MOLECULAR PHYLOGENY AND POPULATION GENETIC STRUCTURE OF NORTHERN RED MUNTJAC *(Muntiacus vaginalis)* IN INDIA

A THESIS

Submitted by

BHIM SINGH

For the award of degree

DOCTOR OF PHILOSOPHY
IN
WILDLIFE SCIENCE

Under the Guidance of

Dr. V.P. UNIYAL

WILDLIFE INSTITUTE OF INDIA
Dehradun, Uttarakhand, India

Saurashtra University
Rajkot, Gujarat, India

JULY 2021

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DECLARATION

I hereby declare that the work conducted under the thesis entitled "**Molecular phylogeny and population genetic structure of Northern red muntjac (*Muntiacus vaginalis*) in India**", is a record of original research work, done by me and subsequently submitted for the award of the degree of doctor of Philosophy in Wildlife Science to Saurashtra University, Rajkot. This research work has been carried out under the guidance and supervision of Dr. Virendra Prasad Uniyal, Scientist-G and co-supervision of Dr. Sandeep Kumar Gupta, Scientist-E, Wildlife Institute of India, Dehardun. The work has not formed the basis for the award of any other degree, diploma or any other qualification. I also declare that the thesis embodies my own work, analysis, observation and understanding and the particulars given in it are true to the best of my knowledge.

(Bhim Singh)

Place: Dehradun

Date: 19-07-2021

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Dated: 19/07/2021

CERTIFICATE

This is to certify that the thesis by **Mr. Bhim Singh** entitled "**Molecular phylogeny and population genetic structure of Northern red muntjac (*Muntiacus vaginalis*) in India**" is an original and independent research work submitted to the **Saurashtra University, Rajkot (Gujarat)**, for the award of the degree of **Doctor of Philosophy** in **Wildlife Science**.

Mr. Bhim Singh has put more than six semesters of research work embodied in this thesis under my guidance and supervision. The work presented in this thesis has not been submitted to any other University or Institute for the award of any degree, diploma or distinction.

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This is to certify that **Mr. Bhim Singh** has made Pre-Ph.D. Presentation as per UGC Guideline "University Grant Commission (Minimum Standard and Procedure for award of Ph.D. Degree) Regulation-2016" and Saurashtra University Ordinance for Ph.D. Programme (O.Ph.D. 8.3), on his research work entitled "**Molecular phylogeny and population genetic structure of Northern red muntjac (*Muntiacus vaginalis*) in India**" at Wildlife Institute of India, Dehradun, Research Centre of Saurashtra University, Rajkot on **15nd January 2021** before all the faculty members and students of the Department for getting feedback and comments.

I certify that the research work was appreciated by all who were present, and the comments made by the faculty and researchers have been appropriately included in the thesis.

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CERTIFICATE OF PLAGIARISM CHECK

It is certified that the Ph.D. thesis entitled **"Molecular phylogeny and population genetic structure of Northern red muntjac (*Muntiacus vaginalis*) in India"** submitted by Mr. Bhim Singh has been examined by us for plagiarism check as per UGC (Promotion of Academic Integrity and Prevention of Plagiarism in Higher Educational Institutions) Regulations. The following inferences are drawn from this check:

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INTRODUCTION

Background

The Order Artiodactyla comprises 10 families of hoofed mammals, including 247 species (Hassanin et al., 2012). The family Cervidae, commonly referred as "deer family" is the second most diverse family consists of 55 species among Artiodactyla (IUCN 2015; Hassanin et al.,2012). Cervids are widely distributed in diverse habitats such as grassland, swamp, forest areas etc. and have adapted to different climatic zones (Mattioli 2011). Based on their distinct foot structure, the family Cervidae is further divided into two subfamilies: New World deer, Capreolinae (*Telemetacapalian*) and Old World deer, Cervinae (*Plesiometacarpalian*). Amongst the New World deer, *Alces* and *Rangifer* inhabit Eurasia and North America, respectively, whereas *Capreolus* and *Hydropotes* are found in Eurasia. The families, *Odocoileus*, *Blastocerus*, *Hippocamelus*, *Mazama, Ozotoceros* and *Pudu* inhabit North and South America. In Old World deer, tribe Cervini is distributed in Eurasia, consists of the following genus: *Axis, Cervus, Dama, Elaphurus, Rucervus* and *Rusa*. However, the genus *Cervus* are also distributed in North America apart from Eurasia. In tribe Muntiacini, the genus *Muntiacus* and *Elaphodus* are distributed in South and South East Asia. The IUCN Red List of Threatened Species classifies 29 species of Cervids in 'Threatened' category, 17 species as ‘Least concern’ and 9 species are in‘Data deficient’ (IUCN 2015).The taxonomic classification of some species in Cervids is still debated due to the lack of robust taxonomic and phylogenetic resolution. However, advancement of molecular tools has been helpful in increasingly elucidating the phylogenetic classification among Cervids.

The genus *Muntiacus* (Rafinesque, 1815) comes under tribe Muntiacinae within family Cervidae. These are widely distributed throughout South and Southeast Asia (Nagarkoti et al., 2007) and exhibit extreme variations in chromosome numbers (Yang et al., 1997, Wang et al., 2007).With increasing number of studies on muntjacs, the numbers of newly recognized species of *Muntiacus* are also growing. For example, based on the differences in skin color, skull and antler morphology, a new endemic species from Borneo (Bornean yellow muntjac or *M. atherod*) was described (Groves et al., 1982), after which the Gongshan muntjac (*M. gongshanensis*) was described from Southwest China and northern Myanmar (Ma et al., 1990, Zang et al., 2021). Previously, the Putao muntjac (*M. putaoensis*) from Myanmar (Amato et al., 1999), Small blackish muntjac (*M. truongsonensis*) from Central Vietnam (Giao et al., 1998), and Roosevelt's barking deer (*M. rooseveltorum*) from Vietnam (Le et al., 2014) has been confirmed with molecular evidence. But the taxonomic classification and validation of species and subspecies is still an ongoing process. Ma et al (1986). reported only five species in the genus, but subsequently, Groves and Grubb (2011) classified 16 species of *Muntiacus* based on their morphological characteristics and geographical distribution ranges. This classification is still controversial since it is difficult to differentiate among species based on morphological data alone as multiple muntjacs share similar morphological characteristics and having overlap distribution ranges. Currently, 13 species of *Muntiacus* are recognized in the IUCN Red List of Threatened Species, whereas 54 % are still data deficient (Zang et al., 2021) (Table 1). Despite taxonomic recognition of many species, further investigation is still warranted due to the unavailability of recently

updated data and lack of uniformity across datasets in previous studies (Groves and Grubb, 1982; Ma et al., 1990; Le et al., 2014). Recently all species of genus *Muntiacus* have been classified into four major groups: *Muntiacus muntjac*, *Muntiacus crinifrons*, New muntjac and *Muntiacus reevesi* (Groves et al., 2011). Red muntjac is placed under *Muntiacus muntjac* group, which is further discussed in this study.

Table 1: IUCN Status and geographical range of *Muntiacus* species

Common name	Scientific name	Geographic range	IUCN status
Northern Red Muntjac	*Muntiacus vaginalis*	Bangladesh; Bhutan; Cambodia; China; Hong Kong; India; Lao People's Democratic Republic; Myanmar; Nepal; Pakistan; Sri Lanka; Thailand; Viet Nam; Malaysia	Least concern
Reeves' Muntjac	*Muntiacus reevesi*	China; Taiwan, Province of China	Least concern
Puhoat Muntjac	*Muntiacus puhoatensis*	Viet Nam; Lao People's Democratic Republic	Data deficient
Large-antlered Muntjac	*Muntiacus vuquangensis*	Cambodia; Lao People's Democratic Republic; Viet Nam	Critically endangered
Sumatran Mountain Muntjac	*Muntiacus montanus*	Indonesia	Data deficient
Roosevelts' Muntjac	*Muntiacus rooseveltorum*	Lao People's Democratic Republic; China; Myanmar; Viet Nam	Data deficient
Black Muntjac	*Muntiacus crinifrons*	China	Vulnerable
Bornean Yellow Muntjac	*Muntiacus atherodes*	Indonesia; Malaysia	Near threatened
Leaf Muntjac	*Muntiacus putaoensis*	India; Myanmar	Data deficient
Fea's Muntjac	*Muntiacus feae*	Myanmar; Thailand;China	Data deficient
Gongshan Muntjac	*Muntiacus gongshanensis*	China; Myanmar; Bhutan; India	Data deficient
Annamite Muntjac	*Muntiacus truongsonensis*	Lao People's Democratic Republic; Viet Nam; China	Data deficient
Southern Red Muntjac	*Muntiacus muntjak*	Brunei Darussalam; Indonesia; Malaysia; Thailand	Least concern

Red muntjac

Red muntjac (*Muntjacs*) comprises two major recognized species: Northern red muntjac (*Muntiacus vaginalis*) and Southern red muntjac (*Muntiacus muntjak*) inhabiting South and South East Asia. The northern red muntjac has wide geographical distribution in comparison to other *Muntiacus* species. They are distributed from north Pakistan to most of India, Nepal, Bhutan, Bangladesh to southern China with Hainan and south Tibet, Myanmar, Thailand, Lao, PDR, Vietnam, Cambodia, and the Thai Malay peninsula.The Southern red muntjac (*M. muntjac*) occupied Thai–Malay peninsula, Java, Bali, Lombok, Borneo, Bangka, Lampung and Sumatra (Timmins et al., 2016) (Fig 1). However, the exact southern range limit of Muntjacs in the Thai–Malay peninsula remains unclear. The Northern red muntjac (*M. vaginalis*) are forest-dwelling species generally found in variety of habitats from forest areas to scrubland, grassland, savannahs and croplands (Prater 1971, Barrette 1977, 2004, Timmins et al., 1998, Duckworth etal., 1999, Teng et al., 2004). However, they are also found in agricultural, plantations, and heavily degraded forests (Oka 1998, Laidlaw 2000, Azlan 2006). The Northern red muntjac (*M. vaginalis*) is commonly found upto 1500 m asl but in some studies, they have also been reported in extreme range in Arunachal Pradesh upto 3000 m asl near Tawang and from Bhutan based on camera-trapping,which was recorded between 2000 m to 3650 m asl (Vernes et al. based on unpublished data pers. comm. 2014, Timmins, 2014). Muntjac is omnivorous, apart from plant matter, sometimes it also feeds on eggs and young birds and hunts small mammals (Kurt, 1981).

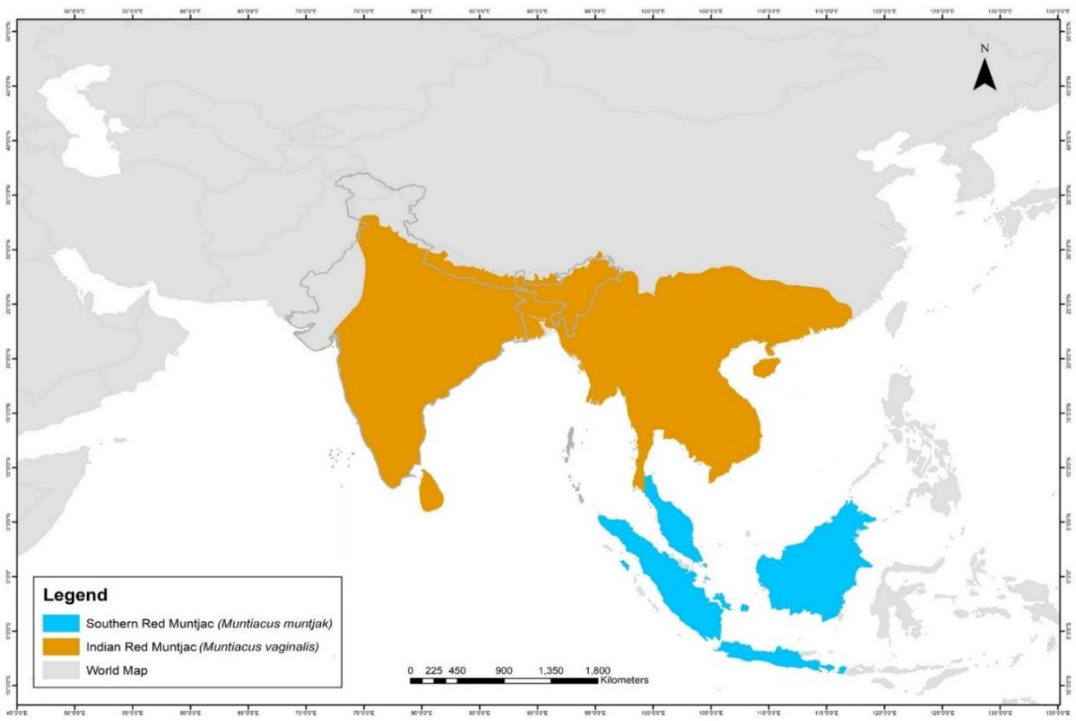

Figure 1: Geographical distribution range of two red muntjac species.

Currently, the population of Northern red muntjac is decreasing across its distribution ranges. The red muntjacis primarily hunted for meat, skin, trophy and used in traditional medicine across its distribution ranges (Srikosamatara et al., 1992, Duckworth et al., 1999, Bennett et al., 2000). The high anthropogenic pressure has resulted in the fragmentation of suitable habitats, affecting the northern red muntjac population across their distribution range. It is currently listed as 'Least Concern' in the IUCN Red List and is protected under Schedule III of the Indian Wild Life (Protection) Act, 1972.

Morphological characteristics

Distinct morphological characters and independent evolutionary historyhave attracted several studies to demystify the red muntjacs. Muntjacs are small in size in comparison

to other deer species. Due to independent evolutionary history, Muntjacs show distinct morphological characteristics like small antlers, long pedicles, and permanent upper canines (enamel coated), like those of *hydropotes* and *moshids*. The large tusk is used as a weapon for territorial defense of resources (Geist 1978a, b). The absence of the metatarsal gland is surprising as it is present in both Old World and New World deer; this shows muntjacs following distinct evolutionary history. The formation of the preorbital fossa is a characteristic feature of the muntjac group and it is a clearly and sharply marked pit, while in other deers, it forms a simple depression, sloping gradually into the facial surface.

Figure 2: Male red muntjac (Jim Corbett National Park**)**

Figure 3: Female red muntjac (Jim Corbett National Park**)**

Chromosomal evolution

Muntjacs are also colloquially called Puzzling deer, as they show similar morphology with a large diversity in their chromosome number. The Chinese muntjac (*M. reevesi*) has the largest number of chromosomes among muntjac species i.e., 2n of 46 in both sexes (Wurster and Benirschke 1967). The Norther red muntjac (*M. vaginalis*) have the lowest number of chromosomes in mammals reaching 2n=6 for females and 7 for males (Wurster and Benirschke, 1970; Wurster and Atken 1972), whereas, the Southern red muntjac (*M. muntjac*) and Black muntjac (*M. criniforns*) bothhave 2n=8 in female and 9 in male (She, 1983). The chromosome number of Gongshan muntjac (*M. gongshanensis*) is 2n=9 (Shi and Ma 1988) and 2n ranges from 12 to 14 in Fea's muntjac (*M. feae*) (Wurster and Benirschke, 1970;Tanomtong et al., 2005).

Taxonomical complexities in red muntjacs

Mattioli classified red muntjacs as a single species, *Muntiacus muntjak* comprising ten subspecies (Mattioli 2011). In 2003, based on karyotype,*M. muntjac* has been differentiated into: the Sundaic form:*M. muntjak* and the northern form:*M. vaginalis* (Groves et al., 2003). However, this classification is not widelyaccepted. International Union for Conservation of Nature (IUCN)has provisionally adopted two species of *Muntiacus: M. vaginalis* (Northern or Indian Red Muntjac) and *M. muntjak* (Southern Red Muntjac). In 2011,Grubb and Groves recognized six species and two subspecies of *Muntiacus* using geographical distributions and morphological characters (Groves et

al.,2011). Recently, the complete mitogenome sequences indicated the presence of three distinct maternal lineages across the distribution range of red muntjacs: Srilankan red muntjac from Western Ghats, India and Sri Lanka; Northern red muntjac in Northern India and Indochina; and Southern red muntjac found in Sundaland (Martins et al.,2017). The samples used in this study belonged to museums, zoos and opportunistically collected samples from Vietnam, Laos, and Peninsular Malaysia (Martins et al.,2017). Due to the inclusion of museum samples, the complete mitogenomes generated from this study contained several ambiguous nucleotides in most samples. Martins et al. (2017) also suggested extensive sampling to unveil taxonomic uncertainties within red muntjacs supplemented with nuclear data (Microsatellites) to examine barriers to gene flow (Martins et al.,2017).

Molecular markers

Molecular markers play a crucial role in population genetic studies for biodiversity conservation. One of the main questions warranting consideration before carrying out any population genetic study is the appropriateness of the molecular markers to address the key question of the study. This depends on the research questions, available resources, time and their cost. Many previous studies have also highlighted the importance of genetic data for successful conservation management and reintroduction plan of threatened species (Gupta et al., 2018). Neutral genetic markers can address the adaptive genetic variation of species, which is essential for analysing evolutionary potential (Hunter, 1996; Frankham et al., 2002). To analyze population genetic structure and diversity of natural populations, prior knowledge about variability of the genetic

marker is prerequisite (Roed 1998). Molecular genetic markers have been increasingly applied to assess genetic differentiation among geographically isolated populations to define the evolutionary significant units for conservation and management purposes, as they aid the revision of the traditionally accepted taxonomic designations (Moritz 1994;Fraser and Bernatchez 2001). Based on transmission and evolutionary patterns, DNA markers are classified as Mitochondrial DNA (mtDNA) and nuclear DNA markers (Park and Moran, 1994). Mitochondrial DNA provides appropriate resolution to phylogenetic and population-level studies and microsatellites are more suited to studies of parentage and mating systems and fine-scale conserved for population-level comparisons (Coulson et al.,1998).

Mitochondrial genome

The mitochondrial genome in ungulates is a circular molecule containing 37 genes and one hyper variable control region. In the 37 genes, 13 genes are categorized as protein-coding gene (PCGs), 22 genes as transfer RNA (tRNA), and 2 genes as ribosomal RNA (rRNA). In mammals, the size of the mitochondrial genome is around 16.5 kb (Fig 4). Mitochondrial DNA (mtDNA) is widely used as genetic markers in phylogenetics and phylogeographic studies due to its inherent haploidic character, which means it is only maternally inherited and lacks recombination. However, it has been observed that short fragments of mitochondrial DNA may lead to inaccurate results but using large-scale mitogenomic data, imprecision may be avoided by improvement of rooting effects in phylogeographic studies (Lari et al., 2011; Hirase et al., 2016). Another benefit of using

the complete mitogenome is its slower mutation rates than non-coding hypervariable regions of the genome, such as microsatellites. Hence, to study the divergence events or geographical structuring among populations, mtDNA is the most appropriate marker (Hofreiter & Stewart, 2009).

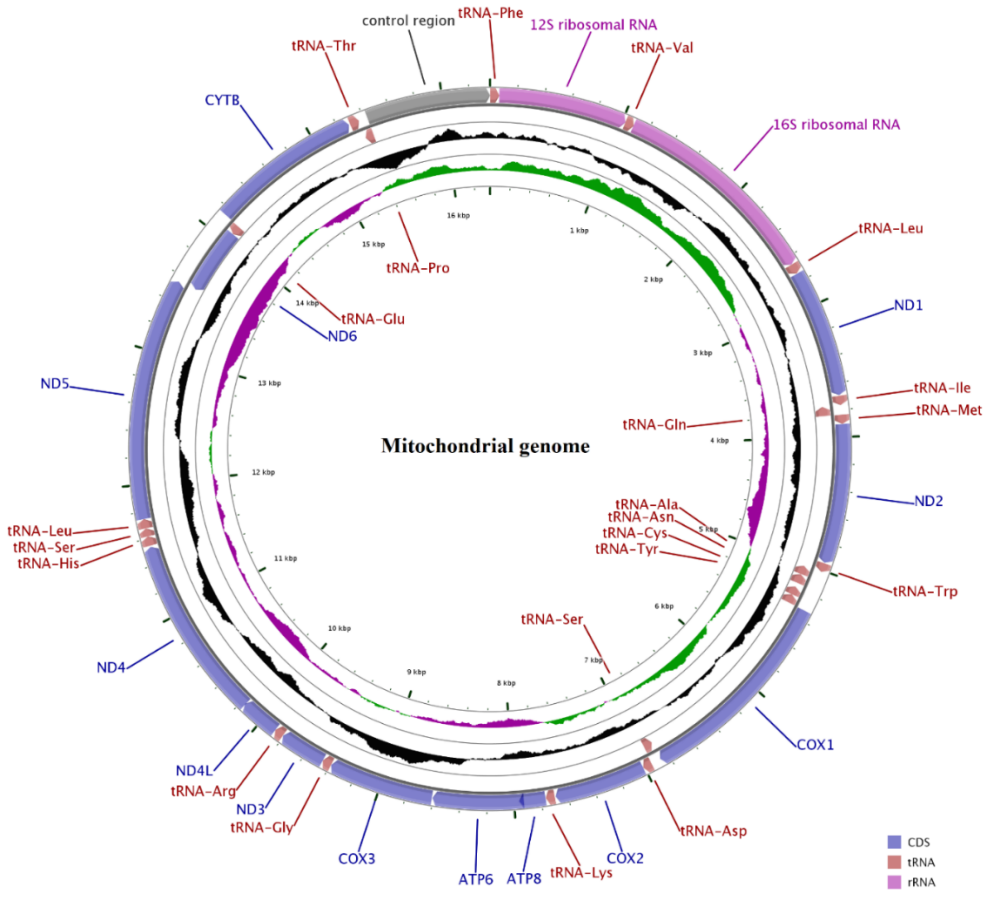

Figure 4: Representation of Mitochondrial genome Structure

Microsatellites Markers

Microsatellites are short tandem repeat arrayed (di-, tri-, or tetra nucleotide) sequences with the size of 1–6 bp repeated several times and flanked by regions of non-repetitive unique DNA sequences (Toth et al.,2000). Microsatellites are multi-allelic in a population and bi-allelic in an individual. They are inherited in a co-dominant Mendelian pattern and can reveal heterozygote (with two different alleles at a locus) and homozygote (with two copies of the same allele at a locus) in each individual, which is used in calculating the allele frequencies of populations. Microsatellites have a high rate of mutation ~10^{-4} events per locus per replication, leading to multiple alleles at each locus. These markers are suitable for studying the recent population genetic events and determining parentage due to the high level of polymorphism.

Research gaps

Recently, advances in the taxonomy of Cervids have improved considerably by introducing molecular systematics (Hassanin and Douzery 2003; Pitra et al., 2004;Gilbert et al., 2006, Ropiquet and Hassanin 2006, Hassanin et al., 2012). The systematic relationships within Cervini have been done multiple times, resulting in better resolution in the taxonomic arrangement (Pitra et al., 2004; Gilbert, Ropiquet & Hassanin, 2006; Hassanin et al., 2012;Croitor 2014). However, Muntiacinae systematics is not well resolved and therefore, phylogenetic relationships are still unclear. Evidence from molecular dating of fossil records suggests that the first appearance of Muntiacinae was around 8 Mya and is consistent and concurrent with paleo-biogeographic data (Gilbert et al., 2006). The taxonomic classification within genus *Muntiacus* has changed over time based on the different schemes of classifications (Ma et al., 1986; Groves et al., 2011; Martins et al., 2017). The red muntjac is distributed in larger geographical areas (South and South East Asia) in comparison to other *Muntiacus* species with overlapping distribution ranges. The similar morphological characteristics and overlapping distribution range create potential misidentification of species and species boundaries. To elucidate this ambiguity, I selected the following objectives to study the genetic diversity, phylogenetics, and the population genetic structure of Indian red muntjac in India and also compared with other existing muntjac.

The objectives of the study are:

1. To assess the genetic diversity of the Indian red muntjacs among different populations using mtDNA and microsatellites markers.
2. To investigate the phylogenetic relationships of Indian red muntjac with respect to other species.
3. To investigate the population genetic structure, gene flow and demographic history of Indian red muntjac.

