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Phylogenetic analysis of Nilgiri langur using mitochondrial cytochrome B gene#

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Abstract

Old world monkeys comprise 28 Langur species which belong to subfamily Colobinae under family Cercopithecidae. Nilgiri Langurs (Trachypithecus johnii) are endemic to the rain forests of the Western Ghats. The current study is an attempt at comparative phylogeny based on mitochondrial CYTB (mtCYTB) gene of Nilgiri Langur with other langurs. Faecal sample was collected from Nilgiri langur and the genomic DNA was isolated. The 1140 bp mitochondrial CYTB was amplified and sequenced using Sanger's di-deoxy method. The amplified sequence along with the 27 sequences of Trachypithecus and Semnopithecus that were retrieved from the GenBank database were used for analysis. The phylogenetic tree was constructed by the maximum likelihood method in MEGA X. The analysis showed the clustering of Nilgiri langur with other langurs of the Semnopithecus sp. as a single clade.

Keywords: *Mitochondria, Langur, CYTB, Trachypithecus*, *Semnopithecus, phylogeny*

Nilgiri langurs are arboreal primates endemic to the Western Ghats and are distributed across the states of Kerala, Karnataka and Tamil Nadu. They are characterized by thick black fur,

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long hairs on the crown and sides of the head with a flaxen mane around their black hairless faces. The young ones are red-brown in colour and this colour turns black and animals attain full adult colouration by 4-5 months. The species has been listed under the Convention on International Trade in Endangered Species of Wild Fauna and Flora (Appendix II). They are protected and covered under the Indian Wildlife Protection Act, 1972. Under the IUCN Red data list, these langurs are listed as vulnerable (Malviya *et al.,* 2011).

Animal mtDNA is maternally transmitted and non-recombining making it valuable for phylogenetic studies. The complete cytochrome b (*CYTB*) gene is 1140 bp and is highly conserved. The mt*CYTB* has been used for species identification in endangered species (Hsieh *et al*., 2001) and to resolve phylogeny in many species like fishes (Farias *et al*., 2001), musk deer (Su *et al*., 1999), sika deer (Kuwayama and Ozawa, 2000) and langurs (Karanth *et al.,* 2008 and Osterholz *et al.,* 2008). Earlier, the Nilgiri langurs were classified as a separate genus Kasi, which was replaced by *Trachypithecus* because of their morphological similarities with other rain forest langurs of Asia (Eudey, 1980). Molecular genetics studies based on mt*CYTB* have grouped Nilgiri langurs under the *Semnopithecus* sp. along with other common langurs of India (Karanth *et al.,* 2008 and Osterholz *et al.,* 2008).

The faecal sample of one Nilgiri langur was collected from Thiruvananthapuram Zoo. Approximately 25 grams of faecal sample was collected, packed in air tight eppendorf tube and transported on ice pack to the molecular genetics laboratory of Department of Animal Genetics and Breeding, College of Veterinary and Animal Sciences, Pookode. Genomic DNA was isolated from the faecal sample using HiPurA™ Stool DNA Purification Kit (Himedia, India) according to the manufacturer's protocol. Polymerase chain reaction was performed for the amplification of 1140 bp fragment of mitochondrial cytochrome b (*CYTB*) gene using the published primers, L14724 and H15915 (Table 1). The PCR reaction was set up in a total volume of 50 µL reaction mixture with EmeraldAmp® GT PCR Master Mix (Cat# RR310B, DSS Takara Bio, India). The PCR cycling conditions comprised of initial denaturation of 94 ºC for 5 min followed by 35 cycles of denaturation at 94 ºC for 40 sec, annealing at 54 ºC for 30 sec, extension at 72 ºC for 90 sec and a final extension at 72 ºC for 10 min. The amplification of the 1140 bp mt*CYTB* was confirmed by 1.75% agarose gel electrophoresis in TAE buffer and Sanger di-deoxy sequencing. The final sequence was submitted to NCBI GenBank database (Accession No. MW717575).

