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A novel real-time PCR coupled with high resolution melting analysis as a simple and fast tool for the entomological authentication of honey by targeting *Apis mellifera* mitochondrial DNA

Mónica Honrado, Ana R. Lopes, M.Alice Pinto, Joana S. Amaral *

Centro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança, Campus de Santa Apolónia, 5300-253 Bragança, Portugal

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ABSTRACT

Honey is one of the foods easily adulterated worldwide. Recently, the analysis of honeybee DNA has been proposed as a useful tool to authenticate the entomological origin of honey. However, the methods proposed so far require more than one polymerase chain reaction (PCR) and the use of agarose gels, making the authentication process laborious and lengthy. In this work, a novel real-time PCR coupled with high-resolution melting (HRM) analysis of a 150 bp fragment of the cytochrome *c* oxidase I (COI) gene is proposed as a fast and simple tool to assess honey's entomological origin by discriminating the mitochondrial DNA lineages of European honey bees (A, M and C lineages). In addition, the new tool allowed the differentiation of honeys produced by different mitotypes of C-lineage ancestry. The method showed high analytical performance and was able to successfully identify the entomological origin of honeys of known origin obtained from research apiaries/beekeepers. Therefore, it was applied to 44 commercial honeys from different countries. It confirmed the entomological authenticity of French PDO honeys that should be produced by the Corse ecotype *A. m. mellifera*. For the remaining honeys, the results were also in good agreement with the declared geographical origin. However, three honeys from Slovenia did not cluster with C2 mitotype *A. m. carnica* as expected, suggesting the mixture of honeys produced by honeybees of different mitotypes.

1. Introduction

Honey is the natural sweet substance produced by honey bees from the nectar of plants or the secretions of living parts of plants (FAO, 2001). Being a product of high dietary value and increasing demand, honey has become a target of economically motivated adulteration (Soares et al., 2017). According to a 2014 European Parliament report on food fraud, honey was ranked as the 6th food product most prone of being adulterated, either by the admixing of honey with lower quality, by the addition of sugars, or by mislabelling of botanical and geographical origins (European Parliament, 2014). Currently, the Knowledge Centre for Food Fraud and Quality (KC-FFQ) of the European Commission considers honey, together with olive oil, milk, saffron, wine, juices and fish, as the most common sources of food fraud (KC-FFQ, 2022Knowledge Centre for Food Fraud and Quality (KC-FFQ) of the European Commission, 2022). Typically, the assessment of honey authenticity has focused mainly on the detection of sugars addition, as this type of fraud has been demonstrated to be one of the most frequent (Aries et al., 2016). However, since consumer's preferences are generally linked to honey's unique organoleptic characteristics that depend primary on their botanical and geographical origins, mislabelling is increasingly considered a relevant type of fraud.

In general, there is a higher demand for monofloral than for multifloral honeys, thus increasing their prices. Furthermore, honeys bearing the label of Protected Designation of Origin (PDO) have a higher commercial value since consumers perceive these honeys as high-quality products and recognize their specific characteristics related to the region and environment from where they originate. While the botanical origin can be determined by well-established methods, such as the microscopic analysis of pollen grains (melissopalynology), the geographical origin is much more difficult to assess. In fact, the geographical origin is frequently inferred from honey's botanical composition based on the peculiarities of the flora that surrounds the apiaries in a certain region (Kaškonienė & Venskutonis, 2010). However, this indirect approach can be inconclusive if no characteristic flora signature is associated with a certain region or if different regions with

* Corresponding author.
E-mail address: jamaral@ipb.pt (J.S. Amaral).

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similar flora are considered. For this reason, an increased attention has been recently paid to honey's entomological origin since it also relates with geographical origin.

In the European Union, honey should be produced by *Apis mellifera* (Directive 2014/63/EU). However, at least 10 different *A. mellifera* subspecies are native to Europe belonging to three major evolutionary lineages, concurrently supported by morphology (Ruttner, 1988) and mitochondrial (mt) DNA (Garnery et al., 1992): the African (A), the western European (M) and the eastern European (C). While in Portugal the predominant mtDNA of the autochthonous subspecies *Apis mellifera iberiensis* belongs to the A-lineage, when moving towards the north-eastern part of Iberia, the A-lineage mitotypes are gradually replaced by M−lineage mitotypes, which are present also in the western European subspecies *Apis mellifera mellifera* (Pinto et al. 2013; Chávez-Galarza et al. 2017). While *A. m. iberiensis* is confined to Iberia, the native distribution of its sister subspecies *A. m. mellifera* expands from the Pyrenees to Scandinavia and from the British Isles to the Ural Mountains. On the other hand, the eastern part of Europe including the Italian and Balkan peninsulas, is occupied by subspecies belonging mainly to C-lineage (Ruttner 1988). Within the C-lineage mtDNA, different mitotypes have been described, with C1 being the predominant in the Italian honeybee *A. m. ligustica* whereas C2 is mostly present in the Carniolan honeybee *A. m. carnica* (Franck et al., 2000; Muñoz et al., 2009; Nedić et al., 2009; Tanaskovic et al. 2021(Tanasković et al., 2021)). Due to the beekeeping activity that has strongly favoured the dissemination of C1 mitotypes from the main breeding area of Emilia-Romagna, currently, mitotypes of C-lineage ancestry are the most frequent in the Italian honeybee (Utzeri et al. 2022). Indeed, although M4 and M7 mitotypes (M-lineage mtDNA) had intermediate frequencies in some regions of Italy over 20 years (Franck et al., 2000), a recent survey found a considerable decrease in M-lineage mitotypes and an increase in C-lineage mitotypes (Utzeri et al., 2022).

Since honeybees naturally occupy allopatric geographical ranges according to their evolutionary lineages, the entomological origin of honey produced in the EU could be correlated with its geographical origin. However, this can only be done to a certain extent because in the last decades the natural distribution of European honeybees has been changing mainly due to queen trading (De la Rúa et al., 2009). On the other hand, some European PDO honeys specify the honeybee subspecies that should be used for their production, which is the case of several PDO honeys from Portugal (*A. m. iberiensis*), Poland (*A. m. carnica*), Croatia (*A. m. carnica*), France (*A. m. mellifera*), and Italy (*A. m. ligustica*) (DG AGRI, 2022). Moreover, in some regions, only specific subspecies are allowed, such as in Emilia-Romagna, Italy, where rearing colonies other than *A. m. ligustica* is forbidden by law (Regional Law, 2019). In all these cases, the entomological origin can be used as an additional authentication parameter to verify the geographical origin of these honeys.

