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COI GENE–BASED MOLECULAR IDENTIFICATION OF HONEY BEES (*APIS SPP.*) FROM BANGLADESH

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ABSTRACT: *Apis* spp. are crucial pollinators that support biodiversity, agriculture, and global food production. Proper identification, understanding genetic diversity, and molecular characteristics of honey bee populations are vital for conservation and sustainable beekeeping practices. This study focuses on the DNA barcoding based on partial mt-*COI* gene sequences of different *Apis* species in Bangladesh. Samples were collected from various locations across Bangladesh, followed by DNA extraction, polymerase chain reaction (PCR) amplification, and sequencing. By comparing the unique nucleotide sequences of the *COI* gene to the reference GenBank database, four honey bee species (*Apis dorsata, A. cerana, A. florea,* and *A. mellifera*) were identified. Different castes (queen, worker, and drone) of *A. mellifera* were also sequenced and identified. Over 82% sequence similarity was observed among different species, with nearly 100% commonality within the *A. mellifera* castes. The mitochondrial A+T content (74%) exceeded G+C (26%). Genetic distance analysis (Kimura's 2-parameter) indicated minimal divergence between *A. dorsata* and *A. cerana* (0.120). Phylogenetic analysis revealed significant genetic variations among different *Apis* species. *A. florea* showed the highest mutation rate (40 steps) from its immediate common ancestor. This study enhances the genotypic and phenotypic identification of honey bee species, including *A. mellifera* castes, in Bangladesh.

Key Words: Honey bee, *Apis*, Castes, DNA barcode, *COI* gene.

INTRODUCTION

Insect pollination plays a vital role as an ecosystem service in agriculture. The production of fruits, vegetables, and seeds in 87 out of the 115 major global food crops relies on animal pollination (Klein *et al.,* 2007). It has been estimated that globally 3/4 of our staple crops and 1/3 of all food crops require pollination (Klein *et al.,* 2007). Insect pollination of crops worldwide was estimated to be worth 153 billion dollars, or 9.5 percent of the value of all agricultural products utilized to supply human nutrition in 2005 (Gallai *et al.*, 2009). High population

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density, foraging behavior, ability to pollinate a wide range of plants, and ease of management in hives make social honey bees indispensable for agricultural productivity and biodiversity conservation (Ollerton, 2017).

Apidae is the biggest family with approximately 5,700 species of bees, such as honeybees, carpenter or orchid bees, cuckoo bees, as well as smaller stingless native bees. Honey bees are social insects that live in large, well-organized colonies. The contemporary honey bees are taxonomically categorized as genus *Apis* (Winston, 1991). In Bangladesh, four species of *Apis* were reported by researchers (Moniruzzaman and Rahman, 2009, Akter *et al.*, 2019), though many parts of Asia have seven *Apis* species (Yadav *et al.*, 2017). In Bangladesh, the identification of *Apis* species has traditionally relied on morphological features such as body size, coloration, wing venation, and other external features. While these traits are generally effective, they can sometimes lead to misidentification due to subtle interspecific differences, intraspecific variation, and caste-based variation, phenotypic plasticity, and the potential for specimen damage or incompleteness. Despite the ecological and economic importance of honey bees in Bangladesh, there has been little to no molecular taxonomic assessment of local *Apis* populations. This represents a critical gap in knowledge. Molecular techniques can complement traditional taxonomy by providing precise species identification, revealing cryptic diversity, and allowing differentiation among castes at the genetic level.

DNA barcoding, utilizing cytochrome c oxidase I (COI) (described by Hebert *et al.*, 2003), is now recognized as a precise method for identifying insects and other biota (Waugh, 2007). The precision of DNA-based identifications is constrained by the prevalence of DNA barcoding reference settings (Villalta *et al.*, 2021). This is since it has not only preserved the first and second codon positions, but also the third position of the codon which is fast evolving making it a common choice by many researchers to conduct population and phylogeographic study of the gene (Marko *et al.*, 2007; Sotka *et al.*, 2004; Hare and Weinberg, 2005).