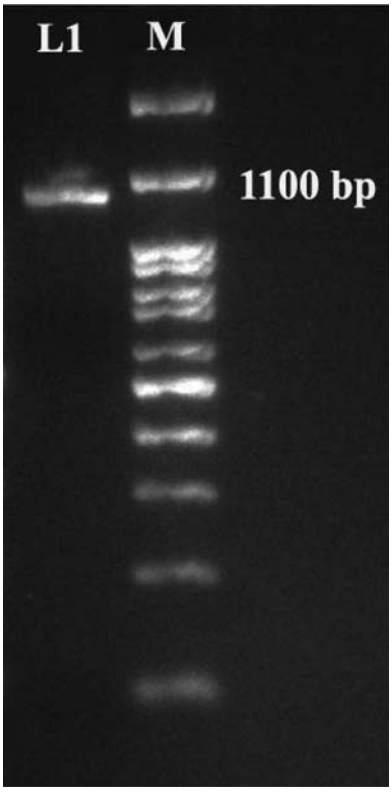

Fig 1. PCR amplification of mt*CYTB* gene of Nilgiri Langur

Table 1. Primer sequences targeting mitochondrial cytochrome b (*CYTB*) gene

Primer name	Strand	Sequence	Reference
L14724	Light	5′-CGAGATCTGAAAAACCATCGTTG–3′	Karanth *et al*., 2008
H15915	Heavy	5′-AACTGCAGTCATCTCCGGTTTACAAGA–3′	

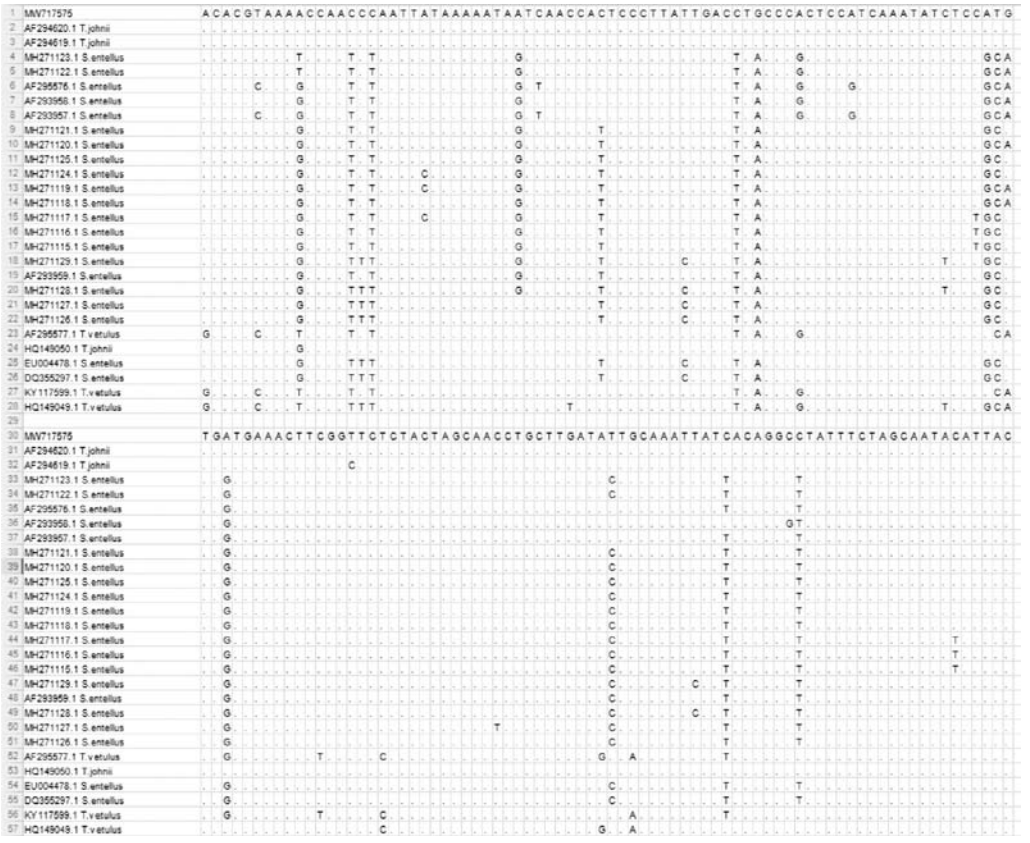

Fig 2. Multiple sequence alignment of mt*CYTB* sequences used in the study.

