Bulgarian Journal of Agricultural Science, 26 (No 1) 2020, 238–242

A preliminary assessment of *trnH-psbA* as DNA barcode for botanical identification of polyfloral honey samples and comparison with *rbcL* marker

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Abstract

Balkanska, R., Stefanova, K., Stoikova-Grigorova, R. & Ignatova, M. (2020). A preliminary assessment of *trnH-psbA* as DNA barcode for botanical identification of polyfloral honey samples and comparison with *rbcL* marker. *Bulg. J. Agric. Sci., 26 (1),* 238–242

Honey is a natural and sweet product produced from *Apis mellifera* L. It can be classified as monofloral and polyfloral. DNA barcoding of pollen in honey can be used to identify the source of honey with high efficiency. Three polyfloral honey samples were used in the study. The *trnH-psbA* barcode was found to be an effective marker as source of additional date in identifying of the botanical origin of honey samples with high level of confidence. The purpose of this study was first to assess the efficiency of *trnH-psbA* marker as a DNA barcode for botanical identification and to compare the data with the obtained results for *rbcL* marker as a complex approach for botanical identification of polyfloral honey samples.

Keywords: polyfloral honey; *trnH-psbA*; marker/barcode; *rbcL*

Introduction

Honey is a natural and sweet product produced from *Apis mellifera* L. It can be classified as monofloral and polyfloral. While monofloral honeys are produced from the nectar of one predominant plant, polyfloral honeys are characterized as a product obtained from nectar from different plants. The botanical origin of honeys is traditionally determined using melissopalynology. This microscopic method detects the presence of pollens of the plants visited by the bees. This analysis was the only tool to identify and classify the pollens based on their morphology (Manivanan et al., 2018). Pollens present in low levels are less likely to be detected with melissopalynology. Several other methods have been proposed for the determination of botanical and geographical origin of honey (Anklam, 1998). Recently, DNA barcoding is a relatively new method of molecular identification of plant species using short sequences of chloroplast DNA. The use of DNA-based analysis has been proposed to identify the botanical composition of honey (Jain et al., 2013; Hawkins et al., 2015; Utzeri et al., 2018). It is well known that they provide unlimited information on the plant composition of honey. They have the potential to reduce processing time and to increase the level of pollen species which could not be identified with melissopalynology. In plants, three regions of the chloroplast genome (*rbcL, matK, trnH-psbA*) have been widely used as DNA barcodes, either separately or in combination (Bell et al., 2016). DNA barcoding studies (Valentini et al., 2010; Hawkins et al., 2015; Manivanan et al., 2018) employed these markers to provide resolution at species level in honey samples. DNA barcoding as an emerging tool in molecular biology can be easily and widely used to investigate floral visitation in honey bees. The *trnH-psbA* spacer (≈450

bp), is the most variable plastid region and is easily amplified across a broad range of land plants. The plastid *trnH-psbA* intergenic spacer as potentially usable DNA region for applying barcoding to flowering plants (Kress et al., 2005) could be proposed as a suitable marker for identification of pollen composition of honey. However, a comprehensive evaluation on the utility of *trnH-psbA* as a single DNA barcode region is lacking for Bulgarian polyfloral honey samples.

The purpose of this study was first to assess the efficiency of *trnH-psbA* marker as a DNA barcode for botanical identification and to compare the data with the obtained results for *rbcL* marker as a complex approach for botanical identification of polyfloral honey samples.

Materials and Methods

The honey samples were collected from beekeepers from Sofia region – Kostinbrod (Ks), Kubratovo (Kb) and Chepinci (Ch) in 2017. The samples were stored at room temperature prior analysis. The total DNA was isolated from 120 g of honey as described by Balkanska et al. (2018) using a GeneJET Genomic DNA Purification Mini Kit (Thermo Scientific).

DNA was amplified using the *trnH-psbA* marker. The primer combination used for *trnH-psbA* was *trnH*: 5'CGCGCATGGTGGATTCACAATCC-3' and *psbA*: 5'GTTATGCATGAACGTAATGCTC-3' (Bruni et al., 2015). The PCR amplification was performed in a 35 µl reaction. The amplification of DNA was done in a thermal cycler QB-96 (Quanta Biotech). The following DNA amplification protocol was used for the *trnH-psbA* spacer comprises denaturation at 94°C for 3 min, 35 cycles of denaturation (45 s at 94°C), annealing at 53°C for 30 s, extension at 72°C for 1 min and a final extension at 72°C for 7 min (Bruni et al., 2015). The resulting PCR products were purified using GeneJET Gel Extraction Kit (Thermo Scientific) following the manufacturer's instructions.