Although morphological identification has traditionally played a central role in honey bee taxonomy, integrating molecular tools such as DNA barcoding can significantly improve species-level resolution, particularly for distinguishing closely related taxa and castes. This study aims to assess the effectiveness of DNA barcoding as a complementary approach to classical taxonomy, with broader implications for ecological research and conservation strategies.

MATERIAL AND METHODS

Sample collection and morphological studies: To ensure broad regional representation, different social honey bees (*Apis* spp.) were systematically

collected from various parts of Bangladesh. They were collected from different bee colonies and flowering plants while visiting for nectar collection. For defining the fine structure of samples, all specimens were photographed under a stereo microscope (Leica EZ4 Stereo Microscope). Collected specimens were preliminarily identified using different characters such as body size, shape, coloration, abdominal banding patterns and hairy appearances and taxonomic keys mentioned in Mustafa *et al.*, 2022. The castes were distinguished by their differentiated taxonomic key characteristics based on their size, head morphology, abdominal structure, and leg morphology (El-Aw *et al.,* 2012 and Shaibi *et al.,* 2009).

*Genomic DNA extraction, PCR amplification, purification, and gene sequencing***:** The molecular procedures for DNA barcode was as mentioned in Aslam *et al.,* 2019a and 2019b. Briefly, somatic tissue of representative samples was used to isolate genomic DNA by using the Wizard® Genomic DNA Purification Kit (USA). The polymerase chain reaction (PCR) was carried out using a good quality DNA with A260/A280 value of approximately 1.8 as measured by a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA).

The following region 658 bp within the 5′ terminus of the *COI* gene was amplified using the following primers- forward primer: (LCO 1490 5′-GGTCAACAAATCATAAAGATATTG G-3′) and reverse primer: (HCO 2198 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer *et al.*, 1994). The PCR amplifications were performed following the conditions: initial denaturation at 94 ℃ for 2 min followed by 35 cycles of 94 ℃ for 1 min (denaturation), 45.5 ℃ for 1 min (annellation), 72 ℃ for 2 min (extension), followed by a final extension at 72 ℃ for 7 min. To confirm the presence, size, and quality of the amplified DNA fragments by separating them based on their length, gel electrophoresis was performed. According to the manufacturer, PCR products were purified with Promega Wizard® SV Gel & PCR Clean-Up System (Promega Corporation, USA).

The purity of PCR-purified products was measured by applying a spectrophotometer and thereafter, DNA sequencing was performed to estimate the nucleotide sequence in the cytochrome oxidase I region. This was done with the BigDye® Terminator v3.1 cycle sequencing kit.

GenBank submission: The quality of the sequenced data was assessed using BioEdit v.7.0.5 software. All sequences were uploaded to GenBank of NCBI (National Center for Biotechnology Information) following the proper procedure and received a unique identifier - accession number.

Biological data processing: FinchTV chromatogram viewer and BioEdit v.7.0.5 software were used to view and edit the sequences. The trimmed sequences were then subjected to BLAST (Basic Local Alignment Search Tool) searches from

NCBI to determine homology with closely related species. COI sequences were aligned using BioEdit v7.2.5 to assess similarities and differences. Phylogenetic analysis, nucleotide composition analysis, and multiple sequence alignment were performed using MEGA (Molecular Evolutionary Genetic Analysis) v. X. Haplotype network analysis of the sequenced samples was conducted in PopART v1.7 employing the TCS network algorithm.

RESULTS AND DISCUSSION

Morphological identification: A total of four social honey bee species of the genus *Apis* were identified in the course of this study. The collected samples were identified to species level using standard diagnostic traits (Fig. 1).

