MITOCHONDRIAL DNA BASED PHYLOGENY AND HAPLOTYPE NETWORKING OF EUROPEAN HONEYBEE *Apis mellifera* L. IN BANGLADESH

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ABSTRACT

The European honeybee, *Apis mellifera* L. is the most widely used species in apiculture. Bangladesh can be suggested as evolutionary significant zone based on the global analysis of its diversity. In the world, there are 33 different *A. mellifera* subspecies, of which 11 are widely utilized. The present study aims to reveal the genetic diversity, distribution pattern and haplotype networking of the available *A. mellifera* subspecies in Bangladesh. The sampling was carried out from 18 localities of Bangladesh based on nectarine sources and the molecular analysis was conducted using 609 bp of mitochondrial Cytochrome oxidase subunit-1 (COI) genes. The phylogenetic analysis based on Neighbor-Joining and Maximum-Likelihood compositions from 18 nucleotide sequences (MW428209-MW428226) of the collected samples revealed 7 types of *A. mellifera* subspecies for the first time in Bangladesh. A total of 82 variable sites of nucleotide composition were observed of which 26 sites were parsimony informative. Remarkable genetic diversity and distribution patterns appeared in central and southern regions of Bangladesh mediated through *A. mellifera adami*, *A. mellifera capensis* and *A. mellifera sicula*. *A. mellifera macedonica*, implying the diversification and habituation of *A. mellifera* subspecies in the Bangladesh context. The haplotype diversity of the identified *A. mellifera* subspecies detected 14 new haplotypes (COIH05-COIH18) for the first time in Bangladesh. Haplotype networking pattern inferred the recent expansion of population in this geographical proximity along with the phylogeographic distribution of the recent *A. mellifera* clades in different parts of the world. The findings of this study will provide reliable molecular information in the world honeybee gene pool about the diversified occurrence of *A. mellifera* subspecies in this country and will shed light on the implication of its uses for modernizing the apiculture sector of Bangladesh.

Keywords: *Apis mellifera*, phylogeny, genetic diversity, haplotype

ABSTRAK

Lebah madu Eropah, *Apis mellifera* L. merupakan spesies yang bertabur secara meluas dalam apikultur. Bangladesh dicadangkan menjadi zon evolusi yang signifikan berdasarkan analisis global kepelbagaiannya. Terdapat 33 subspesies *A. mellifera* di dunia, di mana 11 daripadanya dikaji. Kajian ini bermatlamatkan untuk mengkaji kepelbagaian genetik, corak taburan dan jaringan haplotip pada subspesies *A. mellifera* di Bangladesh. Persampelan telah dijalankan pada 18 lokaliti di Bangladesh berdasarkan sumber nektar dan analisis molekul dijalankan menggunakan 609 pb mitokonria oksidase subunit (*COI*) gen. Analysis filogeni berdasarkan *Neighbor-Joining* dan Maksimum *Likelihood* melibatkan komposisi 18 jujukan nukleotida (MW428209-MW428226) samples telah mempamerkan 7 jenis subspecies *A. mellifera* buat pertama kalinya dari Bangladesh. Sejumlah 82 kawasan bervariasi nukleotida menunjukkan 26 kawasan adalah parsimoni informatif. Hasil kajian menunjukkan kepelbagaian genetik dan corak taburan di kawasan tengah dan selatan Bangladesh di mediated through *A. mellifera adami, A. mellifera capensis* dan *A. mellifera sicula*. *A. mellifera macedonica*, menunjukkan kepelbagaian dan habituasi subspesies *A. mellifera* dari Bangladesh context. Kepelbagaian haplotip ke atas subspesies *A. mellifera* dikenalpasti dari 16 haplotip (COIH05-COIH18) pertama kali dari Bangladesh. Corak jaringan haplotip melibatkan corak inferensi pencapahan populasi dari sudut geografi di sepanjang taburan filogenetik untuk klad *A. mellifera* terkini berbanding klad dari seluruh dunia. Hasil kajian memberikan maklumat molekul berkaitan takungan gen lebah madu mengenai kehadiran kepelbagaian subspesies *A. mellifera* di dunia dan memberikan impak kegunaannya untuk kemodenan sektor apikultur dari Bangladesh.

Kata kunci: *Apis mellifera*, filogeni, kepelbagian genetik, haplotip

INTRODUCTION

Understanding the evolutionary history of *Apis mellifera* is important since it is undoubtedly the most important pollinator of agricultural crops and wild plants. Additionally, it produces a variety of foods with nutritional, therapeutic, and cosmetic applications like honey and propolis. While the European honey bee is native to Europe, Africa, the Middle East, and parts of Asia, the ability of *A. mellifera* to colonize virtually all habitable biomes on Earth and adapt to diverse bioclimatic conditions is living proof of the species' remarkable morphological and behavioral plasticity (Tihelka et al. 2020).

The honeybee *A. mellifera* is a very productive species that can adapt to a wide range of climates and produces 40-50 kg of high-quality honey each year (Islam et al. 2016). Other characteristics of *A. mellifera* that have led to its popularity in apiculture include- ease of domestication, low absconding, and swarming potential.

The European honeybee *A. mellifera* was introduced in 1992, and is currently widely cultured in Bangladesh (Sivaram 2012). Geographic variation has resulted in the establishment of multiple subspecies of *A. mellifera* in various parts of the world, each of which has genetic variation. These subspecies have been described based on morphological and behavioral characteristics (Gupta 2014). Since their distributions correlate to diverse geographic areas, these subspecies are sometimes referred to as "geographic races" (Martimianakis et al. 2011). Selective breeding for honey production, synchronized colony cycle with the native flowering pattern, winter clustering during cold seasons, migratory swarming, and enhanced foraging of

A. mellifera has resulted in 33 different subspecies that are highly adaptable to various ecological conditions around the world (Ilyasov et al. 2020).

