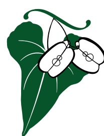
Botanica SERBICA

(2023) 47 (1): 75–85

DOI: https://doi.org/10.2298/BOTSERB2301075B
journal homepage: botanicaserbica.bio.bg.ac.rs

Original Scientific Paper

Assessment of the botanical origin of Bulgarian honey samples using melissopalynological, DNA barcoding and NMR analyses

Ralitsa Balkanska[1,#*], Katerina Stefanova[2#], Radostina Stoikova-Grigorova[3], Dessislava Gerginova[4], Svetlana Simova[4] and Ivan Atanassov[2]

1 Department of Special Branches, Institute of Animal Science, Kostinbrod, Agricultural Academy, Bulgaria
2 Molecular Genetics, Agrobioinstitute, Agricultural Academy, Sofia, Bulgaria
3 Genetics, Breeding and Reproduction, Institute of Animal Science, Kostinbrod, Agricultural Academy, Bulgaria
4 Bulgarian NMR Centre, Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Sofia, Bulgaria
# equally contributed to the work
* Correspondence: r.balkanska@gmail.com

ABSTRACT:
Polyfloral honey samples from Bulgaria were subject to parallel analyses of their botanical origin and composition using traditional melissopalynology, DNA-barcoding based on the plastid rbcL gene and NMR analysis. The obtained datasets were compared with each other to evaluate the information capacity of the applied experimental methods. The results from the melissopalynological and DNA-barcoding studies demonstrated a significantly higher resolution of the latter, revealing the presence of pollen from a total of 17 plant families, 21 plant genera and 5 plant species in comparison to pollen from only 7 plant families, 3 plant genera and 4 plant species identified by melissopalynology. The higher resolution of DNA barcoding allows a more detailed characterisation of the diet and foraging preferences of honey bees, including foraging on plant species growing in lower abundance in the area. The comparison of the quantitative data on floral honey composition for several plant genera and species reveals significant differences between the relative abundance of the pollen grains estimated by melissopalynological analysis and the relative abundance of rbcL clones in rbcL libraries determined after DNA barcoding. All three applied methods confirm the polyfloral botanical origin of the analysed samples and support routine NMR use for the assessment of the floral origin of honey.

Keywords:
rbcL, bee honey, melissopalynology, ^{1}H and ^{13}C NMR

UDC: 638.162(497.2)

Received: 18 June 2021
Revision accepted: 15 December 2022

INTRODUCTION

Honey is a natural product obtained by honey bees (*Apis mellifera* L.) from plant nectar. The composition and properties of honey depend on the botanical origin of the nectar. The major constituents of honey are the sugars fructose and glucose (Siddiqui *et al.* 2017). Additionally, it also contains a very complex mixture of other di- and oligosaccharides, enzymes, organic and amino acids, polyphenols, vitamins and minerals (Habib *et al.* 2014; Vulić *et al.* 2015). Generally, honey is classified as either monofloral or polyfloral. Monofloral honey contains predominantly the nectar and pollen of one plant species, while polyfloral honey contains nectar and pollen from various plants, none of which is predominant. Traditional analysis of the botanical origin of honey includes organoleptic, physicochemical and melissopalynological methods. Melissopalynology is extensively used to determine the geographical and botanical origins of honey (Ramanujam *et al.* 1992; Sajwani *et al.* 2007; Song *et al.* 2012). This traditional method requires considerable skill and experience. The limitation of the

© 2023 Institute of Botany and Botanical Garden Jevremovac, Belgrade

method lies in the fact that pollen analysis is based on the individual knowledge of each pollen analyst. The method needs highly specialised personnel, requires a considerable amount of training and is time-consuming. Some plants from *Lamiaceae* and *Rosaceae* can be particularly difficult to distinguish (Hebda & Chinnappa 1990; Galimberti *et al.* 2014; Kraaijeveld *et al.* 2014; Bruni *et al.* 2015; Hawkins *et al.* 2015). The combination of organoleptic, physicochemical and melissopalynological methods allows a good separation of numerous monofloral honeys (Piro *et al.* 2002; Terrab *et al.* 2003). However, it should be noted that these methods do not always allow discrimination between monofloral and polyfloral honeys (Bogdanov & Gallmann 2008).

In recent years, the use of DNA-barcode based methods for pollen identification has attracted increased interest. These methods have several advantages over melissopalynology. Firstly, DNA-barcode based identification does not require the high level of taxonomic expertise required for microscopic examination. Secondly, a greater number of honey samples can be screened and better resolution for some plant families is provided, thus having the potential to reduce processing time and increase the level of species discrimination. In addition, pollen present in low levels can also be detected (Schnell *et al.* 2010; Valentini *et al.* 2010; Jain *et al.* 2013). The identification of the species origin of pollen in honey has numerous applications, including the characterisation of pollen in polyfloral honey samples, the assessment of plant pollinator networks, honey authentication, allergen monitoring and the detection of pollen from poisonous flowers. Such applications, however, have previously been limited to the microscopy-based identification of pollen, which has low taxonomic resolution. The DNA barcoding method provides an alternative, which could overcome these deficiencies. Both chloroplast and nuclear barcoding markers, or DNA barcoding, use the amplification and sequencing of small fragments, but with different sizes and varying numbers of PCR products to identify single-species samples by matching with known DNA sequences (Bell *et al.* 2019). In plants, three regions (rbcL, matK and trnH-psbA) as well as the nuclear ribosomal ITS region have been widely used as DNA barcodes, either separately or in combination (Chen *et al.* 2010; Hollingsworth *et al.* 2011). The plastid DNA gene rbcL is recommended as the standard DNA barcode marker, based on the availability of universal primers and the high level of taxonomic resolution (Hollingsworth *et al.* 2009). Furthermore, the rbcL region shows one PCR product compared to the trnH-psbA spacer. The reasons for the observed distortions of the estimated diversity from specific plant taxa could be the result of various factors. They include the varying efficiency of DNA isolation from different pollen grains or the efficiency of PCR amplification (non-specific products) and the cloning of mixed PCR fragments from the target trnH-psbA barcoding regions. The trnH-psbA primers often exhibit and have lower levels of universality (Hollingsworth *et al.* 2011; Hawkins *et al.* 2015). This lower universality means that some species within mixed honey samples will not be detected using these primers and others might be detected in biased ratios. In spite of the increased application of DNA barcoding in various areas, the use of this method has been the subject of only a few studies for the characterisation of honey floral composition (Bruni *et al.* 2015; Hawkins *et al.* 2015; Kamo *et al.* 2018). The possibility for its application in qualitative and quantitative analysis remains insufficiently evaluated.