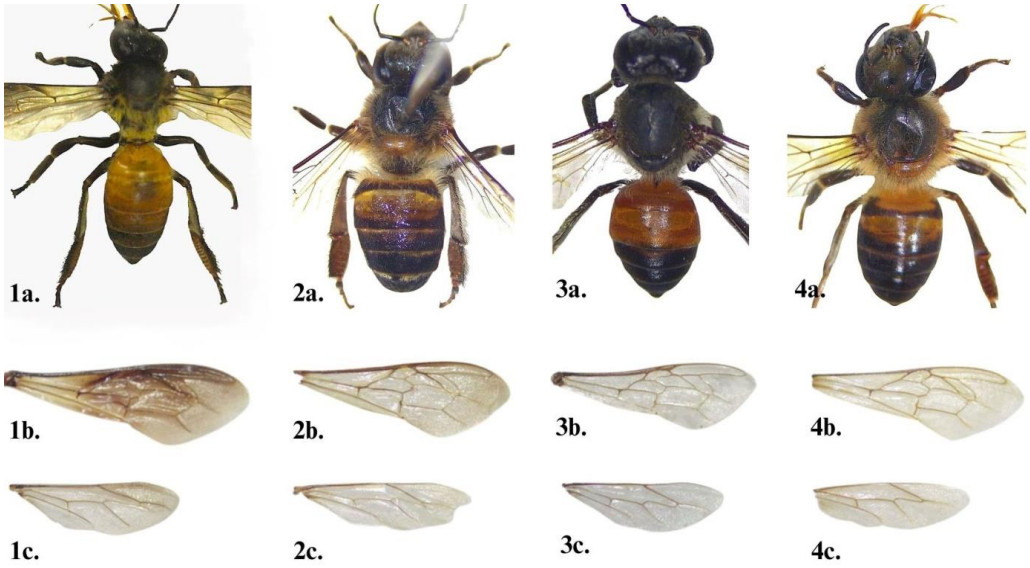

Fig. 1. Comparative morphological structures of 4 social honey bees (not to scale) of Bangladesh; 1. Apis dorsata; 1a. dorsal view, 1b. forewing, 1c. hind wing. 2. A. cerana; 2a. dorsal view. 2b. forewing, 2c. hind wing. 3. A. florea; 3a. dorsal view, 3b. forewing, 3c. hind wing. 4. A. mellifera 4a. dorsal view, 4b. forewing, 4c. hind wing

Apis dorsata is large (~17-20 mm), dark brown with yellow-brown bands; it has a robust body and large wings. Their wings are hyaline at the base, fuscous in the middle, and darker in the anterior margin. *A. florea* is the smallest (~7-10 mm), reddish-brown with thin pale bands, slender body, and small wings. Basal 2 abdominal segments are red. *A. mellifera* is medium (~12-14 mm), golden-brown with black bands, a robust and hairy body, and well-developed wings having a complete, strong venation. *A. cerana* is small to medium (~10-12 mm), dark brown with less distinct bands; has a slender body and smaller wings.

Phenotypic comparison among the castes of *A. mellifera:* Three different castes were observed in the *A. mellifera* colony as queen, drone and worker which possess somewhat distinguishing morphological features (Fig. 2). The queen is the largest with an elongated abdomen and smooth body, workers are the smallest with hairy bodies and pollen baskets, and drones are medium to large with stout bodies, large eyes, and no stinger.