Identification of the subspecies and assessment of the introgression level is used as the basis for the preservation of the gene pool of bee populations (Ilyasov et al. 2021). Beekeepers have long recognized that bee races differ in a number of behavioral traits such as calmness, swarming intensity, honey production, ability to utilize different sources of forage, and resistance to diseases (Tihelka et al. 2020).

Phylogeography study of this species from Albania, Bulgaria, Cyprus, Greece, Italy, Slovenia, Turkey and Greece reveals that the species has much genetic diversity, and migratory beekeeping as well as commercial breeding has resulted in 7 haplotypes in Greece (Martimianakis et al. 2011) and this trend can be reflected in Bangladesh and other Southeast Asian countries as well. In Malaysia, occurrence of pests and predators caused absconding of *A. cerana* honeybee from the existing beehives (Johny et al. 2021), which can also contribute to different haplotype development in honeybees, since migration of honeybee from managed colonies can attribute to hybridization between species (Calfee et al. 2020).

Mitochondrial DNA (mtDNA) is considered as an important tool for studying the phylogeny at the subspecies level. The mitochondrial DNA comprises regions with different rates of evolution, thus it has shown to be a highly helpful molecule for population genetic studies of *A. mellifera* and phylogenetic studies of the genus *Apis* (Martimianakis et al. 2011). A comparative analysis of mtDNA is the most useful tool for phylogenetic analysis of honeybees and other animals (Tan et al. 2011). All members of a colony share the identical mtDNA of the queen, making mtDNA appropriate for phylogenetic analysis of honeybees at the subspecies level. (Avise et al. 1987). This mtDNA can be used to distinguish between subspecies (Pedersen 1996). Biogeographical information of *A. mellifera* has been revealed from the variation in the mtDNA and several evolutionary lineages were identified using morphometric data and, in a few cases, molecular data were used (Oleksa et al. 2021).

mtDNA consists of two ribosomal RNA genes, 22 transfer RNA genes and 13 protein genes (Ilyasov et al. 2021). In various studies, the genes 16s rDNA, CO I, ND 5, COI-COII intergenic region, subunit NADH dehydrogenase (ND2), and COI-ND5 have been utilized to assess the genetic variability of *A. mellifera*. High genetic divergence was found in the COI region (Bouga et al. 2005; Ilyasov et al. 2011; Martimianakis et al. 2011; Özdil & Ilhan 2012; Solorzano et al. 2009). The 16S *r*RNA gene is the most commonly used mtDNA gene in previous phylogenetic studies of bees followed by the COI gene (Kek et al. 2017). The COI region can assess inter and intra-specific diversity, and can also be used to supplement standard morphology-based species identification more accurately (Lombogia et al. 2020).

The evolution of economically significant traits in this species as well as the mechanisms underlying its adaptability to various environmental conditions can both be studied with the aid of a reliable phylogenetic analysis of the European honey bee (Tihelka et al. 2020). The recent phylogeographical study on the weaver ant in Bangladesh implies that Bangladesh is the transitional zone of contact between Indian and South East Asian countries for weaver ant species (Rahman et al. 2017a; 2017b). Therefore, the identical phenomenon could occur in the case of *A. mellifera*. Since migratory beekeeping is practiced in Bangladesh, it is a frequent possibility that the introduction of foreign queens will cause hybridization and alter the mtDNA distribution pattern of local bees. This is because it is known that admixture between lineages increases levels of genetic diversity in honeybee populations (Bouga et al. 2005).

The haplotype networking is important to determine the mutation in the DNA sequences that provides an understanding of the population structure in relation to migration. When compared to the surrounding honeybee population, the results can indicate a hybrid scenario between distinct subspecies, which can be taken into account when developing future conservation strategies and pathogen-parasite-tolerant genetic improvement (Munoz et al. 2012). But in Bangladesh, no phylogenetic approach regarding *A. mellifera* has been followed till now to recognize the available subspecies and also the study of their ancestral relationship along with haplotype diversity has not been performed. The information on genetic diversity thus can be used as a guideline for understanding the distribution pattern of *A. mellifera* subspecies present in Bangladesh. Therefore, the present study was undertaken with the following objectives: to characterize and infer the phylogeny of different subspecies of *A. mellifera* L. population in Bangladesh through mitochondrial DNA analysis, to identify the available haplotype and its networking pattern of *A. mellifera* using Cytochrome oxidase subunit-1 genes and to detect the occurrences of high performing *A. mellifera* L. subspecies in Bangladesh with referring to worldly recognized species.

MATERIALS AND METHODS

Sample Collection and Specimen Preservation

Adult *A. mellifera* workers from 18 colonies were collected from 18 localities in 12 districts of Bangladesh for this experiment. Samples were collected on the basis of topography, geographic features, the availability of nectar and pollen supplies, and apiculture practicing areas. Collection was done from November 2020 to January 2021 (Figure 1). The collected bees were preserved in 99.9% ethanol for molecular characterization. The detailed locality information with GPS coordination was placed in Table 1.

