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Authentication of *Apis Cerana and Apis Mellifera* Derived Honey Using Major Royal Jelly Protein 2 Gene and Spectroscopy

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ABSTRACT

Honey, as a natural health product, encounter great compositional influence by its entomological, botanical and geographical origin. In Bangladesh, apiculture relies on the honey production by mostly the European honeybee, *Apis mellifera*. However, the Asian honeybee *Apis cerana* honey typically costs three times as much as honey from *A. mellifera* in honey market due to the production limit, variation in nutrient composition, being prone to adulteration by mislabeling or incorporation of two honey. *A. mellifera* honey may be mislabeled as *A. cerana* honey or mixed with *A. cerana* honey by dishonest businesses and beekeepers who are motivated by substantial earnings. The current study used the MRJP2 (Major Royal Jelly Protein 2) gene and spectroscopic analysis to differentiate honey from *A. mellifera* and *A. cerana*. Primers for two different species were designed. *A. cerana* and *A. mellifera* exhibited amplification products of 212 and 560 bp, respectively. Duplex PCR was able to identify the presence of as little as 1% *A. mellifera* honey in the mixture. *A. mellifera* and *A. cerana* originated honey was differentiated using MRJP2 gene and UV-VIS spectroscopy. The resulted banding pattern can be used to identify adulterated honey. UV-VIS spectroscopy has been used to determine the concentration of DNA at different wavelength for standardizing the corresponding honey types. Amplifications MRJP2 gene fragments in gel electrophoresis and responsiveness in screening for two honey types with their probable admixtures provided an effective tool for discriminating the honey types. DNA Spectrophotometric Spectrum analysis in different wavelengths and ratios also provided a clear-cut discrimination protocol of these two honey types. The findings of these two methods can be used efficiently apply towards authentication of the honey originated by *A. mellifera* and A. *cerana* in the honey market of Bangladesh.

Keywords: honey authentication, honeybee, mrjp2 gene, social insects.

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I. Introduction

Honey is a popular health product, consumed widely around the world, but it is also most prevalently fabricated among everyday healthcare items [1]. Honey's composition and pricing are strongly influenced by its botanical, geographical, and entomological origins. Honey, sugar, and syrups being counterfeited, as well as labeling error in the flowery source, species of the bees, are all common honey scams nowadays [2], [3]. The western honeybee, *Apis mellifera*, and the eastern honeybee also known as *Apis cerana*, are the two most common honeybee species [4]. In Bangladesh, *A. mellifera* is the common honeybee that the beekeepers use. However, some aesthetically pleasing beekeepers are strengthening their beekeeping skills with the *A. cerana* honeybee. Honey from *A. mellifera* and *A. cerana* are popular in Bangladesh's honey market. The honey derived from *A. dorsata* has also become popular, as about 75 percent of natural honey is produced in the Sundarban forest by this giant bee *A. dorsata* [5]. Recently, honey's entomological origin has drawn a lot of interest in honey quality due to the rising desire for local and traditional goods. Due to low production and regional customer preferences, *A. cerana* derived honey is typically 3–5 times more expensive than *A. mellifera* honey in Asian markets. Because of this, some dishonest beekeepers and honey marketers may combine *A. cerana* derived honey with *A. mellifera* honey or mislabel *A. mellifera* honey as *A. cerana* honey [4], [6].

Honey derived from *A. cerana* has a lot greater nutritious content than honey made from *A. mellifera*; thus, the honey from *A. cerana* is more expensive. As a result, the beekeepers mislabel *A. cerana* derived honey instead of *A. mellifera* or mix *A. cerana* honey with *A. mellifera* honey as a way of adultery. Due to the limited availability of investigations comparing the two types of honey, in order to safeguard the essential interests of consumers and true *A. cerana* beekeepers, it is crucial and necessary to develop analytical methods that can identify *A. cerana* honey from *A. mellifera* honey. Since honey's economic and nutritional benefits are mostly determined by considering its botanical and entomological origins, the majority of works published thus far have been concentrating on those issues [4], [7]. However, as referred, the entomological origin can indirectly provide information regarding the geographical origin of honey. Despite this, very few studies have attempted to identify honey from entomological perspective, either based on protein [8] or DNA analysis [9]–[11].