Research Questions:

1. What is the level of genetic diversity in Indian red muntjacs?
2. How many lineages or evolutionary significant unit (ESU) of Northern red muntjac present in India and how they are genetically different?
3. What is the level of gene flow among the different populations of Indian red muntjacs?

Chapter- 2

TO ASSESS THE GENETIC DIVERSITY OF THE INDIAN RED MUNTJACS AMONG DIFFERENT POPULATIONS USING mtDNA AND MICROSATELLITES MARKERS

Background

Red muntjacs are represent large geographical range and have faced climate fluctuation in the past across their distribution range which has influenced their current genetic diversification (Martins et al.,2017). This has been further affected by subsequent increased anthropogenic activity such as agricultural developments and urbanization. Genetic diversity is important to assess the prevailing population fitness and future viability of species. This plays a key role in studying the evolutionary and population genetic status covering evolutionary patterns, current population structuring of species and impact of the changing climate alteration of habitat on adaptation and viability of species. Genetic diversity for population with small size in highly fragmented habitat may provide insights into inbreeding effects. The inbreeding effects caused by the low genetic diversity may increase the frequency of deleterious recessive alleles due to which genetic drift will be largely affected by the adaptive potential of populations (Lande 1994, Lande 1995, Lynch et al., 1995, Frankham 2002). Many factors like population bottleneck, founder effect, genetic drift, and selection may be responsible for losing a particular population's genetic diversity (Furlan et al., 2012). Population size and its degree of isolation can cause a rapid loss of genetic diversity compared to larger populations. Genetic drift and fixation of the common allele in a small population might reduce the genetic diversity. According to some studies, life-history traits such as fertility may clarify the variation in genetic diversity rather than ecological disturbance and historical contingency (Romiguier et al., 2014, Dutoit et al., 2016). Previously, the taxonomy of red muntjacs has been studied mostly through their morphological characteristics and variation in their chromosome numbers (Groves et al., 2011). However, a recent

study highlighted the molecular phylogeny based on complete mitogenome, which showed three mitochondrial lineages in the red muntjac group across their distribution range (Martins et al., 2017). Unfortunately, there is still a research gap in the genetic diversity of Indian red muntjacs.

The knowledge about their genetic diversity will play a crucial role in building up successful conservation management and reintroduction plan of the species (Frankham 2002). Through the help of heritable genetic markers, we can directly assess the genetic variation, which is linked with the identification and characterization of specific genotype (Avise 2004). The amount of genetic diversity, population structure trends, dispersal patterns, kinship relationships and effect of inbreeding can be estimated with microsatellites markers (Beja-Pereira et al., 2003). The estimation of genetic diversity in mitochondrial DNA is computed using haplotype (*H*), also known as gene diversity and nucleotide diversity (π) which evaluate the average number of nucleotide differences per site in pairwise comparisons among DNA sequences. Microsatellites are highly polymorphic and bi-allelic markers, which are widely used to estimate the genetic variation of populations. The level of heterozygosity, allelic richness and proportion of polymorphic loci can be used to evaluate the genetic variation.The variation within populations is quantified by gene diversity (the heterozygosity expected under Hardy-Weinberg Equilibrium), by the number of distinct alleles per locus, or by the percentage of polymorphic loci (Nei, 1975).

The distribution of genetic variability among individuals correlates with their evolutionary influences of mutation, selection, random genetic drift, fitness, population size and migration (Frankham 1996; Reed and Frankham 2003). Populations' demography and degree of evolutionary isolation can be examined

through different patterns of genetic variation or diversity among the species and populations. The variances in the quantity and quality of alleles, genes, chromosomes, and gene arrangements on the chromosomes present within and among the populations will define genetic variations which are indispensable for the evolution of populations in response to environmental alteration (Allendorf & Leary 1986; Williamson 2001; Çiftci and Okumus 2002). Conservation planning aimed at safeguarding the long-term perseverance of wild populations is efficiently supported, only considering the genetic variability in wild populations (Frankham 1995). To provide useful information for conservation and management of Indian red muntjac, we collected samples from different region of India, and used the both mtDNA and microsatellites markers to analyse the genetic diversity.

Methodology

Sample collection and DNA extraction

In this study, I used 20 samples for mitochondrial analysis and 42 samples for microsatellites analysis of northern red muntjac from a different region of India, includingthe northwest region (NWI=18), mainland (localities of north and central) (MLI=14), northeastern (NEI=3), and southern India (SI=5) and Andaman & Nicobar Islands (AI=2) (Table 2 and Fig. 5). Genomic DNA (gDNA) was extracted from tissue and hair samples using modified DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) protocol, whereas, for antlers and bone samples,we followed Gu-HCl based silica binding method (Gupta et al., 2013). These samples were collected from dead animals from the known localities by the local Forest Department of Indiaand sent to Wildlife Forensic and Conservation Genetic Cell, Wildlife Institute of India, Dehradun. Since the samples were collected from the dead animals, Animal

Ethics Committeeapproval was not required for this study. The authors confirmed that all experiments were performed in accordance with relevant guidelines and regulations.

Table 2: Samples details used for genetic analysis of red muntjac from India. n represents the sample size.

Origin		mtDNA			Microsatellite
	Status	n	Types	n	Types
Northwest India	Wild	5	Tissue	18	Tissue(15), Hairs(3)
Central India	Wild	5	Tissue	14	Tissue(7), Antler(3),Hairs(4)
Northeastern India	Wild	4	Tissue	3	Tissue(2), Bones(1)
Southern India	Wild	4	Tissue	5	Tissue
Andaman & Nicobar Islands	Wild	2	Tissue	2	Tissue

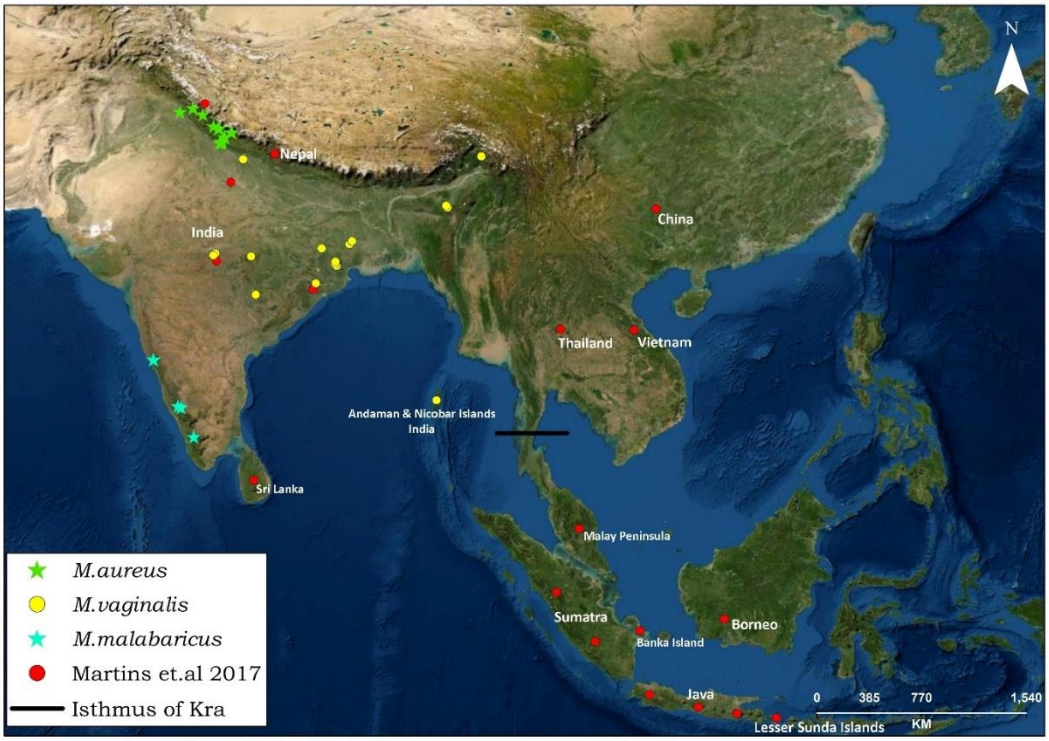

Figure 5: Map depicting the sampling sites and localities of red muntjacs. The horizontalblackline indicates the position of the Isthmus of Kra.

PCR amplification of complete mitogenome and sequencing

Polymerase chain reactions (PCRs) amplifications were carried out in 20µl volumes containing 10–20 ng of extracted genomic DNA containing, 1× PCR buffer (Applied Biosystem), 2.5mM $MgCl_2$, 0.2 mM of each dNTP, 5 pmol of each primer, and 5 units of Taq DNA polymerase (Thermo Scientific). We performed PCR amplification using 23 overlapping fragments of complete mtDNA (Table 3) (Hassanin et al., 2009). Besides, we included the fragments of complete Cytochrome *b* (Gupta et al., 2014) and Cytochrome c oxidase subunit-I gene (Kumar et al., 2017) to increase the overlapping. PCR cycles for DNA amplification were 95°C for 5 min; followed by 35 cycles of 95°C for 40 sec (denaturation), 54-56°C (annealing) for 40 sec, 72°C for 50 sec (extension) and a final extension of 72°C for 15 min. The efficiency and reliability of reactions were monitored using controls. The PCR products were electrophoresed on 2% agarose gel and visualized under UV light in the presence of Ethidium bromide dye. The amplified PCR products were treated with Exonuclease-I and Shrimp alkaine phosphatase (USB, Cleveland, OH) for 15 min each at 37ºC and 80ºC, respectively, to remove any residual primer. The purified amplicons were then sequenced bi-directionally using BigDye Terminator 3.1 on an automated Genetic Analyzer 3500XL (Applied Biosystems, Carlsbad, CA, USA).

Table 3: Details of the primers used in this study to sequence complete mitogenome of red muntjac (Hassanin et al., 2009)

Primer name	Primer Sequences
DLU405	5' -ACCATGCCGCGTGAAACCAGCA-3'
12SL41	5' -GYGYGGATRCTTGCATGTGTA-3'
U1230	5'-CACTGAAAATGCCTAGATGAG-3'
L2226	5'-CTAGGTGTAAACTAGRTGCTT-3'

12SU829	5' -GCACGCACACACCGCCCGTCAC-3'
16SL518	5' -CGCTTTCTTAATTGRTGGCTGC-3'
16SU365	5' -AGCCTGGTGATAGCTGGTTGTCC-3'
16SL1056	5' -AAGCTCCATAGGGTCTTCTCGTC-3
16SU946	5' -CCGTGCAAAGGTAGCATAATCA-3'
N1L64	5'-CCTAGNACTTTTCGTTCNACTA-3'
Uleu	5' -GTGGCAGAGCCCGGTAATTG-3'
IleL	5' -TTACTCTATCAAAGTAACTC-3'
N1U840	5' -TYCGAGCATCHTAYCCHCGATT-3'
N2L492	5'-TGGTTTAGBCCBCCTCAKCCYCC-3'
N2U354	5' -CACTTYTGAGTNCCAGAAGT-3'
AsnL	5'-TAGGGTRTTTAGCTGTTAAC-3'
TrpU	5' -AGACCAAGAGCCTTCAAAGC-3'
C1L339	5' -GCTTCWACTATDGADGATGC-3'
C1U246	5' -GGNGGNTTYGGHAAYTGACT-3'
C1L1017	5' -GAARATRAAGCCTAGRGCTCA-3'
C1U897	5' -TTYACHGTHGGAATAGAYGT-3'
C2L15	5' -GCRTCTTGRAANCCTARTTG-3'
SerU	5' -CCCCCYAYWRYTGGTTTCAAGCCA-3'
A8L1	5' -GTKGAYGTRTCTAGTTGYGGCAT-3'
C2U603	5' -CAATGCTCHGARATYTGYGG-3'
C3L45	5' -GANARDGCTCCYGTDAGNGGTCA-3'
A6U654	5' -GCCTAYGTNTTYACYCTNCTAGT-3'
GlyL	5' -TGATTGGAAGTCARYTGTAC-3'
C3U780	5' -GTHTCYATCTATTGATGAGG-3'
N4L27	5' -CAGGTYAGRGGDATDAGTAT-3'
U213M1	5' -AGCYTGYGAAGCAGCACTAGG-3'
L918M1	5' -GCKGTRGCTCCTATRTARCTTCA-3'
N4U840	5' - AGCTCHATYTGYYTHCGYCAAAC-3'
Leu2L	5' - CCAATTTTTTGGYTCCTAAGRCC-3'
Ser2U	5' -CCGAAAAAGYAYGCAAGAACTGC-3'
N5L652	5' -GCDGATTTTCCDGTKGCDGCTA-3'
N5U501	5' -GACGARCAGAYGCHAAYACAGC-3'
N5L1214	5' -GTDAKTADDAGGGCTCAGGCG-3'
N5U1146	5' -GGMAGCCTNGCNYTAACAGG-3'

N6RL154	50 -AGTTTAATGGDHTDGGDGATTG-3
N6RU102	5' -CCATAACTRTAYAAAGCHGCAA-3'
CBL402	5' -CCTCARAATGATATTTGKCCTCA-3'
CBU162	5' -CAGGMCTATTCCTRGCHATACA-3'
LTHR	5' -CCCTTYTCTGGTTTACAAGACC-3'
U1068	5' -CATCGGACAACTAGCATCTAT-3'
L482	5' -CCTGAAGWAAGAACCAGATG-3'

Microsatellite loci amplification and genotyping

Nine cross-species microsatellite loci: INRA001 (Vaiman et al., 1992), Ca18 (Gaur et al., 2003), BM6506 (Bishop et al., 1994), RT1, RT27, NVHRT48 (Poetsch et al., 2001), CelJP27 (Marshall et al., 1998), and T156, T193 (Jones et al., 2002) were selected for population genetic analysis of the red muntjac. Three sets of multiplex panels were carefully assembled based on molecular size andlabeled fluorescent dyes of loci (Table 4). To avoid ambiguity and amplification errors, for each sample, three multiplex PCR were carried out in 10µl reaction volumes containing 5 µl of QIAGEN Multiplex PCR Buffer Mix (QIAGEN Inc.), 0.2 µM labeled forward primer (Applied Biosystems), 0.2 µM unlabeled reverse primer, and 20–100 ng of the template DNA. PCR cycles for loci amplification were 95°C for 15 min; followed by 35 cycles of 95°C for 45 sec (denaturation), 55°C (annealing) for 1 min, 72°C for 1 min (extension) and a final extension of 60 °C for 30 min.The reliability of reactions was monitored using positive and negative controls. Alleles were resolved in an ABI 3500XL Genetic Analyzer (Applied Biosystems) using the LIZ 500 Size Standard (Applied Biosystems) and analyzed using GeneMaker ver 2.7.4 (Hulce et al., 2011).

Table 4: List of microsatellite markers used in this study

S.No.	STR Marker	Label	Range (bp)	Species	Referenvce
1	RT27	6-FAM	135-155	Reindeer (*Rangifer tarandus*), Red deer (*Cervus elaphus*)	Poetsch et al., 2001
2	RT1	VIC	234-256	Reindeer *(Rangifer tarandus*), Red deer (*Cervus elaphus*)	Poetsch et al., 2001
3	T156	6-FAM	134-234	California Elk (*Cervus elaphus canadensis*)	Jones et al., 2002
4	T193	PET	177-205	California Elk (*Cervus elaphus canadensis*)	Jones et al., 2002
5	NVHRT48	VIC	105-115	Reindeer *(Rangifer tarandus*), Red deer (*Cervus elaphus*)	Poetsch et al., 2001
6	BM6506	PET	191-209	Cattle (*Bos taurus*)	Bishop et al., 1994
7	Ca18	6-FAM	128-136	Chital deer (*Axis axis*)	Gaur et al., 2003
8	INRA011	VIC	148-278	White-tailed deer (*Odocoileus virginianus*)	Vaimen et al., 1992
9	CelJP27	VIC	176-196	Rum Red deer (*Cervus elaphus*)	Marshall et al., 1998

Data Analysis

Mitogenome analysis

A total of 20 complete mitogenome sequences of red muntjac from five different localities of India were generated (Table 2). Sequences obtained from forward and reverse direction were edited and assembled using SEQUENCHER® version 4.9 (Gene Codes Corporation, Ann Arbor, MI, USA). The annotation of complete mitogenome was done using Mitos WebServer (Bernt et al., 2013) and MitoFish (Iwasaki et al., 2013). Careful manual annotation was also conducted by sequence alignment with their related homologs sequences or species for ensuring the gene boundaries. Additionally, 36 sequences of northern red muntjac (*M. vaginalis*) (n=17); southern red muntjac (*M. muntjak*) (n=17) and Sri Lankan (*M. malabaricus*)

(n=2) muntjacs were included from GenBank to cover wide distribution ranges (Table 5, Figure 5). The dataset of 52 sequences of red muntjac was aligned using the CLUSTAL X 1.8 multiple alignment programs (Thompson et al., 1997) and alignments were checked by visual inspection. DnaSP v 5 (Librado et al., 2009) was used to estimate the haplotype (H) and nucleotide (π) diversity.

Table 5: List of complete dataset sequences of complete mitogenome of red muntjac, with information of Accession number and geographic origin

Sample ID	Accession number	Geographic Origin	Reference
BDADM1	MT671398	Andaman, India	Singh et al., 2021
BDADM2	MT671399	Andaman, India	Singh et al., 2021
BDHP1	MT671400	Himachal Pradesh, India	Singh et al., 2021
BDHP2	MT671401	Himachal Pradesh, India	Singh et al., 2021
BDPB1	MT671402	Punjab, India	Singh et al., 2021
BDUK1	MT671403	Uttarakhand, India	Singh et al., 2021
BDUK2	MT671404	Uttarakhand, India	Singh et al., 2021
BDKRT1	MT671405	Karnataka, India	Singh et al., 2021
BDKRT2	MT671406	Karnataka, India	Singh et al., 2021
BDWG1	MT671407	Western Ghats, India	Singh et al., 2021
BDWG2	MT671408	Western Ghats, India	Singh et al., 2021
BDCH1	MT758349	Chhattisgarh, India	Singh et al., 2021
BDNL1	MT758350	Nagaland, India	Singh et al., 2021
BDAP1	MT758351	Arunachal Pradesh, India	Singh et al., 2021
BDMP1	MT758352	Madhya Pradesh, India	Singh et al., 2021
BDMP2	MT758353	Madhya Pradesh, India	Singh et al., 2021
BDN3	MK050505	Nagaland, India	Singh et al., 2018
BDN4	MK050506	Arunachal Pradesh, India	Singh et al., 2018
BDN5	MK050507	Madhya Pradesh, India	Singh et al., 2018
BDN2	MH547032	Chhattisgarh, India	Singh et al., 2018
IND1	KY052109	North India	Martins et al., 2017
IND4	KY052105	India	Martins et al., 2017
IND5	KY052092	North India	Martins et al., 2017

IND6	KY052098	India	Martins et al., 2017
IND7	KY052100	India	Martins et al., 2017
IND10	KY052095	East India	Martins et al., 2017
NEP1	KY052099	Nepal	Martins et al., 2017
THA5	KY052106	Thailand	Martins et al., 2017
THA6	KY052097	Thailand	Martins et al., 2017
VIE5	KY052115	North C. Vietnam	Martins et al., 2017
CHI2	KY052110	Yunnan China	Martins et al., 2017
VIE10	KY052113	North C. Vietnam	Martins et al., 2017
VIE9	KY052111	North C. Vietnam	Martins et al., 2017
JAV1	KY052139	West Java	Martins et al., 2017
JAV2	KY052145	Java	Martins et al., 2017
JAV5	KY052130	East Java	Martins et al., 2017
JAV6	KY052134	West Java	Martins et al., 2017
JAV13	KY052121	West Java	Martins et al.,2017
JAV14	KY052138	West Java	Martins et al., 2017
JAV15	KY052129	Java	Martins et al., 2017
BOR4	KY052133	Borneo	Martins et al., 2017
BOR7	KY052124	Borneo	Martins et al., 2017
LSI1	KY052123	Lesser Sunda Island	Martins et al., 2017
LSI3	KY052153	Bangka Island	Martins et al., 2017
BAL1	KY052120	West Bali	Martins et al., 2017
SUM3	KY052147	Sumatra	Martins et al., 2017
SUM4	KY052136	Sumatra	Martins et al., 2017
SUM6	KY052125	Sumatra	Martins et al., 2017
MAL1	KY052118	Peninsular Malaysia	Martins et al., 2017
MAL2	KY052119	Peninsular Malaysia	Martins et al., 2017
SRI1	KY052116	East Sri Lanka	Martins et al., 2017
SRI2	KY052117	Sri Lanka	Martins et al., 2017

Microsatellite analysis

A total of nine polymorphic loci were used to analyze the 42 samples of red muntjac for population genetic studies. The CERVUS ver 3.0.6 program (Kalinowski et al.,

2007) was used to estimate the polymorphic information content (PIC), the number of alleles per locus, the observed (H_O) and, expected (H_E) heterozygosity. The allelic richness (*Ar*) and mean inbreeding coefficient (F_{IS}) (Weir et al., 1984) was estimated using FSTAT ver 2.9.3 (Goudet et al., 1995). All the loci were checked for under HWE in GenAlEx v6.5 (Peakall and Smouse 2012).

Results

The 20 generated mitogenomes sequences of red muntjac were deposited in GenBank (MT671398-MT671408; MK050505-MK050507; MH547032 and MT758349-MT758353). To elucidate the phylogenetic relationships, we included 32 sequences of Northern, Southern, and Srilankan muntjacs from Martins et al., 2017. The accession numbers and details for each sample are provided in the Table 5. All complete mitogenome sequences grouped into four genetically distinct clusters and represented individual haplotypes with haplotype diversity Hd=1, indicating maternal unrelatedness among the red muntjac samples (Table 6). The nucleotide diversity of *M. vaginalis* (Mainland red muntjac) was π =0.0044 (s.d.=0.0003), *M. aureus* (Himalayan red muntjac) was π =0.0016 (s.d.=0.0002), *M. malabaricus* (Western Ghats& Srilankan red muntjac) was π =0.005 (s.d.=0.0008) and *M. muntjak* (Southern or Sundaland red muntjac) was π =0.0109 (s.d.=0.0006). The overall nucleotide diversity among red muntjacs was π =0.0203 (s.d.=0.001). Among all red muntjacs, the lowest number of segregating sites were found in *M. aureus* (S=67), whereas it was high in *M. muntjak* (S=658).

The genetic diversity of Indian red muntjacs was calculated using nine microsatellite markers (Table 6). The selected microsatellite markers showed high polymorphic information content (PIC>0.5) with a mean value of 0.831; therefore, all used loci

were found to be informative. All the loci significantly deviated from Hardy-Weinberg equilibrium (HWE) and no linkage disequilibrium (LD) was detected ($P>0.05$). No evidence of a large allele drop out was observed, while null alleles at each locus were low in frequency (less than 0.10 per population). The mean number of alleles (Na) in *M. vaginalis, M. aureus* and *M. malabaricus* were 9.33, 7.11 and 6.11 respectively with highest allelic richness (*Ar*) in *M. malabaricus* (*Ar*=6.11) and lowest in *M. aureus* (*Ar*=4.52). The observed heterozygosity (H*o*) and expected heterozygosity (H_E) in *M. aureus* were *H*o: 0.666; H_E: 0.730; for *M. vaginalis*, *H*o: 0.620; H_E: 0.810; whereas, in *M. malabaricus*,it was *H*o: 0.756, H_E: 0.760. The mean inbreeding coefficient (F_{IS}) value for all the red muntjac populations were greater than zero (ranges between 0.096-0.269), indicating a heterozygote deficiency (Table 6), which may be attributed due to Wahlund effect and population not being in HWE.