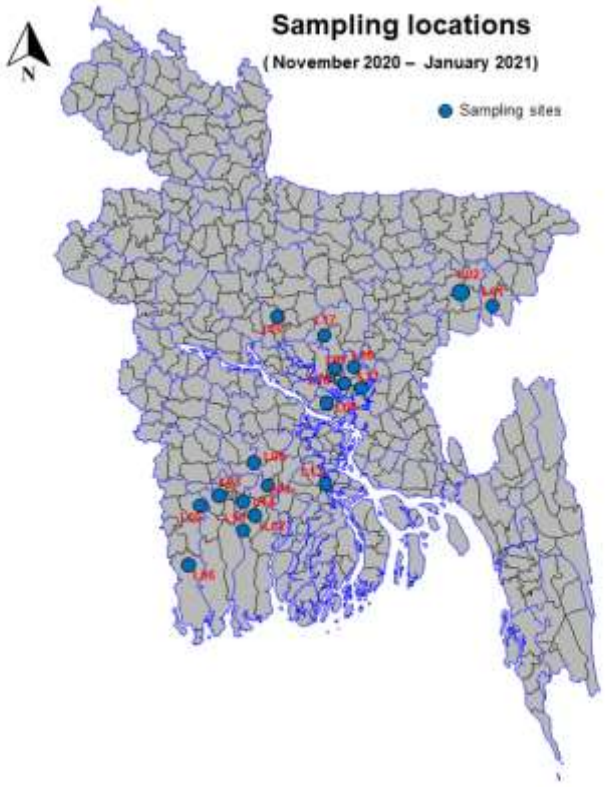

Figure 1. The sampling locations of the collected bee colonies as indicated by L01 to L18

Table 1. Sampling localities of *Apis mellifera* from Bangladesh

Locality no.	Locality Name	Upazilla	District	GPS coordination	
				N	E
L01	Bangladesh Tea Research Institute	Sreemangal	Moulovibazar	24.3047	91.7436
L02	Sylhet Sadar	Sadar	Sylhet	24.9104	91.9308
L03	Dacope Thana porishod	Dacope	Khulna	22.5531	89.5095
L04	Mollarhat bazar	Mollarhat sadar	Mollarhat	22.9291	89.8076
L05	Tala college campus	Tala	Satkhira	22.7496	89.2575
L06	Syamnagor bus stand	Syamnagor	Satkhira	22.4346	89.1046
L07	Mawna bazar	Mawna	Gazipur	24.2257	90.3997
L08	Manikganz hospital periferi	Manikganz sadar	Manikganz	23.8695	90.0006
L09	Narail sadar hospital	Narail sadar	Narail	23.1721	89.4993
L10	Vuapur	Vuapur	Tangail	24.4612	89.8728
L11	Vurulia bazar	Gazipur sadar	Gazipur	24.0135	90.4311
L12	Shat gambuz mosque area	Bagerhat	Bagerhat	22.7515	89.5010
L13	Tungipara	Tungipara	Gopalganj	22.6745	89.7418
L14	Botiaghata	Khulna sadar	Khulna	22.8899	89.8973
L15	Keshobpur	Keshobpur	Jessore	22.9183	89.2112
L16	Kalni	Kalni	Gazipur	23.9975	90.4714
L17	Porabari	Sreepur	Gazipur	24.0493	90.3888
L18	Pirujali	Sreepur	Gazipur	24.1375	90.3693

Extraction of DNA

Genomic DNA was extracted from the legs and thorax of specimens that were preserved in alcohol by using the QIAGEN DNeasy Blood and Tissue kit (Sangon Biotech China) following the manufacturer's instruction. Samples were vortexed by adding 180 µl Buffer ATL and 20 µl proteinase K. and incubated at 55^0C for 48 hours. DNA extraction was completed by adding two wash buffer AW1 and AW2 and buffer AE and lysis buffer AL and elusion buffer AE, as per manufacturer instruction. All centrifugation steps were carried out at room temperature. The colony mates of the specimens used for DNA analysis were preserved in the Advanced Entomology Laboratory of BSMRAU after DNA extraction (Zhao et al 2014). For, mitochondrial DNA analysis, primers for *COI* gene fragment, *COI* 1–3 (5′ ATAATTTTTTTTATAGTTATACC'3) and COI 2–4 (5′ TCCTAAAAAATGTTGAGGAAA'3) were used as forward and reverse primers (Crozier & Crozier 1993).

Polymerase Chain Reaction (PCR)

Amplification of DNA was done by *TaKaRa Ex Taq* PCR kit, according to the manufacturer's instructions. The kit contains 10X *Ex Taq* Buffer (20mM Mg^{2+} plus) and dNTP mixture (2.5mM each). The storage buffer contains 20 mMTris-HCl (pH 8.0), 100 mMKCl, 0.1mM EDTA, 1mM DTT, 0.5% Tween 20, 0.5% Nonidet P-40 and 50% glycerol. dNTP mixtures contain TAPS, KCl, $MgCl_2$, DTT, dATP, dGTP, dCTP with activated salmon sperm DNA. Reaction mixtures for PCR for 50 µl consisted of *TaKaRa Ex Taq* (0.25 µl), 10XExTaq Buffer (5 µl), dNTP mixture (4 µl), a pair of oligonucleotide primers (0.2-1.0 µM). The thermal cycling parameters for COI followed the protocol including 95◦C for 5 min for initial denaturation, 35 cycles of dissociation (92◦C, 1 min), annealing (54◦C, 1 min), and extension (70◦C, 2 min). The detailed primer configuration is presented in Table 2 (Zhao et al. 2014).

Table 2. Primer position of PCR

Region	Name	Direction	Sequence (5'-3')[a]	Position
Mitochondrion Cytochrome oxidase Subunit -1	CO1 1-3[b]	Forward	ATAATTTTTTTTATAGTTATACC	1981-2002
	CO1 2-4[b]	Reverse	TCCTAAAAAATGTTGAGGAAA	3063-3083

[a]Crozier & Crozier 1993
[b]Used for both PCR and sequence

Sequencing and Submission to NCBI GenBank, USA for Accession
The PCR products were sequenced by using the facilities from GENEWIZ, (Azanta life science from China) and they provided the nucleotide sequence data. The obtained sequences were then submitted to NCBI GenBank for receiving the accession number. Following the submission, the accession numbers from NCBI of all the *A. mellifera* samples were received.