Nuclear magnetic resonance (NMR) spectroscopy as a powerful technique for the structure determination of complex mixtures has been successfully used by numerous researchers to evaluate the floral, geographical and entomological origin of honeys (Kortesniemi *et al.* 2016; Zuccato *et al.* 2017; Zhang *et al.* 2020). During the last decade it has become a widely used method for quality control and the determination of organic compounds (carbohydrates, amino acids, phenolic substances) in many foods and natural products. The ^{1}H 1D NMR based metabolomics strategy with different classification models is mostly used for this purpose (Schievano *et al.* 2012). However, ^{13}C and 2D NMR methods have also been applied (Ohmenhaeuser *et al.* 2013; Kazalaki *et al.* 2015; Popescu *et al.* 2016; Khlifi *et al.* 2017). The main advantages of the NMR technique compared to the other most popular metabolomics method of mass spectroscopy are its non-invasive nature, the easy sample preparation and the possibility of the simultaneous identification and quantification of numerous constituents (Consonni & Cagliani 2008; Schievano *et al.* 2019; Popova *et al.* 2021). In combination with chemometric techniques, NMR is one of the most appropriate methods to build useful databases with predictive properties for honey authenticity and quality.

The aim of the present study is to assess the information capacity and resolution for the characterisation of the botanical origin of honey with DNA barcoding and NMR (^{1}H and ^{13}C) spectroscopy when compared to traditional melissopalynology using the parallel application of the three analytical methods on three polyfloral honey samples.

MATERIAL AND METHODS

Samples. Three honey samples collected from apiaries in the vicinity of the town of Kostinbrod, and the villages of Kubratovo and Chepinci, in the Sofia region, Bulgaria with unknown botanical origin were analysed using DNA barcoding, ^{13}C NMR profiling and traditional melissopalynology. Additionally, the chemical profile via ^{13}C NMR of 10 samples with known botanical origin [5 polyfloral (P1-P5) and 5 monofloral (oak, linden, aca-

Table 1. The chemical profiles of the Kb, Ks and Ch honey samples compared with the average values for Bulgarian polyfloral honeys in g/100 g, derived from ^{13}C NMR signal intensities.

Components %	Kostinbrod (Ks)	Kubratovo (Kb)	Chepinci (Ch)	Polyfloral honey (min–max)	Average
			Monosaccharides		
Fructose (F)	35.48	40.26	36.08	24.96–42.24	37.38
Glucose (G)	37.71	32.27	35.23	30.22–42.78	34.37
			Disaccharides		
Gentiobiose (Gb)	0.14	0.13	0.00	0.00–0.57	0.16
Isomaltose (IMa)	0.63	0.81	1.03	0.47–1.41	0.73
Isomaltulose (IMu)	0.26	0.20	0.00	0.00–1.58	0.77
Kojibiose (Kjb)	0.59	0.64	0.92	0.35–0.83	0.59
Leucrose (Lu)	0.08	0.07	0.15	0.04–0.18	0.07
Maltose (Ma)	0.92	0.43	0.63	0.00–1.82	1.04
Maltulose (Mu)	0.58	0.91	0.93	0.34–1.89	0.58
Nigerose (Ng)	0.23	0.30	0.47	0.23–0.49	0.30
Sucrose (Su)	0.11	0.13	0.03	0.03–0.68	0.16
Trehalulose (Tru)	0.55	0.85	0.84	0.00–0.96	0.34
Turanose (Tu)	0.89	1.20	1.41	1.14–3.07	1.75
α,β-Trehalose (αβTr)	0.21	0.22	0.33	0.08–0.23	0.13
αα-Trehalose (ααTr)	0.00	0.00	0.22	0.00–0.42	0.07
			Trisaccharides		
Erlose (Er)	0.22	0.18	0.21	0.00–0.75	0.23
Isokestose (1-Ks)	0.00	0.00	0.00	0.00–0.15	0.05
Melezitose (Mz)	0.00	0.00	0.05	0.00–0.09	0.03
Panose (Pa)	0.19	0.12	0.17	0.00–0.30	0.16
Raffinose (Rf)	0.11	0.09	0.19	0.09–0.46	0.16
			Other compounds		
Meso 2,3-butanediol (mBd)	0.00	0.00	0.00	0.00–0.05	0.01
Proline (Pro)	0.07	0.03	0.09	0.01–0.07	0.03
Quercitol (Q)	0.00	0.00	0.00	0.00–0.06	0.01
Racemic 2,3-butanediol (rBd)	0.00	0.00	0.00	0.00–0.02	0.00
Sum of 16 unidentified compounds	0.76	0.77	0.76	0.55–1.72	0.83

cia, rapeseed and sunflower)] was determined. The samples were collected in sterile jars during July 2017 and stored at room temperature prior to analysis.

Melissopalynological analysis. The melissopalynological analysis was carried out according to the procedures set out in the Bulgarian State Standard for Bee Honey (Bulgarian State Standard 1980) in the Central Laboratory for Veterinary Sanitary Expertise and Ecology, Sofia, Bulgaria. For the pollen analysis 10 g of honey was dissolved in 40 ml distilled water. The solution was centrifuged at 3000 rpm for 10 minutes. The sediment was placed on a glass slide for microscopic examination. Three observations involving 300 pollen grains were carried out for each sample analysis.

DNA rbcL clone libraries. The total DNA was extracted from 120 g of honey as described by Balkanska *et al.* (2018) using a GeneJET Genomic DNA Purification Mini Kit (Thermo Scientific). The DNA extraction for each honey sample was carried out in triplicate as the three DNA samples extracted separately from the portions of one and the same honey sample were combined to obtain the final DNA sample used for further analysis. DNA quantification was carried out using the NanoDrop UV-Vis Spectrophotometer (NanoDrop Technologies). The target region of the plastid rbcL gene was PCR amplified using the primers 1F: 5'-ATGTCACCACAAACAGAAAC and 724R: 5'-TCGCATGTACCTGCAGTAGC. The PCR amplification was performed in a 25 μL volume containing 40 ng of isolated genomic DNA, 1 μL of each primer with a concentration of 10 μM and 12.5 μL of Phusion High-Fidelity PCR Master Mix with HF Buffer (Thermo Scientific). The reaction was performed in a QB-96 thermal cycler (Quinta Biotech) using the following protocol: denaturation 98°C for 30 sec, followed by 35 cycles of 98°C for 10 sec, 50°C for 30 sec and 72°C for 25 sec, with the final extension of 10 min at 72°C. Following agarose gel electrophoresis, the amplified DNA fragments were purified using a GeneJET Gel Extraction Kit (Thermo Sci-

