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PC-F03: High resolution melting analysis of a COI mini-barcode as a simple approach for the entomological authentication of honey

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Honey is highly valued for its taste, aroma, content in bioactive compounds and for being a natural food. In the European Union (EU), market demand for honey is higher than the domestic production and therefore a substantial amount of honey is imported. According to a 2014 European Parliament report on fraud in the food chain, honey was ranked as the 6th food product most prone to adulterateration.[1] Up until now, honey authenticity addressed mainly sugars addition and botanical origin. However, an increased attention has recently been paid to honey entomological origin as it also relates to its geographical origin since honeybees carrying mitochondrial DNA (mtDNA) of distinct ancestries can be found across Europe. While in Portugal the predominant mtDNA of the autochthonous subspecies *Apis mellifera iberiensis* belongs to the A-lineage, when moving towards the northeastern part of the Iberian Peninsula this lineage is gradually replaced by the M-lineage. The native distribution of the M-lineage *A. m. mellifera* expands from the Pyrenees to Scandinavia and from the British Isles to the Ural Mountains while the C-lineage *A. m. ligustica* and *A. m. carnica* are naturally found in the Apennine and Balkan peninsulas, respectively.[2] Currently, several honeys holding the protected designation of origin (PDO) label should be produced with autochthonous *Apis mellifera* subspecies, according to the respective EU geographical indications register. Additionally, consumers are increasingly concerned with ethical and environmental issues, paying attention to issues such as the protection of biodiversity and the mode of production. For these reasons, the development of methodologies for entomological authentication of honey contributes not only to assure consumers rights and avoid unfair competition by the identification of frauds, but also to promote and valorize autochthonous honeybee subspecies. Previous works have been carried out to discriminate among honeys produced by honeybees of each of the three mtDNA lineages (A, M and C). However, the proposed methodology is laborious as it relies on a 2-step approach: a qualitative PCR for the identification of A-lineage followed by a real-time PCR to discriminate between M and C-lineages based on their high-resolution melting profiles. In the present work, we propose a novel methodology based on newly designed primers targeting a 150 bp fragment of the cytochrome c oxidase subunit I (COI) gene to differentiate the three lineages in a single step. DNA was extracted from honeybees of different subspecies, namely *A. m. iberiensis* (lineages A and M), *A. m. mellifera* (lineage M), *A. m. carnica* (lineage C) and *A. mellifera ligustica* (lineage C), using the Ron's Tissue DNA mini kit (Bioron, Germany). Honey samples included honeys of known entomological origin provided by beekeepers and commercial samples. Honeys were submitted to an in-house optimized pre-cleaning step and DNA extracted using the NucleoSpin Plant II kit (Macherey-Nagel, Düren, Germany). The optimized conditions of the real-time PCR allowed establishing an absolute limit of detection (LOD) of 0.1 pg of honeybee DNA, a reaction efficiency of 93.4% and a R^2 of 0.998 (**Fig. 1A and 1B**). The developed HRM analysis allowed successful differentiation of honeybees from lineages A, M and C in three different clusters with high

percentage of confidence (>99%). When applied to honey analysis, the samples provided by beekeepers were clustered according to the information provided proving the efficacy of the proposed methodology. In particular, the honeys provided by the Portuguese beekeepers were clustered with *A. m. iberiensis* lineage A (**Fig. 1C**). However, some commercial samples of honey produced in Portugal did not cluster with any honeybee subspecies, suggesting the presence of a mixture of honeys produced by honeybees of different ancestries, most probably A-lineage with M-lineage. Overall, a new approach, based on a single real-time PCR amplification with the fluorescent dye EvaGreen followed by HRM analysis, is proposed as a simple, fast and cost-effective approach to verify the labeling compliance of PDO honeys that should be produced with specific autochthonous honeybee subspecies.

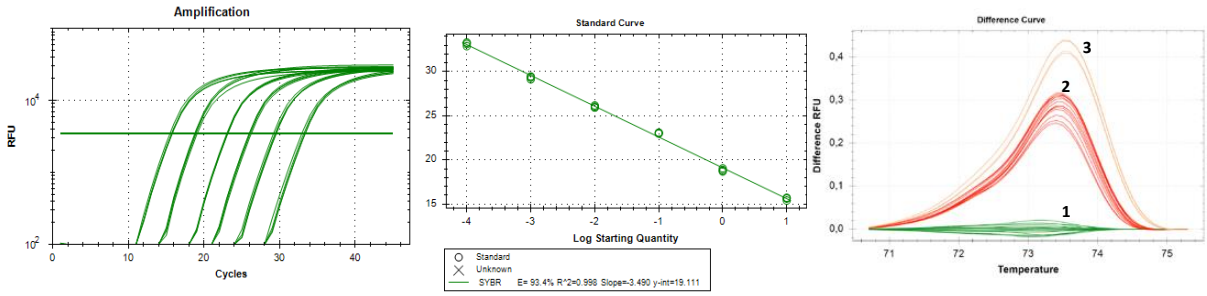

Figure 1: Amplification (A) and calibration (B) curves of the optimized real-time PCR assay with EvaGreen® dye, targeting the COI mini-barcode using 10-fold serially diluted honeybee DNA (10 ng to 0.1 pg). Difference melting curves (C) obtained by HRM analysis of voucher honeybees and honey samples; cluster 1: A-lineage *A. m. iberiensis* and honeys from Portugal; cluster 2: C-lineage *A. m. ligustica* and honeys from Italy; cluster 3: M-lineage *A. m. iberisensis* and honey from Spain.

