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Assessment of Genetic Diversity Index in Feral Colonies of *Apis* Species within Different Eco-Habitats of Bengaluru

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ABSTRACT: The objective of our study was to screen the extent of genetic variability and phylogenetic relations among the three kinds of honey bee species from Bengaluru at different habitats. The Asian honey bee species i.e., *Apis cerana indica, Apis dorsata* and *Apis florea* collected from Bengaluru were studied by using partial sequences from two mitochondrial genes (i) the Cytochrome c oxidase-I (COI) and (ii) the mitochondrial encoded NADH dehydrogenase 5 (NADH5). We then compared their sequences with already submitted sequences from national centre for biotechnology information (NCBI) to deduce the genetic variations among the species. We also studied the rate of mutations across the species by polymorphism studies. The amplified sequences were analysed for evolutionary genetic analysis using neighbor-joining method and Kimura 2-parameter model. Our study confirmed that, there was less genetic variability in *A.florea* species which might be due to its selective pressures. *A.cerana indica* on the other hand was found to consists of more mutations. Throughout our study we found bees within the residential areas showed significant mutation rates than the other habitats which was seen among all the three bee species. Though the significant effect was not remarkable, but still this variability could answer many of the genetic problems of existing and evolving in harsh conditions.

KEY WORDS: *Apis florea, Apis cerana indica, Apis dorsata*, phylogenetic tree, Genetic variability, Polymorphisms

INTRODUCTION

In biology, diversity refers to the variety of organisms that live on all types of surfaces, including terrestrial, marine, and aquatic ecosystems (Harper and Hawksworth, 1994). Ecosystem diversity includes the diversity within and between species. The concept of biological diversity describes all the organisms and their complex ecological relationships within the environment, as well as all ecological processes that contribute to these organisms (Primack,1993).

Organisms are often identified by DNA barcoding. During this procedure, mitochondrial DNA of the COI gene is amplified and sequenced. Nucleotide sequences obtained from the Genome Database and Bold system are compared to those deposited in the databases. While highly conserved in the same taxon, this DNA fragment varies greatly between taxa. While DNA barcoding has been widely accepted, its use for the identification of honey bee subspecies has been limited. In addition to their economic, nutritional, and ecological functions (the bees pollinate a wide range of crops and flora), honey bees (*Apis* spp.) deserve a thorough classification. Currently, 10 species of Apis have been identified, namely i.e. *A. andreniformis, A. binghami, A. cerana, A. dorsata, A. florea, A. koschevnikovi, A. laboriosa, A. mellifera, A. nuluensis*, and *A. nigrocincta* (Arias and Sheppard, 2005).

It is possible to study better the genetic diversity and gene pool of insects by using various molecular marker techniques, and this will allow questions about how to distinguish species based on their morphological characteristics to be answered more clearly. Insect ecological studies have mainly employed mitochondrial DNA, random amplified polymorphic DNA, microsatellites, and amplified fragment length polymorphism markers as relevant molecular techniques (Behura, 2006).

Unlike bees which are classified primarily based on morphology, genetic variation is observed by measuring mitochondrial DNA gene segments (Branchiccela *et al*., 2014; Ostroverkhova *et al.,* 2015), SNPs (Chapman *et al.,* 2016), and allozymes (Smith and Glenn, 1995) to separate races among species. In order to measure the extent of genetic difference between the different honey bee species, molecular analysis is a compelling tool (Meemongkolkiat *et al.,* 2019). Molecular markers, such as mitochondrial genes, are commonly used to study the extent of genetic diversity in insects (Bouga *et al*., 2011) since mitochondrial genes have ten times the mutation rate of nuclear genes (Ballard and Whitlock, 2004).

A. cerana, *A. dorsata*, and *A. florea* are native, while *A. mellifera* is imported (Khan, 2020; Sajid *et al*., 2020; Shakeel *et al.,* 2020). Research on the Asian and western honey bee species found in the country has been very sparse in terms of studies on morphology and molecular biology. Although in honey bee species molecular studies were done based on mitochondrial gene segment i.e., cytochrome c oxidase I (Rizwan *et al.,* 2018) but still polymorphism data was not reported.