Table 2. Melissopalynological and DNA analysis of the honey samples

No	Family	Chepinci (Ch)		Kostinbrod (Ks)		Kubratovo (Kb)	
		Melissopalynological	DNA analysis	Melissopalynological	DNA analysis	Melissopalynological	DNA analysis
1	**Asteraceae**	**5.6±0.3%** *Centaurea* spp. – 4.9%	**12.50%** *Helianthus annuus* – 6.25% (MH536591) *Carthamus* sp. – 3.2% (MH536564) (MH536562)	**32.9±1.7%** *Helianthus annuus* – 28.8%	**63.75%** *Helianthus annuus*-48.75% (MH536591), *Helianthus* sp.– 8.5% (MH536628) (MH536617) (MH536631) (MH536634) (MH536639)	**19.5 ±1.0%** *Helianthus annuus* – 16.7%	**32.5%** *Helianthus annuus* – 18.75% (MH536591) *Helianthus sp.* – 11.00% (MH536589) (MH536603) (MH536604) (MH536609) (MH536612)
2	**Apiaceae**	**11.8±0.6%** *Coriandrum sativum*	**nd**	**nd**	**nd**	**3.6±0.2%**	**nd**
3	**Brassicaceae**	**35.4±1.8%**	**7.50%** *Brassica* sp. – 5% (MH569147) *Raphanus* sp. – 1.25% (MH569148)	**nd**	**1.25%** Brassicaceae sp. (MH569152)	**28.3 ±1.5%**	**8.75%** *Brassica* sp. – 8.75% (MH536592) (MH569150)
4	**Cucurbitaceae**	**nd**	**1.25%** Cucurbitaceae spp. (MH536558)	**nd**	**nd**	**nd**	**3.75%** *Cucumis* sp. – 3.75% (MH536588) (MH536606)
5	**Convolvulaceae**	**nd**	**nd**	**nd**	**nd**	**nd**	**5.00%** *Convolvulus arvensis* – 2.5% (MH536594) (MH536608)
6	**Dryopteridaceae**	**nd**	**nd**	**nd**	**nd**	**nd**	**1.25%** *Polystichum* sp. – 1.25% (MH536581)
7	**Fabaceae**	**26.4±1.4%** *Onobrychis* – 14.6±0.8%, *Trifolium sp.* –2.8±0.1	**7.5%** *Melilotus* sp. – 6.25% (MH536571) (MH536553) (MH536578)	**23.7±1.7%** *Trifolium* sp. – 8.8±0.5%, *Robinia pseudoacacia* -2.4±0.1%	**2.50%** *Melilotus* sp. – 1.25% (MH536637) (MH536640)	**14.3±0.7%** *Lotus corniculatus* – 5.2±0.3%, *Trifolium* sp. – 4.8±0.2%	**1.25%** Fabaceae spp. (MH536593)
8	**Fagaceae**	**nd**	**nd**	**nd**	**nd**	**nd**	**1.25%** *Quercus* sp. – 1.25% (MH536582)
9	**Gesneriaceae**	**nd**	**nd**	**nd**	**nd**	**nd**	**3.75%** *Primulina* sp. – 3.75% (MH536596) or *Oreocharis mileensis*
10	**Lamiaceae**	**nd**	***22.5%*** *Lavandula × intermedia* – 6.25% (MH536556), *Lavandula* sp. – 6.25 % (MH536607), (MH536550) *Hyssopus* sp. – 3.75% (MH536602) (MH536566) *Dracocephalum* sp. – *1.25* % or *Pedicularis* sp. (MH536555) (MH536554) (MH536560) (MH536559) (MH536646) (MH536568)	**nd**	**10%** *Lavandula* sp. – 5% (MH536620) (MH536621) (MH536627) (MH536586)	nd	**5%** *Lavandula × intermedia* – 1.25 % (MH536556), *Lavandula sp.* – 1.25 % (MH536607) *Hyssopus sp.* – 1.25 % (MH536602)
11	**Malvaceae**	**nd**	**nd**	**nd**	**nd**	**2.8±0.1%**	**1.25%** *Tilia sp.* – 1.25% (MH536590)

12	**Oleaceae**	**3.5±0.2%**	**nd**	**nd**	**nd**	**nd**	**2.50%** *Ligustrum* sp. – 2.50% (MH536579)
13	**Poaceae**	**nd**	**5.00%** *Zea* sp. – 5% (MH536585)	**nd**	**3.75%** *Zea* sp. – 2.5% (MH536585) (MH536647) *Sorghum* sp. – 1.25% (MH536630)	**nd**	**13.75%** *Zea* sp. – 10% (MH536585) (MH569149)
14	**Papavaraceae**	**nd**	**1.25%** *Papaver* sp. – 1.25% (MH536561)	**nd**	**nd**	**nd**	**nd**
15	**Rosaceae**	**8.3±0.4%**	**36.25%** *Rosa* sp. – 31.25% (MH536549) (MH536575) (MH536551)	***24.4±1.3%***	*5%* *Rosa* sp. – 2.5% (MH536626) (MH536624) (MH536622)	**22.3±1.2%**	**12.50%** *Malus domestica* – 7.5% (MH536583) *Mamalus* sp. – 1.5% (MH536580) *Rosa* sp. – 3.75% (MH536601) (MH536613)
16	**Simaroubaceae**	**nd**	**3.75%** *Ailanthus altissima* – 2.5 % (MH536595) *Ailanthus* sp. – 1.25 % (MH536563)	**nd**	**nd**	**nd**	**7.50%** *Ailanthus altissima* – 2.5 % (MH536595) *Ailanthus sp.* – 5% (MH536599) (MH536605) (MH536584)
16	**Rhamnaceae**	**nd**	**nd**	**11.2±0.6%**	**10%** *Paliurus* sp. – 5% (MH536618) (MH536619) *Ziziphus* sp. – 1.25% (MH536636) (MH536643) (MH536641)	**3.6±0.2%**	**nd**
17	**Solanaceae**	**nd**	**2.50%** Solanaceae spp. (MH536557) (MH536576)	**nd**	**3.75%** *Capsicum* sp. –2.25% (MH536610) *Solanum* sp. – 1.25% (MH536642) (MH536638)	**nd**	**2.50%** *Capsicum* sp. – 2.50%(MH536610)
Plant family affiliation total:		**91.00%**	**100.00%**	**92.20%**	**100.00%**	**94.40%**	**100.00%**
Plant genus affiliation total:		**22.30%**	**62.50%** + 13,75 %	**8.80%**	**30.75%** + 2,5 %	**4.80%**	**58.75%** + 3,75%
Plant species affiliation total:		**11.80%**	**13.75%**	**31.20%**	**48.75%**	**21.90%**	**32.50%**

nd – not detected; + species with 100% identity were identified, but up to two or three separate species and are assigned in the Table as species or genus – *Rosa × odorata* var. *pseudindica* (Lindl.) Rehder / *Rosa banksiae* R.Br., *Melilotus albus* Medik. / *Melilotus officinalis* Pall., *Capsicum annuum* L. / *Capsicum baccatum* L., *Brassica oleracea* L. / *Brassica napus* L. or *Brassica rapa* L., *Tilia amurensis* Rupr. / *Tilia × europaea* L., *Quercus coccinea* Münch. / *Quercus variabilis* Blume, *Helianthus annuus* L. / *Helianthus tuberosus* L.