Calculation of Nucleotide Frequency
Average nucleotide frequency of the nucleotide sequences was calculated by using MEGA X software. A total of 609 concatenated sequences of mitochondrial *cytochrome oxidase subunit 1* gene were used for this study along with the eight *A. mellifera* subspecies data from GenBank. The sequences of *A. cerana* were used as outgroup to classify the consistency of the nucleotide diversity.

Sequence Alignment and Phylogenetic Analysis
The obtained nucleotide sequences were aligned using Clustal X in the MEGA X software (Kumar et al. 2018). A total of 609 bp of nucleotide sequences were used in the analysis. Neighbor joining tree (Saitou & Nei 1987) and maximum likelihood tree by Tamura-Nei model (Tamura & Nei 1993) were formed using MEGA X software.

For phylogenetic analysis of *Apis mellifera* species, nine genome sequences obtained from NCBI GenBank, were used as reference. Among the 9 standard sequences, eight belonged to *A. mellifera* subspecies. The sequence of *Apis cerana* was used as the outgroup during the phylogenetic analysis of *A. mellifera.* The evolutionary distances were computed using the Maximum Likelihood Estimate of Substitution Matrix (Tamura & Nei 1993). Tajima's Neutrality Test was done to calculate the difference between the mean number of pairwise differences and the number of segregating sites. For observing variable sites in DNA sequences of these subspecies FaBOX (1.5) software was used.

Haplotype Networking
Haplotype diversity was studied using TCS 2.1 and PopART software using the sequences of *COI* genes (Clements et al. 2000). The known sequences of *A. mellifera* subspecies collected from NCBI GenBank were used as reference data for identifying the haplotype distribution in Bangladesh (Kumar et al. 2016; Tamura et al. 2013).

RESULTS

Towards inferring the phylogenetic position of honeybee (*A. mellifera*) in Bangladesh, the study was divided into two sections. First, molecular characterization of *A. mellifera* honeybee from *COI.* Followed by, the haplotype networking of the identified samples to reveal the haplotype diversity of different subspecies in different regions of Bangladesh.

Molecular Characterization

Average Nucleotide Frequency

The average rate of Thymine was observed at approximately 41.9%, with the 12.3%, 32.4%, and 13.4 % for the Cytosine, Adenine and Guanine, respectively (Table 3). The percentage is found to be similar to the reference data that were used from NCBI GenBank. Therefore, it can be said that the estimated sequences were valid and possessed the uniform nucleotide diversity index in all regions.

Table 3. Nucleotide diversity estimation of the *A. mellifera* honeybee samples from Bangladesh

Sequence Name	T(U)	C	A	G	Total
JX982136 *Apis cerana* china	41.87192	11.16585	33.16913	13.7931	609
MG552693.1 *Apis mellifera capensis*	42.03612	12.15107	32.34811	13.4647	609
AY114466.1 *Apis mellifera caucasica*	42.03612	12.31527	32.34811	13.30049	609
AY114483.1 *Apis mellifera sicula*	42.03612	12.15107	32.34811	13.4647	609
AY114473.1 *Apis mellifera macedonica*	41.87192	12.47947	32.34811	13.30049	609
AY114471.1 *Apis mellifera anatoliaca*	41.70772	12.64368	32.34811	13.30049	609
AY114476.1 *Apis mellifera adami*	41.54351	12.31527	32.18391	13.95731	609
NC 001566.1 *Apis mellifera ligustica*	42.03612	12.31527	32.34811	13.30049	609
KX943034.1 *Apis mellifera scutellate*	42.52874	11.65846	32.34811	13.4647	609
Kalni Gazipur	42.03612	12.15107	32.34811	13.4647	609
Porabari Gazipur	42.03612	12.31527	32.18391	13.4647	609
Mawna Gazipur	42.03612	12.31527	32.34811	13.30049	609
Pirujali Gazipur	41.87192	11.98686	32.67652	13.4647	609
Manikganj sadar	41.87192	12.64368	32.18391	13.30049	609
Bagerhat sadar Bagerhat	41.70772	12.64368	32.34811	13.30049	609
Tungipara Gopalganj	41.70772	12.47947	32.51232	13.30049	609
Dacope Khulna	41.87192	12.47947	32.34811	13.30049	609
Tea research center Sreemangal	41.87192	12.47947	32.34811	13.30049	609
Keshobpur Jessore	41.87192	12.15107	32.34811	13.6289	609
Khulna sadar Khulna	41.87192	12.64368	32.18391	13.30049	609
Vurulia Gazipur	41.54351	12.31527	32.18391	13.95731	609
Mollarhat Bagerhat	41.70772	12.47947	32.51232	13.30049	609
Vuapur Tangail	41.05090	12.47947	32.51232	13.95731	609
Syamnagor Satkhira	42.03612	12.31527	32.34811	13.30049	609
Tala Satkhira	42.20033	12.31527	32.01970	13.46469	609
Sylhet sadar Sylhet	41.54351	12.15107	33.00493	13.30049	609
Narail Jessore	41.70772	12.64368	32.34811	13.30049	609
Avg.	**41.85976**	**12.30311**	**32.39068**	**13.44645**	**609**

Maximum Likelihood Estimation

The substitution pattern test was done using the sequences. The performed test is, the Maximum Likelihood Estimate of Substitution Matrix (Table 4). This method estimated the probability of substitution from one base to another. In both methods, the sum of the values was made equal to 100 and the nucleotide frequencies were 32.39% (A), 41.86% (T/U), 12.30% (C), and 13.45% (G). According to the test, the Cytosine (C) was the most conservative nucleotide by showing substitution changes.

