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## Novel diagnostic tools for Asian (*Apis cerana*) and European (*Apis mellifera*) honey authentication

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### ABSTRACT

Honey can be produced by different species of honeybees, with two being of economic importance due to their use in apiculture, namely *Apis mellifera* (known as European honeybee) and *Apis cerana* (known as Asian honeybee). Due to the decline of the wild populations of the Asian honeybee, this honey generally attains much higher market value, being prone to adulteration. This work aims at proposing new tools, based on the use of molecular markers, for the entomological authentication of honey. To this end, new species-specific primers were designed targeting the tRNA<sup>Leu</sup>-cox2 intergenic region and allowing the detection of *A. cerana* DNA by qualitative polymerase chain reaction (PCR). Additionally, a novel real-time PCR method with high resolution melting analysis was developed to target the 16S rRNA gene of both bee species, allowing their discrimination in different clusters. The proposed methodologies were further applied with success in the authentication of Asian and European honey samples by the identification of honeybee DNA, demonstrating the usefulness of these simple and cost-effective new approaches.

### 1. Introduction

According to the standards of the *Codex Alimentarius* (FAO, 2001), honey is the natural sweet substance produced by honeybees. While the European Union legislation stipulates that honey should be produced by *Apis mellifera* bees, in other parts of the world, honey is traditionally obtained from different honeybee species. *Apis mellifera*, also known as the Western or European honeybee, is the only species native to Europe, being also naturally present in Africa and in the Middle East (Ruttner, 1988). However, the genus *Apis* comprises eight more species, all indigenous to Asia, including two dwarf bees, two giant bees and four cavity-nesting bees (Radloff, Hepburn, & Engel, 2011). Behavioural limitations of the dwarf and giant honeybees, particularly their practice of open-air nesting and seasonal migrations, prevent them from being kept commercially by man (FAO, 1990; Robinson, 2012). In contrast, the honeybee species that naturally nest inside cavities can be kept in man-made hives, enabling the colonies to be manipulated for long periods and also being moved between places for foraging. Among the cavity-nesting species, only *A. mellifera* and *Apis cerana* (also known as

the Asian honeybee or the Eastern honeybee) are of economic importance due to their use in apiculture. Until recently, these two closely related species had an allopatric distribution, with *A. cerana* naturally occurring in the East, Southeast and South Asia (Radloff et al., 2011; Ruttner, 1988). However, more than one century ago, human action has introduced *A. mellifera* into much of the geographic range of *A. cerana* (Ruttner, 1988). Since *A. mellifera* is much more productive, it has been introduced and extensively propagated by beekeepers in some regions of Asia, such as in Japan, South Korea, Vietnam, and the Himalayas (Partap & Verma, 1998; Verma, 1990). In some countries where these species are currently sympatric, there is sufficient niche partitioning and both can co-occur successfully (Koetz, 2013). Nevertheless, in other countries, such as Japan and China, there are concerns about the extent of competition between the two species, resulting in the decline of the wild population of *A. cerana* (He et al., 2013).

Due to the production decline of *A. cerana* honey and to a growing demand for local and traditional products, this honey has attained higher market prices than that produced by *A. mellifera*, generally being three- to five-times more expensive (Won, Li, Kim, & Rhee, 2009).

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Although the hive products obtained from *A. cerana* have been, at some extent, commercially neglected for decades, currently, the native Asian honeybee is regarded as an important and valuable genetic resource to preserve and its honey is being increasingly valued. For this reason, *A. cerana* honey is very prone to adulteration either by the admixing with cheaper *A. mellifera* honey or by its total substitution. Therefore, there is a pressing need to develop analytical methodologies for the specific and unequivocal identification of honeybee species, aiming at authenticating the *A. cerana* honey.

While numerous works have focused on the botanical and/or geographical authenticity of honey (Laube et al., 2010), only a few have attempted its entomological authentication (Soares, Amaral, Oliveira, & Mafra, 2017). To differentiate honey produced by stingless bees of different genus of the Meliponini and Trigonini tribes, Vit, Fernandez-Maeso, and Ortiz-Valbuena (1998) suggested the use of concentrations and ratios of three frequently occurring sugars (fructose, glucose and maltose), with particular emphasis on maltose because it is not directly originated from the nectar, but from transglycosylation from enzymatic bee action. Ramón-Sierra, Ruiz-Ruiz, and de la Luz Ortiz-Vázquez (2015) suggested the use of the protein profile obtained by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for determining the entomological origin of honeys produced by *A. mellifera* and by stingless bees (*Trigona* spp. and *Melipona beecheii*). Protein-based methods were also proposed by Won et al. (2009) to detect *A. cerana* honey adulterated with that produced by *A. mellifera*.

DNA-based methods have been considered as the most suitable tools for unequivocal species identification in animal products and processed foods (Amaral, Meira, Oliveira, & Mafra, 2016). Compared to protein-based methods, they present several advantages such as the higher stability of nucleic acids and their ubiquity in every type of cells. Recently, Prosser and Hebert (2017) suggested the use of metabarcoding to examine three gene regions to identify the botanical and entomological origins of honey. These authors screened 6 samples of honey produced by *A. mellifera* and 1 by *Melipona beecheii*. The identification of bee species DNA on honey samples was also achieved by Kek, Chin, Tan, Yusof, and Chua (2017) based on mitochondrial DNA (mtDNA) sequences and phylogenetic analysis by means of forensically informative nucleotide sequencing. However, both reports rely on extensive sequencing analysis, which is not a cost-effective and high-throughput approach. In contrast, the use of polymerase chain reaction (PCR) with species-specific primers is one of the most used approaches for authentication purposes due to its low cost, simplicity, fastness, high specificity and sensitivity, enabling species identification even in complex and processed foods (Amaral et al., 2015; Amaral et al., 2016). Real-time PCR with high resolution melting (HRM) analysis can be regarded as a cost-effective alternative and reliable tool to exploit DNA barcoding because it does not require further sequencing analysis (Fernandes, Costa, Oliveira, & Mafra, 2017). Therefore, the aim of this work was to develop molecular-based approaches, namely species-specific PCR and real-time PCR with HRM analysis, for the entomological identification/authentication of honey. To that end, several honeybee specimens of the two most representative species in apiculture (*A. cerana* and *A. mellifera*) were used for method development.

