PHYLOGENETIC ANALYSIS OF NILGIRI TAHR (*Nilgiritragus hylocrius*) USING MITOCHONDRIAL CYTOCHROME B GENE

SIVASANKAR TAYE
(20-MSVP-11)

DISSERTATION

Submitted in partial fulfilment of the requirement for the degree of

MASTER OF SCIENCE
(Wildlife Studies)
2022

Faculty of Veterinary and Animal Sciences
Kerala Veterinary and Animal Sciences University

KVASU CENTRE FOR WILDLIFE STUDIES
KERALA VETERINARY AND ANIMAL SCIENCES UNIVERSITY
POOKODE, WAYANAD 673576
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DECLARATION

I hereby declare that this dissertation entitled **"Phylogenetic analysis of Nilgiri Tahr (*Nilgiritragus hylocrius*) using mitochondrial Cytochrome b gene"** is a bonafide record of research done by me during the course of research and that the dissertation has not previously formed the basis for the award of any degree, diploma, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

Certified that this dissertation, entitled **"Phylogenetic analysis of Nilgiri Tahr (*Nilgiritragus hylocrius*) using mitochondrial Cytochrome b gene"** is a record of research work done independently by **Sivasankar Taye (Roll no. 20-MSVP-11)** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to him/her.

Place: Pookode
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CERTIFICATE

We, the undersigned members of the advisory committee of **Sivasankar Taye (Roll no. 20-MSVP-11),** a candidate for the degree of Master of Science in Wildlife Studies, agree that this dissertation entitled **"Phylogenetic analysis of Nilgiri Tahr (*Nilgiritragus hylocrius*) using mitochondrial Cytochrome b gene"** may be submitted by **Sivasankar Taye (Roll no. 20-MSVP-11)** in partial fulfilment of the requirement for the degree.

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EXTERNAL EXAMINER

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Sivasankar Taye

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LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
°C	Degree Celsius
CYTB	Cytochrome b
bp	Base pair
BP	Bootstrap percentage
BLAST	Basic local alignment search tool
DNA	Deoxy ribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
Fig	Figure
g	Gram
IUCN	International Union for Conservation of Nature
MEGA	Molecular Evolutionary Genetic Analysis
ML	Maximum likelihood
MP	Maximum parsimony
µL	Microliter
NCBI	National Center for Biotechnology Information
ng	Nanogram
NJ	Neighbor joining
PCR	Polymerase chain reaction
pH	Negative hydrogen ion concentration
PP	Posterior probability
rcf	Relative centrifugal force
RNA	Ribonucleic acid
TBE	Tris borate EDTA
TE	Tris EDTA

INTRODUCTION

1. INTRODUCTION

Tahrs belong to the family Bovidae under the tribe *Caprini sensu lato* (Hassanin and Douzery, 1999), which also includes domestic goats and sheep. Simpson grouped tahrs along with sheep, goats, and their related species, within the tribe *Caprini* in 1945 (Ropiquet and Hassanin, 2004). The tribe *Caprini* represents one of the highly diverged groups within the Bovidae that includes domestic and wild species of goats (*Capra*), sheep (*Ovis*), and related species like Bharals (*Pseudois*) and Aoudad (*Ammotragus*) along with tahrs. Tahrs are gregarious wild ungulates that inhabit the uneven terrain of mountain ranges of India, Oman, and the United Arab Emirates (Sathyakumar *et al*., 2015). They prefer higher altitudes and inaccessible rocky terrain with cliffs and slopes (Kittur *et al*., 2010; Ross *et al*., 2018). Tahrs share numerous morphological features with sheep (*Ovis*) and goats (*Capra*). Hodgson described tahrs as morphologically intermediate between goats and the deer family. Tahrs are characterized by compressed, trigonal horns that are knotted in the front and the presence of a small muffle. Like goats, they are devoid of glands on the face, feet, or inguinal region and have a similar appearance with an odorous body (Jerdon, 1874). The horns are placed closely at the base that curve gradually backward. However, the lack of beard on the male's chin, comparatively smaller horn size, indistinct sexual dimorphism of horn size, and presence of four teats (in the Himalayan tahr) morphologically differentiate tahrs from goats (Lydekker, 1898). At present, three recognized species of tahrs are found. Of the three tahrs, the Nilgiri Tahr (*Hemitragus hylocrius*) (Ogilby, 1838) is present in southern India (Kerala and Tamil Nadu), the Arabian Tahr (*Hemitragus jayakari*) (Thomas, 1894) is endemic to northern Oman and the United Arab Emirates (Ross *et al*., 2018), and along the southern sides of the Greater Himalaya, the Himalayan Tahr (*Hemitragus jemlahicus*) (Smith, 1826) is distributed.

1.1. NILGIRI TAHR

Nilgiri Tahr is generally found on the rocky terrain of high-elevation areas at an altitude over 2500 meters above mean sea level with higher slope angles closer to cliffs and prefers high-altitude shola grassland habitat (Easa and Alembath, 2018). Once, the species was distributed in the mountain ranges of Western Ghats, including the Wayanad and Mysore districts of Kerala and Tamil Nadu (Jerdon, 1874). Currently, Nilgiri tahr is restricted to a small geographic area of the Western Ghats, mainly in three landscapes, i.e. the Nilgiri, the Anamalai, and the Periyar landscapes, of Kerala and Tamil Nadu. The most comprehensive estimate suggests a total population of about 2617–4232 individuals of Nilgiri Tahr throughout its range (Easa and Alembath, 2018). According to the IUCN Red List of Threatened Species 2008, the Nilgiri Tahr is classified as an endangered (EN) species with decreasing trend in its population. Moreover, the species faces a grave threat of extinction due to its distribution in several poorly connected habitat patches. The species is highly prone to the impacts of climate change due to the shrinkage of suitable habitats (Sony *et al*., 2018).

The phylogenic status of tahrs is always under question. Most studies on the phylogeny of tahr based on morphological features either grouped them with goats (Jerdon, 1874) or termed them as a distinct group of caprines allied to goats (Lydekker, 1898). Phylogenetic analyses of Himalayan Tahr based on molecular data indicate tahr's close association with goats (*Capra*) (Groves and Shields, 1996; Gatesy *et al*., 1997; Hassanin *et al*., 1998; Hassanin and Douzery, 1999). Nilgiri Tahr is classified under the genus *Hemitragus,* along with the Arabian and the Himalayan Tahr, under the family Bovidae. A recent phylogenetic analysis including all three species of tahr provided evidence of the genus *Hemitragus* being polyphyletic (Ropiquet and Hassanin, 2005). The authors had proposed a new taxonomy of tahrs assigning Nilgiri Tahr a new genus *Nilgiritragus*. They also hypothesized that Nilgiri Tahr is closely associated with sheep (*Ovis*), not goats (*Capra*). Thus it is necessary to conduct a phylogenetic analysis of tahrs with particular emphasis on Nilgiri Tahr for a

scientific establishment of the species' actual phylogenetic position.

The present study used the mitochondrial cytochrome b gene (Cyb) as a molecular marker. This study was envisaged to sequence the cytochrome b gene of Nilgiri Tahr from fecal samples, investigate the phylogenic position of Nilgiri Tahr, and understand its relationship with the rest of the tribe *Caprini sensu lato* (Hassanin and Douzery, 1999). Moreover, no such study was attempted in Kerala earlier despite the species being endemic to the state. Given the importance of the Nilgiri Tahr as a flagship species with a limited distribution range, this study is imperative.

The present study was undertaken with two objectives:

1. To sequence the mitochondrial cytochrome b gene of Nilgiri Tahr
2. To conduct phylogenetic analysis from the generated sequence data

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1. TAHRS

Tahrs belong to the family Bovidae under the subfamily caprinae, which also includes domestic goats and sheep. The subfamily caprinae comprises three tribes, namely *Caprini*, *Rupicaprini* and *Ovibovini*, including 12 genera of which *Hemitragus* representing tahrs belongs to the tribe *Caprini*. Most species belonging to *Caprini* are mountain dwellers preferring either gentle or steep slopes of Eurasian mountains, some in the North African mountain and American continents (Hassanin *et al*., 1998).

Tahrs are gregarious wild ungulates that inhabit the uneven terrain of mountain ranges of India and Oman, and the United Arab Emirates (Sathyakumar *et al*., 2015). Tahrs prefer higher altitudes and inaccessible rocky terrain with cliffs and slopes (Kittur *et al.*, 2010; Ross *et al.*, 2018). Based on morphology, taxonomists had either placed tahrs along with goats (Jerdon, 1874) or considered them to be allied to the genus *Capra* representing goats (Lydekkar, 1898). Unlike goats, males of tahr do not possess beards; they have a naked muzzle and short sharp backward curving horns. Moreover, unlike other caprids, they show no sexual dimorphism for horn size (Sathyakumar *et al*., 2015). Of the three tahr species, the Nilgiri Tahr (*Hemitragus hylocrius*) (Ogilby, 1838) is present in the southern part of India (Kerala and Tamil Nadu), the Arabian Tahr (*Hemitragus jayakari*) (Thomas, 1894) is endemic to northern Oman and the United Arab Emirates (Ross *et al.*, 2018) and along the southern sides of the Greater Himalaya the Himalayan Tahr (*Hemitragus jemlahicus*) (Smith, 1826) is distributed.

Nilgiri Tahr is the only endemic caprine species of South India. It has a dark reddish-brown coat and is characterized by short, highly backward curving horns closely placed at the base and diverging gradually. The short, thick, and coarse hairs form a short mane at the back of the neck and shoulder in the case of males (Lydekkar, 1898). Mature males are characterized by distinct facial marking and a

white stripe separates the dark brown muzzle from a dark cheek (Abraham *et al*., 2006). It is found generally on the rocky terrain of high elevation areas at an altitude range of 1100-2600 meters with higher slope angles closer to cliffs. They prefer high altitude shola grassland habitat (Easa and Alembath, 2018). According to the IUCN Red List of Threatened Species 2008, the Nilgiri Tahr is an endangered (EN) species with decreasing trend in its population. The Nilgiri Tahr is provided with the highest protection as a Schedule I animal of the Wildlife (Protection) Act of 1972. Presently, the Nilgiri Tahr is distributed in around 5% of the Western Ghats in southern India, Kerala, and Tamil Nadu in several fragmented patches of populations.

2.1.1. Taxonomic Classification

According to the present taxonomic classification, the tahrs are reassigned to three separate genera *Hemitragus*, *Nilgiritragus*, and *Arabitragus* for Himalayan, Nilgiri, and Arabian Tahr respectively (Ropiquet and Hassanin, 2005).

Kingdom	Animalia
Phylum	Chordata
Sub-phylum	Vertebrata
Order	Artiodactyla
Family	Bovidae
Sub-family	Caprinae
Genus	Nilgiritragus
Species	*Nilgiritargus hylocrius* (Ropiquet and Hassanin, 2005)

2.2. MOLECULAR PHYLOGENETIC STUDY

Phylogenetic analysis has gained greater importance in modern-day conservation genetics as it gives an idea of the phylogenetic diversity among species in the habitat instead of a mere idea of species richness (Karanth *et al*., 2019). Moreover, phylogenetic analysis can provide insight into the evolutionary history of species as well the relationship between them. Molecular phylogenetic analysis has become popular nowadays due to the widespread use of DNA sequencing methods, well-established phylogenetic tree construction methods using gene sequences together with the availability of numerous phylogenetic tree construction programs (Horiike, 2016).

A phylogenetic tree consists of branches connected by nodes. It is constructed based on nucleotide sequences which represents the relationship among a group of species, and the nodes represent speciation events. Phylogenetic trees can be constructed either by distance-based methods like Neighbour-Joining (NJ) or character-based methods like Maximum Parsimony (MP), Maximum Likelihood (ML), and Bayesian Index (BI) (Yang and Rannala, 2012).