entific) and cloned using the pJET 1.2 vector of a CloneJET PCR Cloning Kit (Thermo Scientific). A library of 96 rbcL clones was constructed for each sample. The cloned rbcL fragments were sequenced using the vector primers pJETFw: 5' CGACTCACTATAGGGAGAGCGGC and pJET Rev: 5' AAGAACATCGATTTTCCATGGCAG and the Macrogen Europe B.V. sequencing service.

Sequence analysis. The obtained rbcL sequences were manually edited using the Vector NTI v.10 software package (Life Technologies) and searched in the GenBank nucleotide database (standard database non-redundant-nr/nt) using BLASTn (Camacho *et al.* 2009). The top five sequence matches from the BLASTn search for each rbcL clone were considered to determine their phylogenetic affiliation. The rbcL clone sequences which shared over 99% identity at a minimal coverage of 95% with the GenBank (nt) sequences obtained after the BLASTn search were considered affiliated to the plant species corresponding to the sequence hit. The rbcL clone sequences which showed 97% to 99% identity at a minimal coverage of 95% with the GenBank (nt) were affiliated to a particular plant genus if all the top hits of the species were from the same genus, or affiliated to a particular plant family if the top hits corresponded to plant species from different genera but from the same family. The rbcL sequences obtained in this study were deposited in the GenBank database under accession numbers ranging from MH536549 to MH536647 and from MH569147 to MH569152. Sequences MH536556, MH536602, MH536585, MH536595, MH569150 and MH536585 were present in both the Kubratovo and Chepinci samples. MH536625, MH536585 and MH536545 were obtained from the Chepinci and Kostinbrod samples, and MH536610 from Kubratovo and Kostinbrod. Sequence MH536591 has distribution in the samples from all three regions.

NMR spectroscopy and chemometric analysis. For NMR analysis, 0.32 g of honey was dissolved in 0.418 ml distilled water and 0.187 ml deuterated phosphate buffer solution (pH 4.50), containing 0.1 vol% trimethylsilyl propionic acid-d_4 sodium salt (TSPA) and 0.05 vol% of NaN_3 as a preservative. The pH of the samples was adjusted to 4.20 with a small amount of 8.5% H_3PO_4.^{1}H (600.01 MHz) and ^{13}C (150.89 MHz) NMR spectra were acquired using an AVANCE AV600 II+ NMR spectrometer using Topspin v.3.5pl6. ^{13}C spectra were obtained using a 30˚ pulse (zgdc30), a 238 ppm spectral width, 8K scans, 64K data points and a 1.05 s relaxation delay. A solvent suppression pulse sequence (noesypr1d) with a 10.6 ppm spectral width, 256 scans, 16 dummy scans, 64K data points and a 2 s relaxation delay was used to record ^{1}H spectra. All spectra were recorded at 300.0 ± 0.1 K with TSPA-d_4 as an internal reference with chemical shifts at 0.0 ppm and – 2.73 ppm for ^{1}H and ^{13}C, respectively. The assignment of the signals was made on the basis of the gradient enhanced versions of TOCSY, and standard and semi-selective HSQC. The intensities of the non-overlapping signals in the anomeric region of the ^{13}C NMR spectra were used for the quantification of numerous identified and several unidentified compounds as described by Gerginova *et al.* (2020). The quantitative data (in g/100g) were derived from the carbon NMR spectra of 13 samples. The ranges and the average content of the individual components in all the studied honeys are presented in Table 1. One of the widely used unsupervised statistical methods - Hierarchical clustering (HCA, single-linkage clustering) was used for data visualization and the comparison of the honeys. Additionally, a PCA analysis is presented in Supplementary figs. 1–3. The chemometric analysis was performed using Simca 14 software (Umetrics).

RESULTS AND DISCUSSION

Three honey samples designated as Ch (Chepinci), Kb (Kubratovo) and Ks (Kostinbrod) were isolated from single hives in apiaries at three locations in the Sofia region, Bulgaria. The samples were subject to parallel melissopalynological analysis, DNA-barcoding based on the plastid rbcL gene and NMR analysis in order to gain information on their botanical origin. The obtained results were compared to evaluate the information capacity of the applied experimental approaches.

Melissopalynological analysis. The melissopalynological analysis of the three studied honey samples suggested plant family affiliation for a total of 91% to 94.4% of the observed pollen grains (Table 2). Overall, the analysed pollen grains from the three samples were affiliated to seven different plant families. The performed analysis further resulted in plant genus affiliations to part of the pollen grains (total 22.3%; 8.8% and 4.8% for Ch; Ks and Kb respectively) and to plant species affiliations (total 11.8%; 31.2% and 21.9% for Ch; Ks and Kb respectively). Pollen grains from a total of seven plant families, 3 plant genera and 4 plant species were identified in the three analysed samples (Table 2). The melissopalynological analysis demonstrated the presence of larger quantities of pollen grains from *Helianthus annuus* L. (28.8% for the Ks and 16.7% for the Kb samples) and *Coriandrum sativum* L. (11.8% for the Ch sample), related to industrial sunflower and coriander cultivation in the area in the vicinity of the sampled apiaries. In addition, the melissopalynological analysis also revealed the presence of pollen grains from various genera and species of *Fabaceae* in all the honey samples, related to taxa naturally growing in the area, such as *Trifolium* sp., *Onobrychis* sp., *Lotus corniculatus* L. and *Robinia pseudoacacia* L. (Galimberti *et al.* 2014; Bruni *et al.* 2015; Hawkins *et al.* 2015). Pollen grains from plants belonging to the

Brassicaceae and Rosaceae families, present in larger quantities in the studied samples, were identified at family level only. The results from the melissopalynological analysis presented above demonstrated that all three studied samples are polyfloral honeys originating from industrially cultivated and naturally growing plants in the region around the sampled apiaries.