Table 6: Summary of genetic diversity in red muntjacs populations based on complete mitochondrial DNA and microsatellites**.**

	Mitochondrial DNA					Microsatellites					
Population/ Region	**n**	**S**	**H**	**Hd**	***π***	**n**	**Na**	***Ar***	H_o	H_E	F_{IS}
M.aureus	6	67	6	1.00	0.001	18	7.11±0.73	4.52	0.66±0.071	0.73±0.03	0.131
M.vaginalis	23	415	23	1.00	0.004	19	9.33±0.86	5.79	0.62±0.04	0.81±0.02	0.269
M. malabaricus	6	198	6	1.00	0.005	5	6.11±0.56	6.11	0.75±0.06	0.76±0.04	0.096
M. munjak	17	658	17	1.00	0.01	-	-	-	-	-	-

Note: n= number of samples, S= *segregating sites*, H= haplotype, Hd= haplotype diversity, π= nucleotide diversity, Na = number of alleles; *Ar*= allelic richness, H_O= observed heterozygosity; H_E = expected heterozygosity; F_{IS} = inbreeding coefficien

Discussion

The environmental change and high anthropogenic pressure are major cause for continuous loss of biodiversity across the globe which is directly affecting the population size of species (Ceballos et al., 2017). The continuous fragmentation of habitat and reduction in the geographical ranges is a serious challenge for species persistence and may ultimate pose risk of extinctions (Ceballos et al., 2017). To estimate the viability of species population which may face climatic and anthropogenic changes in their natural habitat scientists often use genetic diversity as a tool which perhaps provide evidence about their past evolutionary history and current genetic status. Another necessity is to understand the variability of population (Garner et al., 2005). The valid information about the genetic diversity is crucial and it plays a major role in improving the policy for any conservation priority species' (Groves et al., 2002; Garner et al., 2005). The loss of genetic diversity may cause serious consequences for species by reducing population fitness to adapt environmental changes (Hoffmann & Parsons 1997) and increase the extinction risk (Lacy 1997; Frankham et al. 2002). Indeed, preservation of genetic diversity is one of three global conservation priorities recognized by International Unioun for Conservation of Nature (IUCN) (McNeely et al., 1990).

The climate fluctuation during the last glacial maxima largely affects the demography of South and Southeast Asian region. The consequences of habitat modification and changes in regional environmental conditions responsible for species distribution and diversification pattern in this region (Hughes et al., 2003; Patou et al., 2010; Hughes et al., 2011; Patel et al., 2017). The red muntjacs are highly adaptable and widely distributed in South and Southeast Asian biogeographical region. The expansion of human activities, change in their natural

habitat are major factor for reduction of the population size in red muntjac. Additionally, increasing poaching activities for meat and trophy are major threats for red muntjac population across their distribution range (Higham, 2013; Turvey et al., 2015; Turvey et al., 2016). The decreasing population size across their range and lack of genetic data of species is major concern among management authorities. In this study we evaluated the genetic diversity of red muntjac population based on both mitochondrial and microsatellites markers. Our finding showed that all All complete mitogenome sequences grouped into four genetically distinct clusters and represented individual haplotypes with haplotype diversity $Hd = 1$, indicating maternal unrelatedness among the red muntjac samples. All populations exhibited high haplotypes and nucleotide diversity and microsatellite diversity with significant divergence among them. However, comparatively lower *Ar* and H_E was observed in *M. aureus*, which could be attributed to limited distribution range. Results indicated that currently all populations of red muntjacs are genetically fit and healthy. Nevertheless, continuous change in habitat by high anthropogenic activities and considerable poaching pressure for meat and trophy hunting leads to decline in population size of red muntjac across their distribution range. This study would be helpful for further conservation and restoration plan to maintain genetic integrity and ecological connectivity for red muntjacs.

Chapter-3

TO INVESTIGATE THE PHYLOGENETIC RELATIONSHIPS OF INDIAN RED MUNTJAC WITH RESPECT TO OTHER SPECIES

Background

The Indian subcontinent sustains a diverse ecosystem that supports high faunal richness and diversity, but very little is known about the species diversification and evolutionary history and the role of geo-climatic changes during the Late Pleistocene (Mani. 1974, Roberts et al., 2014). The late Pliocene to early Pleistocene witnessed dramatic climatic shifts in South and Southeast Asia, which led to geographical subdivision with contraction of habitats, influencing the distribution of contemporary species. In old-world deer, the diversification started during the Miocene-Pliocene boundary (3.3 to 7.1 Mya) to early Pleistocene (0.4 - 2.5 Mya) (Douzery and Randy 1997). However, during Miocene-Pliocene, the past climatic changes have played a major role in the diversification of three genera *Axis*, *Cervus* and *Dama* in South Asia (Meijaard and Groves 2004; Pitra et al., 2004; Gilbert et al., 2006). Recently,advanced molecular studies suggest that Muntiacinae have a closed phylogenetic association with Cervinae (Pitra et al., 2004; Gilbert et al., 2006). The genus *Muntiacus* is a taxonomically diverse group, which inhabits the South and South East Asian region. Previous studies based on morphological and geographical area projected large number of species in this genus (Table 7), yet similar morphological features and overlapping geographical range have caused taxonomic ambiguityin this genus. Formally, giant muntjac (*M. vuquangensis*) was assigned in genus *Megamutiacus* (Tuoc et al., 1994), although based on recent studies on morphology and phylogenetic, it has been placed within genus *Muntiacus* (Schaller et al., 1996; Amato et al., 2000). Additionally, the species status of the Putao muntjac (*M. putaoensis*) from Myanmar (Amato et al.,1999), Small blackish muntjac (*M. truongsonensis*) from Central Vietnam (Giao et al.,1998), and Roosevelt's

barking deer (*M. rooseveltorum*) from Vietnam (Le et al.,2014) has been confirmed with molecular studies. In red muntjac group, the northern red muntjac covers a large distribution range and overlapping zone among and with other *Muntiacus* species. Few previous studies highlighted different species in this group based on morphological and molecular characteristics (Ma et al., 1982; Groves and Grubb 2011; Martins et al., 2018). Therefore, the validation of species and sub species of red muntjac is still unclear due to lack of corroborative studies in terms of morphology and molecular aspects and limited number of samples that did not cover the wide range of geographical areas of red muntjac distribution. In this chapter, I shall be discussing the phylogenetic relationship, divergence dating of northern red muntjac with other *Muntiacus* species and study the phylogeographic pattern of red muntjacs.

Table 7: Number of identified species in genus *Muntiacus*.

S.N.	Species name	Ma et al., (1986)	Groves & Grubb., (2011)	IUCN (2018)
1	*M. muntjak* (Zimmermann, 1780)	*M. muntjak*	*M. muntjak*	*M. muntjak*
2	*M. vaginalis* (Boddaert, 1785)		*M. vaginalis*	*M. vaginalis*
3	*M. aureus* (Hamilton Smith, 1826)		*M. aureus*	
4	*M. nigripes* (G. M. Allen, 1930)		*M. nigripes*	
5	*M. malabaricus* (Lydekker, 1915)		*M. malabaricus*	
6	*M. crinifrons* (Sclate, 1885)	*M. crinifrons*	*M. crinifrons*	*M. crinifrons*
7	*M. gongshanensis* (Ma et al., 1990)		*M. gongshanensis*	*M. gongshanensis*
8	*M. feae* (Thomas & Doria, 1889)	*M. feae*	*M. feae*	*M. feae*
9	*M. vuquangensis* (Tuoc et al., 1994)		*M. vuquangensis*	*M. vuquangensis*
10	*M. rooseveltorum* (Osgood, 1932)	*M. rooseveltorum*	*M. rooseveltorum*	*M. rooseveltorum*
11	*M. putaoensis* (Amato et al., 1999)		*M. putaoensis*	*M. putaoensis*
12	*M. truongsonensis* (Giao et al., 1998)		*M. truongsonensis*	*M. truongsonensis*
13	*M. reevesi* (Ogilby, 1839)		*M. reevesi*	*M. reevesi*
14	*M. atherodes* (Groves & Grubb, 1982)		*M. atherodes*	*M. atherodes*
15	*M. montanus* (Robinson & Kloss,1918)		*M. montanus*	*M. montanus*
16	*M. puhoatensis* (Binh Chau, 1997)		*M. puhoatensis*	*M. puhoatensis*

Sequencing and characterization of complete mitogenome of *M. vaginalis, M. aureus,* and *M. malabaricus* and its Phylogenetic relationship with other *Muntiacus* species

Methodology

Sample collection and DNA extraction

We used 20 archival samples of northern red muntjac from a different regions of India, including northwest region (NWI=5), central India (MLI=5), northeastern (NEI=4), and southern India (SI=4) and Andaman & Nicobar Islands (AI=2) (Table 2). Genomic DNA (gDNA) was extracted from tissue and hair samples using modified DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) protocol, whereas, for antlers and bone samples,we followed Gu-HCl based silica binding method (Gupta et al., 2013). These samples were collected from dead animals from the known localities by the local Forest Department of Indiaand sent to Wildlife Forensic and Conservation Genetic Cell, Wildlife Institute of India,Dehradun. Since the samples were collected from the dead animals, Animal Ethics Committee approval was not required for this study. The authors confirmed that all experiments were performed in accordance with relevant guidelines and regulations.

PCR amplification of complete mitogenome and sequencing

Polymerase chain reactions (PCRs) amplifications were carried out in 20µl volumes containing 10–20 ng of extracted genomic DNA containing, 1× PCR buffer (Applied Biosystem), 2.5mM $MgCl_2$, 0.2 mM of each dNTP, 5 pmol of each primer, and 5 units of Taq DNA polymerase (Thermo Scientific). We performed PCR amplification using 23 overlapping fragments of complete mtDNA (Hassanin et al., 2009; Table 3). Besides, we included the fragments of complete Cytochrome *b* (Gupta et al., 2014) and Cytochrome C oxidase-*I* gene (Kumar et al., 2017) to

increase the overlapping. PCR cycles for DNA amplification were 95°C for 5 min; followed by 35 cycles of 95°C for 40 sec (denaturation), 54-56°C (annealing) for 40 sec, 72°C for 50 sec (extension) and a final extension of 72°C for 15 min The efficiency and reliability of PCR reactions were monitored by using control reactions. The PCR products were electrophoresed on 2% agarose gel and visualized under UV light in the presence of ethidium bromide dye. The amplified PCR products were treated with Exonuclease-I and Shrimp alkaine phosphatase (USB, Cleveland, OH) for 15 min each at 37ºC and 80ºC, respectively, to remove any residual primer. The purified Amplicons were then sequenced bidirectionally using BigDye Terminator 3.1 on an automated Genetic Analyzer 3500XL (Applied Biosystems, Carlsbad, CA, USA).

Mitogenome characterization and annotation

The overlapping fragments of DNA sequences were aligned to generate the entire mitogenome of *M. vaginalis* using Sequencher® version 5.4.6 (Gene Codes Corporation, Ann Arbor, MI, USA). The mtDNA annotation was done using Mitos WebServer (Bernt et Al., 2013). We used Draw Organelle Genome Maps (Lohse et al., 2013)for generating a gene map of the complete mtDNA of *M. vaginalis*, *M. aureus*, and *M. malabaricus*. We obtained a total length of 16,352, 16349, and 16356 basepairs, respectively. All sequenced mitogenomes have been deposited in GenBank (MT671400-MT671408, MH547032, MK050505, MK050506, and MK050507).

For the comparative analysis with other *Muntiacus spp.*, complete mtDNA sequences of *M. muntjak* (NC004563), *M. crinifrons* (NC004577), *M. reevesi* (NC008491), *M. vuquangensis* (FJ705435), *M.putaoensis* (MF737190), *M. feae*

(NC041100, MG857663) and *M. gongshanensis* (MK882935) were downloaded from the GenBank. Base compositions and mtDNA genetic code were calculated in MEGA X (Kumar et al., 2018). For estimating the bias in nucleotide composition among the complete mitogenome, skew analysis was carried out using the following method (Perna et al., 1995): AT skew = (A−T) / (A + T), GC skew = (G−C)/(G + C). The intergenic spacer and overlapping regions between genes of complete mitogenome were estimated manually.

Phylogenetic analysis

For phylogenetic analysis, 18 mitogenomes were downloaded from GenBank to reconstruct the phylogenetic relationship between Cervini and Muntiacini. Additionally, the mitogenomes of *Bos javanicus* (JN632606) and *Moschus moschiferus* (FJ469675) were downloaded from GenBank and used as outgroups. A Monte Carlo Markov Chain (MCMC) based Bayesian consensus tree was constructed using BEAST ver 1.7 (Drummond et al., 2012). Bayesian inference analysis ran as four simultaneous MCMC chains for 10 million generations and sampled every 100 generations using a burn-in of 5000 generations. The resulting phylogenetic trees were visualized in FigTree ver 1.4.0 (http://tree.bio.ed.ac.uk/software/figtree).

Results and discussion

Mitogenome organization

The complete mtDNA map of *M. vaginalis,* has been shown in figure 6. However, the mitogenome structure of *M. aureus* and *M. malabaricus* showed similarity with *M. vaginalis*. These sequences exhibited a similarity range between 99.52% to 99.84%. These were consisted of 22 transfer RNA genes, 13 protein-coding genes

(PCGs), two ribosomal RNA genes and a noncoding control region (D-loop region) (Table 8). The arrangement and distribution of these genes are similar to the other *Muntiacus* species (Li et al., 2003, Wang et al., 2006). The total nucleotide composition in mitochondrial genome of *M. vaginalis* was A (33.2%), T (29.0%), C (24.50%) and G (13.30%); in *M. aureus*was A (33.2%), T (28.9%), C (24.5%) and G (13.3%) and in *M. malabaricus* was A (33.2%), T (29.4%), C (24.1%) and G (13.2%). Most of the genes were coded on the H-strand, except for the nad6 gene and eight tRNA genes ($tRNA^{Gln}$, $tRNA^{Ala}$, $tRNA^{Asn}$, $tRNA^{Cys}$, $tRNA^{Tyr}$, $tRNA^{ser}$, $tRNA^{Glu}$, $tRNA^{Pro}$). The control region was located between $tRNA^{Pro}$ and $tRNA^{Phe}$(Table 8). Due to gene compactness and rearrangements of the mitogenome, four pairs of overlapping genes were observed among tRNA-Ile/tRNA-Gln, atp8/atp6, nad4L/nad4, and tRNA-Thr/ tRNA-Pro. These were resulting in 41 overlapping nucleotides (Table 8), which are usually found in other mammalian species (Hassanin et al., 2012, Li et al., 2017).We calculated values of AT skew, GC skew and AT% for identifying nucleotide compositions in the complete mitogenome of all *Muntiacus* species. The AT-skew was positive, whereas GC-skew was negative except in *M. reevesi* (Table 9).

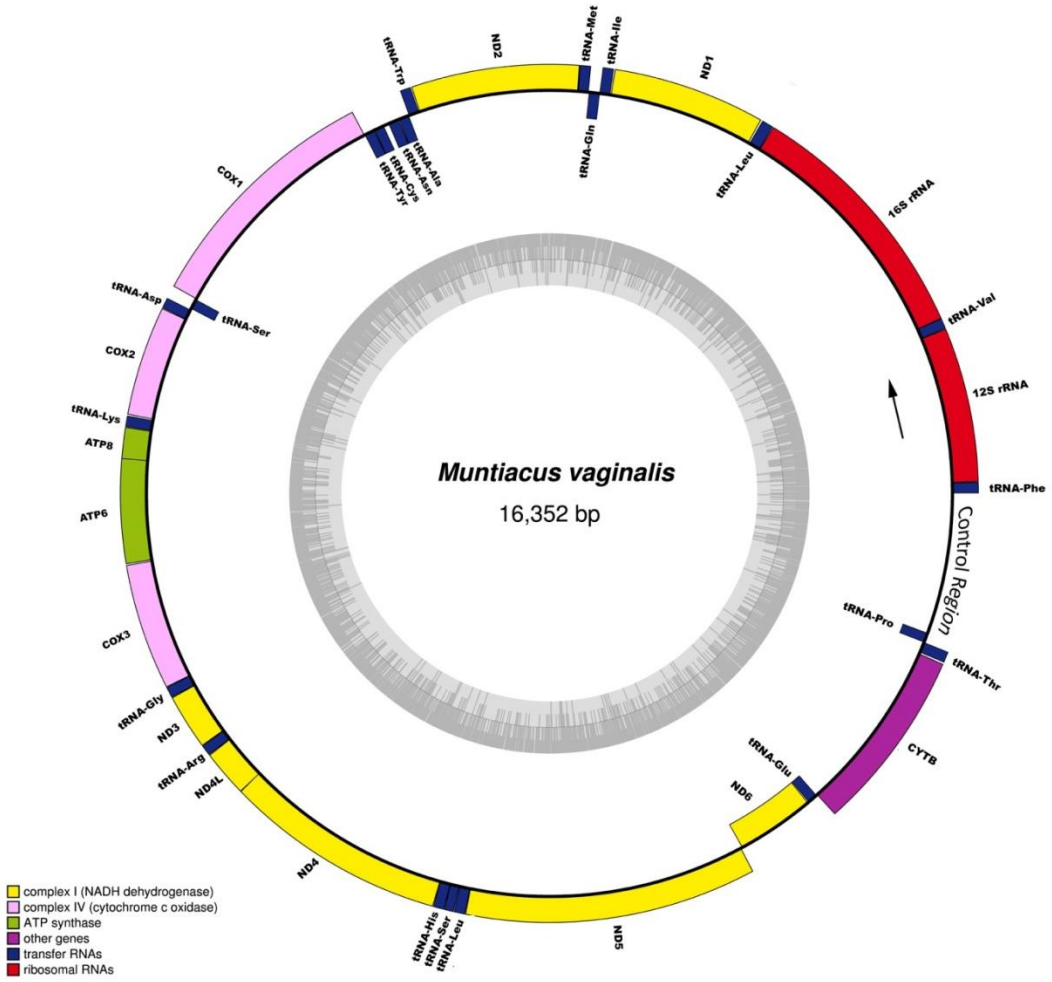

Figure 6. Map of the complete mitochondrial genome of northern Indian red muntjac (*M. vaginalis*). The genes encoded on the heavy and light strand are represented outside and inside the circle, respectively.

Table 8. Tabular organization of the mitochondrial genome of *M. vaginalis*. Partial stop codons* (T-- or TA-) were completed by adding A during the polyadenylation of the premature RNA's. Minus (-) value[#], represent the overlapping number of nucleotides.

	Positions			Codon				
Gene	**Start**	**End**	**Size**	**Start**	**Stop***	**Anti-codon**	**Strand**	**Space/overlap[#]**
tRNA-Phe	1	69	69	-	-	GAA	H	0
12S ribosomal RNA	70	1025	956	-	-	-	H	0
tRNA-Val	126	1093	68	-	-	TAC	H	0
16S ribosomal RNA	1092	2660	1569	-	-	-	H	0
tRNA-Leu	2662	2736	75	-	-	TAA	H	1
ND1	2745	3689	945	ATA	T--	-	H	8
tRNA-Ile	3695	3763	69	-	-	GAT	H	5
tRNA-Gln	3761	3832	72	-	-	TTG	L	-3
tRNA-Met	3835	3903	69	-	-	CAT	H	2
ND2	3904	4941	1038	ATA	T--	-	H	0
tRNA-Trp	4946	5013	68	-	-	TCA	H	4
tRNA-Ala	5015	5083	69	-	-	TGC	L	1
tRNA-Asn	5085	5157	73	-	-	GTT	L	1
tRNA-Cys	5190	5257	68	-	-	GCA	L	32
tRNA-Tyr	5258	5326	69	-	-	GTA	L	0
COX1	5328	6866	1539	ATG	TA-	-	H	1
tRNA-Ser	6870	6938	69	-	-	TGA	L	3
tRNA-Asp	6946	7013	68	-	-	GTC	H	7
COX2	7015	7695	681	ATG	TA-	-	H	1
tRNA-Lys	7702	7770	69	-	-	TTT	H	6
ATP8	7772	7966	195	ATG	TAA	-	H	1
ATP6	7933	8607	675	ATG	TAA	-	H	-33
COX3	8613	9395	783	ATG	T--	-	H	5
tRNA-Gly	9397	9465	69	-	-	TCC	H	1
ND3	9466	9810	345	ATA	T--	-	H	0
tRNA-Arg	9813	9881	69	-	-	TCG	H	2
ND4L	9882	10175	294	ATG	TAA	-	H	0
ND4	10172	11539	1368	ATG	T--	-	H	-4
tRNA-His	11550	11618	69	-	-	ATG	H	10

tRNA-Ser	11619	11678	60	-	-	GCT	H	0
tRNA-Leu	11680	11749	70	-	-	TAG	H	1
ND5	11750	13552	1803	ATA	TAA	-	H	0
ND6	13560	14078	519	ATA	TAA	-	L	7
tRNA-Glu	14082	14150	69	-	-	TTC	L	3
CYTB	14155	15288	1134	ATG	AGA	-	H	4
tRNA-Thr	15298	15367	70	-	-	TGT	H	9
tRNA-Pro	15367	15432	66	-	-	TGG	L	-1
control region	15433	16352	919	-	-	-	H	/

Protein Coding Genes (PCGs)

The total encoding length of 13 protein-coding genes (PCGs) in *M. vaginalis, M. aureus,* and *M. malabaricus* were11319 bp which was 69.26% of the complete mitogenome. The PCGs region of *M. vaginalis, M. aureus,* and *M. malabaricus* were consisted of seven NADH dehydrogenases (nad1−nad6 and nad4L), three cytochrome c oxidases (cox*I*−cox*III*), two ATPases (atp6 and atp8) and one cytochrome *b* (Cyt *b*) gene. All 13 PCGs, except nad6, were encoded on the H-strand (Fig 6). All the 13 PCGs were having putative start codons and started with ATG or ATA. Termination with an incomplete stop codon such as TA- and T-- were found in seven PCGs, those were completed by a post-transcriptional addition of polyadenylation during the mRNA maturation process. The over all AT content of 13 PCGs in *M. vaginalis, M. aureus,* and *M. malabaricus* were 61%-62%. Base skews were estimated to understand the degree of base bias between all PCGs. The AT and GC skew values for the PCGs of *M. vaginalis, M. aureus,* and *M. malabaricus* were compared with other *Muntiacini* mitogenome sequences. Positive AT skewness was obtained for most PCGs, showing that adenines occur more frequently than thymines. GC skewness was observed for the PCGs inall *Muntiacini* that was indicative of C biased nucleotide composition (Table 9). In 13 PCGs, the nad5 gene (1803 bp) was the longest and the atp8 (195 bp) was the smallest in

length. Relative synonymous codon usage and codon number within 13 PCGs of *M. vaginalis* are summarized in Table 10 and Fig 7. 13 PCGs in the *M. vaginalis* mitogenome consist of 3772 codons.

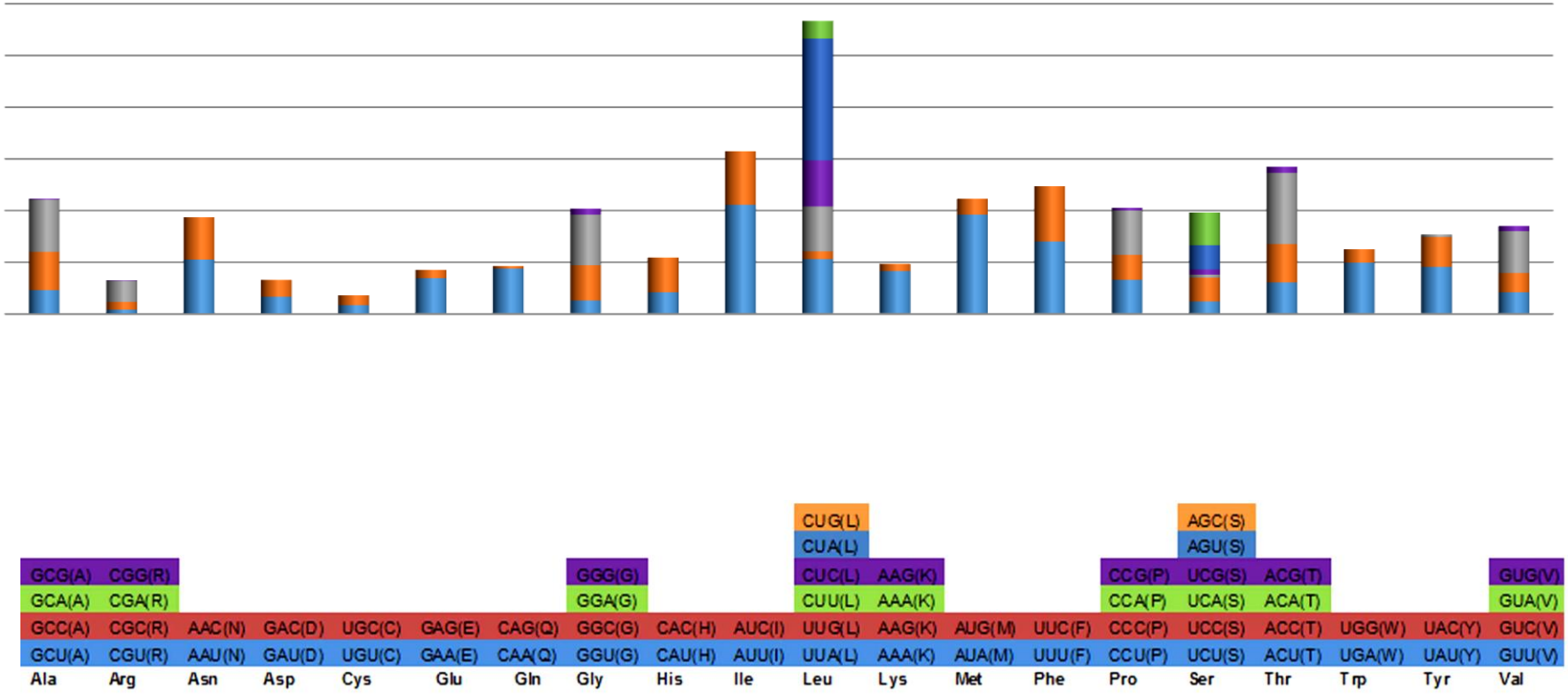

Figure 7: The codon number in the mitogenome of *M. vaginalis*. Codon families are given at X–axis

Table 9: Nucleotide composition indices in different regions of six representative mitogenomes of *Muntiacini*. [a]This study, [b]Shi et al., 2003, [C]Li et al., unpublished, [d]Wang et al., unpublished, [e]Hassanin et al., 2012, [f]Li et al., 2017, [g]Srisodsuk et al., 2018, [h]Zhang et al., 2019.