Table 4. Maximum Likelihood Estimate of Substitution Matrix

	A	**T**	**C**	**G**
A	-	*9.47*	*2.78*	**1.24**
T	*7.33*	-	**11.48**	*3.04*
C	*7.33*	**39.06**	-	*3.04*
G	**2.99**	*9.47*	*2.78*	-

Each entry is the probability of substitution (r) from one base (row) to another base (column). Substitution pattern and rates were estimated under the Tamura & Nei (1993) model.
Rates of different transitional substitutions are shown in bold and those of transversional substitutions are shown in italics.

In this estimation, maximum transitional substitution occurred between T and C. On the other hand, a maximum value of transversion was similar for T substitution with A and G. The overall transition/transversion bias, R obtained was 0.88. The transition T ↔ C was found to dominate the variation in *A. mellifera*. Transitions occur with a higher frequency than transversions. The analysis involved 27 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 609 positions in the final dataset. Evolutionary analyses were conducted in MEGAX.

Tajima's Neutrality Test

The value of Tajima's D=-1.906734<1 represents that a recent population expansion has occurred in our country that gave rise to genetic variation (Table 5).

Table 5. Results from Tajima's Neutrality Test

M	**S**	**Ps**	**Θ**	**π**	**D**
27	82	0.134647	0.034933	0.017707	-1.906734

m = number of sequences, n = total number of sites, S = Number of segregating sites, ps = S/n, Θ = ps/a1, π = nucleotide diversity, and D is the Tajima test statistic.

Determination of Variable Sites:

MEGA X was used to calculate the total number of Conserved sites, Variable sites, and Parsimony informative sites on the sequences of the acquired samples. There were 569 conserved sites, 40 variable sites and 17 parsimony informative sites among the 18 samples. Including the sequences of 9 reference data, the conserved sites decreased to 527 and variable sites as well as parsimony informative sites increased to 82, 26 respectively. Alignments of the 82 variable nucleotide sites of sample as well as reference data along with their nucleotide position are given in Figure 2. The validity of the sequences was confirmed by GenBank's accession number (MW428209-MW428226) for 18 Bangladeshi samples.

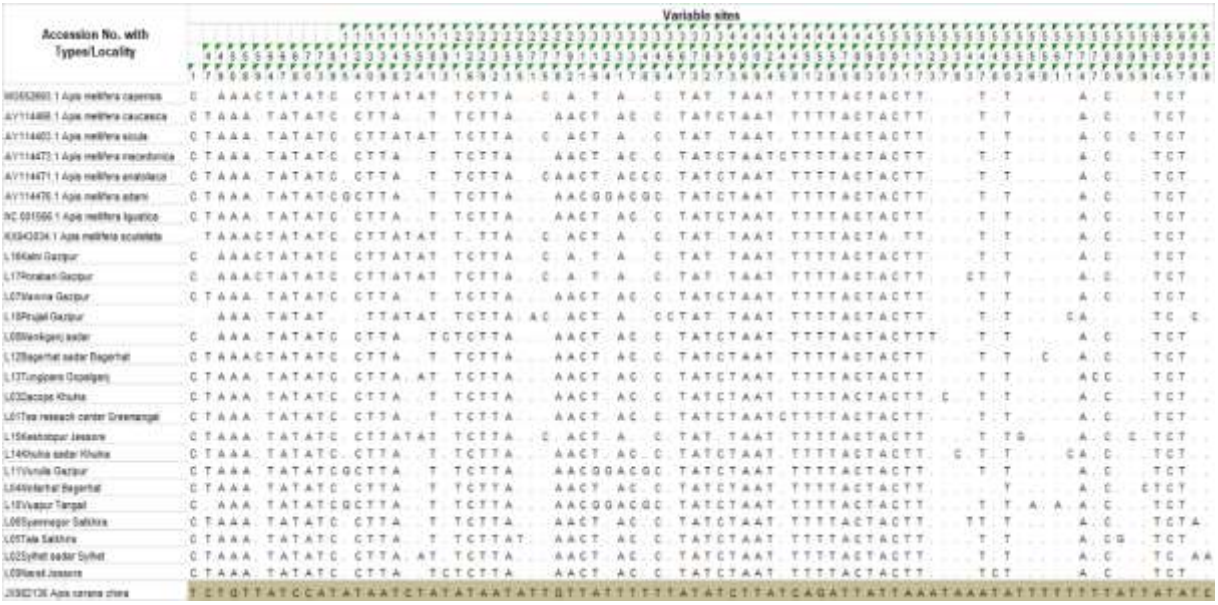

Figure 2. Alignments of the variable sites of nucleotides among the 18 Bangladeshi *A. mellifera* samples with 8 subspecies of *A. mellifera* and *A. cerana* outgroup as references. (Numbers indicating the alignment position above each variable site)

Phylogenetic Study of A. mellifera in Bangladesh

The phylogenetic study of *A. mellifera* in Bangladesh was inferred from both the Maximum likelihood and Neighbor-joining tree using Mega X software. They are presented in Figure 3 and Figure 4. After comparing with the reference data from GenBank, four subspecies were identified in Bangladesh.

This evolutionary history was first inferred by using the Maximum likelihood method and the Tamura-Nei model (Tamura & Nei 1993). The tree with the highest log likelihood (-1360.41) was shown. The bootstrap values of trees in which the associated taxa clustered together was shown next to the branches. This analysis involved 27 nucleotide sequences. There was a total of 609 positions in the final dataset. According to the Maximum likelihood tree topology, sample from Vuapur, Tangail forms the most distinct clade with *A. mellifera adami* with maximum bootstrap value, which also clustered with the sample from Vurulia, Gazipur. Second distinct cluster was seen among the sample from Porabari, Gazipur and Kalni, Gazipur with *A. mellifera capensis*. The sample from Keshobpur, Jessore clustered with the subspecies *A. mellifera sicula* whereas *A. mellifera macedonica* was found in the same cluster with sample from Sylhet in the topology. High bootstrap value supports the proposed topology (Figure 3).

