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Deciphering the molecular phylogenetics of the Asian honey bee, *Apis cerana* and inferring the phylogeographical relationships using DNA barcoding

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Abstract
The Asian honey bee, *Apis cerana* are honey producers and pollinators of cultivated crops and wild plants. They occur in Asia, from Afghanistan to China and from Japan to southern Indonesia. *A. cerana* have yellow stripes on their abdomen and are habituated to Indian plains. These are less aggressive and also display less swarming behavior. Here we report the partial sequence of cytochrome oxidase sub unit I gene (COI) of *A. cerana* (GenBank Accession No. KM230116) and its phylogenetic relationship. The COI gene sequence of *A. cerana* showed considerable variation with other *Apis* species. The COI DNA barcode developed in this study can be used for accurate species identification. The COI partial coding sequence of *A. cerana* showed 2.72% difference over 513 bp nucleotides to *A. cerana* isolated from Indonesia (GU191875) and 6.04% to *A. cerana* isolated from Japan (AF153105). *A. cerana* demonstrates the efficiency of the barcoding gene in discriminating global phylogeographical variants among the *Apis* species complex.

Keywords: *A. cerana,* DNA barcoding, phylogeny, cytochrome oxidase.

1. Introduction
The Asian bee, *Apis cerana* (Hymenoptera: Apidae) is found throughout Asia and across a diverse range of climatic zones [1]. The life cycle of Asian bees is very similar to that of *Apis mellifera* and its life cycle completed within 21 days. The colony is structured with a single fertile female (the queen) several thousand worker bees and seasonally, male bees. Mitochondrial genomes are renowned for mutation hot spots or adaptive substitutions which makes the genome more noteworthy, and results in the heterogeneous evolutionary rates across genes [2]. The average rate of evolution of the mitochondrial genome is known to be 5-10 times higher than that of nuclear genome, in case of mammals [3]. The focus of the current study is to decipher the systematic position of *A. cerana* using mitochondrial and nuclear genes in the order Hymenoptera [4].
Studies on the biology and distribution of races of *A. cerana* in China were done [5]. A morphological analysis of *A. cerana* and populations from Southeast Asia has also been taken [6]. Data on comparative morphology of the dwarf honey bees in Southeast Thailand and Palawan, Philippines has been published [3]. Wongsiri *et al.* (1993) carried out a comparative investigation of some biological characteristics of *A. cerana* bees in China, Thailand and their hybrids for the purpose of using biological measures to control *Varroa* parasitic mites [7]. There is a big gap in information on the genetic diversity of Asian native honey bees. The purpose of this paper is to present some preliminary results on comparative genetic composition of *A. cerana* with some honey bee species, to contribute basic information on certain genetic parameters.

2. Materials and Methods
Asian honey bee, *A. cerana* used in the present study was collected from Kottakkal, Kerala on 21st October 2013. Mitochondrial genomic DNA was extracted from one of the thoracic legs of the experimental insect, *A. cerana*. The tissue was homogenized using a glass pestle and mortar. The genomic DNA in the homogenate was extracted using GeNei Ultrapure Mammalian Genomic DNA Prep Kit (Banglore GeNei, Banglore). About 2 ng of genomic DNA was amplified for mitochondrial cytochrome oxidase subunit (COI) gene using the

forward primer 5'-GGTCAACAAATCATAAAGATATTGG -3' and the reverse primer 5'-TAAACTTCAGGGTGACCAAAAAATCA -3'. The PCR reaction mixture consisted of 2 ng of genomic DNA, 1 µl each forward and reverse primers at a concentration of 2.5 µM, 2.5 µl of dNTPs (2 mM), 2.5 µl of 10X reaction buffer, 0.20 µl of Taq polymerase (3 U/µl) and 11.8 µl H_2O. The PCR profile consisted of an initial denaturation step of 2 minutes at 95 °C, followed by 30 cycles of 5s at 95 °C, 45s at 50 °C and 45s at 72 °C and ending with a final phase of 72 °C for 3 minutes. The PCR products were resolved on 1% TAE-agarose gel, stained with Ethidium bromide and photographed using a gel documentation system. After ascertaining the PCR amplification of the corresponding COI fragment, the remaining portion of the PCR product was column purified using Mo Bio Ultraclean PCR Clean-up Kit (Mo Bio Laboratories, Inc. California). The purified PCR product was sequenced from both ends using the forward and reverse primers used for the PCR using Sanger's sequencing method at SciGenom Labs Pvt. Ltd, Cochin. The forward and reverse sequences obtained were trimmed for the primer sequences, assembled by using ClustalW and the consensus was taken for the analysis. The nucleotide sequence and peptide sequence were searched for its similarity using BLAST programme of NCBI (www.ncbi.nlm.nih.gov/) and Inter and intra specific genetic diversity were calculated using Kimura 2-parameter model with the pair wise deletion option and the difference in the nucleotide in codon usage partial COI sequence of *A. cerana* was analyzed using MEGA6 software.

3. Results and Discussion

DNA sequence based identification technique has revealed the morphological and ecological traits of many species during larval stages [8, 9, 10]. Gurney *et al.* (2000) reported that closely related species have 90% similarity in the standardized DNA sequence and distantly related species have less than 90% similarity in the same genes sequence [11]. Intraspecific divergence of partial coding fragment of COI gene is very efficient for species identification [11]. The COI sequence of *A. cerana* showed close similarity within the species and considerable variation between the species. Therefore the COI sequence of *A. cerana* can be used for the molecular identification in any stage of life cycle. The PCR of the COI gene fragment of *A. cerana* yielded a single product of 513 bp. The BLAST search using the sequence revealed that the sequence obtained in this study is novel. The partial COI DNA sequence of *A. cerana* (GenBank Accession No. KM230116) showed 2.72% difference with that of *A. cerana* (GenBank Accession No. GU191875) isolated from Indonesia and 6.04% difference with *A. cerana* isolated from Japan (GenBank Accession No. AF153105). The partial COI coding sequence generated in this study showed considerable variation with other species.

