. The genetic marker of COI gene is accurate and reliable for this identification as it has direct matching to its reference bee species. Incorporating both chemical and genetic markers affirm the identity of honey.**

***Index Terms*—honey, entomological origin, chemical composition, pattern recognition, DNA marker, phylogenetic analysis**

I. Introduction

Food origin is always a concern in food safety as the authenticity of food is a major factor of its originality, purity and quality. It also affects the price setting and leads to issues of food fraudulence and economically motivates adulteration for financial benefits [1], [2]. Honey is considered as high-value food as it has a variety of positive nutritional and health benefits [3]. It is susceptible to fraud because of its strong economic incentives. The common honey fraud includes substitution with high-fructose corn syrup or low-value honey and mislabelling or declaration of a false origin [4], [5]. Chemical analysis is conventionally used for classifying honeys of various origins based on honey composition and properties where multivariate analysis via the pattern recognition method is applied to interpret large datasets for grouping and detection [6]. The growing need for a rapid, reliable, and reproducible test to verify food origin, the DNA-based method has been developed and offers promising solution for food identification through its species origin [7]. The direct identification of genetic variations of a suitable DNA marker or genetic marker facilitates the differentiation of inter and intra-species which allows identification of species or cultivated varieties for food products [8].

The objective of this study was to develop accurate and reliable methods for identifying the origin of honey following common bee source of *Apis* honey bees or *Trigona* stingless bee using chemical analysis and DNA-based method. The key chemical markers for classifying honeys from bee origin of *Apis* or *Trigona* were identified and the ability of COI gene to serve as genetic marker for identification of honey origin was justified.

II. Materials and Methods

A. Honey Samples

The common types of raw honey in Malaysia, named as *Tualang*, Pineapple, *Borneo*, and *Kelulut* which were produced by either the *Api*s honey bees or *Trigona* stingless bee were obtained as samples. The bee origin of the honey samples was identified by the professional bee collectors or bee keepers (Table I). The *Apis* honey bees consist of three different species which are the *Apis dorsata*, *Apis mellifera*, and *Apis cerana* while for the *Trigona* group, it is only the *Heterotrigona itama* stingless bee.

TABLE I. Honey Samples from Different Bee Sources

Bee origin	Honey type	Bee species
Apis (*n* = 6)	*Tualang* (*n* = 2)	*Apis dorsata*
	Pineapple (*n* = 2)	*Apis mellifera*
	Borneo (*n* = 2)	*Apis cerana*
Trigona (*n* = 3)	*Kelulut* (*n* = 3)	*Heterotrigona itama*

n: number of samples

B. Chemical Analysis

Fifteen honey compositions including moisture content, ash content, protein content, sugar content of fructose, glucose and sucrose, hydroxymethylfurfural content, diastase activity, pH, free acidity, electrical conductivity, colour parameters of *L**, *a** and *b**, and colour intensity

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were measured and analysed. All the parameters were determined following the Harmonised Methods of the International Honey Commission [9] and AOAC Official Methods of Analysis [10] except for colour (*L**, *a** and *b**) which was determined using a Hunter Lab spectrophotometer (UltraScan PRO, Hunter Associates Laboratory, Inc., VA, USA) with D65 illumination, diffuse/8 ° geometry, and 10 ° observer. Colour intensity was determined using the method of Beretta et al. [11] which was based on the net absorbance between 450 nm and 720 nm with a UV-VIS spectrophotometer (Ultrospec 3100 pro, Amersham Biosciences, NJ, USA).

All analyses were performed at least in duplicate and the data were presented as mean ± standard deviation. Data analysis was performed using Statistica for Windows (Version 10, Statsoft, Inc., Tulsa, OK, USA). The significant differences between the groups of *Apis* and *Trigona* for each honey composition were performed by Mann-Whitney test. Unsupervised pattern recognition techniques including principal component analysis (PCA) and hierarchical cluster analysis (HCA) were used to classify and differentiate samples from different bee origin.

C. DNA-based Method

DNA from honey was extracted using DNeasy® *mericon*™ Food Kit (Qiagen, Hilden, Germany) following manufacturer's instructions. The mitochondrial COI gene region was amplified from the extracted DNA using the primers of COI-300F and COI-300R [12]. PCR was performed in a final volume of 50 µL containing 1 × PCR buffer (MyTaq™ Mix 2 ×, Bioline, London, UK), 1–200 ng of DNA template, and 0.4 µM of each primer. The amplified PCR product was sent for sequencing. The identity of partial sequences of COI obtained were determined using NCBI nucleotide BLAST. Phylogenetic tree was generated using the neighbour-joining method with Kimura 2-parameter evolution model for 1,000 bootstrap replications in MEGA program, version 6 [13].

