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A pilot study on genetic diversity in Indian honeybees-*Apis cerana* of Karnataka populations

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ABSTRACT
Unravelling the genetic diversity studies of *Apis cerana,* the indigenous strain of India is very much essential as it provides significant guidance to beekeepers about breeding strategies that would aide in their colonies to survive. There are two predominant species of Indian honey bees, *Apis cerana cerana* (black strain) and *Apis cerana indica* (yellow strain). Currently there are no reports on diversity studies on these subspecies are available. The current research in this paper describes the pilot study undertook to evaluate the genetic diversity of *Apis cerana* from populations of Karnataka. Bee colonies from 12 localities of Karnataka have been genetically characterized through COI gene of mitochondrial genome, providing discreet characteristics for intra-specific diversity studies. This has led to infer the taxonomic status of two subspecies of *Apis cerana*. The results suggest that Indian populations have larger effective size and genetic diversity. The paper discusses the possibility of introduction of honeybees in India in evolutionary time frame and resolving the diversity in Indian honeybees by assessing the phylogeography.

Keywords: Mitochondrial DNA, Genetic diversity, COI gene, Strains of *Apis cerana.*

1. Introduction
The history of Apiculture dates back to several centuries, however the rich distribution and diversity of bees largely remains uncharacterized. The morphological [1, 2, 3] and mitochondrial haplotype [4] diversity across the range of *Apis cerana* does not provide a holistic view of the rich biological diversity in honey bees. Smith & Hagen (1996) based on sequence analysis of mitochondrial DNA, identified three major lineages of *Apis cerana* [4]. These include mainland Asia group (includes the bees from Japan), Sundaland group and Philippine group. However, exhaustive molecular characterization of honeybees needs to be taken up to resolve its rich diversity. The rich pool of mitochondrial DNA provides adequate insights on the origin and the phylogenetic variation in honeybees over evolutionary time scales.
Molecular studies on Asian honeybee-*Apis cerana* is carried out to a limited extent in few countries like China, Japan, Thailand, Philippines, Korea, Burma and India. The maternal inheritance and relatively rapid evolution of mitochondrial DNA has led to its widespread use of genetic marker for studies of maternal gene flow and the dynamics of hybrid zones [5]. Over the past 2 decades, mitochondrial DNA studies have shed light on the biogeography of the Asian cavity nesting honeybee *Apis cerana* Fabricius1793 [6]. The mitochondria of insects contain their own double stranded genomes, the size of which ranges from 14,503bp [7] to 19,517bp [8] in size. With few exceptions, it encodes 37 genes, including 13 protein coding subunits from 3 of the oxidative phosphorylation complexes, plus the 2 ribosomal RNA and 22 transfer RNA genes necessary to translate the protein coding genes [9]. Due to the relative ease of amplification and sequencing, in the last two decades the insect mitochondrial genome has become the most commonly used molecular markers for population genetics, phylogeography and molecular diagnostics.
In India, based on morphological features, two “races” of *Apis cerana* are identified: a black ‘Hill’ morph, that is often said to live at higher elevation and a yellow “plain” morph found at lower elevations [10, 11, 12]. The molecular studies of these two races were earlier identified in Indian samples [13] Analysis of mitochondrial DNA diversity in *Apis cerana* has shown two major haplotype families. The first “Western” haplotype group appears to be confined to India and Sri Lanka and may be associated with yellow plain morphology. The second major haplotype “Eastern” group includes all other *Apis cerana*, *A. nigrocincta*, *Apis nuluensis* [14]. Within the eastern group of haplotypes, there are at least 5 lineages, one of which is the” Mainland Asia” group which has a broad range from India and Japan [4, 6].

One region of the *Apis* mitochondrial genome has proven to be particularly informative for intraspecific studies because the sequence does not appear to be subject to strong purifying selection and accumulate numerous base substitutions and insertions/deletions [15, 16].

Keeping the above in view, an attempt has been made in the present study to recognise geographic and genetic variability of *Apis cerana* within Karnataka through molecular tools and characterize the relationships of *Apis cerana* with samples from various parts including adjacent regions of Karnataka. This novel study would pave way for exemplifying the introduction of honey bees in India in evolutionary time scales and resolving the diversity in Indian honey bees.