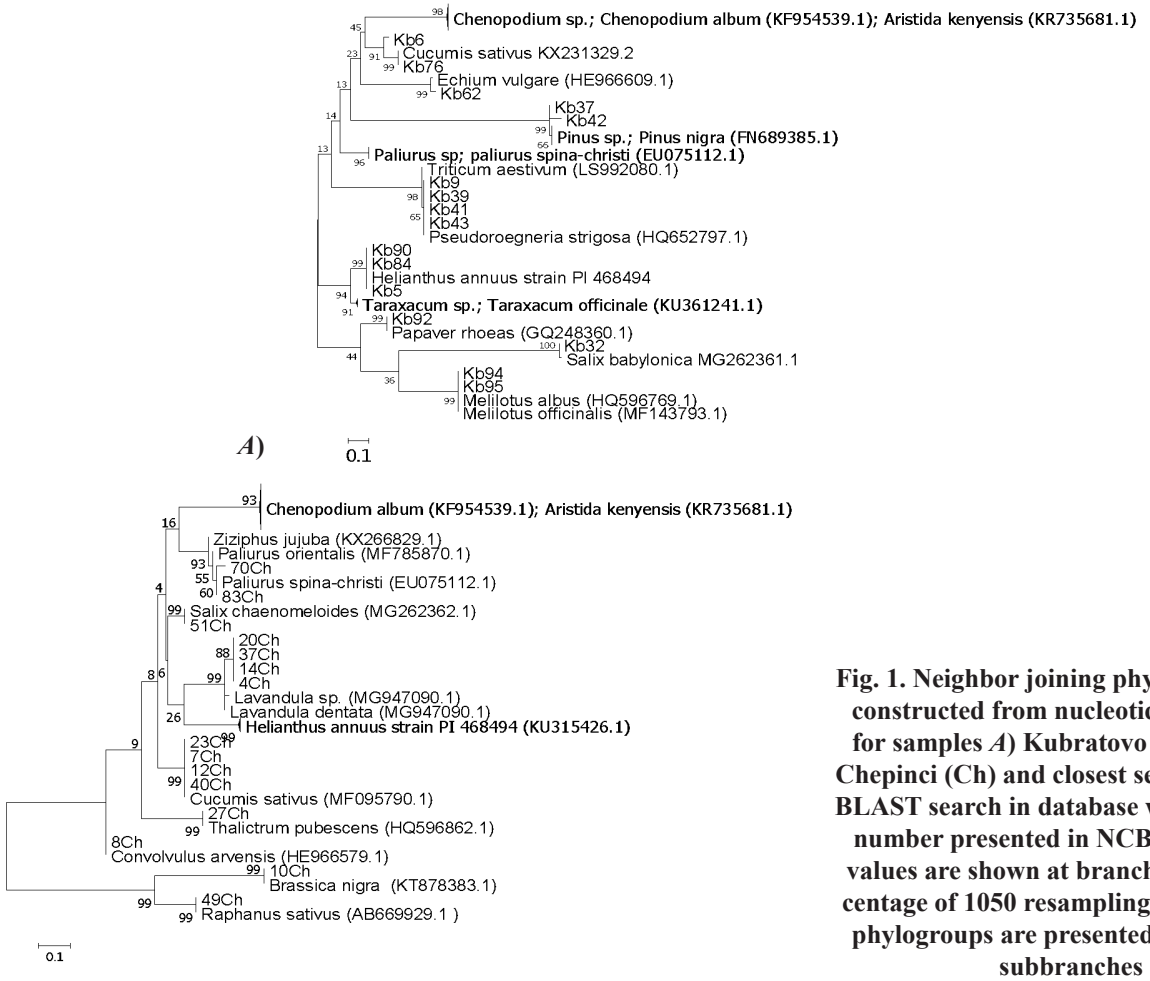

Fig. 1. Neighbor joining phylogenetic tree constructed from nucleotide sequences for samples *A*) Kubratovo (Kb) and *B*) Chepinci (Ch) and closest sequences after BLAST search in database with accession number presented in NCBI. Bootstrap values are shown at branch points (percentage of 1050 resampling). The largest phylogroups are presented as triangles subbranches