To identify *A. cerana* honey adulteration with *A. mellifera* honey, various molecular approaches emphasizing protein and DNA in the honey samples have been applied [6]. However, DNA-based methods have been recognized as the most suitable tools for unequivocal species identification in animal products and processed foodstuffs [12]. DNA-based techniques provide a number of benefits over protein-based methods, including nucleic acids' higher stability and their ubiquitous presence in all cell types. Recently, Prosser and Hebert [10] proposed utilizing metabarcoding to analyze three gene regions to determine the botanical and entomological origins of honey. Based on mitochondrial DNA (mtDNA) sequences and phylogenetic analysis, bee species DNA on honey samples has been identified utilizing the forensically informative nucleotide sequencing [9]. The extensive sequencing analysis used in both studies, meanwhile, is not high throughput or cost-effective. Contrarily, the use of polymerase chain reaction (PCR) with species-specific primers is one of the most popular methods for authentication because of its affordability, ease of use, fastness, high specificity, and sensitivity, which allows for the identification of species even in complex and processed foods [13], [12]. Since real-time PCR with high resolution melting (HRM) analysis does not require subsequent sequencing analysis, it can be a more affordable and reliable approach to exploit DNA barcoding [14]. The research was conducted to develop an authenticated discrimination tool of *A. mellifera* and *A. cerana* originated honey using the Major Royal Jelly Protein 2 gene (MRJP2) and to propose a simple and cost-effective analytical method for detecting the adulteration of *A. mellifera* and *A. cerana* honey through ultraviolet (UV) spectroscopy.

II. Materials and Methods

A. Sampling of Honey

Samples of honey from *A. mellifera* and *A. cerana* were obtained from the apiary located in six districts of Bangladesh (Table I). The hives with identified bee species have been kept under supervision of the contact beekeepers and after that Honey specimens from *A. cerana* and *A. mellifera* were gathered and harvest based on the available pollen and nectar sources in different time of the year.

TABLE I: Sampling Information of A Mellifera and A Cerana Honey from Bangladesh

Locality	Type	District	Geographical position		Date of collection
			N	E	
L01	*A. mellifera*	Gazipur	24.000678°	90.425425°	06/08/21
L02	*A. mellifera*	Tangail	24.229360°	90.636213°	14/08/21
L03	*A. mellifera*	Satkhira	22.737114°	89.330343°	21/09/21
L04	*A. cerana*	Gazipur	24.000678°	90.425425°	07/10/21
L05	*A. cerana*	Tangail	24.229360°	90.636213°	18/11/21
L06	*A. cerana*	Jessore	22.905471°	89.219396°	05/01/22

B. Preservation of Honey

Honey samples were placed in glass vials and kept at room temperature until DNA extraction. The findings of the samples produced from Bangladesh for verification was compared to the genetic information from NCBI GenBank of these two species.

C. Molecular experiments:

1) Extraction of DNA

Genomic DNA was extracted from preserved specimens by *using* QIAGEN DNeasy Blood and Tissue kit, following the manufacturer's instructions. In accordance with the established techniques, DNA was extracted from samples of honey with some modifications [15] where 50 mL of ultrapure water was used to dilute a total of 15 g of each honey samples and homogenized and centrifuged and being incubated at 45°C, [11]. Samples were vortexed after 180 ml of Buffer ATL and 20 ml of proteinase K were added. 48 hours at 55°C were spent incubating the sample. Following the manufacturer's instructions, the maker added two wash buffers (AW1 and AW2), Buffer AE, lysis buffer AL, and elusion buffer AE to finish the DNA extraction. The centrifugation process was finished entirely at room temperature. Following DNA extraction, the samples utilized for DNA analysis were kept at the Entomology lab at BSMRAU.

2) PCR primers preparation and selection

The MRJP2 gene was chosen as a specific location. The MRJP2 gene sequence of *A. cerana* (AY392758.1) and *A. mellifera* (406091) was provided by NCBI. Primer Premier 5.0 was used to create the primers (Premier Biosoft International, USA). The Primer-BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast) was used to determine the specificity of primers.

