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Mitochondrial DNA Diversity Studies in *Apis cerana* populations of Nilgiri Biosphere Reserve

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ABSTRACT

Ecological diversity of the Nilgiri Biosphere Reserve provides an appropriate foraging and nesting substrates for different honeybee species making honey hunting a lucrative activity. The molecular diversity studies of *Apis cerana*, the indigenous strain of India in Nilgiri Biosphere Reserve is very much essential as it provides significant guidance to beekeepers about breeding strategies that would aide in their colonies to survive. The current research in this paper describes the pilot study undertook to evaluate the genetic diversity of *Apis cerana* from populations of Biosphere. Bee colonies from 10 localities of Nilgiri Biosphere have been genetically characterized through COI gene of mitochondrial genome, providing discreet characteristics for intra-specific diversity studies. There are two predominant species of Indian honey bees, *Apis cerana cerana* (black strain) and *Apis cerana indica* (yellow strain). This has led to infer the taxonomic status of two subspecies of *Apis cerana*. The results suggest that the mobile beekeeping in Nilgiris has resulted in genetic recombination of different strains which are grouped together in the phylogenetic clades. 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. Further characterization of genetic diversity is in need with respect to extensive sampling and with different mitochondrial genes.

Introduction:

India, ranked 12th mega biodiversity zone accolades itself with two hotspots (Western Ghats & Eastern Himalayas) of the World. Evidently, the complexities of biodiversity subsidizes production, decomposition, nutrient cycling dynamics thus moving towards stability and elasticity of the system¹. Nilgiri Biosphere Reserve (NBR) was established by UNESCO in September,1986 under Man and Biosphere Programme². NBR region is a seam of 3 Southern states of India i.e., Karnataka, Kerala and Tamil Nadu. Nilgiri Biosphere Reserve inhabits adivasi communities³, whose livelihood is beekeeping.

The ecological diversity of the NBR provides an appropriate foraging and nesting substrates for different honeybee species making honey hunting a lucrative activity in NBR. NBR represents the Asian apicultural situation entirely and is believed to be an area still without exotic *Apis mellifera*, although this species has been introduced to Karnataka, Kerala and Tamil Nadu^{4,5,6}.

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 zones⁷. The high copy number, small size, general conservation gene content, gene arrangement⁸ and lack of recombination have made mt.DNA potential to be used as a hierarchal tool. Over the past 2 decades, mitochondrial DNA studies have shed light on the biogeography of the Asian cavity nesting honeybee *Apis cerana* Fabricius1793⁹. Mitochondrial DNA studies on Asian honeybee - *Apis cerana* is carried out in few countries like China^{10,11}, Japan¹², Thailand¹³, Korea¹⁴, Philippines¹⁵, Burma⁹, Borneo Island¹⁶ but there is an empty niche in characterizing Indian bees with a partial studies carried out^{17,18,19}.

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Based on morphological features, two strains 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 in India^{20,21,22}. The molecular studies of these two races were earlier studied in Indian samples¹⁷. Analysis of mitochondrial DNA diversity in *Apis cerana* has shown two major haplotype families. The first "Western" haplotype (Yellow *Apis cerana*) and "Eastern" group includes all other *Apis cerana* (Black), *A nigrocincta*, *Apis nuluensis*¹⁶. Within the eastern haplotype groups there are at least 5 lineages, one of which "Mainland, Asia" group has broad range from India and Japan^{12,16}. One region of the *Apis* mitochondrial genome has proven to be particularly informative for intraspecific studies and to identify the closely related species^{23,11}.

The current study estimates the level of genetic variability and population differentiation of *Apis cerana* in Nilgiri Biosphere Reserve, which is one amongst few places where the European bees – *Apis mellifera* is not introduced till date. Honey hunting, being the major livelihood of Nilgiri tribes needs extensive studies on diversity to identify a

number of races and sub-races, which differ widely in productivity, behaviour and body size. Identification of diversity further encompasses the colony which shows foraging capacity, pollen and nectar carrying capacity and further its multiplication for honey production.

