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Phylogenetic variations found in Indian honeybee species, *Apis cerana* Fabr. of North Western Ghats of Maharashtra, India

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Molecular systematics of honeybee species *Apis cerana* Fabr. inhabiting North Western Ghats of India have not been investigated till date. This is the first report of phylogenetic variation in *Apis cerana* bees sampled from five diverse ecotypes of North Western Ghats of Maharashtra, *viz*. Pune, Nashik, Mahabaleshwar, Bhimashankar and Wai. Over the years, taxonomy of honeybee has been mostly based on morphometric characters. In the present study, we carried out molecular phylogenetic analysis of mitochondrial DNA sequence with respect to COI gene. It was further aimed to confirm the taxonomical status of *A. cerana* from the Western Ghats of India in comparison with the Asian populations of *A. cerana*.

Keywords: Cytochrome oxidase I, Molecular phylogeny, Western Ghats

The characteristics of geographically distinct species provide important insight to understand how evolutionary forces led to ecotype polymorphism. The geographical adaptation and distribution of species led to the evolution of polymorphic forms. One of the major problems of the contemporary scenario of biodiversity and systematic research has been to assess geographical variance of population at the intraspecific level. The advent of molecular tools in taxonomy has emerged as a useful tool to resolve such issues[1]. In this context, oriental honey bees attracted the attention of molecular evolutionary biologists due to heterogeneity of climatic conditions. *Apis cerana* Fabr., a keystone pollinator exhibiting wide range of distribution has proved to be a suitable model system to address molecular phylogenetic issues pertaining to its adaptation and evolutionary diversification[2].

Over the years, the taxonomy of honeybees has been investigated and has been classified at the subspecies level on the basis of morphometric analysis. Several studies have been conducted on *A. cerana* from the northeastern part of India including the ranges of the Himalaya based on morphometric parameters[3-7]. However, only a few attempts have been made to reveal the genetic variation in *A. cerana* at the molecular level. Nevertheless, there are various reports about studies from many other parts of Asia in both *A. mellifera*[9] and in *A. cerana*[10,11]. In the past, there have been attempts to assess mitochondrial variation of *A. cerana* from the southern states of India[8,12]. However, sequences generated in these studies are not reported in any of the databases, and thus could not be authenticated. Attempts were also made to identify *A. cerana* using DNA barcoding[13]. However, no such attempt has been made to investigate the phylogenetic variation of *A. cerana* bees from the North Western Ghats of India. It is noteworthy that the Western Ghats of Maharashtra has been identified as one of the biodiversity hotspots in the world [14]. Due to its variation within and between species, mitochondrial gene *Cytochrome oxidase I* (COI) is one of the candidate markers to resolve phylogeny, not only in honeybees but also in several other taxa[15,16]. In this study, we explored the taxonomic and phylogenetic position of *Apis cerana* collected from various regions of the North Western Ghats of India.

Materials and Methods

Collection and submission of sample

The present study was carried out in five different locations of the North Western Ghats of Maharashtra, India, *viz.* Pune, Nashik, Mahabaleshwar, Bhimashankar and Wai (Fig. 1). Locality data and sample codes are given in Table 1. These honeybee colonies were wild and captured from natural colonies and later reared in hive boxes in that location itself (study spot). The colonies reared in hive boxes have less probability of dessertation and easy to maintain and handle.

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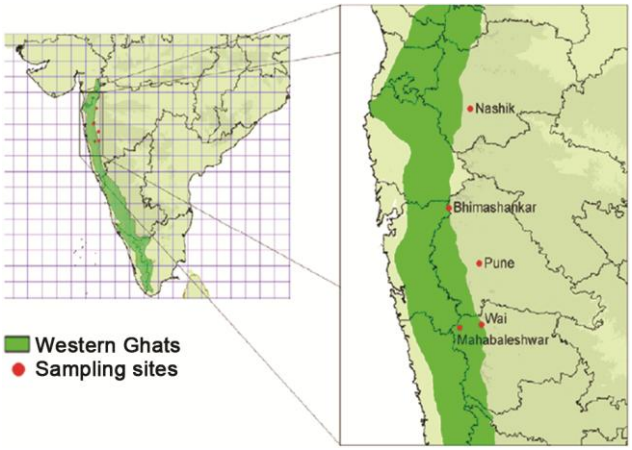

Fig. 1 — Map* representing the sampling site in Western Ghats of Maharashtra, India.

Therefore, the above mentioned in-site rearing was done using hive boxes. Adult worker bees were collected from hive boxes of *A. cerana* from the sampling sites mentioned in Table 1. The samples were preserved in absolute alcohol and stored at 4°C in laboratory. The bees were submitted later to Zoological Survey of India, Western Regional Centre, Akurdi, Pune. The submitted samples were assigned a permanent voucher number (Ent. 6/533 and Ent. 6/534).