2. Materials & Methods

2.1 Sampling of honeybees

Honeybee samples were collected during the year 2012 to 2013 from different regions of Karnataka representing different climatic/ vegetation zones, ranging from 12°00’00.22” N– 17°19’47.03” latitude, 74° 30’00.00”E – 77° 35’40.43”E longitude and 49 - 1236 m elevation level. Adult *Apis cerana* worker honeybees were collected from managed hives (from beekeeping centres) or feral colonies of local origin. A sample size of Yellow and Black strains varied between eight to ten samples of different strains of *Apis cerana*. The collected bees were immediately transferred to vials with 95% alcohol and later stored at -80 °C until DNA extraction.

2.2 DNA Extraction

Genomic DNA was isolated from the legs of each individual using Chromus DNA isolation kit method. The legs were homogenized in suspension buffer followed by RNAase treatment to degrade the RNA content. Lysis buffer was used to lyse the cell contents and the solution was run on the spin column repeatedly as per the Chromous Biotech protocol. Trace of alcohol was removed through wash buffer and DNA was eluted with the elution buffer. Isolated DNA was stored at -20 °C until use.

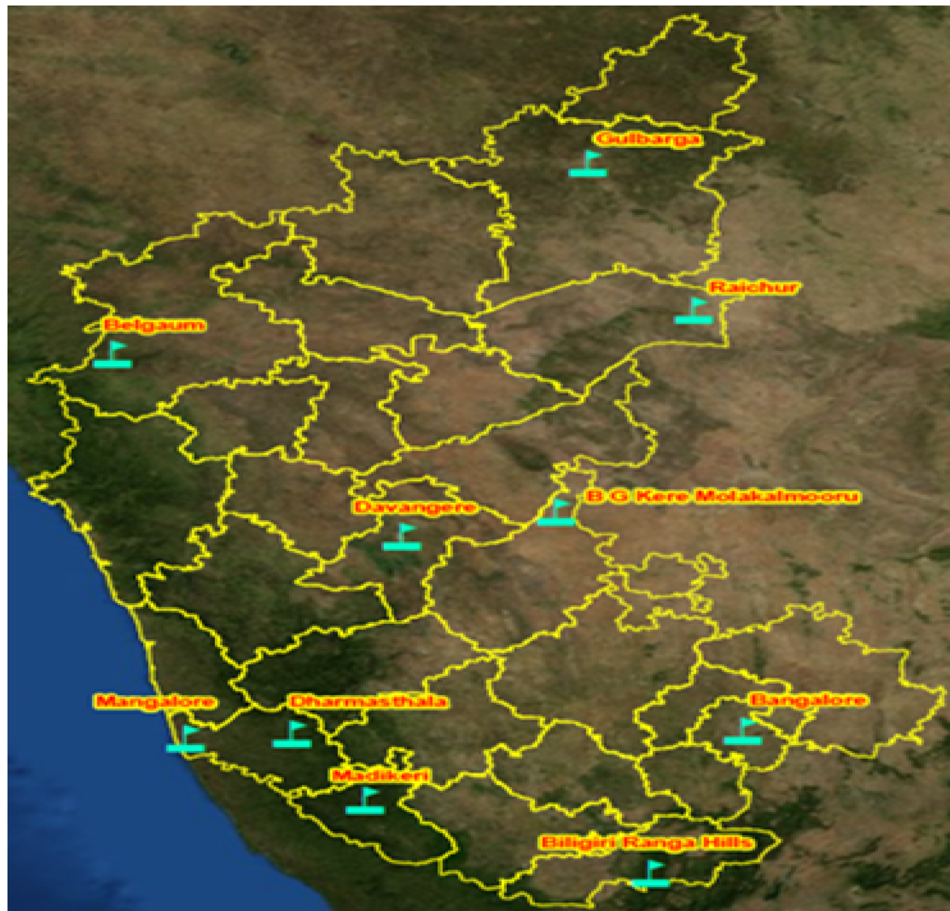

Fig 1: The list of sampled places across Karnataka state for current diversity studies.

2.3. Primers & PCR Amplification

Target DNA from mitochondrial gene, i.e., Cytochrome C Oxidase subunit I (Table 2) was amplified using specific COI primer [17] from samples with following PCR profile. The gene was amplified in 25 µL PCR reaction mixture with 12.5 µL PCR master mix (Fermentas), 1 µL of each forward and reverse primer, ≈1 µg of template DNA and nuclease free water. The PCR cycling conditions for COI is detailed in Table-1. The amplified products were checked under UV light and documented.

