



EVALUATION OF DIFFERENT DNA ISOLATION METHODS FROM PINE HONEY

Mehmet KARAALİ, Bekir ÇÖL*

Muğla Sıtkı Koçman, University, Department of Biology, Muğla, Turkey, 48000
bekircol@gmail.com, mehmetkaraali90@gmail.com

Received: 15.11.2016, Accepted: 01.12.2016

*Corresponding author

Abstract

Honey is a sweet food made by bees and some other insects. Pine honey is a type of honey which is produced by honey bees from the sugary secretions made by the some insect species, such as *Marchalina hellenica*, living on the pine trees. Pine honey is mostly produced in the Mediterranean countries such as Turkey and some regions of Greece. Honey is a highly consumed natural food product and it is associated with numerous health benefits. The knowledge of physicochemical and biological properties of honey as well as its floral origin is very important. Knowing the diversity of pollens, microorganism content of honey or ensuring its GMO (genetically modified organisms) status is significant both in terms of health and economy. To obtain such information, one of the most effective ways is to analyze the DNA of pine honey and identify the biological species it contains. Due to the nature of pine honey such as its viscosity and the presence of inhibitors, there is not a perfect reliable convincing DNA isolation method available to date. In this study, we collected pine honey samples from Mugla region (Turkey) and isolated DNA from the precipitated pollens of the honey using three different DNA isolation approaches. These methods include a modified CTAB method, manual silica dioxide approach and DNeasy Plant Mini Kit. DNA extraction protocols were compared in terms of DNA yield and purity. We demonstrate that the use of DNeasy plant kit has given relatively better results under the conditions of the current study for the Pine honey of Muğla.

Keywords: Pine honey, DNA isolation, PCR, rbcl gene

ÇAM BALINDA FARKLI DNA İZOLASYON TEKNİKLERİNİN DEĞERLENDİRİLMESİ

Özet

Bal, arılar ve diğer bazı böcek türleri tarafından oluşturulan tatlı bir besindir. Çam balı ise, arıların *Marchalina hellenica* gibi çam ağaçları üzerinde yaşayan böceklerin salgılarını kullanarak oluşturdukları bir bal çeşididir. Çam balı üretiminin Akdeniz ülkelerinde özellikle Türkiye ve Yunanistan'ın bazı bölgelerinde olduğu bilinmektedir. En çok tüketilen doğal besinlerin başında gelen bal insan sağlığı açısından birçok faydaya sahiptir. Bu nedenle, balın fizikokimyasal ve biyolojik özellikleri ile birlikte meydana geldiği botanik kökenin belirlenmesi oldukça önemlidir. Baldaki polen çeşitliliği, mikroorganizma varlığı ve GDO gibi statülerin bilinmesi hem ekonomik hem de sağlık açısından önem taşımaktadır. Bu bilgileri elde etmek için en etkili yollardan biri çam balında bulunan DNA'nın analizi ile biyolojik türlerin tespitidir. Fakat doğası gereği oldukça viskoz olan ve içinde bulundurduğu inhibitörlerden dolayı balın DNA analizi kolay değildir. Şu ana kadar yapılan çalışmalar incelendiğinde balın DNA analizi için güvenilir ve verimli sonuç veren bir DNA izolasyon tekniği olmadığı görülmektedir. Bu çalışmamızda Muğla'dan toplanan çam balı örneklerinin presipite edilmiş polenlerinden, üç farklı teknik kullanılarak, DNA izolasyonları yapılmıştır. Bu teknikler arasında; CTAB yöntemi, manuel silika dioksit yöntemi ve DNeasy Plant Mini Kit yöntemi bulunmaktadır. Teknikler DNA saflığı ve kalitesi bakımından karşılaştırılmıştır. Çalışma sonucunda, mevcut koşullar altında Muğla çam balından DNA izolasyonu için en verimli tekniğin DNeasy plant kit olduğu gösterilmiştir.