Species	Accession no.	Whole mitogenome				Protein CodingGenes (PCGs)				RibosomalRNA (rRNA)	
		Length (bp)	AT (%)	AT Skew	GC skew	Length (bp)	AT (%)	AT Skew	GC skew	Length (bp)	AT (%)
M. vaginalis	MH547032[a]	16352	62.3	0.067	-0.293	11319	62.0	0.038	-0.369	2525	62.7
M. vaginalis	MK050505[a]	16352	62.3	0.068	-0.29	11319	62.0	0.038	-0.369	2525	62.8
M. vaginalis	MK050506[a]	16352	62.4	0.067	-0.29	11319	62.0	0.038	-0.370	2525	63.0
M. vaginalis	MK050507[a]	16352	62.3	0.068	-0.29	11319	62.7	0.037	-0.367	2525	62.7
M. aureus	MT671400[a]	16348	62.1	0.068	-0.297	11319	61.9	0.037	-0.367	2525	62.3
M. aureus	MT671401[a]	16349	62.2	0.069	-0.298	11319	62.1	0.038	-0.369	2525	62.3
M. aureus	MT671402[a]	16349	62.2	0.068	-0.296	11319	62.0	0.036	-0.366	2525	62.3
M. aureus	MT671403[a]	16349	62.1	0.068	-0.296	11319	62.0	0.037	-0.366	2525	62.3
M. aureus	MT671404[a]	16348	62.1	0.069	-0.297	11319	61.9	0.037	-0.366	2525	62.3
M. malabaricus	MT671405[a]	16355	62.6	0.063	-0.292	11319	62.6	0.031	-0.362	2525	62.5
M. malabaricus	MT671406[a]	16356	62.6	0.063	-0.292	11319	62.7	0.030	-0.361	2525	62.6
M. malabaricus	MT671407[a]	16356	62.5	0.063	-0.292	11319	62.6	0.031	-0.361	2525	62.4
M. malabaricus	MT671408[a]	16355	62.6	0.063	-0.292	11319	62.6	0.031	-0.361	2525	62.4
M. muntjak	NC004563[b]	16353	62.2	0.071	-0.29	11319	61.9	0.038	-0.366	2524	62.9
M. crinifrons	NC004577[c]	16357	61.9	0.066	-0.288	11319	61.7	0.041	-0.364	2525	62.3
M. reevesi	NC008491[d]	16353	62.1	0.064	-0.29	11319	61.9	0.036	-0.359	2528	62.4

M. vuquangensis	FJ705435[e]	16361	62.3	0.061	-0.284	11319	62.1	0.034	-0.362	2533	62.5
M. putaoensis	MF737190[f]	16349	62.4	0.068	-0.296	11319	62.2	0.028	-0.352	2528	62.2
M. feae	NC 041100[g]	16355	62.0	0.071	-0.292	11319	62.0	0.039	-0.364	2525	62.1
M. feae	MG857663[g]	16356	62.0	0.071	-0.292	11319	62.0	0.039	-0.364	2525	62.1
M. gongshanensis	MK882935[h]	16356	62.0	0.070	-0.293	11319	61.9	0.041	-0.366	2525	62.6

Table 10: Relative synonymous codon usage and codon number within *M. vaginalis* mitochondrial genome (*the termination codons).

Codon	Count	RSCU	Codon	Count	RSCU	Codon	Count	RSCU
UUU(F)	140	1.13	UCU(S)	47	0.97	UAU(Y)	91	1.22
UUC(F)	107	0.87	UCC(S)	63	1.3	UAC(Y)	58	0.78
UUA(L)	106	1.12	UCA(S)	105	2.17	UAA(*)	10	1.29
UUG(L)	15	0.16	UCG(S)	5	0.1	UAG(*)	5	0.65
CUU(L)	87	0.92	CCU(P)	66	1.29	CAU(H)	42	0.77
CUC(L)	89	0.94	CCC(P)	48	0.94	CAC(H)	67	1.23
CUA(L)	236	2.5	CCA(P)	86	1.68	CAA(Q)	88	1.89
CUG(L)	34	0.36	CCG(P)	5	0.1	CAG(Q)	5	0.11
AUU(I)	211	1.34	ACU(T)	61	0.86	AAU(N)	105	1.12
AUC(I)	104	0.66	ACC(T)	74	1.04	AAC(N)	82	0.88
AUA(M)	192	1.72	ACA(T)	138	1.94	AAA(K)	83	1.71
AUG(M)	31	0.28	ACG(T)	12	0.17	AAG(K)	14	0.29
GUU(V)	42	0.99	GCU(A)	46	0.83	GAU(D)	33	1
GUC(V)	37	0.87	GCC(A)	74	1.33	GAC(D)	33	1
GUA(V)	81	1.91	GCA(A)	101	1.81	GAA(E)	69	1.62
GUG(V)	10	0.24	CGA(R)	41	2.52	AGU(S)	24	0.5
UGU(C)	17	0.94	CGG(R)	1	0.06	AGC(S)	46	0.95
UGC(C)	19	1.06	GGC(G)	68	1.33	AGA(S)	6	0.77
UGA(W)	99	1.58	GGA(G)	98	1.92	AGG(S)	10	1.29
UGG(W)	26	0.42	GGG(G)	12	0.24	GGU(G)	26	0.51
CGU(R)	8	0.49				CGC(R)	15	0.92

Ribosomal RNA and transfer RNA genes

The 12s rRNA and 16s rRNA genes in the mitogenome of *M. vaginalis, M. aureus,* and *M. malabaricus* were positioned between tRNA-Pheand tRNA-Val and between tRNA-Valand tRNA-Leu2, respectively. The length of both rRNA genes was 2525 to 2528 bp and accounted for 15.45% of the complete mitogenome. The length of the 12s rRNA and 16s rRNA genes were 956 bp and 1569 bp, respectively. The total AT content of 2 rRNA and 22 tRNA genes were 62.07% and 64.1%, respectively. The base-pair composition of both the rRNAs genes in *M. vaginalis* was A: 37.6%, T: 25.1%, G: 17.0% and C: 20.2%; in *M. aureus*was:A: 37.5%, T: 24.8%, G: 17.0% and C: 20.6% and in *M. malabaricus* was A: 37.5%, T: 24.9%, G: 17.1% and C: 20.5%. The tRNAs genes were found to have an average base composition of A: 33.0%, T: 31.1%, G: 19.5% and C: 16.4%, with the highest AT content. The anticodons of all the tRNAs found in the complete mitogenome of *M. vaginalis* are given in Table 8.

Mitogenome phylogeny of Muntiacus species

The Bayesian inference phylogenetic analysis was performed using the complete mitogenome of eleven species of Cervini and seven species of Muntiacini along with the *M. vaginalis, M. aureus,* and *M. malabaricus*. The results yielded strong statistics support (>99%) within the Cervini and Muntiacini. The clustering pattern of the Muntiacini was broadly consistent with the previous studies (Gilbert et al., 2006, Heckeberg et al., 2016, Le et al., 2014). The newly sequenced *M. vaginalis,*

M. aureus, and *M. malabaricus* nested within Muntiacini, with two *Muntiacus* clades. One clade consisted of *M. reevesi*, *M. putaoensis*, and *M. vuquangensis*, and the other consisted of *M. vaginalis*, *M. aureus*, *M. malabaricus*, *M. muntjak* and *M. crinifrons*. The generated sequences of *M. vaginalis*, *M. aureus*, and *M. malabaricus* were closely related to the *M. muntjak*. *E. cephalophus* as a single species in genus *Elaphodus* of Muntiacinae and formed a sister relationship with *Muntiacus* (Fig. 8).

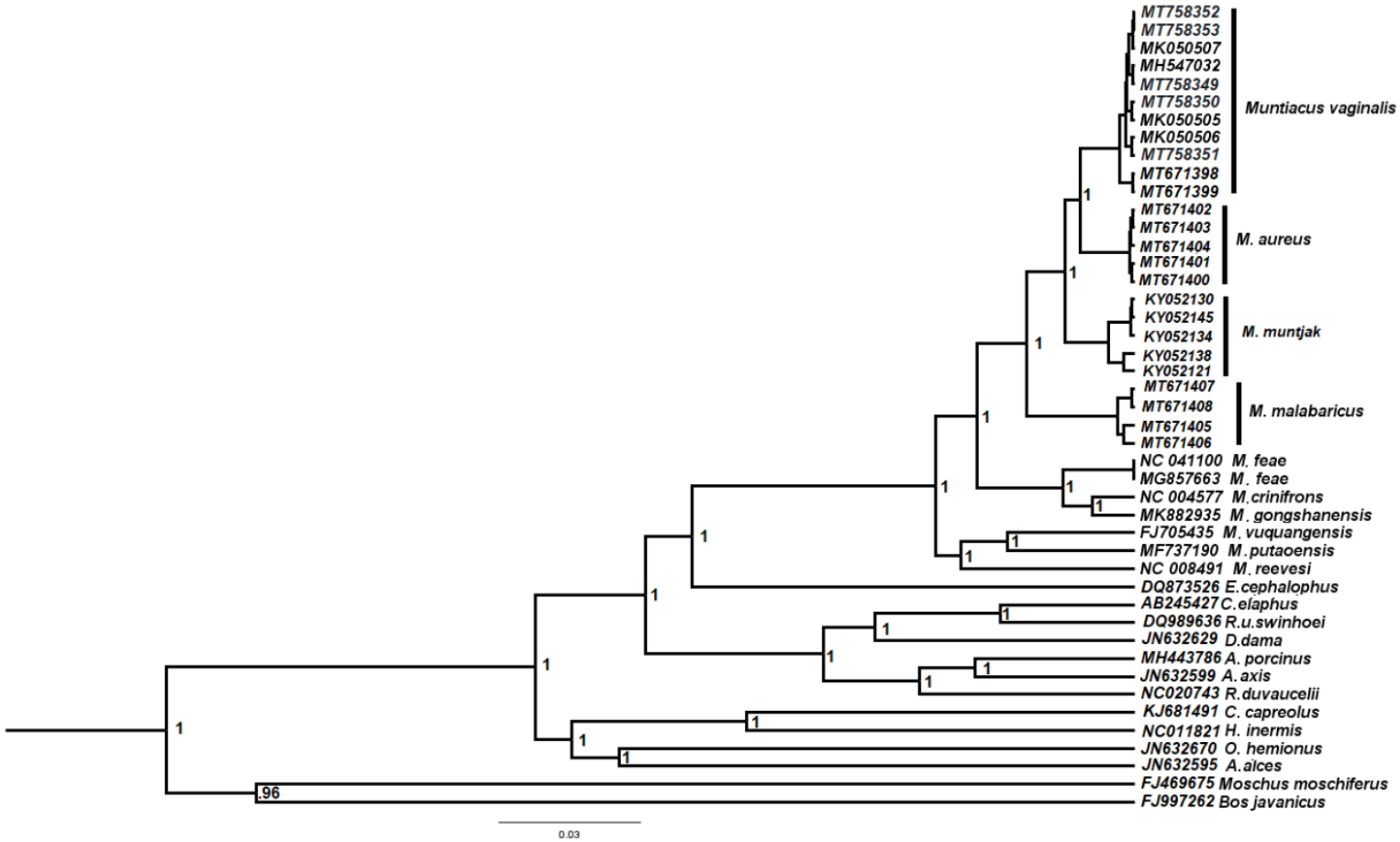

Figure 8: The MCMC Bayesian tree for the phylogenetic resolution between the Cervini and Muntiacini

The number of species and subspecies of muntjac has always been a debatable topic. Few new species of muntjac are included in genus namely, Annamite muntjac (*M. truongsonensis*) (Giao et al., 1998) and Roosevelt's muntjac (*M. rooseveltorum*) from Vietnam (Le et al., 2014), the leaf deer (*M. putaoensis*) from Northern Myanmar (Amato et al., 1999, Rabinowitz et al., 1999), and the Bornean Yellow muntjac (*M. atherodes*) from Borneo (Groves et al.,1982). Recently, it has been recognized that the lineages of *Muntiacus* species are increasing due to in-depth examination in its biogeography and molecular genetics (Martins et al., 2017). The taxonomic stability of muntjak has not been achieved yet, due to the limited molecular studies. Therefore, investigating the mitochondrial genome across the distribution ranges would be crucial to design the proper conservation plan and to identify the evolutionary significant unit. The analysis of newly sequenced mitogenome of *M. vaginalis, M. aureus* and *M. malabaricus* from different landscape of India will provide a basis for conservation studies and determine the existing distribution range of species and subspecies of red muntjac. Moreover, this study will encourage the investigation of phylogeography and evolutionary significance unit (ESU) of red muntjac which will address genetic linkages among these species. However, analysis of more biological's samples from the distribution range of Northern Indian red Muntjac would assist in the identification of more maternal lineages.

Phylogeography of red muntjacs

In this analysis, we used a total of 20 complete mitogenome of red muntjac from India. Additionally, 36 sequences of northern red muntjac (*M. vaginalis*) (n = 17); southern red muntjac (*M. muntjak*) (n = 17) and Sri Lankan (*M. malabaricus*) (n = 2) muntjac were included from GenBank to cover wide distribution ranges (Table 5).The dataset of 52 sequences of red muntjac was aligned using the CLUSTAL X 1.8 multiple alignment programs (Thompson et al., 1997) and alignments were checked by visual inspection.

The phylogenetic analysis was conducted in BEAST ver 1.7 (Drummond et al., 2012). Two outgroup species, *Bos javanicus* (JN632606) and *Moschus moschiferus* (FJ469675) were used to root the phylogenetic tree and the resulting tree was visualized with FigTreever 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/). The spatial distribution of haplotypes was visualized by MJ network created using the PopART software (Leigh et al., 2015).

Estimating divergence dating

To estimate divergence times of red muntjac clades, we inferred genealogies using a strick clock in BEAST ver 1.7(Drummond et al., 2012). We performed the dating estimates using 16 sequences downloaded from NCBI, including the species of Cervidae, Muntiacini, Bovidae and Moschidae, i.e.,the Chital (*Axis axis*, JN632599), Swamp deer (*Rucervus duvaucelii*, NC020743), Red deer (*Cervus elaphus*, AB245427), European Roe deer (*Capreolus capreolus*, KJ681491), Fallow deer (*Dama dama*, JN632629), Water deer (*Hydropotes inermis*, NC011821), Mule deer

(*Odocoileus hemionus*, JN632670), Hog deer (*Axis porcinus*, MH443786), Formosan sambar (*Rusa unicolor swinhoei*, DQ989636), Tufted deer (*Elaphodus cephalophus*, DQ873526), Chinese muntjac (*Muntiacus reevesi*, NC008491), Giant muntjac (*Muntiacus vuquangensis*, FJ705435), Putao muntjac (*Muntiacus puhoatensis*, MF737190), Black muntjac (*Muntiacus crinifrons*, NC004577), Banteng (*Bos javanicus*, FJ997262), and Musk deer (*Moschus moschiferus*, FJ469675).

Divergence times of phylogenetic clades were calibrated using minimum age of fossil record at two points: one point was calibrated at 18 Mya (normal distribution prior, SD=2) as the TMRCA (time to the most recent common ancestor) for the split between Bovidaeand Moschidae, while the other was set to 17.2 Mya (normal distribution prior, SD=2) for the split between Cervidae and Bovidae+Moschidae (Chen et al., 2019, Bibi 2013).We used a Yule-type speciation model and the HKY+I+G substitution rate model for tree reconstruction. We conducted two independent analyses, using MCMC lengths of 10 million generations, logging every 1000 generations. All the runs were evaluated in Tracer ver 1.6. The first 10% per run was discarded as burn-in. Maximum credibility trees were obtained with TREEANNOTATOR (implemented in BEAST ver 1.7 Package) The final phylogenetic tree was visualized in FigTree ver 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

Species delimitation analyses

Species delimitation tests were conducted to validate taxonomic units of *Muntjacs* based on phylogenetic trees derived from whole mitogenome sequences. The analyses were performed using three different approaches: (I) Multi-rate Poisson Tree Processes (mPTP) (Zhang et al., 2013); (II) Bayesian Poisson Tree Processes (bPTP) (Zhang et al., 2013); and (III) Generalized Mixed Yule-Coalescent (GMYC) (Pons et al., 2006).

The mPTP and bPTP analyses were performed on the phylogenetic tree using webserver https://mptp.h-its.org and https://species.h-its.org, respectively. Specified parameters for MCMC, thinning, burn-in and seed value were kept as per default settings. The mPTP model employs a fast approach to estimate the maximum likelihood delimitation from an inferred phylogenetic tree, while the bPTP model adds Bayesian support values to the delimited species inferred from the phylogenetic tree. The GMYC model employed the phylogenetic tree after time calibration using HKY+I+G substitution rate model. It delimits species based on the likelihood approach fitting branching models within and among species to reconstructed phylogenetic tree. The branch lengths were estimated under a relaxed log-normal clock algorithm in BEAST ver 1.7 (Drummond et al., 2012). We used MCMC lengths of 10 million generations, logging every 1000 generations. All the runs were evaluated in Tracer ver 1.6. The first 10% per run was discarded as burn-in. Maximum credibility trees were obtained with TREEANNOTATOR (ver 1.7 BEAST packages (Drummond et al., 2012)). Single threshold of GMYC model was

performed using webserver https://species.h-its.org and the output tree is visualized in Tree view (Page et al., 2003).

Results

Phylogeographical analyses

The Bayesian consensus tree showed that all sequences of red muntjac clustered into four major clades (Fig. 9). Clade-I consisted of the sequences of Mainland red muntjac lineages (*M. vaginalis*), comprising individuals from Northern to Central India, Eastern to Northeastern India, Nepal, Southeast Asia, and Andaman & Nicobar Islands. Clade-II comprised individuals from the Northwestern part of India (*M. aureus*) (i.e., Uttarakhand, Punjab, and Himanchal Pradesh) and Clade-III comprised the sequences from Sunda (*M. muntjak*), mainly from Sumatra, Malay Peninsula, Lombok, Borneo, Java, and Bali's Islands. Clade-IV consisted of individuals from Western Ghats of Southern Indian and Sri Lanka (*M. malabaricus*).

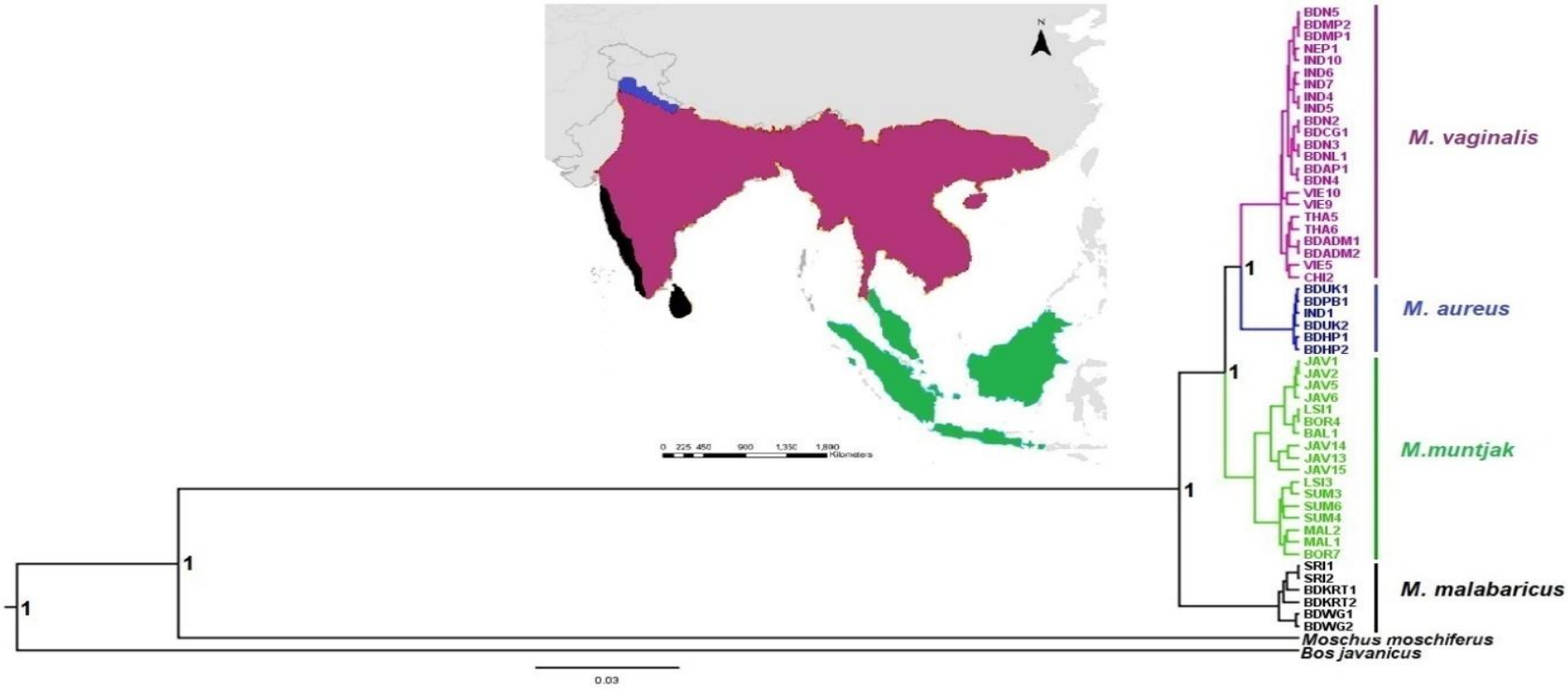

Figure 9: Bayesian inference (BI) phylogenetic tree for red muntjac based on complete mitochondrial DNA. Numbers on clades indicate posterior probability (PP) for the node. The distribution ranges of different lineages are represented by specific colorsin distribution map corresponding to colored clades in a tree topology.

The median-joining (MJ) network of all recognized sequences from India, Southeast AsiaSundaland, and Sri Lanka strongly supported the phylogenetic results, indicating the existence of four geospatial populations from the distribution ranges (Fig. 10). In India, we found three evident clades representing populations of *M.vaginalis* (Clade-I), *M. aureus* (Clade-II), and *M. malabaricus* (Clade-IV), respectively. The phylogenetic and median-joining analyses showed the presence of two sub-groups in the Sunda of Southern red muntjac. It is also noteworthy that the mainland red muntjacs from India exhibited different genetic signature and showed structuringwith respect to other mainlandpopulations that existed in Southeast Asia and Andaman & Nicobar Islands.