Phylogenetics plays a significant role in the field of conservation. Phylogenetic study can determine the phylogenetic rarity or phylogenetic distinctiveness of species that can be utilized in prioritizing conservation activities or identifying species of particular conservation concern. Phylogenetic evaluation of species can determine the extent of loss of evolutionary information due to species extinction which may vary based on the evolutionary age (young or old) and species richness (species-rich or species-poor) of the clade or phylogenetic distinctiveness of the species. It can further help to identify areas with high species richness and high levels of phylogenetic diversity (Winter *et al*., 2013).

2.2.1. Phylogenetic Studies on Wild animals

Phylogenetic studies are being used extensively in resolving taxonomic

confusions of several species of wild animals and birds (Su *et al*., 1999; Ropiquet and Hassanin, 2004; Ropiquet and Hassanin, 2005; Gonzalez *et al*., 2013).

Molecular phylogenetic study can act as a powerful tool for resolving the taxonomic ambiguity of lesser-known species when morphological studies fail to do so. Hassanin and Ropiquet (2004) determined the appropriate taxonomic position of Kouprey, a possibly extinct species of mammal, based on molecular phylogenetic analysis using three molecular markers- the promoter of lactoferrin gene, cytochrome b, and subunit II of cytochrome c oxidase gene. They established that Kouprey belongs to the subtribe *Bovina* along with Banteng and Gaur under the tribe *Bovini*.

Karanth (2003) reviewed the molecular phylogenetic methods are the appropriate approach to understand the disjunct distribution of certain species, including the Nilgiri Tahr. Moreover, phylogenies can determine significant biogeographic phenomena responsible for such species' distribution, which can further explain why a particular species is found in one geographic area but not in another geographic area.

Besides, molecular phylogenetic studies can be used to examine the integrity of taxonomic classifications of larger clades. Agnarsson and May-Collado (2008) performed a large-scale phylogenetic analysis of the order Cetartiodactyla. The result, so generated, was consistent with all known major clades within the order based on previous studies. They observed that phylogenetic analysis using a single mitochondrial gene provides rapid estimates of phylogenetic relationships among species.

Gonzalez *et al*. (2013) performed a detailed phylogenetic analysis of all the 61 extant species of hornbills using Maximum Likelihood (ML), Maximum Parsimony (MP) and Bayesian method. The study, based on both mitochondrial and nuclear gene sequences, provided an insight to the already accepted classification of hornbills and proposed rearrangement of 6 major clades of

hornbills. The study resolved the long-standing confusion of the placement of species like *Penelopides exharatus* and *Tockus hartalaubi* within the family Bucerotidae.

One of the goals of conservation genetics is the assessment of genetic diversity that indicates the genetic well-being of the population. Phylogenetic studies can determine biodiversity in terms of phylogenetic diversity which, along with other matrices, is considered for the immediate management of threatened species (Fernández-García, 2017). Winter *et al*. (2013) reviewed the applicability of phylogenetic study in the field of wildlife conservation. According to the author, instead of conserving all components of biodiversity, phylogenetic diversity may be applied as a criterion for wildlife conservation. Thus, a conservation approach emphasizing phylogenetic rarity, richness, and phylogenetic diversity as a proxy for evolutionary potential was proposed.

Moreover, phylogenetic studies have been employed for testing species barriers of some of the widely distributed cryptic species, like the King Cobras. Shankar *et al*. (2021) performed a phylogenetic analysis of mitochondrial genes using the Maximum Likelihood (ML) method to test for candidate species of King Cobra along its distribution range. They found four lineages of King Cobra based on phylogenetic analysis, and they were considered potential candidate species.

2.3. MITOCHONDRIAL GENES

The mitochondrion is a vital organelle of a eukaryotic cell. It is a double membranous structure- the outer and inner membranes resulting in two compartments within the organelle: intermembranous space and the matrix. The inner membrane acts as a principal site for ATP generation through oxidative phosphorylation. So, mitochondrion is also known as the ‘powerhouse of the cell’. Mitochondria are different from other cellular organelles as they have their genetic component (DNA) apart from the nuclear DNA of the cell, which are of multiple copies per organelle. Like bacterial DNA, mitochondrial DNA is circular

and double-stranded. The size of the mammalian mitochondrial genome is 16,571 base pairs which encode 37 genes, including two ribosomal RNAs, 22 transfer RNAs, and 13 polypeptides required for oxidative phosphorylation (Chinnery and Schon, 2003).

Mitochondrial DNAs are rapidly evolving and are non-recombinational that inherit maternally. The rate of evolution of mitochondrial DNA is ten times faster than nuclear DNA and has a very slow rate of degradation (Mandal *et al*., 2014). Moreover, highly conserved mitochondrial gene sequences along with the above characteristics resulted in the predominant use of mitochondrial genes for deriving phylogenetic hypotheses. The highly conserved sequences provide information on molecular evolution and gene flow patterns (Cook, 2005). Different mitochondrial markers, like 12S rRNA, 16S rRNA, protein-coding sequences, and control region, are employed to resolve phylogenetic relationships at various levels because of the differences in their rate of evolution (Mandal *et al*., 2014).

2.3.1. Cytochrome b Gene

Cytochrome b is a protein coding mitochondrial gene. As compared to the ribosomal genes, protein coding mitochondrial genes have higher evolutionary rate which makes them one of the choicest mitochondrial genes for phylogenetic study. Zardoya and Meyer (1996) classified cytochrome b as one the good protein coding mitochondrial genes for analyzing evolutionary relationship among distantly related species at the levels of family, genera and species. Since cytochrome b contains significant amounts of phylogenetic information at various levels of taxonomy, it has been sequenced for a large number of mammals to perform phylogenetic analysis (Agnarsson and May-Collado, 2008).

2.3.1.1. Cytochrome b Gene in Phylogenetic Study

Cytochrome b is one of the most widely sequenced genes used for the phylogenetic study (Nevo, 2001). Cytochrome b is also a suitable phylogenetic

marker for the reconstruction of the phylogeny of the Bovidae (Hassanin and Douzery, 1999). So, the gene sequence has been used extensively in many studies encompassing a large number of species which can provide a better understanding of the genetic relationships between species, genera, and families.

A phylogenetic study conducted by Wink (1995) using cytochrome b sequences proved that although all vultures (both new world and old world) share common morphological features, vultures as a group are polyphyletic in nature and their shared morphology and behaviour of carrion feeding is a result of convergence rather than that of homology.

A phylogenetic study using cytochrome b gene sequences have provided more insights into the well-known family of ungulates, Cervidae. The previously accepted hypothesis of the monophyletic nature of the antlered deer was found to be invalid as the only antlerless deer species (*Hydropotes inermis*) was closely associated with the Odocolinae sub-family of antlered deer. Moreover, the family Cervidae, irrespective of its four sub-families, was found to consist of three distinct clades (Randi *et al*., 1998).

Cytochrome b gene sequence has been used for resolving long-standing ambiguity on phylogenetic relationship among the members of the musk deer species. With the help of maximum likelihood, maximum parsimony and distance method it was confirmed that all the musk deer species form a monophyletic group consolidating their validity within the order artiodactyla. Moreover, the divergence events of different species of musk deer could be derived by molecular clock (Su *et al*., 1999).

Farias *et al*. (2001) used cytochrome b gene to conduct phylogenetic analysis of the fish family Cichlidae to know whether the gene can be used as molecular marker. Based on the study they suggested that although cytochrome b had certain limitations, it could construct phylogenetic trees which were in agreement with the previously accepted phylogenetic relationships among the

member of the family Cichlidae.

Agnarsson and May-Collado (2008) used Cytochrome b gene as a phylogenetic marker to resolve the phylogeny of 264 extant species of mammals under the order Cetartiodactyla. They found that Cytochrome b as a phylogenetic marker performed impressively well with dense taxon sampling. The result of the study which used Bayesian method of phylogenetic analysis provided the best tool currently available for comparative species-level studies within Cetartiodactyla.

Xiong *et al*. (2013) tried to resolve the phylogenetic ambiguity of Red Goral (*Naemorhedus baileyi*) and to determine its taxonomic status within *Naemorhedus* by using cytochrome b gene sequences. They sequenced the Cytochrome b gene of Red Goral along with other species of gorals, to determine the phylogenetic relationships among them. The study found that the genus *Naemorhedus* comprised three distinct lineages and the Red Goral was an independent species within this genus.

2.4. PHYLOGENETIC STUDY IN CAPRINI

Arai *et al*. (1997) analyzed the phylogenetic relationship of five species within the tribe caprini, including Mouflon (*Ovis musimon*), Pasang (*Capra aegagrus*), Markhor (*Capra falconeri*), domestic goat (*Capra hircus*) and domestic sheep (*Ovis aries*) using complete cytochrome b sequences. They used the neighbor-joining method of phylogenetic analysis. Based on the study, Pasang was found to be the closest relative of domestic goats (99.4% homologous) and Mouflon to that of domestic sheep (99.3% homologous). This study further supported the hypothesis based on morphology, suggesting that domestic goats and sheep originated from Pasang and Mouflon.

Hassanin *et al*. (1998) conducted a phylogenetic analysis of cytochrome b gene sequences of 28 caprine species that comprised all the genera included within the subfamily Caprinae using parsimony analysis. They used differentially

weighted parsimony method. The analysis showed that the monophyly of the subfamily Caprinae holds true only in the absence of the genus *Saiga*, and none of the already recognized tribes including Caprini, under Caprinae was monophyletic. It further showed that tahrs and goats were closely related within the Caprines with the Himalayan Tahr (*Hemitragus jemlahicus*) forming a clade along with Capra and *Pseudois*. They used the cytochrome b sequence of Himalayan tahr as the sole representative of tahrs. This finding was further supported by Hassanin and Douzery (1999).

Based on a phylogenetic study, Hassanin and Douzery (1999) hypothesized that most of the tribes belonging to the family Bovidae resulted from two explosive radiations that occurred in the mid-Miocene period. The tribe caprini which includes the tahrs diverged in the late Miocene or early Pliocene period.

Ropiquet and Hassanin (2006), based on their phylogenetic study, concluded that the origin of wild goats (*Capra*) was a result of interspecific hybridization between the proto-*Hemitragus* and the ancestor of wild goats in the Pliocene epoch. It was followed by widespread diversification of *Capra* in the Plio Pleistocene epoch. The hybridization led to the transfer and later fixation of the maternal mitochondrial genome from proto-*Hemitragus* to *Capra* as a result of positive selection, as it provided a greater adaptive advantage to life at high altitudes with highly variable climatic conditions.

Hassanin *et al*. (2009) also, based on the analysis of mitochondrial genome, confirmed the close association among these three ‘goat-like’ genera (*Capra*, *Pseudois* and *Hemitragus*). Moreover, their study showed that *Ammotragus*, *Arabitragus*, *Budorcus*, *Capra*, *Hemitragus*, *Pseudois*, *Oreamnos*, *Ovis* and *Rupicapra* formed a separate clade within the tribe Caprini.

Zvychaynaya (2010) tried to investigate the phylogenetic relationship among wild goats under the genus Capra based on the cytochrome b gene and intron region of the SRY gene sequences. The author supported the hypothesis

that interspecific hybridization might have played a key role responsible for the greater diversity among Capra species. Moreover, the study demonstrated the recent evidence of interspecies hybridization among the wild and domestic members of the Capra species. The study could further consolidate the presence of two genetically different forms of Siberian Ibex (*Capra sibirica*) in its distribution range, where genetic distance amounts to the species level between these forms.

Urena *et al*. (2018) investigated the phylogenetic relationships between Alpine Ibex (*Capra ibex*) and Iberian Ibex (*Capra pyrenaica*), including different subspecies of Iberian wild goat to know the genetic history of these two caprine species. They analyzed the complete cytochrome b gene sequence from DNA isolated from fossil samples and modern populations as well to analyze the genetic history. They found that apart from other species of Capra, Alpine Ibex and Iberian wild goat were monophyletic in origin. This study further consolidated the findings based only on modern DNA samples. The study also identified *Capra pyrenaica pyrenaica* as an evolutionarily significant unit.