2.4 DNA purification and sequencing

The amplified DNA fragments were extracted from agarose gels and purified using the DNA purification kit “Easy pure” from Biozyme. The primers used were the same primers used in PCR amplification and directly sequenced in “Big dye terminator version 3.1” cycle sequencing kit with sequencing machine–ABI 3500xL Genetic analyser. Then the data analysis of sequences was done using MEGAv5.4.

Table 1: Places of sample collection with geographical location and the Genbank accession numbers of COI gene.

Sl. No	Species Name	Strain (Race)	Sample Location		Geographical Location			Gene Accession No.
			Place	Labelled	Longitude	Latitude	Altitude	COI
1.	*A. cerana*	Black	Bangalore	ACBLR	77° 35’40.43”E	12° 58’17.76” N	913 m	KF760518
2.	*A. cerana*	Yellow	Bangalore	ACBLR	77^0 35’40.43”E	12° 58’17.76” N	913 m	KF760519
3.	*A. cerana*	Black	B.R.Hills	ACBRH	77° 08’18.10”E	12° 00’00.22” N	1236 m	KF760520
4.	*A. cerana*	Black	Dharmastala	ACDSL	75° 22’52.75”E	12° 56’48.66” N	121 m	KF760521
5.	*A. cerana*	Yellow	Madikeri	ACMDK	75° 44’17.47”E	12° 25’27.91” N	1132 m	KF760523
6.	*A. cerana*	Black	Madikeri	ACMDK	75° 44’17.47”E	12° 25’27.91” N	1132 m	KF760524
7.	*A. cerana*	Yellow	Mangalore	ACMLR	74° 51’21.45”E	12° 54’50.91” N	49 m	KF760525
8.	*A. cerana*	Black	B.G.Kere	ACBGK	76° 40’38.81E	14° 39’12.93” N	623 m	Submitted
9.	*A. cerana*	Yellow	Gulbarga	ACGLB	76° 50’03.41”E	17° 19’47.03” N	470 m	Submitted
10.	*A. cerana*	Yellow	Raichur	ACRCH	77° 21’15.70”E	16° 12’25.27” N	410 m	Submitted
11.	*A, cerana*	Yellow	Belgaum	ACBLG	74° 30’00.00”E	15° 52’00.12” N	709 m	Submitted
12.	*A. cerana*	Yellow	Davanagere	ACDVG	75° 55’25.82”E	14° 27’58.84” N	598 m	Submitted

Table 2: List of primers sequences and PCR thermocycling profile with the expected product size

Gene	Primer Sequence	PCR conditions		Product Size
Cytochrome C Oxidase subunit I (Bar code gene)	Forward-LCOF1490 GGTCAACAAATCATAAAGATATTGG Reverse – HCOR2198 TAAACTTCAGGGTGACCAAAAAATCA	94 °C for 1min		710bp
		94 °C for 40sec 45 °C for 40 sec 72 °C for 1min	6 cycles	
		94 °C for 40 sec 50 °C for 40 sec 72 °C for 1min	36 cycles	
		72 °C for 5 min		

2.5 Sequence and Phylogenetic analysis

DNA sequences were aligned using the multiple sequence alignment program CLUSTAL O [18]. MEGA program version 5.4 [19, 20] was used for estimating evolutionary distances [21] and phylogenetic analysis was constructed using Neighbour Joining, DNA Parsimony methods [22]. Analyses were performed on 1000 bootstrapped data sets generated by the program. DNADIST with the Kimura two parameter distance option [23] was used to estimate divergence between sequences with a transition/transversion ratio [24].

3. Results

3.1 Sequence submission and alignment

Seven sequences of *Apis cerana* (table1) are accessed with accession numbers and the remaining accession numbers for sequences are awaited from Genbank. Amongst the aligned sequences, 550 positions of COI were considered. All positions containing gaps and missing data have been eliminated.

3.2 Nucleotide content analysis of COI gene

3.2.1. Relative importance of Transitions and Transversions

The overall mean distance of sequences of COI gene was 12.455. The frequencies of transitions: A↔G is 19.65%, (C↔T) is 33.99% and transversions: A↔T is 18.01%, A↔C is 6.14%, G↔T is 18.01% and C↔G is 6.14% (Table 3). The percentage of sites showing transitions (53.64%) is higher than the number of sites showing transversions (48.30%). The estimated transition/transversion (Ts/Tv) bias of COI **(R) is 0.588**. The nucleotide frequencies are A=33.68 %, T/U = 41.73%, C= 14.09% and G=10.50%.

