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ENHANCED DNA EXTRACTION FROM TRIGONA HONEY: A LOW-VOLUME OF SAMPLE, HIGH-PURITY APPROACH FOR MOLECULAR RESEARCH

***Endang Sulistyarini Gultom*[1], *Rini Hafzari*[2], *Melva Silitonga*[3], *Eva Sartika Dasopang*[4]**

[1]ORCID: 0000-0003-0378-6558
[2]ORCID: 0000-0001-9809-6482
[3]ORCID: 0000-0001-9888-5596
[4]ORCID: 0000-0003-4634-4717
[1–3]Department of Biology, Faculty of Mathematics and Natural Science
State University of Medan, North Sumatra, Indonesia
[4]Program Study Pharmacist
Tjut Nyak Dhien University, Medan Helvetia, Medan, North Sumatra, Indonesia

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Abstract

Bees synthesize honey from floral nectar, with pollen inadvertently incorporated during the foraging process. Pollen constitutes the primary source of plant DNA in honey; however, its extraction is impeded by the high concentrations of sugars, phenolic compounds, and carbohydrates, often resulting in low purity and necessitating substantial sample volumes. This study presents a modified DNA extraction technique specifically optimized for Trigona honey, aimed at enhancing both efficiency and practicality. The protocol involves a pretreatment step where honey is diluted in a 1:4 ratio with distilled water, incubated at 60 °C for 25 minutes, followed by extraction without sample destruction. The quality of the extracted DNA was assessed using a nano spectrophotometer and PCR, demonstrating a high concentration of 1,790 ng/μL with distinct, smear-free bands. This method is straightforward, time-efficient, and resource-conserving, rendering it highly applicable for molecular research and honey authentication.

Introduction

Honey, a natural sweet substance with nutritional and therapeutic properties, has played a central role in traditional medicine for several centuries. It has long been used to treat burns, respiratory diseases, digestive infections, and wounds, thus underscoring its bioactive potential (NORDIN et al. 2018). In addition to its cultural and medicinal importance,

Address: Rini Hafzari, State University of Medan, Jl. William Iskandar Ps. V, Deli Serdang 20221, North Sumatra, Indonesia, phone: +62616613365, e-mail: rinihafzari@unimed.ac.id

honey is a complex biological matrix comprising of carbohydrates, amino acids, minerals, enzymes, vitamins, phenolic compounds, and water. This biochemical richness contributes to both its health-promoting qualities and its significance as a product of its ecological and agricultural value.

While Apis mellifera accounts for the majority of commercial honey, stingless bees (*Trigona* spp.) produce a distinct type of honey known as meliponine honey, pot honey, sugar bag honey, or kelulut honey in Malaysia. Stingless bee honey has been reported to have higher antioxidant activity, approximately 45% greater than that of A. mellifera honey, along with antidiabetic, antimicrobial, and immunomodulatory benefits (AZIZ et al. 2017, KRISHNASREE and UKKURU 2016, ÁVILA et al. 2019). Its unique flavor, medicinal attributes, and cultural heritage emphasize the growing need for the robust authentication and molecular characterization of stingless bee honey.

The quality and identity of honey are primarily determined by the floral sources visited by bees. Honey can be categorized as monofloral or multifloral depending on its botanical origin. Monofloral honeys are derived predominantly from a single plant species and are valued for their distinctive aroma, taste, and biological properties, whereas multifloral honeys are produced from diverse floral sources (SCHIEVANO et al. 2016). The composition of floral sources directly influences the phenolic and flavonoid content, shaping both the nutritional and therapeutic properties of honey. For example, antibacterial honey is often derived from *Corymbia calophylla, Eucalyptus marginata,* and *Leptospermum polygalifolium* (IRISH et al. 2011). Geographic location, seasonal variation, and nectar sources further contribute to differences in honey phytochemistry (VALDÉS-SILVERIO et al. 2018, ZAWAWI et al. 2021, LARSEN and AHMED 2022).

Accurate identification of floral origins is therefore essential not only to ensure quality and prevent adulteration, but also to explore honey as a natural archive of environmental DNA (eDNA). DNA-based molecular techniques have become preferred tools for species identification because of their speed, accuracy, and reproducibility (GULTOM et al. 2023; GULTOM et al. 2025, HAFZARI et al. 2024). Studies such as those by WIRTA et al. (2021) and SOARES et al. (2017) have demonstrated the ability of DNA metabarcoding to identify pollen sources down to the species level, reconstruct floral diversity, and detect plant taxa such as *Calluna vulgaris, Lavandula* spp., and *Eucalyptus* spp. from honey. Recent advances have highlighted honey-derived DNA as a promising source for environmental monitoring and plant-pollinator interaction studies in different ecosystems (ULLAH et al. 2024).