2.4.1. Phylogenetic Studies on Tahrs

There are very few studies focusing solely on the phylogeny of the tahrs. The taxonomic position of the tahrs was always under question and the tahr phylogeny being ambiguous. Most of the previous authors hypothesized that tahrs were close relatives of goats (Groves and Shields, 1996; Hassanin and Douzery, 1999; Ropiquet and Hassanin, 2006; Hassanin *et al*., 2009;). Particularly, the close association between tahr, bharal and wild goats was reiterated by a few authors (Hassanin *et al*., 1998, 2009; Hassanin and Douzery, 1999). Moreover, all the authors dwelt on the hypothesis that tahrs belong to single genera *Hemitragus*. Until recently, all the previous studies on phylogeny of tahrs were mainly confined to the Himalayan Tahr (*Hemitragus jemlahicus*) which was used as a sole representative of all the three species of tahrs. The findings proved a close association between tahrs and goats, indicating association between the Himalayan Tahr and goats.

One of the earliest works includes phylogenetic analysis of the mitochondrial cytochrome b gene sequences of caprines by Groves and Shields (1996). Their analysis showed a strong association between tahr (*Hemitragus jemlahicus*) and domestic goats (*Capra hircus*). Later, similar results were obtained by Gatesy *et al*. (1997) based on the analysis of the mitochondrial ribosomal DNA sequences of bovids which showed the Himalayan Tahr (*Hemitragus jemlahicus*) as a close relative of goats (Capra). The authors mentioned tahrs were relative species of goats.

The most comprehensive molecular phylogenetic studies of tahrs till date were performed by Ropiquet and Hassanin (2005), who, for the first time, included all three species of tahr- the Nilgiri Tahr, the Himalayan Tahr, and the Arabian Tahr, for analysis. They analyzed 3165 nucleotide characters derived from the mitochondrial genes- 12S rRNA , cytochrome b and subunit II of the cytochrome c oxidase, and an intron of the nuclear gene protein kinase c iota. The result showed that, unlike its previous belief, the genus *Hemitragus* representing the three species of tahr is polyphyletic, where the Nilgiri Tahr, Himalayan Tahr, and Arabian Tahr are associated with *Ovis*, *Capra*, and *Ammotragus*, respectively. They further reclassified the three species and reassigned them to three genera as *Nilgiritragus*, *Hemitragus*, and *Arabitragus*, respectively.

Yang *et al*. (2013) also supported the new taxonomy that tahrs were polyphyletic and each tahr (the Himalayan, the Arabian, and the Nilgiri Tahr) belonged to separate genera.

Joshi *et al*. (2018) conducted the one and only phylogentic study from the Western Ghats on Nilgiri Tahr. They studied two Nilgiri Tahr populations of the north and south of the Palakkad Gap using the cytochrome b gene. They found that, although the two populations were genetically different, the divergence was shallow with a mean pairwise genetic distance of 0.007. This study showed that the Palakkad gap was not a significant barrier for these two populations.

Ross *et al*. (2020) first analyzed the population genetic structure of the endangered Arabian Tahr of Oman using microsatellites. Their study found the presence of poor genetic connectivity between the northern and southern populations of the Arabian Tahr due to anthropogenic activities. They also reported lower genetic diversity in the southern population due to the small population size and lack of connectivity.

MATERIALS AND METHODS

3. MATERIALS AND METHOD

3.1. SITE OF STUDY

Fecal samples of Nilgiri Tahr were collected from the Eravikulam National Park of Idukki district, Kerala, India. The Eravikulam National Park, with an area of 97 square kilometres, is located in the southern part of Kerala in the Devikulam Taluk of Idukki District on SH17 (Udumalpet Road) 10^0 10' - 10^0 20' North and 77^0 0' - 77^0 10' East. The park is more than 2100 meters above mean sea level. It holds the highest population of Nilgiri Tahr with better visibility due to the presence of suitable habitat for the species. The vegetation type of the park is shola grasslands with steep slopes and rocky cliffs. The park is also known for the Anamudi Peak, the highest peak south of the Himalayas with a height of 2695 metres above the mean sea level.

3.2. SAMPLE COLLECTION

Faecal samples from eight individuals of Nilgiri Tahr were collected from the resting places of the animals, mainly rocky beds and cliffs, from the tourism zone of the Eravikulam National Park. The faecal samples were stored in Lysis buffer (Longmire *et al*., 1997) and 95 per cent ethanol until DNA isolation.

3.3. CHEMICALS USED FOR THE STUDY

All the chemicals used in this study were molecular biology grade. The reagents used were Agarose (M/s. HiMedia Laboratories, India CAT# MB002); Nuclease-Free Water (Thermo Fischer Scientific, USA, CAT# AM9932); Ethidium bromide (M/s. Sisco Research Laboratories, India, CAT# 17220); 100 bp DNA ladder (M/s. HiMedia Laboratories, India, CAT# MBT049).

A

B

C

Plate 1. Photographs from the field. **(A)** An adult male Nilgiri Tahr at the Eravikulam National Park. **(B)** Fecal pellets of Nilgiri Tahr. **(C)** Collection of fecal pellets using sterile, wooden toothpicks.

3.4. REAGENTS AND BUFFERS

The reagents and buffers used in the study are given in the Annexure I.

3.5. GLASSWARES AND PLASTICWARES

The glasswares and plasticwares used in the study were procured from M/s. Borosil, Mumbai, India, and M/s. Tarson, Kolkata, India.

3.6. KITS

DNA isolation kit, procured from M/s HiMedia Laboratories, India, was used in this study.

3.7. SOFTWARES

FinchTV v.1.5 (Geospiza, Inc.; Seattle, WA, USA; http://www.geospiza.com), Unipro UGENE v.45.0 (Okonechnikov *et al*., 2012), and NCBI-BLAST (Altschul *et al*., 1990) were used for visualization of chromatograms and sequence analysis, MEGA11: Molecular Evolutionary Genetics Analysis version 11 (Tamura *et al*., 2021) was used for sequence alignment and phylogenetic analysis, raxmlGUI 2.0 (Edler *et al*., 2020) was used for phylogenetic analysis using the maximum likelihood method and MrBayes 3.1.2 (Ronquist *et al*., 2012) was used for phylogenetic analysis of the sequences using the Bayesian method. For selecting the best fit model of nucleotide substitution jModelTest v2.1.10 (Darriba *et al*., 2012; Guindon and Gascuel, 2003) was used. Genetic diversity of the sampled individuals was calculated using the dnaSP v6 (Rozas *et al.*, 2017) and the haplotype network analysis was carried out in popART v1.7 (Bandelt *et al*., 1999).

3.8. EQUIPMENT

The equipment/instruments used in this study were thermal cycler (M/s Bio-Rad, USA), gel documentation system G:BOX F3 (M/s SYNGENE, USA), horizontal electrophoresis apparatus with powerpack (M/s BioRad, USA), Nanodrop 2000C spectrophotometer (M/s Thermo Scientific, Massachusetts, USA), electronic balance (M/s Sartorius, Gottingen, Germany), Vortex (M/s REMI Group REMI Electrotechnics Limited), laboratory cooling centrifuge (iFuge UC02R , M/s Neuation Technologies Pvt. Ltd., India), water bath (M/s LabLine Equipment Pvt. Ltd., India), microwave oven (IFB India), Autoclase (Equitron) and variable volume micropipettes (M/s Transferpette, M/s Thermo Scientific and M/s Tarsons).

3.9. ISOLATION OF DNA

Fecal samples were transported to the Molecular Genetics Laboratory of the Department of Animal Genetics and Breeding, College of Veterinary and Animal Sciences, Kerala Veterinary and Animal Sciences University, Pookode. Genomic DNA from the fecal samples was isolated using HiPurA^{TM} Stool DNA Purification Kit (HIMEDIA, Mumbai, India, Cat. No. MB544) according to the manufacturer's protocol.

3.9.1. Isolation of DNA using kit

The DNA isolation procedures were performed as per the prescribed protocol of HiPurA^{TM} Stool DNA Purification Kit (HiMedia, India, Cat. No. MB 544). Fecal samples were centrifuged at 8000 g for 3 minutes in an iFUGE UC02R (Neuation Technologies Pvt. Ltd., India) centrifuge machine. The supernatant was discarded, and the pellet was resuspended in 500 µl of Lysis Solution AL). To 200 µl the resuspended solution, 20 µl of Proteinase K solution (20 mg/mL) was added. Then it was incubated at 55°C for 30 minutes to obtain a homogenous mixture. Stool lysis buffer (SL1) was added to the solution and allowed to lyse by incubation for 10 minutes at 70°C. 250 µl of Inhibitor Removal Solution (IRSH) was added to the lysate and was kept for 5 minutes at 4°C. The

tube containing the lysate was centrifuged at 10000 rcf for 1 minute at room temperature, and the supernatant was carefully transferred to a clean microcentrifuge tube (1.5 µl). Then 200 µl of Binding Solution (SB) was added to the lysate. The lysate was loaded on the HiElute Miniprep Spin Column (capped) and centrifuged at 10000 g for 1 minute. The flow-through was discarded, and the spin column was placed into the collection tube. Two-step washing was done by adding 500 µl of already diluted Wash Solution (WSP) to the spin column and centrifugation at 10000 g for 1 minute at room temperature. The flow-through was discarded every time. Additional centrifugation of the spin column was done at 10000 g for 1 minute to remove residual ethanol. The spin column was transferred to a new 2 mL uncapped collection tube. The DNA was eluted by adding 30 µl of Elution Buffer (ET) to the center of the spin membrane and centrifugation at 10000 rcf for 1 minute. The eluate-containing DNA was transferred to a 0.5 mL microcentrifuge tube and stored at -20°C till further use.

3.10. ASSESSMENT OF PURITY AND CONCENTRATION OF DNA

The concentration and purity of the genomic DNA were assessed by spectrophotometric analysis and agarose gel electrophoresis. Spectrophotometry was done in the NanoDrop 200C Spectrophotometer (Thermo Scientific, USA). The purity of DNA was assessed by noting the ratio of absorbance at 260 nm to absorbance at 280 nm (260/280 value).

The quality of the genomic DNA was determined by 1 per cent agarose gel electrophoresis. The DNA bands were visualized in the Gel Documentation System (G:BOX F3, M/s SYNGENE, USA).

3.11. POLYMERASE CHAIN REACTION

The template DNAs were subjected to PCR amplification using universal primers for ungulates (Gupta *et al*., 2014). The PCR amplicons were visualized in 2 per cent agarose gel electrophoresis in a gel documentation system (M/s

Syngene, USA).

3.11.1. Primers

All the primers used for the PCR amplification of genes were custom synthesized from Sigma-Aldrich Chemicals Private Limited. The details of the primers used in the study are shown in Table 1.

Table1. Details of primers used in the study

Target gene	Primer name	Oligonucleotide sequence (5'-3')	Product size	References
Cytochrome b gene (Cytb)	MC b F1 103	5′ CATTATTCTCACATGGAATCTAACC 3′	503 bp	Gupta *et al*., 2014
	MC b R1 560	5′ GCTCCTCAGAATGATATTTGTCCTC 3′		

3.11.2. PCR amplification

All PCR reactions were conducted in an automated thermal cycler with a heated lid (Bio-Rad MJ Mini Gradient Thermal Cycler, USA). The PCR reactions were set up in a total volume of 25µl using EmeraldAmp GT Master Mix (Cat. No. RR310B, DSS Takara Bio, India). The reaction mixture for PCR amplification is detailed in Table 2.