Anahtar Kelimeler: Çam balı, DNA izolasyonu, PCR, rbcl geni

1 Introduction

Honey is a natural sweet substance, produced by the bees, when the nectar and sweet secretions from the plants are collected and elaborated in the honey sac [1, 2]. Honeys can either be unifloral or multifloral, depending on whether the honey collected is from the nectar of the same plant or various plants. Non-floral honey is produced by the bees from sweet material of living tissues of plant and/or from the secretions of plant-sucking insects [3]. Honeybees then collect these sweet secretions and convert them into honey. Pine honey is a type of honey which is produced by honey bees from the sugary secretions made by the some insect species, such as *Marchalina hellenica*, living on the pine trees. Pine honey is mostly produced throughout Mediterranean countries such as Turkey and some regions of Greece. The chemical component and biological properties of honey depend on the source of plants and the features of the plants such as color, aroma, flavor, density and physical and chemical properties are reflected in honey [4]. Furthermore, different geographical locations and various sources of plants have an impact on honey quality in

terms of different nutritional and biological properties [5]. Some plants may produce toxic compounds that can be carried in honey [6, 7]. Therefore, identification of the plants contributing to the botanical origin of honey has an economical value and potentially provides health benefits for humans [8]. For the determination of botanical origin of honey, it is possible to utilize some traditional methods such as pollen analysis [9]. However these approaches are very tedious and require skilled labor [10, 11]. Alternatively, using the sequence of barcode genes can help identify the plant species honeys possess [12, 13]. Nowadays, with the development and wide spreading of molecular biology techniques, the studies based on DNA have become fast, reliable and precise [14]. Up to now, all the DNA based studies on honey were carried out using a number of different extraction methods and yet there is no convincingly optimized technique for DNA extraction from honey. Variable results may be obtained from using different techniques. Therefore, the present study aimed to test some of available DNA isolation methods with the goal of finding the most efficient method involving some optimization steps

incorporated into already available DNA isolation methods. For this purpose, we have initially selected three of these approaches namely CTAB, silica dioxide and DNeasy Plant Mini kit isolation methods to isolate genomic DNA (gDNA) from naturally collected pine honey. The performance of each method was tested by DNA quality and amount as well as amplification of *rbcl* gene using standard PCR.

2 Material and Methods

2.1 Sample Preparation

The honey samples utilized in this study were collected from Muğla region in Turkey in 2015. For each honey sample, 50 g of honey was weighed and distributed into four sterile 50 ml centrifugation tubes (12,5 g honey per tube) and heated for 1 hour at 37°C to dissolve the honey. The samples were then centrifuged at 8000xg for 10 min. The supernatants were discarded and the pellets were washed in 20 ml distilled water followed by centrifugation at 8000xg for 5 min and this step was repeated 3 times to wash the pellets. The pellets were then diluted in 5 ml of distilled water and all of the four samples for one honey sample were combined into 1 tube, centrifuged at 8000xg for 5 min and flow-through was discarded. The pellets were immediately used for DNA isolation or kept at -20 °C until further use.

2.2 DNA Extractions

2.2.1 CTAB Extraction Method

For the CTAB method, the prepared pellets were dissolved in 1 ml sterile distilled water and sonicated 4 cycles (20% power) for 20 seconds (Bandelin Sonopuls HD 2200, Germany). The samples were transferred to fresh 2 ml centrifugation tubes with 4 pieces of 0.5 mm glass beads and vortexed harshly for 2 min. The tubes were incubated initially for 15 min at 65°C and for 15 min at -80°C. Then, the glass beads were removed following centrifugation. In the next step, 1 ml CTAB extraction buffer (2% CTAB, 1.4 M NaCl, 0,5 M EDTA, 1 M Tris-HCl and β-mercaptoethanol) were added and the tubes were well-mixed. 5 µl RNase A (10 mg/ml) were added to the mix and incubated at 65°C for 15 min. After the incubation, 30 µl proteinase K (20 mg/ml) was added and the samples were incubated at 65°C for 15 min and centrifuged at 16000 g for 10 min. 700 µl of clear supernatants were transferred to new tubes and 500 µl chloroform-isoamylalcohol (24:1) were added. After mixing, the samples were centrifuged at 16000 g for 15 min and 500 µl of the clear watery phase were transferred into new tube pre-filled with 500 µl pre-cooled propanol. The mixture was incubated for 30 min at room temperature and subsequently centrifuged at 16000 g for 15 min. Supernatants were discharged and the pellets were washed by ethanol (70%), centrifuged at 16000 g for 5 min and supernatant was discarded. The pellets were dried at room temperature and resuspended in 100 µl sterile distilled water. Extracted DNA was then stored at -20°C.