Despite its promise, DNA extraction from honey remains technically challenging. High concentrations of sugars, phenolics, and polysaccharides

frequently inhibit enzymatic reactions and reduce the DNA yield and quality (RIBANI et al. 2022, UTZERI et al. 2018). Established protocols often require large sample volumes (up to 50 mL), specialized glass-bead grinding equipment, and prolonged pretreatment steps, limiting their practicality for large-scale molecular studies (RIBANI et al. 2022). Previous studies WITA et al. (2021) emphasized the necessity of optimizing extraction protocols; however, a gap remains in developing a method that is simple, cost-effective, and efficient, while still producing high-quality DNA from small honey volumes.

This study aimed to address this gap by presenting a modified DNA extraction protocol specifically optimized for stingless bee honey (*Trigona* spp.). Key innovations include reducing the required sample volume, shortening the incubation duration, and optimizing lysis and washing steps. By improving the efficiency without compromising DNA integrity, this method seeks to provide a practical tool for molecular studies, honey authentication, and broader applications in biodiversity monitoring and food traceability.

Materials and Methods

Collection of honey *Trigona* sp. sample

The honey samples used in this study were Trigona and multiflora honey types obtained from forests in Riau.

DNA extraction: improve method

The DNA extraction procedure has been modified, as LOWE et al. (2022) referenced. DNA extraction from honey samples involves several stages: pretreatment of the honey sample, incubation, centrifugation to isolate fractions for sample pellets, DNA extraction utilizing a modified commercial kit, and assessment of DNA quality through electrophoresis and a nano spectrophotometer. The pretreatment of honey samples involves diluting the sample with sterile distilled water. The dilution ratio of honey to distilled water is 1:4 (1 ml of honey to 4 ml of distilled water). Honey diluted with distilled water is incubated at 60 °C for 25 minutes. The subsequent phase involves partitioning the incubated honey sample into four tubes (1,250 µl each), followed by centrifugation at 10,000 rpm for five minutes. The supernatant is discarded, and the particle is retained. The pellet is diluted by adding 25 µl of sterile distilled water. Subsequently, vortex momentarily at moderate velocity. Subsequently, centrifuge and amalgamate

the pellets from the four diluted tubes into a single tube. Moreover, the pellets were directly extracted utilizing the Dneasy Plant Mini kit (QIAGEN), omitting the need for glass beads for sample disruption.

The diluted pellets combined in one tube were supplemented with 400 µl of AP1 buffer and 5 µl of RNase A, then vortexed and incubated for 10 minutes at 65 °C. Introduce 130 µl of P3 buffer. Incubate at -20 °C for 3 minutes. Centrifuge for 7 minutes at 13,500 revolutions per minute. Transfer the supernatant to the QIA shredder spin column and centrifuge at 13,500 rpm for 4 minutes. Transfer the supernatant to a fresh tube, then incorporate 1.5 ml of AW1 buffer. Transfer 650 µl of the mixture to a DNeasy Mini spin column and centrifuge for 1 minute at 8000 rpm. Insert the spin column into a fresh collecting tube. Add 500 µl of Buffer AW2 and centrifuge for 1 minute at 8000 rpm. Eliminate the supernatant. Introduce 500 µl of Buffer AW2 and centrifuge at 13,500 rpm for 3 minutes. Relocate the spin column to a fresh tube; add 50 µl of Buffer AE, incubate for 5 minutes at ambient temperature, and centrifuge at 8000 rpm for 1 minute. The concentration of DNA was quantified with a nano spectrophotometer and PCR amplification.

Evaluation of Polymerase Chain Reaction (PCR) amplification

DNA was amplified using universal primers for the target genes *ITS2* and *rbcl*. The primers used refer to (URUMARUDAPPA et al. 2020). The primer sequences used can be seen in Table 1. PCR was performed with a final reaction volume of 25 µl. A total of 2.5 µl of DNA template was mixed with 12.5 µl of HotStart Taq Polymerase master mix (QIAGEN), 0.5 µl of each primer (5 µM), 1.0 µl of BSA (10 µM) and eight µl of nuclease-free water. The PCR cycle was carried out by following the program as follows: predenaturation at 95 °C for 5 minutes, followed by denaturation at 95 °C for 40 seconds, annealing at 48 °C for 60 seconds, extension at 72 °C for 30 seconds for 35 cycles, and a final extension at 72 °C for 5 minutes and 30. Visualization of PCR results was carried out using electrophoresis. This process was carried out using 1.5% agarose gel in TAE buffer 1X at 70 volts for 70 minutes. After completion, the gel was stained with Gel Red and visualized under

Table 1

List of primer sequences to be used for amplification and metabarcoding

Target gene	Name of primer	Primer sequence 5'-3'
ITS2	SF	5'-ATGCGATACTTGGTGTGAAT-3'
	SR	5'-GACGCTTCTCCAGACTACAAT-3
rbcl	RBF	5'-ATGTCACCACAAACAGAAAC-3'
	RBV	5'-TCGCATGTACCTGCAGTAGC-3'

a UV transilluminator to see the amplified DNA bands. The Thermo Scientific GeneRuler 100 bp DNA Ladder was used to determine the size of the DNA bands. The electrophoresis results were then photographed using gel documentation (BALKANSKA et al. 2020).