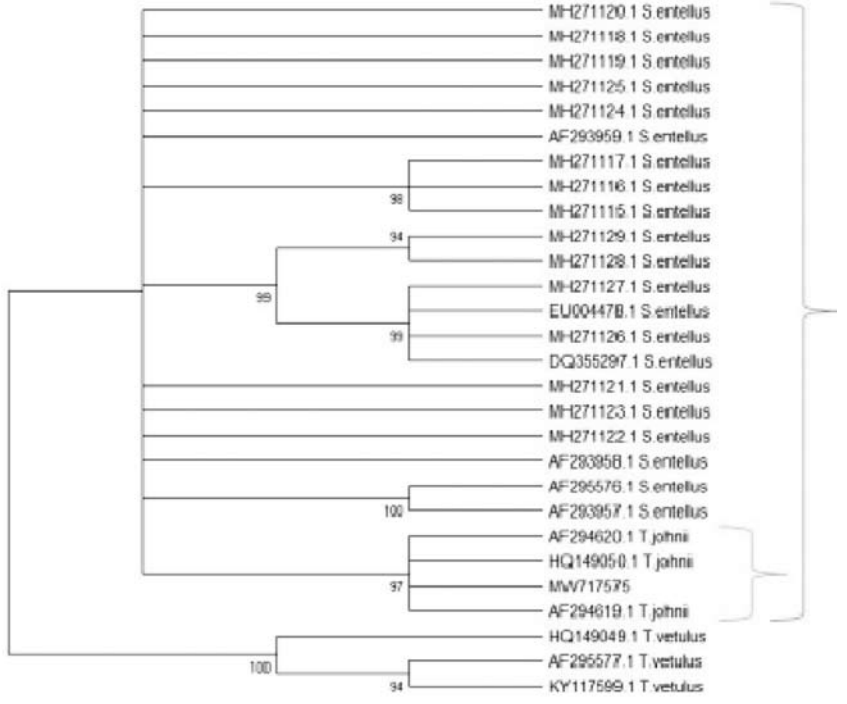

Fig 3. Phylogenetic tree constructed using maximum likelihood method

Multiple sequence alignment was done using the Clustal W algorithm using default parameters in MEGA X (Kumar *et al.,* 2018). Twenty-seven *CYTB* sequences of langur species of *Semnopithecus* and *Trachypithecus* species (Accession nos. AF294620.1, AF294619.1, MH271123.1, MH271122.1, AF295576.1, AF293958.1, AF293957.1, MH271121.1, MH271120.1, MH271125.1, MH271124.1, MH271119.1, MH271118.1, MH271117.1, MH271116.1, MH271115.1, MH271129.1, AF293959.1, MH271128.1, MH271127.1, MH271126.1, AF295577.1, HQ149050.1, HQ149049.1, DQ355297.1, KY117599.1, EU004478.1) were retrieved from the GenBank database. The multiple sequence aligned file (.meg) was used to predict the best model for phylogenetic analysis using the best DNA/Protein model module in MEGA X. Phylogenetic tree was constructed by maximum likelihood method and Hasegawa-Kishino-Yano model with gamma distribution (Hasegawa *et al*., 1985). 1000 bootstrap replications were done with cut off value of 90 was used for bootstrapping.

PCR confirmed the amplification of 1140 bp mt*CYTB* gene (Fig. 1). The chromatogram (.ab1) files were visualized by Finch TV 1.4.0 for sequence analysis. The multiple sequence alignment file was generated for constructing phylogenetic tree (Fig. 2). Phylogenetic analysis showed that Nilgiri langur sequence from this study (MW717575) having clustering as sub tree with rest of *Trachypithecus* sequence from GenBank. In general, all the *Semnopithecus* and *Trachypithecus* sequences were found to cluster as single clade (Fig. 3). The sequences of *Trachypithecus vetulus* was found to form a seperate clade. These animals are endemic to Sri Lanka. Karanth *et al.* (2008) based on mt*CYTB*, protamine P1 and lysozyme genes, resolved the phylogeny among the different colobine species. They found that Hanuman Langurs (*S. entellus*) are inter- related with Nilgiri Langur and Purple-faced-Langur. Osterholz *et al.* (2008) used Y chromosomal and 573 bp mitochondrial sequence data to resolve the phylogeny in langurs and found that *T. vetulus* clustered within *Semnopithecus* sp. They observed paraphyly in *T. vetulus* and polyphyly of *Semnopithecus* genus which was split into 3 groups (*S. entellus* of North India, *S. entellus* of South India along with *T. johnii*, and *S. entellus* of Sri Lanka along with *T. vetulus*). This study supports the reports of Karanth *et al.* (2008) and Osterholz *et al.* (2008) in the placing both Nilgiri Langur and other langurs of India under same genera.

Summary

Nilgiri Langurs (*Trachypithecus johnii*) are endemic primates of the rain forests of the Western Ghats. Faecal sample was collected from Nilgiri langur and the genomic DNA was isolated. The 1140 bp mitochondrial *CYTB* was amplified and sequenced using Sanger's di-deoxy method. The amplified sequence along with the 27 sequences of *Trachypithecus* and *Semnopithecus* that were retrieved from the GenBank database were used for analysis and the Phylogenetic tree was constructed by the maximum likelihood method in MEGA X. Phylogenetic analysis showed that the Nilgiri langur sequence from this study (MW717575) had clustering as sub tree with the rest of the *Trachypithecus* sequence retrieved from the GenBank. The analysis has shown the clustering of Nilgiri langur with other langurs of *Semnopithecus* sp. as a single clade.

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Conflict of interest

The authors have no conflicts of interest to declare that are relevant to the content of this article.

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