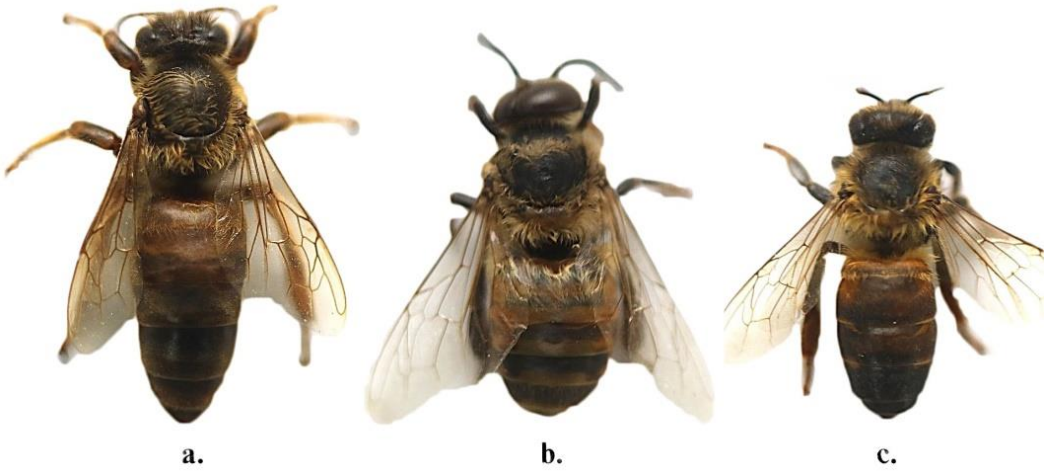

Fig. 2. Different castes of *Apis mellifera* colony; dorsal view of a. queen, b. drone and c. worker

Table 1. BLAST analysis result of the sequenced samples, along with information on GenBank accession numbers

Voucher ID	GPS Location	BLAST Analysis Result			Acquired Accession Number
		Accession Number	Homology	Identified Species	
AP1	23.8490N 90.2580E	KU212345.1	99.54%	*Apis dorsata* (1)	MG587946.1
HY46	23.77 N 90.37 E	OR563676.1	99.07%	*Apis dorsata* (2)	OP435372.1
HY8	23.8218N 90.4125E	OK632479.1	99.69%	*Apis cerana*	MG587944.1
HY34	23.7695N 90.3731E	KT960841.1	99.39%	*Apis florea*	MH378769.1
HY37	23.9580N 89.9322E	MT188686.1	99.85%	*Apis mellifera*	MH388489.1
HY42 (queen)	23.87 N 90.00 E	OK355187.1	100.00%	*Apis mellifera*	OP435367.1
HY43 (drone)	23.87 N 90.00 E	MF543425.1	99.38%	*Apis mellifera*	OP435369.1
HY41 (worker)	23.87 N 90.00 E	OM203280.1	100.00%	*Apis mellifera*	OP435365.1

Molecular characterization: Eight samples of honey bees were sequenced. BLAST analysis revealed that all the obtained sequences shared 99-100% similarity with the genes of GenBank, and they belonged to four species under the Apis

genus (Table 1). Following BLAST, the sequences were deposited in the NCBI GenBank (BankIt) and received a unique identifier termed as GenBank accession number for each sample (Table 1). The results showed that species could be distinguished using COI barcoding.

