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DNA FINGERPRINTING STUDY OF SOME OF THE INDIAN NON-HUMAN PRIMATE HAIRS

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INTRODUCTION

There have been some studies using molecular aspects such as that of Zhang and Shi (1993) on phylogeny of rhesus in China and that of Melnick *et al.* (1993) on rhesus from India and some of its neighbouring countries. Karanth *et al.* (2008) have researched on molecular phylogeny and biogeography of langurs and leaf monkeys (Colobine) of South Asia and Zhang and Ryder(1998) have worked on old world monkeys including Asian Colobines. There have been studies on new world monkeys as well (Ascunce *et al*., 2007). Many of these molecular studies have been done using blood samples or hair samples (Ascunce *et al*., 2007; Karanth, 2008). Earlier, Ascunce *et al. (*2003) have demonstrated amplification of mitochondrial CoII gene from DNA extracted from hair samples of some species of New World monkeys. However, the present study is an attempt on molecular aspects of taxonomy on the basis of DNA fingerprinting using only hair samples. The objectives of the present study included - extraction and amplification of DNA from available non-human primate hair samples and secondly to process the available DNA sequences using appropriate softwares to align sequences independently for each gene and to conduct phylogenetic and molecular evolutionary analysis.

METHODS

Initially hair samples obtained from National Zoological Collection , Zoological Survey of India, Kolkata were sent to CCMB, Hyderabad for DNA fingerprinting. However, as these samples did not give good amplifiable DNA, so fresh hair samples of monkeys provided by Zoo were sent to CCMB for analysis. Out of this also, DNA could not be obtained from two samples, so the DNA isolated from exhibits A, B, C and F were subjected to PCR (Polymerase Chain Reaction)amplification using the universal Primers (Verma and Singh, 2003; US Patent No. 71,41,364) to generate species specific molecular signature. The following protocol was used for extraction of DNA from hairs

Ten to fifteen hair pieces with hair roots about 0.5 cm were cut and placed into a 1.5 ml eppendorf tube. A lysis buffer of 50ul consisting of 1 mm Tris pH 8.3, 50 mM KCL and 0.5% Tween was used. Besides, 10 l of 20 g/ml solution of Proteinase K in 10 mM Tris-HCL (pH 7.5) was also added to the above. This was vortexed for 30 seconds. Then, ultracentrifuge was done 1t 13000 rpm for one second. It was then put in incubator for overnight in a 56-60 degree centigrade waterbath. Thereafter, it was incubated overnight for 10 minutes and cooled down to room temperature. Again it was ultracentrifuged at 13000 rpm for one second. The DNA so extracted was used for PCR.

Sequences were aligned independently for each gene using MUSCLE (Edgar, 2004). Phylogenetic and molecular evolutionary analyses were conducted using MEGA5 (Tamura *et al*., 2011). Best fit model for nucleotide substitution was selected based on minimum

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Akaike Information Criterion (AIC) value (Posada & Crandall, 2001). We constructed phylogenetic trees based on maximum parsimony (MP) and maximum likelihood (ML). Reliability of the phylogenetic tree was estimated using bootstrap values run for 1000 iterations. ML tree was used for further predicting the molecular time scale of species divergence using the calibration points 29.2 myr divergence between *Hylobates* and *Macaca* and 16.7 myr divergence between *Semnopithecus* and *Macaca* based on time tree (Hedges *et al*., 2006). *Hylobates hoolock* was used as an outgroup. A molecular clock test was performed to find out whether the substitution rates were uniform (Tamura *et al*., 2011).

RESULTS

The DNA sequences obtained from different hair samples are shown in Table 1. Samples A, B and F are similar to *Macaca mulatta*. They are monophyletic with a high bootstrap support (Fig. 1). While sample C belongs to *Macaca nemestrina* which is also supported by high bootstrap value. In the cladogram, *Hylobates hoolock* (an ape) was used as an outgroup and other old world monkey species were used for comparison and it was found that none of the samples came from *Semnopithecus entellus* (Hanuman langur). Maximum likelihood method (Fig. 2) showed similar results as Maximum parsimony method as in the former method again three samples belong to *Macaca mulatta* and one sample belonged to *Macaca nemestrina*. The sample in question was 100% same as *Macaca nemestrina nemestrina* (Gen Bank accession Number AF350394). It was first tested whether the molecular clock hypothesis is true. Table 2 shows that the null hypothesis of equal evolutionary rate throughout the tree was accepted (p=0.05). No nucleotide divergence was found among samples A, B and F as compared to *Macaca mulatta* (Table 3).