## 2. Materials and methods

### 2.1. Samples

Voucher specimens of *A. cerana* individuals from three Asian countries, including China ( $n = 7$  colonies), Thailand ( $n = 3$ ) and Vietnam ( $n = 6$ ), and honeybee individuals of five different *A. mellifera* subspecies representative of European honey production [*A. m. iberiensis* of African (A) mtDNA evolutionary lineage from Portugal ( $n = 24$ ) and Spain ( $n = 5$ ), *A. m. iberiensis* of western European (M) lineage from Spain ( $n = 7$ ), *A. m. mellifera* M lineage from France ( $n = 5$ ), *A. m. carnica* C lineage from Croatia ( $n = 1$ ) and Serbia

( $n = 3$ ), and *A. m. ligustica* C lineage from Italy ( $n = 4$ )] were used in this work (see Chávez-Galarza et al., 2017 for further details on *A. mellifera* evolutionary lineages). All honeybee individuals were collected from distinct colonies and apiaries and their tissues stored in absolute ethanol at  $-20\text{ }^{\circ}\text{C}$ .

Authentic honey samples were collected by beekeepers directly from the hives, namely honey produced by *A. cerana* from Vietnam ( $n = 2$ ), and honey produced by *A. mellifera* from Portugal ( $n = 2$ ) and Vietnam ( $n = 1$ ). Additionally, commercial honeys ( $n = 3$ ) acquired from different European countries were tested.

### 2.2. DNA extraction

Honeybee tissues were homogenised in reaction tubes, stored at  $-20\text{ }^{\circ}\text{C}$  overnight and submitted to DNA extraction using the Wizard method, as described by Mafra et al. (2008). Additionally, for sequencing purposes, total DNA was extracted with the phenol/chloroform/isoamyl alcohol (25:24:1) method (Sambrook, Fritsch, & Maniatis, 1989) from the thorax of honeybee individuals (namely for *A. m. mellifera*, *A. m. carnica* and *A. m. ligustica*).

Regarding honey samples, prior to DNA extraction, a pre-treatment was initially performed to discard possible interferences. Briefly, a total of 20 g of each honey was frozen ( $-80\text{ }^{\circ}\text{C}$ ) and subsequently diluted in 45 mL of distilled water. The mixture was heated to  $45\text{ }^{\circ}\text{C}$  for 5 min, homogenised and centrifuged at  $18,500 \times g$  for 25 min. Then, the supernatant was discarded, the pellet was suspended in 1 mL of distilled water and again centrifuged at  $17,000 \times g$  for 10 min. The supernatant was discarded and the pellet stored at  $-20\text{ }^{\circ}\text{C}$  until DNA extraction.

DNA from honey pellet was extracted using the commercial kit NucleoSpin® Plant II (Macherey-Nagel, Düren, Germany), according to the manufacturer instructions with some minor modifications, as described by Soares, Amaral, Oliveira, and Mafra (2015). All extractions were performed in duplicate assays for each sample.

The yield and purity of the extracts were assessed by UV spectrophotometry using a SynergyHT multi-mode microplate reader (BioTek Instruments, Inc., Vermont, USA) with a Take 3 micro-volume plate accessory. The absorbance was measured at 260 and 280 nm to estimate DNA content and purity using the nucleic acid quantification protocol with sample type defined for double-stranded DNA in the Gen5 data analysis software version 2.01 (BioTek Instruments, Inc., Vermont, USA).

The quality of the extracted DNA was further evaluated by electrophoresis in a 1.0% agarose gel containing Gel Red 1 × (Biotium, Hayward, CA, USA) for staining and carried out in SGTB 1 × (GRiSP, Research Solutions, Porto, Portugal) for 20 min at 200 V. The agarose gel was visualised under a UV light tray Gel Doc™ EZ System (Bio-Rad Laboratories, Hercules, CA, USA) and a digital image was obtained with Image Lab software version 5.1 (Bio-Rad Laboratories, Hercules, CA, USA).

### 2.3. Selection of the target regions and design of oligonucleotide primers

Using DNA extracts obtained from the European honeybee subspecies *A. m. mellifera*, *A. m. carnica* and *A. m. ligustica*, the mtDNA region located between the tRNA<sup>leu</sup> and cytochrome *c* oxidase subunit II genes (commonly known as the tRNA<sup>leu</sup>-cox2 intergenic region) was PCR-amplified using the primers and reaction conditions detailed by Garnery, Solignac, Celebrano, and Cornuet (1993). The amplicons were sent to Macrogen (Seoul, Korea) for direct Sanger sequencing in both directions. The sequences were checked for base calling and aligned with sequences available in GenBank (<http://www.ncbi.nlm.nih.gov/>) using MEGA 6.06 (Tamura, Stecher, Peterson, Filipinski, & Kumar, 2013). The haplotypes were identified and named following the nomenclature system revised by Chávez-Galarza et al. (2017). The sequences were deposited in GenBank with accession numbers MF428428 (*Apis m. mellifera* haplotype M8), MF428426 (*A. m. ligustica* haplotype C1),

MF428427 (*A. m. carnica* haplotype C3a). Sequences from *A. m. iberiensis* of lineages A and M ancestry were obtained as part of a previous work (deposited in GenBank with accession numbers KX463813.1 and KX463907.1, respectively). Sequences from *A. cerana* for the tRNA<sup>leu</sup>-cox2 intergenic region were also retrieved from GenBank (accession numbers KP065103.1, AP017314.1). All sequences were aligned and compared to design species-specific primers, namely AC1-F (TCT GAA TTC AAA CTC AAA GTA AAA) and AC1-R (ATA ATA TGA GTT TGA TTC TTG AAA), to produce DNA fragments of 111 bp of *A. cerana* species (Fig. S1, Supplementary material).