Table 3: Frequency percentage (%) of transitions and transversions and Transition/Transversion ratio (Ts/Tv) of COI gene.

	Transitions (%)		Transversions (%)								Ts/Tv ratio
	G/A	C/T	A/T	A/C	G/T	G/C	T/A	T/G	C/A	C/G	
COI gene	19.65	33.99	5.97	2.07	5.97	2.07	12.04	12.04	4.07	4.07	0.588

3.2.2. Base composition at each Codon positions
The high numbers of polymorphic sites verified in the COI gene were evenly distributed among the 3 codon positions. The A+T bias was pronounced in general for this region for all codon positions (Table 4). The nucleotide content of all the samples and the total C+G and A+T were calculated using MEGA software (5.4).

Table 4: Mean frequencies (%) for base compositions at different codon positions for COI region.

Samples	First Codon				Second Codon				Third Codon				Total	
	A	C	G	T	A	C	G	T	A	C	G	T	C+G	A+T
ACGLB_COIB	30.4	15.7	17.4	37	17.4	23.0	14.8	45	49.8	9.2	1.7	39	27.266	**72.866**
ACRCH_COIY	29.4	14.0	17.8	39	17.7	24.2	14.0	44	50.9	3.3	1.9	44	25.166	**74.9**
ACBGM_COIY	26.4	15.0	19.1	40	23.6	22.3	11.8	42	48.6	3.2	2.7	45	24.66	**75.5**
ACBGK_COIY	53.3	2.6	0.0	44	29.5	12.3	18.9	39	16.7	23.3	14.1	46	23.733	**76.166**
ACDVG_COIY	18.3	23.0	14.8	44	53.5	3.0	0.0	43	29.3	14.0	18.3	38	24.366	**75.366**
ACMLR_COIY	30.9	13.5	18.3	37	17.4	23.9	14.3	44	50.7	4.8	1.3	43	25.366	**74.33**
ACDSL_COIB	29.1	15.7	18.7	37	17.4	23.9	14.8	44	51.1	6.1	1.7	41	26.966	**73.2**
ACMDK_COIB	52.2	3.9	1.3	43	30.4	12.6	19.1	38	17.0	24.0	14.4	45	25.1	**75.2**
ACMDK_COIY	53.0	3.5	1.7	42	30.9	12.6	17.8	39	17.0	23.6	15.3	44	24.833	**75.3**
ACBRH_COIB	29.2	14.4	15.9	41	15.5	24.7	13.9	46	55.2	6.3	1.0	38	26.033	**74.133**
ACBLR_COIB	28.8	14.8	18.3	38	16.6	24.0	14.8	45	52.4	6.1	1.3	40	26.433	**73.6**
ACBLR_COIY	31.0	13.5	18.3	37	18.0	22.8	14.0	45	50.9	3.9	1.3	44	24.6	**75.3**

3.3 Phylogenetic analyses of COI sequences:
Phylogenetic analyses of COI datasets were carried out to identify two criteria: (i) genetic divergence from total populations of Karnataka and (ii) sequence divergence amongst and between two strains of *Apis cerana* populations.