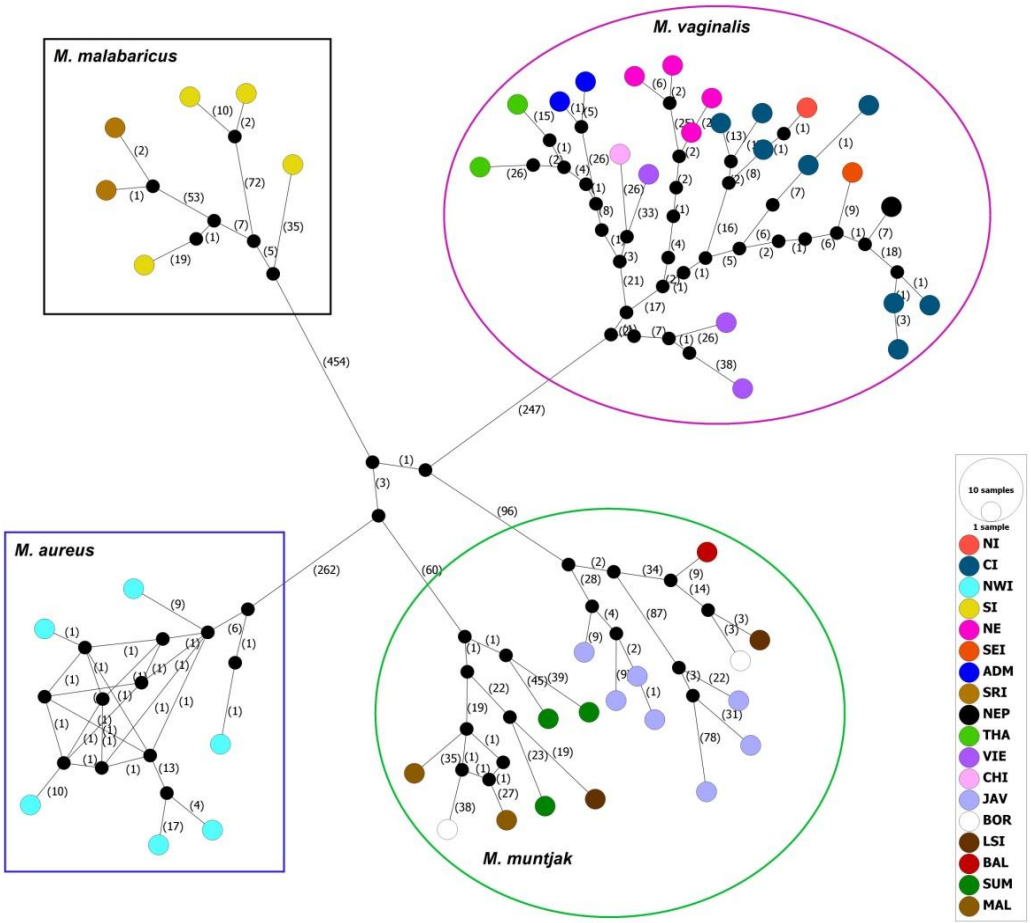

Figure 10: A median-joining (MJ) network of full mitogenome of red muntjac lineages. The number in bracket (n) represent the number of mutations separating the haplotype. The size of each circle indicates the relative frequency of the corresponding haplotype in the whole dataset.

Estimating genetic divergence

We calibrated the root age (TMRCA of Bovidae and Moschidae) to 18 ± 2 Mya ($CI_{95\%}$: 14.74–22.94) and the split between Cervidae and Bovidae+Moschidae was set at 17.2 ± 2 Mya ($CI_{95\%}$: 16.04–24.08). Our divergence results suggested that the split between the red muntjacs and black muntjac (*M. criniforns*) occurred in the Late Pliocene, around 3.29 Mya ($CI_{95\%}$:2.57–4.16). Within red muntjac, group diversification started during the Pleistocene. The *M. malabaricus* split earlier ~2.2 Mya ($CI_{95\%}$:1.67–2.77),and thereafter, the clade of *M. muntjak* of Sunda split around 1.4 Mya ($CI_{95\%}$:1.05–1.76). Within the northern lineages, the split between the *M.vaginalis* and *M. aureus* was estimated to have occurred at ~1.01 Mya ($CI_{95\%}$:0.75–1.27) (Fig. 11). Our analysis indicated the Northwestern red muntjac to be the youngest among the red muntjac.

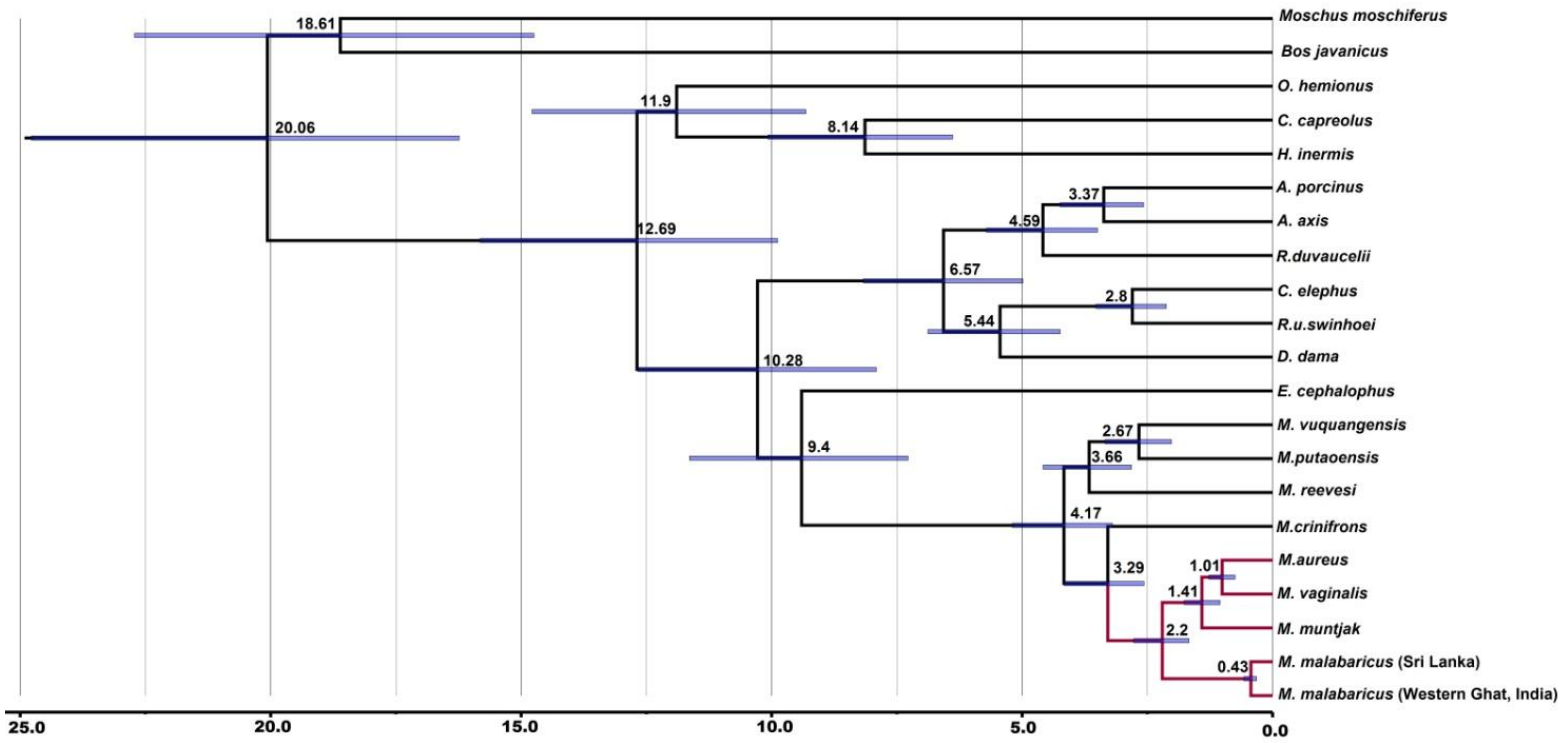

Figure 11: Divergence time estimationbased on maximum credibility tree using complete mitogenome generated from BEAST analysis.

Species delimitation

The bPTP and GMYC analyses indicated four species in our dataset whereas the mPTP analysis indicated seven. All the analyses delimited the same taxonomic units as inferred from BEAST phylogenetic analysis, namely: *M. vaginalis*, *M. aureus*, *M.muntjak* and *M. malabaricus*, while the mPTP model additionally expanded the *M.muntjak* group from Sundaland into 4 subsets (Fig. 12). The result of bPTP and GMYC analyses supported the previously recognized taxonomic subdivisions, also corroborated by our analyses.

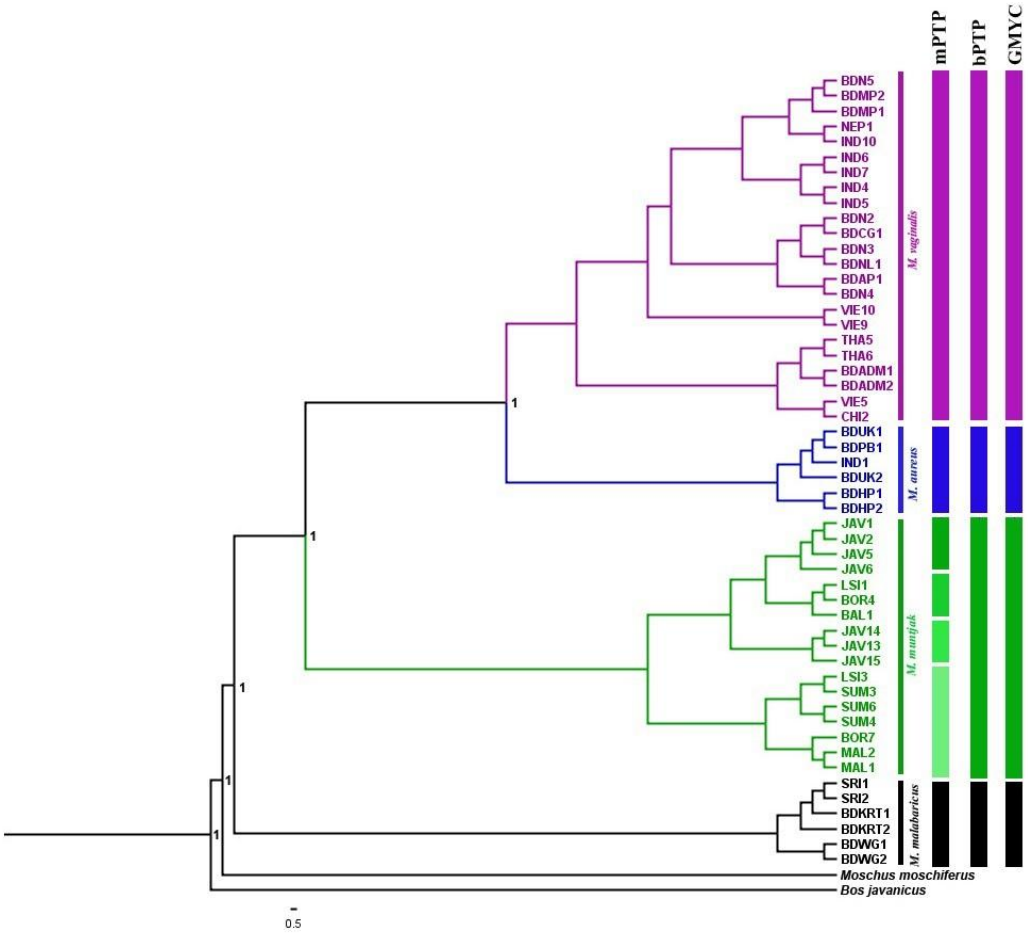

Figure 12: Bayesian phylogeny constructed based on complete mitochondrial genomes representing the result of three different molecular species delimitation methods. Multi-rate Poisson Tree Processes (mPTP), Bayesian Poisson Tree Processes (bPTP) and Generalized Mixed Yule-Coalescent (GMYC).

Discussion

Phylogeographic studies of red muntjac showed a clear division between Northern and Southern lineages, indicating physical barriers to gene flow resulting from extremely dry climatic conditions caused by global ice advances (Martins et al., 2017). As the taxonomy of muntjacs is considerably fragile and still debated (Groves et al., 2011), ongoing research aims to resolve the phylogenetic complexities to elucidatethe exact number of lineages. The population genetic studies on red muntjacs will act as definitive tools to understand the lineage diversification, genetic structuring, and diversity, resulting in developing appropriate conservation and management strategies for this enigmatic species.Previously, Martins et al., (2017) reported three mitochondrial lineages: The Srilankan red muntjac (*M. malabaricus*), Northern red muntjac (*M. vaginalis*) and Southern red muntjac (*M. muntjak*) (Martins et al., 2017).The species delimitation methods supported taxonomic resolution findings among *Muntjacs*, corroborating the divergence between *M. vaginalis* and *M. aureus*as distinct taxonomic units while grouping the *M. malabaricus* from the Western Ghats and Sri Lanka within a single taxonomic unit. The mitogenomic data estimated the split between the Northwestern and Mainland lineages to late Pleistocene ~1.01 Mya ($CI_{95\%}$:0.75–1.27), indicating the most recent split among red muntjacs. The Himalayan red muntjac inhabits the foothills of the Himalaya in the Northwestern part of the Indian states of Uttarakhand, Himachal Pradesh, and Punjab. The Northwest lineage was suspected to be distributed in

Northwestern and Central India and Myanmar (Groves et al., 2011). The presence of distinct genetic lineage from the Northwestern Indian region was also speculated by Martins et al. based on a single sequence (*IND1*) from Himachal Pradesh Province that formed a distinct placement (Martins et al., 2017). However, due to the unavailability of sufficient sample size, explicit depiction of the lineage could not be done. Hence, we confirmed the previously suspected hypothesis and provided molecular evidence for the Northwestern lineage from India.

The rise of anthropogenic activities in the late Quaternary was a key factor that changed the global biodiversity pattern (Meijaard & Groves., 2006). Qinghai-Tibetan Plateau (QTP) upliftment played a significant role in faunal and floral diversification and evolution in Himalayan ranges (Favre et al., 2015). The upliftment of the Himalayas followed by the Plio-Pleistocene glaciation led to the evolution of high altitudinal elements shaping biogeographic evolution in the Indian Himalayan region (Mani 1974). The Himalayan region enabled allopatric speciation through geographic isolation and adaptive diversification across altitudinal gradients (Doebeli et al., 2003, Körner 2007). This diversification was also driven by pre-adapted lineages immigrating and undergoing subsequent speciation (Johansson et al., 2007,Price et al., 2014). Long-term isolation in fragmented habitat srestricted gene flow and caused genetic divergence (Liu et al., 2018, Pyron et al., 2010, Ye et al., 2013) that contributed to the evolution of genetically distinct lineages (Ye et al., 2013). It was followed by rapid civilization in the Indo-Gangetic plain of North

India, causing extensive destruction of natural habitat, altering plants and animals' ecological and distributional patterns (Mani 1974).

In India, the Western Ghats lineage was genetically more diverse than the Mainland, Northwestern and Sundaland populations. The phylogenetic analysis indicated that the Srilankan red muntjac (*M. malabaricus*) is the oldest red muntjac lineage showing genetic similarity with India's Western Ghats population. The genetic divergence results suggested that the Srilankan red muntjac split from the mainland lineage during Pleistocene ~2.2 Mya when the climatic condition was quite complex in India (Martins et al., 2017). Despite the present biogeographic separation between Southern India and Sri Lanka, both populations are genetically similar at the mitogenomic level. A similar genetic relationship was observed in *Paradoxurus* Palm civets,where Southern India and Sri Lanka clustered with each other (Patou et al., 2010). The Western Ghats and Sri Lankan lineages' common origin might be due to the historical connectivity between these two landscapes. Thereafter, changes in sea levels may have led to isolation causing local endemism (Patou et al., 2010, Bossuyt et al., 2004, Manamendra et al., 2005). In India, the discontinuity of Western Ghats with mainland population could be due to unfavorable habitat conditions that culminated in isolated patches forming the refugia population inhabiting Western Ghats biodiversity hotspot (Karanth 2003). The barrier formed by India's central dry zone restricted the gene flow between the Western Ghats and the rest of the Indian population of red muntjac.The restriction in species distribution is probably due to pronounced climate fluctuation in the last glacial maxima that

caused the contraction of the rainforest,with forest-dwelling species restricted to the available habitats in high elevation areas (Meijaard & Groves., 2006, Patou et al., 2010).

The mainland red muntjac (*M.vaginalis*) is distributed in a larger landscape in India (i.e, Chhattisgarh, Odisha, West Bengal, North East India, and Andaman & Nicobar Islands) and Indo-Chinese region (Vietnam, China and Thailand). The Andaman & Nicobar Islands individuals were genetically closer to the population of Indo-Chinese red muntjac. Unsuitable conditions limited the mainland population to small humid forest patches. Interglaciation led to the emergence of forests in drier areas that enabled former distributions to reoccupy and extend the red muntjac range. The Southern red muntjac inhabited the Sudanic region (Java, Sumatra, Bali and Borneo) and the lineage diversification has been described by Martins et al., 2017 (Martins et al., 2017). The major transition zone between the Indochinese and Sundaic zoogeographic subregion is the Isthmus of Kra located on the Malay/Thai Peninsula that might act as a possible barrier preventing gene flow between populations (Meijaard 2004, Woodruff & Turner 2009). It is also speculated that rather than geo-physical barrier, repeated rapid sea-level changes may have resulted in species' isolation (Woodruff & Turner 2009). A similar diversification pattern has also been observed in South and Southeast Asian species, like rodents, masked palm civet, common palm civet and *Macaca spp.,* where the formation of the contemporary phylogeographic and genetic structure occurred during the same period (Gorog et al., 2004, Patou et al., 2009, Patou et al., 2010, Ziegler et al., 2007, Liedigk et al.,

2015).The Southern red muntjac split around 1.4 Mya from the Indochinese Mainland population. This divergence estimation is congruent with the lineage's diversification in Amphibians (Inger &Voris 2001); Birds (Hughes et al., 2003); Mammals (Woodruff & Turner 2009); bats (Hughes et al., 2011) and Leopard cat (Patel et al., 2017) that occurred in the Indo chinese and Sundaic region. Faunal diversification between the Indochinese and Sundaic regions might have resulted from fluctuation inthe Indian summer monsoon (Zhisheng et al., 2015) and sea level rise (Woodruff & Turner 2009, Miller et al., 2005).

Chapter-4

TO INVESTIGATE THE POPULATION GENETIC STRUCTURE, GENE FLOW AND DEMOGRAPHIC HISTORY OF INDIAN RED MUNTJAC

Background

The pattern of genetic variation in species is largely affected by change in original habitat especially by formation of physical barrier like mountains and rivers, which may restrict the population in small fragmentation and reduce the geneflow between populations for long time leading to substantial differentiation in population's genetic structure (Macfarlane et al., 2016). Genetic drift and selection can cause populations to diverge. Geneflow encourages homogenization of populations, while population can be in divergence if the current selection is robust (Hemmer-Hansen et al., 2007; Nadachowskaand Babik 2009; Pavey et al., 2010; Richter-Boix et al., 2010). Inversely, if the level of gene flow exceeds the strength of selection, then local adaptation will be hindered by the continued introduction of alleles from other populations. The climatic fluctuation during Plio-Pleistocene largely affects the population genetic structure and phylogeographic pattern of many species in South and Southeast Asia. During the glaciation period, many forest-dependent species migrated to high elevation area due to the accessibility of favorable habitats (Meijaard 2004; Patou et al., 2010). These populations were confronted with modified habitats, gene flow reduction, genetic drift, and natural selection, whichled to loss in their genetic diversity and created the genetic differentiation between populations. However, many factors like temporal changes in geography, demographic history of populations, gene flow, genetic drift, and selection are cumulative factors that shape the populations' genetic variations (Hewitt 1999;

Geffen et al., 2004; Thanou et al., 2017). In novel environmental condition formed after glaciation period, species face strong selection pressure and the ability to adapt these novel conditions are challenging to achieve (Chevin and Lande 2009). Many evolutionary, ecological and anthropogenic factors are responsible for adaptive diversification and population structuring occurring in different temporal and spatial scales. The genetic makeup of populations changes during the evolutionary process by change in their allele frequencies with time. In view of this, I shall be discussing the population genetic structure, gene flow and demographic history of Indian red muntjac using both mtDNA and microsattelite markers.

Methodology

Sample collection and DNA extraction

We used 42 archival samples of northern red muntjac from different regions of India, including the Northwestern region (NWI=18), Mainland (localities of north and central) (MLI=14), northeastern (NEI=3), Western Ghats (SI=5) and Andaman & Nicobar Islands (AI=2) (Table 2 and Fig. 5). Genomic DNA was extracted from tissue and hair samples using modified DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) protocol, whereas, for antlers and bone samples, we followed Gu-HCl-based silica binding method (Gupta et al., 2013). These samples were collected from dead animals by the local Forest Department from India's known localities and sent to Wildlife Forensic and Conservation Genetic Cell, Wildlife Institute of India, Dehradun.

Mitogenome Amplification

Polymerase chain reactions (PCRs) amplifications were carried out in 20µl volumes containing 10–20 ng of extracted genomic DNA containing, 1× PCR buffer (Applied Biosystems), 2.5mM $MgCl_2$, 0.2 mM of each dNTP, 5 pmol of each primer, and 5 units of Taq DNA polymerase (Thermo Scientific). We performed PCR amplification using 23 overlapping fragments of complete mtDNA (Hassanin et al., 2009). Besides, we included the fragments of complete Cytochrome *b* (Gupta et al., 2014) and Cytochrome c oxidase subunit-I gene (Kumar et al., 2017) to increase the overlapping. PCR cycles for DNA amplification were 95°C for 5 min; followed by 35 cycles of 95°C for 40 sec (denaturation), 54-56°C (annealing) for 40 sec, 72°C for 50 sec (extension) and a final extension of 72°C for 15 min. The efficiency and reliability of reactions were monitored using controls. The PCR products were electrophoresed on 2% agarose gel and visualized under UV light in the presence of Ethidium bromide dye. The amplified PCR products were treated with Exonuclease-I and Shrimp alkaine phosphatase (USB, Cleveland, OH) for 15 min each at 37ºC and 80ºC, respectively, to remove any residual primer. The purified amplicons were then sequenced bidirectionally using BigDye Terminator 3.1 on an automated Genetic Analyzer 3500XL (Applied Biosystems, Carlsbad, CA, USA).

Microsatellite loci amplification and genotyping

Nine cross-species microsatellite loci: INRA001 (Vaiman et al., 1992), Ca18 (Gaur et al., 2003), BM6506 (Bishop et al., 1994), RT1,RT27, NVHRT48 (Poetsch et al., 2001),CelJP27 (Marshall et al., 1998), and T156, T193 (Jones et al.,2000) were selected for population genetic analysis of red muntjac. Three sets of multiplex panels were carefully assembled based on molecular size and labeled fluorescent dyes of loci. To avoid ambiguity and amplification errors, for each sample, three multiplex PCR were carried out in 10 µl reaction volumes containing 5 µl of QIAGEN Multiplex PCR Buffer Mix (QIAGEN Inc.), 0.2 µM labeled forward primer (Applied Biosystems), 0.2 µM unlabeled reverse primer, and 20–100 ng of the template DNA. PCR cycles for loci amplification were 95°C for 15 min; followed by 35 cycles of 95°C for 45 sec (denaturation), 55°C (annealing) for 1 min, 72°C for 1 min (extension) and a final extension of 60 °C for 30 min.The reliability of reactions was monitored using positive and negative controls. Alleles were resolved in an ABI 3500XL Genetic Analyzer (Applied Biosystems) using the LIZ 500 Size Standard (Applied Biosystems) and analyzed using GeneMaker ver 2.7.4 (Hulce et al.,2011).

