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Of bees and buzz: towards validated NGS-based methods in honey authentication

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Honey, a food with a long history, has evolved into a substantial global market. Its production relies on honeybees, which are also important in pollination and agriculture. Honey adulteration threatens this sector, requiring analytical methods to assess its authenticity, with Next Generation Sequencing emerging as a promising innovative solution. However, like any authentication method, its effectiveness must be evaluated via rigorous testing, ring trials, and comparison with existing methods.

Humans have been consuming honey for thousands of years. The global honey market is now valued at approximately 8 billion euros in 2022, projected to grow to 12 billion euros by 2030¹. Honey, with its distinct flavour and potential health benefits, is considered a popular alternative to sugar. However, the growing demand for this natural sweetener often overshadows the role that honeybees play in pollinating crops, an important ecosystem service that is essential to global agriculture. As major pollinators in natural habitats, bees - including both wild bees and honeybees - play a crucial role in the reproduction of these plants, ultimately contributing to global food supplies through their role in crop pollination².

Honey adulteration has become a matter of growing concern over the last few years, as highlighted by the EU Coordinated Control Plan “From the Hives”, which found that 46% of analysed samples raised suspicions³. These adulterations can be done by directly adding sweeteners (corn syrup, rice syrup) to the honey, mixing highly priced monofloral honey with polyfloral honey or mislabelling the geographical origin, botanical composition or organic status of the product. It can also be related to any practice that would fail to meet the legal standards regulating honey quality⁴. To identify adulterations in honeybee products, a range of analytical methods is employed, encompassing diverse methodologies such as physicochemical analysis, chromatography or electrochemistry⁴. This also includes DNA analysis, such as real-time quantitative PCR for the botanical identification of Manuka honey, or spectroscopy methods such as elemental analysis, isotope-ratio mass spectrometry⁵ (EA-IRMS) and liquid-chromatography IRMS⁶ (LC-IRMS).

To ensure its reliability, an authentication method needs to be validated. For quantitative methods (such as ¹H NMR or LC-IRMS), this implies the assessment of key performance parameters such as Limit of Detection (LOD), Limit of Quantification (LOQ), specificity, robustness and trueness. If possible, their performance should also be compared with validated orthogonal methods. Several quantitative methods have ranges for

their responses (e.g. enzyme activity, concentrations of certain substances), and samples exceeding these ranges are considered adulterated. Validating these methods confirms that the analytical results are trustworthy, which in turn ensures that the assessment of whether a sample is adulterated or not is reliable.

However, other approaches (such as Raman spectroscopy or Next Generation Sequencing (NGS) analysis) do not determine adulteration by comparing a single measurement to predefined limits. Instead, they assess whether the overall response pattern resembles that of authentic samples, making them qualitative rather than quantitative approaches. Qualitative approaches must also be validated to demonstrate how well they perform and how reliable their findings are. This validation can be carried out by evaluating the false-positive and false-negative rates, which enable the assessment of the method's detection capability⁷ (sensitivity, selectivity, efficiency). The minimum concentration at which an adulterant can be reliably identified (e.g., LOD) must also be established. As honey adulterations are diverse and continually adapting, new experimental approaches are being explored.

NGS is a technology allowing the high-throughput parallel sequencing of DNA fragments. In the context of honey authentication, NGS-based analysis can be conducted using two different methodologies: either targeted (metabarcoding), by sequencing specific regions of DNA enabling species identification, or untargeted (whole genome sequencing), to provide a comprehensive overview of the genetic material present (shotgun metagenomics).

Both approaches have already been applied to study honeybee products and honeybee-associated microbiomes⁸, yet efforts to standardise these methods for the purpose of honey authentication are still required.