DISCUSSION

Molecular taxonomy is a useful tool. Cytochrome b gene (also used in the present study) is widely used in identifying vertebrate species. Parson *et al*. (2000) have used nucleotide sequence analysis of cytochrome b gene to identify 44 different animal species belonging to 5 major vertebrate groups. Earlier workers have used molecular approaches to study primates.

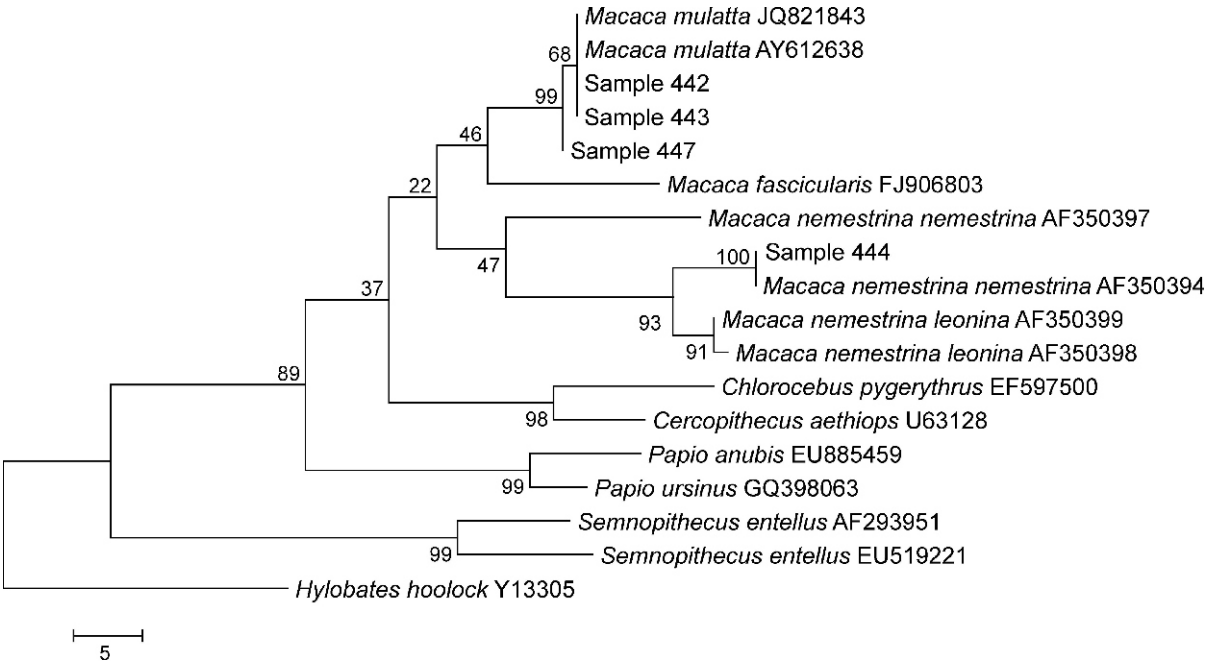


Figure 1: Maximum parsimony tree of samples and related species. Sample numbers as per Table 2. There were 310 bases in final analysis. Bootstrap values are based on 1000 iterations. *Hylobates hoolock* is used as an outgroup. GenBank axccession numbers are provided after the species name.