For HRM analysis, the primers AM1-F (AGC TAA TTA AAA CAA CAA TAC A) and AM1-R (AAG GTA GTA AAT GTT GAA TCA TT) were designed for the amplification of a 16S rRNA common fragment, based on the sequences retrieved from GenBank for *A. cerana* (accession numbers GQ162109.1 and AP017314.1) and *A. mellifera* (NC.001566.1, KJ396191.1, KM458618.1, KJ601784.1) (Fig. S2, Supplementary material).

Primers were designed using the software Primer-BLAST tool (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). Primer specificity was assessed using this tool, which reveals the homologies in relation to all sequences available in Genbank. Primer properties and the absence of hairpins and self-hybridisation were assessed using the software OligoCalc (<http://www.basic.northwestern.edu/biotools/oligocalc.html>). The designed primers were synthesised by STABVIDA (Lisbon, Portugal).

#### 2.4. End-point PCR conditions

PCR amplifications were carried out in a total reaction volume of 25 µL, containing 2 µL of DNA extract (10 ng), 67 mmol/L Tris-HCl (pH 8.8), 16 mM of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1% of Tween 20, 200 µmol/L of each dNTP, 1.0 U of SuperHot Taq DNA Polymerase (Genaxxon Bioscience GmbH, Ulm, Germany), 3.0 mmol/L of MgCl<sub>2</sub> and 300 nmol/L of each primer. The reactions were performed in a thermal cycler MJ Mini™ Gradient Thermal Cycler (Bio-Rad Laboratories, USA) using the following programs: (i) initial denaturation at 95 °C for 5 min; (ii) 35 cycles at 95 °C for 30 s, 52 °C for 30 s and 72 °C for 30 s, for primers AM1 and 40 cycles at 95 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s, for primers AC1; (iii) and a final extension at 72 °C for 5 min. Each extract was amplified at least in duplicate assays.

The amplified fragments were analysed by electrophoresis in a 1.5% agarose gel containing Gel Red 1 × (Biotium, Hayward, CA, USA) for staining and carried out in SGTB 1 × (GRiSP, Research Solutions, Porto, Portugal). The agarose gel was visualised under a UV light tray Gel Doc™ EZ System (Bio-Rad Laboratories, Hercules, CA, USA) and a digital image was obtained with Image Lab software version 5.1 (Bio-Rad Laboratories, Hercules, CA, USA).

#### 2.5. Real-time PCR and HRM analysis

To verify the specificity and sensitivity of the proposed primers AC1-F/AC1-R, a DNA extract from *A. cerana* was 10-fold serially diluted to cover 6 levels of concentrations from 10 to 0.0001 ng of DNA. The real-time PCR assays were performed in 20 µL of total reaction volume, containing 2 µL of DNA extract (10–0.0001 ng or 10 ng for primers AC1-F/AC1-R or AM1-F/AM1-R, respectively), 1 × of SsoFast™ Evagreen® Supermix (Bio-Rad Laboratories, USA) and 400 nmol/L of each primer. All real-time PCR assays were carried out on a fluorometric thermal cycler CFX96 Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA). The following conditions were used for amplification with AM1-F/AM1-R primers: 95 °C for 5 min, 50 cycles at 95 °C for 20 s, 52 °C for 20 s and 72 °C for 30 s. The temperature conditions for AC1-F/AC1-R primers were: 95 °C for 5 min, 50 cycles at 95 °C for 15 s and 60 °C for 45 s. For both cases, the fluorescence signal was collected at the end of each cycle and the data was processed using the software Bio-Rad CFX Manager 3.1 (Bio-Rad, Hercules, CA, USA).

For HRM analysis, PCR products were denatured at 95 °C for 1 min and then annealed at 57 °C for 5 min to allow the correct annealing of the DNA duplexes. These two steps were followed by melting curve ranging from 57 to 85 °C with temperature increments of 0.2 °C every 10 s. The fluorescence data were acquired at the end of each melting temperature.

The collected fluorescence data were processed using the Precision Melt Analysis Software 1.2 (Bio-Rad Laboratories, Hercules, CA, USA) to generate melting curves as a function of temperature and difference curves for easier visual identification of clusters. Cluster detection settings were defined targeting high sensitivity and threshold yields, with the melting curve shape sensitivity parameter being adjusted to a value > 98% and T<sub>m</sub> difference threshold parameter set as a default value of 0.15. DNA extracts were analysed in two independent assays using four replicates in each one.

### 3. Results and discussion

Considering that honey is a very complex matrix, in which the remaining DNA is highly degraded and fragmented, in the present work, the amplification of small fragments from two mtDNA regions was performed, aiming at identifying the entomological origin of honeys produced by two different honeybee species (*A. cerana* and *A. mellifera*). Besides presenting the advantage of existing in several copies per cell and having a higher evolutionary rate compared to nuclear DNA, mtDNA is maternally inherited, being therefore transmitted intact by the queen to all her offspring (workers and drones). Based on the available information from honeybee genetic studies (Chávez-Galarza et al., 2017; Engel & Schultz, 1997; Meixner et al., 2013; Michel-Salzat, Cameron, & Oliveira, 2004), the tRNA<sup>leu</sup>-cox2 intergenic region and the 16S rRNA gene were the two selected regions for primer design.