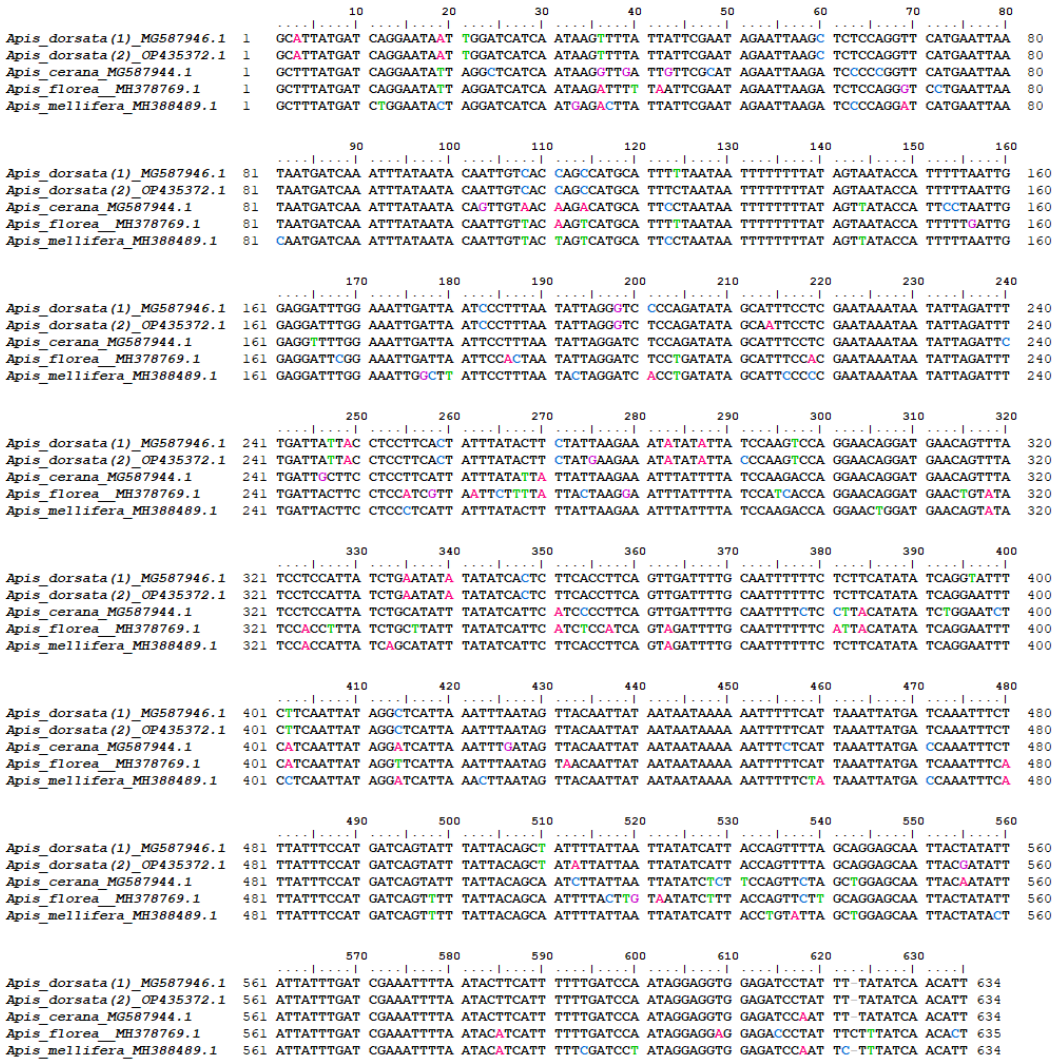

Fig. 3. Multiple sequence alignment based on *COI* gene sequences of four social honey bees. Colored letters denoted the non-conserved region among these five nucleotide sequences.

Alignment of sequenced COI genes: Multiple sequence alignment of *COI* gene nucleotide sequences of social honey bees is shown in Fig. 3. This alignment illustrates both conserved and variable regions across the different species. The analysis revealed that the overall sequence similarity exceeded 82%, indicating a relatively high degree of homology among the studied species. This level of

similarity suggests that these species share a recent common evolutionary origin, supporting the hypothesis of phylogenetic relatedness within the *Apis* genus. The residues per row were 80, where the colored ATCG letters denoted the non-conserved region among sequences, and the identical nucleotides are shown in black letters.

Another multiple sequence alignment of *COI* genes from different castes of *Apis mellifera* was conducted to examine if there are any differences (Fig. 4). The alignment showed nearly 100% of similarity among the castes, confirming that they belong to the same species with no differences in *COI* gene nucleotides.