1) Polymerase chain reaction (PCR)

Amplification of DNA was done by polymerase chain reaction (PCR) TaKaRa Ex Taq PCR kit, according to the manufacturer's instructions. The primer selection was implemented in accordance with Table II. The molecular analyses were conducted in the Advanced Entomology laboratory of Bangabandhu Sheikh Mujibur Rahman Agricultural University.

TABLE II: Primer Information with Size

Honey	Primer	DNA Sequences	Product Size (bp)
A. cerana	C-F C-R	TTTAACAATAAAAATAA TCAGAAGA TTACATCCTAATTGATTT TAATGCG	212
A. mellifera	M-F M-R	GCCATCCCTTGAAATTG TCACTCGT TCTGCAAACGACCAATC AGGATAT	560

Thermal cycle was performed, consisting of 35 cycles of initial denaturation at 95 C for 5 min, dissociation at 92 C for 1 min, annealing at 54 C for 1 min, and extension at 70 C for 2 min. Illustra and ExoProStar were used for catalytic PCR and sequenced clean-up in accordance with the manufacturer's instructions, GE Healthcare. Exonuclease (1 reaction µl−1, Solution in 20 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 1 mM DTT, 50% (v/v) glycerol) and Alkaline phosphate (1 reaction µl−1, Solution in 20 Mm HEPES-NaOH (pH 7.4), 1 mM MgCl2, 0.1 mM ZnCl2, 0.1% (v/v) Triton X-100, 50% (v/v) glycerol were added to the PCR product and incubate 37◦C for 15 min and 80◦C for 15 min to inactivate the enzymes. For cycle sequencing, ABI PRISM Big Dye Terminator v3.1 cycle sequencing kits from Applied Biosystems were used in an automated sequencer. Sequencing reactions were performed using ABI 3100 Avant (Applied biosystem). For conducting the PCR, a PCR machine (GTQ-Cycler 96) was used from the Department of Horticulture of BSMRAU.

2) Gel electrophoresis

After the PCR, the results were verified using gel electrophoresis. Gel was made by boiling 100 ml of 1XTAE buffer, 1.0 g of agarose, and 10 l of ethanol bromide for 1.30 minutes at 800 watts. The PCR product was then placed onto the gel of the gel electrophoresis plate for 20 minutes after being combined with 6X coding buffer to compare the results with the standard product that contained a 100 bp DNA ladder.

3) Spectrophotometry

DNA quantification is usually done by measuring the absorbance at 260 nm on a sample of DNA in solution with a UV spectrophotometer. The amount of light that passes through the solution is calculated. Spectrophotometric study of DNA solutions necessitates the use of a nuclear source or another method of detecting UV absorbances (below 320 nm). Nucleic acids absorb maximum at around 260 nm, depending on the base makeup. At 280 nm and 230 nm, proteins absorb the light [16]. Quartz cuvettes are commonly used to retain samples. The instrument was zeroed using TE buffer, and the DNA samples was diluted properly in TE buffer as well. Readings were typically collected at 260, and 280 nm. For conducting the spectrophotometry analysis, a T80+ uv/vis spectrometer was used from the Department of Entomology of BSMRAU. Table III provides the 260/280 ratio values that indicate the purity of DNA, RNA, and protein [16].

TABLE III: Approximate Purity Based on A260/A280 Ratio (Specific Nucleic Acids Are not Clearly Distinguished from Each Other Using this Ratio)

Nucleic Acid Type	Approximate A260/A280 Ratio
Pure DNA	1.8
Pure RNA	2.0
Pure Protein	0.57

III. Results and Discussion

A. PCR-Based MRJP2 Gene Identification in Honey

In honey, a gene called MRJP2 was found by utilizing PCR technique with specified primers after DNA extraction from all tested samples. Agarose gel electrophoresis with staining was used to identify amplified DNA products (Fig. 1). The columns 3 and 5 were used for the DNA template of *A. cerana* honey, while columns 2 and 3 were used for *A. cerana* primers. Columns 1 and 2 were used for the DNA template of *A. mellifera* honey, while lanes 1 and 5 were used for *A. mellifera* primers. Only columns 1 and 3 have amplified DNA templates present. Only the matched honey samples with the relevant primers were able to identify *A. cerana* DNA. Negative amplifications for *A. mellifera*-derived honeys and vice versa were found, in contrast. All of the honey and bee worker samples corroborated these findings. The PCR results were sequenced after electrophoresis. The amplified fragments were identified as *A. cerana* and *A. mellifera* MRJP2 gene products. For *A. cerana*, the size of the amplified fragment was 212 bp, while for *A. mellifera*, it was 560 bp.