Establishing the entomological origin of honey can not only be used to authenticate the honeys, and further valorise them, but also to contribute for preserving autochthonous honeybee subspecies. Together with introgression phenomena due to the use of foreign queens, honeybees are increasingly threatened by several interacting factors, including climate change, loss of habitat, among others. Thus, the preservation of autochthonous subspecies in their native ranges, to which they are better adapted as a result of evolution, and the valorisation of the honey produced by sustainable beekeeping based on conservation strategies, is perceived as being of utmost importance. For these reasons, the development of methodologies for the entomological authentication of honey will contribute not only to assure consumers rights and avoid unfair competition by the identification of frauds, but also to promote and valorise autochthonous honeybee subspecies.

Several works are available on honeybees' genetics aiming to characterize different species and subspecies (Garnery et al., 1998; Henriques et al., 2019; Meixner et al., 2013; Pinto et al., 2014). However, they generally rely on the amplification of large DNA fragments, which is not adequate for application to honey samples due to DNA degradation. Moreover, while some molecular-based approaches have been proposed to distinguish honeys produced by different honeybee species (Prosser and Hebert, 2017; Kek et al. 2017; Soares et al. 2018; Wang et al., 2019; Zhang et al., 2019; (Moškrič et al., 2021)Moškrič, 2021), only a few have attempted to differentiate among *A. mellifera* subspecies. Utzeri et al. (2018) and Soares et al. (2019) were able to differentiate the three mitochondrial lineages (A, M and C) typical of the European subspecies in honeys using molecular-based approaches. Utzeri et al. (2018) designed new primers to amplify short fragments of the popular $tRNA^{leu}$-COX2 intergenic mitochondrial region, which takes advantage of a length polymorphism to distinguish lineages A, M and C. Soares et al. (2019) used a two-step approach, which includes a qualitative polymerase chain reaction (PCR) for the specific identification of A lineage using agarose gel electrophoresis followed by a real-time PCR to discriminate between M and C-lineages, based on their high-resolution melting (HRM) profile. More recently, Utzeri et al. (2022) proposed a follow-up method to further distinguish between C1 and C2 mitotypes for the differentiation of *A. m. ligustica* and *A. m. carnica.* However, this approach is also multi-step as it relies on the use of a qualitative PCR and a high percentage agarose (4.5%) gel electrophoresis to identify the three lineages, as proposed by Utzeri et al. (2018), followed by a second PCR using specific primers to amplify a region of 170 bp that contains an informative single nucleotide polymorphism. The products are then Sanger-sequenced to distinguish between C1 and C2 mitotypes. Considering that the methodologies proposed up until now are laborious and time consuming, this work aimed at developing a novel and simple approach based on the high-resolution analysis of an informative short fragment of the cytochrome *c* oxidase I (COI) gene to verify the entomological origin of honey. Moreover, further application to commercial honey samples from a wide range of countries was also envisaged.

2. Materials and methods

2.1. Samples

A total of 22 samples corresponding to voucher specimens of honeybee workers from different subspecies of *A. mellifera* and haplotypes (Table 1), were used for developing the real-time PCR method. Those included the most representative of European honey production namely: C-lineage *A. m. carnica* from Serbia (n = 2) and Croatia (n = 3); C-lineage *A. m. ligustica* from Italy (n = 3); A-lineage *A. m. iberiensis* from Portugal (n = 8) and Spain (n = 1); M−lineage *A. m. iberiensis* from Portugal (n = 2) and Spain (n = 2); M−lineage *A. m. mellifera* from France (n = 1). All honeybee individuals were collected from distinct apiaries (Table 1), in the framework of previous studies, and their tissues were stored in absolute ethanol at −20 °C. In the previous works, DNA was extracted using the phenol–chloroform method and the haplotypes were determined based on the *Dra*I test, according to Garnery et al. (1993).

Authentic honey samples of different botanical origin (including monofloral and multifloral honey) and of known entomological origin were collected by beekeepersin 2020 either from research apiaries or apiaries recently studied within the scope of other projects, including Portugal (n = 5), Spain (n = 2) and Italy (n = 4), corresponding to A-lineage *A. m. iberiensis*, M-lineage *A. m. iberiensis* and C-lineage *A. m. ligustica*, respectively. In addition, the authentic honeys from A and M lineages were also used to prepare mock mixtures of 25:75, 50:50, and 75:25 (A:M) proportions. Additionally, a total of 44 commercial samples produced in different countries were purchased in 2021 on local Portuguese stores and on e-commerce international markets, namely: Portugal (n = 19), Spain (n = 4), Italy (n = 2), France (n = 4), Norway (n = 1), Germany (n = 3), Sweden (n = 1), Estonia (n = 1), Finland (n = 1), Slovenia (n = 4), Latvia (n = 2), Lithuania (n = 1) and New Zealand (n = 1).

Table 1
Results of real-time PCR coupled with HRM analysis targeting a 150-bp fragment of the COI gene of *A. mellifera* applied to honeybee specimens of different subspecies.

Honeybee subspecies	Lineage	Haplotype	Origin	Coordinates		Real-time PCR Ct ± SD[a]	HRM	
				Latitude (Y)	Longitude (X)		Cluster	Level of confidence ± SD (%)[a]
A. m. iberiensis	A	A1	Portugal	41.809	−6.712	21.90 ± 0.05	1	99.9 ± 0.1
A. m. iberiensis	A	A9b	Portugal	41.809	−6.712	18.83 ± 0.09	1	99.8 ± 0.0
A. m. iberiensis	A	A11b	Portugal	41.809	−6.712	22.18 ± 0.17	1	99.3 ± 0.6
A. m. iberiensis	A	A14a	Portugal	37.752	−25.588	22.01 ± 0.02	1	99.8 ± 0.0
A. m. iberiensis	A	A20	Portugal	41.809	−6.712	23.22 ± 0.04	1	99.7 ± 0.0
A. m. iberiensis	A	A3	Portugal	41.574	−7.213	20.26 ± 0.05	1	99.8 ± 0.0
A. m. iberiensis	A	A33	Portugal	39.052	−7.437	23.01 ± 0.07	1	99.4 ± 0.1
A. m. iberiensis	A	A41	Portugal	41.809	−6.712	19.32 ± 0.27	1	99.5 ± 0.2
A. m. iberiensis	A	A2	Spain	40.680	−3.208	23.59 ± 0.05	1	99.5 ± 0.1
A. m. iberiensis	M	M4g	Portugal	41.809	−6.712	23.33 ± 0.02	4	99.9 ± 0.0
A. m. iberiensis	M	M8	Portugal	41.809	−6.712	24.07 ± 0.08	4	99.7 ± 0.1
A. m. iberiensis	M	M4a	Spain	40.680	−3.208	21.98 ± 0.01	4	99.8 ± 0.0
A. m. iberiensis	M	M8	Spain	40.680	−3.208	24.20 ± 0.13	4	99.1 ± 0.4
A. m. mellifera	M	M4a	France	48.477	−5.067	22.80 ± 0.02	4	98.6 ± 0.3
A. m. carnica	C	C2	Croatia	45.800	15.717	23.91 ± 0.07	2	99.8 ± 0.1
A. m. carnica	C	C2	Croatia	45.800	15.717	19.36 ± 0.02	2	99.7 ± 0.0
A. m. carnica	C	C2	Croatia	45.800	15.717	21.21 ± 0.01	2	99.7 ± 0.1
A. m. carnica	C	C1b	Serbia	45.250	19.867	21.74 ± 0.16	2	99.9 ± 0.1
A. m. carnica	C	C2	Serbia	45.250	19.867	20.30 ± 0.13	2	99.9 ± 0.0
A. m. ligustica	C	C1	Italy	44.480	11.401	20.85 ± 0.07	3	99.2 ± 0.4
A. m. ligustica	C	C1	Italy	44.480	11.401	20.54 ± 0.09	3	98.3 ± 0.5
A. m. ligustica	C	C1	Italy	44.480	11.401	21.29 ± 0.02	3	98.3 ± 0.3

