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Molecular Approach to Detecting Pollen Types In Honey: DNA Barcoding

Balda Polen Tiplerini Belirlemek için Moleküler Yaklaşım: DNA Barkodlama

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ABSTRACT

Nowadays, the demand for bee products has also increased due to the growing interest in natural products. Although it is quite easy to adulteration of honey, which is one of these products, it is also very difficult to detect adulteration. In the point of detecting forgery, studies are being carried out specifically to identify the plant sources of honey. Melissopalynological and chemical analyses are methods commonly used in order to identify the botanical origin of honey. Despite the fact that the detection of botanical origin of honey by DNA-based methods which provide faster, simpler and more reliable results are being carried out in recent years, these researches are very less in Turkey. Unlike morphological methods, which require the visual examination of pollen grains, the recently developed genetic methods have the potential to increase the resolution and scale of pollen analyses. In this study, the aim was to present cumulative data by compiling the results of the studies conducted using molecular techniques on honey, and the advantages and disadvantages of this technique were evaluated.

Key Words

DNA barcoding for honey, plant diversity, pollen analysis of honey, melissopalynologic analysis.

ÖZ

Günümüzde doğal ürünlere olan ilginin artmasına bağlı olarak arı ürünlerine olan talep de artmıştır. Bu ürünlerden biri olan balın tağşişi oldukça kolay olmasına rağmen tağşişin tespit edilmesi de bir o kadar zordur. Sahteciliği tespit etme noktasında özellikle baldaki bitki kaynaklarını tespit etmeye yönelik çalışmalar yapılmaktadır. Balın botanik orijinini tanımlamak için yaygın olarak kullanılan yöntemler, melissopalinolojik analiz ve kimyasal analizlerdir. Son yıllarda ise daha hızlı, basit ve güvenilir sonuç sunan DNA tabanlı yöntemler ile balın bitki kaynakları tespit edilmeye başlanmasına rağmen bu konuda yapılan çalışmalar Türkiye'de yok denecek kadar azdır. Bireysel polen tanelerinin görsel olarak incelenmesini gerektiren morfolojik tanımlama yöntemlerinin aksine, yakın zamanda geliştirilen genetik yaklaşımlar, polen analizlerinin ölçeğini ve çözünürlüğünü artırma potansiyeline sahiptir. Bu çalışmada balda moleküler teknikler kullanılarak yapılan çalışmaların sonuçları derlenerek toplu bir veri sunulması amaçlanmış, yöntemin avantaj ve dezavantajları değerlendirilmiştir.

Anahtar Kelimeler

Bal, DNA barkodlama, bitki çeşitliliği, balın polen analizi, melissopalinolojik analiz.

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INTRODUCTION

Honey is defined as a natural product in which the honeybees change the plant nectar, the secretion of the plants' live sections or the secretion of the plant-sucking insects living on the live sections of the plants by combining them with their own substances after they are collected by the honeybees, decrease the water content and store and ripen them in the honeycomb [1]. Approximately 95% of the dry weight of honey is comprised of carbohydrates (mostly glucose and fructose). A small proportion (5%) consists of polyphenols and flavonoids, proteins, minerals, enzymes, aroma components, organic acids, amino acids, fatty acids, vitamins, pollen and other solid substances that are mixed in during the honey harvest [2].

Unlike morphological methods, which require the visual examination of pollen grains, genetic methods have the potential to increase the resolution and scale of pollen analyses. With the increase in the world population, the effort to improve the quality of life has increased the demand for especially traditional products [3]. Honey, one of the products called as a functional food in the literature, has been used frequently since the history of mankind because of its medicinal aspects such as antimicrobial, antiviral, antiparasitic, anti-inflammatory, antioxidant and antitumor properties [2]. The chemical content, plant source and appearance of honey vary depending on various factors (region, season, bee race, etc.). However, the physicochemical and biological properties of honey are basically linked to its plant composition [4]. For this reason, it is very important for human health to know especially the plant sources of this product consumed by people for its therapeutic effects as a food product. For example, the antimicrobial activity of honey is associated with its plant origin. Similarly, some plant groups such as Apocynaceae, Boraginaceae, Eupatorieae, Senecioneae and the genus of Crotalaria from Fabaceae have been reported to produce pyrrolizidine alkaloids which are toxic for humans and these alkaloids can be found in honey and pollen products [5-7]. This implies that the consumption of honey containing toxic compounds poses a threat to human health, and at thus it is very important to know the plant sources of honey. Knowing the conditions such as pollen diversity, microorganism presence and genetically modified organism (GMO) in honey is important economically and in terms of health, but the DNA isolation from honey is not easy due to its natural viscosity and its inhibitors [8].