Table 2. Reaction mixture for PCR

Components	Volume/ reaction
2X PCR Premix	12.5 µl
Forward primer	1 µl
Reverse primer	1µl
Template DNA	Approx. 100 ng
Sterile Nuclease free water	Make up the volume to 25 µl

3.11.2.1. Amplification of Cytochrome b (Cytb) Gene

PCR reaction conditions for amplification of the Cytochrome b gene (503 base pair) were as follows- initial denaturation at 95°C for 10 minutes followed by 35 cycles each of denaturation at 95°C for 45 seconds, annealing at 55°C for 1 minute and extension at 72°C for 1 minute 30 seconds. The final extension was done at 72°C for 10 minutes (Gupta *et al*., 2014).

3.11.3. Agarose Gel Electrophoresis and Gel Documentation

Agarose gel electrophoresis was carried out to check the amplified PCR product in a 2 percent agarose gel. Agarose powder (M/s. Sigma Aldrich Chemicals Pvt. Ltd., Bengaluru, India) of 800 mg was suspended in 40 mL of 1X Tris Borate EDTA (TBE) buffer prepared from 5X Tris Borate EDTA (TBE) buffer. The suspension was dissolved by heating in a microwave oven and then cooled. 1µL (10mg/mL) of Ethidium bromide (M/s Himedia, Mumbai, India) solution was added to the solution and mixed thoroughly. Finally, the mixture was poured into a gel casting tray placed with an acrylic comb (to form wells) and allowed to solidify. After solidification, the comb was carefully removed and the gel was immersed in the electrophoresis tank containing 1X TBE buffer. The PCR products were loaded into separate wells. A 100bp DNA ladder (M/s. HiMedia Laboratories Private Limited, India) was used as a molecular marker. Electrophoresis was carried out at 70V, 400mA power supply for 90 minutes. The amplified PCR products were visualized by gel documentation system (Ms Syngene, USA).

3.12. PCR PRODUCT SEQUENCING

PCR products were sequenced at Eurofins Genomics India Pvt Ltd., Bangalore, India, on automated Sanger di deoxy nucleotide sequencing platform.

3.12.1. Sequence Analysis

The nucleotide sequences obtained were analysed for their identity using FinchTV v.1.4.0 (Geospiza, Inc.; Seattle, WA, USA; http://www.geospiza.com), Unipro UGENE v. 45.0 (Okonechnikov *et al*., 2012) and NCBI-BLAST (Altschul *et al*., 1990).

3.13. RETRIEVING NUCLEOTIDE SEQUENCES

An additional 34 cytochrome b gene sequences representing all the genera within the tribe *Caprini* (Hassanin and Ropiquet, 2004) were retrieved from GenBank, NCBI (www.ncbi.nlm.nih.gov) database using the keywords Cytochrome b, Cytb and species name. The details of the retrieved sequences are given below (Table 3).

Table 3. Details of Sequences Downloaded from NCBI

Sl.No.	**Species Name**	**Common name**	**Accession no.**
1	*Ammotragus lervia*	Aoudad	MZ507934
2	*Budorcas taxicolor*	Takin	MK748332
3	*Capra falconeri*	Markhor	AF034736
4	*Capra hircus*	Domestic Goat	MK234706
5	*Capra hircus*	Domestic Goat	*OQ117029
6	*Capra ibex*	Alpine Ibex	MF139822
7	*Capra nubiana*	Nubian Ibex	AF034740
8	*Capra aegagrus*	Wild Goat	DQ246781.1
9	*Capra sibirika*	Asiatic Ibex	DQ246799
10	*Capra caucasica*	West Caucasian Tur	DQ246801.1
11	*Capra cylindricornis*	East Caucasian Tur	DQ246776.1
12	*Capra walie*	Walia Ibex	EU368863.1
13	*Capricornis crispus*	Japanese Serow	LC316130.1
14	*Capricornis rubidus*	Burmese Red	NC_045205.1

Sl.No.	Species Name	Common name	Accession no.
		Serow	
15	*Capricornis swinhoei*	Taiwan Serow	OL814589.1
16	*Capricornis sumatraensis*	SumatranSerow	MH155202.1
17	*Hemitragus hylocrius*	Nilgiri Tahr	AY846792
18	*Hemitragus hylocrius*	Nilgiri Tahr	*OQ117027
19	*Hemitragus hylocrius*	Nilgiri Tahr	*OQ117028
20	*Hemitragus hylocrius*	Nilgiri Tahr	*OQ117030
21	*Hemitragus hylocrius*	Nilgiri Tahr	*OQ117031
22	*Hemitragus hylocrius*	Nilgiri Tahr	*OQ117032
23	*Hemitragus hylocrius*	Nilgiri Tahr	*OQ117033
24	*Hemitragus hylocrius*	Nilgiri Tahr	*OQ117034
25	*Hemitragus hylocrius*	Nilgiri Tahr	*OQ117036
26	*Hemitragus jayakari*	Arabian Tahr	AY846791
27	*Hemitragus jemlahicus*	Himalayan Tahr	AF034733
28	*Naemorhedus goral*	Himalayan Goral	EU259118
29	*Naemorhedus caudatus*	Long-tailed Goral	EU259117.1
30	*Naemorhedus baileyi*	Red Goral	JX506310.1
31	*Ovis ammon*	Gobi Argali	MW677290
32	*Ovis aries*	Domestic Sheep	MG407528.1
33	*Ovis canadensis*	Bighorn Sheep	FJ936181.1
34	*Ovis aries musimon*	European Mouflon	FR873151.1
35	*Ovis nivicola*	Snow Sheep	MW737010.1
36	*Ovis dalli*	Dall Sheep	EU365992.1
37	*Ovis vignei*	Urial	EU366043
38	*Oreamnos americanus*	Rocky mountain goat	AF190632
39	*Ovibos moschatus*	Muskox	FJ207536.1
40	*Pantholops hodgsonii*	Chiru	NC_007441.1
41	*Pseudois nayaur*	Bharala	JX10165
42	*Rupicapra pyrenaica*	Pyrenian Chamois	AF034726

Sl.No.	Species Name	Common name	Accession no.
43	*Rupicapra rupicapra*	Alpine Chamois	AF034725
44	*Cervus elaphus*	Red Deer	MT747183.1

*Sequence of this study

Cytochrome b gene sequences of *Cervus elaphus* was used as outgroup in the phylogenetic analysis.

3.14. MULTIPLE SEQUENCE ALIGNMENT

The nucleotide sequences were aligned using the Clustalw Algorithm (Higgins *et al*., 1994) in MEGA11 software (Tamura *et al*., 2021) using default parameters.

3.15. MODEL SELECTION FOR PHYLOGENETIC ANALYSIS

The jModelTest 2.1.10 (Darriba *et al*., 2012; Guindon and Gascuel, 2003) software was used for choosing the best fit nucleotide substitution model for the dataset. GTR+G+I model was selected based on Akaike Information Criterion (AIC) for phylogenetic analysis.

3.16. PHYLOGENETIC ANALYSIS

In addition to 43 Cytochrome b sequences of Caprini species including 9 sequences of this study, one sequence of *Cervus elaphus* was used as outgroup to root the phylogenetic tree. The GTR+G+I model was used for phylogenetic analysis of the sequences. The sequences were analysed using Maximum Likelihood, Bayesian Inference and Neighbour-Joining methods.

3.16.1. Tree Topology

Phylogenetic analysis using Maximum Likelihood, Bayesian Inference and

Neighbor-Joining methods. The model suggested by jModelTest 2.1.10 was used in all cases.

3.16.2. Maximum Likelihood

Maximum Likelihood Analysis of the aligned sequences were performed usingraxmlGUI 2.0 (Edler *et al*., 2020) software with 1000 bootstraps.

3.16.3. Bayesian Method

Bayesian method of phylogenetic analysis was carried out in MrBayes 3.2.7a (Ronquist *et al*., 2012) software. The evolutionary model used was Genereal Time Rversible (GTR) substitution model with gamma-distributed rate variation across sites and a proportion of invariable sites (GTR+G+I). The Bayesian analysis was performed with four independent Markov Chains Monte Carlo (MCMC) run for one million generations. The chain was sampled every 500 generations and the first 25% of the samples were discarded as burn-in. At program termination, the average standard deviation of split frequencies was 0.008320.

3.16.4. Neighbor-Joining Method

The Neighbor-Joining method was also used for analysing the sequences. It was performed in MEGA11 using default parameters.

3.17. GENETIC DIVERSITY AND HAPLOTYPE NETWORK ANALYSIS

Genetic diversity of a fraction of the Nilgiri Tahr population (eight individuals) from the Eravikulam National Park, Kerala, was calculated using DnaSPv6.12.03 (Rozas *et al.*, 2017) and the haplotype network analysis was carried out in PopART v1.7 (Bandelt *et al*., 1999).

RESULTS

4. RESULTS

4.1. SAMPLE COLLECTION AND STORAGE

A total of 8 fecal samples of Nilgiri Tahr were collected from the Eravikulam National Park, Idukki, Kerala. The fecal samples were kept in Lysis Buffer (Longmire *et al*., 1997) and 95% ethanol at room temperature till further processing. Both the storage conditions yielded satisfying results with a good percentage of successful amplification during PCR reaction.

4.2. POLYMERASE CHAIN REACTION AND SEQUENCE ANALYSIS

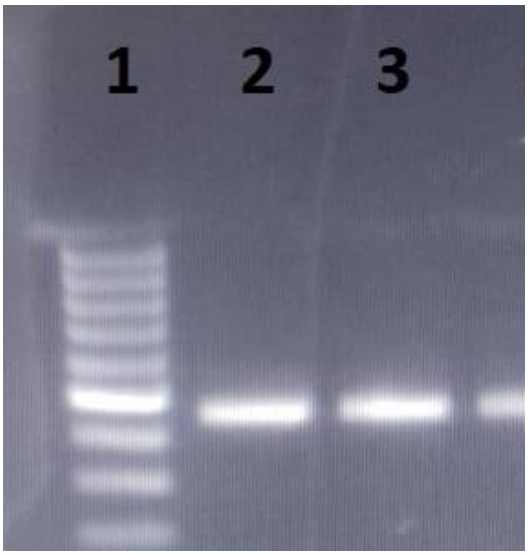

Figure 1. PCR amplification of a fragment (503bp) of the cytochrome b gene

The genomic DNA isolated from fecal samples was used as a template for PCR amplification of the 503 bp fragment of the cytochrome b gene (Figure 1). Sequences of the PCR products sequenced using automated sanger sequencing technology was used for phylogenetic analysis.

4.3. CYTOCHROME B SEQUENCE OF NILGIRI TAHR

Eight sequences of cytochrome b gene were finally sequenced from the amplified PCR products. For confirmation, the sequences were compared with the already available cytochrome b sequence by running a nucleotide BLAST in NCBI (Altschul *et al*., 1990). It showed a high percent identity value (Per Id) with the only available sequence of Nilgiri Tahr. The nucleotide BLAST results of the sequences are given below (Table 4).

Table 4. NCBI-BLAST results of the generated cytochrome b gene sequences

Isolate	**Accession no.**	**Query cover (%)**	**Percent identity (%)**
NT2	OQ117027	100	99.30
NT7	OQ117028	100	99.53
NT8	OQ117030	100	99.30
NT9	OQ117031	99	99.53
NT10	OQ117036	99	96.44
NT13	OQ117032	100	99.30
NT14	OQ117033	100	99.53
NT15	OQ117034	99	99.30

4.4. PHYLOGENETIC ANALYSIS

An attempt was made to understand the phylogenetic relationship of Nilgiri Tahr with the rest of the tahrs and other members of the extended tribe *Caprini sensu lato* (Hassanin and Douzery, 1999) with intensive species sampling. An effort was made to increase the sample size by including a maximum number of species of the genera belonging to this tribe. The cytochrome b gene was chosen as a molecular marker for phylogenetic analysis. The cytochrome b gene of Nilgiri Tahr sequenced in this study (Accession no. OQ117027, OQ117028, OQ117030, OQ117031, OQ117033, OQ117032, OQ117034, OQ117036) and

retrieved from GenBank NCBI (https://www.ncbi.nlm.nih.gov) along with that of other species were used for phylogenetic analysis. The details of the sequences are given inTable3.