[a] mean values ± standard deviation (SD) of n = 3 replicates; Ct, cycle threshold.

2.2. DNA extraction

DNA extraction from honeybees: Each sample was composed by a pool of 10 workers collected from a single colony. Total DNA was extracted from pools of 20 forelegs (two per worker) using the Ron's Tissue DNA Mini Kit (Bioron), following manufacturer instructions with slight modifications.

DNA extraction from honey: Before the extraction step, each sample (25 g) of pre-heated (56 °C, 15 min) and homogenized honey was submitted to a pre-treatment to eliminate possible interferents such as sugars and polyphenols. The pre-treatment step consisted in a series of washing steps, followed by centrifugation for DNA concentration, as previously described by Soares et al. (2015) with some modifications. The obtained pellet was frozen overnight at −20 °C, then stored at −80 °C for 3 h and then heated at 56 °C for 10 min in a thermoblock with the aim of inducing cell lysis through a thermal shock. Subsequently, the pellet was centrifuged at 16,000 *g* for 15 min at 4 °C, then washed with ultrapure water and concentrated by centrifuging. The DNA was extracted from the pellet using the NucleoSpin® Plant II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer instructions with minor modifications, as described by Soares et al. (2015). DNA extracts were kept at −20 °C until further analysis.

The yield and purity of the extracts were assessed by UV spectrophotometry using a SPECTROstar® *Nano* microplate reader (BMG Labtech, Offenburg, Germany) with a LVis plate accessory. The absorbance was measured at 260, 280 and 230 nm to estimate DNA content and purity using the Multi-user Reader Control and MARS Data Analysis Software (LVis; BMG Labtech, Offenburg, Germany).

2.3. Selection of the target gene and oligonucleotide primers

The DNA sequences of the mitochondrial genome obtained from previous works (Henriques et al., 2019) by whole-genome sequencing on the Illumina HiSeq 2500 platform of a total of 95 honeybees of different lineages (A (n = 55), M (n = 35) and C (n = 5)), in addition to the representative COI sequence of the reference mitogenome (NC001566.1) retrieved from NCBI database (GenBank). The 95 mitogenomes plus the reference genome were aligned and informative regions with nucleotide differences between the subspecies of different lineages were identified. PCR primers targeting the COI gene were then manually designed to anneal in conserved regions, flanking the informative region, and allowing the amplification of short fragments (Fig. 1): amsCOI-F (5′-CAATGAGACTTATTATTCGAATAG-3′) and amsCOI-R (5′-GCCAATTTCCAAATC CTCCAA-3′). Primer properties and the absence of hairpins and self-hybridization were assessed using the software OligoCalc (https://biotools.nubic.northwestern.edu/OligoCalc.html). Primer specificity was assessed using the Primer-BLAST tool to reveal homologies in relation to all sequences available in GenBank. The designed primers were synthesised by Metabion International AG (Planegg, Germany).

2.4. Real-time PCR and HRM analysis

The real-time PCR assays were performed on a fluorometric thermal cycler CFX96 Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA) using a total volume of 10 μL per reaction containing 1 μL of DNA extract (5 ng DNA of honey samples and 0.1 ng DNA of honeybee samples), 1 × of SsoFast™ Evagreen® Supermix (Bio-Rad Laboratories, USA) and 400 nM of each primer amsCOI-F/amsCOI-R. The following temperature profile was used in the real-time PCR: 95 °C for 5 min, 45 cycles at 95 °C for 20 s, 60 °C for 45 s, with the collection of fluorescence signal at the end of each cycle. Each sample was amplified at least in triplicate. Immediately after the PCR, the products were submitted to HRM analysis. For this purpose, the PCR products were denaturated at 95 °C for 1 min and then annealed at 60 °C for 5 min. Subsequently, for melt curve analysis, the temperature was increased from 60 °C to 95 °C increments of 0.2 °C every 5 s, with fluorescence data being acquired at the end of each melting temperature increment. The data of real-time PCR were collected and processed using the software Bio-Rad CFX Maestro 1.1 and the melt curve analysis was processed using the Precision Melt Analysis Software 1.3 (Bio-Rad Laboratories, Hercules, CA, USA). Cluster detection settings were defined with the melting curve shape sensitivity parameter set as a default value of 50% and Tm difference threshold parameter set as a default value of 0.15.

2.5. Sequencing of PCR products

The real-time PCR products obtained for the honey samples that were not clustered with any of the reference honeybees were sent to a specialised research facility (STABVIDA, Lisbon, Portugal) for Sanger

Fig. 1. Alignment of the mitochondrial reference genome NC_001566.1 retrieved from the NCBI database with sequences obtained from mitogenomes of *A. m. iberiensis*, *A. m. mellifera*, C1 mitotype *A. m. ligustica* and C2 mitotype *A. m. carnica*, demonstrating the amplified COI fragment containing the discriminating SNPs. The arrows indicate the forward and reverse primers (amsCOI-F/amsCOI-R) located at the positions 1882 and 2011 on the mitochondrial reference genome NC_001566.1, highlighting the region used in HRM analysis. For each honeybee sequence, information on the country of origin and haplotype is given in parenthesis.

sequencing. In addition, for confirmatory purposes, a PCR product per each type of identified honey was also Sanger sequenced. After purification, to remove interfering components such as the EvaGreen dye, both strands of each product were sequenced in opposite directions. The results of both strands were used to obtain a consensus sequence with good quality. The sequencing data were analysed and aligned by BioEdit v7.2.5 (Ibis Bio- sciences, Carlsbad, CA, USA).