DNA isolation and sequencing

The preserved worker bees were removed from alcohol and dried using tissue paper to remove excess alcohol. Few dorsal (tergal) plates were removed and alcohol was allowed to evaporate from the sample. The worker bees were dissected without saline. Tissue isolated from abdominal plates were used for isolation of DNA. Commercial DNA isolation kit by MO-BIO, U.S.A. was used. The isolated DNA was electrophoresed on 1% agarose gel and was confirmed by ethidium bromide staining and also spectrophotometrically. Subsequently, the eluted DNA was used for sequencing (3730 DNA analyzer, ABI, Hitachi). Primers[17] were used for the amplification of COI genes were: LCO1490:5'-GGTCAACAAATCATAAA GATATTGG-3' and HCO2198:5'-TAAACTTCAGGGT GACCAAAAAATCA-3'. PCR reaction was carried out in total volume of 25 µL containing 2 µL DNA template, 10 pmol of each primer and 200 µM of dNTP and 0.2 µL of Taq polymerase (Bangalore Genei, India). Thermocycling conditions involved: one initial cycle of 5 min at 94° followed by 35 cycles of 94° for 1 min, 46° for 1 min 30 s, 72° for 1 min 15 s, with final step of 72° for 5 min. The PCR products obtained were checked on 1% agarose gel

Table 1 — Sampling sites with co-ordinates and their code

Ecotype	Coordinates	Code
Nashik	$20^0$00' N $73^0$47' E	N1
Pune	$17^0$55' N $72^0$43' E	P1
Bhimashankar	$19^0$4' N $73^0$32' E	B1- B3
Mahabaleshwar	$17^0$00' N $73^0$40' E	M1-M2
Wai	$17^0$93' N $73^0$9' E	W1

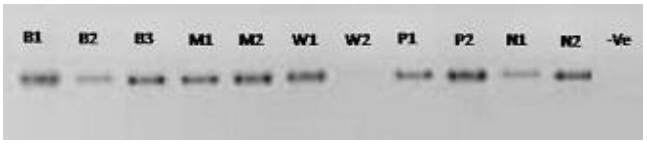

Fig. 2 — PCR products of COI gene amplification of honey bee samples resolved in 1% agarose gel. The sample codes (also refer Table 1 for correspondence): Bhimashankar 1 (BI), Bhimashankar 2 (B2), Bhimashankar 3 (B3), Mahabaleshwar 1 (M1), Mahabaleshwar 2 (M2), Wai ((W1), Wai (W2), Pune (P1), Pune (P2), Nashik (N1), Nashik (N2), Negative control (-ve).

and were purified using PEG-NaCl method (Fig. 2). These PCR products were sequenced with both forward and reverse primers using an automated sequencer, these are the same primers which was used for PCR. The sequences obtained were edited through ChromosPro 3.1 software, and a Fasta format was generated.

Results and Discussion

Analysis of sequences

The Fasta format generated after sequencing was used for BLAST search at NCBI and species identification tool at BOLD[18]. Accession numbers of the sequences uploaded to NCBI are: Nashik (N1- KP255460, 648 bp); Pune (P1- KP255461, 627 bp); Bhimashankar (B1- KP255462, 648 bp); Bhimashankar (B2- KP255463, 643 bp); Bhimashankar (B3- KP255464, 620 bp); Mahabaleshwar (M1- KP255465, 648 bp); Mahabaleshwar (M2-KP255466, 640 bp) and Wai (W1- KP255467, 643 bp). Conspecific sequences of the *A. cerana* were also downloaded from the database. Neighbour Joining (NJ) clustering analysis was performed using MEGA 6 software. *A. mellifera* was used as an out group. The sequence of about 600 bp was obtained, with no indels or stop codons in it. BLAST analysis and BOLD identification tool for all samples showed 99% similarity with *A. cerana indica* (GQ162109) from the database suggesting correct identification.

The present study is the first attempt to resolve the phylogenetic position of *Apis cerana*. NJ (Neighborhood Joining) clustering analysis based on COI gene showed monophyly of the *A. cerana*.