Phylogenetic analysis using Bayesian Inference (BI), Maximum Likelihood (ML), and Neighbor-Joining (NJ) methods yielded a similar topology except for a few differences in branch support of certain branching events (Figure 4, 5 and 6).

In the case of Bayesian Inference, the basal branches were strongly supported with high posterior probability (PP) values but weakly supported in the Maximum Likelihood and Neighbor-Joining trees. Three major clades with strong support values (BP, PP) were observed in this analysis, they were

Sheep (*Ovis*) and Nilgiri Tahr (*Hemitragus hylocrius*) clade (PP=0.99, BP_{ML}=76, BP_{NJ}=81),

Goats (*Capra*) and Himalayan Tahr (*Hemitragus jemlahicus*) clade (PP=1.0, BP_{ML}=95, BP_{NJ}=95) and

Goral (*Naemorhedus*), Serow (*Capricornis*) and Muskox (*Ovibos moschatus*) clade (PP=1.0, BP_{ML}=77, BP_{NJ}=90)

The phylogenetic analysis showed that *Pantholops hodgsonii* was the first to diverge from the rest of the *Caprini sensu lato* group. This node was supported by a strong posterior probability value in Bayesian inference (1.0).

The genus *Ovis* was paraphyletic as it was associated with *Hemitragus hylocrius* forming the sheep-Nilgiri Tahr clade. Within this clade, the *Ovis ammon-Ovis vignei-Ovis aries-Ovis aries musimon* group was found to form a sub-clade along with *Hemitragus hylocrius,* indicating their close association with Nilgiri Tahr. All the cytochrome b sequences of Nilgiri Tahr sequenced in this study (Accession No, OQ117027, OQ117028, OQ117030, OQ117031, OQ117033, OQ117032, OQ117034, OQ117036) clustered with the members of

the genus *Ovis* sharing a common ancestor with sheep (*Ovis*). This association was shown with strong node support in all the trees (PP=0.99, BP_{ML}=76, BP_{NJ}=81).

The genus *Capra* was paraphyletic. The Himalayan Tahr (*Hemitragus jemlahicus*) formed a close association with goats (*Capra*) within the *Capra-Hemitragus jemlahicus* clade. The association was strongly supported in both Bayesian and maximum likelihood (ML) analyses (PP=1.0, BP_{ML}=95, BP_{NJ}=95). Besides, many *Capra* species shared a recent ancestor with Himalayan Tahr.

The Arabian Tahr (*Hemitragus jayakari*) was closely associated with Aoudad (*Ammotragus lervia*), which was shown in all three analyses supported by high posterior probability value in Bayesian analysis (PP=0.95) but low bootstrap percentage in maximum likelihood and neighbor-joining analysis (BP_{ML}=59).

The genus of interest, *Hemitragus,* was found to be polyphyletic as none of the members included in this genus shared a recent common ancestor and all of them were associated with different genera within the tribe *Caprini sensu lato* (Hassanin *et al*., 1998).

The third major clade was formed by *Naemorhedus*, *Ovibos*, and *Capricornis* (Goral, Serow, and Muskox) with robust node support in all the cases (PP=1.0, BP_{ML}=77, BP_{NJ}=90). All the species currently recognized under the genera *Namorhedus* and *Capricornis* were closely associated with *Ovibos moschatus* (Muskox).

All phylogenetic trees indicated a close association of Takin (*Budorcas taxicolor*) with *Pseudois nayaur*, and both were associated with *Capra*.

The only monophyletic genus recovered from this phylogenetic analysis with strong node support, *Rupicapra* (Chamois) (PP=1, BP_{ML}= 95, BP_{NJ}=98), formed a close association with the *Ammotragus-Hemitragus jayakari* group (PP=0.6).

The Rocky Mountain Goat (*Oreamnos americanus*) was associated with the *Naemorhedus-Capricornis-Ovibos* clade (PP=0.74).

Besides, none of the genera *Ovis* and *Capra,* along with *Hemitragus,* was found monophyletic.

4.5. GENETIC DIVERSITY ANALYSIS

The genetic diversity of a small fraction of Nilgiri Tahr population (eight) from the Eravikulam National Park, Idukki, Kerala, India, was analysed in this study. The genetic diversity analysis was carried out in DnaSP v6.12.03 software (Rozas *et al.*, 2017). A total of eight mitochondrial cytochrome b gene sequences each of 423bp long were analyzed for single nucleotide polymorphism. The analyzed sequences were devoid of insertion/deletion (InDels). A total of 15 polymorphic sites could be identified of which 14 were single variable sites and one was parsimony informative site.

The haplotype diversity (Hd) of the sub-population was 0.679 ±0.122 and the nucleotide diversity (Pi) was 0.00963. The average number of nucleotide differences (K) of the sub-population was 4.071 (Table 5).

Table 5. Results of genetic diversity analysis

Cytochrome b gene	Eravikulam National Park Sub-population
Aligned sequence length	423 base pair
No. of polymorphic (Segregating) sites (S)	15
Number of haplotypes (H)	3
Haplotype diversity and standard deviation (Hd ± SD)	0.679 ± 0.122
Nucleotide diversity (Pi)	0.00963
Average number of nucleotide differences (K)	4.071

Despite the small sample size, neutrality test was carried out to understand the demographic structure at molecular level. Tajima's D, Fu and Li's D and Fu and Li's F values were -1.51567, -1.74048 and -1.87508, respectively. All the values were found to be not significant.

Table 6. Results of neutrality test

Tests	**Results**
Tajima's D	-1.51567, P > 0.10
Fu and Li's D	-1.74048, 0.10 > P > 0.05
Fu and Li's F	-1.87508, 0.10 > P > 0.05

4.6. HAPLOTYPE ANALYSIS

The eight 423bp long cytochrome b sequences were further used for haplotype analysis. The analysis could identify 3 haplotypes in accordance with the variable site distribution. Haplotype H2 showed the highest frequency which was shared by four sequences and haplotype H3 had the lowest frequency which was found in only one sequence.

Table 7. Results of haplotype diversity analysis

Haplotype	**Sequences (Accession no.)**	**Haplotype frequency**
Hap 1	OQ117031, OQ117028 and OQ117033	3
Hap 2	OQ117030, OQ117027, OQ117034 and OQ117032	4
Hap 3	OQ117036	1

4.7. HAPLOTYPE NETWORK ANALYSIS

The haplotype network analysis of the eight sequences was carried out in PopART version 1.7 (Bandelt *et al*., 1999). A median-joining haplotype network was created for the three haplotypes. The haplotype network analysis showed that haplotype 1 (Hap 1) and haplotype 2 (Hap 2) differed from each other by only one segregating sites while haplotype 1and haplotype 3 (Hap3**)** differed by 14 nucleotide segregating sites (Figure 7)