3. Results and discussion

3.1. Target selection, primers design and method development

The mitogenomes of 96 honeybees belonging to different mtDNA lineages and respective subspecies under study, namely *A. m. iberiensis* (A-lineage and M-lineage), *A. m. ligustica* (C-lineage, C1 mitotype), *A. m. carnica* (C-lineage, C2 mitotype), and *A. m. mellifera* (M-lineage), were aligned to allow the identification of regions containing single nucleotide polymorphisms (SNPs) that could be used to distinguish among the different lineages/subspecies (Fig. 1). Considering that the honeybee DNA present in honey is expected to be degraded, at the same time we targeted for the amplification of short DNA fragments, which are the most adequate for HRM analysis. After careful examination of the alignments, a single 150-bp fragment of the COI gene could meet those criteria (Fig. 1). Moreover, this region carried a A/G SNP, associated with C2 and C1 mitotypes, respectively. In 2003, the COI gene was proposed as a universal barcode for species identification in the animal kingdom (Herbert et al., 2003(Hebert et al., 2003)). Since then, the number of complete or partial COI gene sequences available in public databases, such as GenBank or BOLD (https://www.boldsystems.org/), has increased tremendously, as well as the COI-based barcoding studies. In this study, the COI barcode gene also revealed to be well-suited for designing an assay for entomological honey authentication.

Both the forward and reverse primers were designed to anneal in conserved regions of the 150-bp fragment of the COI gene, allowing its amplification in all the subspecies under study. The effectiveness of the proposed primer set was verified experimentally by end-point PCR using DNA extracts obtained from different subspecies of honeybees (data not shown). Subsequently, a real-time PCR methodology using the EvaGreen dye was optimized regarding temperature and time profile. After establishing the optimal conditions, the developed real-time PCR methodology was evaluated for the acceptance criteria previously established for real-time PCR assays, as described by Bustin et al. (2009) and ENGL (2015). For that purpose, a honeybee DNA extract was 10-fold serially diluted from 10 ng to 0.1 pg, thus covering six concentration levels (Fig. 2A). As expected, the corresponding melting curves showed a single melting peak at 73.7 ± 0.1 °C, consistent with the absence of unspecific amplicons (Fig. 2B). Fig. 2C shows the obtained real-time PCR calibration curve, demonstrating its good agreement with the established acceptance criteria (Bustin et al., 2009; ENGL, 2015), namely the slope (-3.490) was within the range of −3.6 to −3.1, the PCR efficiency (93.4%) was within the range of 90% to 110% and the correlation coefficient (0.998) was > 0.98. The limits of detection (LOD) and of quantification (LOQ) were established also as described by Bustin et al. (2009) and ENGL (2015), thus the former corresponded to the lowest amplified level for 95% of the replicates and the last to the lowest amplified level within the linear dynamic range of the calibration curve since it covered >4 orders of magnitude. Therefore, the LOD was established as being 0.01 pg of honeybee DNA while the LOQ corresponded to 0.1 pg of honeybee DNA, which was the lowest value within the linear dynamic range (Fig. 2). The cycle threshold (Ct) values corresponding to LOD and LOQ were 37.06 ± 1.23 and 33.25 ± 0.07, respectively.

To develop the proposed method, the EvaGreen dye was selected because it provides enhanced fluorescence when binding to DNA, very good stability and can be used at high concentration without inhibiting the PCR reaction, therefore being highly suitable for HRM analysis (Grazina et al., 2021). The real-time PCR coupled to HRM analysis was subsequently applied to several honeybee DNA extracts aiming for their discrimination. The five honeybee subspecies assessed herein exhibited very good amplification with Ct values ranging from 18.74 to 24.33 (Table 1). The application of HRM analysis allowed to differentiate lineages A, M and C. Fig. 3A and 3B show the normalised and difference HRM curves obtained for the five subspecies assayed evidencing their differentiation into four distinct clusters with high confidence levels: the

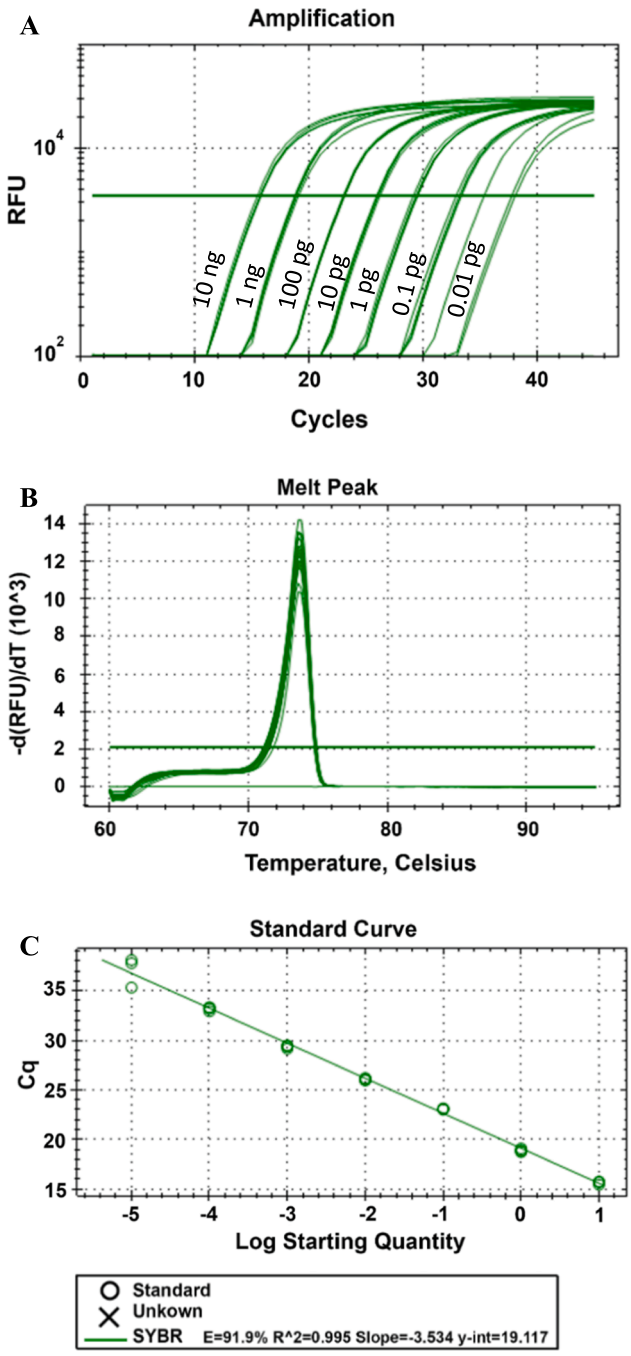

Fig. 2. Amplification (A), melting (B) and calibration (C) curves of the optimized real-time PCR assay with EvaGreen® dye targeting the COI fragment, obtained using a 10-fold serially diluted honeybee DNA (10 ng to 0.1 pg).