Materials & Methods:

Sampling of honeybees:

Honeybee samples were collected during the year 2013 to 2014 from different regions of Nilgiri Biosphere region representing different climatic/ vegetation zones, ranging between 76° to 77°15' E longitude, 11° 15' to 12° 15' N latitude and an altitude from 33m to 2670m. Adult *Apis cerana* worker honeybees were collected from managed hives (from beekeeping centres) or feral colonies of local origin. Sample size of Yellow and Black strains from eight to ten. The collected bees were immediately transferred to vials containing 95% alcohol and stored at -20°C until DNA extraction.

Table 1 - Places of sample collection with geographical location

Sl. no	Species Name	Strain (Race)	Sample Location			Geographical Location		
			Place	Labelled	State	Longitude	Latitude	Altitude
1.	<i>A. cerana</i>	Yellow	Vazhithottam	ACVZT	Tamilnadu	77°49'33.63"E	8°20'52.21" N	33m
2.	<i>A. cerana</i>	Yellow	Nilambur	ACNIL	Kerala	76°14'23.23"E	11°16'45.96"N	40m
3.	<i>A. cerana</i>	Yellow	Palakkad	ACPAL	Kerala	76°39'17.25"E	10°47'12.23"N	84m
4.	<i>A. cerana</i>	Yellow	Banagudi	ACBNG	Tamilnadu	77°34'3.63"E	8°18'53.72" N	108m
5.	<i>A. cerana</i>	Yellow	Gundlupete	ACGDP	Karnataka	76°41'30.33"E	11°48'29.09"N	795m
6.	<i>A. cerana</i>	Yellow	Chokkanahalli	ACCKH	Tamilnadu	76°38'33.77"E	11°34'19.84"N	942m
7.	<i>A. cerana</i>	Black	B.R.Hills	ACBRH	Karnataka	77° 08' 18.10"E	12° 00' 00.22" N	1236m
8.	<i>A. cerana</i>	Black	Coonor	ACCNR	Tamilnadu	76°82'E	11°35'N	1850m
9.	<i>A. cerana</i>	Black	Kotagiri	ACKTG	Tamilnadu	76°51'33.03"E	11°25'50.65"N	1989m
10.	<i>A. cerana</i>	Yellow	Ooty	ACOTY	Tamilnadu	76°41'40.76"E	11°24'42.30"N	2232m

DNA Extraction:

Genomic DNA was isolated from the legs of each individual using Chromus DNA isolation kit. 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 centrifuged on spin column repeatedly as per the Chromous Biotech protocol. Trace of alcohol was removed using wash buffer and DNA was eluted with the elution buffer. Isolated DNA was stored at -20°C until use.

Primers & PCR Amplification:

Target DNA from mitochondrial gene i.e., Cytochrome C Oxidase subunit I (Table 2) was amplified using specific COI primer⁽²⁴⁾ from samples with following PCR profile. The gene was amplified in 25µL PCR reaction mixture containing 12.5 µL PCR master mix (Fermentas), 1 µL each forward and reverse primer, ≈1 µg of template DNA and nuclease free water. The PCR cycling conditions for COI is detailed in Table2. Gel was documented under UV light.

Table 2- List of primer 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 ⁰ C for 1min		710bp
		94 ⁰ C for 40sec 45 ⁰ C for 40 sec 72 ⁰ C for 1min	6 cycles	
		94 ⁰ C for 40 sec 50 ⁰ C for 40 sec 72 ⁰ C for 1min	36 cycles	
		72 ⁰ C for 5 min		

DNA purification and sequencing:

The amplified DNA fragments were extracted from agarose gels and purified using the DNA purification kit “Easy pure” from Biozyme. PCR amplification primers were used 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 MEGA5.4.

Sequence and Phylogenetic analysis:

DNA sequences were aligned using the multiple sequence alignment program CLUSTAL O⁽²⁵⁾. MEGA program version 5.4^(26,27) was used for evolutionary distances estimation⁽²⁸⁾ and phylogenetic analysis was constructed using Neighbour Joining, DNA Parsimony methods⁽²⁹⁾. Analysis were performed on 1000 bootstrapped data sets generated by the program. DNADIST with the Kimura two parameter distance option was used to estimate divergence between sequences with a transition/transversion ratio⁽³⁰⁾.

Results and Discussion:

Sequence alignment and submission:

The sequences were aligned to check for insertions, deletions and mutations. Gaps and missing data positions were eliminated from 550 positions of COI. All the sequences of *Apis cerana* (table1) are awaited for accession numbers from Genbank.