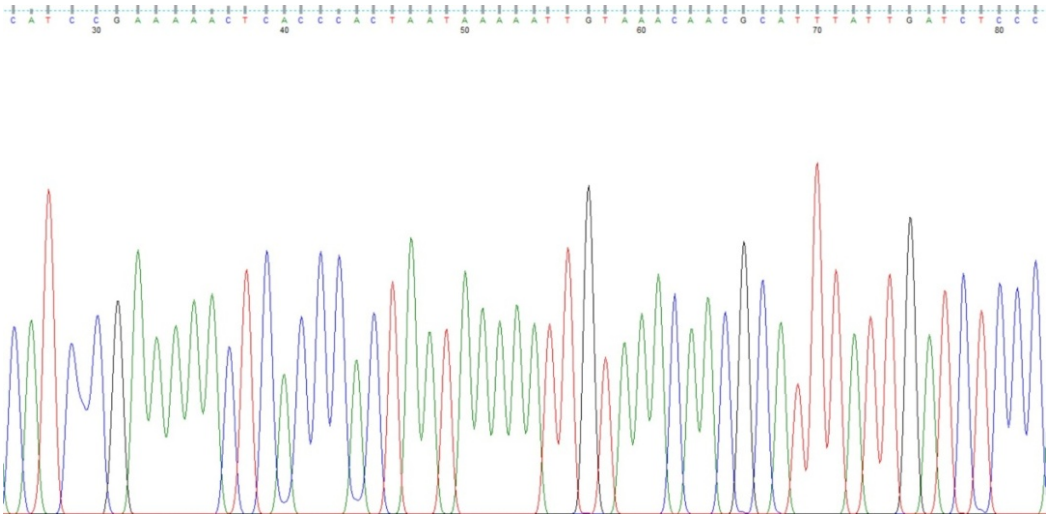

Figure 2. Chromatogram showing OQ117027 sequence of cytochrome b gene

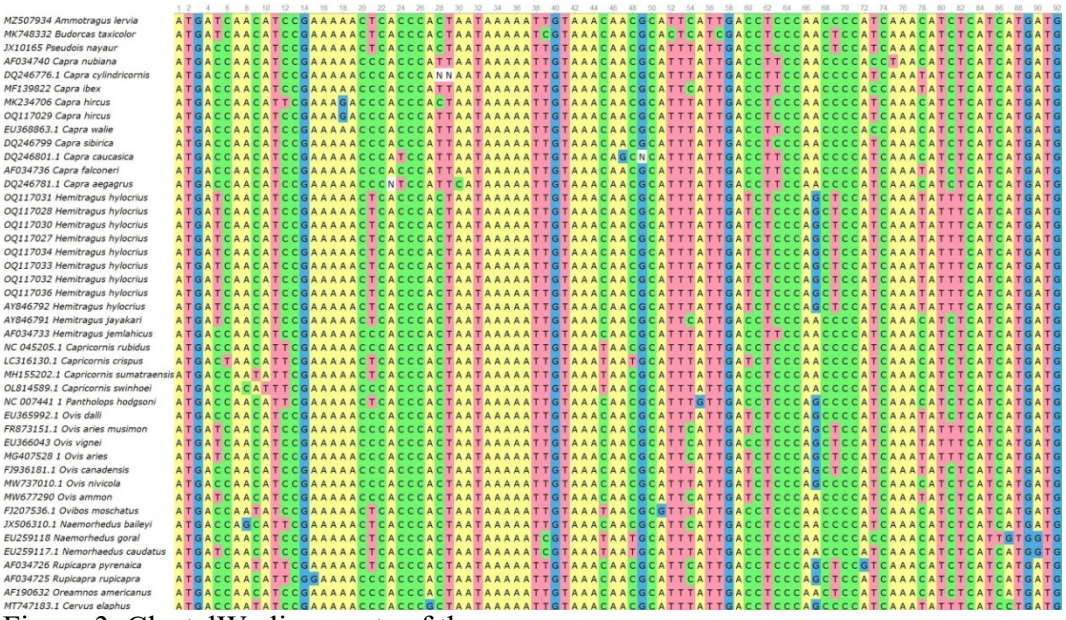

Figure 3. ClustalW alignments of the sequences

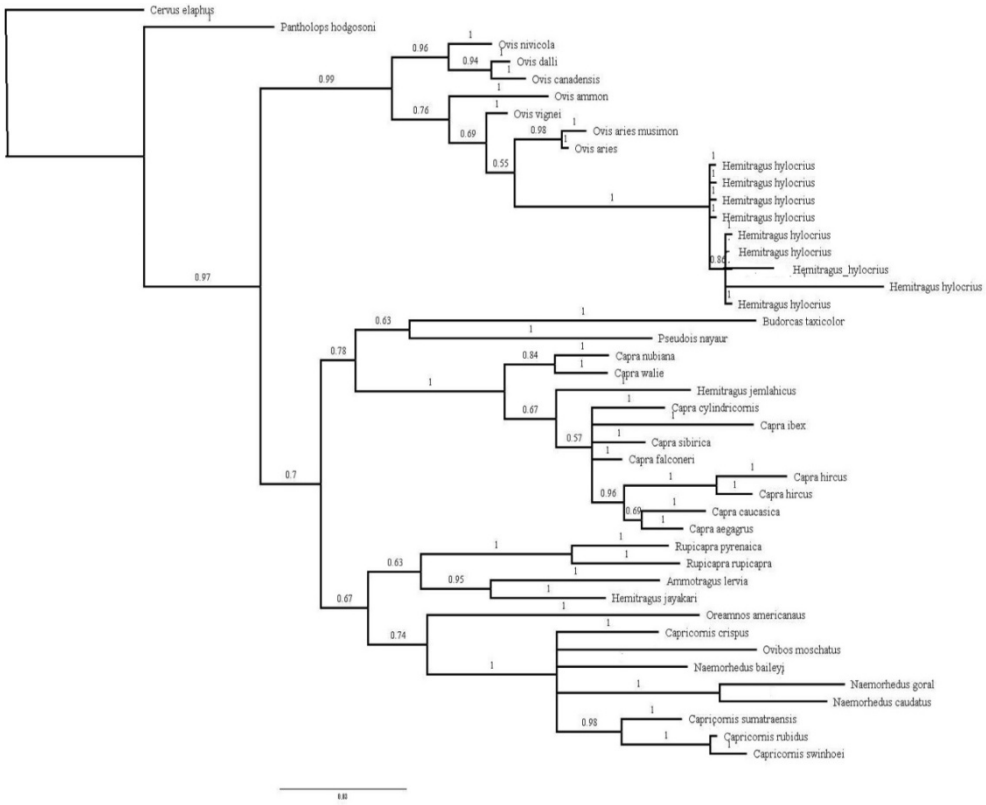

Figure 4. Bayesian method of phylogenetic anlaysis; the values above thebranches are posterior probability (PP)

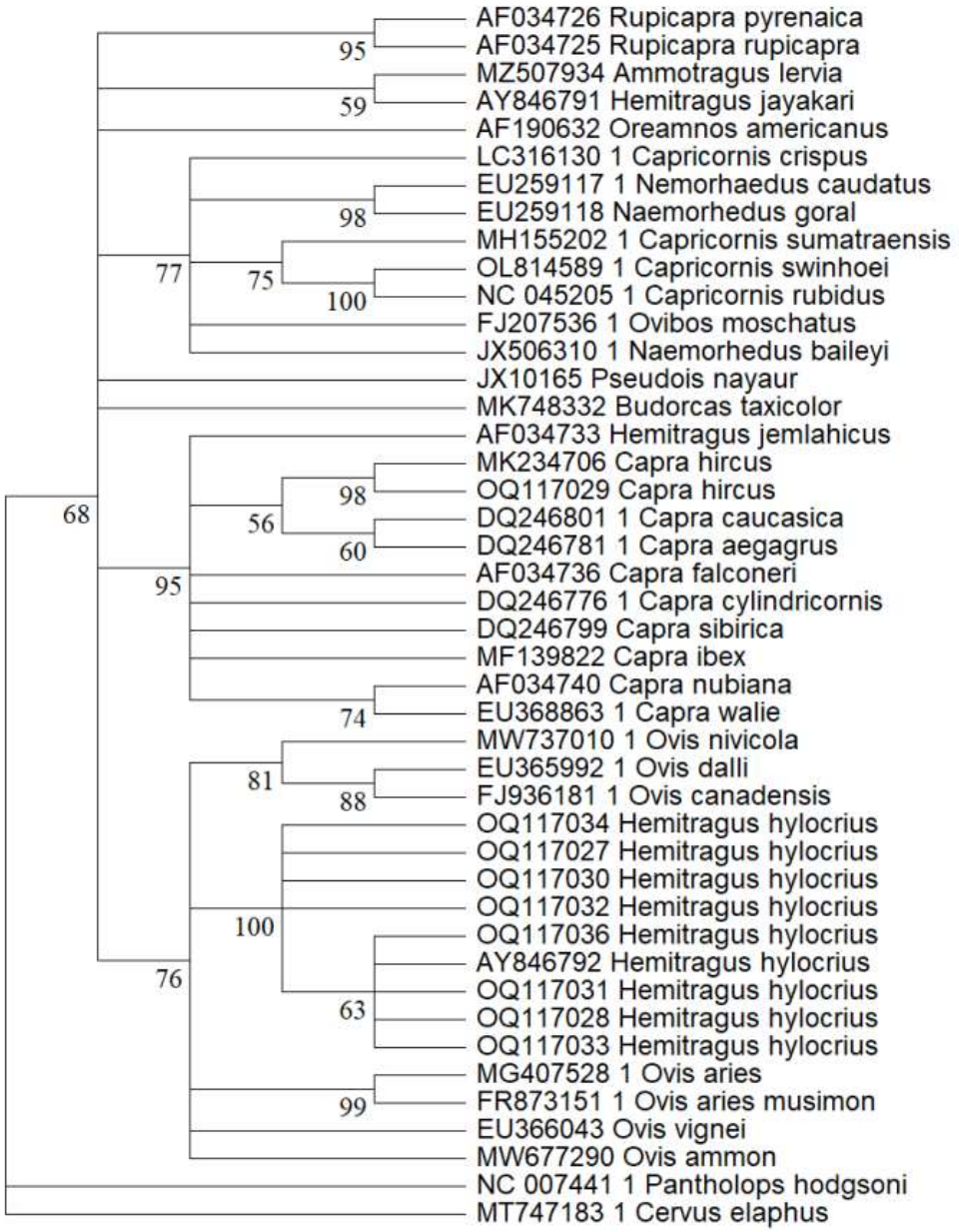

Figure 5. ML method of phylogenetic anlaysis; the values in front of the nodes are bootstrap percentage (BP)

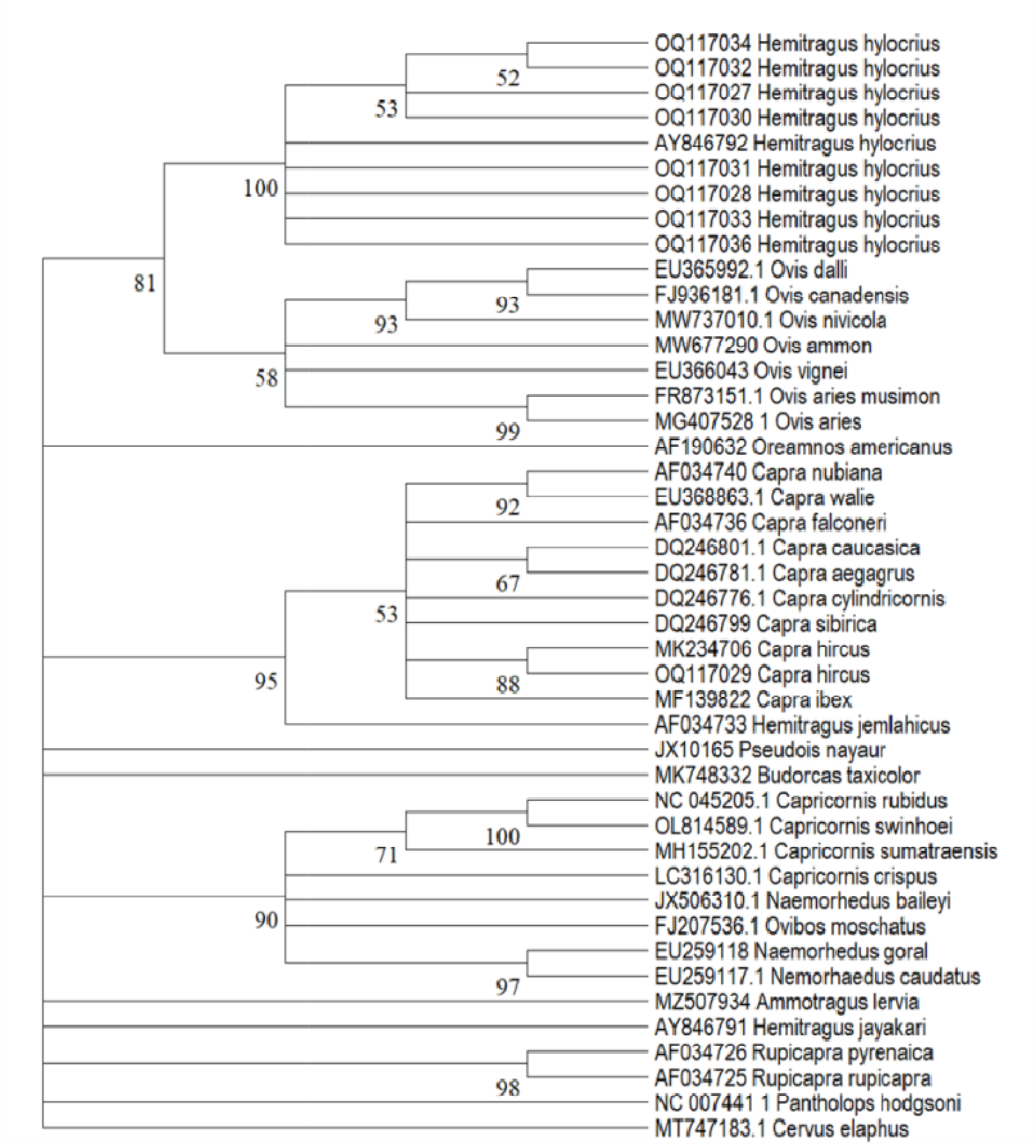

Figure 6. Neighbour-Joining method of phylogenetic anlaysis; the values in front of the nodes are bootstrap percentage (BP)

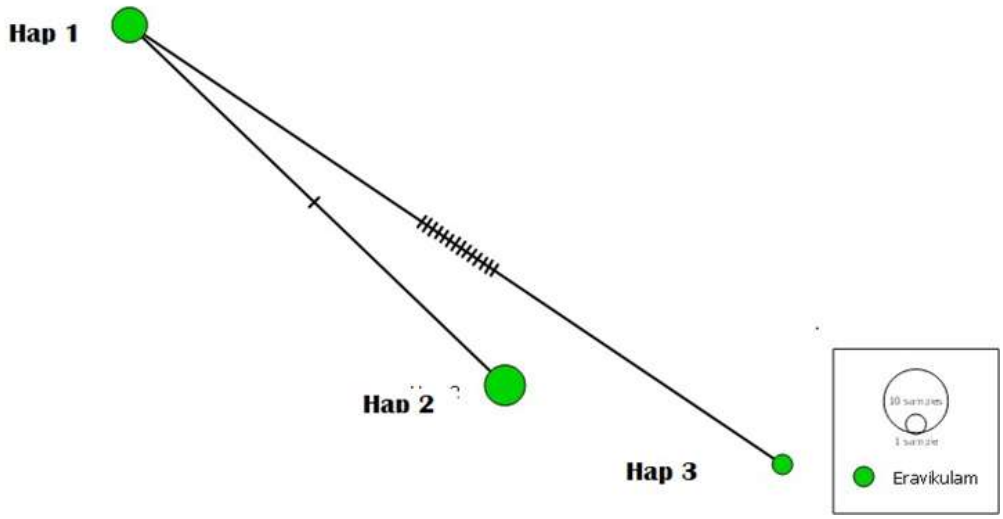

Figure 7. Haplotype network of the eight individuals from the Eravikulam National Park, Kerala

DISCUSSION

5. DISCUSSION

A phylogenetic analysis of Nilgiri Tahr and its related species within the tribe *Caprini sensu lato* (Haasanin and Douzery, 1999) was carried out using the cytochrome b gene as a molecular marker. A total of 43 cytochrome b sequences, including eight sequences of Nilgiri Tahr and one that of domestic goat, were analyzed in this study. Emphasis was given to determining the phylogenetic position of Nilgiri Tahr and understanding the intergeneric relationship of the member of the tribe *Caprini sensu lato* (Hassanin and Douzery, 1999) with intensive species sampling. Fecal samples of Nilgiri Tahr were collected for the isolation of host DNA from the Eravikulam National Park in the Idukki district of Kerala, India, which hosts the largest population of the species.