2.2.2 Silica dioxide Method

For the silica dioxide method, the prepared pellets were resuspended in 600 µl lysis buffer (0,1M Tris-HCl pH 8, 1,4 M NaCl, 20 mM EDTA pH 8 and β-mercaptoethanol) and 100 µl urea was added. 5 pieces of glass beads (size 0.5 mm) were added to each tube, vortexed and incubated at 65°C for 15 min. After incubation, the samples were vortexed and incubated at -80°C for 15 min. The beads were removed and 280 µl resuspension buffer (50 mM Tris, 10 mM EDTA, pH 7,6) were added. The samples were incubated on ice for 5 min. After incubation, 280 µl cell lysis buffer (200 mM NaOH and 1 g SDS) and 280 µl neutralization buffer (29,44 g potassium acetate, in final volume of 100 ml dH2O, pH 5.5) were added and were mixed by turning the tube up and down. The samples were

incubated at room temperature for 5 min and centrifuged at 17000xg for 10 min. The clear supernatants were transferred into new 1,5 ml tubes and supplemented with 140 µl P4 solution (2,5% SDS in isopropanol). The samples were incubated at -20°C for 40 min followed by centrifugation step at 17000 g for 10 min. The supernatants were transferred into new tubes and the pellets were resuspended in 50 µl sterile distilled water. 196 µl silica dioxide were added to the supernatants and mixed by turning the tube up and down. The samples were incubated at room temperature for 15 min and then centrifuged at 10000xg for 5 min. The supernatants were discarded and the pellets were washed with 1500 µl ethanol (70%) followed by centrifugation step at 10000 g for 5 min. The last two steps were repeated twice. The DNA pellets were dried at 37°C for 10 min. At the end, the samples were resuspended in 50 µl sterile distilled water and stored at -20°C.

2.2.3 DNeasy Plant Mini Kit

For DNeasy Plant kit (Qiagen, Germany), 400 µl AP1 buffer and 4 µl RNase A (10 mg/ml) were added to the prepared pollen pellets, vortexed and incubated for 10 min at 65°C. The tubes were inverted 2-3 times during incubation. 130 µl AP2 buffer was added, mixed and incubated on ice for 5 min. The lysate was then pipeted into QIAshredder mini spin column and centrifuged for 2 min at 14000 rpm. The flow through was transferred to new tube without disturbing the pellet and 1.5 volumes of buffer AP3/E added and mixed by pipetting. 650 µl of mixture was transferred into DNeasy mini spin column, centrifuged for 1 min at 8000 rpm and flow through was discarded. The spin columns were placed into new tubes. 500 µl buffer AW was added and centrifuged for 1 min at 8000 rpm. The flow through was discarded. After addition of 500 µl AW, centrifugation was carried out for 2 min at 14000 rpm. The spin columns were transferred to new 1,5 ml tube and 100 µl buffer AE was added, incubated for 5 min at room temperature and centrifuged for 1 min at 8000 rpm. The DNAs were stored at -20°C.

2.3 DNA Concentration, Purity and Quality

DNA concentrations and quality were estimated by measuring the absorbance at 260 nm (A260) and 280 nm (A280) using MaestroGen MN-913 spectrophotometer (MaestroGen, Taiwan). Purity was determined by calculating the ratio of absorbance at 260 nm and absorbance at 280 nm (A260/ A280) and the ratio at 260 nm and 230 nm (A260/A230).