#### 3.1. Quality of DNA extracts

The quality assessment of DNA extracts from honeybee tissues showed high yields (72–552 ng/µL) and adequate purity (1.8–2.2). The DNA extraction from honey samples was more difficult to accomplish due to the matrix complexity, with high levels of sugars and plant secondary compounds that frequently affect DNA recovery and promote PCR inhibition (Soares et al., 2015). Even so, the extracts from honey presented adequate concentrations (ranging from 2.3 to 303.9 ng/µL), although with variable purity (ranging from 1.1 to 2.6). In order to assess amplification capacity, all DNA extracts were tested by PCR with the universal eukaryotic primers 18SEU-F/18SEU-R, as described by Fajardo et al. (2008). All extracts showed positive amplification, confirming the absence of false negative results that might occur due to PCR inhibition (Tables 1 and 2).

#### 3.2. Specific detection of *A. cerana* targeting the tRNA<sup>leu</sup>-cox2 intergenic region

##### 3.2.1. Real-time PCR method development and validation

The PCR amplification of the non-coding tRNA<sup>leu</sup>-cox2 intergenic region followed by digestion with *Dra*I restriction enzyme (commonly known as the *Dra*I test) is the most popular assay for honeybee mtDNA identification, in particular for identifying haplotypes of different *A. mellifera* subspecies (Garnery et al., 1993; Meixner et al., 2013). This is mainly due to its relative low cost and scoring simplicity, combined with the high information content of this region (Meixner et al., 2013). The high resolution power of the *Dra*I test is explained by the existence of an important length polymorphism, based on a variable number of copies (1 to 5 in *A. mellifera* and absent in *A. cerana*) of a 192–196 bp sequence (Q element) and the complete or partial deletion of a 67 bp sequence (P element) (Garnery et al., 1993). Due to this polymorphism, while the tRNA<sup>leu</sup>-cox2 intergenic region has been reported to vary between 570–1414 bp in *A. mellifera* (Chávez-Galarza et al., 2017;

**Table 1**

Results of qualitative and real-time PCR with HRM analysis applied to the analysis of different species and subspecies of honeybees.

Samples	Species	Origin	End-point PCR			HRM analysis	
			18SEU <sup>a</sup>	AC1 <sup>b</sup>	AM1 <sup>c</sup>	Cluster	Level of confidence (%) <sup>*</sup>
Asian honeybee	<i>A. cerana</i>	Thailand	+	+	+	1	99.9 ± 0.1
Asian honeybee	<i>A. cerana</i>	China	+	+	+	1	99.5 ± 0.8
Asian honeybee	<i>A. cerana</i>	Viet Nam	+	+	+	1	99.4 ± 0.3
Asian honeybee	<i>A. cerana</i>	Viet Nam	+	+	+	1	99.8 ± 0.2
Asian honeybee	<i>A. cerana</i>	Viet Nam	+	+	+	1	99.9 ± 0.0
Asian honeybee	<i>A. cerana</i>	Thailand	+	+	+	1	99.8 ± 0.1
Asian honeybee	<i>A. cerana</i>	China	+	+	+	1	99.3 ± 0.3
Asian honeybee	<i>A. cerana</i>	China	+	+	+	1	99.7 ± 0.1
Asian honeybee	<i>A. cerana</i>	Thailand	+	+	+	1	99.8 ± 0.2
European honeybee	<i>A. m. mellifera</i>	France	+	–	+	2	99.7 ± 0.4
European honeybee	<i>A. m. carnica</i>	Croatia	+	–	+	2	99.9 ± 0.2
European honeybee	<i>A. m. iberiensis</i> (lineage A)	Spain	+	–	+	2	99.8 ± 0.4
European honeybee	<i>A. m. iberiensis</i> (lineage M)	Spain	+	–	+	2	99.9 ± 0.1
European honeybee	<i>A. m. iberiensis</i> (lineage A)	Portugal	+	–	+	2	99.9 ± 0.1
European honeybee	<i>A. m. ligustica</i>	Italy	+	–	+	2	99.9 ± 0.1

\* mean values ± standard deviation of n = 3 replicates; + positive amplification; – negative amplification; amplification was performed using primers.

<sup>a</sup> 18SEU-F/18SEU-R targeting eukaryotes.<sup>b</sup> AC1-F/AC1-R targeting *A. cerana*.<sup>c</sup> AM1-F/AM1-R targeting *Apis* spp.

Rortais, Arnold, Alburaki, Legout, & Garnery, 2011), it is much shorter in *A. cerana* (around 86–93 bp), mainly because of the absence of the Q element (Lee et al., 2016). Considering the remarkably high variability reported for this region, which is able to distinguish the two *Apis* species and the four evolutionary lineages (A, C, M and O) within *A. mellifera* (Ruttner, 1988), it was selected for primer design. For this purpose, several sequences of *A. cerana* and *A. mellifera* subspecies were aligned, allowing the design of primers AC1-F/AC1-R targeting a 111 bp specific amplicon in *A. cerana* mitogenome (Figs. 1A and S1, Supplementary material).

The specificity of the new primers was firstly *in silico* assayed by the use of the primer-BLAST tool and subsequently tested experimentally using several DNA extracts obtained from *A. cerana* (n = 16) originating from a wide geographical origin and from different subspecies of *A. mellifera* (n = 49). As expected, only *A. cerana* samples were positively amplified (Table 1). The absence of the specific 111 bp fragment across all *A. mellifera* samples confirmed the specificity of these primers for *A. cerana* (Fig. 1B and Table 1). Since plant DNA is also present in honey samples (Soares et al., 2015), a wide range of plant species (n = 36), whose pollen is frequently present in monofloral and multifloral honeys, was also tested for cross-reactivity purposes, with negative amplification being attained in all cases (Table S1, Supplementary

material). The amplification capacity of DNA extracts from plants was confirmed using primers EG-F/EG-R targeting a universal eukaryotic gene as described by Villa, Costa, Oliveira, and Mafra (2017).

