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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 adulteteration.[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 valorise 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 labelling compliance of PDO honeys that should be produced with specific autochthonous honeybee subspecies.

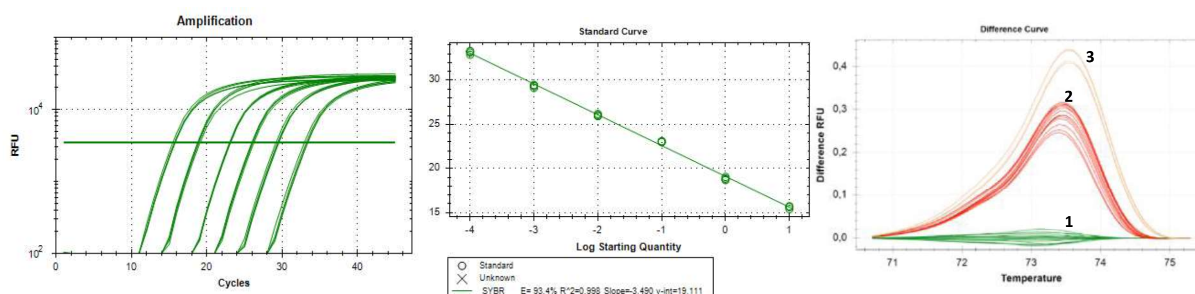

Figure 1: Amplification (A) and calibration (B) curves of the optimized real-time PCR assay with EvaGreen® dye, targeting the COI mini-barcode using 10-fold serially diluted honeybee DNA (10 ng to 0.1 pg). Difference melting curves (C) obtained by HRM analysis of voucher honeybees and honey samples; 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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