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PRELIMINARY STUDY OF DNA EXTRACTION FROM BULGARIAN HONEYS AND ITS AMPLIFICATION BY PCR FOR BOTANICAL IDENTIFICATION

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ABSTRACT

The physiochemical and biological properties of honey are directly associated to its floral origin. Some current commonly used methods for identification of botanical origin of honey involve melissopalynological analysis or other analytical methods. However, these methods can be less sensitive and time-consuming. DNA-based methods have become popular due to their quickness and reliability. In this respect, the purpose on the present study is to compare two DNA extraction kits – DNeasy Plant Mini Kit (Qiagen) and GeneJET Genomic DNA Purification Mini Kit (Thermo Scientific) and CTAB method combined with different pre-treatment procedures and applied to 3 multifloral and 2 monofloral honey samples. The DNA extraction and amplification was carried out for all honey samples with similar results in each replication. The extracted DNA was amplified by PCR using plant specific primers rbcL gene and trnH-psbA spacer. The result obtained confirming that the DNA extracted has good quality for analysis of PCR products and that it can be used for botanical identification of honey.

Keywords: Honey, rbcL, trnH-psbA spacer, DNA extraction, Botanical identification

Introduction

There are a number of methods available for the determination of botanical origin of the honey. The traditional method for honey botanical determination (melissopalynology) is based on microscopic analysis of honey pollen composition. However, this method is time-consuming, requires experts in the botany with adequate skills and experience to identify pollen grains based on its different morphologies. In the recent years, in the process of development are other research approaches for determination of honey botanical origin such as volatile compounds (Guyot et al., 1999; Radovic et al., 2001; Soria et al., 2004). Furthermore, combination of some physicochemical parameters, elemental composition and protein content may be used for honey identification (Liberato et al., 2013). Interesting research task is determination of mineral and trace elements in honey for determination of botanical origin (Fernandez-Torres et al., 2005; Hernandez et al., 2005; Latorre et al., 1999). The markers for identification of different honey types are flavonoids, aromatic compounds and sugars (Wang and Li, 2011). In recent years, modern DNA methods for determination of honey botanical origin are needed. DNA barcode is a technology enables the identification of all plant and animal species by amplification and sequencing information from genes or intergenic target region belonging outside the nuclear genome. A number of authors (Matsuki et al., 2008; Longhi et al., 2009; Galimberti et al., 2014) indicate that molecular-based approaches show great potential to overcome the limitations of previously applied methods. For example, the analysis of certain DNA regions is successfully applied in the identification of plant species in bee pollen (Wilson et al., 2010; Bruni et al., 2012; De Mattia et al., 2012; Parducci et al., 2012; Galimberti et al., 2014). Recent studies indicate that the combination of two plastid regions as genetic markers – rbcL and trnH-psbA are the most effective for determination of plant species in bee pollen gathered by the bees. Galimberti et al. (2014) investigated the effectiveness of the DNA barcode for the characterization of pollen composition. In Bulgaria the melissopalynological analysis are limited (Atanassova et al., 2004; Atanassova and Kondova, 2004; Atanassova et al., 2009; Atanassova et al., 2012) and DNA methods for determination of honey botanical origin are needed. In this respect, the purpose on the present study is to compare two DNA extraction kits – DNeasy Plant Mini Kit (Qiagen) and GeneJET Genomic DNA Purification Mini Kit (Thermo Scientific) and CTAB method combined with different pre-treatment procedures and applied to five honey samples.

Materials and Methods

A total of five honey samples were analyzed. Three multifloral honey samples were collected from Bulgarian beekeepers in 2016. The apiaries are located near to Kostinbrod (Sofia region), Kubratovo (Sofia region) and Debelets (Veliko Tarnovo region). Two monofloral honey samples were purchased from the market.

The melissopalynological analysis was carried out using the method established by Bulgarian State Standard for Bee Honey 3050-80 and 2673-80. The pollen analysis was done in the Central Laboratory of Veterinary Control and Ecology, Sofia, Bulgaria.

Two bee pollen samples (BP 1 and BP 2) were used as a control for the procedure for DNA extraction with flash freeze in liquid nitrogen. The samples were multifloral and were collected from the Experimental apiary of the Institute of Animal Science, Kostinbrod by using a pollen trap.

