A DNA BASED METHOD TO DETERMINE THE ORIGIN OF HONEY PRODUCED BY *APIS CERANA*

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Abstract

The value of a product is derived from various components, such as its functionality, availability on the market, origin, raw material, exclusivity; in case of food products not least taste and health effects. In this approach honey has an outstanding position because of its reputation in the food and health industry. The honey of the Asian honey bee is unique and of great value, and because of this it is often prone to adulteration. Asian honey is restricted to those countries where the Asian honey bee is endemic. Beekeeping with this species of bee in these areas is part of cultural tradition. The idea of this work is to design a fast and accurate detection method

for adulteration, in the interest of preserving the value of the honey produced by the Asian honey bee (*Apis cerana*). The purpose of the study was to point out the genetic differences between two close honey bee species (*A. cerana* and *A. mellifera*) and to use mitochondrial loci to develop a molecular method to confirm the entomological origin of honey deriving from a Cerana apiary. The markers designed besides being suitable per se can be adopted as a part of a genetic analysis panel.

Keywords: *Apis cerana*, ethnic honey, molecular marker, honey authenticity, adulteration

Összefoglalás

Egy termék értékének megállapításában számos tényező részt vesz. Ilyen paraméterek az adott produktum piaci felhasználhatósága, elérhetősége, eredete, alapanyagának minősége, exkluzivitása. További kritériumok merülnek fel élelmiszeripari termékek esetében, mint például az ízvilág és az egyre népszerűbb egészségre gyakorolt hatás. Ebből a megközelítésből a méz kiemelkedő helyet foglal el, mivel elismerten jelentős termék az élelmiszeripar és az egészségügy szempontjából egyaránt. Az ázsiai méh (*Apis cerana*) méze egyedülálló és különlegesen értékes, és emiatt gyakran van kitéve hamisításnak. Az ázsiai méh által termelt méz korlátozottan elérhető, leginkább csak azokban az országokban, ahol ez faj endemikus. Az ázsiai méh első sorban Kínában, Szibériában, Indiában és Japánban terjedt el. Ezeken a területeken a méhészkedés hozzátartozik a kulturális hagyományokhoz. Ezen munka középpontjában a keleti méh által termelt méz értékmegóvása áll, egy olyan módszer fejlesztése által, ami gyors és pontos módja a hamisítás kimutatásának. A tanulmány célja két közeli rokon méhfaj (*Apis cerana* és *Apis mellifera*) genetikai különbségeinek kimutatása és mitokondriális lókuszok segítségével olyan marker fejlesztése, aminek segítségével kimutatható a méz keleti

méhtől való származása. Mindamellett, hogy önmagukban is használhatóak a tervezett primer párok, beilleszthetőek már meglévő genetikai vizsgálati panelekbe is.

Kulcsszavak: *Apis cerana*, helyi méz, molekuláris marker, méz eredetiség, hamisítás

Introduction

Currently, food fraud is an increasing and spreading problem, causing economic loss, and nutritional drawbacks, and because of this, it may lead to health consequences. In a narrower interpretation, Economically Motivated Adulteration (EMA), is intentional fraud for a financial gain (Everstine et al., 2013). The most popular articles of food exposed for adulteration are olive oil, milk and honey (Moore et al., 2012). According to the Codex Alimentarius FAO (2001), honey is a sweet viscous fluid obtained from the nectar of flowers or from secretions of living parts or excretions of plant-sucking insects on the living parts of plants, and produced in the honey sac of bees. Being a valuable, high-quality food with many health benefits, honey is a product that is often subjected to adulteration, through mixing with cheaper honey, or sugars, or even mislabeling the product. Despite being unethical this can also be harmful to human health. The adding of different foreign matters, colorants, aroma may lead to hyperglycemia, causing in many cases type II diabetes, obesity, hypertension (Ajibola et al., 2013). Moreover the added alduterants of unknown origin can have harmful effects on organs (Shapiro et al., 2008; Soares et al., 2017) and may even cause death by increasing visceral fat and total body fat (Samat et al., 2017).