The amplified product was more substantial than anticipated, which might be explained by the inclusion of an intron fragment. Honey might have a lot of damaged bee DNA. According to Utzeri, Ribani, and Fontanesi [15], the PCR primer pair was designed to amplify fragments of less than 500 bp in size. All of the samples of honey from the PCR were successfully examined when primers were employed. These results showed that the species-specific primers could be used to detect the entomological origins of the 2 types of honey as well as the entomological origin of the honey produced by *A. cerana* and *A. mellifera* could be determined based on variations in their MRJP2 genes.

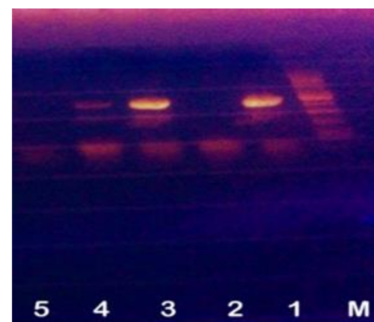

Fig. 1. DNA that was amplified using PCR with primers specific to *A. cerana* honey and *A. mellifera* honey on an agarose gel electrophoresis. DNA marker in column M, *A. mellifera* honey DNA extracts in column 1 and 2 (C-F and C-R), and *A. cerana* honey DNA extracts in column 3 and 5 (M-F and M-R).

Standard PCR may be used for a variety of purposes. It does not need a costly device and is reasonably simple to use. Only specific primers for *A. cerana* were available for

standard PCR analysis, whereas efficient *A. mellifera* honey primers capable of detecting *A. mellifera* DNA directly using a standard PCR procedure is absent. However, without appropriate *A. mellifera* primers, identification may be erroneous. The DNA analysis was unaffected by the geographical or botanical origin of honeys, and the results were corroborated by honey samples gathered. Using a standard PCR approach, it is possible to distinguish *A. cerana* honey from *A. mellifera* honey.

B. *Responsiveness towards Screening*

DNA extracted from *A. mellifera* was serially diluted and employed to screen the effectiveness of this PCR detection technique. Using M-F/M-R primers, PCR conditions achieved responsiveness. As the amount of DNA template decreased, the brightness of the *A. mellifera* honey's identifying strip diminished. Even with low DNA template concentration, the strip was still observed (lane 1), demonstrating the method's high specificity. Honey has enough DNA to fulfill the minimum quantity of DNA templates necessary for identification (Fig 2).

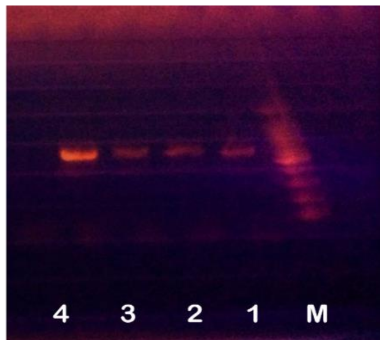

Fig. 2. PCR results with A. mellifera honey-specific primers and a serial dilution of A. mellifera honey DNA extract was separated by electrophoresis on an agarose gel. DNA marker in column M.

C. *Development of Real-Time PCR Method*

A real-time PCR test utilizing the same primers was also proposed for additional delicate and faster recognition. After real-time PCR amplification, the Amplified products of both sides were sequenced. Using BLAST, the amplified fragments were confirmed to be MRJP2 gene products from *A. cerana* and *A. mellifera*. Primers can only amplify DNA templates from the same species of bee. Electrophoresis on an agarose gel verified this conclusion. The DNA analysis was unaffected by the honey's geographical or botanical origin, and samples validated the results. Compared to the duplex PCR method, the real-time PCR test offers a faster and more accurate means of applying DNA barcoding without a further electrophoresis process.