Data Analysis

A total of 20 complete mitogenome sequences of red muntjac from five different localities of India were generated (Table 5 and GenBank accession No. MT671398-MT671408; MT758349-MT758353). Sequences obtained from forward and reverse direction were edited and assembled using SEQUENCHER®version 4.9 (Gene Codes Corporation, Ann Arbor, MI, USA). The annotation of complete mitogenome

was done using Mitos WebServer (Bernt et al., 2013) and MitoFish (Iwasaki et al., 2013). Careful manual annotation was also conducted by sequence alignment with their related homologs sequences or species for ensuring the gene boundaries. Additionally, 36 sequences of northern red muntjac (*M. vaginalis*) (n=17); southern red muntjac (*M. muntjak*) (n=17) and Sri Lankan (*M. malabaricus*) (n=2) muntjac were included from GenBank to cover wide distribution ranges (Table 5).The dataset of 52 sequences of red muntjac was aligned using the CLUSTAL X 1.8 multiple alignment programs (Thompson et al., 1997) and alignments were checked by visual inspection. The genetic distance between lineages was calculated based onthe Tamura-3 parameter with a discrete Gamma distribution (TN92+G) with the lowest BIC score value implemented in MEGA X (Kumar et al., 2018).The neutrality statistical approaches Tajima's D (Tajima et al., 1989) and Fu's *F*s (Fu et al., 1997) were used to investigate the demographic history of each population using Arlequin ver 3.5 (Excoffier et al., 2010).

To determine the most likely number of the genetic cluster within India, we performed a Bayesian clustering implemented in STRUCTURE 2.3.4 (Pritchard et al., 2000). We analyzed our data using the admixture model and allele frequencies were assumed to be independent with a burn-in of 10,000 followed by 100,000 MCMC (Markov chain Monte Carlo) replications. Ten independent runs were carried out for each cluster set (*K*) from 1 to 10. We also used the ΔK metric to determine the statistically most support number of clusters (*K*) in web interface STRUCTURE HARVESTER (Earlet 2012). Further, DAPC method was also

implemented to identify the number of genetic clusters of the population using the ADEGENET package in R (Jombart et al., 2010). DAPC is a multivariate and model-free approach that maximizes the genetic differentiation between groups with unknown prior clusters, thus improving populations' discrimination without requiring the population to be in HWE. FCA was also performed using the GENETIX 4.05 software package (Belkhir 2004). CONVERT 1.31 (Glaubitz 2004) was used to convert the required input file format. The pairwise F_{ST} values among the populations were calculated using GenAlEx ver 6.5 (Peakall et al., 2006).

Spatial genetic analysis

The correlation between the pairwise genetic and geographic distances was performed to detect the pattern of isolation by distance between the disjointed areas, according to Mantel's test implemented in Alleles in Space 1.0 (Miller et al., 2005).

Results

Population genetic structure and genetic differentiation

The Bayesian clustering analysis identified the highest ΔK when *K* was set at 2 (Mean LnP(K)= -1584.64; ΔK=9.6) under the admixture model. As recommended by Evanno et al. (2005) interpret *K* with caution, I further analyzed accordingly to identify the possible hidden substructure for each predefined cluster (Evanno et al., 2005). Therefore, we adopted the second-highest value of *K*=3 (Mean LnP(K)= -1565.47; ΔK=5.9), where most individuals were assigned to three different clusters, which differentiated among Northwestern (*M. aureus*), Mainland (*M. vaginalis*), and

Western Ghats population (*M. malabaricus*) (Fig. 13). Although the genetic structure of the mainland population indicated some alleles from the Himalayan and Western Ghats populations, none of the alleles were found vice-versa. The multivariate Discriminant Analysis of Principal Components (DAPC) (Fig. 14) andfactor correlation analysis (FCA) (Fig. 15) also supported the Bayesian clustering method that differentiated the populations into three genetic clusters. We found strong concordance between microsatellite and mitogenome data using different Bayesian and non-Bayesian clustering methods used in this study.

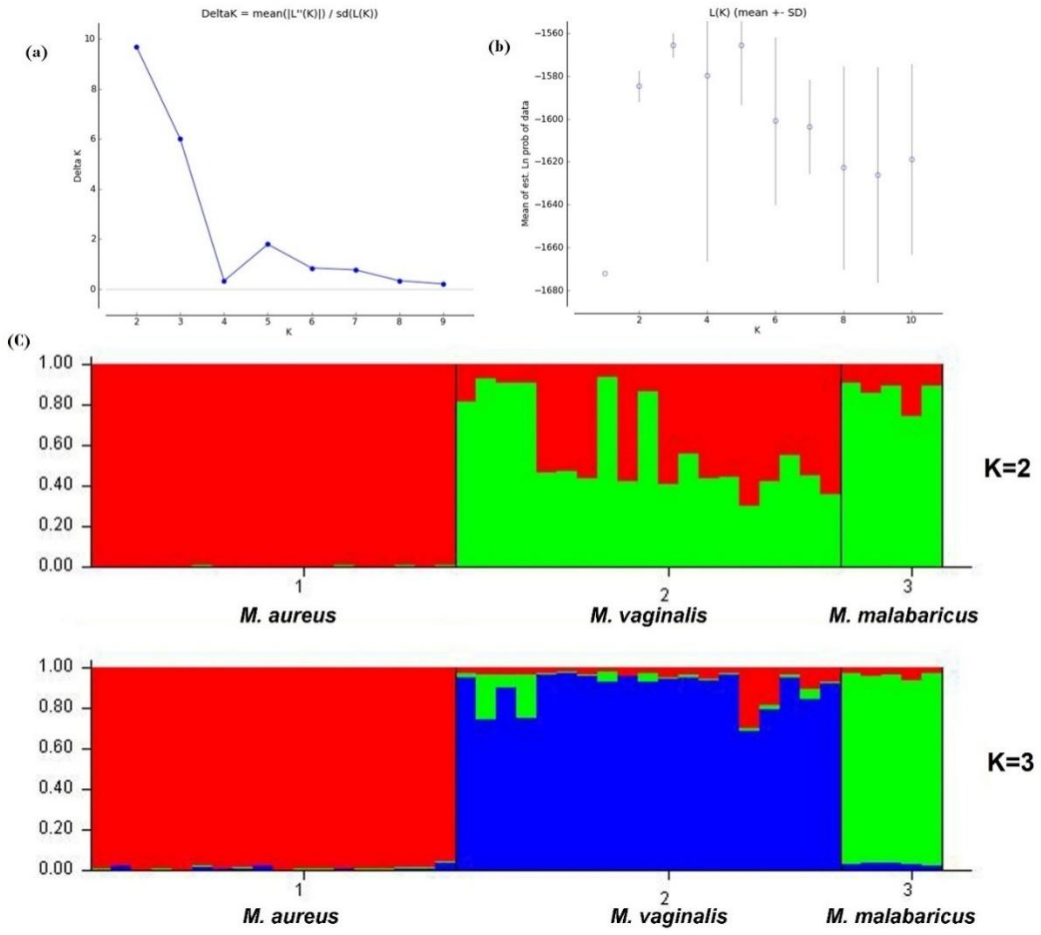

Figure 13: Results ofgenetic clusters inferred from Structure 2.3.4. (a) Delta K values with respect to K (b) Mean of estimates Ln probability of data with respect to K. (c) Barplot indicating the genetic structure at K=2 and K=3, showing the population assignments for each individuals of *M. aureus*, *M. vaginalis* and *M. malabaricus*, respectively. The Y axis is depicting the proportion derived from each cluster.

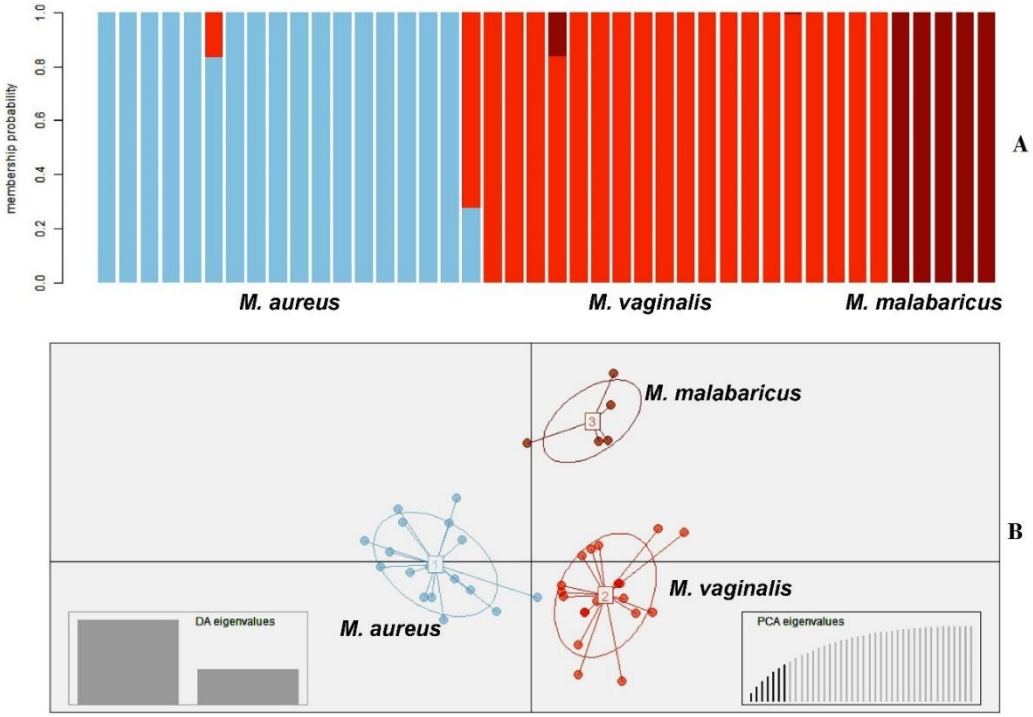

Figure 14: The bar plot results show genetic clustering implemented in Discriminant Analysis of Principal Components (DAPC) (A). Each column along the X-axis represents one red muntjac individual. The Y-axis represents the assignment of the membership probability of each individual. Scatterplots of DAPC

using a hierarchical islands model and shown by different colors and inertia ellipses (B). The DA and PCA eigenvalues of the analyses are displayed in the inset.

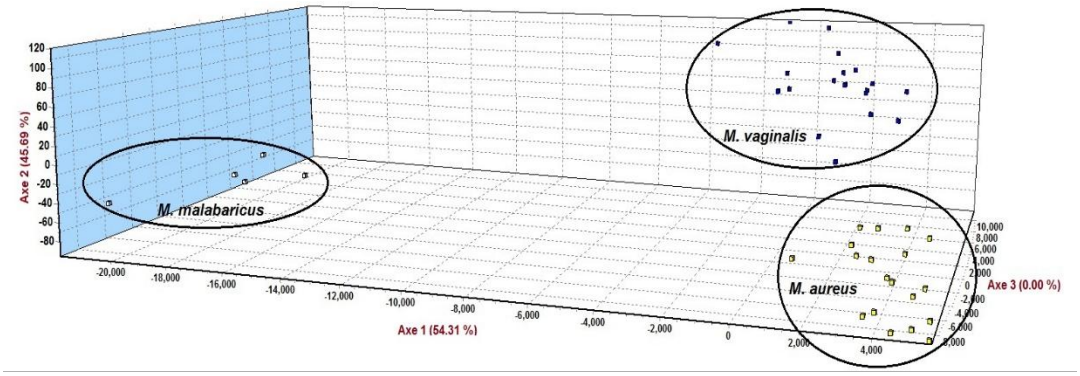

Figure 15: Results of factor correlation analysis (FCA) using microsatellite markers, indicating three major clusters in the Red muntjac population in India.

The analysis based on pairwise F_{ST} for red muntjac using complete mitogenome demonstrated significant genetic differentiation of *M. vaginalis* from *M. aureus* (0.0208) and *M. malabaricus* (0.0396). The genetic differentiation of *M. aureus* from *M. vaginalis* and *M. muntjak* was almost similar (0.026), whereas a comparatively high level of differentiation was observed between *M. muntjak* and *M. malabaricus* (0.039). A parallel pattern was observed in microsatellite analysis where the observed genetic differentiation between the *M. aureus* and the *M. vaginalis* was 0.062, and *M. malabaricus* was 0.105, whereas *M. vaginalis* to *M. malabaricus* was 0.080 (Table 11).We also calculated genetic differentiation with other *Muntiacus* species and recovered a comparatively low level of genetic differentiation

between red muntjac lineages with *M. criniforns* (0.056 to 0.057) than with other species such as *M. reevesi* (0.065 to 0.068), *M. vuquangensis* (0.060 to 0.068) and *M. putaoensis* (0.067 to 0.069). Tajima's D and Fu's F_S test were non-significantly different from zero ($P > 0.01$), which indicated that red muntjac populations had not undergone an expansion (Table 12).The spatial genetic analysis revealed a significant correlation between the pairwise genetic distances among geographical subsets and geographical distances (Mantel test statistic, *rM*=0.513; *P*=0.0009, Fig. 16). This pattern of isolation by distance (IBD) was strongly influenced by the genetic differentiation and major geographical distance between the red muntjac lineages.

Table 11: Genetic differentiation among red muntjac and other Muntiacus species. The pairwise F_{ST} values based on the complete mitogenome and microsatellite loci (in bold) are shown above and above the diagonal, respectively.

Species	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
M. vaginalis	-	**0.062**	**0.080**					
M. aureus	0.0208	-	**0.105**					
M. malabaricus	0.0396	0.0401	-					
M. muntjak	0.0269	0.0264	0.0399	-				
M. criniforns	0.0557	0.0572	0.0562	0.0571	-			
M. reevesi	0.0668	0.0657	0.0682	0.0685	0.0651	-		
M. vuquangensis	0.0679	0.0683	0.0681	0.0681	0.0665	0.0606	-	
M. putaoensis	0.0693	0.0690	0.0695	0.0695	0.0692	0.0624	0.0486	-

Table 12: Result of Tajima's D and Fu's F_S test for demographic analysis([a]all P-values > 0.01 (not significant))

Population/Region	Tajima's D[a]	Fu's *Fs*[a]
M.aureus	− 0.49	0.46
M.vaginalis	− 1.33	− 2.7
M. malabaricus	0.48	1.9
M. munjak	− 0.37	− 0.01

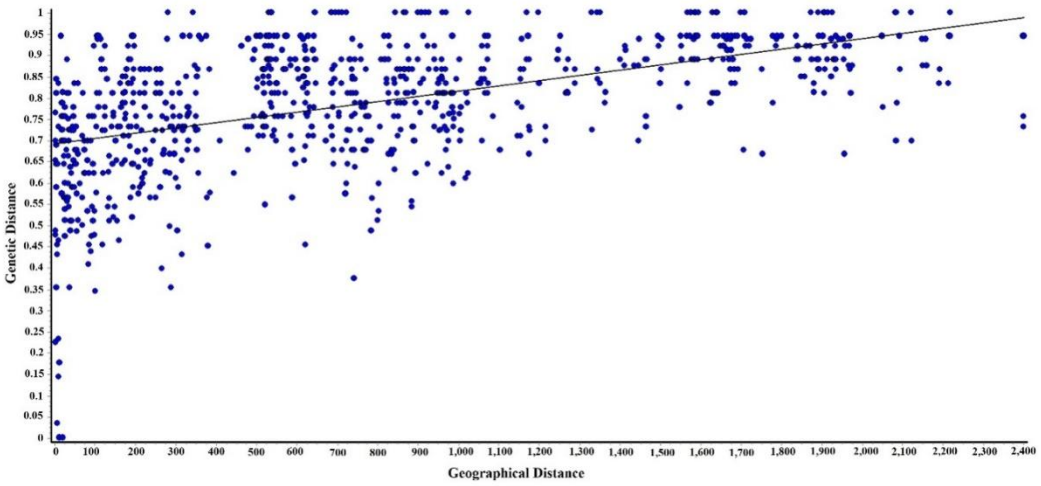

Figure 16: Correlation of genetic and geographical distance in kilometer between red muntjac population using microsatellite data (Mantel test, *rM* = 0.513; *P* = 0.0009).

Discussion

The past climatic fluctuation during the Pleistocene largely affect the distribution and demography of species which includes habitat fragmentation, range contraction and local extinction (Gaubert et al., 2016, Khanal et al., 2018). The study brings together the extensive analyses of complete mitochondrial and microsatellite loci variation to understand the Quaternary climatic fluctuations and geological events on the probable influence on the demographic pattern and population genetic structure among the red muntjac groups. The present study on genetic structure and differentiation among red muntjac populations exhibited four genetically distinct lineages from its geographical distribution range in South and Southeast Asia. Structure, DAPC and FCA analyses supported the result with three genetic clusters of red muntjac from India.We detected clear genetic signatures of the Northwestern and Western Ghats lineages of red muntjac, whereas few alleles of these lineages were observed in the Indian mainland red muntjac indicating historical gene flow, while the current populations have undergone well-defined structuring.Our genetic differentiation result based on both Mitochondrial and microsatellites showed significant genetic differentiation among red muntjac population. However, we observed comparatively high level of differentiation of *M. malabaricus* with other red muntjac population.Tajima's D and Fu's F_S test were used to check the population expansion.

Additionally, we found values of both analysis to be non-significantly different from zero ($P > 0.01$), which indicated that red muntjac populations had not undergone an expansion.The result of the spatial genetic analysis showed significant correlation between the pairwise genetic distances among geographical subsets and geographical distances. This pattern of isolation by distance (IBD) was strongly influenced by the genetic differentiation and major geographical distance between the red muntjac lineages.

The late Quaternary was played an important factor that changed the global biodiversity pattern after rise of antropogenic activites (Meijaard & Groves., 2006). The faunal and floral diversification and evolution in Himalayan ranges was emerged after the upliftment of Qinghai-Tibetan Plateau (QTP) (Favre et al., 2015). The upliftment of the Himalayas shaped biogeographic evolution that leds to allopatric speciation through geographic isolation and adaptive diversification of species during the Plio-Pleistocene glaciations period (Mani 1974; Doebeli et al., 2003, Körner 2007). This diversification may driven the pre-adapted lineages immigrating and undergoing subsequent speciation in red muntjac lineages.

Chapter-5

CONCLUSION

The genus *Muntiacus* is an useful model for identify cryptic lineages and study South and Southeast Asian biogeography. In present IUCN only recognized two species of red muntjac Northern red muntjac *Muntiacus vaginalis* and Southern red muntjac *M. muntjak* across their distribution range, which are classified based on their chromosomal variations the Norther red muntjac (*M. vaginalis*) have lowest number of chromosomes in mammals reaching at 2n=6 for female and 7 for male (Wurster and Benirschke 1970; Wurster and Atken 1972; Shi 1976). Whereas, the Southern red muntjac (*M. muntjak*) have 2n=8 in female and 9 in male (She 1983). However, previous studies proposed several sub-species and species in red muntjac group. Mattioli classified red muntjacs as single species *Muntiacus muntjak* comprising ten subspecies (Mattioli et al., 2011). Grubb and Groves recognized six species and two subspecies of *Muntiacus* using geographical distributions and morphological characters (Grubb and Groves., 2011). These taxonomic classifications are not universally adopted because the pervious classifications are mainly based on morphological traits which are shared by species across their distribution range.

This thesis has helped to accomplish my objectives towards fulfilling my research aim to study the Molecular phylogenetic relationship of Northern red muntjac with other *Muntiacus* species and test the phylogeographic pattern of red muntjac group along with the study of population genetic structure in India. In chapter 1 introduction part discussed about taxonomic and phylogenetic complexsities of red

muntjac. Chapter 2 of the thesis comprises the study about the genetic diversity of red muntjac lineages and Chapter 3 compiled the study regarding phylogenetics and phylogeography of red muntjacs. Lastly, Chapter 4 focused on the population genetic structure, gene flow pattern and demographic history of red muntjacs in India.

To assess the genetic diversity of the Indian red muntjacs among different populations using mtDNA and microsatellites markers:

The objective 1, complied in chapter 2 describes the level of genetic diversity in red muntjacs based on extensive genetic sampling using whole mitogenome and microsatellite markers. The data of microsatellite markers provided in this thesis is novel and will act as a baseline for future studies of red mutjac. Globally, expansion of human population is a major cause of large-scale habitat fragmentation leading to degradation of suitable survival habitat of wildlife. Loss of genetic diversity has been seen in many species due to continuous fragmentation of habitat owing to limited geneflow. Similarly, red muntjac population is depleting due to high anthropogenic pressure causing species to restrict itself in confinement. In the result, we found high haplotype and nucleotide diversity among the red muntjac population. This shows stability in population even though the continuous decline in apparent. Novel microsatellite data show that red muntjac population is healthy but it also displays deficiency of heterozygosity in few lineages. This indicates that all three population of red muntjacs in India is restricted in their geographical habitat

restricting the gene flow maintenance with each other. Hence, the outcome of this objective will be helpfulto predict the future viability and fitness of the red muntjac population through which conservation management authorities can plan better conservation schemes.

To investigate the phylogenetic relationships of Indian red muntjac with respect to other species:

The objective 2, complied in chapter 3 details the phylogenetic relationship of Indian red muntjac with other species of *Muntiacus* through a comprehensive mitogenomic phylogeny. Earlier studies of *Muntiacus* phylogeny were largely based on comparative morphological characters (Ma et al., 1986, Mattioli.,2011, Grubb and Groves., 2011). However, few studies have also highlighted phylogeny using partial fragment of mitochondrial DNA (Cao et al., 2002; Wang & Lan, 2000; Yi et al., 2002). Recently, for some species, mitogenomes have been used for phylogenetic reconstruction of *Muntiacus* (Li et al., 2017; Martins et al., 2017; Srisodsuk et al., 2018; Zhang et al., 2019a). Comparison of phylogeny mitigates issue of branching resulted from rapid radiations (Chen et al., 2019; Jiang et al., 2019).Therefore, mitogenomes have been taken up to explore the interspecific relationships within *Muntiacus*. Our phylogenetic results confirmed the presence of Himalayan red muntjac (*M. aureus*) from the north-western part of India. The study represents the whole mitogenome of Himalayan red muntjac (*M. aureus*), Northern red muntjac (*M. vaginalis*) and Sri Lankan red muntjac (*M. malabaricus*) and to be crucial for

further evolutionary study of genus *Muntiacus*. The study by Martins et al., (2017), used the samples from historical museum and reported the presence of three distinct mitochondrial lineages in red muntjac group: The Sri Lankan red muntjac (*M. malabaricus*), Northern red muntjac (*M. vaginalis*) and Southern red muntjac (*M. muntjak*) . However, the result of this chapter confirmed that the red muntjac population has clustered into four distinct clades rather than three clades. Complete mitogenomes contain increased phylogenetic signals compared to partial fragments and therefore provide more comprehensive insight into the phylogeny of taxa.

The diversification in red muntjac has occurred in the late Pliocene where the split between the red muntjacs and black muntjac took place. However, the climatic and topographical changes during Pleistocene were largely responsible for current diversification within red muntjacgroup. Result of divergence analysis displayed the split between the *M. vaginalis* and *M. aureus* to have occurred at ~ 1.01 Mya. This indicated that the North-western red muntjac (*M. aureus*) to be the youngest among the red muntjacs. This confirmation of *M. aureus* posed new questions regarding their conservation status in IUCN. Through this objective we aim to place *M. aureus* in the IUCN assessment list so that management of this evolutionary significant unit (ESU) can be planned strategically.

To investigate the population genetic structure, gene flow and demographic history of Indian red muntjac:

The objective 3, complied in chapter 4 has detailed information about the population genetic structure, genetic differentiation and demography history of red muntjac in India.Confirmation of three distinct clades of red muntjac in India poses a necessity to retrieve information concerning their genetic structure and differentiation. Evidence from genetic structure and differentiation within the three populations will provide patterns of their gene flow. The findings of this objective suggest that the three populations of red muntjac from India are well structured and genetically well differentiated from each other. However, microsatellite analysis showed mixing of some alleles in between population of *M. vaginalis* to *M. malabaricus* and between *M. vaginalis* to *M. aureus*. This result is significant for future planning of *in-situ* translocation of population. On the other hand, baseline data will be helpful to identify the source of confiscated sample of red muntjac since it is highly poached for meat and trophy hunting.