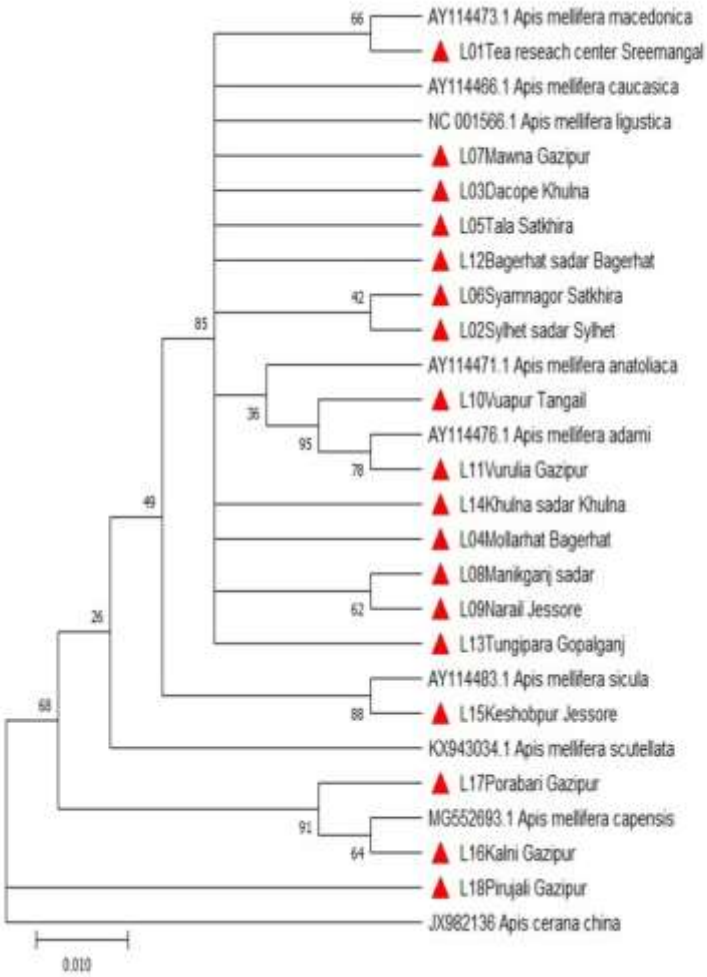

Figure 3. Molecular Phylogenetic analysis by Maximum Likelihood method (The taxon with red triangle denotes the sample from Bangladesh and the samples with accession numbers were used from GenBank for reference. *A. cerana* in the tree was used as outgroup)

The neighbor-joining tree also showed almost similar trends of phylogenetic distribution like the maximum likelihood tree topology. Moreover, it clearly indicates the occurrence of certain subspecies with high bootstrap value to some particular locality of the country. Variation in this tree topology was observed in case of *A. mellifera anatoliaca*, which was found in the same cluster with sample from Vuapur, Tangail and Vurulia, Gazipur (Figure 4). The results of this study denote the divergence type of this European honeybee as mentioned by the Bouga et al. (2005) and Cánovas et al. (2008).

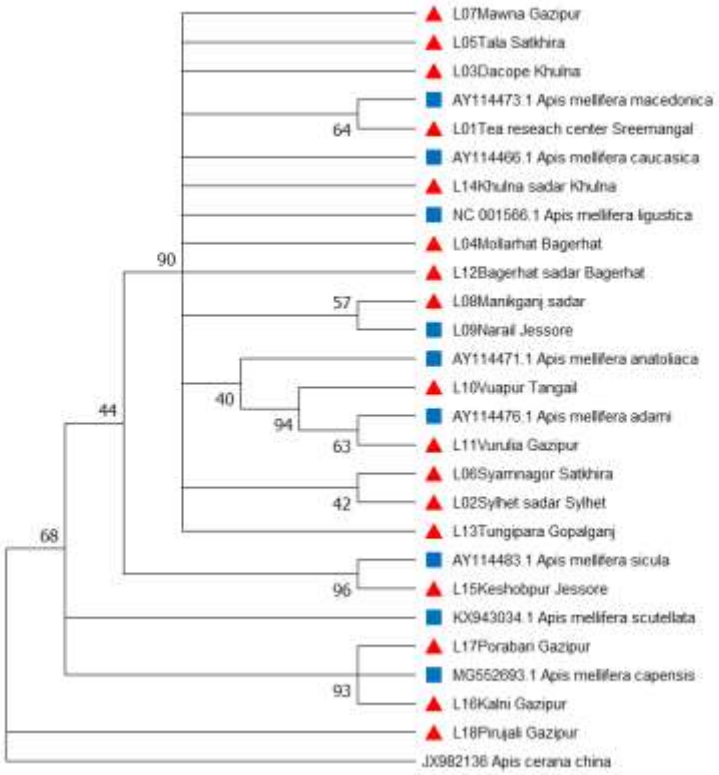

Figure 4. Evolutionary relationships of taxa by Neighbor Joining tree. (The taxon with red triangle denotes the sample from Bangladesh and the samples with blue square and accession numbers are used from GenBank for reference. *A. cerana* in the tree was used as outgroup)

Distribution of A. mellifera Subspecies in Bangladesh

The distributions of different subspecies of *A. mellifera* from 18 localities of Bangladesh are shown in Figure 5. A total of four subspecies were detected from 18 localities. *A. mellifera sicula* was observed in L15, *A. mellifera adami* in L10 and L11, *A. mellifera capensis* in L16 and L17 and *A. mellifera macedonica* on L01.