A-lineage *A. m. iberiensis* in cluster 1 (>98.7% of confidence), *A. m. carnica* (C-lineage, C2 mitotype) in cluster 2 (>99.6% of confidence), *A. m. ligustica* (C-lineage, C1 mitotype) in cluster 3 (>97.8% of confidence) and the M−lineage *A. m. iberiensis* and *A. m. mellifera* in cluster 4 (>98.3% of confidence; Table 1). The clear separation among the different clusters demonstrates the effectiveness of the proposed method.

3.2. Application of the developed method to honey samples

The developed real-time PCR assay targeting an informative region of 150 bp of the COI gene coupled with HRM analysis was then applied to honey samples. Overall, the pre-treatment step and DNA extraction produced good quality and purity extracts, as most of them were successfully PCR-amplified. The yield and purity of the honey DNA extracts, determined by spectrophotometry, ranged from 5.2 to 504.0 ng/μL and from 1.3 to 2.0, respectively. It is worthy of note that the estimated concentration of DNA in honey extracts includes not only DNA from honeybees but mainly DNA from pollen grains and viruses such as the *Apis mellifera* filamentous virus, as well as DNA from bacteria and fungi (Bovo et al., 2018). Thus, honey extracts presented a lower amount of honeybee DNA and consequently exhibited higher Ct values. Therefore, in an attempt to produce more balanced Ct values during amplification, lower concentrations were used for DNA extracts from honeybees (0.1 ng) than for honey samples (5 ng) in the PCR assays.

The method developed herein was first applied to 11 honeys with known entomological identity, obtained from previously studied apiaries, to evaluate its applicability in establishing the entomological origin of honey. The obtained results, displayed in Table 2 and Fig. 3C and 3D, clearly show the ability of the method in discriminating the three mtDNA lineages with high confidence levels. As can be observed, all honey samples clustered with the honeybee samples that matched the lineages indicated by the beekeepers. Following this validation step, the method was then applied to 44 commercial honeys from different geographical origins, including from 12 European countries and New Zealand. The obtained results are summarized in Table 3. Of the 19 analysed honeys from Portugal, the entomological origin of 14 (all from the south, Alentejo, and central, Lousã, regions) matched the autochthonous A-lineage *A. m. iberiensis*, congruent with the maternal composition of the Portuguese honeybee populations (Pinto et al. 2013; Chávez-Galarza et al. 2017). The remaining five honeys did not cluster with any of the reference honeybee subspecies used in the assay. This could be due to the commercial practice of mixing honeys from various colonies and apiaries, which can include colonies headed by queens of different lineages. In fact, for sample *H*10 (collected in the region of Bragança), the beekeeper confirmed having in her apiary colonies of *A. m. iberiensis* from both A and M lineages. This can be explained because Bragança is located near the border with Spain and thus both A and M lineages of *A. m. iberiensis* may coexist, possibly due to queen trading between the two countries (Pinto et al. 2013). To assess whether honey blends originating from honeybees of different mtDNA lineages produce different clusters, mock mixtures were prepared using authentic honeys from A and M−lineages at the following proportions: 25:75, 50:50 and 75:25 (A:M, m/m). The results of the HRM analysis showed the formation of three new clusters corresponding to each one of the mock mixtures (Fig. S1, supplementary material), suggesting that the samples that do not cluster with any of the four reference clusters correspond to blends of honeys produced by honeybees of varying ancestries. To further confirm this observation, the real-time PCR products were Sanger sequenced together with honey samples identified as being from lineage A and M ancestries. The obtained electropherograms evidenced an overlapping pattern thereby confirming the presence of DNA sequences characteristic from both A and M lineages (Fig. 4). Likewise, sequencing suggested that the samples H11 and H12, also from the Bragança region, were mixtures of A and M lineages. These two samples were included in different clusters (Fig. 3E and 3F) possibly because of varying proportions from each lineage in their composition, a pattern consistent with the results of the mock honey mixtures (Fig. S1, supplementary material). For sample H14 from the Faial Island of the Azores archipelago, Sanger sequencing indicated a mixture of the A-lineage *A. m. iberiensis* with the C-lineage *A. m. ligustica*, which is in good agreement with the existence of an admixed honeybee population on this island (Ferreira et al., 2020). Sample H15, also from the Azores (São Miguel Island), produced patterns of higher complexity, suggesting a mixture of all lineages. This is also in good agreement with the work of Ferreira et al. (2020), which showed a high diversity of honeybees in São Miguel Island.

The four samples originating from Spain gave similar results to those of Portugal, with two of them (H20, H21) assigned to the A-lineage

