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Research Article

MITOCHONDRIAL CYTOCHROME B GENE BASED PHYLOGENETIC ANALYSIS OF NILGIRI LANGUR (*SEMNOPITHECUS JOHNII*) SAMPLED FROM PERIYAR TIGER RESERVE, KERALA

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ABSTRACT

The Western Ghats, a biodiversity hotspot in peninsular India, serve as a critical habitat for the vulnerable Nilgiri langur (*Semnopithecus johnii*), endemic to the region. This study explores the phylogenetic placement of *S. johnii* populations based on the mitochondrial *CYTB* gene (857 bp) sequences. Briefly, twelve faecal samples were collected from three sites within Periyar Tiger Reserve (PTR), Kerala. The DNA was isolated from the faecal samples and the *CYTB* gene was amplified and sequenced. The sequences were subjected to phylogenetic analyses using Maximum Likelihood, Bayesian Inference and Neighbour-Joining methods. All the methods revealed a well-supported monophyletic clade comprising PTR sequences and additional *S. johnii* and *S. priam* sequences retrieved from NCBI sampled from Aanamalai, Silent Valley and Walayar. The PTR populations clustered into three subclades with moderate support, while one sequence formed a distinct lineage, indicating genetic differentiation within populations. These findings corroborate earlier molecular studies, supporting the classification of *S. johnii* under the genus *Semnopithecus*.

Keywords: CYTB, monophyly, Nilgiri langur and Phylogenetic analysis.

INTRODUCTION

Western Ghats (Sahyadri Mountains) form a continuous 1,600 km mountain range along the western coast of peninsular India, spanning 140,000 km² across five states. This region, recognised for its ecological significance, is home to three langur species, including the Nilgiri langur (*Semnopithecus johnii*), which is endemic to the area. *S. johnii* is distributed across various forest types, from high-altitude evergreen rainforests to low-altitude deciduous forests, with notable populations in Brahmagiri Hills, Silent Valley National Park and Walayar (Singh *et al*., 2020). The Nilgiri langur is listed as vulnerable on the IUCN Red List, protected under Appendix II of CITES and the Indian Wildlife Protection Act, 1972, with an estimated population of fewer than 20,000 individuals, including less than 10,000 mature individuals (Molur *et al*., 2003; Singh *et al*., 2020). Periyar Tiger Reserve (PTR), a biodiversity hotspot in Kerala's Western Ghats, provides a crucial habitat for the Nilgiri langur. Mitochondrial DNA (mtDNA), with its

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high copy number and lack of recombination, is widely used in phylogenetic studies. The mitochondrial *CYTB* gene has been widely used for species identification in endangered species (Hsieh *et al*., 2001) and for resolving phylogenies in various taxa, including langurs. The taxonomy of langurs in the Indian sub-continent has been contentious, with early classifications relying on subjective assessments of morphological features. Groves (2001) classified Nilgiri langurs of the Western Ghats and Purple-faced langurs of Sri Lanka under *Trachypithecus*, citing their shared habitat in high rainfall regions while placing Hanuman langurs (*Semnopithecus sp.*) in dryland habitats to justify their separation into distinct genera. Phylogenetic analyses using mitochondrial and nuclear markers revealed their polyphyly with Nilgiri and Purple-faced langurs, prompting the provisional split of Hanuman langurs into three species to align taxonomy with evolutionary relationships (Karanth *et al*., 2008, 2010; Osterholz *et al*., 2008). Thus, this study was undertaken with the objective to use phylogenetic techniques in addressing the taxonomical classification of langurs.

MATERIALS AND METHODS

The study was conducted in three locations in Periyar Tiger Reserve viz., Parakulam (Latitude: 9.597557°N, Longitude: 77.177657°E), PTR bamboo groove (9.601038°N, Longitude: 77.167087°E and PTR landing (Latitude:9.578590°N, Longitude: 77.180358°E) (Figure 1.).