Nucleotide content analysis of COI gene:

Nucleotide polymorphism levels are expected to correlate with evolutionary rate under the neutral theory and is a relationship between the transition and transversion ratio within populations and long term evolutionary rate is expected.

Comparative significance of Transitions and Transversions:

The overall mean distance of sequences of COI gene is 13.985. The frequencies of transitions and transversions is mentioned in Table 3. The percentage of sites showing transitions (77.47%) is higher than the number of sites showing transversions (22.53%). The estimated transition/transversion (Ts/Tv) bias of COI (**R**) is **0.388**. The nucleotide frequencies are A=33.68 %, T/U = 41.49%, C= 10.70% and G=14.13%.

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	T/C	A/G	A/T	A/C	T/A	T/G	C/A	C/G	G/T	G/C	
COI gene	27.72	29.52	8.77	10.15	4.93	1.69	4.02	1.27	4.02	1.27	4.93	1.69	0.388

The transition/transversion (Ts/Tv) ratio is influential in deducing the magnitude and direction of natural selection. The transitions (77.47%) and transversions (22.53%) depicts ratio of 3:1. Transitions do not donate heavily to genetic divergence, whereas transversions generate significant impact on evolution of the species. The current Ts/Tv value of 0.388 signifies the presence of minor neutral selection in *Apis cerana* of Nilgiri biosphere reserve. The 2 main reasons for establishment of relationship between the transition and transversion ratio within the populations and long term

evolutionary rates are: (i) more diverged sequences might be expected to have higher proportions of transversional substitutions and (ii) less constrained regions may tolerate transversions more often. The current results suggested divergence between the bees of nilgiri biosphere. The ratio greater than 1 implies positive or Darwinian selection and less than 1 implies purifying selection and equal to one indicates neutral selection. However the positive and purifying selection at different points within the gene or at different times along its evolution may cancel each other

giving an average value that may be less than, equal to or higher than actual value, thus it could be inferred that the honeybees of Nilgiri Biosphere reserve are still at the verge of diversification. The analogous values of transition/transversion ratio in the present study suggest the possible genetic divergence over evolutionary time scale.

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 all codon positions of this region (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	T	G	A	C	T	G	A	C	T	G	C+G	A+T
ACOTY_COI	53.4	7.3	38	0.9	29.0	15.6	37	18.6	18.2	22.9	45	14.3	79.6	250.2
ACKTG_COI	34.4	14.6	37	14.2	24.0	21.8	40	13.8	41.3	11.1	42	5.8	81.3	218.7
ACGDP_COI	18.1	22.8	44	15.1	53.2	3.0	44	0.0	29.0	13.9	39	18.6	73.4	227.3
ACVZT_COI	27.1	15.4	38	19.6	21.4	22.8	44	12.1	48.6	3.3	47	1.4	74.6	226.1
ACCKH_COI	27.7	14.5	39	19.1	21.4	22.7	45	11.4	49.1	2.7	46	1.8	72.2	228.2
ACBNG_COI	49.1	5.9	41	4.1	28.5	16.7	36	19.0	21.3	22.6	44	12.2	80.5	219.9
ACNIL_COI	29.7	12.5	39	19.0	17.3	23.8	45	14.3	53.2	3.0	43	0.4	73	227.2
ACPAL_COI	17.4	23.0	45	14.8	52.2	3.5	43	9.0	29.3	13.1	39	18.8	82.2	225.9
ACBRH_COI	30.4	14.1	39	16.3	16.2	24.3	45	14.6	54.9	6.6	37	1.1	77	222.5
ACCNR_COI	52.2	3.9	43	1.3	30.4	12.6	38	19.1	17.0	24.0	45	14.4	75.3	225.6

In correspondence with Willis *et al.*,⁽³¹⁾, honeybees are AT biased, the average of A+T and C+G content ratio from the findings is 3:1. Willis *et al.*, further concluded that AT rich sequences may have resulted due to several factors comprising selection drift, mitochondrial polymerase inefficiency and small effective population size. Antique evidence also points to rapid morphological change in *Apis* and predicts the rapid changes during approximately 10 million years ago between Upper Eocene and the Oligocene (35-40 mya). The percentage of AT in ACOTY is very high compared to other samples. The AT bias described for different codons has been explained either as a mutational process which further favours accumulation of those nucleotides or the result of an ineffective repair system.