DNA Extraction

Three extraction procedures were employed. Two commercial kits DNeasy Plant Mini Kit (Qiagen) and GeneJET Genomic DNA Purification Mini Kit (Thermo Scientific) and CTAB method were used for DNA extraction. Honey (120 g) was diluted with 360 mL MiliQ water, incubated at 45°C for 10 min in a water bath and after that was filtrated through membrane filter (pore size 8 µm). The filter was placed in a sterile petri dish (diameter 5 cm). A volume of 1200 µl of Buffer AP1 from a DNeasy Plant Mini Kit (Qiagen) or 950 µl of Lysis Buffer A (GeneJET Genomic DNA Purification Mini Kit, Thermo Scientific) had been added and shaked for 15 min. After that 400 µL or 350 µL into 3 new tubes (2 mL volume) were transferred. The samples were disrupted using a Tissue Lyser II (Qiagen GmbH) with 3 mm metal beads for 4 min at 30 l/s and then incubated for 10 min at 65°C and 60°C in a water bath. The subsequent steps of the DNeasy Plant Mini Kit were followed according to the manufacturer's instructions, with the exception that the QIAshredder column and second wash stage were omitted. The GeneJET Genomic DNA Purification Mini Kit (Thermo Scientific) was followed according to the manufacturer's instructions without any modifications.

The pre-treatment procedures include – sample disruption with a lysis buffer for DNA extraction or grinding the sample in liquid nitrogen at -70°C.

The pollen samples (0.5 g) were flash freezed in liquid nitrogen and after that 1200 µl of Buffer AP1 from a DNeasy Plant Mini Kit (Qiagen) were added. The next steps were the same as for honey samples.

The DNA concentrations of samples were determined by UV spectrophotometry (Nano Drop 2000). The CTAB-based method was performed as described by Murray and Tompson (1980) at 60°C with some modifications (110 mM DTT was added) as described by Lalhmangaihi et al. (2014).

The extracted DNA was stored at -20°C prior to subsequent analysis. Each extraction or amplification method was performed in triplicate.

PCR Amplification

DNA was amplified using the rbcL and trnH-psbA DNA barcode marker region. One of the internationally agreed core DNA barcodes for plants is rbcL whilst trnH-psbA is recognized as valuable additional marker. For PCR amplification of rbcL, the primer combination was 1F: 5'-ATGTCACCACAAACAGAAAC-3' and 724R: 5'-TCGCATGTACCTGCAGTA GC-3'. The primer combination used for trnH-psbA was trnH: 5'-CGCGCATGGTGGATTCACAATCC-3' and psbA: 5'-GTTATGCATGAACGTAATGCTC-3' (Bruni et al., 2015). Conventional PCRs were performed in a total volume of 25 µL. The reaction mixture contained PCR mix Taq polymerase (DNA Polymerase, Bioline), primers, and template DNA solution. Amplification was performed in the Thermal Cycler (Mastercycler-Eppendorf). PCR cycles consisted of an initial denaturation step for 3 min at 94°C, 35 cycles of denaturation (45 s at 94°C), annealing (30 s at 50°C for rbcL and 53°C for trnHpsbA), extension (1 min at 72°C) and a final extension at 72°C for 7 min (Bruni et al., 2015). The DNA extraction and length of amplification products obtained were checked by electrophoresis on 1.8% (w/v) agarose gel and Bioline HyperLadder 100bp. The gel was visualized after being stained with 20 µL GelRed (Biotium) in 200 mL distilled water for 2 hours. The concentration of DNA was check with lambda DNA (50, 25, 12.5, 6.25, 3.13 ng).

The statistical analysis was performed using SPSS Statistical Package, version 21 for Windows. All results are presented as minimal and maximal value, means ± standard deviation.

Results and Discussion

First, the botanical origin of the honey was certified. Pollen analysis was used to specifically identify and confirm the botanical origin of the honey samples. In general, 3 honey samples analyzed could be considered as multifloral and 2 honey samples as monofloral according to pollen contribution of the botanic species. The results are presented in Table 1.