The amplification products obtained were subsequently cloned CloneJET PCR Cloning Kit (Thermo Scientific). Recombinant plasmids were isolated using GeneJET Plasmid Miniprep Kit (Thermo Scientific). For each honey sample at least 100 clones were selected and sequenced at Macrogen Inc. DNA quantification was carried out using the Nano Drop UV-Vis Spectrophotometer (Nano Drop Technologies). The obtained sequences were analyzed, using Vector NTI v.10 software package (Life Technology). The clones containing correct inserts were compared to the sequences in GenBank database by using standard Nucleotide-Blast search tools to determine their close relatives and approximately phylogenetic affiliation.

Results and Discussion

Three multifloral honey samples (Ch, Kb and Ks) were subject of DNA-barcoding based on plastid *trnH-psbA* spacer in order to gain information about their botanical origin. The obtained results were further compared to evaluate the information capacity of the applied experimental approaches.

DNA-barcoding based on trnH-psbA spacer

Three *trnH-psbA* clone libraries were constructed for three honey samples, followed by PCR amplification of *trnH-psbA* spacer region using universal *trnH-psbA* (*trnH F* and *psbA R*) primers and cloning into plasmid vector. Sequence analysis of the obtained *trnH-psbA* clones resulted in identification of totally 49 different *trnH-psbA* sequences. The BLAST search of GenBank with the obtained *trnH-psbA* clone sequences show that 43.62% of them have above 99% identity with the GenBank sequences of known plant species and could be affiliate to them. The rest 36.67% of the obtained *trnH-psbA* clone sequences show high level homology (95% to 99% identity) to the *trnH-psbA* spacer sequences of known plant species. The analyzed *trnH-psbA* clone sequences and retrieved higher homology to GenBank sequences were further subject to phylogenetic analysis. The constructed phylogenetic trees show formation of 10 clusters in Ch sample, 6 in Ks and 11 in Kb related to distinct plant families, (Figure 1A and B).

Taking together the results from GenBank search and phylogenetic analysis makes possible affiliation of all *trnH-psbA* sequences to total 15 plant families, 17 plant genus and 5 plant species. The results from sequence analysis of *trnH-psbA* clones together with the abundance of identified clone sequences in the *trnH-psbA* libraries were used to estimate the relative abundance from identified plant families, genus and species (Table 1). The results show that 15%, 32.91% and 50% of the *rbcL* clones from Ch, Ks and Kb libraries were affiliated to particular genus and additional 60%, 64.55% and 14% of the *trnH-psbA* clones were affiliated to distinct plant species.

The comparison of the DNA barcoding data for honey botanical origin of the samples from Ks, Kb and Ch with *trnH-psbA* and *rbcL* demonstrate two main features:

Table 1. Plant families and species presented in the polyfloral honey samples from Chepinci, Kostinbrod and Kubratovo

	Family	Chepinci (Ch)	Kostinbrod (Ks)	Kubratovo (Kb)
1	*Asteraceae*	*Helianthus annuus*	*Helianthus annuus; Helianthus praecox; Helianthus sp.*	*Helianthus annuus; Helianthus praecox; Taraxacum sp.*
2	*Apiaceae*	nd	*Conium sp.*	nd
3	*Brassicaceae*	*Brassica sp.*; *Raphanus sp.*	nd	nd
4	*Boraginaceae*	nd	nd	dt
5	*Cucurbitaceae*	*Cucumis sativus*	nd	*Cucumis sativus*
6	*Chenopodiaceae*	*Chenopodium album*	*Chenopodium album*	*Chenopodium album Chenopodium sp.*
7	*Convolvulaceae*	nd	nd	*Convulvus arvensis*
8	*Fabaceae*	nd	*Gleditsia sp.*	*Melilotus sp.*
9	*Lamiaceae*	dt	nd	nd
10	*Poaceae*	*Aristida kenyensis*	nd	*Aristida kenyensis; Pseudoroegneria sp.*
11	*Papavaraceae*	nd	nd	*Papaver sp.*
12	*Pinaceae*	nd	*Pinus sp.*	*Pinus sp.*
13	*Salicaceae*	nd	*Salix sp.*	*Salix sp.*
14	*Rhamnaceae*	*Paliurus sp.*	*Paliurus sp.*	*Paliurus* sp.
15	*Ranunculaceae*	*Thalictrum sp.*	nd	nd