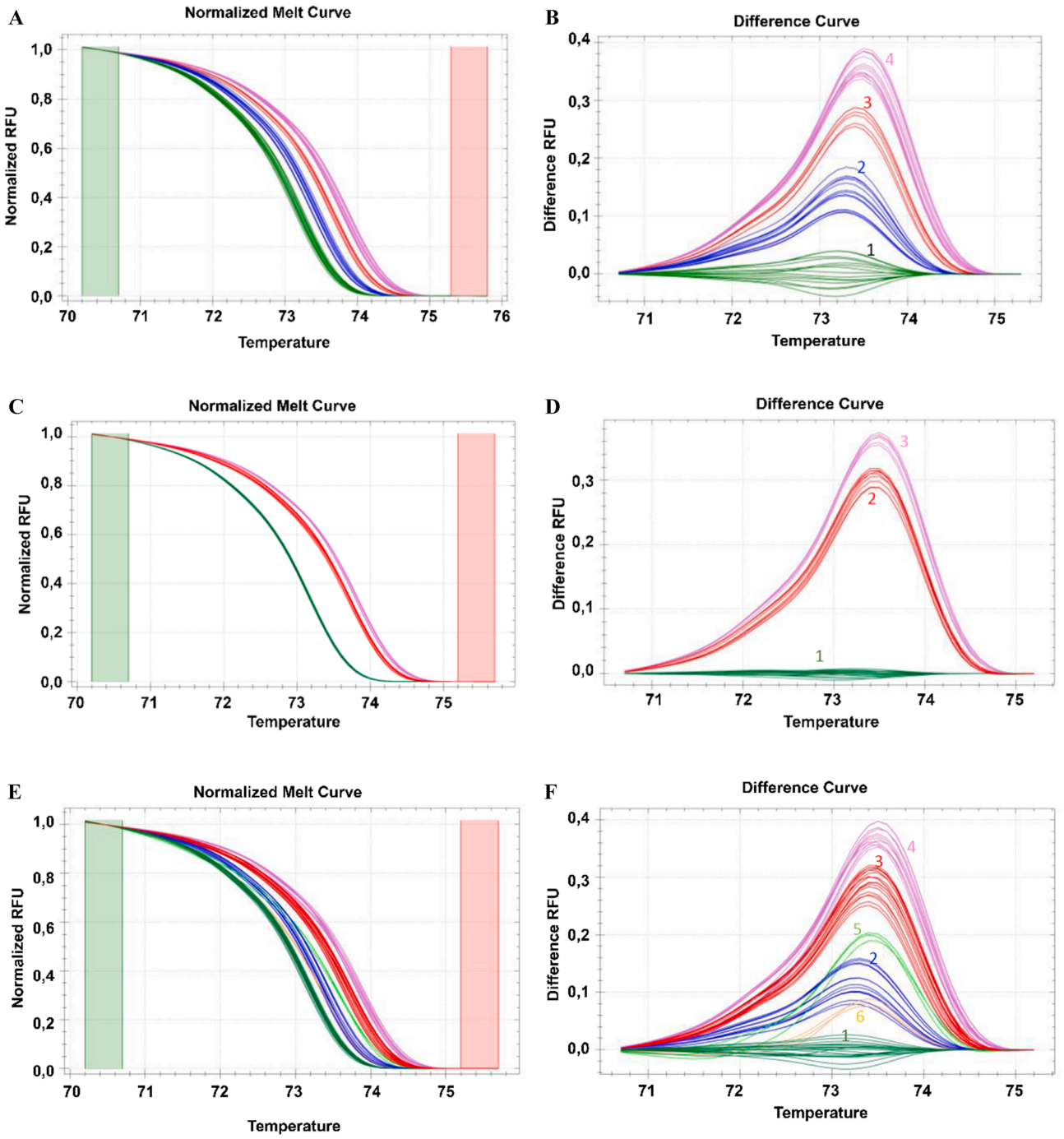

Fig. 3. Normalised melting curves (A, C, E) and difference curves (B, D, F) obtained by real-time PCR amplification with EvaGreen dye and HRM analysis targeting the COI gene applied to honeybee subspecies (A and B), honeys of known entomological origin (C and D) and commercial honeys (E and F). Clusters as indicated in Table 1 (A and B), Table 2 (C and D) and Table 3 (E and F).

cluster while the other two (H22, H23) did not cluster with any reference honeybee. Again, Sanger sequencing confirmed the mixture of honeys produced by *A. m. iberiensis* from lineages A and M. The Spanish honeys were produced in the province of Alicante, on the outskirts of Sierra de Mariola. Previous works comprising a large-scale population survey across the Mediterranean coast of Spain, using the sequence data of the $tRNA^{leu}$-COX2 intergenic region, demonstrated the presence of *A. m. iberiensis* from both A- and M-lineages in this region (Chávez-Galarza et al., 2017), therefore supporting the herein obtained results.

The remaining honey samples were assigned to C-derived entomological origin, except for the four samples from the French island of Corsica. According to the product specification file of "Miel de Corse" available in the EU geographical indications register (DG AGRI, 2022), this French PDO honey should be produced by honeybees of the Corse ecotype, which belongs to the *A. m. mellifera* subspecies (M-lineage). Therefore, the results here obtained confirm the entomological authenticity of the four analysed samples in terms of mtDNA lineage as they all clustered with M-lineage honeybees (Table 2).

The honey sample from New Zealand (H24) was classified as being produced by the mitotype C1 typical of *A. m. ligustica,* a result that was also in good agreement with the most common subspecies reported in this country. Since the 1980s until 2015, it was very usual to import

Table 2
Results obtained by real-time PCR coupled with HRM analysis targeting a 150-bp fragment of the COI gene of *A. mellifera* applied to the analysis of authentic honey samples obtained directly from apiaries previously studied in other works.

Honey sample	Geographical origin	Botanical origin	Entomological origin	Real time PCR Ct ± SD[a]	HRM		
					Cluster	Lineage	Level of confidence mean ± SD (%)[a]
1	Portugal, Alentejo	Multifloral	*A. m. iberiensis A-lineage*	25.64 ± 0.23	1	A	100.0 ± 0.1
2	Portugal, Alentejo	Multifloral	*A. m. iberiensis A-lineage*	25.75 ± 0.24	1	A	100.0 ± 0.0
3	Portugal, Alentejo	Multifloral	*A. m. iberiensis A-lineage*	24.18 ± 0.18	1	A	99.9 ± 0.1
4	Portugal, Alentejo	Multifloral	*A. m. iberiensis A-lineage*	24.84 ± 0.21	1	A	99.9 ± 0.1
5	Portugal, Alentejo	Multifloral	*A. m. iberiensis A-lineage*	25.36 ± 0.09	1	A	99.6 ± 0.4
6	Spain, Tarragona	Multifloral	*A. m. iberiensis M-lineage*	22.88 ± 0.05	3	M	97.6 ± 0.4
7	Spain, Tarragona	Multifloral	*A. m. iberiensis M-lineage*	27.74 ± 0.18	3	M	99.7 ± 0.1
8	Italy, Bologna	Multifloral	*A. m. ligustica*	30.34 ± 0.19	2	C (*A. m. ligustica*)	99.1 ± 0.8
9	Italy, Bologna	Multifloral	*A. m. ligustica*	28.62 ± 0.14	2	C (*A. m. ligustica*)	98.0 ± 0.8
10	Italy, Bologna	Acacia	*A. m. ligustica*	27.09 ± 0.37	2	C (*A. m. ligustica*)	98.7 ± 0.3
11	Italy, Bologna	Lime	*A. m. ligustica*	26.09 ± 0.28	2	C (*A. m. ligustica*)	99.1 ± 0.9

[a] mean values ± standard deviation (SD) of n = 3 replicates; Ct, cycle threshold.

Table 3
Results obtained by real-time PCR coupled with HRM analysis targeting a 150-bp fragment of the COI gene of *A. mellifera* applied to the analysis of commercial honey samples from different countries.