To optimise the amplification conditions of the proposed species-specific qualitative PCR assay, a ten-fold serially diluted DNA extract (100 ng to 0.0001 ng) obtained from *A. cerana* was used. The optimised PCR conditions using AC1-F/AC1-R primers allowed achieving a sensitivity of 0.01 ng (Fig. 1A).

The applicability of the novel AC1-F/AC1-R primers to detect *A. cerana* DNA was subsequently assayed with authentic honey samples, collected by beekeepers from *A. cerana* and *A. mellifera* apiaries. As expected, the developed methodology allowed the detection of *A. cerana* DNA in the corresponding honey samples, while negative amplifications were observed for honeys produced by *A. mellifera* (Fig. 1B). The methodology was further applied in the analysis of honeys produced in the EU. As expected, negative results for the presence of *A. cerana* DNA were obtained since EU honeys should be produced by *A. mellifera* in accordance with the legislation.

### 3.2.2. Real-time PCR method development and validation

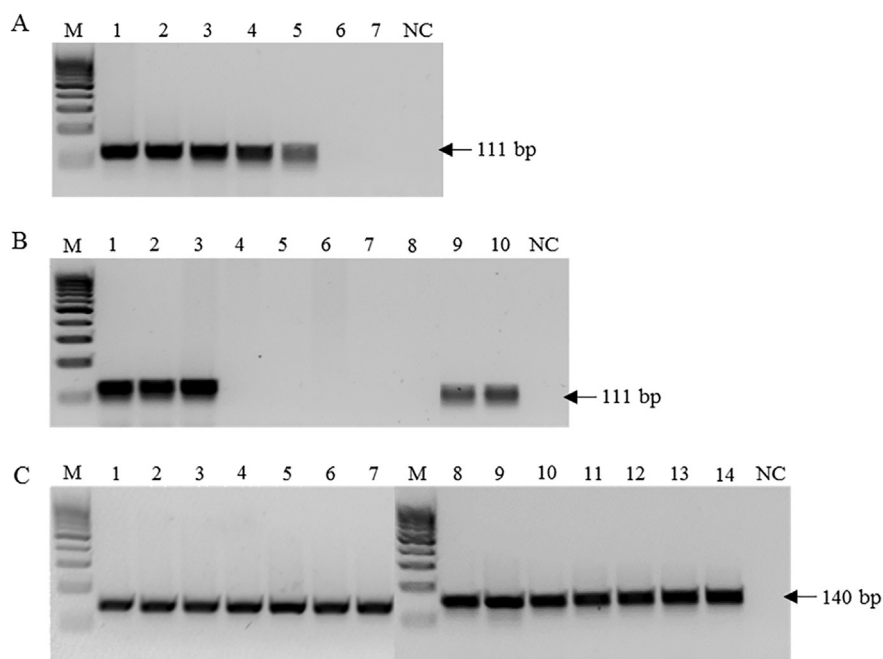
Given the complexity of the honey matrix and that DNA from honeybees can be present at trace amounts, a real-time PCR assay using

**Table 2**

Results obtained by qualitative and real-time PCR with HRM analysis applied to the analysis of voucher samples of honeybees, authentic and commercial honey samples.

Samples	Species	Origin	End-point PCR			HRM analysis	
			18SEU <sup>a</sup>	AC1 <sup>b</sup>	AM1 <sup>c</sup>	Cluster	Level of confidence (%) <sup>*</sup>
Asian honeybee	<i>A. cerana</i>	Viet Nam	+	+	+	1	99.9 ± 0.4
Honey (apiary)	<i>A. cerana</i>	Viet Nam	+	+	+	1	98.8 ± 0.2
Honey (apiary)	<i>A. cerana</i>	Viet Nam	+	+	+	1	99.2 ± 0.3
European honeybee	<i>A. m. carnica</i>	Croatia	+	–	+	2	99.9 ± 0.2
European honeybee	<i>A. m. iberiensis</i> (lineage M)	Spain	+	–	+	2	99.7 ± 0.2
European honeybee	<i>A. m. ligustica</i>	Italy	+	–	+	2	99.8 ± 0.1
Honey (apiary)	<i>A. mellifera</i>	Viet Nam	+	–	+	2	99.6 ± 0.1
Honey (commercial)	<i>A. mellifera</i> **	Italy	+	–	+	2	99.7 ± 0.1
Honey (commercial)	<i>A. mellifera</i> **	France	+	–	+	2	99.4 ± 0.8
Honey (commercial)	<i>A. mellifera</i> **	Portugal	+	–	+	2	99.5 ± 0.3

\* mean values ± standard deviation of n = 3 replicates; + positive amplification; – negative amplification; \*\* samples not labelled, but in compliance with legislation, EU honey should be produced by *A. mellifera*; amplification was performed using primers.<sup>a</sup> 18SEU-F/18SEU-R targeting eukaryotes.<sup>b</sup> AC1-F/AC1-R targeting *A. cerana*.<sup>c</sup> AM1-F/AM1-R targeting *Apis* spp.