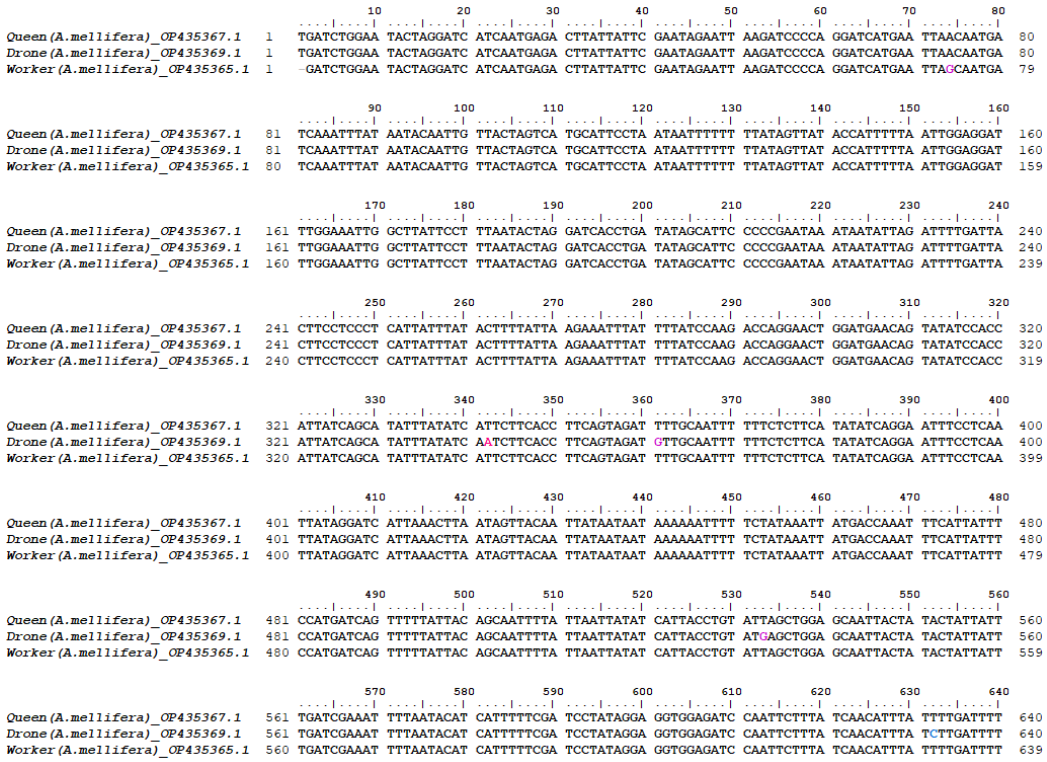

Fig. 4. Multiple Sequence Alignment of sequenced *COI* gene of queen, drone, and worker (*Apis mellifera).*

Table 2. The nucleotide base composition of the sequenced bee species

Species Name		T%	C%	A%	G%	T+A %	C+G%	Total
Apis dorsata 1		42.3	13.8	33.4	10.6	**75.7**	24.4	653
Apis cerana		40.7	15.1	32.7	11.6	73.4	26.7	649
Apis florea		41.6	14.0	33.4	11.0	75.0	25.0	652
Apis mellifera		40.7	15.5	32.7	11.0	73.4	26.5	670
Castes of	Queen	40.6	15.2	33.1	11.0	73.7	26.2	643
Apis	Drone	39.9	15.4	33.4	11.2	73.3	26.6	641
mellifera	Worker	40.5	15.3	33.1	11.1	73.6	26.4	640
Average		40.9	14.9	33.1	11.0	74.0	26.0	649

Nucleotide composition of COI gene sequences: Retrieved sequences were subjected to analyzed for nucleotide composition. Codon positions included were 1st + 2nd + 3rd + non-coding. The COI fragment's total nucleotide base composition differed between specimens, exhibiting an AT bias as expected, with an average value of 74% (T+A content) and 26% (C+G content) (Table 2).

Genetic distance analysis: The MEGA-X algorithm estimated the pairwise distance using Kimura's 2-parameter (K2P) genetic distance approach to demonstrate partial sequence divergences between and within species. The interspecific nucleotide difference among the species ranged from 0.120 to 0.160. The lowest distance between *Apis dorsata* 1 and *A. cerana* was 0.12, representing their closely related ancestral state. Very high genetic dissimilarity was observed between *A. florea* and *A. mellifera* (drone), and the distance was 0.160. The intraspecific nucleotide difference among the castes of *A. mellifera* ranged from 0.002 to 0.008 (Table 3).