2.4 PCR Conditions

The PCR amplifications were carried out in a total of 50 µl volume, containing 3 µl of template DNA, 10X Taq buffer, 2 mM MgCl₂, 10 pmole of each primer *rbcl1f/rbcl724R*, 0,2 mM dNTP mix (Thermo Scientific, Germany) and 5 U of Taq DNA polymerase (Thermo Scientific, Germany). The primers used in this study are listed in Table 2.1.

Table 2.1. The primer sequences used in PCR

Gene	Name	5'-3' sequence	Reference
Ribulose-1,5 biphosphate-carboxylase/oxygenase	<i>rbcl-1f</i>	ATGTCCACCACAA ACAGAAAC	[15]
	<i>rbcl-724R</i>	TCGCATGTACTT GCAGTAGC	

The reactions were performed in Mastercycler gradient (Eppendorf, Germany) using the following program; initial denaturation at 94°C for 3 min, amplification 35 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 2 min, final extension at 72°C for 10 min. The PCR products were analyzed by electrophoresis on a 0,8% agarose gel containing ethidium bromide. The gels were visualized under UV light and the

images were obtained by MiniBIS pro (DNR Bioimaging systems, Israel)

3 Results

3.1 DNA Extractions

In this study, three different extraction methods were applied to the selected honey samples. Corn seeds were used as positive control for each method. The quality of all the samples was analyzed by gel electrophoresis and absorbance measurements with a UV spectrophotometer. The results of gel analysis indicate that visible DNA was obtained only for positive controls (lanes 3 and 6, Figure 3.1). gDNA from honey samples were not apparent on the gels. In addition, PCR analysis was conducted later to test indirectly the presence of honey gDNA.

3.2 DNA Concentration, Purity and Quality

The efficiency of the extraction methods was also determined by the amount and quality of the DNA extracted from honey samples using a spectrophotometer. The values of A260/A230 and A260/A280 are given in Table 2. According to the results, DNA isolation from corn seeds seems successful both in terms of quantity and quality. Silica method did not produce satisfactory results. For honey samples, however, DNA is not in a pure quality as suggested by the ratios. Absorbance measurements for DNA concentration and quality are apparently not an ideal and suitable way for honey DNA. It is possible that the contamination or carryovers in honey such as sugary substances and the fact that there is a very limited amount of DNA that can be isolated from honey samples most likely limit the efficiency of this technique. Therefore, other approaches determining the amount and quality control of isolated honey DNA should be incorporated in order to reach relatively more accurate and conclusive results. One of these methods is PCR, where amplification of a plant gene is used as we have preferred in this study.

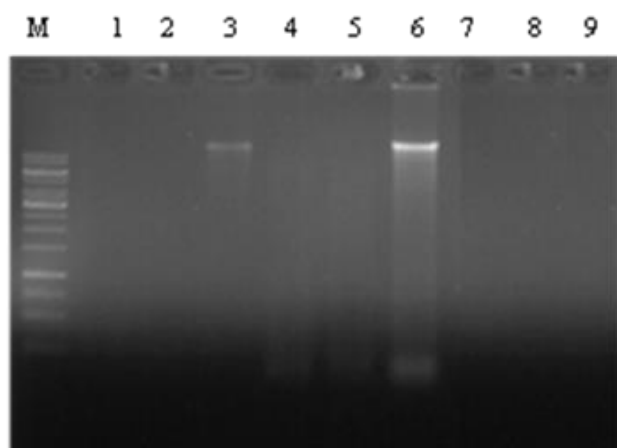


Figure .1. Result from agarose gel electrophoresis analysis of DNA extracted from honey samples. Lanes; M, marker (Gene Ruler 1 kb DNA ladder, Thermo Scientific, Germany), gDNA of honey samples 1 (lane 1), 2 (lane 2) and corn DNA as positive control (lane 3) isolated using DNeasy Plant Mini Kit; CTAB method (lanes 4, 5, 6) and manual silica dioxide method (lanes 7, 8, 9).