Results

DNA concentration and purity

The extraction of genomic DNA is a crucial step in molecular analysis, particularly for species identification and phylogenetic studies. The findings of this study demonstrate that DNA extraction from honey samples can be effectively performed using a modified protocol with reduced sample volume and simplified processing. The DNA concentration varied among the tested honey samples, with the highest concentration obtained from multiflora honey (1,790 ng/µl), followed by Trigona honey with red coloration (965 ng/µl) and Trigona honey with yellow coloration (580 ng/µl). The DNA purity, measured by the A260/A280 ratio, ranged from 1.43 to 1.57, which is slightly below the optimal range of 1.8–2.0 required for high-purity DNA (LUCENA-AGUILAR et al. 2016).

Despite achieving high DNA concentrations, the suboptimal purity suggests the presence of residual contaminants such as proteins, phenols, or carbohydrates, which may interfere with downstream molecular applications. Previous studies have reported that insufficient washing steps during DNA precipitation could contribute to lower purity values (BABADI et al. 2022). Increasing the number of washing steps or incorporating additional purification techniques may enhance DNA purity. However, the high DNA yield obtained in this study highlights the effectiveness of the modified protocol in extracting sufficient DNA for molecular applications, especially when sample availability is limited. Elevating the temperature during sample pre-treatment is recognized to enhance the efficacy of cell lysis (MENCHHOFF et al. 2020)

Polymerase Chain Reaction (PCR) amplification

To further validate the applicability of the extracted DNA, PCR amplification was performed using *ITS2* and *rbcl* gene-specific primers. The PCR results demonstrated clear and well-defined DNA bands with no visible smearing, confirming the suitability of the extracted DNA for molecular analysis (Figure 1). The absence of smear indicates that

the extracted DNA was of adequate quality for enzymatic reactions, despite its relatively low purity. This finding is consistent with previous research suggesting that PCR performance can remain unaffected when DNA purity is slightly below the ideal range, provided that contamination levels do not inhibit polymerase activity (RODRÍGUEZ-RIVEIRO et al. 2022).

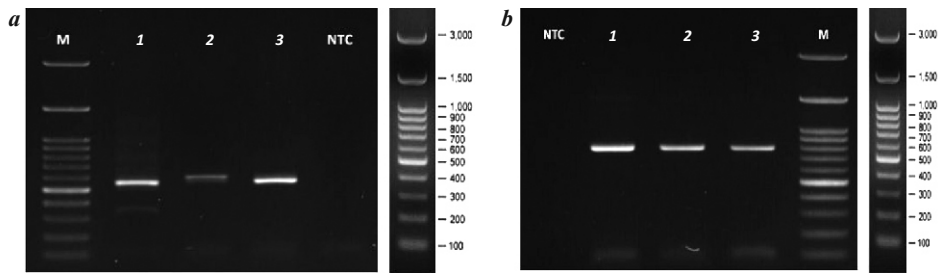

Fig. 1. DNA amplification results using a template from a modified extraction method
Explanation: *a* – *ITS2* gene amplification; *b* – *rbcl* gene amplification; *1* – yellow trigona honey; *2* – red trigona honey; *3* – multiflora honey

Several factors likely contributed to the successful PCR amplification observed in this study. First, the low level of contaminants allowed the Taq polymerase enzyme to function efficiently. Second, the high DNA concentration compensated for minor purity deficiencies, ensuring sufficient template availability for amplification. Third, the inclusion of PCR additives such as bovine serum albumin (BSA) and dimethyl sulfoxide (DMSO) may have mitigated the effects of potential inhibitors, enhancing amplification efficiency (SUTANTA et al. 2022).

Discussion

The results of this study demonstrate that the modified DNA extraction method provides an efficient and reliable approach for extracting DNA from honey samples, particularly Trigona and multiflora honey. This method successfully reduces the sample volume requirement, processing time, and complexity, making it more accessible for laboratories with limited resources. The ability to extract DNA without mechanical disruption (e.g., glass beads grinding) enhances its practicality for routine analysis. However, while the DNA concentration obtained was relatively high (up to 1,790 ng/µl), the purity values (1.43–1.57) were slightly below the ideal range of 1.8–2.0 (LUCENA-AGUILAR et al. 2016), suggesting the presence of residual contaminants such as proteins, phenols, or polysaccharides that could interfere with downstream applications.