5.1. PHYLOGENY OF TAHRS

The phylogeny of tahrs was always ambiguous, with an unstable taxonomic status. The morphological affinity of tahrs with goats led many taxonomists to place tahrs along with goats. Earlier taxonomists classified them along with wild goats (Jerdon, 1874). Lydekker (1898) differentiated tahrs from goats based on morphological features. Nilgiri Tahr was initially named *Kemas hylocrius* by Ogilby in 1837 and later was included in *Capra* as *Capra warayattu* by Gray in 1843. The present name *Hemitragus hylocrius,* was assigned by Blyth in 1859. Even after that, Sclater named Nilgiri Tahr as *Capra hylocrius* (Lydekker, 1898).

This study investigated the phylogeny of the tahrs, especially Nilgiri Tahr, in the light of additional cytochrome b sequences. This study attempted to provide a comprehensive phylogenetic analysis by including all three species of tahr. This analysis showed that sheep (*Ovis*) is the closest relative of Nilgiri Tahr (*Hemitragus hylocrius*) as all the species of *Ovis* are grouped with the sequences of Nilgiri Tahr forming the sheep-Nilgiri Tahr clade. The result of our analysis upholds the finding of Ropiquet and Hassanin (2005). However, among the sheep, Urial (*Ovis vignei*), domestic sheep (*Ovis aries*) and Mouflon (*Ovis aries*

musimon) are the nearest relatives of Nilgiri Tahr. The discovery of sheep-Nilgiri Tahr association is recent in the phylogeny of Caprines as none of the studies before 2005 included Nilgiri Tahr in the phylogenetic analysis. The close association between the Himalayan Tahr (*Hemitragus jemlahicus*) and Capra and, paraphyly of the latter was shown in almost all the previous investigations (Groves and Shields, 1996; Hassanin *et al*., 1998; Hassanin and Douzery, 1999; Ropiquet and Hassanin, 2005). Our study also reaffirms these findings. Similarly, the sister taxa relationship of Arabian Tahr (*Hemitragus jayakari*) with Aoudad (*Ammotragus lervia)* was strongly supported in this study.

The association of Nilgiri Tahr with the rest of the Caprines has not been investigated much in phylogenetic analyses. Before 2005, all authors used the Himalayan Tahr (*Hemitragus jemlahicus*) as the sole representative of tahr in phylogenetic analysis. So, the phylogenetic position of Nilgiri Tahr was unclear; additionally, the outcomes of these analyses were extrapolated to the rest of the tahrs and suggested tahrs to be close relatives of goats (*Capra*). Similarly, the relatedness of sheep with Nilgiri Tahr, in particular, was not mentioned in the old literature, although there are minute differences between these two genera. Unlike goats (*Capra*), tahrs lack beards on the chin of males; have smaller horns as compared to goats and the sexual dimorphism of horn size in tahrs is not as prominent as that of goats. However, morphological features like the absence of glands on the face, in the groin, or between the hooves of tahrs bring them closer to goats. But the absence of a beard on the male's chin of tahrs shows their inclination towards sheep (Lydekker, 1898). Since the reliability of these morphological features for differentiating a species is questionable, Ropiquet and Hassanin (2005) reasoned karyotype and horn conformation of Nilgiri tahr for assessing its relatedness with sheep (*Ovis*). Based on the inference of Bunch *et al.* (2000), the common ancestor of *Ovis* had the same number of chromosomes as that of Nilgiri Tahr i.e. 58. On the other hand, the lack of prominent keels in front of the horns of *Ovis* and Nilgiri Tahr and the marking of deep, transverse wrinkles showed their close association.

This study convincingly indicates that the genus *Hemitragus* is polyphyletic as all three species of tahrs conventionally included within this genus, namely the Himalayan Tahr, Nilgiri Tahr and the Arabian Tahr, are distantly related to each other. Moreover, they associate with three different genera within the tribe *Caprini sensu lato* (Hassanin and Douzery, 1999). The polyphyly of this genus was first shown by Hassanin and Ropiquet (2005). Based on that, they proposed a new taxonomy for tahrs and reassigned two additional genera, each to Nilgiri Tahr and Arabian Tahr. According to the new taxonomy, the Nilgiri Tahr is named *Nilgiritragus hylocrius*, and the Arabian Tahr is *Arabitragus jayakari*, whereas the Himalayan Tahr retains the old name.

Finally, the present study reveals three prominent clades within the said tribe. They are the Nilgiri Tahr along with all the species of *Ovis* clade, the Himalayan Tahr with all the species of *Capra*, and the Arabian Tahr with Aoudad (*Ammotragus lervia*).

5.2. INTER-GENERIC RELATIONSHIP AMONG THE MEMBERS OF CAPRINI SENSU LATO (HASSANIN AND DOUZERY, 1999)

This study used the cytochrome b gene as a phylogenetic marker to investigate the inter-generic relationships, in general, and phylogeny of tahrs in particular, with members of one of the most controversial groups of ungulates, the *Caprini sensu lato* (Hassanin and Douzery, 1999). Ropiquet and Hassanin (2005, 2006) and Yang *et al*. (2013) proposed the close association of *Ovis* with Nilgiri Tahr. Ropiquet and Hassanin (2004, 2005, 2006) also suggested that *Ovis* is monophyletic based on the representative species of *Ovis* included in their studies. However, this study, encompassing almost all the currently recognized species of the genus, shows that *Ovis* is not monophyletic, nor associated with *Budorcas* as suggested by previous studies (Groves and Shields, 1996; Hassanin *et al*., 1998; Hassanin and Douzery, 1999; Ropiquet and Hassanin, 2004). This analysis clearly shows that *Ovis* is closely associated with Nilgiri Tahr (*Hemitragus hylocrius*)

and *Ovis aries*, *Ovis vignei*, and *Ovis aries musimon* form a sister taxa relationship with Nilgiri Tahr, thereby making *Ovis* paraphyletic. However, the paraphyly of *Ovis* needs further analysis using additional molecular markers, as this result cannot be conclusive in this regard due to the relatively short length of the analysed sequences. The complete cytochrome b sequences of all the species within *Ovis* need to be investigated further for a conclusion.

The association of *Budorcas* with *Pseudois nayaur* is observed in all the analyses. Based on mitochondrial genome analysis, Kumar *et al*. (2019) confirmed that *Budorcas taxicolor* is more closely related to *Pseudois nayaur* than *Capra* species. This study further confirms the finding of Ropiquet and Hassanin (2005) regarding the sister group relationship of *Pseudois* with the Capra-Himalayan Tahr clade. However, the association of *Oreamnos* with *Budorcas*, as described by Ropiquet and Hassanin (2005) and Yang *et al*. (2013) was not observed. On the contrary, the Rocky Mountain Goat (*Oreamnos americanus*) is likely to be associated with the *Naemorhedus-Capricornis-Ovibos* clade.

Genus *Pantholops* is associated with the rest of the caprines as *Pantholops hodgsonii* was the first to diverge from the tribe *Caprini sensu lato* (Hassanin and Douzery, 1999). This result is consistent with the previous investigations (Ropiquet and Hassanin, 2004, 2006; Yang *et al*., 2013).

This study indicated the close association of *Ovibos* (Muskox) with *Naemorhedus* (Goral) and *Capricornis* (Serow) since these three genera form a clade with robust node support. The Muskox-Goral-Serow clade appeared invariably in past investigations based on a range of molecular markers, including the cytochrome b gene (Groves and Shields, 1996; Hassanin *et al*., 1998; Ropiquet and Hassanin, 2004, 2005, 2006; Yang *et al*., 2013). So, this group is very likely to originate from a common ancestor.

The phylogenetic position of *Rupicapra* (Chamois) is not clear from our analysis. However, the genus shows an association with *Ammotragus lervia*

(Aoudad) and *Hemitragus jayakari* (Arabian Tahr).

5.3. GENETIC DIVERSITY OF NILGIRI TAHR

The genetic diversity of a given population reveals the population's health, as the degree of genetic diversity can indicate how long the population can sustain without undergoing an inbreeding depression. The fragmentation of a large population contributes to the reduction of genetic diversity in local populations (Gibbs, 2001). Ross *et al*. (2020) showed that size and loss of connectivity between populations have a significant impact on the genetic diversity of Arabian Tahr populations distributed in a range of fragmented habitats. Nilgiri Tahr is a geographically limited species distributed in several fragmented populations (metapopulations) throughout its habitat range with limited connections between populations (Easa and Alembath, 2018). A genetic diversity analysis of a small subpopulation of Nilgiri Tahr from the Eravikulam National Park, Kerala, was carried out in this study. A total of 15 variable sites are present in eight individuals. A population genetic study conducted by Joshi *et al*. (2018) did not find intra-population variable sites while analyzing two Nilgiri Tahr populations of Mukurthi National Park and Annamalai Tiger Reserve. Compared to their result, the Nilgiri Tahr population of the Eravikulam National Park showed higher intrapopulation variability despite the low sample size. The haplotype diversity ranges from 0.557-0.801, and nucleotide diversity stands at 0.00963. However, due to a lack of studies on the intra-population genetic diversity of Nilgiri Tahr, the findings of this study could not be compared. So, further studies on the population genetics of Nilgiri Tahr with increased sample size are needed to validate the result of this analysis.

SUMMARY

6. SUMMARY

Molecular phylogenetic analysis of Nilgiri Tahr was carried out using the cytochrome b gene as a molecular marker. Fecal samples of Nilgiri Tahr were collected from the Eravikulam National Park, Kerala, and DNA was isolated from these samples using commercial fecal DNA isolation kits. Universal primers for ungulates were used to amplify a fragment (503 bp) of the cytochrome b gene. The amplified PCR products were sequenced, and sequences were analyzed for confirmation. A total of 43 cytochrome b sequences, including 12 genera and 34 species of the tribe *Caprini sensu lato* (Hassanin and Douzery, 1999) were analyzed for the reconstruction of the phylogenetic tree. Red Deer (*Cervus elaphus*) was included as an outgroup. All three species of tahrs, the Himalayan Tahr, Nilgiri Tahr, and Arabian Tahr, were included in this study. Phylogenetic trees were constructed by using Bayesian Inference, Maximum Likelihood, and Neighbor-Joining methods. The genetic diversity analysis of Nilgiri Tahr was carried out, and a haplotype network was constructed.

All three methods showed a similar tree topology except for a few differences in the node supports (posterior probability and bootstrap percentage values) among the trees.

This study shows that *Pantholops* is associated with the rest of the Caprines; however, it doesn't form an association with any particular genera or species. The phylogenetic analysis clarified the association of tahrs with members of the tribe *Caprini sensu lato*. The study has confirmed the association of *Ovis* with Nilgiri Tahr as it maintains a paraphyletic relationship with the latter. Ovis is monophyletic only with the absence of Nilgiri Tahr. Similarly, the Himalayan Tahr clusters with *Capra* making the genus paraphyly. The association of the Arabian Tahr with Aoudad (*Ammotragus lervia*) is also prominent in this study.

This study further confirms the grouping of *Ovibos* with *Naemorhedus* and *Capricornis*. Similarly, *Budorcas* is more closely related *to Pseudois nayaur* than

any other member of the tribe *Caprini sensu lato*. However, this study cannot confirm the phylogenetic status of the genus *Oreamnos*.

Three clades stand out from the present study; they are: (i) the *Ovis*-*Hemitragus hylocrius* clade, (ii) the *Capra*- *Hemitragus jemlahicus* clade, and (iv) the *Ovibos*-*Naemorhedus*-*Capricornis* clade.