There are several methods for adulteration, which mainly focus on the chemical composition of the honey. Consisting mainly of carbohydrates and water (Bogdanov, 2009), the most obvious way of EMA is the mixing of different sugars in the honey, this way increasing the volume, but lowering the quality. The sugar profile of honey can be analyzed with Gas

Chromatography (GC) and Liquid Chromatography (LC); this way a doubtful origin can be detected (Ruiz-Matute et al., 2010). Another technique for evaluating honey adulteration is Near Infrared Transflectance Spectroscopy, showing beet invert and corn syrup residues in honey (Zhu et al., 2010). The Fourier transform infrared (FTIR) spectroscopy and attenuated total reflection (FTIR-ATR) approach is an effective method for detecting the botanical origin of the honey, making the classification possible based on melissopalynological data (Svečnjak et al., 2015). Another important characteristic of honey is its protein profile. The level of protein in honey highly depends on the type of flora and hence it is variable. Honey of different plant origin can be tracked by their pollen protein content, using it as a chemical marker. Silver staining SDS-PAGE is an analytical method for identifying and isolating protein molecules from pollens (Won et al., 2008).

A current diagnostic method used in honey analysis for food control is DNA based techniques. PCR based methods use exponential amplification of target-specific DNA and a signal can be detected only if the target DNA is present. In the case of honey analysis, this applies to the genetic material of the honey bee and floral DNA as well (Laube et al., 2010). HRM (High-Resolution Melting) is adequate in the case of honey samples because it allows genotyping and serotyping of pathogenic microorganisms and detection of food allergens (Druml and Cichna-Markl, 2014).

For identifying the origin of the honey not only its botanical origin has to be examined, since entomological authentication can provide valuable information about the product (Kek et al., 2017; Soares et al., 2017; Utzeri et al., 2018). Recently, a real-time PCR method using the tRNAleu-cox2 intergenic region was designed by Soares et al. (2018) to distinguish between species *A. cerana* and *A. mellifera*.

The Asian honey bee produces a significant amount of honey too Hisashi (2010). In comparison to the European honey bees the collected amount is lesser, but its characteristics justify its importance on the honey market (Partap and Verma, 1998; Verma, 1990). According to the study of Won et al. (2009) the price of the honey produced by the indigenous honey bees in Asia can be up to five times higher than the one produced by *A. mellifera.* However, from the beginning of the 20th century, the western honey bee started to spread on the continent, thanks to beekeepers who imported the species in the hope of a greater profit (Partap and Verma, 1998). Because of this, the western honey bee poses a threat to the endemic species, causing the decline of *A. cerana* colonies, potentially leading to extinction in its native environment (He et al., 2013; Partap and Verma, 1998; Theisen-Jones and Bienefeld, 2016). Although the European honey bee shows a more effective foraging behavior, the Asian honey bee has beneficial traits that make them valuable for beekeepers in the East. The imported species *A. mellifera* integrated itself instantaneously in the new environment, especially the Himalayan region, but fortunately beekeepers in Nepal and Bhutan still prefer to opt for the endemic species, *A. cerana* being suitable for beekeeping in the mountains (Partap and Verma, 1998).

Moreover, due to its natural *Varroa* resistance, *A. cerana* requires no treatment with acaricides, meaning that the honey will not be exposed to miticide related chemical residues. *A. cerana* honey is sometimes considered to be of superior quality compared to that of *A. mellifera*, especially in parts of China and India, with increasing demand (Abrol, 2013; Hu, 2015 and Puttaraju, 2015, personal communication). *A. cerana* colonies can also be reproduced and scaled up with very little additional input: strong colonies can be divided easily (Abrol, 2013). Because of the higher quality and price, honey produced by Eastern honey bees is potentially exposed to EMA on a global scale. Our aim was to preserve the value of Cerana honey, by

developing a fast and cost-effective molecular, PCR-based detection method, which can be a useful tool against food fraud, causing serious economic damage.

Material and method

Sample collection

Honey from two distant points of the distribution area of the Asian honey bee was used, collected in Japan (apiary at Tamagawa University, Japan) and India (organic apiary Uttarkhand, India). Japanese honey and honey bee were collected in a private apiary while the certified organic honey produced by the Himalayan subspecies of *A. cerana* derived from an apiary in India. For negative control honey of western honey bee was used (Hungary, private apiary). We used honey bee individuals of each species as the positive control.