Table - 1: Cytochrome b gene sequence of four samples

S N	Exhibit	BLAST outcome (GenBank Accession No. and species)	Maximum identity	Sequence
1	A 442	JQ821843 (*Macaca mulatta*)	100%	GGCTTTCATGGGTTATGTTCTCCCATGAGGCCAAATATCATTCTGGGGAGCAACAGTAATCACAAACCTGC TATCAGCAATCCCGTATATCGGAACCAATCTCGTCCAATGAATCTGAGGAGGATACGCCATCGACAGCCCT ACTCTCACACGATTCTTCACCTTACACTTTATCCTACCCTTCATCATCATCGCCCTCACAACCGTGCACCTA CTATTCCTGCACGAAACAGGATCAAACAACCCTTGCGGAATCTCCTCCGACTCAGACAAAATCGCCTTCCA CCCCTACTACACAACCAAAGACATCCTGGGCCTAGTCCTCCTTCTCTTCATCCTAGCAACACTAACACTACT CTCACCCAACCTCCTAAACGACCCAGACAACTACATTCCAGCCGACCCATTAAACA
2	B 443	AY612638 (*Macaca mulatta*)	100%	CACGATTCTTCACCTTACACTTTATCCTACCCTTCATCATCATCGCCCTCACAACCGTGCACCTACTATTCCT GCACGAAACAGGATCAAACAACCCTTGCGGAATCTCCTCCGACTCAGACAAAATCGCCTTCCACCCCTAC TACACAACCAAAGACATCCTGGGCCTAGTCCTCCTTCTCTTCATCCTAGCAACACTAACACTACTCTCACCC AACCTCCTAAACGACCCAGACAACTACATTCCAGCCGACCCATTAAACACTCCCCCACATATCAAACCAG AGTGATACTTCCTATT
3	C 444	AF35039 (*Macaca nemestrina nemestrina*)	100%	CACCCTACACTTCATCCTACCCTTCATCATCGTCGCCCTCACAACCGTACACCTACTTTTCCTACACGAAAC AGGATCAAACAACCCCTGCGGAATTTCCTCCGACTCGGATAAAATCACCTTTCACCCCTATTATACAATCA AAGACATCCTAGGCCTAATCCTCCTTCTCTTTGCCCTAACAATACTAACACTATTCTTACCCAACCTCCTAA ACGACCCAGACAACTACATTCCAGCTGACCCACTAAATACCCCCCCACATATCAAGCCAGAGTGATACTT CCTATTTGCATACACAATCCTACGAT
4	F 447	JQ821843 (*Macaca mulatta*)	99%	TCTCACACGATTCTTCACCTTACACTTTATCCTACCCTTCATCATCATCGCCCTCACAACCGTGCACCTACTA TTCCTGCACGAAACAGGATCAAACAACCCTTGCGGAATCTCCTCCGACTCAGACAAAATCGCCTTCCACCC CTACTACACAATCAAAGACATCCTGGGCCTAGTCCTCCTTCTCTTCATCCTAGCAACACTAACACTACTCTC ACCCAACCTCCTAAACGACCCAGACAACTACATTCCAGCCGACCCATTAAACACTCCCCCACATATCAAA CCAGAGTGATACTTCCTATTTGCATACACAATCCTACGATCCATCCCCAACAAACTGGGAGGCGTACTAGC ACTCTTTCTATCGATCCTCATTCTAGCAGCCATCCCT

