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Mitochondrial DNA based molecular phylogeny of Indian honeybee, *Apis cerna* F. in Bangladesh

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Abstract
The Indian honey bees (*Apis cerana*) are considered as an important component of pollination and honey production however, it's genetic diversity with reference to phylogeny is not being studied yet and thus, resulted only the involvement of *Apis mellifera* population in apiculture of Bangladesh. The climatic oscillation influenced the genetic diversity of different honeybee species through its wide range of genetic distribution. This study aims to reveal the mitochondrial DNA based genetic information of *A. cerana* population from Bangladesh. Sampling was executed from 7 localities of Bangladesh based on the foraging sources and analysis were based on the mitochondrial Cytochrome oxidase subunit-1 (COI) gene of 623 bp from 11 concatenated sequences (GenBank accession number: MZ558037-MZ558043).The phylogenetic tree resulted that the *Apis cerana* population from the southern part of country belonging to the clade of *Apis cerana indica* however, the central part of the country has the population structure similar to the *Apis cerana japonica* types. *A. cerana cerana* representing as the ancestral derivatives among the subspecies. This is the first report of occurring two subspecies in Bangladesh. These findings reveal the differentiation and the demographic expansion of *Apis cerana* population in Bangladesh with its divergent gene flow with anthropogenic effects. This genetic information will led to introduce *Apis cerana* species to the beekeeper's of Bangladesh along with the existing *Apis mellifera* population.

Keywords: genetic diversity, *Apis cerana*, phylogeny, mtDNA

1. Introduction
Nowadays, beekeeping for honey production is a successful agricultural activity across every area of the world. Apiculture is closely tied with agriculture as well as the cultural and natural heritage of rural people in Bangladesh. Although beekeeping skills have recently developed in Bangladesh, the selection tactics of modern honeybee species have not incorporated. *Apis cerana* is proven as an effective bee species for profitable beekeeping in many South Asian countries [1]. But primarily European honeybee *Apis mellifera* L. (Hymenoptera, Apidae) is being commonly applied by Bangladeshi beekeepers. *Apis cerana* Honeybees are the economically most precious pollinators for crop-monocultures around the world [2]. Moreover, *A. cerana* is primarily found in the Eastern Asian part of the world, whereas *A. mellifera* is found in the Western European and African regions. The native range of *A. cerana* F. (also known as the Asian honeybee, Asiatic bee, Indian honeybee, Chinese bee, Eastern honeybee, and Fly Bee) extends over temperate and tropical Asia, from Afghanistan to Japan, north into the Himalayan foothills, and south to Indonesia [3, 4, 5] where it has been utilized for thousands of years for honey production and pollination activities [6]. For commercial bee product manufacturers, the eastern honeybee is also a valuable economic resource [7]. Due to the frequent disease pest infestation, usage of vulnerable bee species, and temporal changes, the beekeepers in Bangladesh have various difficulties in optimizing their honey production and pollination activities. *A. cerana* is highly resistant to diseases and also coevolved with the mite *Varroa jacobsoni* [8] and as a result, it grooms more carefully than *A. mellifera*, and it might be able to find dispel the bee mites from their colonies [9]. *A. cerana* nectar is predominant to other nectar since of its long nectar cycle and an assortment of nectar sources [10]. From the perspective of Bangladeshi beekeepers, *A. cerana* can itself be more successful.
Mitochondrial DNA has been usually applied in phylogenetic studies of *Apis mellifera*, because in particular the complete DNA sequence of mtDNA is available for *A. mellifera* [11]. Although it is more likely to be used in *A. cerana* [12, 13, 14, 15].

The recent phylogeography study using mitochondrial *COI* genes [16] reveal the high genetic diversity of *A. cerana* in this subcontinent [17]. Mitochondrial DNA diversity analysis of *A. cerana* revealed two main haplotype groups in India [18]. This molecular phylogeny study will be based on mtDNA that can reveal the genetic diversity of *A. cerana* in Bangladesh and introduce more profitable bee species to Bangladeshi beekeepers to provide nutritional security and pollination operations. In Bangladesh this species of western bees (*A. cerana*) is likely to evolve a potential haplotype. Lineage mixing raises nuclear genetic diversity levels in honeybee populations [19].