In our study we used whole mitogenome as well as microsatellite's data to resolve the phylogenetic complexities of red muntjac group and revealed the presence of four distinct lineage across their distribution range. In addition, we also confirm the presence of new lineage of red muntjac, Himalayan red muntjac (*M. aureus)* from North western part of India. Our divergence result showed that Himalayan red muntjac (*M. aureus)* split ~1.01 Mya which is indicated *M. aureus* is youngest lineage among the red muntjac. Moreover, the Sri Lankan red muntjac (*M. malabaricus*) which is also found in western ghats of India is most primitive lineage among red muntjac group which is split during Pleistocene ~2.2 Mya. Genetic

confirmation of *M. aureus* from North-Western part of India will provide help in taxonomic revision of the red muntjac group. The study will directly help conservation authority to prepare effective future management plans along with strategical planning of *in-situ* or *ex-situ* conservation of different lineages in India. Since IUCN recognized only two species i.e., *M. vaginalis* and *M. muntjak* in the RedList, therefore, based on the findings of this studyit is recommend to IUCN to reassess the taxonomic classification of red muntjac group. Apart from this, field conservation studies are required to assess the *M. aureus* and *M. malabaricus* to attain a requisite conservation status in IUCN red list.

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PUBLICATIONS

Peer Reviewed Articles

1. **Singh B,** Kumar A, Uniyal VP, Gupta SK. Complete mitochondrial genome of northern Indian red muntjac (*Muntiacus vaginalis*) and its phylogenetic analysis. *Molecular biology reports*. 2019 Feb; 46(1):1327-33.
2. **Singh B,** Kumar A, Uniyal VP, Gupta SK. Phylogeography and population genetic structure of red muntjacs: evidence of enigmatic Himalayan red muntjac from India. *BMC ecology and evolution*. 2021 Dec; 21(1):1-5.

Conferences and Workshops

1. **Singh B,** Kumar A, Uniyal VP, Gupta SK. Phylogeography and Phylogenetics Phylogeography of red muntjacs. *International Conference on Environment*, *Agriculture*, *Human and Animal Health* (#ICEAHAH2021).
2. **Singh B,** Participation in *International Conference on Environment*, *Agriculture*, *Human and Animal Health* (#ICEAHAH2021).
3. **Singh B,** Kumar A, Uniyal VP, Gupta SK. Molecular evidence of genetically distinct lineage of red muntjac from Uttarakhand, India. 15th Uttarakhand state science and technology congress 2020-21 (Submitted).
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SHORT COMMUNICATION

Complete mitochondrial genome of northern Indian red muntjac (*Muntiacus vaginalis*) and its phylogenetic analysis

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Abstract
We report complete mitochondrial genome of Northern Indian red muntjac, *Muntiacus vaginalis*, and its phylogenetic inferences. Mitogenome composition was 16,352 bp in length and its overall base composition in the circular genome was A = 33.2%, T = 29.0%, C = 24.50% and G = 13.30%. It exhibited a typical mitogenome structure, including 22 transfer RNA genes, 13 protein-coding genes, two ribosomal RNA genes and a major non-coding control region (D-loop region). All the genes except *ND6* and eight tRNA's were encoded on the heavy strand. Phylogenetic analyses showed that *M. vaginalis* is closely related to *M. muntjak* and formed a sister relationship with *Elaphodus cephalophus*. In view of the unclear distribution range and escalating habitat loss, it is important to identify its population genetic status. The complete mitogenome described in this study can be used in further phylogenetics, identification of extant maternal lineage, evolutionary significance unit and its genetic conservation.

Keywords Northern Indian red muntjac (*Muntiacus vaginalis*) · Complete mitogenome · Maternal lineage · Phylogenetics

Introduction

The Northern Indian red muntjac, *Muntiacus vaginalis* are widely distributed in India and Southeast Asia [1, 2]. It is classified as "least concern" in the red list of International Union for Conservation of Nature (IUCN). In India, this species is protected under Schedule-III of the Indian Wildlife (Protection) Act, 1972. The numbers of recognized *Muntiacus* species are still unclear and have been debated in several studies [3, 4]. Based on morphological variations the northern muntjac was divided into four species, *M. vaginalis, M. malabaricus, M. aureus and M. nigripes* [3]. Recently, based on the mitochondrial DNA (mtDNA) genetic variations, the red muntjacs separated into three geographically distinct groups, which indicated the presence of three distinct matrilineal lineages i.e. *M. vaginalis, M. malabaricus*, and *M. muntjak* in Southeast Asia [4]. Probably due to the use of the museum samples, mtDNA sequences generated by Martin et al. contained a substantial amount of ambiguous nucleotide with several incomplete or missing genes [4]. The population size of muntjacs is sharply declining across its distribution ranges due to the habitat destruction and poaching for meat and their body parts. Therefore, assessment of sequence variation from the complete and unambiguous mtDNA would enable us to identify the evolutionary significance units (ESU's) in its distribution range.

A complete mitogenome of *M. vaginalis* is lacking in the GenBank for the comparison with the other *Muntiacus* species. Therefore, the aims of this study were to generate and characterize the complete mitogenome sequence of the northern Indian muntjac, *M. vaginalis* and investigate the molecular phylogenetics of the species to confirm its taxonomic positions among Muntiacinae.

Materials and methods

Sample collection and DNA extraction

We used four muscle sample collected from the carcasses of wild *M. vaginalis* from Central (N = 2) and Northeast Indian landscape (N = 2). These samples were stored in − 80 °C

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until DNA extraction. Genomic DNA was extracted from the sample using the DNeasy Blood Tissue Kit (QIAGEN, Germany) in a final elution volume of 100 µl. The extracted DNA was quantified by QIAxpert system spectrophotometer, followed by 0.8% agarose gel electrophoresis to examine the quality of DNA. Isolated DNA was diluted in a final concentration of 40 ng/µl for the PCR amplification.

PCR amplification and sequencing

PCR amplification and sequencing of the entire mtDNA genome was performed using primers described earlier [5–7]. PCR reactions were carried out in 20 µl reaction volumes using 1 × PCR buffer (10 mM Tris–HCl, pH 8.3, and 50 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 3 pmol of each primer, 0.5 units of AmpliTaq Gold DNA Polymerase (Applied Biosystems) and 1 µl (~ 30 ng) of template DNA. The PCR conditions were initial denaturation at 95 °C for 10 min, followed by 35 cycles of denaturation at 95 °C for 45 s, annealing at 55 °C for 40 s and extension at 72 °C for 75 s. The final extension was at 72 °C for 10 min. PCR amplification was confirmed by electrophoresis on 2% agarose gel stained with ethidium bromide (0.5 mg/ml) and visualized under a UV transilluminator. The amplified PCR products were treated with exonuclease-I and shrimp alkaline phosphatase (Thermo Scientific Inc.) at 37 °C for 20 min for the removal of any residual primer and dNTPs and followed by inactivation of enzymes at 85 °C for 15 min. The purified fragments were sequenced directly in an Applied Biosystems Genetic Analyzer 3500 XL from the forward and reverse direction using BigDye v3.1 Kit.

Muntiacus vaginalis genome characterization and annotation

The overlapping fragments of DNA sequences were aligned to generate the entire mitogenome of *M. vaginalis* using Sequencher®version 5.4.6 (Gene Codes Corporation, Ann Arbor, MI, USA). The mtDNA annotation was done using Mitos WebServer [8]. We used Draw Organelle Genome Maps [9] for generating a gene map of the complete mtDNA of *M. vaginalis*. We obtained a total length of 16,352 bp from four *M. vaginalis* mitogenome that has been deposited in GenBank (MH547032, MK050505, MK050506, and MK050507).

For the comparative analysis with other *Muntiacus* spp., complete mtDNA sequences of *M. muntjak* (NC004563), *M. crinifrons* (NC004577), *M. reevesi* (NC008491), *M. vuquangensis* (FJ705435) and *M. putaoensis* (MF737190) were downloaded from the GenBank. Base compositions and mtDNA genetic code were calculated in MEGA 7 [10]. For estimating the bias in nucleotide composition among the complete mitogenome, skew analysis was carried out using the following method [11]: AT skew = (A − T)/(A + T), GC skew = (G − C)/(G + C). The intergenic spacer and overlapping regions between genes of complete mitogenome were estimated manually.

Phylogenetic analysis

For phylogenetic analysis, 16 mitogenomes were downloaded from GenBank to reconstruct the phylogenetic relationship between two groups, Cervini and Muntiacini. Additionally, the mitogenomes of mouse deer (*Moschiola indica* KY290452) were downloaded from GenBank and used as outgroups. A Monte Carlo Markov Chain (MCMC) based Bayesian consensus tree was constructed using BEAST version 1.7 [12]. Bayesian inference analysis ran as four simultaneous MCMC chains for 10 million generations and sampled every 100 generations using a burn-in of 5000 generations. The resulting phylogenetic trees were visualized in FigTree v1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/).

Results and discussion

Mitogenome organization

The complete mtDNA map of *M. vaginalis* (MH547032, MK050505, MK050506, and MK050507) has been shown in Fig. 1. These sequences exhibited a similarity range between 99.52 and 99.84%. These were consisted of 22 transfer RNA genes, 13 protein-coding genes (PCGs), two ribosomal RNA genes and a noncoding control region (D-loop region) (Table 1). The arrangement and distribution of these genes are similar to the other *Muntiacus* species [13, 14]. The total nucleotide composition of *M. vaginalis* mitochondrial genome was A (33.2%), T (29.0%), C (24.50%) and G (13.30%). Most of the genes were coded on the H-strand, except for the ND6 gene and eight tRNA genes ($tRNA^{Gln}$, $tRNA^{Ala}$, $tRNA^{Asn}$, $tRNA^{Cys}$, $tRNA^{Tyr}$, $tRNA^{ser}$, $tRNA^{Glu}$, $tRNA^{Pro}$). The control region was located between $tRNA^{Pro}$ and $tRNA^{Phe}$ (Table 1). Due to gene compactness and rearrangements of the mitogenome, four pairs of overlapping genes were observed among tRNA-Ile/tRNA-Gln, atp8/atp6, nad4L/nad4, and tRNA-Thr/ tRNA-Pro. These were resulting in 41 overlapping nucleotides (Table 1) which are usually found in other mammalian species [15, 16]. We calculated values of AT skew, GC skew and AT% for identifying nucleotide compositions in complete mitogenome. The AT-skew was positive whereas GC-skew was negative except in *M. vuquangensis* (Table 2). The A + T and G + C content were 62.2% and 37.8%, respectively.

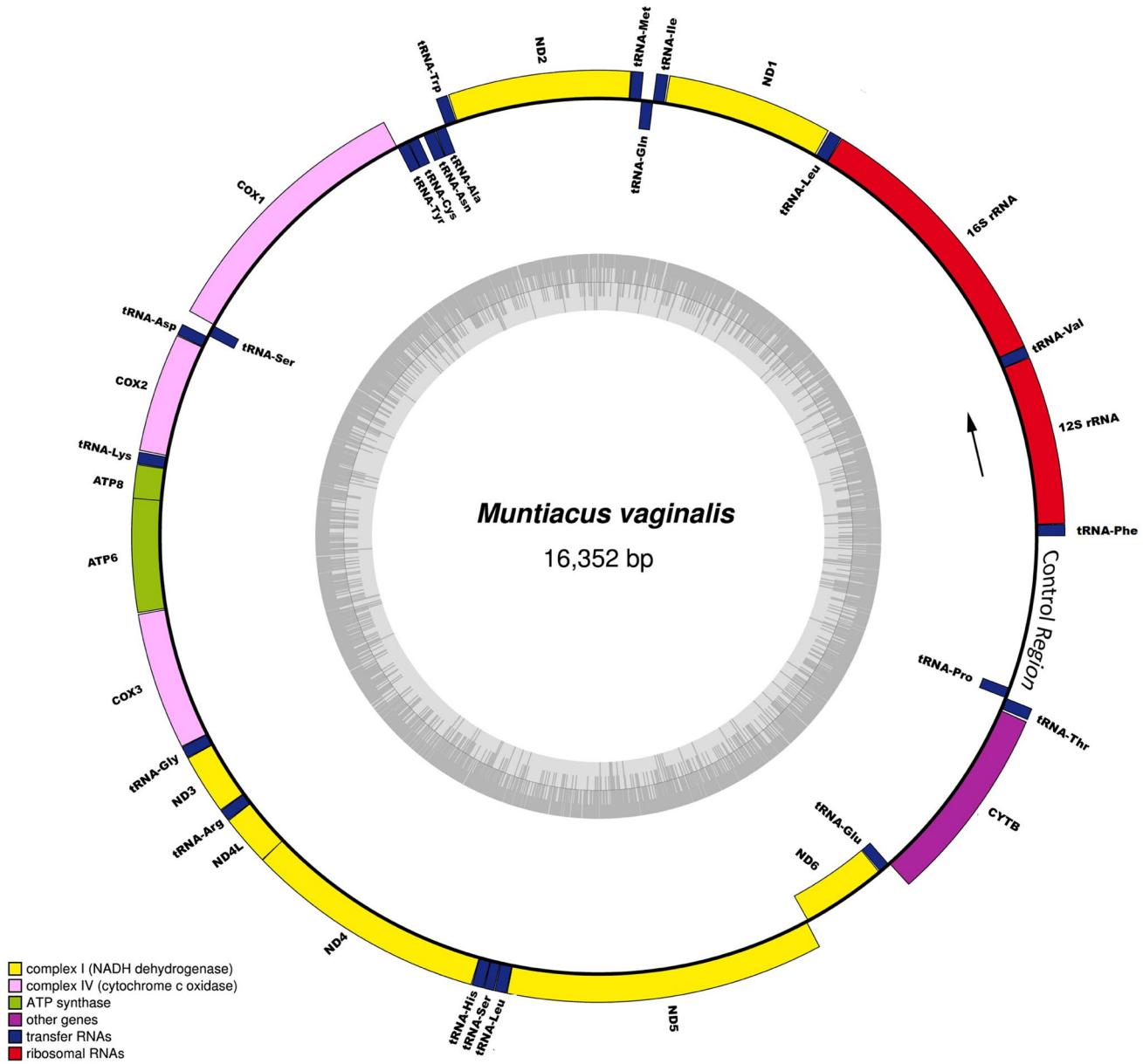

Fig. 1 Map of the complete mitochondrial genome of northern Indian red muntjac (*M. vaginalis*). The genes encoded on the heavy and light strand are represented outside and inside the circle, respectively

Protein coding genes (PCGs)

The total encoding length of 13 protein-coding genes (PCGs) in *M. vaginalis* was 11,319 bp that was 69.26% of the complete mitogenome. The PCGs region of *M. vaginalis* consisted of seven NADH dehydrogenases (nad1–nad6 and nad4L), three cytochrome c oxidases (cox1–cox3), two ATPases (atp6 and atp8) and one cytochrome *b* (cyt *b*) genes. All 13 PCGs, except NAD6, encoded on the H-strand (Fig. 1). All the 13 PCGs were having putative start codons and started with ATG or ATA. Termination with an incomplete stop codon such as TA– and T– were found in seven PCGs, those were completed by a post-transcriptional addition of polyadenylation during the mRNA maturation process. The overall AT content of 13 PCGs in *M. vaginalis* was 62%. Base skews were estimated for understanding the degree of base bias between all PCGs. The AT and GC skew values for the PCGs of *M. vaginalis* were compared with other *Muntiacini* mitogenome sequences. Positive AT skewness was obtained for most of the PCGs, showing that adenines occur more frequently than thymines. Negative GC skewness was observed for the PCGs in all *Muntiacini* except *M. reevesi* (0.29) that was indicative of C biased nucleotide

Table 1 Tabular organization of the mitochondrial genome of *M. vaginalis*

Gene	Positions			Codon				
	Start	End	Size	Start	Stop*	Anti-codon	Strand	Space/overlap[#]
tRNA-Phe	1	69	69	–	–	GAA	H	0
12S ribosomal RNA	70	1025	956	–	–	-	H	0
tRNA-Val	1026	1093	68	–	–	TAC	H	0
16S ribosomal RNA	1092	2660	1569	–	–	–	H	0
tRNA-Leu	2662	2736	75	–	–	TAA	H	1
ND1	2745	3689	945	ATA	T--	–	H	8
tRNA-Ile	3695	3763	69	–	–	GAT	H	5
tRNA-Gln	3761	3832	72	–	–	TTG	L	−3
tRNA-Met	3835	3903	69	–	–	CAT	H	2
ND2	3904	4941	1038	ATA	T--	–	H	0
tRNA-Trp	4946	5013	68	–	–	TCA	H	4
tRNA-Ala	5015	5083	69	–	–	TGC	L	1
tRNA-Asn	5085	5157	73	–	–	GTT	L	1
tRNA-Cys	5190	5257	68	–	–	GCA	L	32
tRNA-Tyr	5258	5326	69	–	–	GTA	L	0
COX1	5328	6866	1539	ATG	TA-	–	H	1
tRNA-Ser	6870	6938	69	–	–	TGA	L	3
tRNA-Asp	6946	7013	68	–	–	GTC	H	7
COX2	7015	7695	681	ATG	TA-	–	H	1
tRNA-Lys	7702	7770	69	–	–	TTT	H	6
ATP8	7772	7966	195	ATG	TAA	–	H	1
ATP6	7933	8607	675	ATG	TAA	–	H	− 33
COX3	8613	9395	783	ATG	T--	–	H	5
tRNA-Gly	9397	9465	69	–	–	TCC	H	1
ND3	9466	9810	345	ATA	T--	–	H	0
tRNA-Arg	9813	9881	69	–	–	TCG	H	2
ND4L	9882	10,175	294	ATG	TAA	–	H	0
ND4	10,172	11,539	1368	ATG	T--	–	H	−4
tRNA-His	11,550	11,618	69	–	–	ATG	H	10
tRNA-Ser	11,619	11,678	60	–	–	GCT	H	0
tRNA–Leu	11,680	11,749	70	–	–	TAG	H	1
ND5	11,750	13,552	1803	ATA	TAA	–	H	0
ND6	13,560	14,078	519	ATA	TAA	–	L	7
tRNA-Glu	14,082	14,150	69	–	–	TTC	L	3
CYTB	14,155	15,288	1134	ATG	AGA	–	H	4
tRNA-Thr	15,298	15,367	70	–	–	TGT	H	9
tRNA-Pro	15,367	15,432	66	–	–	TGG	L	−1
Control region	15,433	16,352	919	–	–	–	H	/

Partial stop codons* (T-- or TA-) were completed by the addition of A during the polyadenylation of the premature RNA's. Minus (−) value[#], represent the overlapping number of nucleotides

composition (Table 2). Of these 13 PCGs, the ND5 gene (1803 bp) was the longest and the ATP8 (195 bp) was the smallest in length. Relative synonymous codon usage and codon number within 13 PCGs of *M. vaginalis* are summarised in Supplementary Table ST1 and Fig. SF1. 13 PCGs in the *M. vaginalis* mitogenome consist of 3772 codons.

Ribosomal RNA and transfer RNA genes

The 12s rRNA and 16s rRNA genes in the mitogenome of *M. vaginalis* were positioned between tRNA-Phe and tRNA-Val and between tRNA-Val and tRNA-Leu2, respectively. The length of both rRNA genes was 2525 to 2533 bp and

Table 2 Nucleotide composition indices in different regions of six representative mitogenomes of *Muntiacini*

Species	Accession no.	Whole mitogenome				Protein Coding Genes (PCGs)		Ribosomal RNA (rRNA)	
		Length (bp)	AT (%)	AT Skew	GC skew	Length (bp)	AT (%)	Length (bp)	AT (%)
M. vaginalis	MH547032[a]	16,352	62.3	0.067	−0.293	11,319	62	2525	62.7
M. vaginalis	MK050505[a]	16,352	62.3	0.068	−0.29	11,319	62	2525	62.8
M. vaginalis	MK050506[a]	16,352	62.4	0.067	−0.29	11,319	62	2525	63
M. vaginalis	MK050507[a]	16,352	62.3	0.068	−0.29	11,319	62.7	2525	62.7
M. muntjak	NC004563[b]	16,353	62.2	0.071	−0.29	11,319	61.9	2524	62.9
M. crinifrons	NC004577[c]	16,357	61.9	0.066	−0.288	11,319	61.7	2525	62.3
M. reevesi	NC008491[d]	16,353	62.1	0.064	0.29	11,319	61.9	2528	62.4
M. vuquangensis	FJ705435[e]	16,361	62.3	0.061	−0.284	11,319	62.1	2533	62.5
M. putaoensis	MF737190[f]	16,349	62.4	0.068	−0.296	11,319	62.2	2528	62.2

[a]This study

[b]Shi et al. [13]

[c]Li et al. [17]

[d]Wang et al. [18]

[e]Hassanin et al. [19]

[f]Li and Quan [20]

accounted for 15.45% of the complete mitogenome. The length of the 12s rRNA and 16s rRNA genes in *M. vaginalis* were 956 bp and 1569 bp, respectively. The total AT content of 2 rRNA and 22 tRNA genes were 62.07% and 64.1%, respectively. The base pair composition of both the rRNAs genes was A: 37.6%, T: 25.1%, G: 17.0% and C: 20.2%. The tRNAs genes were found to have an average base composition of A: 33.0%, T: 31.1%, G: 19.5% and C: 16.4%, with the highest AT content. The anticodons of all the tRNAs found in the complete mitogenome of *M. vaginalis* are given in Table 1.

Mitogenome phylogeny

The Bayesian inference phylogenetic analysis was performed using the complete mitogenome of eight species of Cervini and seven species of Muntiacini along with the four sequences of *M. vaginalis*. The results yielded strong statistics support (> 99%) within the Cervini and Muntiacini. The clustering pattern of the Muntiacini was broadly consistent with the previous studies [19, 21, 22]. The newly sequenced *M. vaginalis* nested within Muntiacini, with two *Muntiacus* clades. One clade consisted of *M. reevesi, M. putaoensis*, and *M. vuquangensis*, and the other consisted of *M. vaginalis, M. muntjak*, and *M. crinifrons*. The four generated sequences of *M. vaginalis* were most closely related to the *M. muntjak*. *E. cephalophus* is the single species in genus *Elaphodus* of Muntiacinae and formed a sister relationship with *Muntiacus* (Fig. 2).

The number of species and subspecies of muntjac has always been a debate. Few new species of muntjac are included in genus namely, Annamite muntjac (*M. truongsonensis*) [2] and Roosevelt's muntjac (*M. rooseveltorum*) from Vietnam [23], the leaf deer (*M. putaoensis*) from Northern Myanmar [24, 25], and the Bornean Yellow muntjac (*M. atherodes*) from Borneo [26]. Recently, it has been recognized that the lineages of *Muntiacus* species are increasing due to in-depth examination in its biogeography and molecular genetics [4]. The taxonomic stability of muntjak has not been achieved yet, due to the limited molecular studies. Therefore, investigating the mitochondrial genome across the distribution ranges would be crucial to design the proper conservation plan and to identify the evolutionary significant unit. The analysis of four newly sequenced mitogenome of *M. vaginalis* from two landscape of India will provide a basis for conservation studies and determine the existing distribution range of northern subspecies. Moreover, this study will help spur investigation of phylogeography, evolutionary significance unit (ESU) and to address genetic linkages among this species. However, analysis of more biologicals samples from the distribution range of Northern Indian red Muntjac would assist in the identification of more maternal lineages.