The present study was designed to explore the extent of the genetic variability and phylogenetic inference among three kinds of honey bee species (*A. cerana, A. dorsata, and A. florea*) across five different habitats (Micro-forest, Botanical garden, Residential area, industrial area and public parks) from Bengaluru. Successfully amplified the partial sequence of COI and ND5 from honey bee samples. The amplified fragments were sequenced and analysed using BLASTn and phylogenetic variability along with polymorphisms were screened to assess the number of genetic variations.

METHODS

The three honey bee species i.e., *Apis cerana indica*(the Indian honey bee), *A. dorsata* (the giant/rock honey bee), and the *Apis florea* (Dwarf bee) collected from different habitats in and around Bengaluru were studied using partial sequences from two mitochondrial genes Cytochrome *c* oxidase I (COI) and NADH dehydrogenase 5 (NADH5) and then sequentially compared with other honey bees sequences from the National centre for biotechnology information (NCBI).

Extraction of genomic DNA: Freshly emerged adult bees were collected from honeycombs and were frozen at -20°C. Body segment thorax was used for the gDNA extraction. Thorax region dissected was placed in a centrifuge tube containing BSA (250 mg/ml) and 3.2mm stainless steel beads (2-3nos). The tissues were then homogenized by a homogenizer. 285µl of 6M NaCl was added to the contents and homogenized thoroughly for 5min. following homogenization, the contents were centrifuged at maximum speed and supernatant was collected [Khalid Ali Khan, 2021]. To the supernatant 3 volumes of 20% SDS was added and vortexed. The contents were then centrifuged at maximum speed for 20min at 4°C. Aliquots of 150µl of cleared supernatant was then added with ice cold isopropanol to precipitate the DNA. The tubes were centrifuged to pellet the DNA and the DNA pellet was dissolved in 10x TE buffer (100mMTris HCl, 25mMEDTA). Resuspended DNA was then checked for purity in UV spectrophotometer (Shimadzu 1800).

PCR was executed with an Eppendorf MasterCycler Personal cycler. Each PCR reaction mixture contained 2.5µL of 10× reaction buffer (HiMedia), 1µL of 10mM dNTPs, 1µL of 10µM forward primer, 1µL of 10 µM reverse primer, 3µL of 25mM Mg^{2+}, 2µg of template DNA, 2.5 units of thermostable Taq DNA polymerase (HiMedia), and deionized water (up to 25µL). PCR regime included initial denaturation at 95^0C for 4 min; 35 cycles of denaturation at 95^0C for 20s, annealing at 53^0C for 45s, elongation at 72^0C for 90s; and final elongation at 72^0C for 10min.

Table 1: A list of primers used in the study.

Gene Name		**(5'->3')**	**Length**	**Tm**	**GC%**	Product Length	**Reference**
COI	FW	GGTCAACAAATCATAAAGATATTGG	20	60.4	50	450	Vrijenhoek, 1994
	RV	TAAACTTCAGGGTGACCAAAAATCA	20	58.7	50		
NADH5 (AD)	FW	ATTCAAATTTGCAACTAAACCA	24	59.8	55	510	Self designed
	RV	GAAACTGTAAAAATAGTTCCAACA	21	59.8	55		
NADH5 (AC)	FW	GAACTGTAAAAATAGTTCCAACA	19	60.2	55	440	Self designed
	RV	GGATGAGATGGTTTAGGATT	24	60.2	50		
NADH5 (AF)	FW	ATGTTGAATAAGCAACAACCT	20	59.67	55	505	Self designed
	RV	GGTTGAGATGGATTAGGATTA	20	59.7	50		

PCR products were purified from an agarose gel with a Clean-up Standard kit (HiMedia) and sequenced with an Applied Bio systems 3500 genetic analyser using the Big Dye Terminator v3.1 Cycle Sequencing Kit using the same DNA barcoding primers. Sequence alignment was performed with the Clustal Omega tool (The European Bioinformatics Institute, Hinxton, UK, http://www.ebi.ac.uk/Tools/msa/clustalo).