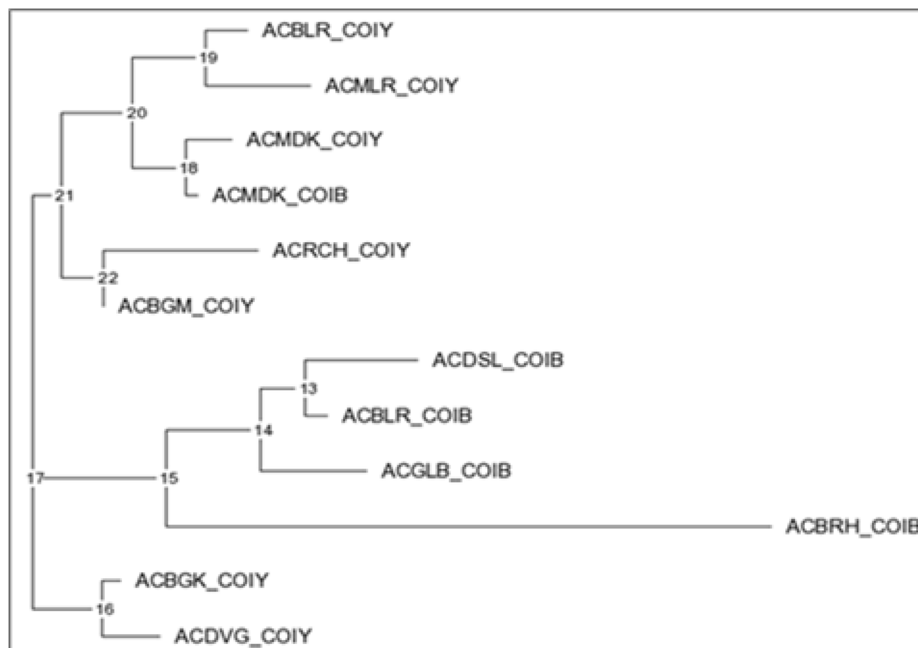

Fig 2: Phylogenetic tree of COI constructed by DNA Parsimony using sequences.

a) DNA Parsimony tree:
The tree has 3 haplotypes of which 2 yellow strains form distinct clusters followed by black strains (Figure 2). However, it was interesting to note that the black strain ACMDK_B merged with the yellow strains ACBLR_Y, ACMLR_Y, ACMDK_Y, ACRCH_Y and ACBGM_Y.DNA Parsimony tree length inferred the highest branch length from root to the node as 0.234565 for ACBRH_COIB and the least length as 0.002778 for ACBGM_COIY.

(b) Neighbour Joining tree: The NJ tree was quite similar to the DNA Parsimony tree, in which the ACMDK_COIY stood as an out-group. Amongst three haplotypes constructed, the Black and Yellow were separated from each other, but one haplotype had a Black strain along with two Yellow strains. Figure 3 illustrates the Neighbour joining phylogenetic tree of COI gene.
The distance matrix calculated using the NJ method amongst the strains (Black & Black and Yellow & Yellow) and between strains (Black and Yellow) is illustrated in Table 5

Table 5: Illustrates the distance matrix amongst and between the strains

Combination	COI	Difference of distance
Apis cerana Black & Black	0.026090 to 0.088401	0.06231
Apis cerana Yellow & Yellow	0.004418 to 0.04674	0.04233
Apis cerana Black & Yellow	0.027005 to 0.89995	0.87295

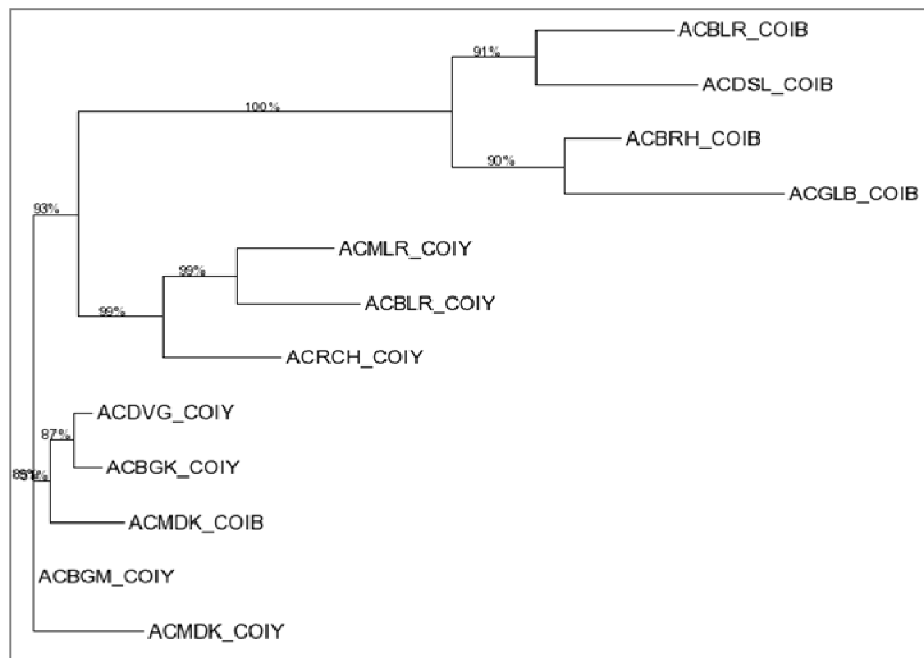

Fig 3: Phylogenetic tree of COI constructed by Neighbour Joining method.