Table 3. Genetic distance among 4 bee species using Kimura 2 Parameter (K2P)

Species	1	2	3	4	5	6
Apis dorsata 1	-					
Apis cerana	**0.120**	-				
Apis florea	0.159	0.148	-			
Apis mellifera	0.141	0.135	0.151	-		
Apis mellifera (queen)	0.123	0.133	0.149	0.000	-	
Apis mellifera (drone)	0.131	0.144	**0.160**	0.006	0.006	-
Apis mellifera (worker)	0.126	0.135	0.151	0.002	**0.002**	**0.008**

Phylogenetic analysis: Phylogenetic analysis applied under the context of the DNA barcoding and *COI* gene analysis serves to identify evolutionary relationships, confirm the species identification, and also measure evolutionary divergence between the various species of *Apis*. The Maximum Likelihood approach was applied to the Tamura-Nei model to determine the evolutionary history. The tree is drawn to scale with the number 0.10 reflecting the length of the branch that represents an extent of genetic distance on the basis of eight experimental sequences (acknowledging with the name Bangladesh) and other sequences of Apis species in other countries that were found on the GenBank under NCBI. All four species originated from distinct clades. *A. dorsata* and *A. cerana* originated from the sister clade showing genetic similarities, which indicates their same origin, while *A. mellifera* (including the sequenced castes) and *A. florea* belonged in another sister clade. (Fig. 5).

Haplotype network analysis: Statistical parsimony (TCS) haplotype network was performed based on *COI* gene sequences of four *Apis* species to know genetic diversity among them (Fig. 6). *Apis dorsata, A. cerana, A. florea,* and *A.*

mellifera were separated from their immediate common ancestors by 27, 30, 40, and 31 mutational steps respectively. They share a very common ancestor between the species with few mutational steps. The lower mutational steps are very common within the castes of similar species.

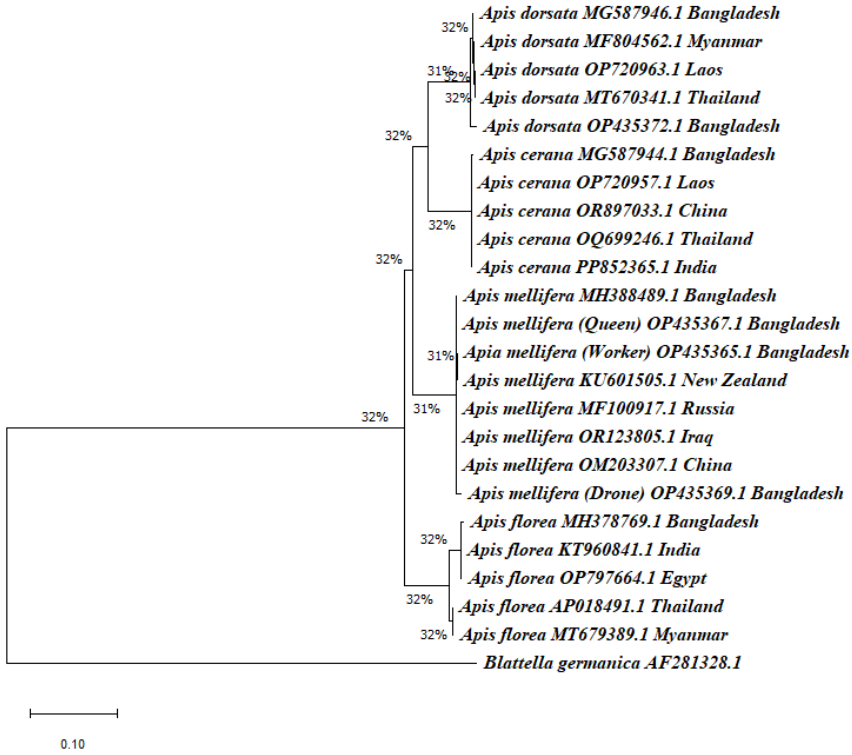

Fig. 5. Maximum Likelihood phylogenetic tree based on *COI* gene sequences of different *Apis* species. Sequences from different countries were compared with the studied samples. The genetic distance scale is shown at the bottom.