Table 3.2. Concentration and purity of DNA preparations

Sample	Ext. Method	Conc. ng.µl ⁻¹	A260/A280	A260/230
Sample 1	Qiagen Kit	8,54	1,472	0,585
Sample 2	Qiagen Kit	15,94	1,424	0,836
Corn	Qiagen Kit	24,44	1,718	1,305
Sample 1	CTAB	97,61	1,162	0,963
Sample 2	CTAB	60,17	1,310	1,105
Corn	CTAB	548,44	1,801	1,703
Sample 1	Silica dioxide	93,8	1,056	0,955
Sample 2	Silica dioxide	37,24	1,179	0,844
Corn	Silica dioxide	59,92	1,236	0,892

3.3 PCR Amplification

Absence of visible proof of DNA presence on agarose gels does not actually mean proof of absence. If the amount of isolated DNA is below the minimum amount that can be detected by agarose gel analysis and UV-EtBr staining, then no DNAs can be seen on the gels. However, sometimes there is sufficient DNA present in the tubes that can be used to amplify specific gene products. Therefore, in this study, we have used the capacity of amplification of a plant gene namely *rbcl* as a way to show isolated DNA from honey samples.

The results indicate that DNA isolation using DNeasy plant kit produced *rbcl* PCR bands for both of the honey samples (Figure 2; lanes 1 and 4). Although the intensity of PCR bands was less than the band produced using the positive control corn DNA (Figure 2; lane 7), the amount obtained by the methods is sufficient and has good quality to conduct PCR and use in sub-cloning studies. The other methods used in this study did not result in successful PCR amplification under tested conditions (Figure 2; Lanes 2 and 5 for the silica method, Lanes 3 and 6 for the CTAB method).

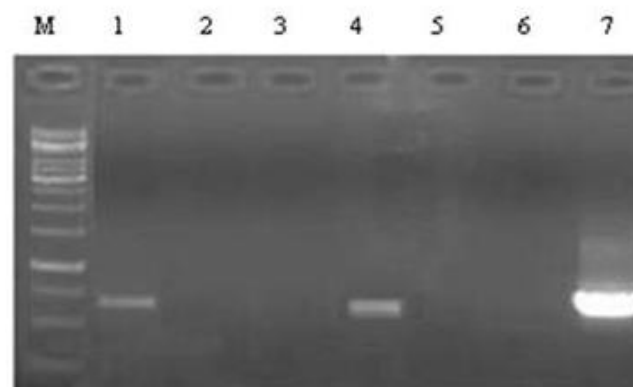


Figure 2. Results of PCR amplification. PCR products obtained with primers *rbcl1f/rbcl724R* are present in Lanes 1, 4 and 7.

Lanes; M, marker (GenRuler 1 kb DNA ladder, Thermo Scientific, Germany), The template DNA was obtained from the isolation using DNeasy plant kit for honey samples 1 and two (Lanes 1 and 4), and the silica method (Lanes 2 and 5) and the CTAB method (Lanes 3 and 6). Corn DNA was used as the positive control (Lane 7).

4 Discussion

Analysis of honey DNA has increasingly become important due to several reasons. The physical and biological properties of honey are in part dependent upon the environment where it is obtained. DNA extracted from honey is used to determine the botanical origin of honey, its GMO status, microorganism

content and provide indirect information about some allergic compounds. The most widely used method of DNA isolation from the plants in the literature is CTAB based isolation [16-18], however this method is relatively labor intensive and time-consuming. In addition to CTAB, commercial kits are modified and utilized in order to isolate honey DNA. We have used three different isolation methods in this study for the honey samples collected from Muğla. According to the results, we suggest the use of DNeasy plant kit compared to the other two methods. Although we have not detected DNA by the two methods and agarose gel analysis of genomic DNA in honey and UV measurements did not provide reliable evidence regarding the presence of DNA, PCR amplification of *rbcl* gene was satisfactory. Furthermore, as an alternative or in addition to DNeasy plant kit, we also suggest that other means of DNA isolation methods should be investigated and tested in the future studies such as magnetic beads- based DNA isolation.

5 Acknowledgment

This study was financially supported by Muğla Sıtkı Koçman University Scientific Research Projects Unit (BAP project No: 15/128).