Table 1. Pollen content in the honey samples, (n=5)

Sample	Plant families and species
Sample 1 Multifloral Honey	*Asteraceae* – 44.7±2.3%; (*Helianthus annuus* – 36.9 ±1.9%); *Fabaceae* – 33.0 ±1.7%; (*Melilotus sp.* – 30.0±1.6%); *Brassicaceae* – 3.9 ±0.2%; *Rosaceae* – 2.9 ±0.2%; *Violaceae* (*Viola tricolor* 1.9 ±0.1%); *Poaceae* – 7.8±0.4%; *Betulaceae* – 1.9 ±0.1%; *Chenopodiaceae* – 1.9 ±0.1%.
Sample 2 Multifloral Honey	*Rosaceae* – 65.6 ±3.4%; *Tiliaceae* – 7.9 ±0.4%; *Fabaceae* – 13.2 ±0.7% (*Robinia pseudoacacia* – 5.7 ±0.3%; *Trifolium sp.* – 3.5 ±0.2%; *Melilotus sp.* – 1.3±0.1%); *Asteraceae* – 7.0 ±0.4% (*Helianthus annuus* – 5.3 ±0.3%; *Taraxacum sp.* – 0.88±0.04%); *Poaceae* – 2.2 ±0.1%; *Plantaginaceae* 0.88±0.04%.
Sample 3 Multifloral honey	*Rosaceae* – 31.5 ±1.6%; *Brassicaceae* – 26.8 ±1.4%; *Fabaceae* – 21.4 ±1.4% (*Lotus corniculatus* – 9.5 ±0.5%; *Melilotus sp.* – 5.4 ±0.3%; *Trifolium sp.* – 3.0 ±0.2%); *Tiliaceae* – 7.1 ±0.4%; *Asteraceae* – 4.8 ±0.2% (*Helianthus annuus* – 2.4 ±0.1%).
Sample 4 Sunflower Honey	*Asteraceae* – 81.9 ±4.3% (from them *Helianthus annuus* – 76.1 ±4.0%).
Sample 5 Rape Honey	*Brassicaceae* – 78.5 ±4.1%.

DNA Extraction and Quantitative Analysis

In the present study, we made modifications to select DNA isolation systems to simplify and improve their efficiency in DNA isolation. The yield and quality of isolated DNAs were assessed via gel electrophoresis and spectrophotometric measurement. The DNA isolation from bee honey as a complex matrix of sugars, mineral elements and enzymes has remained very challenging.

1. DNA isolation with DNeasy Plant Mini Kit (Qiagen)

As a first step different pre-treatment procedures were applied for DNA isolation of honey samples with use of only Proteinase K or RNase A. Furthermore, flash freeze in liquid nitrogen was applied for honey sample from Kostinbrod, Kubratovo and Debelets. Two bee pollen samples (P1 and P2) were used as a control. The pretreatment procedure for DNA extraction with flash freeze in liquid nitrogen is suitable but it is not optimal for DNA isolation from honey samples. A further disadvantage of the grinding method with the use of liquid nitrogen is the need to have a continuous source of liquid nitrogen. The results showed that better results are obtained with the both use of Proteinase K and RNase A, without freezing in liquid nitrogen. Figure 1 presents DNA extracted from honey samples with 2 µL Proteinase K (100 mg/mL) and 6 µL RNase A (100 mg/mL). Similar results are obtained for the samples from Debelets and for 2 monofloral honeys (Sunflower and Rape).

2. DNA isolation with GeneJET Genomic DNA Purification Mini Kit (Thermo Scientific)

According to the second method, the results of agarose gel electrophoresis did not show clear visible DNA compare to the result of DNeasy Plant Mini Kit (Qiagen), (Fig. 2). Similar results are obtained for Sunflower and Rape honey samples.

3. CTAB method

The CTAB method is the most widely used method for extracting DNA from plants. The results of agarose gel electrophoresis showed that visible DNA was obtained with CTAB method (Fig. 3) and to a higher extent with the two commercial kits. The CTAB method exhibited the highest DNA intensity for the all honey samples. It was allowed obtaining higher DNA quantity which was particularly evidenced in the studied honey samples.

As can be seen from Fig. 3 (A, B) the CTAB method can extract much more DNA quantity compare to the two commercial kits. Furthermore, the use of CTAB method with common laboratory reagents for DNA extraction instead of commercially available kits make this extraction process more cost effective.