DNA-barcoding. Three rbcL clone libraries were constructed after DNA isolation from pollen preparations of the studied honey samples, followed by PCR amplification of the rbcL gene region using universal rbcL (1F and 724R) primers and cloning into plasmid vectors. The sequence analysis of the obtained rbcL clones resulted in the identification of 105 different rbcL sequences. The BLAST search of GenBank with the obtained rbcL clone sequences showed that 44.76% of them have over 99% identity with GenBank sequences of known plant species and could be affiliated to them. The remaining 55.24% of the obtained rbcL clone sequences show a high level of homology (97% to 99% identity) to the rbcL gene sequences of known plant species and were affiliated to corresponding plant genera and families as described in Material and Methods. Considered together, the results from the BLAST search show the possible affiliation of all the rbcL clone sequences to a total of 17 plant families, 21 plant genera and 5 plant species (Table 2). In addition, 7 species with 100% identity were identified, but up to two or three separate species of genus. The results also show that 62.5%, 30.75% and 58.75% of the rbcL clones from the Ch; Ks and Kb libraries were affiliated to a genus and an additional 13.75%, 48.75% and 32.5% of the rbcL clones were affiliated to distinct plant species (Table 2). Further comparison of the botanical origin and pollen composition of the studied honey samples determined by the melissopalynological analysis and rbcL based DNA-barcoding demonstrated and support the higher resolution and information capacity of the DNA-barcoding reported in several studies (Bruni *et al.* 2015; Hawkins *et al.* 2015). Generally, the DNA-barcoding confirms the presence in the studied samples of pollen grains from the plant families, genera and species identified by the melissopalynological analysis (Table 2). At the same time, the DNA-barcoding also suggests the presence of pollen grains from plant species and genera from an additional 10 plant families not detected by the melissopalynological analysis. For example, the DNA-barcoding suggests the presence of members of the Lamiaceae, Poaceae and Solanaceae families in all the studied honey samples, while no pollen grains from these families were identified in the melissopalynological analysis. Closer observation of the DNA barcoding data shows the higher complexity of the diet and foraging preferences of honey bees. In addition to the main groups of plants determined by the melissopalynological analysis, the DNA barcoding also identified a number of additional minor groups of plants present in low abundancy in the Sofia region including naturally growing trees and shrubs (*Quercus* sp., *Tilia* sp., *Ailanthus altissima* Miller, *Paliurus* sp. and *Ziziphus* sp.) (Galimberti *et al.* 2014; Bruni *et al.* 2015; Hawkins *et al.* 2015), weeds (*Primulina* sp., *Papaver* sp. and *Convolvulus arvensis* L.), herbs (*Lavandula* sp., *Hyssopus* sp. and *Dracocephalum* sp.), plants cultivated in the local gardens (*Cucumis* sp., *Capsicum* sp., *Solanum* sp. and *Sorghum* sp.) and cereals grown at relatively low intensity (*Zea* sp.). Therefore, the demonstrated higher information capacity of DNA barcoding over the melissopalynological analysis could be particularly beneficial for a detailed characterisation of the diet and foraging preferences of honey bees, including foraging on plant species growing in lower abundance in the area or plant species less preferred honey bees.

The results from the sequence analysis of the rbcL clones together with the abundant identified clone sequences in the rbcL libraries were further used to estimate the relative abundance of the pollen grains from the identified plant families, genera and species (Table 2). The comparison of the pollen abundance for different plant families, genera and species evaluated by the melissopalynological analysis and DNA barcoding showed sample-independent distortion of the estimated relative abundance of pollen grains from several plant families. Thus, the DNA barcoding estimation of the relative pollen abundance from the *Asteraceae* family / *Helianthus* sp. was almost twice as high as that revealed by the melissopalynological analysis (Table 2).Conversely, melissopalynology showed the presence of a much larger proportion of pollen grains affiliated to the Brassicaceae and Fabaceae families in comparison to their relative abundancy estimated by the parallel DNA barcoding analysis (Table 2). Additionally, whereas the DNA barcoding demonstrated the presence of pollen from the Lamiaceae and Poaceae families in all the samples, no pollen grains affiliated to these families were observed in the melissopalynological analysis (Table 2). The reasons for the observed directional sample-independent distortions of the estimated relative abundance for pollen grains from specific plant taxa has been reported in a few studies (Galimberti *et al.* 2014; Bruni *et al.* 2015; Hawkins *et al.* 2015) applying both experimental approaches and could be the result of various factors, including the efficiency of DNA isolation from pollen of different plant taxa or PCR amplification bias of the target DNA barcoding region from these species. The above also brings into question the direct use of DNA barcoding data for the assessment of the botanical origin of honey instead of the current well-regulated usage of melissopalynological analysis. For example, the application of both methods will result in a different designation for the Ks honey in this study, since the melissopalynological analysis describes it as polyfloral,