The phylogeny of *Apis cerana* in Bangladesh is poorly understood. At the molecular level, only a few attempts have been performed to elucidate the genetic diversity in *A. cerana*. Analyses of morphological variation extend well into the past into the southern Himalayan region and surround [20, 21]. Molecular phylogenetic studies on the eastern honeybee have only recently started and have shown limited results [17]. *A. cerana* derived from numerous locations has shown geographic variation [22]. Two investigations on mtDNA divergence in *A. cerana* populations were undertaken in 2006 and 2007 using samples from colonies spread over mainland China and Hainan Island [23, 24]. According to the study remarkable diversity exists across different geographic populations, and a phylogeny consisting of few intraspecific lineages has been suggested [22, 25, 26]. To understand the vast variety of honeybees, extensive molecular characterization is required [18] and many issues remain unanswered [23, 24]. Smith and Hagen (1996) [27] and Smith *et al*. (2000) [28] surveyed a large sample of eastern honeybees using the noncoding intergenic region of mitochondrial DNA. Phylogenetic analysis of these data showed a significant geographical pattern in the distribution of mitochondrial DNA (mtDNA) haplotypes [28]. The mitochondrial genome of insects has become one of the most widely used molecular markers in population genetics, phylogeography, molecular diagnostics and also offers sufficient information on the origins and phylogenetic diversity in honeybees throughout evolutionary time periods [18].

The purpose of this work is to look into the genetic variability and phylogenetic analysis of *A. cerana* at the mitochondrial level utilizing the COI region [29]. The current study aimed to recognize geographical and genetic variability of *A. cerana* using molecular techniques, as well as characterize the connections of *A. cerana* populations with its established subspecies patterns for entomological authentication.

2. Materials and Methods

2.1 Sampling of honeybees and preservation

In light of geography, geological component, accessibility of nectar and pollen sources, *Apis cerana* samples from managed hives and natural sources were collected from 7 localities of Bangladesh. Honeybee samples were collected from the year 2020 to 2021. For each geographic population, 30 bees from each colony were collected. Locality and sample information are given in Table 1. Honeybee specimens were caught directly on the comb and immediately transferred to vials with 99.9% ethanol. Later, all samples were stored at room temperature for DNA extraction.

Table 1: Sampling information's of *A. cerana* honeybee from Bangladesh and the Genbank accession numbers

Locality no.	Type	District Name	Geographical location		Date of Collection	GenBank Accession no.
			N	E		
L01	*A. cerana*	Gazipur	24.000678°	90.425425°	08/09/2020	MZ558042
L02	*A. cerana*	Tangail	24.229360°	90.636213°	18/09/2020	MZ558043
L03	*A. cerana*	Satkhira	22.737114°	89.330343°	25/09/2020	MZ558041
L04	*A. cerana*	Manikganj	23.810167°	90.012817°	14/10/2020	MZ558040
L05	*A. cerana*	Faridpur	23.601870°	89.833422°	04/12/2020	MZ558037
L06	*A. cerana*	Jessore	22.905471°	89.219396°	09/12/2020	MZ558039
L07	*A. cerana*	Moulovibazar	24.489277°	91.750267°	12/01/2021	MZ558038

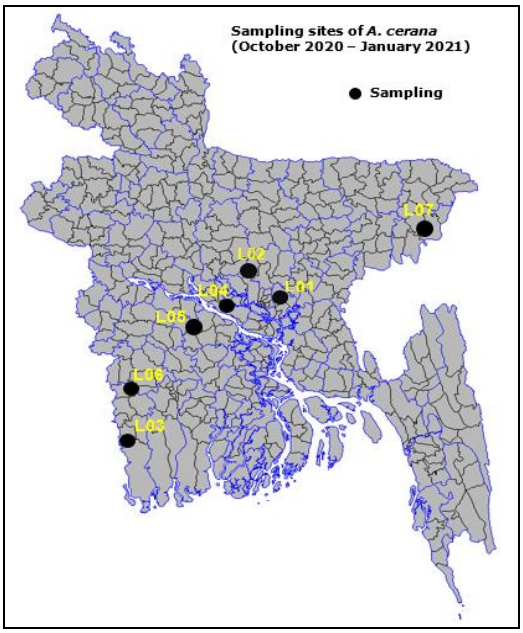

Fig 1: Sampling sites of *A. cerana* in Bangladesh. The details information of the locality is corresponding to the table 1.