As a next step, the quality of all obtained DNA extracts was assessed by UV spectrophotometry. As shown in Table 2, for each extraction method we have reported the yield and the ratios of spectrophotometric absorbance at 260 and 280 nm. In fact, the ratio A260/280 is usually employed to measure the purity level of DNA and some other nucleic acids and nucleotides. The absorbance A260/280 up to 1.90 generally indicates a “pure” DNA sample. Generally, data were in an agreement with the electrophoretic analysis. As can be expected, the spectrophotometric analyses revealed the presence of DNA with good quality in all samples extracted with DNeasy Plant Mini Kit (Qiagen). Surprisingly, the results of agarose gel electrophoresis of DNA extracted with GeneJET Genomic DNA Purification Mini Kit (Thermo Scientific) did not show clear visible DNA. Anyway, spectrophotometric analyses showed similar DNA concentrations (Table 2) but a bit lower ratios of absorbance (A260/280 <1.90) for the most honey samples.

The DNA concentration observed by the CTAB method was higher due to high presence of RNA. The concentration of DNA was additionally checked with lambda DNA (50, 25, 12.5, 6.25, 3.13 ng), (Figure 3A). DNA extracted with the CTAB method from all honey samples showed high concentration. According to the results from the agarose gel and DNA standards it is not more than 3 – 12 ng visible DNA in 5 µL. Similar results for DNeasy Plant Mini Kit (Qiagen) and CTAB method are received by Soares et al. (2015) for honey samples.

PCR Amplification

To evaluate the extracted DNA from the five honey samples with two kits and CTAB method all extracts were amplified by PCR. In the present study, rbcL DNA barcode marker was amplified successfully from the DNA isolated from honey samples (Fig. 4). The amplification results showed strong PCR products (10 µL loaded on agarose gels). As can be seen from Fig. 4 the results obtained after PCR amplification are comparable for the DNA extraction with two commercial kits and CTAB method. With the CTAB method was observed co-precipitation of DNA and yellow pigment which can result in less DNA amplification. The DNA extracted with GeneJET Genomic DNA Purification Mini Kit (Thermo Scientific) as shown on Figure 2 is not clear visible. However, it could be easily amplified by PCR demonstrating that the DNA extract is proved adequate and shows strong PCR product in all honey samples (Figure 4).

DNA barcoding analysis was performed with the trnH-psbA intergenic spacer. The trnH-psbA marker was easily amplified in all honey samples.

Figure 5 shows that there is a difference in the number and length of the amplified fragments. Probably, it is due to the better amplification of DNA isolated with GeneJET Genomic DNA Purification Mini Kit (Thermo Scientific) compared to faint and defused bands obtained with DNesay Plant Mini Kit (Qiagen). Furthermore, this was an expected results considering that the multifloral honey samples might contain different pollen grains.

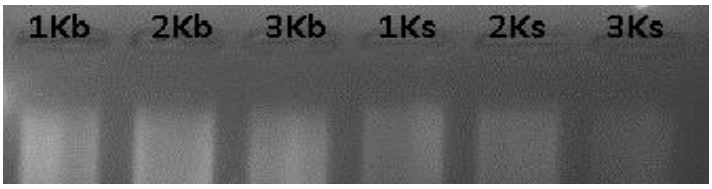


Figure 1. Agarose gel electrophoresis of DNA extracted from honey samples from Kubratovo (1Kb, 2Kb, 3Kb) and Kostinbrod (1Ks, 2Ks, 3Ks), DNeasy Plant Mini Kit (Qiagen)