**Fig. 1.** Agarose gel electrophoresis of PCR products using (A) primers AC1-F/AC1-R applied to a serial dilution of a *A. cerana* DNA extract, (B) primers AC1-F/AC1-R applied to the amplification of DNA extracts from different *Apis* species and honey samples, and (C) primers AM1-F/AM1-R applied to different subspecies of *A. mellifera* (lanes 1 to 7 and 14) and *A. cerana* DNA extracts (lanes 8 to 13). Legend (A): lane 1, 100 ng; lane 2, 10 ng; lane 3, 1 ng; lane 4, 0.1 ng; lane 5, 0.01 ng; lane 6, 0.001 ng; lane 7, 0.0001 ng; NC, negative control; M, 100 bp DNA molecular marker (Bioron, Ludwigshafen, Germany). Legend (B): lane 1, *A. cerana* HB from Thailand; lane 2, *A. cerana* from China; lane 3, *A. cerana* from Vietnam; lane 4, *A. m. mellifera*; lane 5, *A. m. iberiensis* (lineage M); lane 6, *A. m. iberiensis* (lineage A) from Portugal; lane 7, honey of *A. mellifera* from Portugal; lane 8, honey of *A. mellifera* from Vietnam; lanes 9 and 10, honey of *A. cerana* from Vietnam. Legend (C): lane 1, *A. m. iberiensis* (lineage A) from Spain; lane 4, *A. m. iberiensis* (lineage M); lane 5, *A. m. mellifera*; lane 6, *A. m. carnica*; lane 7, *A. m. ligustica*; lanes 8 and 9, *A. cerana* from Thailand; lanes 10 and 11, *A. cerana* from China; lanes 12 and 13, *A. cerana* from Vietnam; lane 14, *A. m. iberiensis* (lineage A) from Portugal; NC, negative control; M, 100 bp DNA molecular marker (Bioron, Ludwigshafen, Germany).

the fluorescent EvaGreen dye was also proposed using AC1-F/AC1-R primers. For this purpose, the guidelines of “Minimum Information for Publication of Quantitative Real-Time PCR Experiments” (Bustin et al., 2009) and the requirements set by the European Network of GMO Laboratories (ENGL) document “Definition of minimum performance requirements for analytical methods of GMO testing” (ENGL, 2015) were taken into consideration. The amplification results by real-time PCR of a serially diluted DNA extract from *A. cerana* (10 ng to 0.0001 ng) are presented in Fig. 2A. The depicted calibration curve is showed in Fig. 2B, covering a dynamic range with 6 orders of magnitude of the target analyte. Over the whole dynamic range the relative repeatability, expressed as the relative standard deviation of the cycle threshold values obtained for each concentration level, was always lower than 2.1%, which is in good agreement with the maximum value recommended of 25% (ENGL, 2015). A limit of detection (LOD) of 0.0001 ng was obtained considering the lowest concentration level with amplification of at least 95% of the times, meaning that all the 4 replicates performed for each level were amplified. Since the LOD value was within the linear range of the calibration curve, it could also be established as the limit of quantification (LOQ). The real-time PCR assay revealed high performance because the values of PCR efficiency (91.6%), slope (−3.541) and correlation coefficient (0.981) (Fig. 2B) are within the acceptance criteria for this type of methods (Bustin et al., 2009; ENGL, 2015). The subsequent melting curve analysis highlights the occurrence of a single melt peak temperature (68.60 °C) for all fragments, indicating one group of PCR products and the absence of primer dimer or other non-specific fragments (Fig. 2C). These results suggested that, as expected, real-time PCR exhibited better sensitivity than species-specific qualitative PCR.

Accordingly, a real-time PCR method targeting *A. cerana* DNA was successfully developed and applied. The specificity was fully demonstrated *in silico* and based on the exclusive amplification of *A. cerana* DNA. Besides, the analytical performance of the method was effectively demonstrated in terms of precision, complying with the acceptance criteria of linearity and PCR efficiency.

### 3.3. Differentiation of *A. mellifera* from *A. cerana* by HRM analysis of 16S rRNA gene

In this work, a second approach to differentiate *A. mellifera* from *A.*

*cerana* was attempted, based on the use of a coding region. To date, a variety of mitochondrial genes has been employed in phylogenetic studies of Hymenoptera, including 16S rRNA, 12S rRNA, ND (1–6), cytochrome *b*, COI and ATPase (Mandal, Chhakchhuak, Gurusubramanian, & Kumar, 2014; Meixner et al., 2013). Among those, the 16S rRNA has been the marker of choice for estimating phylogenies across a wide range of insect taxa, and is considered the most informative for studying closely related species (Whitfield & Cameron, 1998). More specifically for *Apis* species, data recovered from 16S rRNA sequences were found to be strongly congruent with those from morphological data (Engel & Schultz, 1997). Therefore, the available sequences of the 16S rRNA gene for *A. mellifera* and *A. cerana* were aligned to design new primers. Given that sequences for *A. m. iberiensis* of African ancestry were unavailable, sequences of the sub-Saharan *A. m. scutellata* and the North African *A. m. intermissa*, both belonging to the African lineage, were included in the analysis (Fig. S2, Supplementary material). As previously reported for many holometabolous insects (Whitfield & Cameron, 1998), the 16S rRNA gene of the selected species was also found to be highly AT-rich, causing an increased possibility of hairpins and self-annealing and, therefore, severely restricting the possibilities for primer design. For this reason, the new primers AM1-F/AM1-R were designed to amplify different species of the genus *Apis* and used in real-time PCR assays with HRM analysis. HRM is a technique that measures the rate of dissociation of double stranded to single-stranded DNA through small increments of temperature (0.01–0.2 °C/s), which allows the discrimination of two fragments of similar size with minor nucleotide differences (in theory, a single nucleotide substitution is sufficient for discrimination) (Drumil & Cichna-Markl, 2014; Martín-Fernández et al., 2016; Reed, Kent, & Wittwer, 2007). The results obtained by qualitative PCR using the primers AM1-F/AM1-R evidenced positive amplification for all tested DNA extracts of *A. cerana* and *A. mellifera* subspecies (Fig. 1C). These primers were also tested for cross-reactivity against 36 plant species, showing negative results in all cases (Table S1, Supplementary material). Subsequently, DNA extracts of honeybees, including *A. cerana* from distinct geographical origins and several *A. mellifera* subspecies (*A. m. mellifera*, *A. m. carnica*, *A. m. ligustica* and *A. m. iberiensis*), were analysed by real-time PCR, confirming the previously obtained results by qualitative PCR. After real-time PCR amplification and melting curve analysis (Fig. 3A), all data were further analysed using the Precision