Honey sample	Labelled geographical origin	Labelled botanical origin	Real time PCR Ct ± SD[a]	HRM		
				Cluster	Lineage	Level of confidence ± SD (%)[a]
H1	Portugal, Terceira Island (Azores)	Multifloral	29.79 ± 0.08	1	A	99.0 ± 0.7
H2	Portugal, Alentejo	Multifloral	23.85 ± 0.11	1	A	99.6 ± 0.3
H3	Portugal, Alentejo	Multifloral	23.68 ± 0.15	1	A	99.5 ± 0.4
H4	Portugal, Alentejo	Multifloral	26.40 ± 0.03	1	A	99.8 ± 0.1
H5	Portugal, Alentejo	Multifloral	24.13 ± 0,04	1	A	99.6 ± 0.1
H6	Portugal, Alentejo	Multifloral	26.15 ± 0.54	1	A	98.9 ± 0.6
H7	Portugal, Alentejo	Multifloral	22.69 ± 0.52	1	A	99.7 ± 0.1
H8	Portugal, Alentejo	Multifloral	25.67 ± 0.36	1	A	99.4 ± 0.0
H9	Portugal, Alentejo	Multifloral	26.82 ± 0.06	1	A	98.8 ± 1.0
*H*10	Portugal, Bragança	Multifloral	25.24 ± 0.02	5	n.d (mixture)	97.8 ± 0.2
*H*11	Portugal, Bragança (PDO)	Multifloral	35.17 ± 0.04	6	n.d (mixture)	97.3 ± 0.6
H12	Portugal, Bragança (PDO)	Rosemary	26.80 ± 0.03	5	n.d (mixture)	95.7 ± 1.2
H13	Portugal, Azores (PDO)	Incense	24.90 ± 0.08	1	A	98.0 ± 0.3
H14	Portugal, Faial Island (Azores)	Multifloral	27.53 ± 0.05	5	n.d (mixture)	97.1 ± 0.2
H15	Portugal, S. Miguel (Azores)	Incense	34.74 ± 0.35	5	n.d (mixture)	97.6 ± 0.5
H16	Portugal, Lousã	Orange	29.07 ± 0.06	1	A	99.7 ± 0.1
H17	Portugal, Lousã	Heather	26.90 ± 0.01	1	A	99.7 ± 0.1
H18	Portugal, Lousã	Eucalyptus	26.49 ± 0.08	1	A	99.4 ± 0.4
H19	Portugal, Lousã (PDO)	Heather	25.10 ± 0.09	1	A	99.2 ± 0.1
H20	Spain, Alicante	Multifloral	33.13 ± 0.07	1	A	99.0 ± 0.4
H21	Spain, Alicante	Avocato	21.92 ± 0.08	1	A	99.6 ± 0.3
H22	Spain, Alicante	Carob tree	23.56 ± 0.03	6	n.d	97.7 ± 0.1
H23	Spain, Alicante	Medlar	25.20 ± 0.02	5	n.d (mixture)	96.7 ± 0.1
H24	New Zealand	Manuka	31.03 ± 0.03	3	C (*A. m. ligustica*)	98.4 ± 1.0
H25	Italy	Multifloral	26.79 ± 0.04	5	n.d (mixture)	97.3 ± 0.1
H26	Italy, Belluno	Multifloral	31.53 ± 0.29	6	n.d (mixture)	93.8 ± 5.6
H27	France, Corse (PDO)	Clementine flowers	30.85 ± 0.09	4	M	98.5 ± 0.9
H28	France, Corse (PDO)	Autumn maquis of ivy flowers	33.04 ± 0.05	4	M	95.5 ± 0.2
H29	France, Corse (PDO)	Spring maquis	30.58 ± 0.04	4	M	92.9 ± 0.5
H30	France, Corse (PDO)	Autumn maquis	28.31 ± 0.02	4	M	99.2 ± 0.1
H31	Germany, north	Dandelion	25.17 ± 0.02	3	C (*A. m. ligustica*)	99.6 ± 0.2
H32	Germany, north	Acacia	26.39 ± 0.00	5	n.d (mixture)	97.3 ± 0.0
H33	Germany, north	Summer flowers	24.60 ± 0.50	2	C (*A. m. carnica*)	99.9 ± 0.1
H34	Sweden	Raspberry	26.72 ± 0.06	2	C (*A. m. carnica*)	99.6 ± 0.1
H35	Estonia	Wild fruits	25.58 ± 0.07	2	C (*A. m. carnica*)	99.9 ± 0.0
H36	Slovenia	Chestnut	24.45 ± 0.04	2	C (*A. m. carnica*)	64.3 ± 1.1
H37	Slovenia	Acacia	24.88 ± 0.06	5	n.d (mixture)	97.2 ± 0.1
H38	Slovenia	Fir	24.17 ± 0.14	5	n.d (mixture)	98.0 ± 0.4
H39	Slovenia	Linden	29.28 ± 0.02	5	n.d (mixture)	97.6 ± 0.1
H40	Latvia	Buckwheat	29.53 ± 0.04	5	n.d (mixture)	97.5 ± 0.1
H41	Latvia	Heather	29.79 ± 0.21	5	n.d (mixture)	97.8 ± 0.0
H42	Finland	Summer flowers	29.40 ± 0.12	6	n.d (mixture)	97.7 ± 0.1
H43	Lithuania	Wild	27.91 ± 0.11	5	n.d (mixture)	98.0 ± 0.0
H44	Norway	Mountain flowers	29.78 ± 0.10	5	n.d (mixture)	97.4 ± 0.3

[a] mean values ± standard deviation (SD) of n = 3 replicates; Ct, cycle threshold; n.d., not determined.

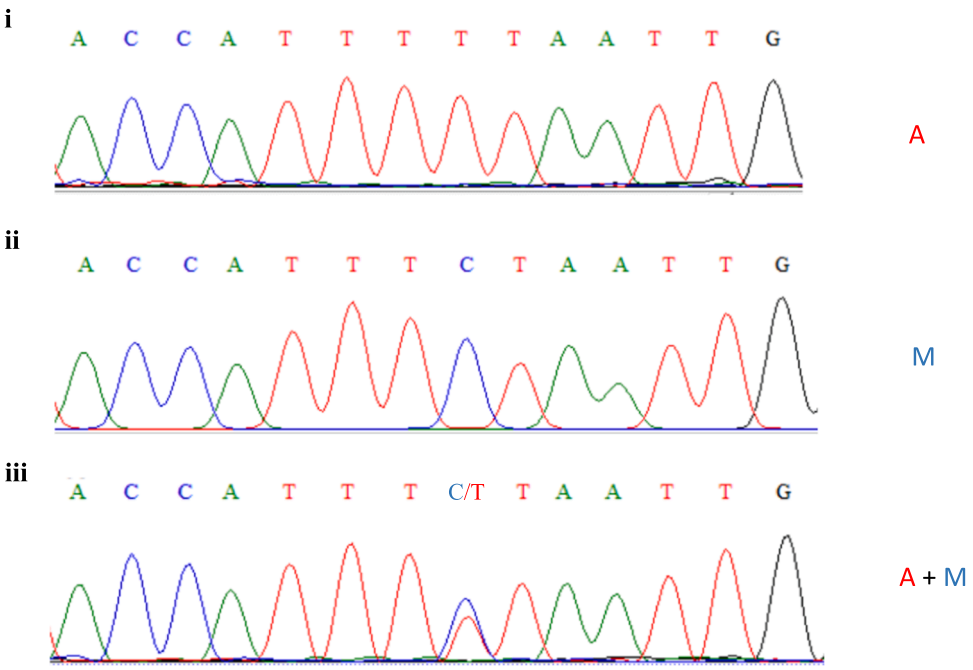

Fig. 4. Electropherogram obtained from the sequencing of real-time PCR fragments amplified from: i) honey that had only DNA from A-lineage honeybees; ii) honey that had only DNA from M−lineage honeybees; iii) honey that had a mixture of both A- and M−lineages, evidencing the presence of both T (characteristic of A-lineage) and C (characteristic of M−lineage) at the position 2007 on the mitochondrial reference genome NC_001566.1).