D. *Application in adulteration*

Honey from both *A. cerana* and *A. mellifera* were combined in different amounts. Their DNA was tested to see if this PCR approach could detect adulteration of *A. mellifera* honey. The PCR technique was used to detect the combined samples, and the results are presented in Fig 3. The proportion of *A. mellifera* honey in the combination was 100,70,60,1,80,0, 10, and 20 percent from left to right. When *A. mellifera* honey made up just 1% of the mixture, it still had positive results and the species-specific band (560 bp) of *A. mellifera* honey was observed. With the rise in *A. mellifera* honey, the luminance of this band intensified.

Real-time PCR testing, as opposed to duplex PCR, provides a quicker and more precise way to apply Molecular biology techniques without a subsequent electrophoresis procedure. Therefore, the results of this PCR analysis may be utilized to confirm the authenticity of the entomological origin of the two varieties of honey.

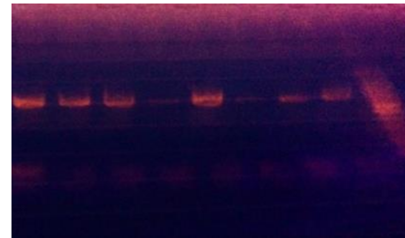

Fig. 3. DNA amplified by PCR from genomic DNA extracts of *A. cerana* and *A. mellifera* honey mixes was electrophoresed on an agarose gel. The proportion of *A. mellifera* honey- 100, 70, 60, 1, 80, 0, 10, and 20 percent (from left to right).

E. *Spectrophotometric Analysis of DNA*

An agarose gel is commonly used to determine the concentration of DNA in a sample as well as its condition. When many bands of DNA are detected, such concentration calculations are at best semi-quantitative, time-consuming, and confusing. A UV spectrophotometer is widely used for DNA solutions in DNA Spectrophotometric Spectrum. Table IV contains the measured Spectrophotometric Spectrum for a more precise estimation of the concentration of DNA in a sample.

An analysis of the solution's optical density values at different wavelengths can be used to evaluate the purity of a DNA solution. Table V presents the measured 260/280 nm ratio. For pure DNA 260/280 ratio is near 1.8. Increased ratios are frequently indicative of the presence of RNA, that can be verified by passing 1 g of the sample over an agarose gel. A protein or phenol contamination is commonly present when the 260/280 ratio is less than 1.8 [16]. It is difficult to determine whether the contamination is protein, phenol, or both. Once confirmed that sample includes pure DNA, in that case, the concentration of DNA can be calculated with precision.

TABLE IV: DNA SPECTROPHOTOMETRIC SPECTRUM

Wavelength (nm)	260	280
A. mellifera	0.195	0.102
A. cerana	0.364	0.158
A. mellifera	0.139	0.078
A. cerana	0.411	0.153

TABLE V: 260/280 RATIO VALUES FOR A MELLIFERA AND A. CERANA HONEY SAMPLES OF DNA

Sample	260/280 ratio
A. mellifera	1.91
A. cerana	2.30
A. mellifera	1.78
A. cerana	2.68

IV. Conclusions

The entomological origin of honey made by *A. cerana* and *A. mellifera* based on the MRJP2 gene of the two species of bees is postulated in the current work using a DNA-based test. Using the duplex PCR method, it is possible to identify even 1% inclusion of *A. mellifera* honey in *A. cerana* honey.

In this study, the entomological origin of honey has been successfully determined by analyzing the MRJP2 genes upon analyzing the nucleotide diversity of the two honeybee species. Experimental results derived from UV-VIS spectroscopy towards authenticating the honey samples of its entomological origin provided a magnificent insight.

For entomological authentication of honey samples, these two techniques can satisfy different needs with various instruments and equipment. Both the extension of the species' breeding business as well as the utilization of A. cerana honey resources will benefit from this study. This study might potentially be used as a reference to distinguish honey made by various bee species.

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Conflict of Interest

We declare that we do not have any conflict of interest.

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