2.2 Extraction of DNA

Genomic DNA was extracted from alcoholic preserved specimens by using *QAGEN DNeasy Blood and Tissue kit*, following the manufacturer's instructions. Samples were vortexed with adding 180 µl Buffer ATL and 20 µl proteinase K. Sample was incubated at 550 C for 48 hours. DNA extraction was completed by adding two wash buffer AW1 and wash buffer AW2 and Buffer AE and lysis buffer AL and elusion buffer AE, as per manufacturer instruction. All centrifugation steps were completed at room temperature. The colony mates of the specimens used for DNA analysis were preserved in the Entomology laboratory BSMRAU after DNA extraction.

Amplification of DNA was done by polymerase chain reaction (PCR) *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 contain 20 mM Tris-HCl (pH 8.0), 100 mM KCl, 0.1mM EDTA, 1mM DTT, 0.5% Tween 20, 0.5% Nonidet P-40 and 50% glycerol. dNTP mixtures contains TAPS, KCl, $MgCl_2$, DTT, dATP, dGTP, dCTP with activated salmon sperm DNA. Reaction mixtures for PCR for 50 µ l,

TaKaRa Ex Taq (0.25 µl), 10XExTaq Buffer (5 µl), dNTP mixture (4 µl), a pair of oligonucleotide primers (0.2-1.0 µM; Table 2) and sterilized distilled water (up to 50 µl) will run in Biometra Genomics and Proteomix analyzer with the following instructional status. The list of primers will be followed according to table 2. The molecular analyses were conducted Advanced Entomology laboratory of Bangabandhu Sheikh Mujibur Rahman Agricultural University. 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 as cited by Crozier and Crozier (1993) [13]. The thermal cycling parameters for COI basically followed the protocols established by Crozier and Crozier (1995) and Sameshima *et al*. (1999) [30], including 95 ^{0}C for 5 min for initial denaturation, 35 cycles of dissociation (92 ^{0}C, 1 min), annealing (54 ^{0}C, 1 min), and extension (70 ^{0}C, 2 min). For Enzymatic PCR and sequencing clean-up, Illustra and ExoProStar were followed according to the instruction of the manufacturer GE Healtcare. 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 ^{0}C for 15 min and 80 ^{0}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).

Table 2: Primer information with position

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

[a] (1) Crozier *et al*. 1994 [31], [b] Used for both PCR and sequence

2.3 Submission of the final data to NCBI GenBank
The sequenced data of both mtDNA derived from mitochondrial COI region of *Apis cerana* in Bangladesh was submitted to GenBank of NCBI, for worldwide reference.

2.4 Phylogenetic inferences
For the phylogenetic analysis of *A. cerana* populations, 11 samples for COI genes have been used with 623 bp. In addition, sequence data of COI were used from NCBI were used for references. The sequence data of COI from *A. dorsata* were used as outgroup in this analysis. The sequencing analysis was done by using Vector NTI Advance ver. 11.5 software. The sequences of COI were aligned by using MEGA 7.0 software [31]. Phylogenetic trees were inferred from 11 concatenated matrix sequences COI genes., For the selection of best– fit model, MrModeltest 2.3 was performed with PAUP*4.0b10. The substitution model, GTR + I + G was used in mitochondrial COI genes were used the alignment was partitioned into 1st, 2nd and 3rd nucleotide positions. The nucleotide sequences for COI were deposited in the GenBank with accession number (Table 1).
DNA sequences were aligned using MEGA 7.0 by the multiple sequence alignment program CLUSTAL W [32]. MEGA 7. 0 was used for estimating evolutionary distances and phylogenetic analysis was conducted using both Neighbor-joining tree and Maximum likelihood tree [33, 34, 35, 36, 37, 38]. The reference data were used from GenBank for identifying the suitable *Apis cerana* species in Bangladesh.

3. Results
3.1 Nucleotide diversity
The genetic diversity of *A. cerana* from 07 localities of Bangladesh are presented in Table. 3. A total of 623 concatenated sequences of mitochondrial Cytochrome oxidase subunit 1 gene were used for this study. The sequences of *A. cerana* was used in this analysis for reference to find the consistency of the nucleotide diversity.