The genetic diversity analysis showed a higher number of variable sites in the analyzed population comparing the sample size. However, the remaining parameters could not be evaluated due to the lack of similar studies on Nilgiri Tahr.

The present study confirms the polyphyletic nature of the genus *Hemitragus* under which tahrs are grouped. Since all the species of tahr associate with different members of the tribe *Caprini sensu lato*, this study strongly advocates the taxonomic reclassification of tahrs. Given the polyphyly of tahrs, it is opined that the new taxonomy of tahrs proposed by Ropiquet and Hassanin (2005) may be accepted. However, the paraphyly of *Ovis* needs further confirmation by using a complete cytochrome b sequence with a larger sample size.

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7. REFERENCES

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ABSTRACT

PHYLOGENETIC ANALYSIS OF NILGIRI TAHR (*Nilgiritragus hylocrius*) USING MITOCHONDRIAL CYTOCHROME B GENE

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ABSTRACT

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8. ABSTRACT

Nilgiri Tahr is an endemic species of wild goat distributed in the Western Ghats of India, which belongs to the tribe *Caprini sensu lato*. This tribe, representing a highly diverged group of ungulates, includes domestic and wild species of sheep, goat, and their relatives, along with tahrs. Despite being an endangered species, the phylogenetic relationship of the Nilgiri Tahr with members of the tribe *Caprini sensu lato* is still ambiguous. The present study investigated the phylogenetic status of Nilgiri Tahr and the intergeneric relationships within the tribe *Caprini sensu lato* by analyzing 34 species of 12 genera currently described in the tribe. The cytochrome b gene was amplified from template DNA isolated from the fecal samples of Nilgiri Tahr, and phylogenetic analysis of a fragment of the cytochrome b gene (423base pair) sequences was carried out using the Bayesian Inference, Maximum Likelihood, and Neighbor-Joining method. Genetic diversity analysis of a small population of Nilgiri Tahr from the Eravikulam National Park, Kerala, India, was also conducted. The phylogenetic analysis showed the presence of three distinct clades within the tribe *Caprini sensu lato*. The Nilgiri Tahr (*Hemitragus hylocrius*) with *Ovis*, the Himalayan Tahr (*Hemitragus jemlahicus*) with *Capra*, and the *Ovibos* with *Naemorhedus* and *Capricornis* formed clades within the tribe. The study further confirmed the association of Aoudad (*Ammotragus lervia*) with Arabian Tahr. Thus, the genus *Hemitragus,* representing tahrs, is found to be polyphyletic. Our result supports the taxonomic reclassification of tahrs proposed by Ropiuet and Hassanin (2005). In contradiction with previous investigations, the genus *Ovis* appears to be paraphyletic from this study. The genetic diversity analysis showed a higher intra-population variation of the sampled individuals. However, the remaining parameters could not be evaluated due to the lack of similar studies on Nilgiri Tahr.

ANNEXURES

ANNEXURE 1

REAGENTS AND BUFFERS

5X TBE buffer (1000ml):

27.5g of Boric acid,

25g of Tris base,

20 mL of 0.5M EDTA (pH 8)

Autoclaved triple glass distilled water (up to) 1000.0 mL

1X TBE buffer (500mL)

100 mL of 5X Tris Borate EDTA (TBE) buffer

400 mL of distilled water

LYSIS BUFFER (Longmire *et al*., 1997)

975 mL double-distilled water

100 ml of 1 M Tris-HCl, pH 8.0

200 ml of 0.5 M EDTA, pH8.0

2 ml of 5 M NaCl

25 ml of 20% SDS (w/v) Filter the buffer with an autofill PES bottle top filtration device (sterile 500ml, 0.22 µm)

Acad. Form No.19a

KERALA VETERINARY AND ANIMAL SCIENCE UNIVERSITY

Faculty of Veterinary and Animal Sciences

PROGRAMME OF RESEARCH WORK FOR DISSERTATION FOR MASTER OF SCIENCE DEGREE

1. Title of dissertation

   Phylogenetic analysis of Nilgiri Tahr (*Nilgiritragus hylocrius*) using mitochondrial cytochrome b gene

2. a) Title of the department /KVASU research:

   Not Applicable

   b) Project of which this forms a part

   Not Applicable

   c) Code No. if any, and order by which the departmental/KVASU research project is approved

   Not Applicable

3. a) Name of student

   Sivasankar Taye

   b) Admission No.

   20-MSVP-11

   c) Name of the discipline

   M.S. (Wildlife Studies)

4. a) Name of Guide

   Dr. Muhasin Asaf V. N.

   b) Address:

   Assistant Professor,

   Department of Animal Breeding and Genetics,

   College of Veterinary and Animal Sciences,

   Pookode,Wayanad -673576

5. Objectives of the study

1. To sequence the mitochondrial cytochrome b gene of Nilgiri Tahr
2. To conduct phylogenetic analysis from the generated sequence data

6. Practical /Scientific utility

Tahr are wild ungulates related to sheep and goat. They inhabit the uneven terrains of the mountain ranges. The tahrs found across the globe includes the Nilgiri Tahr (*Nilgiritragus hylocrius),* Himalayan Tahr (*Hemitragus jemlahicus*) and the Arabian Tahr (*Arabitragus jayakari*). Nilgiri Tahr is endemic to Western Ghats and is characterized as endangered (EN) with decreasing population trend by the International Union for Conservation of Nature (IUCN). The Nilgiri Tahr was classified under the genus *Hemitragus* along with other two species of tahr and was believed to be closely related to goats. Ropiquet and Hassanin (2005) proposed taxonomy with the genus *Hemitragus* being restricted to the Himalayan Tahr, and two new genera were created with *Arabitragus* for the Arabian Tahr and *Nilgiritragus* for the Nilgiri Tahr.

Phylogenetic study has particular importance in the conservation of wild animal species. Determining the phylogenic status of a species is of utmost importance for taking steps in the conservation of the species. Molecular phylogenetic analysis using mitochondrial DNA has provided a better understanding of several previously confusing questions on phylogeny. Mitochondrial *CYTB* is widely used for resolving the evolutionary relationships.

7. Important publications on which the study is based

Hassanin *et al*. (1998) analysed a total of 32 complete cytochrome b sequences of all the genera within Caprinae by using differentially weighted parsimony. They found that the tribe Saigini is not corroborated by the study and suggested that Saiga should be excluded from Caprinae.

Hassanin and Douzery (1999) used mitochondrial cytochrome b gene as phylogenetic marker to reconstruct the Bovidae phylogeny and established the position of Pelea, Pantholops and Saiga.

Ropiquet and Hassanin (2004) studied the molecular phylogeny of caprines by analysing 2469 characters drawn from three distinct molecular markers viz., mitochondrial cytochrome b, mitochondrial 12S rRNA and exon 4 of the κ-casein gene. The study suggested that the Caprines shared a common ancestor with Alcelaphini and Hippotragini in the middle-late Miocene.

Ropiquet and Hassanin (2005) constructed a molecular phylogeny of the tribe *Caprini sensu lato* for determining the taxonomic status of the three species of tahr. Based on the results observed, they proposed a taxonomy with the genus *Hemitragus* being restricted to the Himalayan Tahr, and two new genera were created with *Arabitragus* for the Arabian Tahr and *Nilgiritragus* for the Nilgiri Tahr.

Yang *et al*. (2013) supported the taxonomy with genus *Hemitragus* being restricted to the Himalayan Tahr, *Arabitragus* for the Arabian Tahr and *Nilgiritragus* for the Nilgiri Tahr based on complete mitochondrial genome analysis.

Luis *et al*. (2017) used microsatellite markers to assess the population genetics of Nilgiri Tahr. The study was conducted on 50 pellet samples on 19 microsatellite markers. The observed heterozygosity was found to be 0.4280 ± 0.2376 while the expected heterozygosity was 0.4464 ± 0.2265. The F_{IS} value was found to be 0.0138 ($p = 0.63$).

Joshi *et al*. (2018) studied the populations of Nilgiri Tahr (*Nilgiritragus hylocrius*) north to the Palghat gap and south to the Palghat gap using mitochondrial CYTB gene. The study revealed the presence of two diverged populations of Nilgiri Tahr in Western Ghats, India.

Ross *et al*. (2020) studied the population structure, genetic diversity and landscape connectivity of the Arabian Tahr using mitochondrial control region and 14 microsatellite loci. They identified the most likely location of corridors connecting Arabian Tahr populations.

8. Outline of the technical programme

A minimum of eight faecal / hair / post-mortem samples will be collected from the Nilgiri Tahr from their habitats in the Western Ghats region. The collected samples will be transported to laboratory for DNA isolation. DNA will be isolated using standard protocols. Mitochondrial cytochrome b (*CYTB*) gene will be amplified using polymerase chain reaction (PCR). The PCR amplicons will be sequenced by Sanger di-deoxy method and the sequence data will be analysed using appropriate software. The generated sequence data will be subject to multiple sequence alignment. The aligned sequences will be subjected to phylogenetic analysis using appropriate software. Bioinformatics analysis will be conducted from the data generated from the current study as well as data retrieved from databases wherever necessary.

9. Main items of observations to be made

    1. PCR amplicons of mitochondrial *CYTB* gene of Nilgiri Tahr
    2. Sequence data of mitochondrial *CYTB* gene of Nilgiri Tahr
    3. Phylogenetic tree

10. Duration of research work

    One semester

Signature of student

Project coordination group proposed: NIL

Place:

Date:

Signature of Guide

Name, address and signature of other members of the Advisory committee:

1. Dr. George Chandy
   Special Officer
   Centre for Wildlife Studies
   Kerala Veterinary and Animal Sciences University
   Pookode, Wayanad -673576

2. Dr. Renjith Sebastian
   Assistant Professor
   Department of Veterinary Biochemistry
   College of Veterinary and Animal Sciences
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11. References:

Hassanin, A. and Douzery, E.J.P. 1999. The Tribal Radiation of the Family Bovidae (Artiodactyla) and the Evolution of the Mitochondrial Cytochrome b Gene. *Mol. Phylogenet. Evol.* **13**: 227-243.

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Joshi, B.K., Matura, R., Predit M.A., Rahul, D., Pandav, B., Sharma, V., Nigam, P. and Goyal, S.P. 2018. Palghat gap reveals presence of two diverged populations of Nilgiri Tahr (*Nilgiritragus hylocrius*) in Western Ghats, India. *Mitochondrial DNA Part B.* **3**: 245-249

Luis, A., Davidar, P. and Reddy, P.A. 2017. Potential of cross-species microsatellite markers to assess population genetics of the endemic, endangered Nilgiri Tahr (*Nilgiritragus hylocrius*). *Eur. J. Wildl. Res.* **63**: 16.

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Yang, C., Xiang, C., Qi, W., Xia, S., Tu, F., Zhang, X., Moermond, T. and Yue, B. 2013. Phylogenetic analyses and improved resolution of the family Bovidae based on complete mitochondrial genomes. *Biochem. Syst. Ecol*. **48**: 136-143.

CERTIFICATE

Certified that the research project has been formulated observing the stipulations laid down under the prevention of Cruelty to Animals Act (Amendment, 1998).

Place: **Dr. Muhasin Asaf V.N.**

Date: (Guide)

CURRICULUM VITAE

1.	Name of the candidate	Sivasankar Taye
2.	Date of Birth	01-01-1998
3.	Place of Birth	Sivasagar, Assam
4.	Marital Status	Unmarried
5.	Permanent Address	Thekeratol, Bharalua tiniali, Sivasagar, Assam, 785664
6.	Major field of Specialization	Veterinary Science, Wildlife Studies
7.	Educational Status	Bachelor of Veterinary Science and Animal Husbandry
8.	Professional Experience	Registered veterinary practitioner for 1 year
9.	Publications Made	NIL
10.	Membership in Professional Societies	Assam Veterinary Council