Although various methods have been developed for the determination of honey's plant origin in recent years, pollen analyses are currently being carried out through the melissopalynological method, which is traditionally based on microscopic identification of pollen grains in honey [9]. Some chemical methods which are based on free amino acids, aroma compounds or minerals and trace elements are also used for the identification of honey's floral origin, however, these methods are sophisticated and require expensive instruments [10-12]. Moreover, all of these methods provide insufficient information on the plant origins of honey. Even though the melissopalynological approach, which requires significant taxonomic expertise, cannot distinguish many plant species [13], it is a powerful diagnostic tool especially when used with other methods [14]. With the advancement in DNA sequencing technology, the examination of the geographical and botanical origin of honey is much easier, and also it is precise, fast and reliable. This approach is based on 'DNA barcoding', where the species composition of mixed matrices is determined by comparing the sequences of the same DNA region with the reference database [15-17].

If the source of honey intensely consists of one plant species, it is called monofloral; if it consists of more than one plant sources, then it is called multifloral honey [4]. Monofloral honey is named according to the plant source (chestnut honey, lavender honey, rhododendron honey etc.) while multifloral honey can be referred to with the name of the region (Bayburt honey, Anzer honey, Kars honey etc.) where it is produced. The fact that a single plant species is not found to be intense compared to monofloral honey limits the discovery of the plant origins of multifloral honey in detail. Honey produced in different regions has different characteristics due to floral differences. When assessed in this respect, it is very important to reveal the differences of honey scientifically. Therefore, this new molecular method, which can be applied to honey products, also provides information about the plant sources of honey and its reliability as food (whether it contains toxic/GMO plants).

In recent years, scientists in different countries have started to use the molecular approach in order to identify especially the plant sources of honey and have published different researches [5,17,18]. However, molecular fingerprint studies have been started for honey with two studies conducted recently although there are currently no articles published in parallel with these de-

velopments in Turkey, which is one of the leading countries in honey production. Ozkök et al. [19,20] showed that the DNA isolation could successfully be applied on honey in a project they executed in 2015-2016. They carried out microscopic analysis and determined the pollen numbers in pine, sunflower, chestnut, canola, citrus, clover, rhododendron, oak, astragalus, mullein and multifloral (Ardahan, Anzer) honey samples with the new-generation sequence analysis. Then, they isolated DNA from honey samples with high pollen quality, which were found to be unifloral (monofloral). As a result of the PCR, the samples from only two gene regions (rbcL and trnH-psbA) were sent to the sequence analysis.

Even though studies conducted to detected the plant sources of honey are quite limited in not only Turkey but also the whole world, the selection of the universal informative markers is quite significant to specify the botanical origin of honey and differentiate the pollen taxons [17]. The DNA markers like Nuclear 18S rDNA [18] and plastid trnL gene [5] were used to determine the plant species from different honey samples [15,17,21]. Early metabarcoding, which uses the trnL-UAA intron marker, was not enough to identify many plants beyond the family level [5]. Similarly, Hawkins et al. [14] applied a different marker (rbcLa) to increase the classification, but they could do identification in terms of species only for one third of the taxon. Galimberti et al. [21] and Bruni et al. [17] analyzed both the rbcLa and trnH-psbA region and could increase the taxonomic resolution in the study for which they used a reference library. Likewise, the rbcL and matK [22]; ITS2 and rbcL [23]; trnL-UAA [24] gene regions have been used to identify the origin in honey recently.

Laube et al. [25] developed the real-time PCR system in order to distinguish the different plant species (acacia, linden, citrus, clover, heather, rosemary, sunflower) commonly found in Korskian honey. They revealed that this method requires prior knowledge regarding the plant species that might exist in honey and honey must be adjusted to its geographical origin.

Valentini et al. [5] suggested a DNA barcoding approach which combined the universal primers and massive parallel pyrosequencing to identify the plant and geographical origin of 2 commercial honey samples (Pyrenean honey and wild flower honey). Researchers stated that the trnL approach was an appropriate method for the determination of the plant diversity in honey and no prior knowledge was needed about a probable plant species composition. They reported that this approach was fast, easy and did not require expertise for analysis, and it was more reliable than the classic methods.

Lalhmangaihi et al. [26] presented a method for the DNA isolation from a low amount of honey sample in their study. They implemented a traditional PCR-based method, which made it possible to identify the plant species from a low amount of honey sample as less as 3 ml. An anionic detergent was used to lyse pollen shell and the DDT was used for the isolation of the thiolated DNA. They stated that the chloroplast matK gene was successfully multiplied from the DNA isolated from honey samples with this method. Researchers reported that, in this study, they developed a method enabling easy, adequate and effective DNA isolation from honey with the traditional phenol-chloroform method contrary to the isolation through kit utilization in different studies.