Table 3: The nucleotide diversity estimation of the *A. cerana* honeybee samples from Bangladesh

Locality information	Nucleotide diversity				Total
	T(U)	*C*	*A*	*G*	
1.*Apis cerana cerana*	40.9	15.1	33.1	10.9	623
2.*Apis cerana* Indica	40.8	15.2	32.9	11.1	623
3.*Apis cerana* japonica	40.9	15.1	32.9	11.1	623
4. *Apis cerana* Faridpur (BD)	40.8	15.2	32.7	11.2	623
5. *Apis cerana* Moulovibazar (BD)	41.1	14.8	33.1	11.1	623
6. *Apis cerana* Jessore (BD)	40.9	15.1	32.9	11.1	623
7. *Apis cerana* Manikganj (BD)	40.8	15.2	33.1	10.9	623
8. *Apis cerana* Satkhira (BD)	40.8	15.2	32.9	11.1	623
9. *Apis cerana* Gazipur (BD)	40.8	15.2	32.9	11.1	623
10. *Apis cerana* Tangail (BD)	41.1	14.9	32.9	11.1	623
Avg.	40.9	15.1	32.9	11.0	623.0

The average rate of Thiamine was observed as 40.9%, with the 15.1%, 32.9%, and 11.0% for the Cytosine, Adenine and Guanine, respectively as represented in Table 3. The diversity of the nucleotides observed from Bangladeshi samples are corresponding to the overall nucleotide genome sequencing of the *Apis cerana* species in the world except the samples of *Apis cerana* from Moulovibazar and Tangail. Interestingly, 3 *A. cerana* subspecies populations are completely matched with the samples of Bangladesh thus, three subspecies have been identified in Bangladesh sharing the same diversity as mentioned in the reference data from NCBI GenBank.

3.2 Phylogenetic study of *A. cerana* in Bangladesh
The phylogenetic study of *A. cerana* in Bangladesh were

inferred from both Maximum likelihood and Neighbor-joining tree generated by MEGA 7 are presented in Fig. 2 and Fig. 3 respectively.

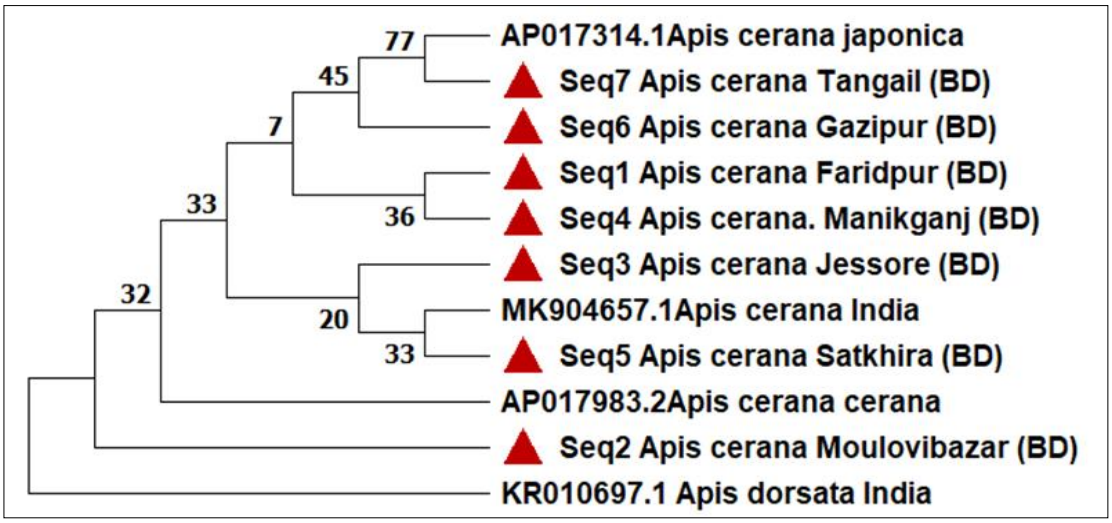

Fig 2: Maximum likelihood tree of *A. cerana* in Bangladesh. The taxon name with red triangle denotes the sample from Bangladesh. The evolutionary history was inferred using Tamura-Nei model [33]. *Apis dorsata* in the tree are used as outgroup.