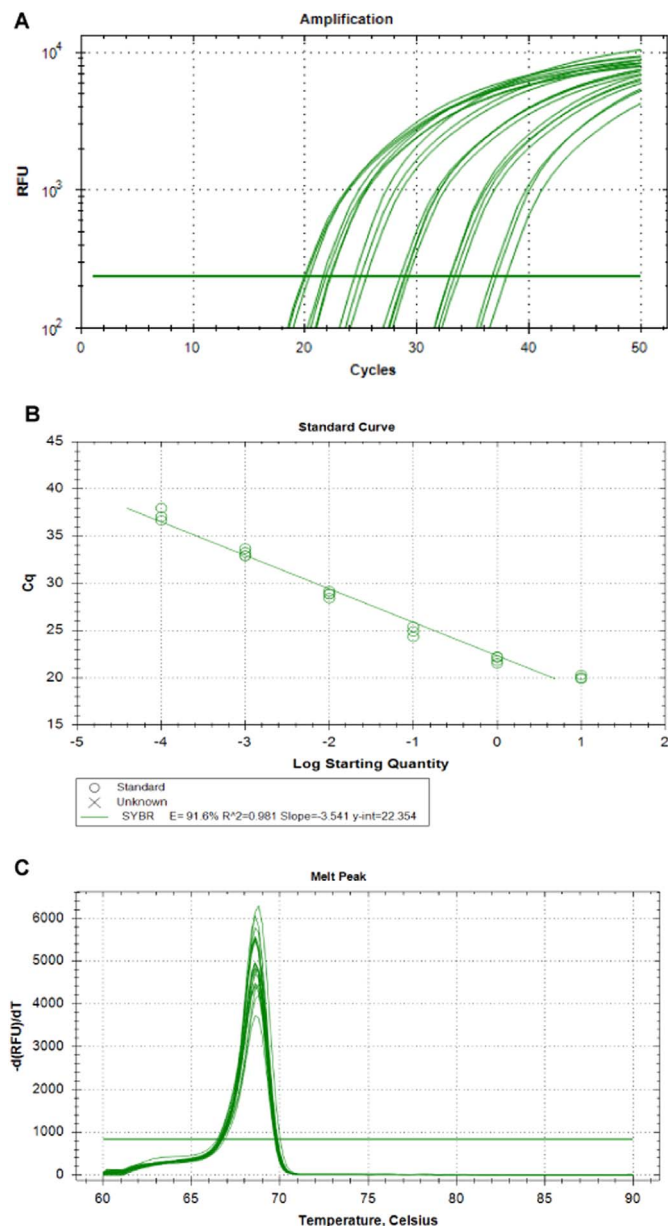


Fig. 2. Amplification curves (a), calibration curve (b) and melting curve analysis (c) obtained by real-time PCR with EvaGreen dye amplification using specific primers AC1-F/AC1-R applied to a *A. cerana* DNA extract serially diluted (10 ng, 1 ng, 0.1 ng, 0.01 ng, 0.001 ng and 0.0001 ng) ( $n = 4$  replicates).

Melt Analysis software, allowing the discrimination of two clusters (Fig. 3B and C, Table 1). Cluster 1 was defined as the reference cluster and included all the *A. cerana* specimens, with confidence levels above 99.3%. Cluster 2 comprised the different *A. mellifera* subspecies, also with a high confidence levels (99.7%) (Fig. 3B and C, and Table 1).

The developed HRM method was further applied to honey samples, enabling their entomological authentication (Fig. 4 and Table 2). The amplicons exhibited a good resolution, both by conventional melting curve analysis, with single melt peaks for each species (72.6 °C for *A. cerana* and 73.8 °C for *A. mellifera*) (Fig. 4A), and HRM analysis, with clear separation into two clusters in normalised and in difference curves (Fig. 4B and C). The honey samples produced by *A. cerana* (from Vietnam) were clustered with *A. cerana* (cluster 1), while the authentic honey samples produced by *A. mellifera* (from Portugal and Vietnam) were grouped in the cluster of *A. mellifera* (cluster 2), with confidence levels above 99.2% and 99.4% for cluster 1 and 2, respectively (Table 2). The commercial honey samples produced in Europe were