III. Results and Discussion

A. Identification by Chemical Markers

Table II summarises the composition of the honey samples grouped following the bee origin of *Apis* and *Trigona*. These 15 honey composition are commonly included to establish the identity and for the quality control of honey [14], [15]. The moisture content, fructose content, glucose content, sucrose content, free acidity, and colour intensity were found to be significant ($p < 0.05$) for the differentiation of honeys between *Apis* and *Trigona* bee species. PCA and HCA analyses were performed considering these six variables.

Two principal components were fitted by 10-fold cross-validation that explained the 88.86% (PC1) and 7.14% (PC2) of total variance. Fig. 1 shows that the nine honey samples clearly differentiated into groups of *Apis* and *Trigona*. The *Kelulut* honey samples from *Trigona* stingless bee showed positive PC1 which had highest positive loadings of moisture content, sucrose content, free acidity, and colour intensity on PC1 (Fig. 2). Fructose and glucose contents were the variables with greatest negative loadings associated to *Apis* bee honeys that included *Tualang*, Pineapple and *Borneo* on PC1 (Fig 2). It shows that the moisture, fructose, glucose, sucrose, free acidity, and colour intensity are possible parameters for use when classifying honeys to their bee origin.

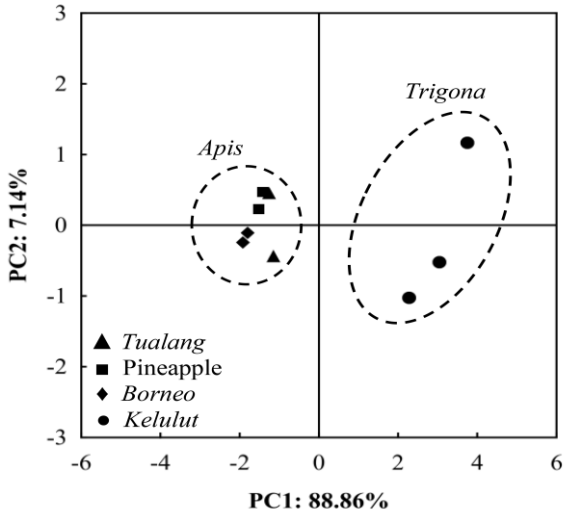

Figure 1. PCA score plot showing the classification between honey samples following its bee origin

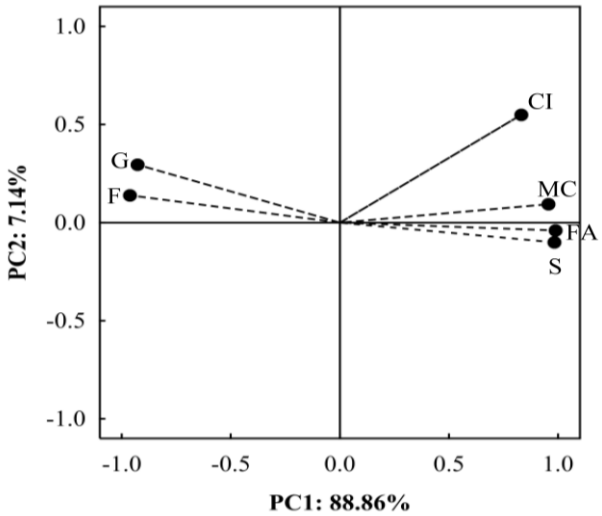

Figure 2. PCA loading plot of six variables, MC: moisture; F: fructose; G: glucose; S: sucrose; FA: free acidity; CI: colour intensity

In line with the PCA results, the dendrogram of HCA in Fig. 3 shows that the honey samples are grouped into two major classes for the *Apis* and *Trigona* on the basis of similarities. The smaller the value of linkage distance, the higher similarity occurs between the samples. It is suggested that the differences in multivariable of moisture, fructose, glucose, sucrose, free acidity, and colour intensity in honeys produced by *Apis* honey bees and *Trigona* stingless bee contributed to this separation. These six properties are suggested as markers for classification and differentiation of honeys from the PCA and HCA analyses.