nd – non detected

dt – detected but not affiliated with genus or plant species in the samples

(a) the applied *trnH-psbA* barcoding analysis identify 15 plant families and 17 genus in comparison to the 17 plant families and 21 genus, as well as members of 5 versus 5 plants species revealed by the *rbcL* DNA barcoding assay from the previously described study.

(b) the comparison of the observed different plant families, genus and species revealed by *trnH-psbA* the analysis with relative abundance of corresponding plant taxa in the *rbcL* libraries constructed from the same honey samples show some distinct differences from the application of both methods. An important result from the comparative study is the observed sample-dependent distortion in relative abundance of the corresponding sequences and such as in all plant families and genus revealed by the two applied analysis. Thus, *trnH-psbA* DNA barcoding of sample Ks was able to estimates 4 plant families and 3 genera from previously observed 7 plant families using *rbcL* barcode. Three new families (*Pinaceae, Apiaceae, Chenopodiaceae*) were identified with *trnH-psbA* marker. For the samples Ch and Kb *trnH-psbA* DNA barcoding identified only 6 plant families and 7 genera versus 16 plant families (19 genus) established with *rbcL* DNA barcoding. In addition, only 4 new plant families (*Chenopodiaceae, Pinaceae, Salicaceae* and *Rhamnaceae*) in the both samples were established with *trnH-psbA* marker.

The reasons of the observed distortions of the estimated diversity from specific plant taxa could be the result from various factors. It includes different efficiency of DNA isolation from different pollen grains or efficiency of PCR amplification (non-specific products) and cloning of mixed PCR fragments of 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. Further, comparison of relative abundance of sequenced *trnH-psbA* clones reveals 25%, 3.83%, 36% non-specific amplification that were not established, using *rbcL* barcoding in Ch, Ks and Kb samples. In addition, phylogenetic analysis shows that only 3.47% from totally sequenced clones from studied samples could not be affiliated with plant genus or species.

The presented results also confirm the high information capacity of the *trnH-psbA* barcoding analysis on qualitative characterization of botanical origin of multifloral honey samples reported by Bruni et al. (2015) and Hawkins et al. (2015). It has to be pointed out that although more detailed information derived from DNA barcoding in this study, one part of the *trnH-psbA* clones were affiliated with only particular plant genus, without identification of corresponding plant species. Such still limited identification could be explained also with (i) that in some groups of plants, *trnH-psbA* is not sufficiently variable to distinguish among closely related species (Whitlock et al., 2010) as well as (ii) the lack of sequenced *trnH-psbA* spacer regions for plants species represented in the database which are typical for Bulgaria.

Although using multiple barcode markers provides high resolution of analysis at genus and species level, the results additionally complicate the interpretation of the quantitative data and estimation of different plant species. The above raises the question on the direct use of *trnH-psbA* DNA barcoding data for quantitative assessment the botanical origin of honey instead of the presently well-regulated application of melissopalynological analysis.

Conclusion

The presented results confirm the high information capacity of the *trnH-psbA* with *rbcL* barcoding analysis on qualitative characterization of botanical origin of polyfloral honey samples as a successful complex approach. Five additional plant families (*Pinaceae, Apiaceae, Chenopodiaceae, Salicaceae* and *Rhamnaceae*) were identified with *trnH-psbA* marker compared to the *rbcL* marker. The *trnH-psbA* is a suitable additional marker for analyzing plant diversity in honey. The results indicated that pollen composition of honey was largely influenced by floristic local biodiversity. However, there is a lack of sequenced *trnH-psbA* spacer regions for plants species represented in the database which are typical for Bulgaria.

Acknowledgements

This work was supported by the National Science Fund of the Bulgarian Ministry of Education and Science, Sofia (grant number DM 06/3 17.12.2016).

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Received: January, 2, 2019; *Accepted:* May, 10, 2019; *Published:* February, 29, 2020