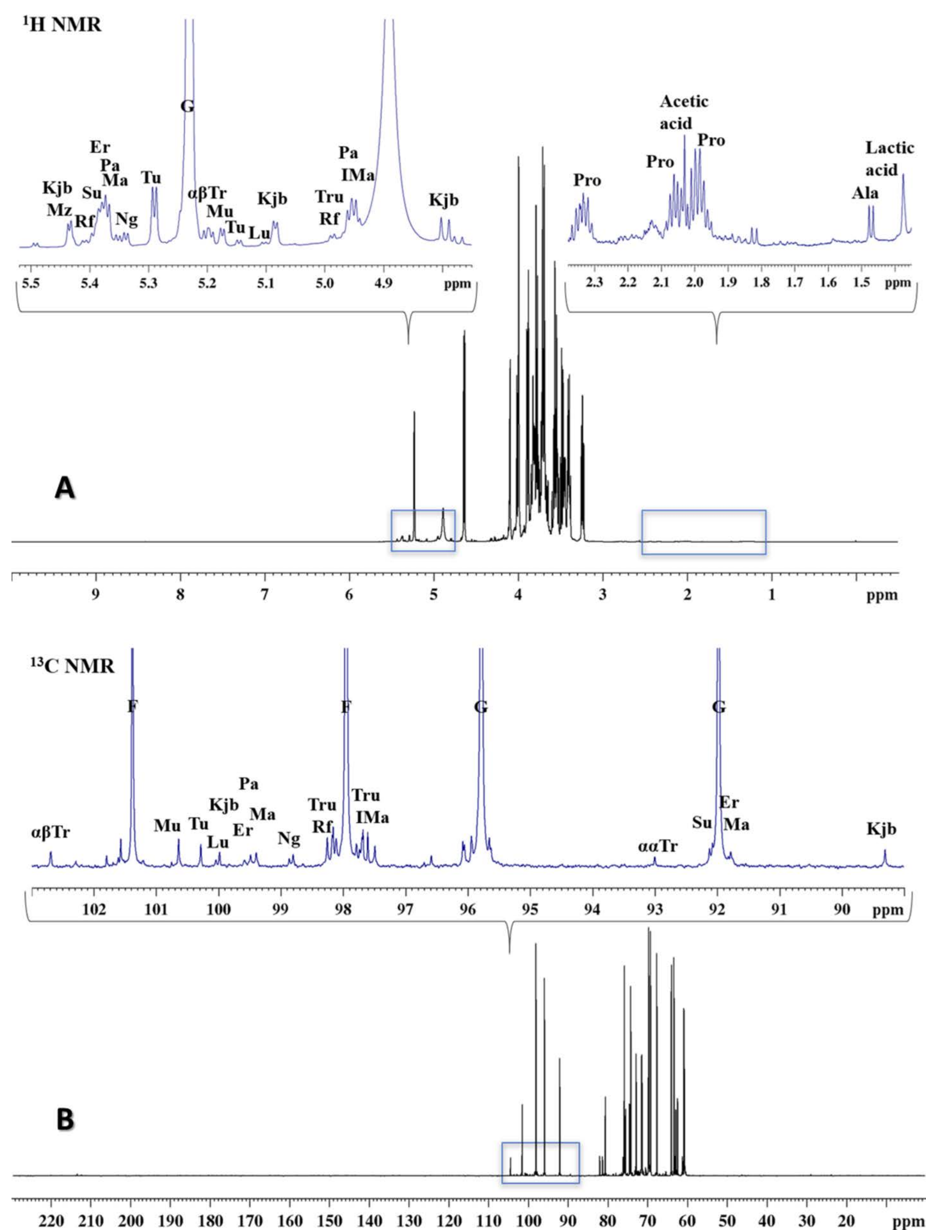

Fig. 1. ¹H (A) and ¹³C (B) NMR spectra of the polyfloral honey from Chepinci (Ch) with annotation of the metabolites (Acetic acid; Lactic acid; Acronyms: Ala—Alanine; Pro—Proline; F—Fructose; G—Glucose; IMa—Isomaltose; Kjb—Kojibiose; Lu—Leucrose; Ma—Maltose; Mu—Maltulose; Ng—Nigerose; Su—Sucrose; Tru—Trehalulose; Tu—Turanose; ααTr—ααTrehalose; αβTr—αβTrehalose; Er—Erlose; Mz—Melezitose; Pa—Panose; Rf—Raffinose).

with no abundance of pollen from any particular plant species exceeding 45%, while the parallel DNA barcoding will identify the Ks honey as monofloral with 48.75% estimated abundance of *Helianthus annuus* L. pollen. This illustrates the need for additional research and consideration of a larger pool of experimental data prior to DNA barcoding which can be routinely used in parallel with melissopalynological analysis in quantitative honey evaluation, related to the established standards and guidelines on the floral composition of the honey varieties.

NMR profiling. In Fig. 1 the ¹H (A) and ¹³C (B) NMR spectra of the honey from Chepinci are presented with the annotation of numerous metabolites. The sugar profile of the three analysed samples (Ch, Ks, Kb) is compared with the profile of five Bulgarian polyfloral honeys (P1-P5). The results are presented in Table 1 and demonstrate similarities in the content of all the samples. The semiquantitative data for samples Ch, Ks, Kb, P1-P5 and five known monofloral types of honey (rapeseed, acacia, sunflower, linden and oak) are applied in the unsupervised hierarchical cluster analysis and PCA for the identification of the groups of samples with similar characteristics. The graphical representation of the HCA (dendrogram, Fig. 2) illustrates six distinct groups – one group including all the polyfloral samples and a separate group for the monofloral honey varieties. Analogously,

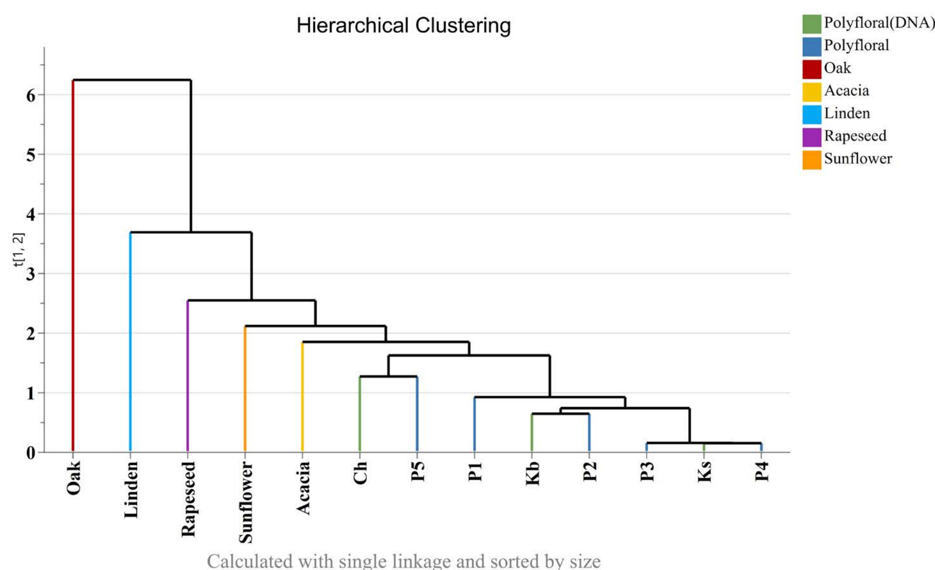

Fig. 2. A graphical representation of the hierarchical clustering of Kb, Ks and Ch with 10 honey samples of known botanical origin – 5 mono- and 5 polyfloral

the PCA analysis (the scores and loading scatter plots and biplot are presented in Supplementary Figs. 1–3) indicates the differentiation of the polyfloral and monofloral honeys and suggests the polyfloral origin of the three samples of unknown origin. Accordingly, the application of NMR chemometrics and the comparison of the obtained data for the three analysed honey samples with data from a small set of mono- and polyfloral samples was sufficient to suggest their polyfloral origin, revealed by the parallel melissopalynological and DNA barcoding analyses.