A central focus of this study is the impact of pre-treatment conditions on the efficiency of DNA extraction. The elevated temperature during the incubation phase (60 °C for 25 minutes) likely enhanced cell lysis and DNA release, as corroborated by previous research (MENCHHOFF et al. 2020). Nonetheless, it is plausible that higher temperatures also facilitated the co-extraction of undesirable compounds, thereby reducing DNA purity (BABADI et al. 2022). To mitigate this issue, additional modifications such as enzymatic digestion (e.g., proteinase K treatment) or supplementary ethanol precipitation steps could further enhance purity (RODRÍGUEZ-RIVEIRO et al. 2022).

Despite the slightly lower purity values, PCR amplification of *ITS2* and *rbcl* genes was successfully achieved, indicating that the extracted DNA was of sufficient quality for molecular analysis. The clear, well-defined bands in gel electrophoresis suggest that Taq polymerase activity was not significantly inhibited, even though impurities were present. This is in agreement with previous research showing that DNA purity is not always the limiting factor for PCR success, provided that sufficient DNA concentration and optimized reaction conditions are maintained (SOARES et al. 2017). In this study, the inclusion of PCR additives such as BSA and DMSO may have contributed to overcoming the effects of minor inhibitors, enhancing amplification efficiency (SUTANTA et al. 2022).

Comparisons with existing DNA extraction methods

Traditional honey DNA extraction methods, such as those reported by LALHMANGAIHI et al. (2014), typically require large sample volumes (≥50 ml) and long incubation times (≥1 hour), making them labor-intensive and costly. The method developed in this study significantly improves upon these approaches by:

– reducing sample volume (1 ml honey per reaction instead of ≥50 ml);

– shortening the incubation time (25 minutes instead of ≥1 hour);

– eliminating the need for glass beads or mechanical disruption, making it more practical for routine use;

– using a modified DNeasy Plant Mini Kit approach, optimizing buffer composition to enhance DNA recovery.

Compared to DNA-based authentication techniques used in honey botanical origin studies (WIRTA et al. 2021), this study demonstrates that high DNA concentrations can be obtained even from small sample sizes, making it suitable for large-scale honey authentication, pollination source identification, and biodiversity assessment.

Implications for honey authentication and molecular research

The ability to efficiently extract DNA from honey has important applications in food safety, authenticity testing, and conservation biology. DNA metabarcoding techniques, which rely on high-quality DNA extraction, are increasingly used to detect honey adulteration, verify botanical origin, and monitor ecosystem biodiversity (URUMARUDAPPA et al. 2020). The method developed in this study provides a practical solution for researchers and industry stakeholders seeking to implement genetic traceability methods for honey products, particularly in regions where fraudulent labeling is a concern.

Furthermore, the results of this study underscore the potential of honey-derived DNA as a valuable resource for reconstructing plant biodiversity. Previous research has demonstrated that pollen DNA extracted from honey can effectively reflect floral diversity across different geographical regions (SOARES et al. 2017). More recently, a study on Southeast Asian honey from Apis cerana and Heterotrigona itama in Karangasem, Indonesia, employed pollen DNA metabarcoding (ITS2 amplicon sequencing) to trace the botanical and geographical origins of honey, identifying dominant plant taxa such as Schleichera and Syzygium (ULLAH et al. 2024). These findings suggest that the method described herein can be further adapted for environmental DNA (eDNA) analyses, enabling researchers to investigate pollinator–plant interactions and monitor ecosystem dynamics using honey samples.

Conclusions

This study introduces an innovative method for extracting DNA from Trigona honey. This approach requires a smaller sample size, is time-efficient, and does not necessitate specialized equipment. Although the purity of the extracted DNA is relatively low (1.43–1.57), the yield is substantial (up to 1,790 ng/μL), and it performs effectively in PCR assays, yielding clear results. This demonstrates the method's reliability for subsequent molecular investigations.

The method's simplicity and cost-effectiveness make it accessible to laboratories with limited resources, facilitating large-scale honey studies. It addresses a significant challenge in honey research by simplifying the extraction of DNA from complex samples. This advancement can aid in assessing honey quality, tracing its geographical origin, and exploring biodiversity through environmental DNA.

Future research should aim to enhance DNA purity, evaluate the method across various honey types, and integrate it with advanced sequencing

technologies. These enhancements will strengthen the utility of honey DNA in food safety, ecosystem studies, and environmental monitoring.

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