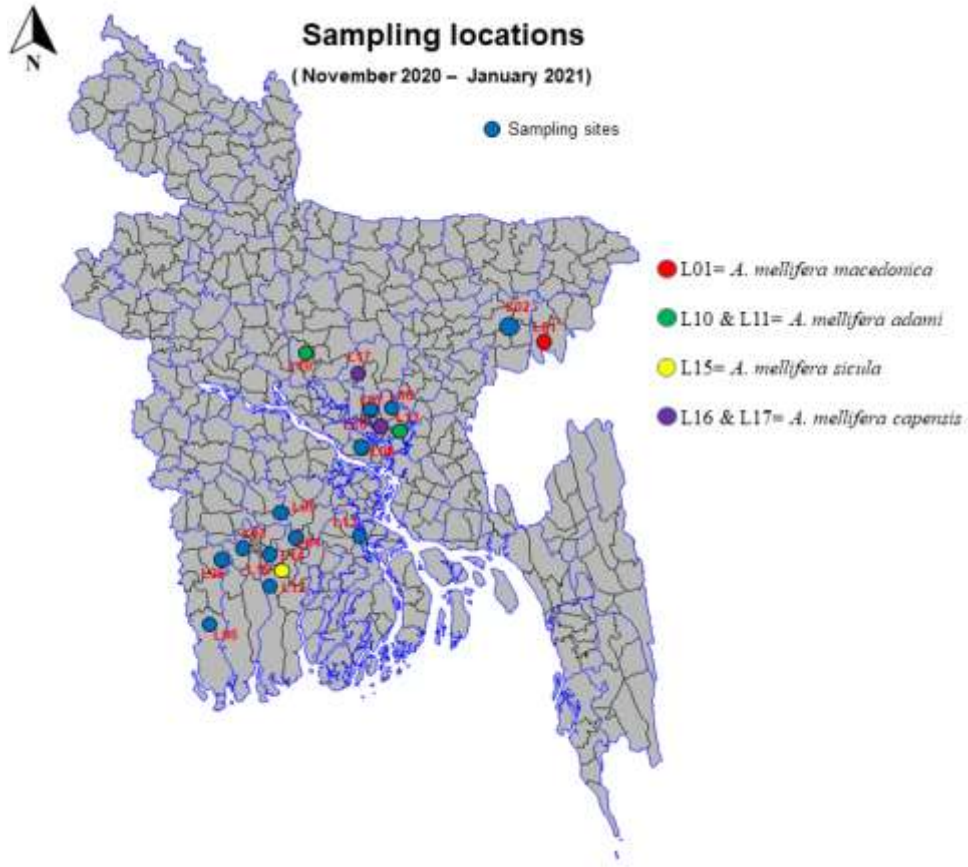

Figure 5. The distribution pattern of *A. mellifera* in the selected areas of Bangladesh

Haplotype Diversity and its Networking Pattern of A. mellifera in Bangladesh

A total of 18 haplotypes (COIH01 - COIH18) were identified in this study out of which, fourteen were unique (COIH05-COIH18). The detailed list is provided in Table 6. Sample from 4 locations collapsed with the reference sequence.

The results showed that the samples from Kalni, Gazipur represent *A. mellifera capensis*, haplotype from Vurulia of Gazipur represents *A. mellifera adami*, and the sample of Mawna, Gazipur showed most diverse haplotype pattern that represent both *A. mellifera caucasica* and *A. mellifera ligustica*. The haplotype of *A. mellifera* sample from Bangladesh Tea Research Institute of Sylhet represents *A. mellifera macedonica*. The rests of the samples were unique haplotype identified from Bangladesh.

The networking pattern of the identified haplotypes were presented in Figure 6. Long mutational gaps were found between the samples from Gazipur and Jessore to the other localities of the country.