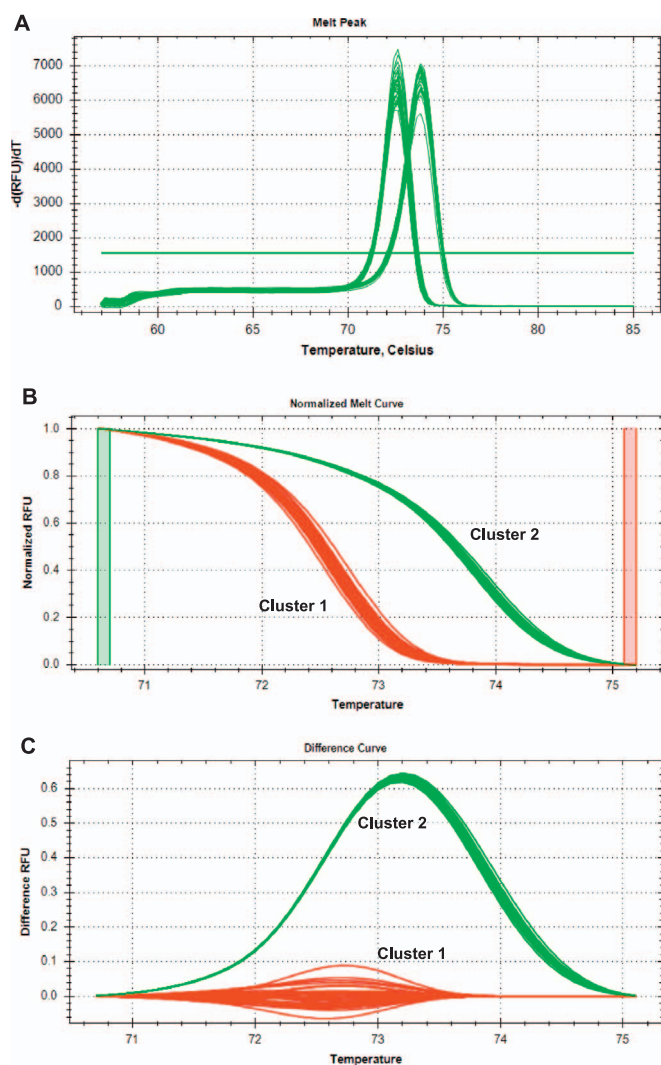
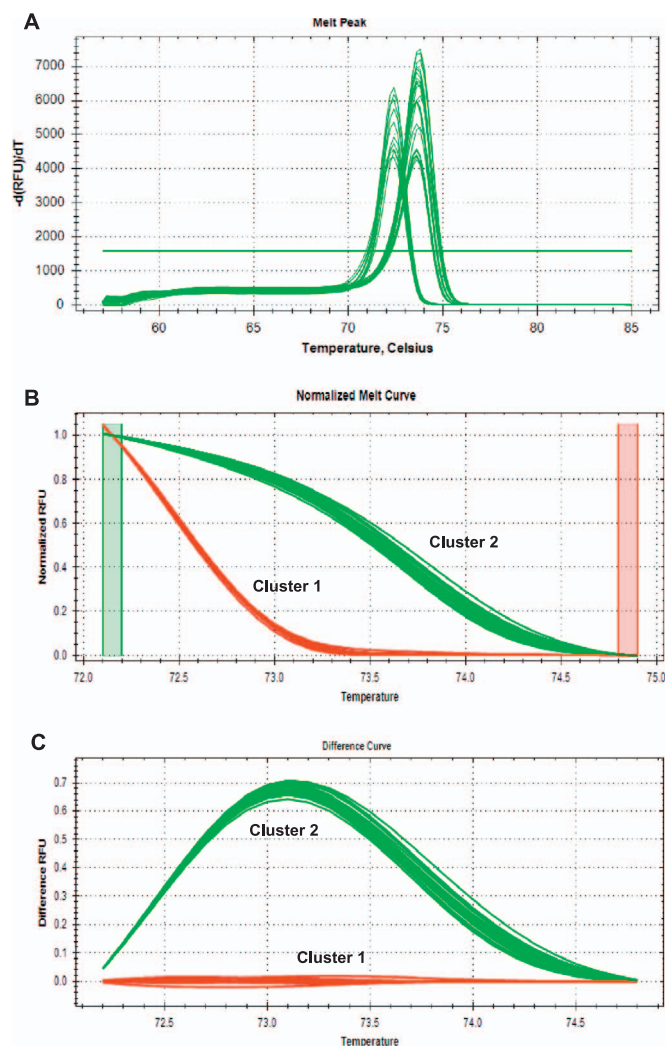


Fig. 3. Conventional melting (A), normalised melting (B) and difference (C) curves obtained by real-time PCR amplification with EvaGreen dye and HRM analysis targeting 16S rRNA gene ( $n = 3$  replicates). Legend: cluster 1, *A. cerana* from China, Thailand and Vietnam; cluster 2, *A. m. mellifera*; *A. m. carnica*; *A. m. iberiensis* (lineages A and M) and *A. m. ligustica*.

grouped in the cluster of *A. mellifera*, suggesting the effectiveness of the technique for identifying the entomological origin of honey.

#### 4. Conclusions

In the present work, two mitochondrial DNA regions, the tRNA<sup>leu</sup>-cox2 intergenic region and the 16S rRNA gene, were extensively evaluated for the discrimination of the two honeybee species used in apiculture, the European honeybee *A. mellifera* and the Asian honeybee *A. cerana*. Different approaches were followed for each mtDNA region, namely (i) the development of a species-specific PCR for the identification of *A. cerana* DNA, based on the length polymorphisms of the intergenic region; and (ii) a real-time PCR system targeting the 16S rRNA gene coupled with HRM analysis, based on sequence variation in this coding region. Although *A. cerana* and *A. mellifera* are closely related species, thereby showing only slight genetic differences, the obtained results proved the ability of the developed methods herein to distinguish these two important honeybee species. In particular, the species-specific PCR approach allows identification of *A. cerana* DNA, while the HRM analysis allows identification of both *A. cerana* and *A. mellifera* DNA. The developed methodologies were further applied to



**Fig. 4.** Conventional melting (A), normalised melting (B) and difference (C) curves obtained by real-time PCR amplification with EvaGreen dye and HRM analysis targeting 16S rRNA gene ( $n = 3$  replicates). Legend: cluster 1, *A. cerana* from Vietnam and authentic honey samples produced by *A. cerana* from Vietnam; cluster 2, *A. m. carnica*; *A. m. iberiensis* (lineage M); *A. m. ligustica*, authentic honey samples produced by *A. mellifera* from Vietnam, and commercial honey samples from Italy, France and Portugal.

analyse honey samples of known origin, allowing their correct identification and proving the effectiveness of both techniques for entomological authentication of honey samples.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodres.2017.11.081>.

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