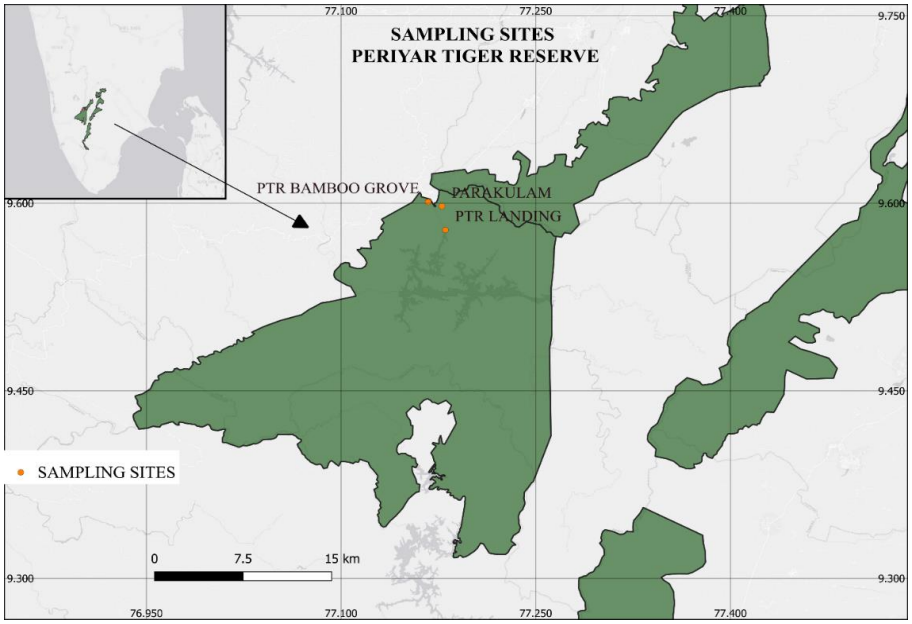

Figure 1. The study site in the Periyar Tiger Reserve

Fresh faecal samples, collected during peak activity hours (08:00–12:00 and 14:00–18:00), were preserved in sterile vials containing Longmire buffer (Longmire *et al*., 1997) and stored at room temperature. Observations were supplemented with GPS coordinates and photographs. Twelve faecal samples from *S. johnii* were collected and DNA was isolated using QIAamp Fast DNA stool mini kit (Qiagen). DNA purity and concentration were checked via NanoDrop spectrophotometry. The partial mitochondrial *CYTB* gene (874 bp) was amplified using specific primer set using primer sets 5′-CGAGATCTGAAAAACCATCGTTG–3′ and 3′-AACTGCAGTCATCTCCGGTTTACAAGA–5′ (Karanth *et al.*, 2008) and a PCR thermal cycling protocol 94°C for 5 min; 35 cycles of 94°C for 40 s, 54°C for 30 s, 72°C for 90 s and final extension at 72°C for 10 min. Amplicons were visualised via gel electrophoresis, purified and sequenced using the Sanger method. Chromatograms were analysed and sequences were validated using NCBI-BLAST. The phylogenetic relationship was inferred using Maximum likelihood (HKY+G+I -model) and Neighbour-joining (TN93 - model) in MEGA 11 (Tamura *et al*., 2021) and Bayesian inference analysis using MrBayes (GTR+G+I model) (Ronquist *et al*., 2012). For all the analysis sequences of *S. priam* (Accession nos. JQ734694.1 and JQ734745.1) and *S.johnii* (Accession nos. AF294619.1 and JQ734755.1) retrieved from NCBI were used. *Trachypithecus phyrei* (AF294621.1) was used as an outgroup.

RESULTS AND DISCUSSION

Three sites within Periyar Tiger Reserve were surveyed for *Semnopithecus johnii*. The largest troop, located at PTR Landing, consisted of 24 individuals with all age classes well-represented. Smaller troops were recorded at Parakulam (13 individuals) and PTR Bamboo Groove (19 individuals) (Table 1.).

Table 1. Troop composition of *Semnopithecus johnii* troops from Periyar Tiger Reserve.

Sampling site	Adult male	Adult female	Subadult	Young one	Juvenile	Troop size
Parakulam	2	3	4	1	2	13
PTR bamboo groove	2	4	6	1	4	19
PTR landing	2	6	7	4	5	24

The *CYTB* sequences of Nilgiri langur from PTR formed a well-supported clade, subdivided into three sub-clades with moderate support (BPML = 71). Sequence 5 remained distinct (Figure. 2).

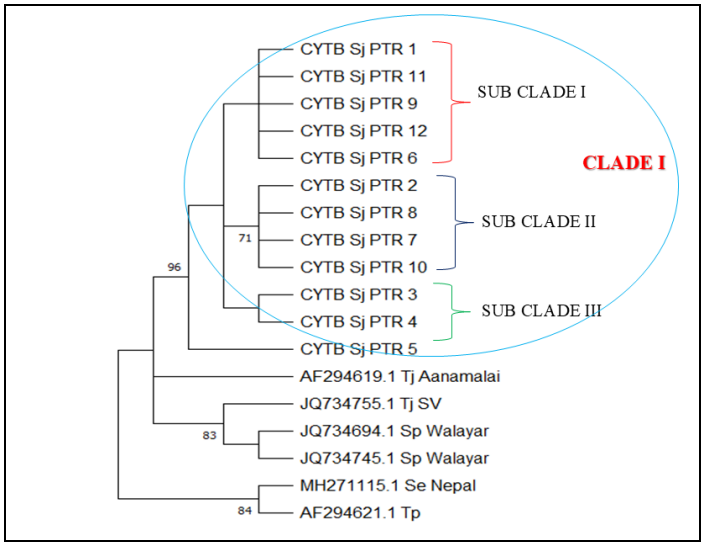

Figure 2. Phylogenetic analysis of samples from PTR on CYTB gene using Maximum likelihood method.