DNA extraction

Extraction of DNA was preceded by dissection of the honey bees. *A. mellifera* and *A. cerana* bee individuals were stored frozen at -70°C right after sampling respectively preserved in RNA later. For DNA extraction, we used muscle tissue from hind legs bees using QIAGEN DNeasy Tissue Kit (QIAGEN GmbH, Hilden, Germany) following the manufacturer's protocol. Honey samples needed preprocessing before extracting total DNA. Therefore 5 grams of honey has been measured from each sample. Tubes containing the honey were filled with 45 ml distilled water and put in 40°C water bath for 30 minutes with constant shaking. Subsequently, we centrifuged the tubes at 4,000 rpm for 30 minutes. Supernatant was discarded and the pellet was used for the DNA extraction, using the kit above, according to the manufacturer's instructions. Yield and purity of the extracts were determined by spectrophotometry (260/280 nm ratio and

absorbance spectrum) using NanoDrop™ 2000/2000c Spectrophotometer (Thermo Scientific™, Waltham, MA, USA).

Primer design and PCR amplification

To detect the genetic differences between *A. cerana* and other Apis species primers were designed to target a part of the *cytb* region of two honey bee species. The sequences used were downloaded from GenBank and are shown in an additional table (*Table 1.*).

Table 1. Accession numbers of the A. mellifera and A. cerana honey bee sequences used for primer design.

Apis mellifera	*Apis cerana*
EF184057.1	FJ229480.1
EF184058.1	FJ229478.1
GU979492.1	FJ229476.1
EF184041.1	FJ229475.1
EF184043.1	FJ229473.1
EF184042.1	FJ229471.1
JQ778297.1	EF467437.1
JQ778298.1	EF180095.1
JQ778299.1	F180094.1
JQ778301.1	F180093.1
JQ778300.1	EF180092.1
JQ778302.1	EF180091.1
JQ778303.1	EF180090.1
EF184046.1	
EF184047.1	
GU979494.1	

The *cytb* sequences were aligned and compared using BioEditv. software, version 7.0.4. (Hall, 1999) to detect species-level differences. Primers were designed using the Primer3web version 4.1.0. primer designer website. Two *Apis cerana* species-specific primer pairs (Acer_short forward 5'-TGAGGTGCAACAGTAATTACAAATTTAC-3', Acer_short reverse 5'-ACTTCACTTTATTTTACCTTTAGTAATT-3'; Acer_long forward 5'-TGAGGTGCAACAGTAATTACAAATTTAC-3', Acer_long reverse 5'-

ATAATTAATTTTCAATATCCTTATTATTT-3') were meant to result in different fragment lengths. The primer pair indicated as 'short' resulted in a 143 bp amplicon, whilst the 'long' one generated a 300 bp product.

Reactions were performed in Rotor-Gene Q 5Plex HRM (Qiagen, Germany) appliance, using the following cycling profile: initial denaturation at 95 ºC for 3 min; 40 cycles at 95 ºC for 30 s, 55ºC for 60 s, 72 ºC for 60 s and 72ºC for 10 min for final extension. The PCR-mixture (Thermo Scientific™, Waltham, MA, USA) contained5 µl 10X DreamTaq Green Buffer, 1 µl of forward and reverse primers, 5 µl dNTP Mix, 1 µl DreamTaq DNA Polymerase, 36 µl nuclease-free water, 2 µl DNA template in a total volume of 50 µl.

The amplified fragments were visualized in a 1% agarose gel containing Ethidium bromide 1x (Merck, Germany) for staining and carried out in TBE 1x (Duchefa, Netherlands) Agarose Electrophoresis Buffer. The fragments were chemically purified using the QIAquick GelExtraction Kit (Qiagen, Germany) according to the protocol of the manufacturer and 10 µl was loaded into the wells of the gel per each sample. The results were confirmed by Sanger sequencing on 3130 Genetic Analyzer (Thermo Fisher Scientific, USA) using the same primers listed above.

Results

DNA extraction

The honey samples were of different floral origin, thus containing large amount of plant DNA. Consequently, we had to extract a greater amount of DNA, to ensure it includes residues from the honey bee. The quantitation of the extracts resulted in yield between 11 and 128 ng/µl with a purity ranging from 1.9 to 2.2, which was proved to be adequate.

Primer design and PCR amplification

Using the primer pairs designed, the expected mitochondrial region of *cytb* gene was successfully amplified in the *A. cerana* bee and honey sample as well. The primer pair Acer_short resulted in an amplicon of the size of 143 bp, while Acer_long primers exhibit a band on the gel of the size of 300 bp. This fragment seemed unreliable for detection, as the fragment is barely visible on the gel electrophoresis image, thus we did not use it in further reactions. In case of the *A. mellifera* bee and honey samples, which were used as negative controls, no amplification was detected (*Fig. 1*).