The tree implied the phylogenetic results of appearing three *A. cerana* subspecies in Bangladesh. The samples from Tangail was identified as *A. cerana* japonica while it was considered as the derivatives of the sample from Gazipur, Faridpur and Manikganj. The samples from Satkhira, the southern part of Bangladesh is clustered with the same branch of *A. cerana cerana* with slight nucleotide diversity changed with the samples from *A. cerana* collected from Jessore. The samples from Moulovibazar, the eastern part of the country provided a bit different results that predicted the chance of occurrence as distant species than the rest samples. Similar results were also observed using the neighbor joining tree (Fig. 3). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. This analysis involved 11 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 623 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

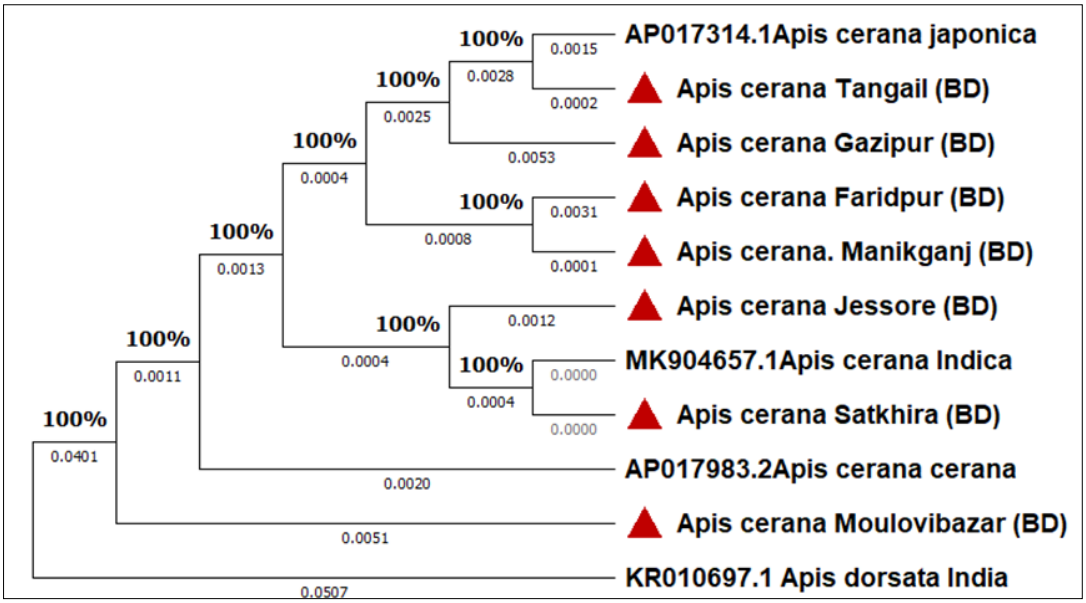

Fig 3: Neighbor-joining tree of *A. cerana* in Bangladesh. The red triangles denotes the *A. cerana* samples from Bangladesh. *A. dorsata* were used as outgroup. 11 Concatenated sequences of 627 bp were used in the analysis to infer the phylogenetic tree. % indicating the ration of data coverage among the internal nodes

3.3 Variable nucleotide sites among three *A. cerana* subspecies of Bangladesh

In Bangladesh, three distinct *A. cerana* subspecies were identified with this study and its nucleotide variables with the reference samples from NCBI GenBank as presented in Fig. 4. The figures denotes that haplotype pattern in the sequences among the Bangladeshi samples.