A neighbor-joining tree was constructed using the Kimura 2-parameter method [Saitou, 1987] in MEGA7. A phylogenetic tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Jones-Taylor-Thornton (JTT) model was used in both the cases with Site Coverage Cut-off at 95%. The percentage of duplicate trees in which the related taxa clustered together in the bootstrap test (500 replicates with pairwise deletion of missing/gap data and inclusion of all replacements (transversions and transitions)) are showing in next to the branches. *Apis dorsata (*GI1772614101*), Apis cerana* (GI1619282822) and *Apis florea (*GI1787270937) were chosen as the outgroup.

Genetic diversity: Mitochondrial gene diversity was studied on two gene segments among the three honey bee species in the study and also across the habitats. The study was carried out using the DNA sequence polymorphism Dna SP v 5.10 (Librado and Rozas, 2009).

RESULTS

Three bees of each species at different habitats were initially studied. We performed classical DNA barcoding using the primers specified in the table. We amplified a total of 45 nucleotide sequences which are aligned using the MEGA7 software, to deduce the phylogenetic relation and species confirmation.

For mitochondrial COI gene fragment, there was 98.85% (total score 1543; E value = 0.0–0.0), 96.57% (total score 752; E value = 0 .0–0.0), and 99.05% (total score 1203; E value = 0.0–0.0) nucleotide identity for *A. dorsata*, *A. cerana*, and *A. florea* sequences respectively. For mitochondrial ND5 gene fragment, there was 97.85% (total score 1290–1345; E value = 0.0–0.0), 94.55% (total score 734–870; E value = 0 .0–0.0), and 98.19% (total score 765–771; E value = 0.0–0.0) nucleotide identity for *A. dorsata*, *A. cerana*, and *A. florea* sequences respectively.

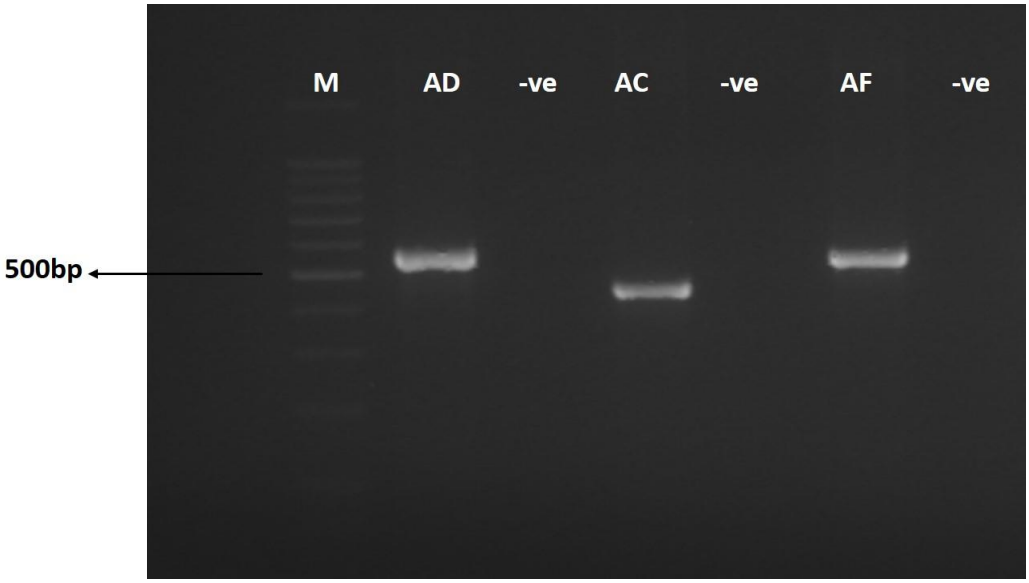

Figure 1: Agarose gel showing the amplified fragments of NADH for the 3 species in the study. AD: *Apis dorsata*; AC: *Apis cerana*; AF: *Apis florea*

The amplified fragments were run on 0.8% agarose gel and showed bands approximately at expected molecular size. The bands were shown at 510, 440 and 505bp for *Apis dorsata, Apis cerana* and *Apis florea* respectively [Figure].