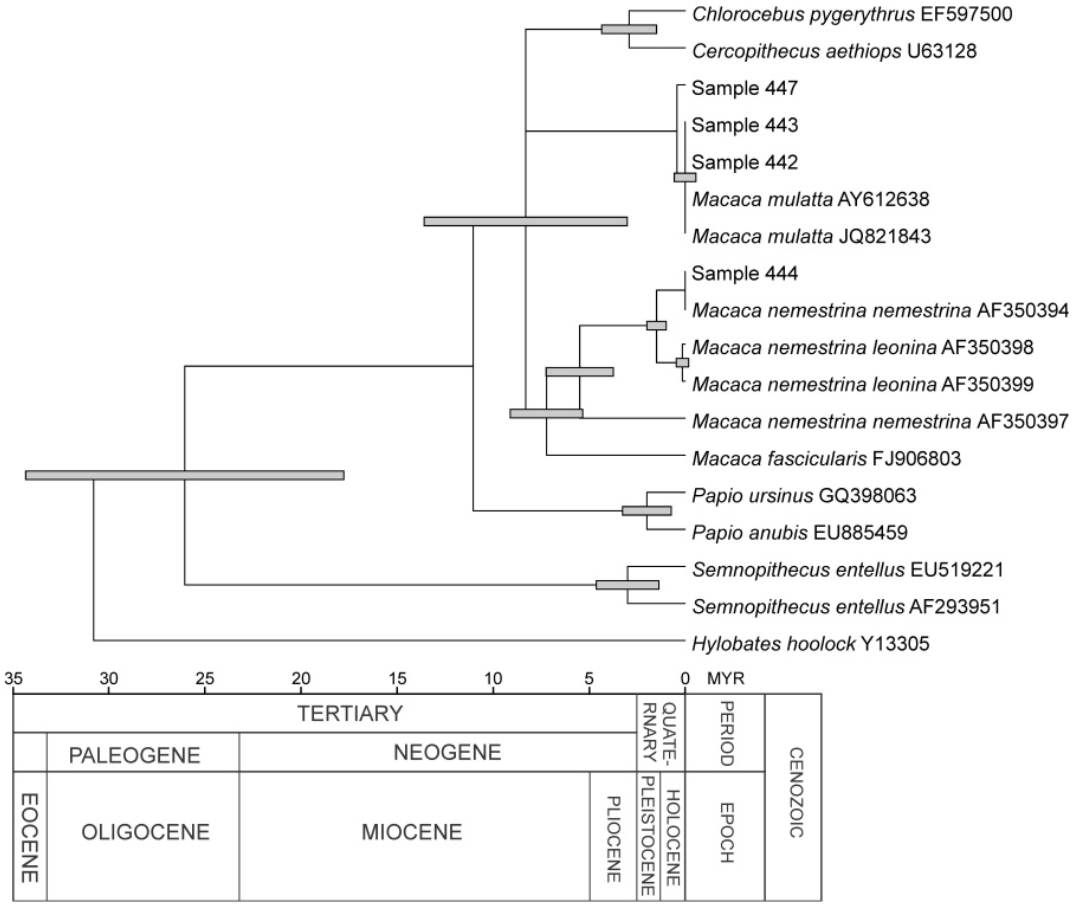
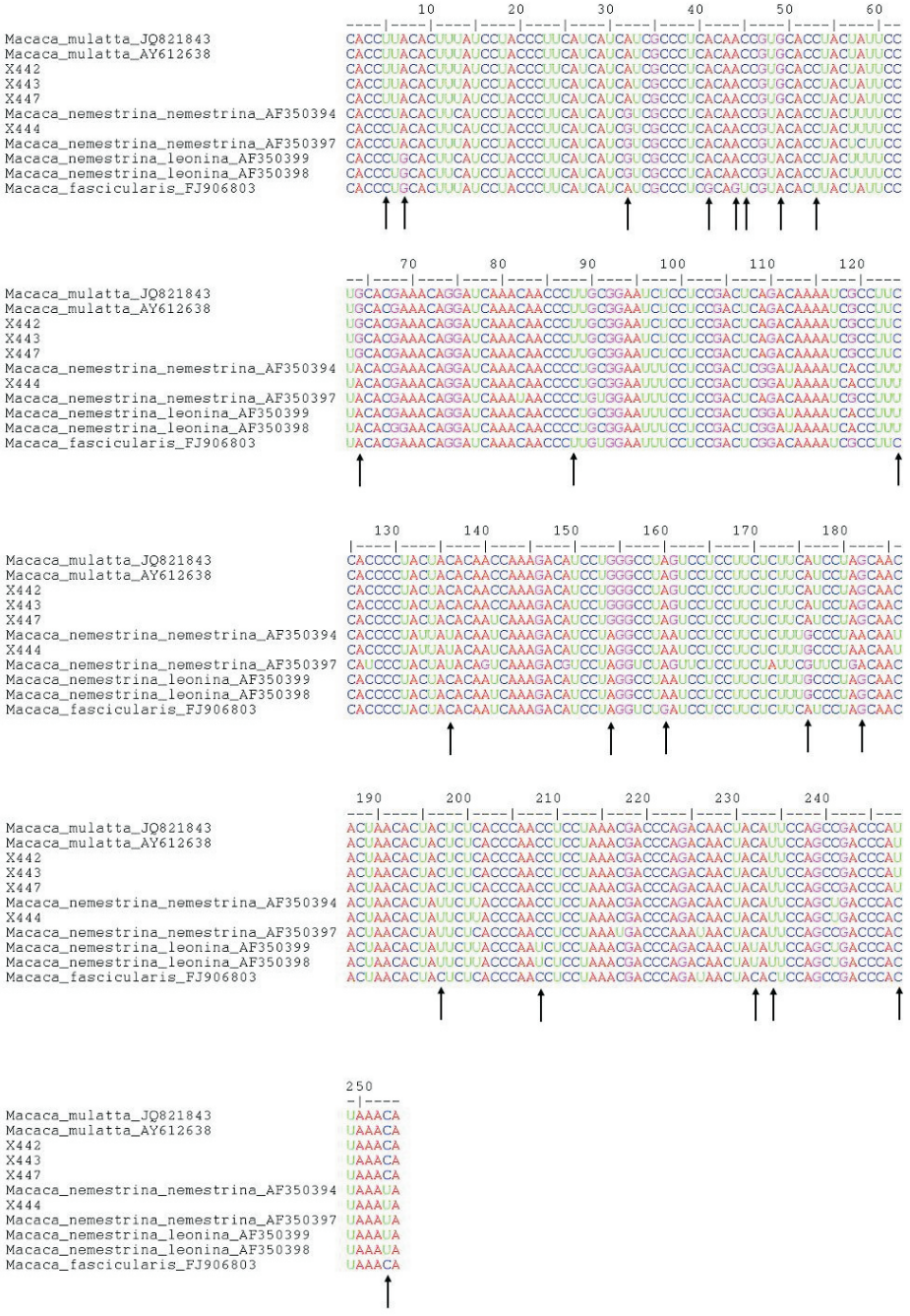