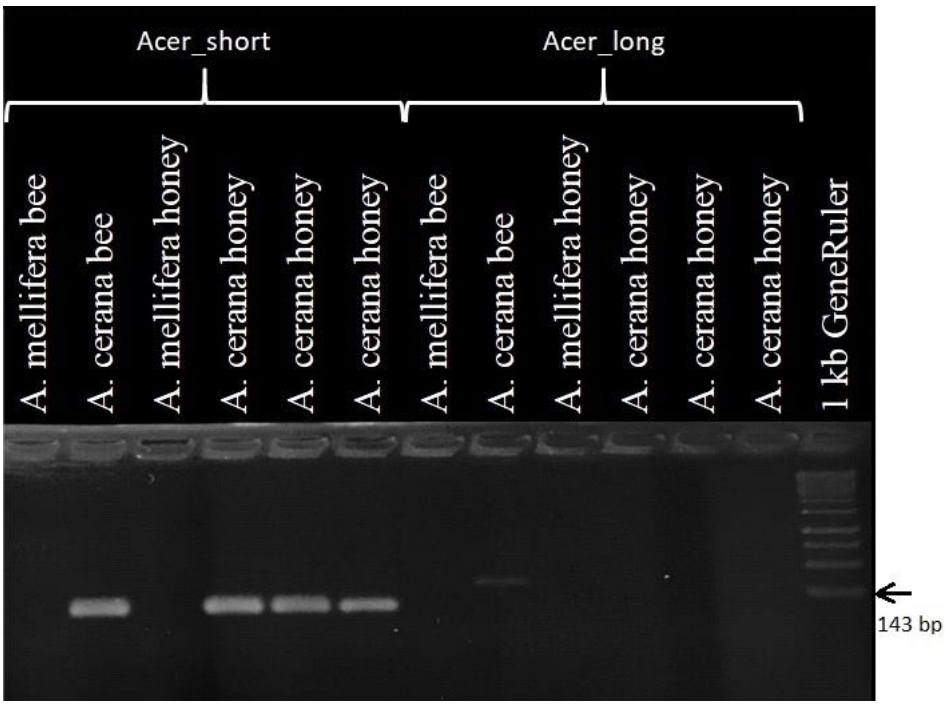

Figure 1 The gelelectrophoresis image of the fragment samplified with the primer pairs designed.

Discussion

The amount of the honey bee's DNA in honey depends mostly on beekeeping and honey extraction technology. Technology applied for western honey bee uses honey extractors leaving the honeycomb undestroyed, thus being recyclable. In case of the eastern honey bee,

honeycomb is removed from the hive and honey can be extracted by crushing and straining (Reuber, 2015). This implies that honey of *A. cerana* may contain an increased amount of DNA coming from larvae and pupae tissue.

Soares et al. (2018) used the $tRNA^{leu}$-cox2 intergenic region to differentiate between the two honey bee species concerned. A recent study focused on the entomological differences between Apis species as well, using qualitative end-point PCR using EPIC markers coupled with specific fragment restriction (Moškrič et al., 2020) In addition to this, our group had focused on *cytb* mitochondrial gene. A completion to the previous study a confirmatory application was designed to a possible further profiling panel. Meta-barcoding of mixed pollen samples has revealed that multi-locus approach is more reliable than single-locus analyses (Sickel et al., 2015). Our primers resulting the short amplicon proved to be accurate, it amplified the target region, and we did not detect any non-specific fragments in honey produced by *A. mellifera* honey bees. The long amplicon cannot be used as a confirmatory marker, we can assume, that DNA in honey is mostly fragmented and the chance of longer fragments deriving from honey bees remaining in the samples is low, DNA of bee origin representing the lesser portion of the nucleic acid content of the honey DNA. Despite resulting in a fragment in case of the bee samples, long amplicon primers yielded in variable amount and inconsequently in honey.

Conclusion

The development of a simple method for the detection of *Apis cerana* honey in mixed honey samples was successful. This novel DNA marker may be used in a multiplex PCR system with previous primers designed for the same reason but targeting other loci. It allows confirming the entomological origin of the honey to protect this unique product. PCR amplification being

exponential even for samples of small and degraded DNA content as DNA based methods can be highly sensitive.

In practice the method provides quick and reliable information about Asian honey bee derived honey exposed to fraudulent procedures, this way it may prevent beekeepers from economic damage.

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