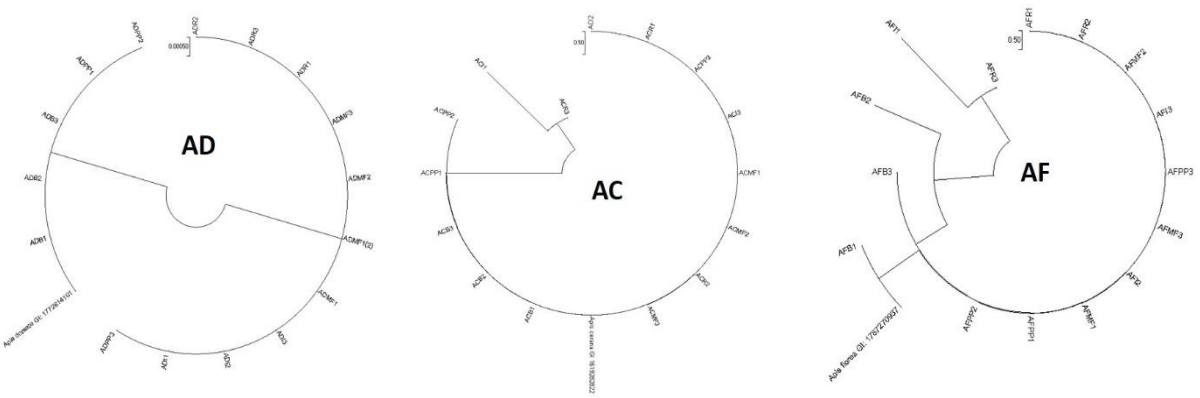

Figure 2: Image showing the evolutionary tree of the DNA sequences using the Neighbor-Joining method [Jones DT, 1992]. The distances were calculated using the Maximum Composite Likelihood method and analysis involved 40 nucleotide sequences. All the gaps and missing data were removed and the final dataset contained about 630, 741, 697 positions for AD, AC and AF respectively. Phylogenetic analyses were done with MEGA7 [Kumar S, 2016]. AD: *Apis dorsata*; AC: *Apis cerana*; AF: *Apis florea*.

Phylogenetic tree: The phylogenetic tree built using MEGA7 showed close relation with their respective strains. *Apis dorsata, Apis cerana* and *Apis florea* sequences from NCBI data base were used as reference sequences with their respective counter parts. All the sequences showed similarity without much substitutions stating the minimal variations within the species. This might be due to the narrow range of geographical distribution [Figure 2 and Figure 3].