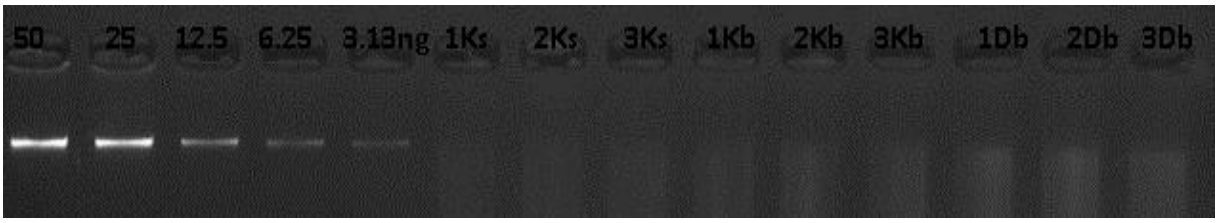
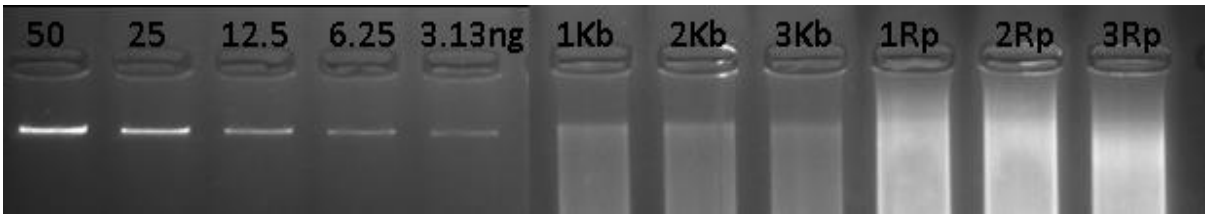
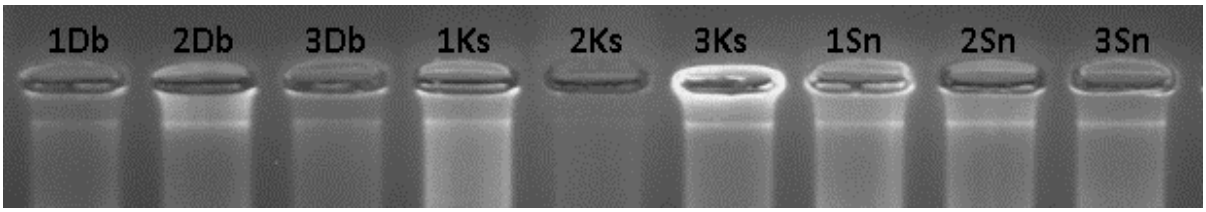


Figure 2. Agarose gel electrophoresis of DNA extracted from honey samples from Kostinbrod (1Ks, 2Ks, 3Ks), Kubratovo (1Kb, 2Kb, 3Kb), Debelets (1Db, 2Db, 3Db), GeneJET Genomic DNA Purification Mini Kit (Thermo Scientific), Lambda DNA (50, 25, 12.5, 6.25, 3.13 ng)

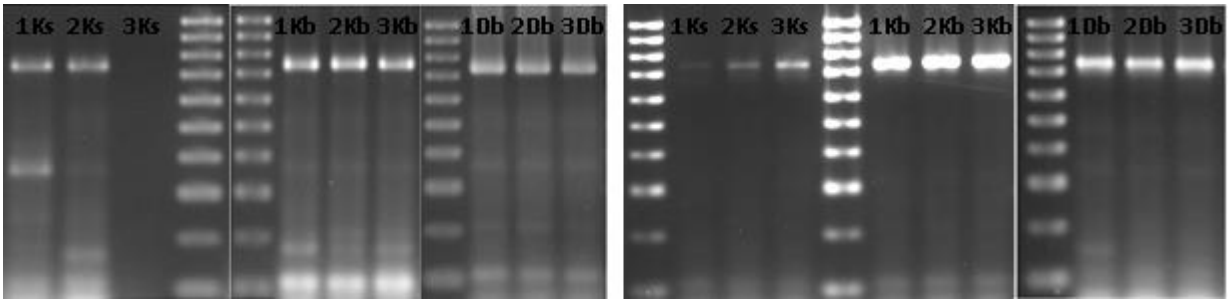


A)

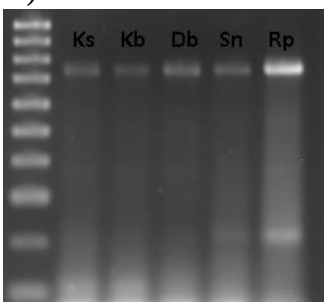


B)

Figure 3. Agarose gel electrophoresis of DNA extracted from honey samples (CTAB method)
A) Lambda DNA (50, 25, 12.5, 6.25, 3.13 ng), Kubratovo (1Kb, 2Kb, 3Kb), Rape honey (1Rp, 2Rp, 3Rp) B) Debelets (1Db, 2Db, 3Db), Kostinbrod (1Ks, 2Ks, 3Ks) and Sunflower honey (1Sn, 2Sn, 3Sn)