A comparison of the three methods. All the three applied methods revealed the polyfloral origin of the three analysed honey samples and demonstrated the ability to differentiate between monofloral and polyfloral honey. During the last decade NMR analysis has been increasingly and efficiently applied to honey authentication studies including the development of markers for the origin of botanical honey. Since honey composition is determined by complex factors related to the local environment and flora, NMR alone is unlikely to be sufficiently efficient for a detailed determination of the floral composition of honey. Instead, it needs to be further developed as a routine method to test for honey adulteration, the distinction of monofloral and polyfloral honey and possibly the identification of the main floral constituents of honey if sufficient datasets from the analysis of a wide range of honey are available. At the same time, both melissopalynological and DNA barcoding pollen analysis have the capacity for the qualitative characterisation of the floral composition of honey based on pollen analysis. The results from the present and several other studies demonstrate the higher resolution of DNA barcoding, especially for the identification of the presence of pollen grains from plant species growing in lower abundance in the area or plant species less preferred by honey bees. Therefore, DNA barcoding should be considered for studies requiring a more detailed characterisation of the diet and foraging preferences of honey bees, especially for the evaluation of the minor impact of different plant taxa. The need for skilled laboratory staff and the relatively high cost of carrying out the DNA barcoding still limits the application of this method for the routine characterisation of the authenticity and botanical origin of honey. Additionally, the results of the present study show substantial differences in the estimated relative pollen grain abundances for several plant families after parallel melissopalynological and DNA barcoding analyses. This suggests that more parallel characterisation of a wide range of honey samples by both methods should be carried out to evaluate and take into consideration the expected discrepancies in the estimated relative pollen grain abundances for some plant families in the routine application of DNA barcoding.

CONCLUSIONS

The comparison of melissopalynological and DNA barcoding analyses proves that DNA barcoding provides improved resolution for a detailed qualitative characterisation of the floral composition of honey, especially in the assessment of the minor impact of the botanical composition of the honey. Our quantitative data demonstrated significant sample-independent distortions between the pollen grain abundance determined by the melissopalynological analysis for several plant families and the corresponding rbcL clone abundance determined by DNA

barcoding. The observed differences in the estimated pollen abundance reveals an important drawback for the characterisation of the floral composition of honey which should be addressed by further parallel studies of a wider range of honey samples of different composition. The results of the NMR analysis confirm the polyfloral botanical origin of the analysed samples, suggested in the other two analyses in the present study.

Acknowledgements – This work was supported by the National Science Fund at the Ministry of Education and Science, Sofia, Bulgaria within the "Competition for financial support for junior researchers – 2016" (grant number DM 06/3 17.12.2016 and instrumental grants UNA-17/2005, DRNF-02-13/2009 and INFRAMAT).

REFERENCES

Balkanska R, Stefanova K, Stoikova-Grigorova R & Manolova V. 2018. Preliminary study of DNA extraction from Bulgarian honeys and its amplification by PCR for botanical identification. *Food and Health* **4**(3): 194–201.

Bell KL, Burgess KS, Botsch JC, Dobbs EK, Read Timothy D & Brosi Berry J. 2019. Quantitative and qualitative assessment of pollen DNA metabarcoding using constructed species mixtures. *Molecular Ecology* **28**(2): 431–455.

Bogdanov S & Gallmann P. 2008. Authenticity of honey and other bee products state of the art. *ALP Science* **520**: 1–12.

Bruni I, Galimberti A, Caridi L, Scaccabarozzi D, de Mattia F, Casiraghi M & Labra M. 2015. A DNA barcoding approach to identify plant species in multiflower honey. *Food Chemistry* **170**: 308–315.

Bulgarian State Standard. 1980. *Rules for sampling and tasting methods*. Qualitative indicators on honey 3050–80.

Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K & Madden T. 2009. BLAST+: architecture and applications. *BMC Bioinformatics* **10**: 421.

Chen S, Yao H, Han J, Liu C, Song J, Shi L, Zhu Y, Ma X, Gao T, Pang X, Luo K, Li Y, Li X, Jia X, Lin Y & Leon C. 2010. Validation of the ITS2 region as a novel DNA barcode for identifying medicinal plant species. *PLoS One* **5**(1): e8613.

Consonni R & Cagliani L. 2008. Geographical characterization of polyfloral and acacia honeys by nuclear magnetic resonance and chemometrics. *Journal of Agricultural and Food Chemistry* **56**(16): 6873–6880.

Galimberti A, de Mattia F, Bruni I, Scaccabarozzi D, Sandionigi A, Barbuto M, Casiraghi M & Labra M. 2014. A DNA barcoding approach to characterize pollen collected by honey bees. *PLoS One* **9**(10): e109363.

Gerginova D, Simova S, Popova M, Stefova M, Stanoeva J & Bankova V. 2020. NMR profiling of North Macedonian and Bulgarian honeys for detection of botanical and geographical origin. *Molecules* **25**(20): e4687.

Habib HM, Al Meqbali FT, Kamal H, Souka UD & Ibrahim WH. 2014. Physicochemical and biochemical properties of honeys from arid regions. *Food Chemistry* **153**: 35–43.

Hawkins J, Vere N, Griffith A, Ford CR, Allainguillaume J, Hegarty MJ, Les Baillie L & Adams-Groom B. 2015. Using DNA metabarcoding to identify the floral composition of honey: A new tool for investigating honey bee foraging preferences. *PLoS One* **10**(8): e0134735.

Hebda RJ & Chinnappa CC. 1990. Studies on pollen morphology of Rosaceae in Canada. *Review of Palaeobotany and Palynology* **64**(1): 103–108.

Hollingsworth ML, Clark AA, Forrest LL, Richardson J, Pennington RT, Long DG, Cowan R, Chase MW, Gaudeul M & Hollingsworth PM. 2009. Selecting barcoding loci for plants: evaluation of seven candidate loci with species-level sampling in three divergent groups of land plants. *Molecular Ecology Resources* **9**(2): 439–457.

Hollingsworth PM, Graham SW & Little DP. 2011. Choosing and using a plant DNA barcode. *PLoS One* **6**(5): e19254.

Jain SA, Jesus FT, Marchioro GM & Divino de Araújo E. 2013. Extraction of DNA from honey and its amplification by PCR for botanical identification. *Food Science and Technology (Campinas)* **33**(4): 753–756.

Kamo T, Kusumoto Y, Tokuoka Okubo S, Hayakawa H, Yoshiyama M, Kimura K & Konuma A. 2018. A DNA barcoding method for identifying and quantifying the composition of pollen species collected by European honeybees, *Apis mellifera* (Hymenoptera: Apidae). *Applied Entomology and Zoology* **53**(3): 353–361.