4. Discussion
The indigenous hive bees *Apis cerana* of Asia, occurs in two different strains *viz.*, black and yellow strain. The current studies confirmed the occurrence of these two strains which are morphologically different. Earlier studies by Oldroyd [13] suggest that mitochondrial COI genes are ideally suited for characterization of genetic divergence within species. Meixner *et al.* [25] have previously shown that this technique is effective in inferring bee evolutionary subspecies identification and human mediated contemporary history. The current study was taken up in similar lines to characterize the genetic diversity in 12 populations of *Apis cerana* honeybees from Karnataka state.
The transition/transversion ratio is instrumental in inferring the direction and magnitude of natural selection. The Ts/Tv value of 0.588 suggests that there is insignificant neutral selection in honeybees of Karnataka. The ratio value of greater than 1, it implies positive or Darwinian selection; and if less than 1, it implies purifying selection and a ratio of one indicates neutral (i.e., no) selection. However the positive and purifying selection at different points within the gene or at different times along its evolution may cancel each other out giving an average value that may be lower, equal or higher than, and thus it could be inferred that the honeybees of Karnataka might be in the verge of diversification. Additionally, the exact transition (53.64%) and transversion (46.36%) suggests no significant difference indicating frequent gene flow, migration, mutations among these populations. Transitions do not contribute heavily to genetic divergence, whereas transversions create significant impact on the evolution of the species. The comparable values of transitions and transversions in the current study suggest the possible occurrence of genetic divergence over evolutionary time scales.
In congruence with Willis *et al.* [26], suggesting honeybees are AT-biased, the average of A+T and C+G content of the findings is in the ratio of 3:1 respectively. Willis *et al.* [26] further opined the AT-rich sequence may be the result of various factors comprising selection drift, small effective population size and mitochondrial

polymerase inefficiency. Historical evidence also points to rapid morphological change in *Apis* and predicts the rapid changes during approximately 10 million years between the Upper Eocene and the Oligocene (35-40 Mya.) [27]. The variation in nucleotide content almost completely resides in the third position, which is also rich with AT, compared to the first and second position of codons (Table-4). The A+T bias described for different codons has been explained either as a mutational process favouring the accumulation of those nucleotides or the result of an ineffective repair system.

The phylogenetic characterization of the COI genes through DNA Parsimony tree and Neighbour Joining tree revealed the distance matrix amongst and between the strains and the evolutionary status of strains of *Apis cerana* respectively. However, the DNA Parsimony and Neighbour Joining trees showed similar topology with slight variations in terminal branch resolution, indicating the need for exclusive studies on the black and yellow strain with respect to the altitude (above the sea elevation level). The phylograms exemplify the black and yellow strains to form separate clusters; the third cluster with yellow includes a black strain. The distance matrix clearly signifies the vast distance between the strains and least difference amongst the strains. The distance between strains proves that there is diversity between them.

The strains of *Apis cerana* i.e., the Black and Yellow were identified with the morphological studies. The first three tergites of the abdomen of the bee were main criteria to discriminate them, followed with the mitochondrial haplotype studies. The study is in congruence with the previous reports of Oldroyd [13] with little queries on the ACMDK black with the other yellow strains. Though these strains are reproductively isolated, there might be the possibilities of genetic recombination and strain diversity due to human mediated migratory beekeeping.

Indian sub-continent is rich in bio-diversity due to varied ecological constraints and elevations from mean sea levels. The temperate and tropical climatic zones of India further enhance this genetic divergence. When the current study, which is limited to a particular state of India is showing such great divergence, a detailed sampling of honeybees across India with different agro-climatic zones would provide interesting insights on the genetic diversity. The pioneering study will be of impeccable importance in this direction as would be influential in filling up the empty niche of characterizing the genetic diversity of *Apis cerana* in India.

5. Conclusion

The current study identifies the Western and Eastern group of *Apis cerana* in 12 populations of Karnataka. *Apis cerana* in India occurs in two strains viz., *Apis cerana indica* (the yellow plain morphs) and the *Apis cerana cerana* (the black hill morph). The investigations involved characterization of honeybees through morphological and molecular studies. The morphometric microscopic examination of the tergites of abdomen and mitochondrial DNA-COI gene characterization revealed the presence of two strains of *Apis cerana* in Karnataka populations. The pilot study is of vital importance in establishing the genetic diversity of the indigenous honeybees of *Apis cerana* in the Indian sub-continent.

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