TABLE II. COMPOSITION OF HONEY FROM DIFFERENT BEE ORIGIN

Parameter	*Apis* (n = 6)	*Trigona* (n = 3)	p-value
Moisture (g/100 g)	24.05 ± 2.17	33.24 ± 2.93	< 0.05
Ash (g/100 g)	0.14 ± 0.09	0.08 ± 0.02	> 0.05
Protein (g/100 g)	0.51 ± 0.14	0.85 ± 0.47	> 0.05
Fructose (g/100 g)	42.30 ± 2.59	15.77 ± 2.68	< 0.05
Glucose (g/100 g)	34.57 ± 6.18	9.22 ± 2.70	< 0.05
Sucrose (g/100 g)	0.25 ± 0.61	32.30 ± 2.38	< 0.05
HMF (mg/kg)	14.78 ± 8.78	23.42 ± 19.79	> 0.05
Diastase (DN)	2.85 ± 1.05	2.12 ± 0.20	> 0.05
pH	3.56 ± 0.31	3.26 ± 0.17	> 0.05
Free acidity (meq/kg)	50.4 ± 5.4	136.8 ± 8.8	< 0.05
EC (mS/cm)	0.70 ± 0.26	1.08 ± 0.43	> 0.05
L^*	26.56 ± 0.85	24.90 ± 1.58	> 0.05
a^*	1.64 ± 0.52	1.90 ± 0.55	> 0.05
b^*	3.07 ± 0.46	2.52 ± 1.21	> 0.05
CI (mAU)	477.6 ± 107.8	990.3 ± 438.7	< 0.05

HMF: hydroxymethylfurfural; EC: electrical conductivity; CI: colour intensity.

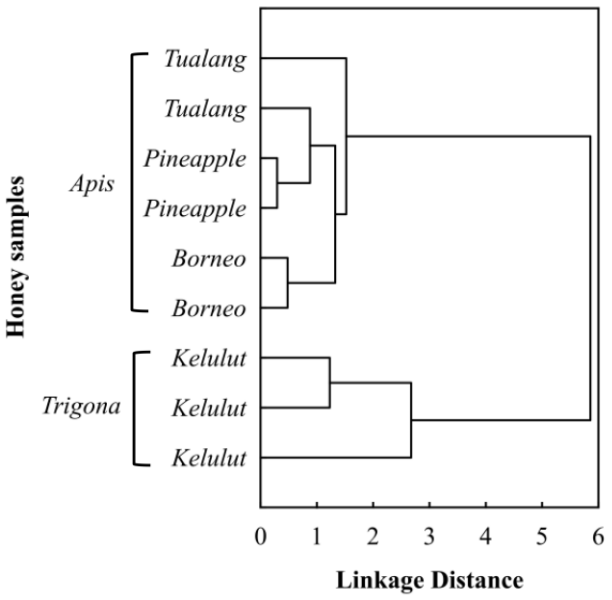

Figure 3. HCA dendrogram of honey samples from different bee origin

B. Identification by Genetic Markers

The phylogenetic tree constructed from the COI gene sequences shows that all the honey samples are correctly grouped to their belonging bee species into the same cluster (Fig. 4). The segregation defined by each bee species was supported with a high bootstrap value of 99–100%. The *Tualang*, Pineapple, *Borneo*, and *Kelulut* honeys were identified from bee sources of *Apis dorsata*, *Apis mellifera*, *Apis cerana*, and *Heterotrigona itama*, respectively. Two major groups were observed in the phylogenetic tree where *Heterotrigona itama* from *Trigona* stingless bee was grouped distantly from the *Apis* group which included *Apis dorsata*, *Apis mellifera*, and *Apis cerana* (Fig. 4). The genetic marker of COI gene is capable of differentiating honeys from the different bee species and tracing the honey origin to its bee source accurately. Phylogenetic result by DNA-based analysis is similar to the classification results generated by chemical pattern recognition methods with PCA and HCA. Although chemical and genetic methods are two rather distinct analyses, their results are in agreement with each other suggesting the use of suitable markers enable classification and identification of honey origin.

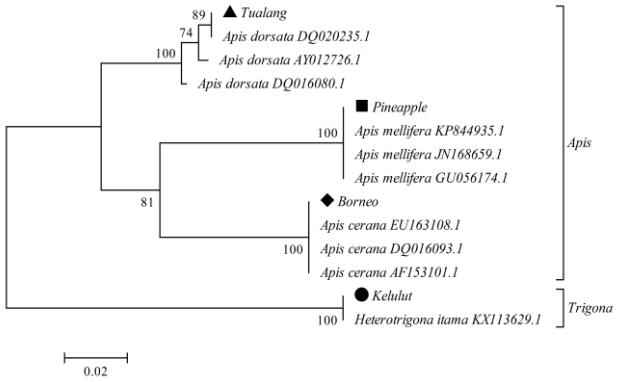

Figure 4. Phylogenetic tree of COI gene partial sequences from honey samples and NCBI GenBank sequences of closely related bee species constructed using neighbour-joining method and Kimura 2-parameter model. Bootstrap values of 1000 replicates are shown next to the branches

IV. CONCLUSION

Honeys were successfully classified into two major groups of bee origins, *i.e.* the *Apis* honey bees or *Trigona* stingless bee by using chemical markers via PCA and HCA analyses and subsequently reinforced with DNA-based method using genetic marker of COI gene via phylogenetic analysis. The use of suitable markers has presented a reliable and novel method for identifying honey origin via its bee species instead of by its floral/nectar origin. These identified chemical and genetic markers can be used synergistically for tracing honey origin and source.

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