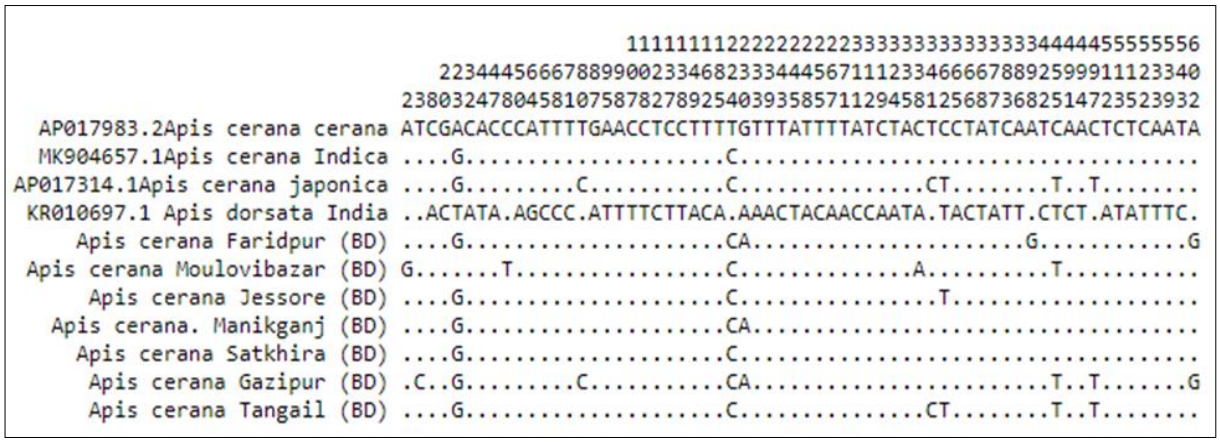

Fig 4: Alignments of the variable sites of nucleotides among the seven Bangladeshi *A. cerana* samples with 3 sub species of *A. cerana* as references. Numbers indicating the alignment position above each variable site

4. Discussion

This study investigated the pattern of genetic diversity among the seven *Apis cerana* populations collected from 7 localities of Bangladesh. Those seven localities have a diverged agri-environmental situation with varied nectarine sources that might be the major consideration for the bee species. As, in Bangladesh, no previous phylogenetic study was performed, hence, this study carried the first significant findings of occurrence three *A. cerana* populations in Bangladesh. In addition, we found three deeply divergent intraspecific lineages while compared with the published reference sequences. These results were correlated and found consistent with the phylogeographic structure proposed by smith (2011) [25] who identified four major mtDA lineages or groups of *A. cerana* (a Mainland Asian group, a Sundaland group, an Oceanic Philipines group and an Indian group). Our findings clearly mentioning the successful occurrences of Indian and SE Asian groups in the country. Bangladesh is often considered as one of the most important transitional zone of genetic diversity of many social insects including honeybees and ants, hence, the findings of this study strongly supporting its divergence pattern in the tropic. However, Hepburn *et al*. (2001) hypothesized that *A.cerana indica* of Thailand, Borneo and Malaysia are certainly not the same bees which occur in the India and Sri Lanka [39]. Under this circumstances, further molecular studies with more detailed extensive sampling are necessary for reconfirming the genetic diversity status of *A. cerana* population in Bangladesh. The recent phylogeographic studies on insects, plants, amphibians or fishes shown the significance of Pleistocene climate changes and biogeographic barriers like mountains, rivers, seas and desserts alter the diversification, radiation and isolation of new genetic lineages within many species [40]. This climatic oscillation played the major role of forming such distribution of species by changing the genetic structure and diversity [41]. Regarding the regions of East Asia, the glacial influence were not so extensive due to influence of monsoons formed by the Pacific Ocean and the presence of biotic zone at higher northern latitude. However, the fossil and biogeographic evidence showed the dramatic effects of climate changes thorough Asia and its impact on the distribution of several animal species is under consideration. The case of the distribution pattern of *A. cerana* population in Bangladesh can be correlated with the distribution pattern of Asian elephants. Vidya *et al*., (2009), explained that in the case of the elephant haplotypes in Myanmar, rather suggested these haplotypes did not arise within Myanmar, but instead resulted from a northward range expansion of beta clade haplotypes during warm period from both Sri Lanka and the Sundaland region followed by subsequent admixture in this region [42].

5. Conclusion

The present study identified the three *A. cerana* subspecies in Bangladesh among the 7 identified samples. The southern part of the country are dominating with the haplotypes corresponding to *A. cerana indica*, and the central part of the country have it's similarity with *A. cerana japonica*. The eastern part of the country, is not so clearly indicating the haplotype occurrences and its flows but it's clustering with the subspecies *A. cerana cerena*. The diversity inferred from mtDNA analysis indicating that Bangladesh with all these three *A. cerena* bee diversity, can easily be considered as the perfect zone for introducing the *A. cerana* honeybee along with the commonly cultivated A. mellifera species in the beekeeping sector to modernize and updating the apiculture sector more profitable.

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