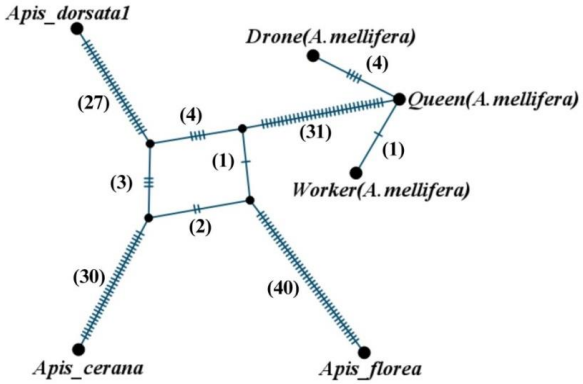

Fig. 6. TCS haplotype network based on mitochondrial *COI* gene sequences of social honey bee species, constructed using PopART 1.7. Each large circle represents a distinct haplotype, while small black circles indicate hypothetical common ancestors. The numbers on the lines between haplotypes represent the number of mutational steps.

While the presence of *Apis dorsata*, *A. cerana*, *A. florea*, and *A. mellifera* in Bangladesh has been reported by Bhuiya and Miah (1990) and Akter *et al.* (2019), our study is the first to validate these records using both morphological and COI gene-based molecular techniques, offering a more reliable taxonomic assessment. DNA barcoding has emerged as a highly reliable tool for the identification of Hymenopteran insects, offering greater accuracy and consistency than traditional morphological keys (Blaxter, 2003). For instance, in Pakistan, *A. dorsata*, *A. mellifera*, and *A. florea* were successfully identified and characterized using mitochondrial *COI* gene sequences (Mustafa *et al.*, 2022). In a broader context, Yadav *et al.* (2017) reported the presence of seven different honey bee species across various regions of Asia and other parts of the world, highlighting the global diversity within the genus *Apis*.

In this study, the COI gene fragment showed a typical AT bias, consistent with patterns observed in the mitochondrial genomes of many insect species (Das *et al.*, 2020; Rain and Aslam, 2023; Rain *et al.*, 2023; Willis, 1992). Factors such as selective constraints, replication mechanisms, thermal stability, and species-specific genetic requirements have all been proposed to influence the higher AT content in mitochondrial genes like *COI* (Smith, 2012).

Genetic distance analysis revealed a close evolutionary relationship between *Apis dorsata* and *A. cerana*, indicated by a relatively low K2P distance (0.120). A similar finding was reported by Bandral *et al.* (2023), who observed a genetic distance of 0.0867 between these two species, further supporting the genetic proximity reflected in our results. Phylogenetic tree analysis also represented the similar relationship between *Apis dorsata* and *A. cerana* which are grouped together within a sister clade. A similar phylogenetic pattern among these four *Apis* species was reported by Garnery *et al.* (1991) using COXII gene barcoding, where the branching topology showed comparable divergence relationships.

CONCLUSION

The present study successfully identified four species of social honey bees present in Bangladesh. This current research successfully utilized the *COI* gene as a DNA barcode to identify and distinguish between various *Apis* species, including *A. mellifera*, *A. cerana*, *A. dorsata*, and *A. florea*. The findings confirm the utility of the *COI* gene as a reliable molecular marker for species identification within the *Apis* genus, providing clear evidence of species-specific nucleotide variations. The DNA barcode reference library developed in this study, based on both molecular data and morphological validation, can serve as a useful resource for more accurate identification of *Apis* species in Bangladesh. Future research could expand the application of DNA barcoding to other genetic

markers or include additional *Apis* species to gain deeper insights into honeybee phylogenetics and evolution.

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