6 References

- [1] Al-jabri AA, "Honey, milk and antibiotics", African Journey of Biotechnology, 4: 1580-1587. 2005.
- [2] Barth, M. O. "Melissopalynology in Brazil: a review of pollen analysis of honeys, propolis and pollen loads of bees", Scientia Agricola, v. 61, n. 3, p. 342-350, 2004.
- [3] Casiraghi, M., Bruni, I., De Mattia, F., Galimberti, A., Galasso, G., Banfi, E., & Labra, M., "Identification of poisonous plants by DNA barcoding approach", International Journal of Legal Medicine, 124(6), 595-603. 2010.
- [4] Etzold E, Lichtenberg-Kraag B, "Determination of the botanical origin of honey by Fourier-Transformed Infrared Spectroscopy: An approach for routine analysis", Eur Food Res Technol; 227 (2): 579-586. 2007.
- [5] Fay, M. F., Bayer, C., Alverson, W. S., de Bruijin, A. Y., & Chase, M. W. "Plastid *rbcl* sequence data indicate a close affinity between *Diegodendron* and *Bixa*", Taxon, 47: 43-50, 1998.
- [6] Fleischmann, A.; Heubl, G. "Overcoming DNA extraction problems from carnivorous plants", Anales del Jardín Botánico de Madrid, v. 66, n. 2, p. 209-215, <http://dx.doi.org/10.3989/ajbm.2198>, 2009.
- [7] Galimberti, A., Bruni, I., De Mattia, F., Galasso, G., Banfi, E., Casiraghi, M., & Labra, M. 2014, "A DNA barcoding approach for identify plant species in multiflower honey", Food Chemistry 170: 308-315, 2015.
- [8] Jain, S.A. et. al. "Extraction of DNA from honey and its amplification by PCR for botanical identification", Food Sci. Technol, Campinas, 33(4): 753-756, 2013.
- [9] Koca, I.; Koca, A 'F. "Poisoning by mad honey", Food and Chemical Toxicology, v. 45, p. 1315-1318, 2007.
- [10] Liberato, M. C. T. C. Moaris, M. S. Magalhaes, C. E. C. Magalhaes, I. H. Cavalcanti, D. B. Silva, M. M. O. "Physicochemical properties and mineral and protein content of honey samples from Ceará state", Northeastern Brazil, Food Science and Technology, v. 33, n. 1, p. 38-46, 2013.
- [11] Marguitas L, Stanciu O, Dezmirean D, Bobis O, Popescu O, Bogdanov S. and Campos M. "In Vitro antioxidante capacity of honeybee-collected pollen of selected floral origin harvested from Romania". Food Chemistry 115: 878-883, 2009.
- [12] Moyo, M. Baiuru, M. W. Amoo, S. O. Van Staden, J. "Optimising DNA isolation for medicinal plants", South African Journal of Botany, v. 74, n. 4, p. 771-775.
- [13] Namias N 2003, "Honey in the management of infections. Surg. Infect", 4: 219-226, 2008.
- [14] Ramirez R, Montenegro YG "Certificación Del Origen botánico y polen corbicular perteneciente a la comuna de Litueche", VI Región de Chile', Cien. Inv. Agric., 31: 197-211, 2004.
- [15] Sharma, P.; Purohit, S. D "An improved method of DNA isolation from polysaccharide rich leaves of *Boswellia serrata* Roxb", Indian Journal of Biotechnology, v. 11, p. 67-71. 2012.
- [16] Stanimirova, I. Üstün, B. Caijka, C. Riddelova, K Hajslova, J. Buydens, L. M. C. Walczak, B. "Tracing the geographical origin of honeys based on volatile compounds profiles assessment using pattern recognition techniques", Food Chemistry, v. 118, n. 1, p. 171- 176, <http://dx.doi.org/10.1016/j.foodchem.2009.04.079>, 2010.
- [17] Subrahmanyam M "Topical application of honey for burn wound treatment-an overview", Ann. Burns Fire Disasters. 20: 3. 2007.
- [18] Wang, J.; Li, Q. "Chemical Composition, Characterization, and Differentiation of Honey Botanical and Geographical Origins', Advances in Food and Nutrition Research", v. 62, p. 89-137, 2011.