The BLAST analysis of 513 bp of the insect *A. cerana* showed significant homology with *A. cerana* from Indonesia. The phylogenetic NJ tree was carried out using MEGA6 software. The NJ tree was constructed based on the multiple aligned sequence data for different *Apis* species. The tree separates the genomes into 3 main clades. All *A. mellifera* species were included in one clade, *A. cerana* species in other clade and *A. koschevnikovi* and *A. florea* in another clade.

The estimated transition/transversion bias (R) is 0.46. The average nucleotide composition across the species is T=42.2%; A=32.4%; C=15.3%; G=10.1%. These results show that analysis based on mitochondrial gene can be useful for unraveling phylogenetic relationships within the species *A. cerana*. The percentage of A+T was higher than that of G+C which reflected further in the codon usage. The second codon position contained 70.9% of AT nucleotides and decreased to 59.7% in third codon position. AT nucleotide composition of *A. cerana* from Kerala was higher than that of Indonesia (Table 1). The branch length of *A. cerana* (Indonesia) (GU191875) was less compared to *A. cerana* Kerala (GenBank Accession No. KM230116), indicating less divergence from its ancestor. The phylogeny analysis using NJ tree revealed the sharing of common ancestor of these two species (Figure 1).

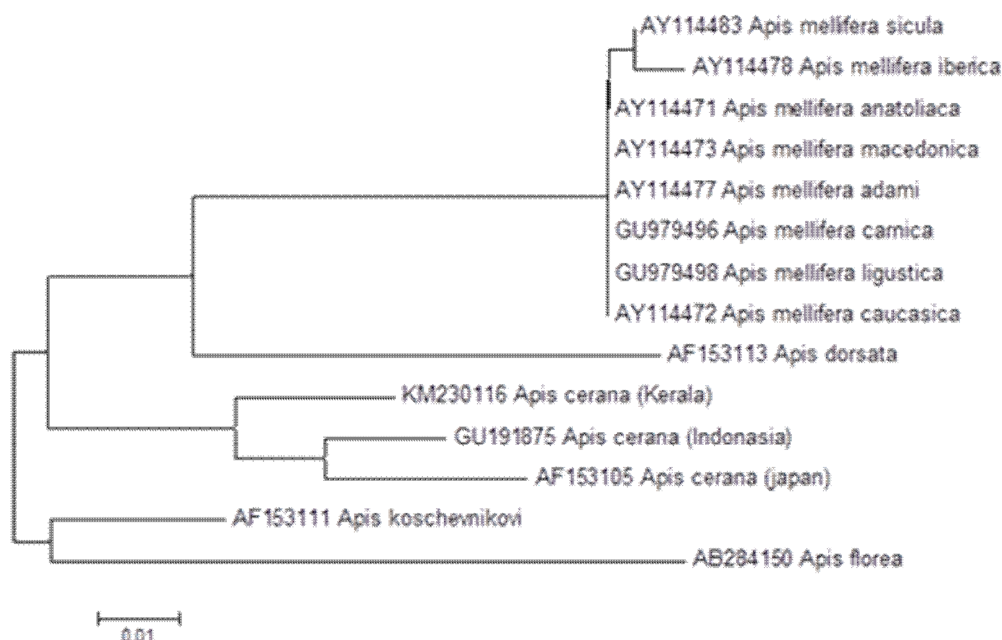

Fig 1: Phylogenetic tree of Apis cerana using NJ method

Table 1: Evolutionary divergence between COI sequences of *A. cerana* with other *Apis* species

Serial No.	Species name with Accession Number	% of divergence
1	*Apis cerana* (Indonesia)(GU191875)	2.72
2	*Apis cerana* (Japan) (AF153105)	6.04
3	*Apis koschevnikovi* (AF153111)	6.53
4	*Apis mellifera ligustica* (GU979498)	8.54
5	*Apis mellifera carnica* (GU979496)	8.54
6	*Apis mellifera adami* (AY114477)	8.54
7	*Apis mellifera macedonica* (AY114473)	8.54
8	*Apis mellifera caucasica* (AY114472)	8.54
9	*Apis mellifera anatoliaca* (AY114471)	8.54
10	*Apis mellifera sicula* (AY114483)	8.93
11	*Apis mellifera iberica* (AY114478)	9.38
12	*Apis dorsata* (AF153113)	10.36
13	*Apis florea* (AB284150)	11.97

4. Conclusion

Most of the phylogenetic studies are based on individual gene or a few genes with similar evolutionary rate or entire mitochondrial genome. DNA barcoding techniques have been used to demarcate the phylogeographical variants. In mitochondrial gene, the phylogeny and phylogeography of *A. cerana* have been resolved. Geographically India is nearest to Indonesia than Japan. So the high intraspecific nucleotide distance observed is due to the geographical isolation of these populations. The phylogenetically close species of *A. cerana* is *A. mellifera*. This region has good discrimination power for *A. cerana*. This study reveals that the former region is capable of differentiating the phylogeographical variation of *Apis* species found in world.

5. Acknowledgements

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