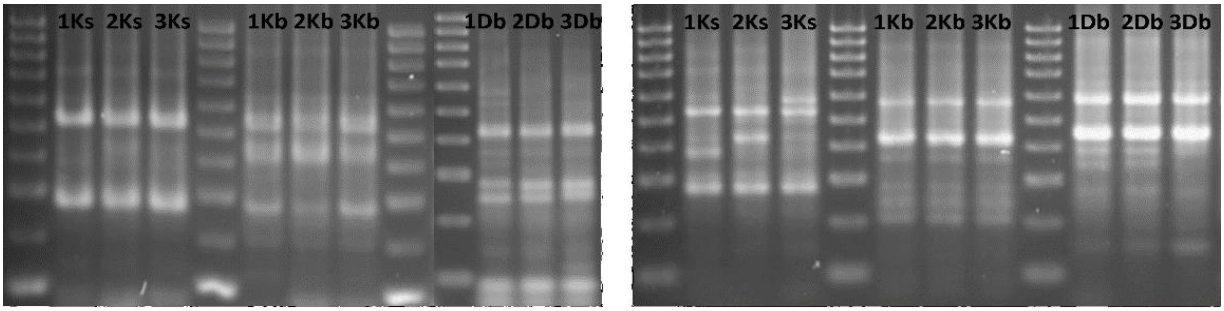
A) B)



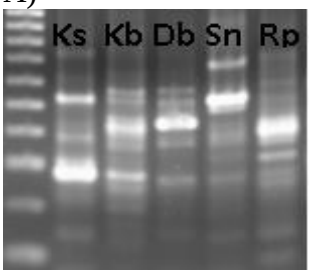
C)

Legend: A) Kostinbrod (1Ks, 2Ks, 3Ks), Kubratovo (1Kb, 2Kb, 3Kb), Debelets (1Db, 2Db, 3Db), DNeasy Plant Mini Kit (Qiagen); B) Kostinbrod (1Ks, 2Ks, 3Ks), Kubratovo (1Kb, 2Kb, 3Kb), Debelets (1Db, 2Db, 3Db), GeneJET Genomic DNA Purification Mini Kit (Thermo Scientific); C) CTAB method – Ks, Kb, Db, Sn, Rp

Figure 4. Agarose gel electrophoresis of PCR products of rbcL DNA barcode marker region amplified from honey samples prepared with two commercial kits and CTAB method



A) B)



C)

Legend: A) Kostinbrod (1Ks, 2Ks, 3Ks), Kubratovo (1Kb, 2Kb, 3Kb), Debelets (1Db, 2Db, 3Db), DNeasy Plant Mini Kit (Qiagen); B) Kostinbrod (1Ks, 2Ks, 3Ks), Kubratovo (1Kb, 2Kb, 3Kb), Debelets (1Db, 2Db, 3Db), GeneJET Genomic DNA Purification Mini Kit (Thermo Scientific); C) CTAB method – Ks, Kb, Db, Sn, Rp

Figure 5. Agarose gel electrophoresis of PCR products of trnH-psbA DNA barcode marker region amplified from honey samples prepared with two commercial kits and CTAB method

The length of the PCR product reflects to the amplification efficiency of our DNA samples. Also, these results are in a good agreement with the pollen analysis. It shows the presence of plant species in which the genetic marker trnH-psbA has a variable and high fragment length ~ 395 and ~ 573 bp. This PCR fragment for *Helianthus annuus* has a length ~ 517 bp, for *Melilotus sp.* ~ 395 bp (in honey sample from Kostinbrod) and for *Robinia pseudoacacia (Fabaceae)* ~ 543 bp, *Rosa canina* 433 bp and *Crataegus monogyna* ~ 394 bp (*Rosaceae*), (in honey sample from Kubratovo and Debelets). On Figure 5 C at lanes 4 (Sn) and 5 (Rp), strong PCR products were observed with ~ 573 bp and ~ 357 bp, respectively. It can be assumed that this marker can be used in studies including molecular identification of botanical origin of monofloral honey samples.

According to Bruni et al. (2015) the plastid spacer trnH-psbA was the most suitable marker to determine the plant species composition of honey. Furthermore, the DNA analysis is faster than melissopalynological analysis and can be used for routine use.

Conclusion

This study describes for the first time DNA extraction from Bulgarian multifloral and monofloral honey samples. According to the results obtained, we can concluded that all tested DNA isolation procedures and commercial kits presented, result in isolation of sufficient amount of pollen DNA from multifloral and monofloral honey samples. Thus, extracted DNA is successfully used for PCR amplification of rbcL gene and trnH-psbA spacer regions. The obtained results will be in a further use for routine application of PCR based molecular methods for identification and verification of botanical origin of the honey or determining the presence or absence of plant species.

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