The Bayesian Inference analysis resulted in a similar phylogenetic tree with strong support for the basal branches, with a high posterior probability value (PP = 0.99). Subclade I consisted of sequences 1, 6, 9, 11 and 12 (PP = 0.96). Subclade II included sequences 2, 7, 8 and 10 (PP = 0.96). Similarly, Subclade III comprised 3 and 4 (PP = 0.97). Sequence 5 remained a distinct lineage, not grouping with any other sequences (Figure 3.).

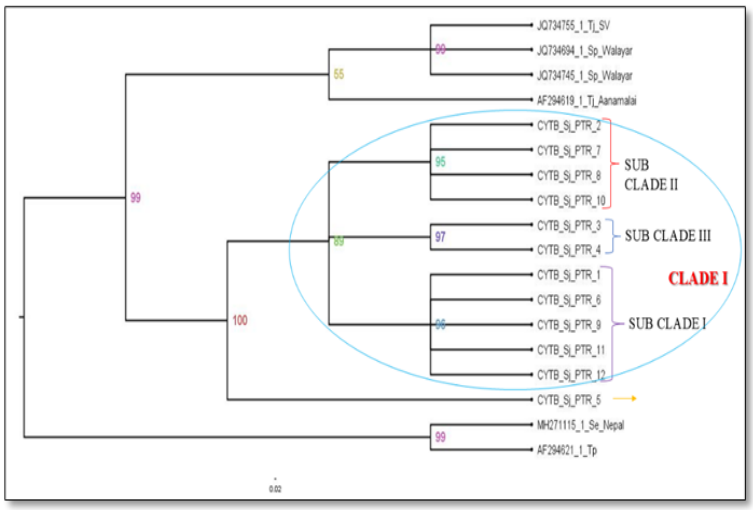

Figure 3. Phylogenetic analysis of samples from PTR on CYTB gene using Bayesian phylogenetic.

Neighbour-joining analyses showed similar clades with strong support (PP = 0.99), but weak support for Subclade III in NJ (BPNJ < 70) (Figure. 4).

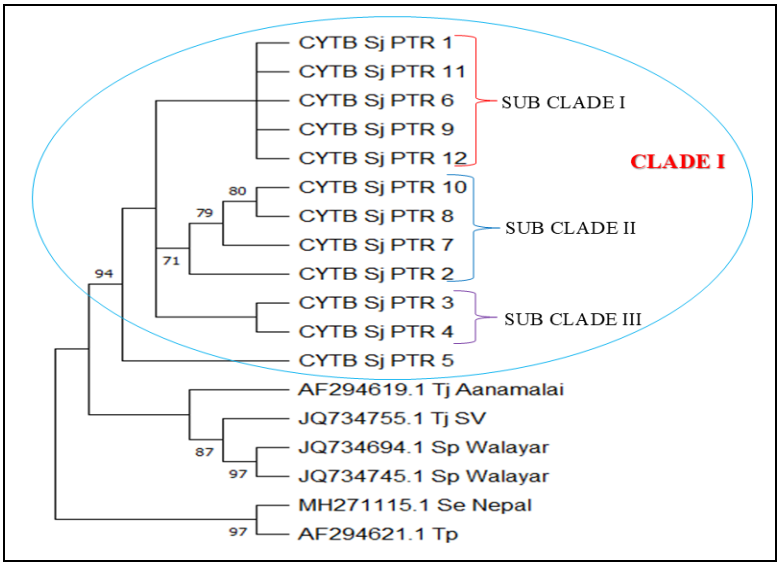

Figure 4. Phylogenetic analysis of samples from PTR on CYTB gene using Neighbour-joining method.

In all the analyses, the sequences from Parakulam and PTR bamboo grove cladded together. These PTR sequences were monophyletic, grouping with two sequences of *S. johnii* (from Aanamalai and Silent Valley) and two sequences of *S. priam* (from Walayar) retrieved from NCBI. This indicated langurs of WG belonging to the Semnopithecus genus are monophyletic. This study supports the reports of Karanth *et al*. (2008) and Osterholz *et al*. (2008) in placing both Nilgiri Langur and other langurs of WG under the same genera.

CONCLUSION

The findings affirm the taxonomic placement of *S. johnii* under the genus *Semnopithecus*, as previously proposed by molecular studies. The genetic data corroborate the hypothesis that langurs of the Western Ghats belong to a monophyletic group, supporting their classification under a single genus despite earlier morphological differences. The observed genetic differentiation within PTR populations highlights the potential impact of habitat fragmentation on population structuring. Given the vulnerable status of *S. johnii* on the IUCN Red List and its restricted distribution in the Western Ghats, extensive studies using molecular markers within and across populations are crucial for long-term conservation. The genetic differentiation among PTR populations highlights the importance of enhancing habitat connectivity and implementing conservation strategies to address fragmentation.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest

ETHICS APPROVAL

Not applicable

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