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PC-F07: Next-generation sequencing as a promising approach for assessing the entomological origin of honey

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Honey is a food widely consumed worldwide and much appreciated for its nutritional and organoleptic properties as well as for its beneficial health effects. However, honey is also considered one of the foods most prone to be adulterated either by the admixing of honey with lower quality, by the addition of sugars, or by mislabeling of botanical and geographical origins, among other possible frauds.[1] Therefore, typically, honey authentication has focused mainly on the development of techniques targeting these types of frauds. Recently, increased attention has been paid to honey's entomological origin since it also relates with geographical origin whose label non-compliances are difficult to detect. Moreover, in the current context where native honeybees are increasingly threatened by introgression, due to the use of exotic queens, preservation of honeybee subspecies in their native ranges, to which they are better adapted, is perceived as of high importance. In this sense, valorisation of the honey produced by native subspecies has been suggested as a possible approach to generate higher income for beekeepers, contributing to the development of rural regions and of sustainable beekeeping based on conservation strategies.[2] Within the project Autent+, new approaches are being explored and tools developed for the authentication of honey produced by the native Portuguese honeybees (*Apis mellifera* subsp. *iberiensis*, mitochondrial DNA lineage A) and its discrimination from honey produced by honeybees of different maternal lineages. Lately, DNA-metabarcoding is emerging as a promising alternative for species identification since high-throughput sequencing (also known as next generation sequencing, NGS) platforms are able to yield millions of reads due to massive parallel sequencing. In the present work, the use of NGS was attempted to identify the entomological origin of honey samples. To that end, the mitogenomes obtained from previous works by whole genome sequencing of 121 individuals belonging to different subspecies and mitochondrial lineages, namely *A. m. iberiensis* (lineages A and M), *A. m. mellifera* (lineage M), *A. m. carnica* (lineage C) and *A. mellifera ligustica* (lineage C), were used to select the most promising regions for primers design. Based on the potential to discriminate *A. m. carnica* from *A. m. ligustica*, primers were designed targeting a 406 bp fragment of the cytochrome c oxidase subunit I (COI) gene. This gene has been proposed as a universal barcode for animal species identification.[2] A total of 35 samples of honey, including samples of known entomological origin provided by beekeepers from Portugal, Spain and Italy, and honeys commercially acquired in supermarkets were submitted to DNA extraction using an in-house optimized pre-treatment step to eliminate interferents and the NucleoSpin Plant II kit (Macherey-Nagel, Düren, Germany). After optimizing PCR conditions, DNA extracts were first amplified using the newly designed primers attached to suitable adapters for subsequent NGS. PCR products were amplified in a second PCR with a set of appropriate indexes and sequenced on the Illumina MiSeq platform. The obtained sequences were analyzed using a bioinformatics pipeline tailored for assigning sequencing reads to the different mitochondrial lineages and corresponding *Apis mellifera* subspecies. After applying several filters, the total number of reads used for species identification varied from 140 to 5001,

with most samples presenting over 1000 usable reads. The proposed methodology has the advantage of allowing identification of mixtures of DNA in the same sample. Honey may contain DNA of different maternal lineages when it is harvested from different hives headed by queens from different lineages and it is then combined. In particular, some samples from Spain showed the presence of mixtures of DNA from lineages A and M, which is consistent with the distribution of *A. m. iberiensis* (lineages A and M) in the Iberian Peninsula. For a sample collected in Faial, Azores, a mixture of DNA from lineages A and C was detected, which is also consistent with the subspecies used in beekeeping in that island. The obtained number of sequence reads was used to estimate the % of each subspecies in Faial honey, allowing to conclude that there was a predominance of C-lineage *A. m. ligustica* DNA (68%) over A-lineage *A. m. iberiensis* DNA from (32%). In general, the obtained results corroborated the information provided by beekeepers for the samples of known origin and the commercial samples were in good agreement with data previously generated using other approaches. To the best of our knowledge, this is the first study proposing a DNA metabarcoding approach for identifying honeybee subspecies and/or mitochondrial lineages in honey samples. Overall, our results suggest that COI metabarcoding offers a reliable and high-throughput alternative to establish the entomological origin of honey.

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