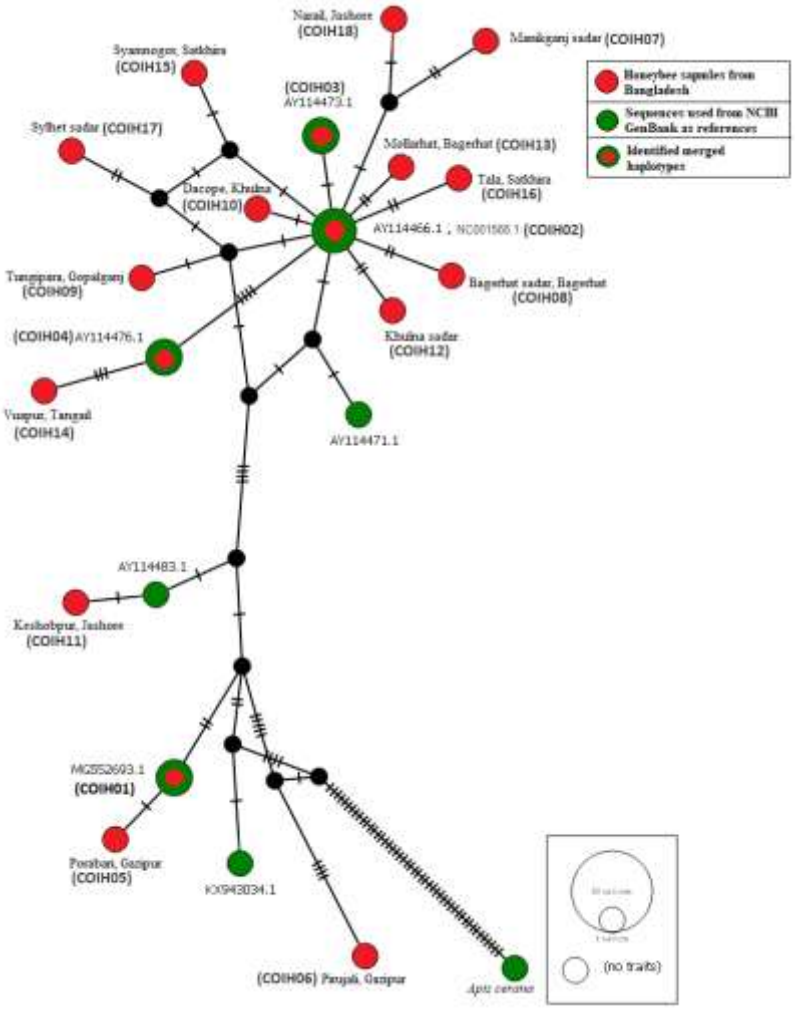

Figure 6. Haplotype diversity and networking pattern of *A. mellifera* in Bangladesh

Table 6. Available haplotypes of *A. mellfera* and their distribution in Bangladesh (The bold letter denotes the haplotypes from Bangladesh)

Haplotype Number	**Locality and Subspecies Name**
COIH01	Kalni, Gazipur (MG552693.1 *Apis mellifera capensis*)
COIH02	Mawna, Gazipur (AY114466.1 *Apis mellifera caucasica*, NC 001566.1 *Apis mellifera ligustica*)
COIH03	Bangladesh Tea Research Institute, Sreemangal (AY114473.1 *Apis mellifera macedonica*)
COIH04	Vurula, Gazipur (AY114476.1 *Apis mellifera adami*)
COIH05	Porabari, Gazipur
COIH06	Pirujali, Gazipur
COIH07	Manikganj sadar
COIH08	Bagerhat sadar, Bagerhat
COIH09	Tungipara, Gopalganj
COIH10	Dacope, Khulna
COIH11	Keshobpur, Jossore
COIH12	Khulna sadar, Khulna
COIH13	Mollarhat, Bagerhat
COIH14	Vuapur, Tangail
COIH15	Syamnagor, Sathira
COIH16	Tala, Satkhira
COIH17	Sylhet sadar, Sylhet
COIH18	Narail, Jossore

DISCUSSIONS

Although migratory beekeeping is practiced in Bangladesh, there is no record of importing specific *A. mellifera* subspecies for apiculture, yet four different subspecies were observed in different regions. The variation in this natural distribution pattern may be the result of the introduction of improved technology in modern beekeeping. In Bangladesh, migratory bee keeping is followed by apiculturists. In this process, the importation of foreign queens and the movement of hives in the short interval from one location to another location for pollen, and nectar source can also affect the genetic structure of available local bee species by genetic introgression (Garnery et al. 1998). In India, in the case of the diamond back moth, researchers also suggested almost similar phenomena and according to that study, from the geographical perspective, high rates of migration between populations indicate that dispersal of gene flow over considerable distances is a major factor in the development of genetic variability in the species (Ojha et al. 2016). Another study suggested that the recent migratory beekeeping and commercial breeding plays an important role in genetic differentiation (Bouga et al. 2005). Natural migration of bee species from surrounding regions for climatic adaptation can also contribute to this diverse distribution pattern that is supported by one of the theories of the origin of *A. mellifera* that states the development of climatic adaptations allowed the expansion of honeybee into Europe from Africa (Wilson 1971). The distribution pattern of different subspecies was found interesting and thus, it can be said that, due to geographical and geological aspects such as temperature, humidity, flowering pattern, Bangladesh is the transitional zone of different honeybee species and subspecies. *A. mellifera* populations collected from 18 localities of Bangladesh showed genetic diversity. These localities vary from each other in geographical and environment conditions, as well as pollen and nectar sources, are different. It can be considered as a reason for this variation. Another reason can be hybridization between the resident and introduced bee species that were found to occur in America since 1957 (Collet et al. 2006). According to Han et al. 2012, shared genetic variation between *A. mellifera* subspecies suggests that they were not exposed to a long period of isolation.

When it comes to the pattern of distribution, the central part, mainly Gazipur has the predominance of different *A. mellifera* subspecies and it might be due to the main focusing migratory beekeeping activities by the beekeeper. In Gazipur, where a vast majority of litchi and mustard is grown and thus the availability of nectar and pollen can be seen all year-round with diverged flowering patterns. Beekeepers from different parts of the country migrate there with beehives during the mustard flowering season and so, the chance of occurring diverged bee species in that zone is very high.

In case of haplotype networking, the star like sub networking pattern was observed, that is similar to the haplotype networking pattern observed in case of hypothermal Shrimp along the Mid-Atlantic Ridge (Teixeira et al. 2011). The star like pattern denotes the recent expansion of the populations that is to be distributed from the middle part, namely, Manikganj, Tangail and Gazipur to the Satkhira and Khulna region of the county.

CONCLUSION

In this study, for the first time in Bangladesh, four *A. mellifera* subspecies were identified through mitochondrial DNA analysis. The identified subspecies, *A. mellifera adami* (L10, L11), *A. mellifera sicula* (L15), *A. mellifera capensis* (L16, L17) and *A. mellifera macedonica* (L01), showed the bootstrap value ranging from 60-95% with 26 parsimony informative sites. Total

22 haplotypes were identified. Fourteen haplotypes were discovered to be distinct to Bangladesh within these haplotypes. The haplotype networking pattern proposed a recent expansion of *A. mellifera* subspecies in our country. This is the first molecular approach for characterizing the *A. mellifera* subspecies using *mt*COI region. Addition of Cytochrome b region with the existing data may provide more precious results for subspecies identification. More extensive sampling throughout the country and their detailed nuclear DNA study is suggested to determine the parental composition and hybridization status among the subspecies.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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