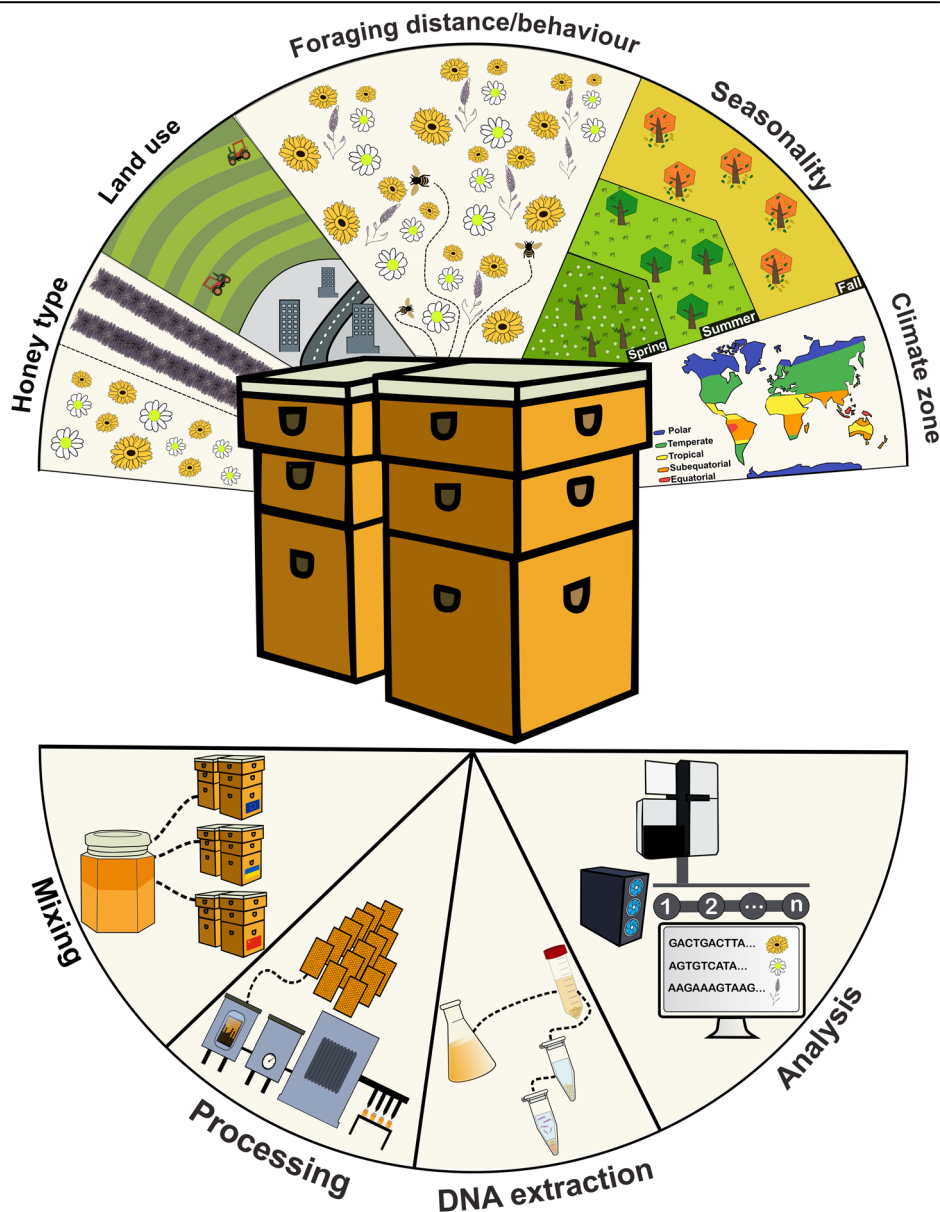
Recently, the use of metagenomics for honey authentication has garnered significant media attention, with some reports suggesting that up to 80% of tested samples were counterfeit^{9,10}. However, these claims have been met with scepticism¹¹ as the method used was not validated and honey's complexity poses significant challenges for authentication^{12,13}. While NGS analysis, either based on metagenomic or metabarcoding, holds promises for authenticating food through its DNA content, assessing its effectiveness for honey authentication is essential.

Here, we emphasise how the intricate composition of honey affects its DNA profile, highlighting the need for rigorous testing to validate NGS-based honey authentication methods and the development of dedicated reference databases to guarantee accurate species identification.