Clustering analysis further showed five ecotypes with the South East Asian clade. The specimens studied here were closely related to the haplotypes from the South East Asian countries. Further, distinct clades were formed by the haplotypes from Taiwan, Malaysia and Southern India (Clade I and Clade II). Comparative analysis of the samples revealed high nucleotide divergence (>3%) of these haplotypes. Previous studies based on COI gene sequences (Hebert *et al.*[1]) showed interspecies nucleotide divergence of more than 3%. Therefore, specimens investigated in the present study represent possible cryptic species that warrants further studies. The specimens studied here revealed five haplotypes of *A. cerana* belonging to respective geographical regions. The samples from the five ecotypes were clustered into South East Asian clade (Fig. 3). The samples from the Mahabaleshwar and Wai ecotypes were placed in one group. The Wai ecotype also showed similarity with sequences (KF861941 and KF760521) from the South Indian bees. The Pune and Bhimashankar ecotypes were placed in one group while the Nashik ecotype formed a separate group. NJ clustering analysis showed monophyly of *A. cerana* studied in these ecotypes. The data from the phylogeny has further confirmed that the species is *A. cerana*, when compared with the COI haplotypes of the other Asian honeybees. The three groups formed in the phylogenetic tree were based on their geographic locations, i.e. how far they would be separated from each other. The Mahabaleshwar and Wai site formed one group due to their close proximity; similarly, the Pune and Bhimashankar formed another group, while the Nashik location got isolated from the rest of the groups. The distance between Nashik and Bhimashankar ecotype was more as compared to the Bhimashankar and Pune ecotype. Pune and Bhimashankar forming one group could be due to migration of colonies from Pune to Bhimashankar and through transfer of colonies by the beekeepers for commercial purpose, which might have led to the mixing of colonies in these two

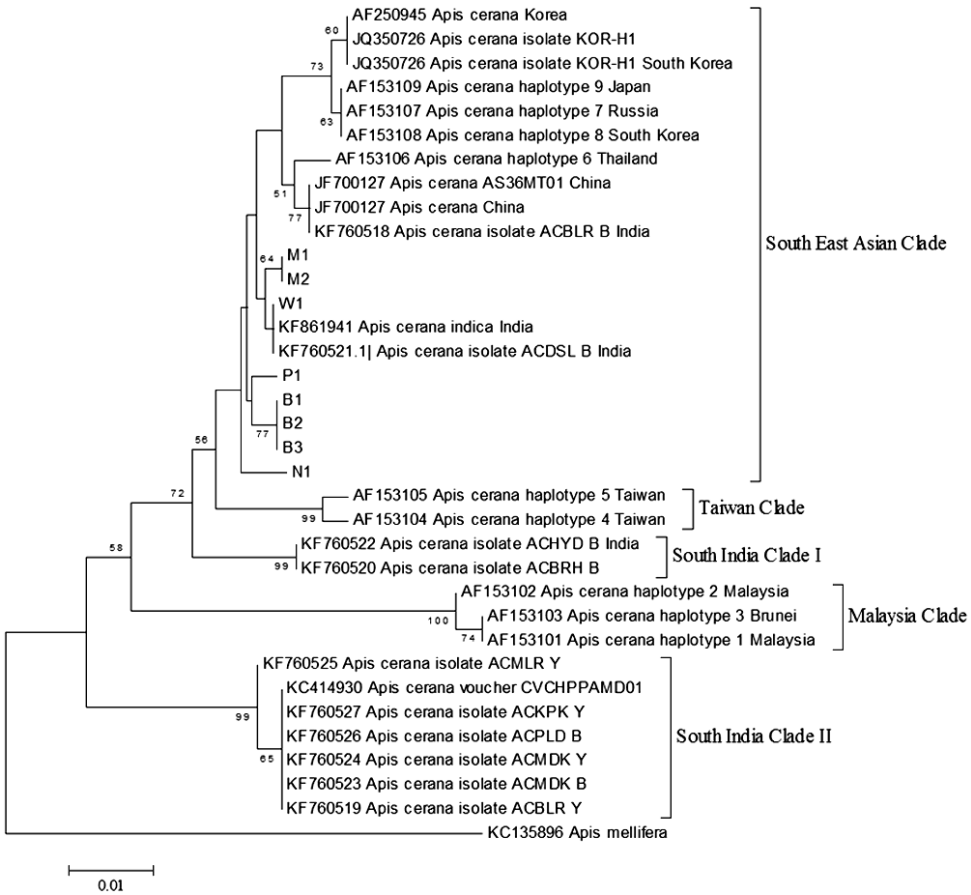

Fig. 3 — Clustering analysis of *A. cerana* based on COI gene sequences. *A. mellifera* has been used as an out group. Distinct clades can be seen belonging to respective geographic region (shown on the right of each clade). The clustering analysis was performed using MEGA 6 software.

ecotypes. The present study bears significance for being the first attempt to investigate the phylogenetic variation of *A. cerana* inhabiting the North Western Ghats of India. Our study confirmed that the species of these bees are *A. cerana*. However, when sequences from other regions of the world were compared with the samples studied here, they showed cryptic divergence suggesting further investigation of the Asian population of *A. cerana*.

From a global perspective, honey bee population is under severe ecological crisis[19]. The impact has also been realized in the altered population dynamics and genetic diversity of wild population of Indian *A. cerana*[20]. These issues provide impetus to carry out future molecular phylogenetic investigations of *A. cerana* population from other parts of India and provide insights for microevolutionary trends under prevailing ecological regime.

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