A. m. ligustica queens into New Zealand (Beard, 2015). Moreover, more recently, Nakagawa et al. (2018) sequenced the complete mitochondrial genome of the New Zealand yellow coloured *A. mellifera* and found that it was most closely related to the Italian subspecies *A. m. ligustica*. The two honeys from Italy revealed to be mixtures of the two C-lineage mitotypes. Although one of the samples did not indicate the region of provenance, the other originated from Belluno region. According to the EU geographical indications register, the PDO honey "Miele delle Dolomiti Bellunesi" should be produced by the local ecotype of *A. mellifera*, which results from the natural cross-breeding of the Italian and the Carniolan honeybee that over time adapted particularly well to the characteristics of the mountain environment of the Belluno region and can provide good yields of honey. Therefore, despite not being labelled as PDO, sample H26 gave a result well in line with the declared geographical origin (Belluno region).

Unexpectedly, among the four analysed honeys from Slovenia (H36-H39), only H36 was classified as being produced by C2 mitotype, more common in the Carniolan honeybee *A. m. carnica*, the subspecies indigenous to Slovenia. When joining to the European Union, Slovenia declared its intention to continue applying appropriate measures to ensure the preservation of its autochthonous Carniolan honeybee and prohibited the import of any other subspecies (European Comission, 2003). HRM results indicated the presence of mixtures since the honey did not cluster with any tested lineage or mitotype, which was further confirmed by Sanger-sequencing as being mixtures of C1 and C2 mitotypes. Considering that *A. m. carnica* is naturally found in Slovenia with the prevalence of mitotype C2 (Tanaskovic et al. 2021) it was expected that samples H37, H38 and H39 also cluster with this mitotype as in sample H36. However, since both C1 and C2 mitotypes were present in these samples, and C1 mitotype is most frequently present in *A. m. ligustica*, the admixing with honey from other countries cannot be excluded.

The honeys from northern Europe were all assigned to the C-lineage cluster, despite their origin in the native range of the M-lineage *A. m. mellifera* subspecies. The Swedish sample (H34) was classified as being of C2 mitotype while the honeys from Finland (H42) and Norway (H44) revealed to be mixtures of C1 and C2 mitotypes (Table 2). Results for the three samples from Germany suggested the usage of both C-lineage subspecies in this country since both mitotypes were detected. The samples from Lithuania and Latvia were also identified as being a mixture of honeys from C-lineage honeybees while the honey from Estonia was included in the cluster of the C2 mitotype most commonly present in *A. m. carnica*. These results are consistent with the described practices of commercial beekeeping and introduction of queens from C-lineage ancestries in those countries.

Since the beginning of the 20th century, *A. m. carnica* and *A. m. ligustica* subspecies have been expanding their native ranges in the Balkan and Italian peninsulas to other regions, such as central and northern European countries, as a result of intense queen trading (De la Rúa et al. 2009). This has been motivated by their gentle behaviour and higher productivity making them favoured for commercial bee breeding purposes (Sušnik et al., 2004; Jensen et al., 2005). Recurrent beekeeper-mediated movements from eastern to western and northern Europe have led to C-derived introgressive hybridization and even replacement of the native *A. m. mellifera* in many countries (Groeneveld et al., 2020). In Germany, the massive introduction of *A. m. carnica* has virtually driven the native *A. m. mellifera* to extinction (Jensen et al., 2005). A similar scenario has been reported for Scandinavian countries, where currently the majority of honeybee colonies are derived from *A. m. carnica* and *A. m. ligustica,* and the number of native *A. m. mellifera* colonies is very low (Groeneveld et al., 2020). In Lithuania, *A. m. mellifera* was bred in reservation areas from 1971 up to 1995. However, this practice was discontinued due to the difficult management and high hybridization levels, so that, currently, beekeeping mainly relies on Carniolan honeybees (Ceksteryte et al., 2012). In summary, native diversity patterns have changed dramatically in large tracts of western and northern Europe due to intense queen trading and commercial beekeeping. Accordingly, honeybees of C-lineage ancestry are more likely to be found than the native M-lineage, which is consistent with the results obtained in this work.

4. Conclusions

To detect possible frauds, the entomological origin has been recently

proposed as an additional tool that can complement other methodologies towards establishing the authenticity of honey, particularly in what concerns to its geographical origin. In this work, a one-step approach based on HRM analysis of a 150 bp fragment of the COI gene was developed to establish the entomological origin of honey by discriminating A, M and C mtDNA lineages and differentiating a SNP associated with a high frequency of C1 or C2 mitotypes in the Italian honey bee *A. m. ligustica* and the Carniolan honey bee *A. m. carnica*. The method is also capable of indicating the mixture of honeys produced by honey bees of different lineages, although not allowing to identify the lineages or mitotypes in the mixture.

The method is particularly useful for evaluating honeys that have a clear link between geographical and entomological origins, such as some PDO honeys for which the honeybee subspecies that should be used in their production is clearly established or honeys produced in regions that enforce by law the use of autochthonous subspecies, such as in the Italian region of Emilia Romagna where rearing colonies other than *A. m. ligustica* is forbidden. When this direct connection does not exist, this method still adds information that can assist, together with other parameters, in detecting honey frauds.

The application of the method confirmed the entomological authenticity of the analysed PDO honeys that specify the honeybee subspecies in their production. Moreover, this work further highlights the prevalence of C-lineage subspecies introduced by commercial beekeeping in many regions where *A. m. mellifera* is native.

CRediT authorship contribution statement

Mónica Honrado: Methodology, Writing – original draft. **Ana R. Lopes:** Methodology. **M. Alice Pinto:** Conceptualization, Resources, Writing – review & editing, Supervision, Funding acquisition. **Joana S. Amaral:** Conceptualization, Resources, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2022.111761.

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