The dynamic composition of honey: implications for DNA analysis and authentication

Honey production by honeybees relies on the collection of “the natural sweet substance produced by *Apis mellifera* bees from the nectar of plants or from secretions of living parts of plants or excretions of plant-sucking insects on the living parts of plants, which the bees collect, transform by

Fig. 1 | Environmental and processing factors influencing honey DNA profiling. The honey DNA profile results from a complex interplay of environmental, commercial and processing factors, requiring careful consideration in the development of authentication methods.



combining with specific substances of their own, deposit, dehydrate, store and leave in honeycombs to ripen and mature¹⁴. The floral landscape surrounding beehives is shaped by a complex interplay of environmental factors. This includes abiotic factors such as the seasonal patterns, geographical location and climate regimes that create variations in temperature, precipitation, and daylight hours, affecting the timing and duration of flowering periods as well as the availability of blooming flowers for pollination over time. Additionally, human activities such as agriculture and urbanisation can significantly alter soil quality, leading to changes in nutrient availability and diversity of floral species¹⁵. This affects the complexity and composition of plant communities, ultimately influencing the richness and variety of plants that are available to pollinators, such as honeybees, and contributing to the specificity of the honey produced in a specific area. The foraging behaviour and distance of honeybees is also

influenced by a range of environmental factors such as the morphology and scent of flowers, which will attract honeybees to varying degrees, as well as the energy trade-off of foraging, the plant physiological state, the community diversity and the competition with pathogens or other pollinators¹⁶. The environmental and pollen DNA found in a honey jar is derived from this dynamic interplay, leading to a DNA composition changing over time rather than remaining static¹⁷ and implying that the honey DNA profile from a single beehive can vary significantly throughout the season.

In addition, mixing honeys from different hives is legal. In the European Union, current legislation allows mixing of honeys of different countries if the countries of origin are clearly mentioned on the label, together with the percentage of mass of each country¹⁸. This implies that a typical sales unit of honey found in the market is often a mix of different geographical and botanical sources. Finally, honey processing, such as

heating and filtration, alter further the DNA composition and may prevent the detection of certain species initially present. This will therefore increase the variation in the DNA profile observed, even for fully authentic honey samples. As a result, NGS-based authentication methods applied to honey, like any honey authentication method, are impacted by these factors (summarised in Fig. 1).

Towards NGS-based honey authentication methods

Metagenomics and metabarcoding exhibit methodological differences but share key dependencies: high-quality DNA extraction from collected samples and bioinformatic analysis—particularly the use of comprehensive reference databases—to analyse and interpret the resulting sequences. Consequently, three major factors can affect the final DNA profile: (1) the amplicon-based or genomic reference database used^{19,20} (2) the bioinformatic analysis of the obtained sequences^{19,20}, and (3) the DNA extraction and library preparation methods^{19,20}. These factors present challenges to honey authentication that must be resolved before determining a sample's authenticity. Addressing them requires a coordinated strategy that begins with the creation of a dedicated honey-DNA reference library. This library should be assembled from a large, well-documented set of authentic honeys that captures the full spectrum of botanical origins, geographic regions, seasons and the typical blends found in commercial products. Each sample must be unquestionably authentic, either by sourcing from certified producers or by confirming authenticity with orthogonal methods such as EA-IRMS, LC-IRMS, ¹H-NMR screening, or melissopalynology²¹.

Although existing databases such as BEEexact²² and BeeRoLaMa²³ focus on bee-associated microbial communities, they demonstrate the feasibility of curated repositories, and guidelines for constructing food-authenticity databases^{24,25} provide a practical framework for sampling, metadata capture, and data-submission standards. Validation of the workflow should be achieved through interlaboratory comparison in which each laboratory receives identical honey extracts and evaluates both DNA-extraction efficiency and bioinformatic pipelines against the shared reference set. False-positive and false-negative rates, as well as the LOD, can be established by spiking authentic honeys with known quantities of sugar syrup (e.g., 0.5%–10% w/w) and assessing classification performance. Although individual laboratories may maintain their own proprietary reference databases, a publicly accessible reference platform would provide a common benchmark that facilitates method development, enables consistent performance evaluation, and promotes regulatory acceptance. Altogether, these advantages allow reliable discrimination between natural variability and intentional adulteration.

Both metagenomics and metabarcoding hold promise in enhancing the authenticity and quality of honey products. However, unlocking this potential requires recognising and overcoming the biological and methodological challenges inherent in honey analysis, as well as demonstrating the method's reliability against existing standards. By doing so, the NGS technologies will become a valuable tool for informing regulatory agencies, consumers, and supporting the long-term sustainability of the honey industry.

Data availability

No datasets were generated or analysed during the current study.

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Author contributions

C.P. and T. P. J. L. wrote and edited the manuscript. L.A. provided supervision.

Competing interests

The authors declare no competing interests.

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