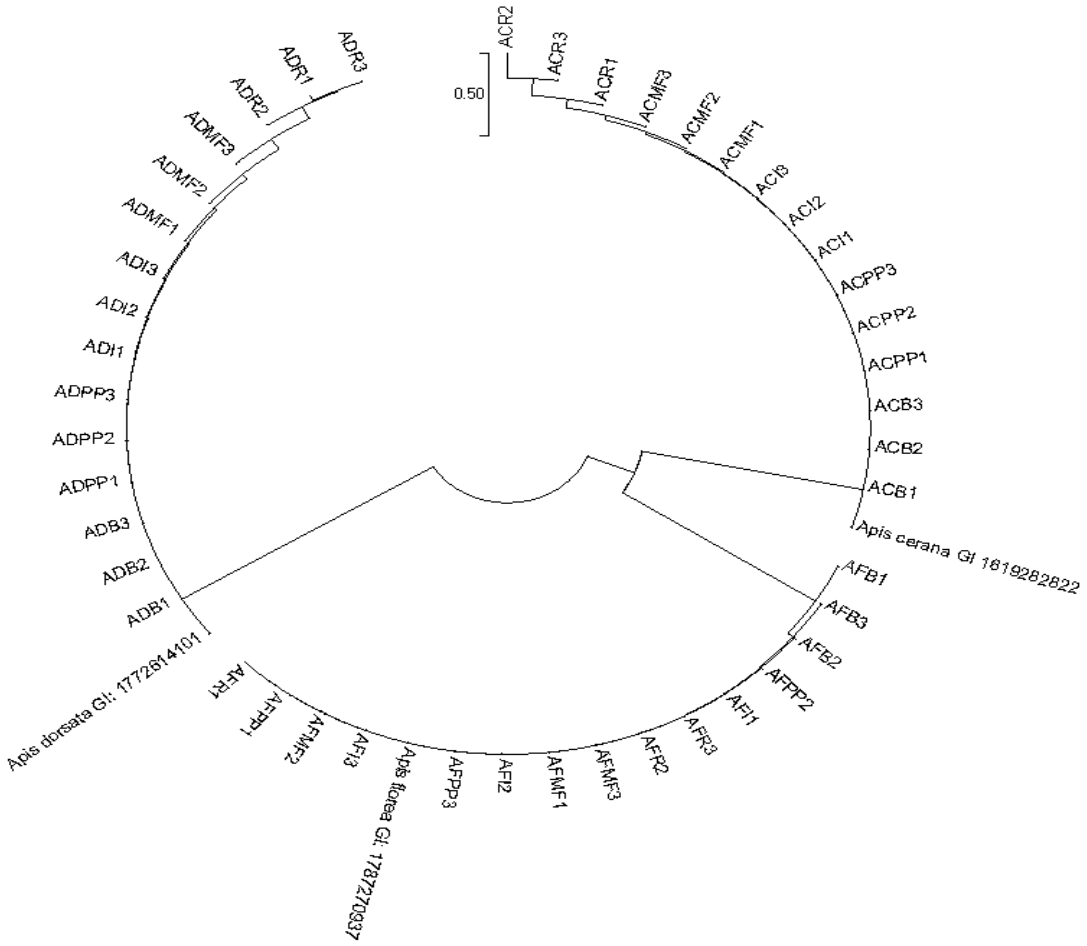

Figure 3: Image showing the evolutionary tree of the DNA sequences using the Neighbor-Joining method [Jones, 1992]. The optimal tree with the sum of branch length = 4.92720112 is shown. The distances were calculated using the Maximum Composite Likelihood method and analysis involved 40 nucleotide sequences. All the gaps and missing data were removed and the final dataset contained about 562 positions in the final dataset. Phylogenetic analyses were done with MEGA7 [Kumar, 2016].

Genetic diversity among honey bee species: The three honey bee species i.e., *A. cerana* (the Indian honey bee), *A. dorsata* (the giant honey bee), and the *A. florea* (Dwarf bee) collected from different habitats in and around Bengaluru were studied for genetic diversity among the species and also within the habitats. Though the genetic differences were not too high to be marked, but still their existed traces of significant variations across the habitats and species.

On analysing, within COI gene sequences, the highest genetic difference (as seen from Eta value) was found noted in *A. cerana* followed by *A. dorsata* within residential areas showing up to 39 and 31 mutations respectively. This trend was seen across all the habitats and within the species. *A. florea* seems to have a smaller number of variations among all the habitats. The next highest genetic difference (as seen from Eta value) was found noted in *A. cerana* followed by *A.dorsata* within industrial areas showing up to 29 and 25 mutations respectively [Figure 4].

The number of mutations observed were more in residential area followed by industrial area, public park, botanical garden and micro forest. Our results are in accordance to the foraging pattern and immune gene expression data (not shown in this paper). Overall *A.cerana* showed more variations or diversity across all the habitats ($p<0.05$).