Kazalaki A, Misiak M, Spyros A & Dais PH. 2015. Identification and quantitative determination of carbohydrate molecules in Greek honey by employing ^{13}C NMR spectroscopy. *Analytical Methods* **7**: 5962–5972.

Khlifi S, Mauve C, Glab N, Gilard F, Hodges M, Mouilled G & Farjon J. 2017. An easier analysis of complex mixtures with highly resolved and sensitivity enhanced 2D quantitative NMR, *Analytical Methods* **9**: 2328–2333.

Kortesniemi M, Slupsky CM, Ollikka T, Kauko L, Spevacek AR, Sjövall O, Yang B & Kallio H. 2016. NMR profiling clarifies the characterization of Finnish honeys of different botanical origins. *Food Research International* **86**: 83–92.

Kraaijeveld K, de Weger LA, Ventayol García M, Buermans H, Frank J, Hiemstra PS & Den Dunnen JT. 2014. Efficient and sensitive identification and quantification of airborne pollen using next-generation DNA sequencing. *Molecular Ecology Resources* **15**: 8–16.

Ohmenhaeuser M, Monakhova YU, Kuballa TH & Lachenmeier D. 2013. Qualitative and quantitative control of honeys using NMR spectroscopy and chemometrics. *Hindawi ISRN Analytical Chemistry* **2013**: 825318.

Piro R, Guidetti G, Persano Oddo L & Piazza MG. 2002. Mathematical diagnosis of unifloral honeys. In: Sabatini AG, Bolchi Serrini G, Frilli R & Porrini C (eds.), *Il ruolo della ricerca in apicoltura*, pp. 235–239, Litosei, Bologna.

Popescu R, Geana E, Dinca O, Sandru C, Costinel D & Ionete R. 2016. Characterization of the quality and floral origin of Romanian honey. *Analytical Letters* **49**(3): 411–422.

Popova M, Gerginova, Trusheva B, Simova S, Tamfu AN, Ceylan O, Clark K & Bankova V. 2021. Chemical profiling of honey, cerumen and propolis of the African stingless bee *Meliponula ferruginea*. *Foods* **10**: e997.

Ramanujam CGK, Kalpana TP & Fatima K. 1992. Melittopalynology and recognition of major nectar and pollen sources for honey bees in some districts of Andhra pradesh. In: Venkatachala BS, Jain KP & Awasthi N (eds.), *Proceedings of the Birbal Sahni Birth Centenary Palaeobotanical Conference*, pp. 261–271, Palaeobotanical Society.

Sajwani A, Farooq SA, Patzelt A & Eltayeb EA. 2007. Melissopalynological studies from Oman. *Palynology* **31**(1): 63–79.

Schievano E, Stocchero M, Morelato E, Facchin CH & Mammi ST. 2012. An NMR-based metabolomic approach to identify the botanical origin of honey. *Metabolomics* **8**: 679–690.

Schievano E, Stocchero M, Zuccato V, Conti I & Piana L. 2019. NMR assessment of European acacia honey origin and composition of EU-blend based on geographical floral markers. *Food Chemistry* **288**: 96–101.

Schnell I, Fraser M, Willerslev E & Gilbert MT. 2010. Characterisation of insect and plant origins using DNA extracted from small volumes of bee honey. *Arthropod-Plant Interactions* **4**(2): 107–116.

Siddiqui AJ, Musharraf SG, Choudhary MI & Rahman A. 2017. Application of analytical methods in authentication and adulteration of honey. *Food Chemistry* **217**: 687–698.

Song XY, Yao YF & Yang WD. 2012. Pollen analysis of natural honeys from the central region of Shanxi, North China. *PLoS One* **7**(11): e49545.

Terrab A, González AG, Díez MJ & Heredia FJ. 2003. Characterisation of Moroccan unifloral honeys using multivariate analysis. *European Food Research and Technology* **218**(1): 88–95.

Valentini A, Miquel C & Taberlet P. 2010. DNA barcoding for honey biodiversity. *Diversity* **2**(4): 610–617.

Vulić J, Čanadanović-Brunet J, Ćetković G, Djilas S & Šaponjac VT. 2015. Antioxidant and sensorial properties of polyfloral honey with dried apricots after one year of storage. *Hindawi Journal of Chemistry* **2015**: 1–7.

Zhang J, Chen H, Fan C, Gao S, Zhang Z & Bo L. 2020. Classification of the botanical and geographical origins of Chinese honey based on ^{1}H NMR profile with chemometrics. *Food Research International* **137**: 109714.

Zuccato V, Finotello C, Menegazzo I, Peccolo G & Schievano E. 2017. Entomological authentication of stingless bee honey by 1H NMR-based metabolomics approach. *Food Control* **82**: 145–153.

REZIME

Botanica SERBICA

Procena botaničkog porekla bugarskih uzoraka meda korišćenjem melisopalinoloških analiza, DNK barkodiranja i NMR analiza

Ralitsa Balkanska, Katerina Stefanova, Radostina Stoikova-Grigorova, Dessislava Gerginova, Svetlana Simova i Ivan Atanassov

Uzorci poliflornog meda iz Bugarske podvrgnuti su paralelnim analizama botaničkog porekla i sastava korišćenjem tradicionalne melisopalinološke studije, DNK-barkodiranja na osnovu plastidnog rbcL gena i NMR analize. Dobijeni skupovi podataka su međusobno upoređeni da bi se procenio informacioni kapacitet primenjenih eksperimentalnih metoda. Rezultati melisopalinoloških i DNK-barkodnih studija pokazali su značajno veću rezoluciju ovog poslednjeg, otkrivajući prisustvo polena iz ukupno 17 biljnih porodica, 21 roda i 5 vrsta u poređenju sa polenom iz samo 7 porodica, 3 roda i 4 vrste identifikovane melisopalinologijom. Veća rezolucija DNK-barkodiranja omogućava detaljniju karakterizaciju ishrane medonosnih pčela i preferencija za ishranu, uključujući ishranu medonosnih pčela na biljnim vrstama koje rastu u manjoj količini u tom području. Poređenje kvantitativnih podataka o sastavu cvetnog meda za nekoliko biljnih rodova i vrsta otkriva značajne razlike između relativne zastupljenosti polenovih zrna procenjene melisopalinološkom analizom i relativne zastupljenosti rbcL klonova u rbcL bibliotekama utvrđenih nakon DNK barkodiranja. Sve tri primenjene metode potvrđuju poliflorno botaničko poreklo analiziranih uzoraka i podržavaju rutinsku NMR upotrebu za procenu cvetnog porekla meda.

Ključne reči: rbcL, pčelinji med, melisopalinologija, ^{1}H i ^{13}C NMR.