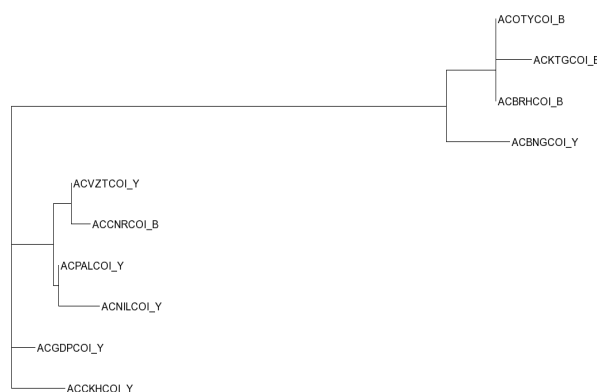
Phylogenetic analyses of COI sequences:

Phylogenetic tree was constructed mainly to address the classification of *Apis cerana* species in Nilgiri Biosphere reserve.

a) Neighbour joining tree:

The tree has 2 haplotypes, one with the black haplotype and second with the yellow haplotype. However, it was quite curious to see one yellow strain i.e., ACBNGCOIY among black strains i.e., ACKTGCOIB, ACOTYCOIB, ACBRHCOIB and a black strain i.e., ACCNRCOIB was merged with the yellow strains i.e., ACNILCOIY, ACPALCOIY, ACVZTCOIY, ACGDPCOIY and ACCKHCOIY.

Figure 1: Phylogenetic tree constructed by Neighbour Joining method using COI gene



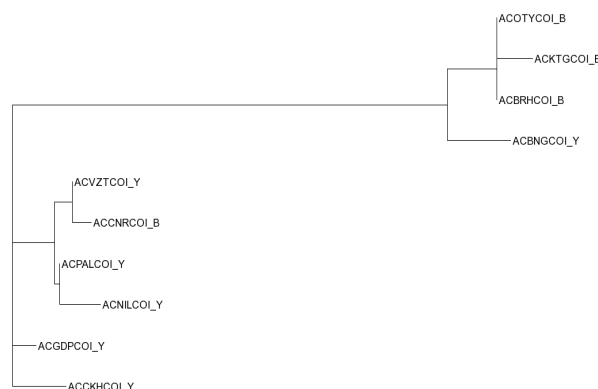
b) Maximum Likelihood tree:

The maximum likelihood tree was quite similar to the Neighbour joining tree in which two haplotypes were obtained, rather the tree length varied with different samples. The black haplotype had an additional yellow strain i.e., ACBNGCOIY and a yellow haplotype with a black strain i.e., ACCNRCOIB.

The molecular phylogenetic analysis seems necessary to classify the evolution of the Asian cavity nesting species.

The phylogenetic tree construction using COI genes through Neighbour joining (NJ) method and Maximum likelihood (ML) method revealed the diversity of *Apis cerana* species in Nilgiri biosphere reserve. However, the NJ tree and ML tree showed similar topology with slight variations in terminal branch resolution, indicating the need for extensive studies on the black and yellow strains at different altitudes levels. Oldroyd (2006) made first attempt in studying the molecular aspects of Indian bees, where the black and yellow stood in separate haplotypes depicting that they were reproductively isolated. But the trees constructed in the current study is not in support to previous studies for which, reasons may be many. Migratory beekeeping, a human mediated deed is an exclusive reason for merging of black and yellow strains together which would have caused the genetic recombination between the strains. The branch length very clearly depicts the distance between and amongst the strains, which further paves way for extensive sampling and study of diversity with more mitochondrial genes.

Figure 2: Phylogenetic tree constructed by Maximum Likelihood method using COI gene



Conclusion:

The current study identifies the Western and Eastern group of *Apis cerana* in 10 populations of Nilgiri biosphere reserve. *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 molecular studies in support to the previous morphological studies. The morphometric microscopic examination of the tergites and molecular examination with mitochondrial DNA-COI gene characterization revealed the presence of two strains of *Apis cerana* in Nilgiri biosphere reserve populations. These studies partially contribute to enhancement of the tribal livelihood. The molecular diversity studies with more number of mitochondrial genes and extensive sampling would contribute much to the beekeeping practice in the ecologically diversified region, Nilgiri biosphere reserve.

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