Figure 2: Molecular time clock for the evolution of Macaca and related species based on maximum likelihood method with Tamura and Nai (1993) model of nucleitide substitution and invariant sites. *Hylobates hoolock* is used as an outgroup. GenBank axccession numbers are provided after the species name. Calibration points are 29.2 myr divergence between *Hylobates* and *Macaca* and 16.7 myr divergence between Semnopithecus and Macaca based on time tree (Hedges *et al.,* 2006).

Table - 2: Results from a test of molecular clocks using the Maximum Likelihood method. The null hypothesis of equal evolutionary rate throughout the tree was accepted (P = 0.05).

	ln*L*	Parameters	(+*G*))
With Clock	-1273.248	23	0.371
Without Clock	-1259.835	39	0.36

Table - 3: Nucleotide Sequence of Various *Macaca* Species.



Zhang and Shi (1993) classified rhesus in China into 6 sub-species using mitochondrial DNA. Work of Karanth *et al. (*2008) using two unlinked nDNA markers (lysozyme and protamine P1) and one mtDNA (cytochrome b) marker on langurs and leaf monkeys from South Asia support the classification of langurs of Indian sub-continent (Hanuman, Nilgiri and Purple-faced langurs) in the genus *Semopithecus* and leaf monkeys from South-East Asia in the genus *Trachypithecus*.

Macaca mulatta and *Macaca nemestrina* diverged around 9 m years ago i.e. mean divergence is 9 m years ago (confidence interval 4-14 m years ago, because one gene only was used so confidence interval was very large.

Ascunce *et al.* (2007) have used blood and hair samples for their molecular study on New World Monkey species *Alouatta caraya* and Karanth *et al. (*2008) have used blood, tissue and hair samples. The present work is based exclusively on DNA extracted from hair samples. So, the approach used in the present study is non-invasive based on hair samples which may be useful in identification of seized material. Besides, it may also be useful in studies involving free-living animals (removing the problem of catching the individuals for taking out blood samples).

SUMMARY

The present work uses a non-invasive approach for study of taxonomy of some of the Indian non-human primates based on DNA sequences obtained through cytochrome b gene from hairs. The DNA isolated was subjected to PCR (Polymerase Chain Reaction) amplification using the universal Primers to generate species specific molecular signature. The DNA sequences sent by CCMB, Hyderabad were analysed .

Reliability of phylogenetic tree was estimated using bootstrap values run for 1000 iterations. A molecular clock test to check whether substitution rates were uniform showed that the null hypothesis of equal evolutionary rate throughout the tree was accepted (p=0.05). Maximum likelihood method showed results similar to that of Maximum Parsimony method. Nucleotide substitution sites in cytochorme–b gene among different study species were also found out. No nucleotide divergence was found among *Macaca mulatta* samples in question. Future studies incorporating more genes and larger number of samples and species may provide further insight.

DNA based taxonomy is useful in wildlife forensics and non-invasive method is particularly useful in free-living and zoo primates as it does not involve catching them.

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