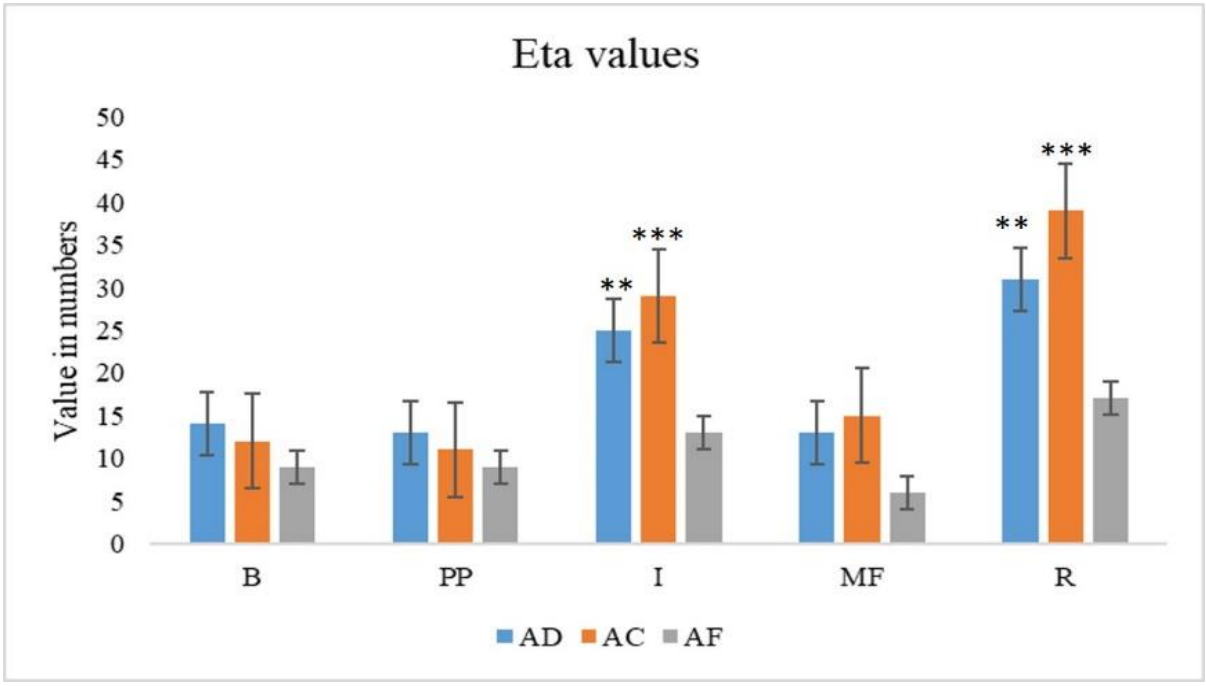

Figure 4: Graph showing the Eta values obtained on analysing genetic diversity across the habitats and species. Eta = Total number of mutations. AD: *Apis dorsata*; AC: *Apis cerana*; AF: *Apis florea.* (n=15 for all the three species studied). Value are represented as value ±SD. Data was analysed on DnaSP 5.1 (Free ware).

A two-way ANOVA was done to trace the significant effect of mutation rate between the species and habitats. There was a significant effect of the number of mutations between the species and habitats remembered at the $p<0.05$ level. The significance effect of the mutations on the species and habitats was found to be [$F(2,8) = 152.2667$, $p = 0.01133$] and [$F(4,8) = 201.7333$, $p=0.002498$].

DISCUSSION AND CONCLUSION:

Our study identified genetic diversity among the species and within the habitats. We studied on three honey bee species at five different habitats. Our investigation involved amplifying the two gene fragments (COI and NADH5) from the three types of be samples and identifying the genetic variations among them at different habitats. Sequencing studies confirmed of the strains on BLAST analysis and on comparison of the polymorphisms we found *A.florea* showed lessor no mutations when compared to other species. Our previous studies on immune gene expression and foraging patterns also showed similar findings wherein *A.florea* exhibited little changes or response.

Similar works were done by Smith *et al*. (2011), wherein they stated *A. cerana* colonies are more able to survive infestations against pathogens and surroundings. Such healthy systems are going to aid in improving the honey bee colony health. The honey bee, *A. cerana* not only shows greater hygienic behaviour but also is more disease resistant and as such could co-adapt and maintain stability within its genetic content (Lin, 2016). Studies done earlier also reported that, *A. florea* colonies were more resistant and less subjected to pressures from predators. They do not suffer a great loss in terms of habitat loss or colony collapse

disorders. In other words, environmental influences are too less on *A. florea* species which makes it less vulnerable for colony collapse (Julia¸ 2007).

Reports from Oldroyd (2006) suggested that, mitochondrial COI genes are mostly used in characterizing the genetic divergence within species. Meixner *et al*. (2013) reported that, this technique is quite useful to infer the bee evolutionary subspecies identification and also of the human mediated intervention. Our work confirmed of the detailed genetic and phylogenetic interactions within the honey bee species found in Bengaluru and among the habitats. Among these *Apis* species, the mitochondrial NADH5 area could aid in solving the interactions among the species. Further confirmation could be done to evaluate the findings with more mitochondrial genes so as to conclude the variations and relationship with their habitats.

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