Bruni et al. [17] examined the plant sources of four multifloral honey products, which had been produced in different places of a floristically rich area in the northern Italian Alps, using the rbcL and trnH-psbA plastid regions as barcode markers. Researchers formed a wide reference database (consisting of 315 plants) of the barcode sequences for the local flora in order to identify the taxonomic content of honey. In honey samples, they identified thirty-nine plant species comprised of plants from *Castanea* sp., *Quercus* sp., *Fagus* sp. and a few herbaceous taxons. They identified at least one endemic plant species in four honey samples and stated that this clearly revealed the geographical identity of the honey. Furthermore, they reported that DNA barcoding was important to test the reliability of honey by determining the DNA of Atropa belladonna, which is a toxic plant, in a honey sample. Consequently, it is understood with the study conducted by these researchers that the taxonomic resolution increased when the plant references were used with the honey samples, resolution was provided till the level of species, and contribution was made to revealing the geographical identity of honey.

Hawkins et al. [14] identified the floral origin of the honey products with the DNA barcoding approach by using the rbcL DNA barcode marker and 454-pyrosequencing.

Meanwhile, they compared the results by applying melissopalynological analysis. Researchers specified that both of the DNA barcoding and melissopalynological techniques were enough to identify the floral source which had the biggest contribution to the honey content, however, the DNA barcoding technique was more advantageous because it did not require high-level taxonomic expertise.

Col and Karaali [8] implemented DNA isolation from precipitated pollen of honeydew honey collected in Muğla by using three different techniques (CTAB method, manual silica dioxide method and DNeasy Plant Mini Kit). They reported as a result of the study that the DNeasy plant kit was the most efficient technique for DNA isolation from the honeydew honey of Muğla under the present conditions. Similarly, Jain et al. [4] aimed at preparing a protocol for DNA isolation from honey and showing that the molecular analysis of the obtained DNA could be used for the botanical diagnosis. In their research, they modified the original CTAB-based protocol used for DNA extraction from plants and used it for DNA isolation. They conducted DNA isolation from different honey samples giving similar results in every replication and multiplied the isolated DNA with the PCR by using plant-specific primers. In the study they conducted using different types of honey, Bruni [17] defined a protocol enabling the DNA barcoding of three gene regions (trnH-psbA, rbcLa and COI) to provide information about the botanical and entomological origins of honey. Pollens were examined with the nuclear trnH-psbA (apprx. 350 bp), and the rbcLA plastid gene of the non-pollen plant material and the sources of the bee species were examined using the mitochondrial cytochrome c oxidase subunit I (COI). The rbcL and trnH-psbA primers, which were distinctive for several plants, were selected to determine the pollen source of honey. It was stated as a result of the study that no plant and insect sources were encountered in five samples and no plant or insect sources were discovered in the other two samples. In their study, Laha et al. [27] researched the floral origin of 20 honey samples in Northeast India with the DNA sequencing approach by using the three gene regions rbcL, matK and ITS2, and compared the results with the melissopalynological analysis. As a result, they revealed that melissopalynological and DNA sequencing approach were both successful at identifying the intense plant species, however, the molecular approach set forth a higher plant diversity compared to different techniques. Similarly, Soares et al. [28] expressed in their study that the chloroplast matK gene could be used successfully to identify the botanical origin of the lavender honey. Considering all these results obtained in different regions of the world, the number of professional researchers in melissopalynological analysis in Turkey is quite insufficient. For this reason, molecular analyzes that do not require high-level taxonomic expertise should be developed for the determination of plant origin in honey.

CONCLUSION

Today, plant sources of honey are determined with the help of microscopic and some chemical methods. In the microscopic method, the pollen in the honey composition are examined morphologically with the light microscope, and thus, information is obtained about the plant origin. However, method is tiring and time-consuming and require dull taxonomic expertise in terms of identifying the pollen morphologies. Moreover, non-discovery of the distinctive properties for the pollen of some families under light microscope causes insufficiency in differentiating a lot of plants in terms of species. On the other hand, various methods have also been suggested in association with the aroma compounds [29] and the mineral content [11] in order to determine the botanical origin of honey. Although all these methods give good results for identifying the geographical origin of honey and differentiating honey products from different botanical origins, they present insufficient information about the exact plant diversity of honey [5]. The fact that researches have gained momentum in recent years suggests that the plant origin of honey can be identified with molecular techniques. Hence, more reliable results can be obtained by spending less time. Therefore, studies on this topic are quite significant.

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