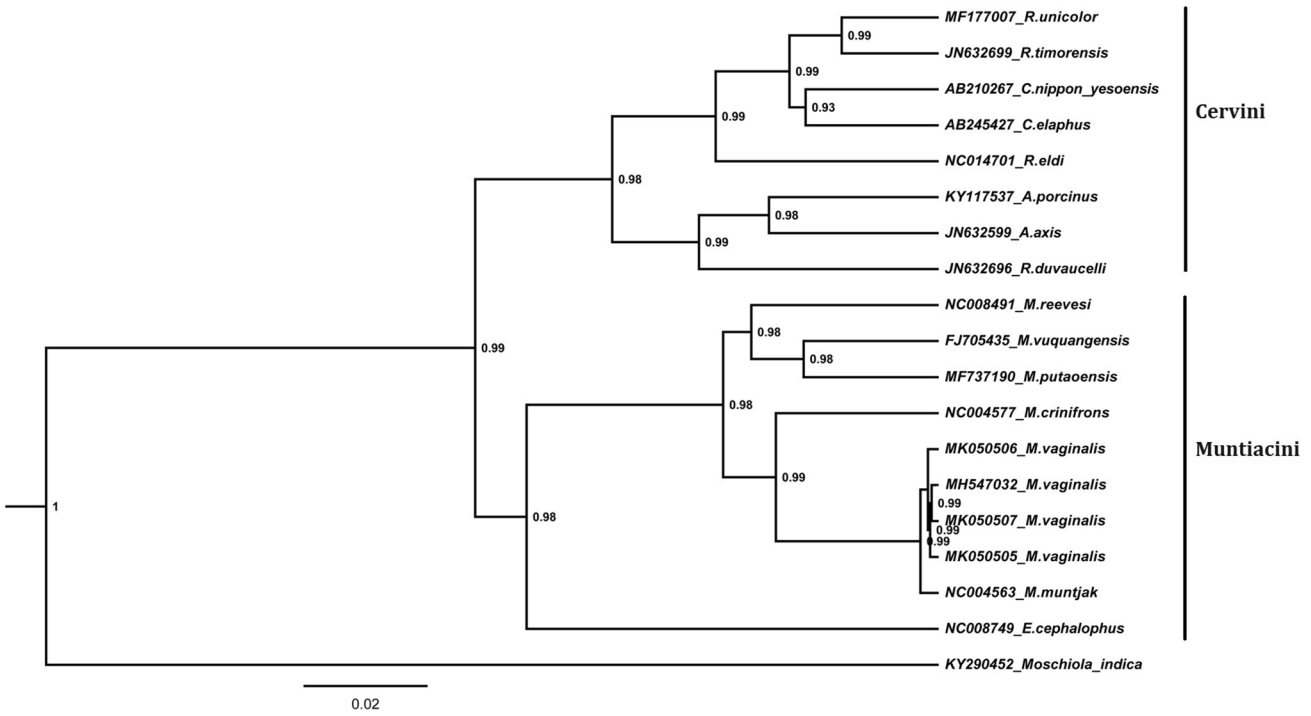

Fig. 2 The MCMC Bayesian tree for the phylogenetic resolution between the Cervini and Muntiacini. The *Moschiola indica* was used as outgroup. Numeric value at each node represent the posterior probability

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Compliance with ethical standards

Conflict of interest The author declare that they have no conflict of interest.

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RESEARCH ARTICLE

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Phylogeography and population genetic structure of red muntjacs: evidence of enigmatic Himalayan red muntjac from India

Bhim Singh, Ajit Kumar, Virendra Prasad Uniyal and Sandeep Kumar Gupta*

Abstract

Background: Identifying factors shaping population genetic structure across continuous landscapes in the context of biogeographic boundaries for lineage diversification has been a challenging goal. The red muntjacs cover a wide range across multiple vegetation types, making the group an excellent model to study South and Southeast Asian biogeography. Therefore, we analysed mitogenomes and microsatellite loci, confirming the number of red muntjac lineages from India, gaining insights into the evolutionary history and phylogeography of red muntjacs.

Results: Our results indicated the Northwestern population of red muntjac or the Himalayan red muntjac (*M. aureus*) in India as genetically diverse and well-structured, with significant genetic differentiation implying a low level of gene flow. The phylogenetic, population genetic structure, as well as species delimitation analyses, confirm the presence of the lineage from Western Himalayan in addition to the previously identified red muntjac lineages. Relatively low genetic diversity was observed in *M. aureus* compared to *M. vaginalis*, *M. malabaricus* and *M. muntjak*. The *M. aureus* and *M. vaginalis* lineages have split during the late Pleistocene, ~ 1.01 million years ago (Mya), making *M. aureus* the youngest lineage; whereas, *M. malabaricus* split earlier, ~ 2.2 Mya and appeared as the oldest lineage among red muntjacs.

Conclusions: Pronounced climate fluctuations during the Quaternary period were pivotal in influencing the current spatial distribution of forest-dwelling species' restriction to Northwestern India. Our finding confirms the distinct Himalayan red muntjac (*M. aureus*) within the red muntjac group from Northwestern India that should be managed as an Evolutionary Significant Unit (ESU). We recommend a reassessment of the conservation status of red muntjacs for effective conservation and management.

Keywords: Phylogeography, Red muntjacs, *M. aureus*, Mitogenome, Microsatellite, Evolutionary Significant Unit

Background

The genus *Muntiacus* belongs to tribe Muntiacini within the family Cervidae. It is widely distributed throughout South and Southeast Asia [1], and exhibits extreme variations in chromosome numbers [2, 3]. The taxonomic classification and validation of species and subspecies is still controversial. Mattioli classified red muntjacs as single species *Muntiacus muntjak* comprising ten subspecies [4]. Grubb and Groves recognized six species and two subspecies of *Muntiacus* using geographical distributions and morphological characters [5]. International Union for Conservation of Nature (IUCN) has provisionally adopted two species of *Muntiacus: M. vaginalis* (Northern or Indian Red Muntjac) and *M. muntjak* (Southern Red Muntjac). While the former is widely distributed from northern Pakistan to most of India, Nepal, Bhutan, Bangladesh to southern China with Hainan and south Tibet, and into Myanmar, Thailand, Lao, PDR, Vietnam,

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Cambodia, the latter is limited to the Thai–Malay peninsula, Java, Bali, Lombok, Borneo, Bangka, Lampung and Sumatra [6]. However, the exact southern range limit of Muntjacs in the Thai–Malay peninsula remains unclear.

For highly adapted mammals such as red muntjac, whose extensive distribution ranges are linked to different political boundaries, contemporary genetic variation and population structure may be shaped by both natural and anthropogenic factors [7]. With the increasing number of studies on muntjacs, the number of newly recognized *Muntiacus* species is also increasing. For example, based on the differences in skin color, skull and antler morphology, a new endemic species from Borneo (Bornean yellow muntjac or *M. atherod*) was described [8], after which the Gongshan muntjac (*M. gongshanensis*) was described from Southwest, China and northern Myanmar [9, 10]. Previously, the Putao muntjac (*M. putaoensis*) from Myanmar [11], Small blackish muntjac (*M. truongsonensis*) from Central Vietnam [12], and Roosevelt's barking deer (*M. rooseveltorum*) from Vietnam [13] have been confirmed with molecular studies. In a recent study, the complete mitogenome sequences indicated the presence of three distinct maternal lineages across the distribution range of red muntjacs: Srilankan red muntjac from Western Ghats, India and Sri Lanka; Northern red muntjac in Northern India and Indochina; and Southern red muntjac found in Sundaland [14]. Distinct morphological and genetic characters are a center of attraction to study the mystifying red muntjacs. It is of urgent importance when anthropogenic activities such as habitat fragmentation and destruction with poaching have drastically influenced the population size, distribution ranges and population genetic structure of several deer species in the last few centuries [15–17]. The Indian subcontinent sustains a diverse ecosystem that supports high faunal richness and diversity, but very little is known about the species diversification and evolutionary history and the role of geo-climatic changes during the Late Pleistocene [18, 19]. The late Pliocene to early Pleistocene witnessed dramatic climatic shifts in South and Southeast Asia, which led to geographical subdivision with contraction of habitats, influencing the distribution of contemporary species. The persistence of rainforests in high elevation areas led to many refugia populations inhabiting the high mountainous region [20–22]. The wide distribution range of the Northern red muntjac across multiple biogeographic zones makes it an excellent model to study the biogeography and diversification of red muntjacs in South and Southeast Asia.

The Northern red muntjac is currently listed as "Least Concern" in the IUCN Red List and protected under Schedule III of the Indian Wild Life (Protection) Act, 1972. Extensive genetic characterization of Northern red muntjac from India can depict population boundaries and genetic structuring for its appropriate classification and formulation of management strategies. Recently, Martins et al. (2017) investigated the geographic distribution of mtDNA lineages among red muntjac populations using museums, zoos, and opportunistically collected samples from Vietnam, Laos, and Peninsular Malaysia [14]. Due to museum samples, the complete mitogenomes from this study contained several ambiguous nucleotides in most of the sequences. Martins et al. (2017) also suggested extensive sampling to unveil taxonomic uncertainties within red muntjacs with nuclear data analysis to examine barriers to gene flow [14].

Moreover, Groves et al. [5] and Martins et al. [14] have previously suspected the presence of a distinct lineage from Northwestern India, Central India, and Myanmar. Therefore, to investigate the phylogeography and molecular ecology of red muntjacs from India, we combined newly generated complete mitogenome sequences with data by Martins et al., [14] to gain insights into the evolutionary history and phylogeographic pattern of red muntjacs. We further employed nuclear markers to substantiate the characterization of the Indian muntjacs.

Results

Genetic diversity

The 16 generated mitogenomes sequences of red muntjac were deposited in GenBank (MT671398-MT671408; MT758349-MT758353). To elucidate the phylogenetic relationships, we included four sequences of *M. vaginalis* from Singh et al. [23] and 32 sequences of Northern, Southern, and Srilankan muntjacs from Martins et al. [14]. The accession numbers and details for each sample are provided in the Additional file 1: Table S1. All complete mitogenome sequences grouped into four genetically distinct clusters and represented individual haplotypes with haplotype diversity Hd = 1, indicating maternal unrelatedness among the red muntjac samples (Table 1). The nucleotide diversity of *M. vaginalis* (Mainland red muntjac) was $\pi = 0.0044$ (s.d. = 0.0003), *M. aureus* (Himalayan red muntjac) was $\pi = 0.0016$ (s.d. = 0.0002), *M. malabaricus* (Western Ghats & Srilankan red muntjac) was $\pi = 0.005$ (s.d. = 0.0008) and *M. muntjak* (Southern or Sundaland red muntjac) was $\pi = 0.0109$ (s.d. = 0.0006). The overall nucleotide diversity among red muntjacs was $\pi = 0.0203$ (s.d. = 0.001). Among all red muntjacs, the lowest number of segregating sites were found in *M. aureus* (S = 67), whereas it was high in *M. muntjak* (S = 658). Tajima's D and Fu's FS test were non-significantly different from zero ($P > 0.01$), which indicated that red muntjac populations had not undergone an expansion.

Table 1 Summary of genetic diversity in red muntjacs populations based on microsatellites and complete mitochondrial DNA

Population/Region	Mitochondrial DNA							Microsatellites					
	n	S	H	Hd	π	Tajima's D[a]	Fu's *Fs*[a]	n	Na	*Ar*	H_O	H_E	F_{IS}
M.aureus	6	67	6	1.00	0.001	− 0.49	0.46	18	7.11 ± 0.73	4.52	0.66 ± 0.071	0.73 ± 0.03	0.131
M.vaginalis	23	415	23	1.00	0.004	− 1.33	− 2.7	19	9.33 ± 0.86	5.79	0.62 ± 0.04	0.81 ± 0.02	0.269
M. malabaricus	6	198	6	1.00	0.005	0.48	1.9	5	6.11 ± 0.56	6.11	0.75 ± 0.06	0.76 ± 0.04	0.096
M. munjak	17	658	17	1.00	0.01	− 0.37	− 0.01	–	–	–	–	–	–

n number of samples, *S* segregating sites, *H* haplotype, *Hd* haplotype diversity, *π* nucleotide diversity, *Na* number of alleles, *Ar* allelic richness, H_O observed heterozygosity, H_E expected heterozygosity, F_{IS} inbreeding coefficient, [a]all P-values > 0.01 (not significant)

The genetic diversity of Indian red muntjacs was calculated using nine microsatellite markers (Table 1). The selected microsatellite markers showed high polymorphic information content (PIC > 0.5) with a mean value of 0.831; therefore, all used loci were found to be informative. All the loci significantly deviated from Hardy–Weinberg equilibrium (HWE) and no linkage disequilibrium (LD) was detected (P > 0.05). No evidence of a large allele drop out was observed, while null alleles at each locus were low in frequency (less than 0.10 per population). The mean number of alleles (Na) in *M. vaginalis, M. aureus* and *M. malabaricus* were 9.33, 7.11 and 6.11 respectively with highest allelic richness (*Ar*) in *M. malabaricus* (*Ar* = 6.11) and lowest in *M. aureus* (*Ar* = 4.52). The observed heterozygosity (*Ho*) and expected heterozygosity (H_E) in *M. aureus* were *Ho*: 0.666; H_E: 0.730; for *M. vaginalis, Ho*: 0.620; H_E: 0.810; whereas, in *M. malabaricus*, it were *Ho*: 0.756, H_E: 0.760. The mean inbreeding coefficient (F_{IS}) value for all the red muntjac populations were greater than zero (ranges between 0.096 and 0.269), indicating a heterozygote deficiency (Table 1), which may be attributed due to the Wahlund effect and population not being in HWE.

Phylogeography of red muntjacs

The Bayesian consensus tree showed that all sequences of red muntjac clustered into four major clades (Fig. 1). Clade-I consisted of Mainland red muntjac lineages (*M. vaginalis*), comprising individuals from Northern to Central India, Eastern to Northeastern India, Nepal,

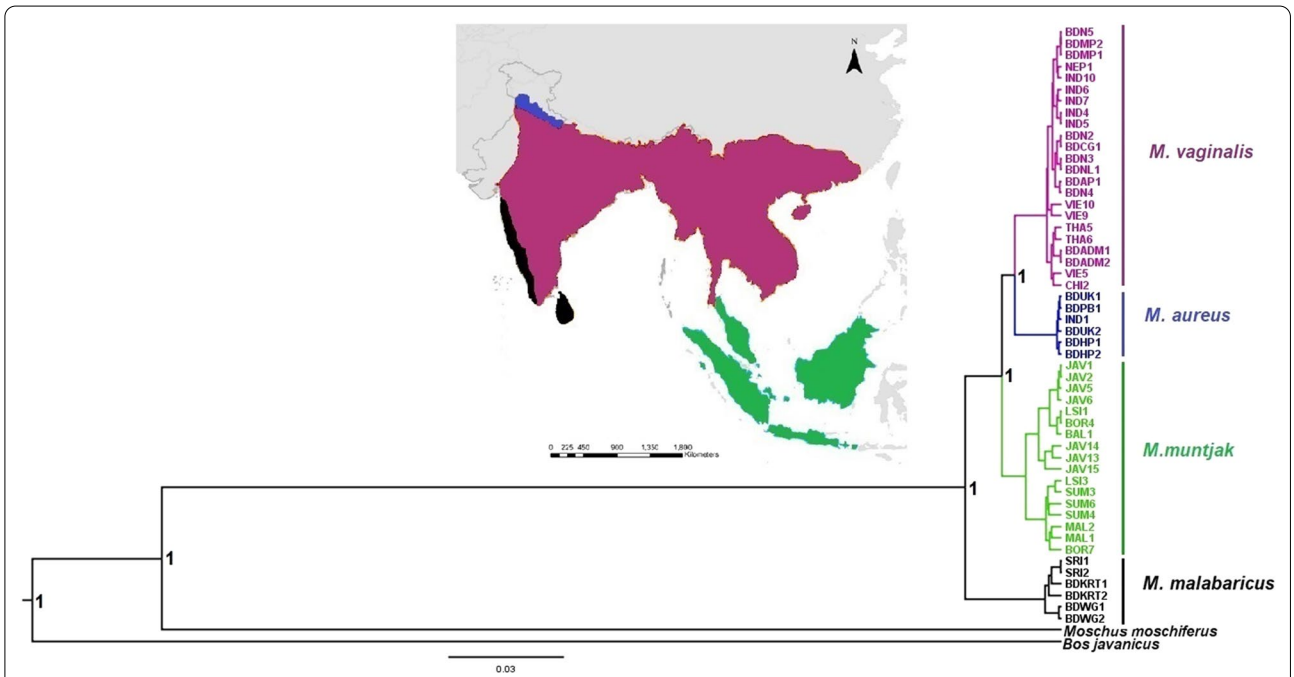

Fig. 1 Bayesian inference (BI) of phylogenetic tree for red muntjac based on complete mitochondrial DNA. Numbers on clades indicate posterior probability (PP) for the node. The distribution ranges of different lineages are represented by specific colors in distribution map corresponding to colored clades in a tree topology

Southeast Asia, and Andaman & Nicobar Islands. Clade-II comprised the individuals from the Northwestern part of India (*M. aureus*) (i.e., Uttarakhand, Punjab, and Himanchal Pradesh) and Clade-III comprised the sequences from Sunda (*M. muntjak*), mainly from Sumatra, Malay Peninsula, Lombok, Borneo, Java, and Bali's Islands. Clade-IV consisted of individuals from Western Ghats of Southern Indian and Sri Lanka (*M. malabaricus*). The median-joining (MJ) network of all recognized sequences from India, Southeast Asia Sundaland, and Sri Lanka strongly supported the phylogenetic results, indicating the existence of four geospatial populations from its distribution ranges (Fig. 2). In India, we found three evident clades representing populations of *M. vaginalis* (Clade-I), *M. aureus* (Clade-II), and *M. malabaricus* (Clade-IV). The phylogenetic and median-joining analyses showed the presence of two sub-groups in the Sunda of Southern red muntjac. It is also noteworthy that the mainland red muntjacs from India exhibited different genetic signature and showed structuring with respect to other populations in Southeast Asia and Andaman & Nicobar Islands.

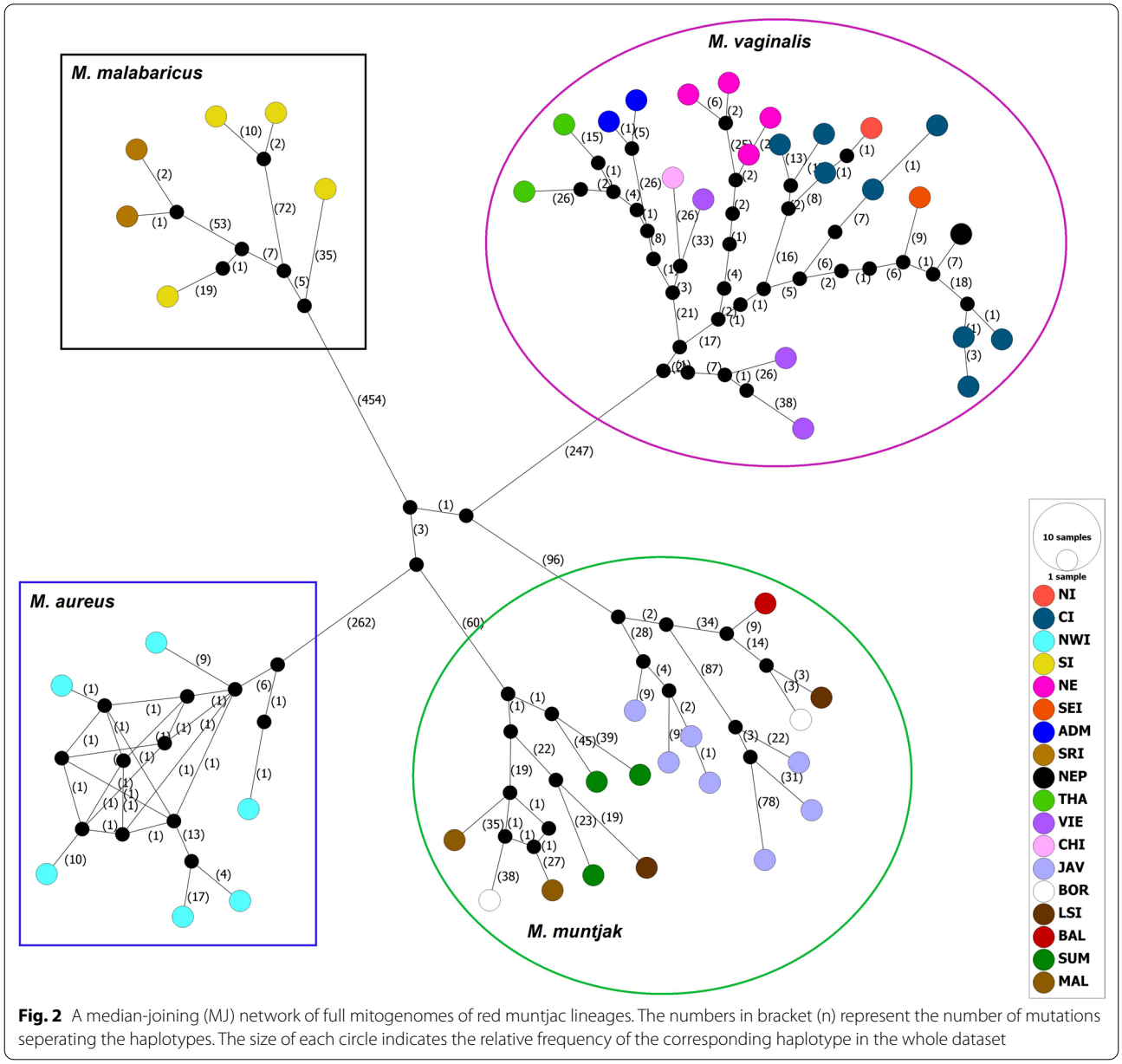

Fig. 2 A median-joining (MJ) network of full mitogenomes of red muntjac lineages. The numbers in bracket (n) represent the number of mutations seperating the haplotypes. The size of each circle indicates the relative frequency of the corresponding haplotype in the whole dataset

Estimating genetic divergence

We calibrated the root age (TMRCA of Bovidae and Moschidae) to 18 ± 2 Mya ($CI_{95\%}$: 14.74–22.94) and the split between Cervidae and Bovidae + Moschidae was set at 17.2 ± 2 Mya ($CI_{95\%}$: 16.04–24.08). Our divergence results suggested that the split between the red muntjacs and black muntjac (*M. criniforns*) occurred in the Late Pliocene, around 3.29 Mya ($CI_{95\%}$: 2.57–4.16). Within red muntjac, group diversification started during the Pleistocene. The *M. malabaricus* split earlier ~ 2.2 Mya ($CI_{95\%}$: 1.67–2.77), and thereafter, the clade of *M. muntjak* of Sunda split around 1.4 Mya ($CI_{95\%}$: 1.05–1.76). Within the northern lineages, the split between the *M. vaginalis* and *M. aureus* was estimated to have occurred at ~ 1.01 Mya ($CI_{95\%}$: 0.75–1.27) (Fig. 3). Our analysis indicated the Northwestern red muntjac to be the youngest among the red muntjac.

Species delimitation

The bPTP and GMYC analyses indicated four species in our dataset, whereas the mPTP analysis indicated seven. All analyses delimited the same taxonomic units as inferred from BEAST phylogenetic analysis, namely: *M. vaginalis*, *M. aureus*, *M.muntjak* and *M. malabaricus*, while the mPTP model additionally expanded the *M. muntjak* group from Sundaland into 4 subsets (Fig. 4). The result of bPTP and GMYC analyses supported the previously recognized taxonomic subdivisions, also corroborated by our analyses.

Population genetic structure and genetic differentiation

The Bayesian clustering analysis identified the highest ΔK when *K* was set at 2 (Mean LnP(K) = − 1584.64; ΔK = 9.6) under the admixture model. As recommended by Evanno et al. (2005) interpret *K* with caution, we further analyzed accordingly to identify the possible hidden substructure for each predefined cluster [24]. Therefore, we adopted the second-highest value of *K* = 3 (Mean LnP(K) = − 1565.47; ΔK = 5.9), where most individuals were assigned to three different clusters, which differentiated among Northwestern (*M. aureus*), Mainland (*M. vaginalis*), and Western Ghats population (*M. malabaricus*) (Fig. 5). Although the genetic structure of the Mainland population indicated some alleles from the Himalayan and Western Ghats populations, none of the alleles were found vice-versa. The multivariate Discriminant Analysis of Principal Components (DAPC) (Fig. 6) and factor correlation analysis (FCA) (Additional file 1: Figure S1) also supported the Bayesian clustering method that differentiated the populations into three genetic clusters. We found strong concordance between microsatellite and mitogenome data using different Bayesian and non-Bayesian clustering methods used in this study.

The analysis based on pairwise F_{ST} for red muntjac using complete mitogenome demonstrated significant genetic differentiation of *M. vaginalis* from *M. aureus* (0.0208) and *M. malabaricus* (0.0396). The genetic differentiation of *M. aureus* from *M. vaginalis* and *M. muntjak* was almost similar (0.026), whereas a comparatively high level of differentiation was observed between *M.*

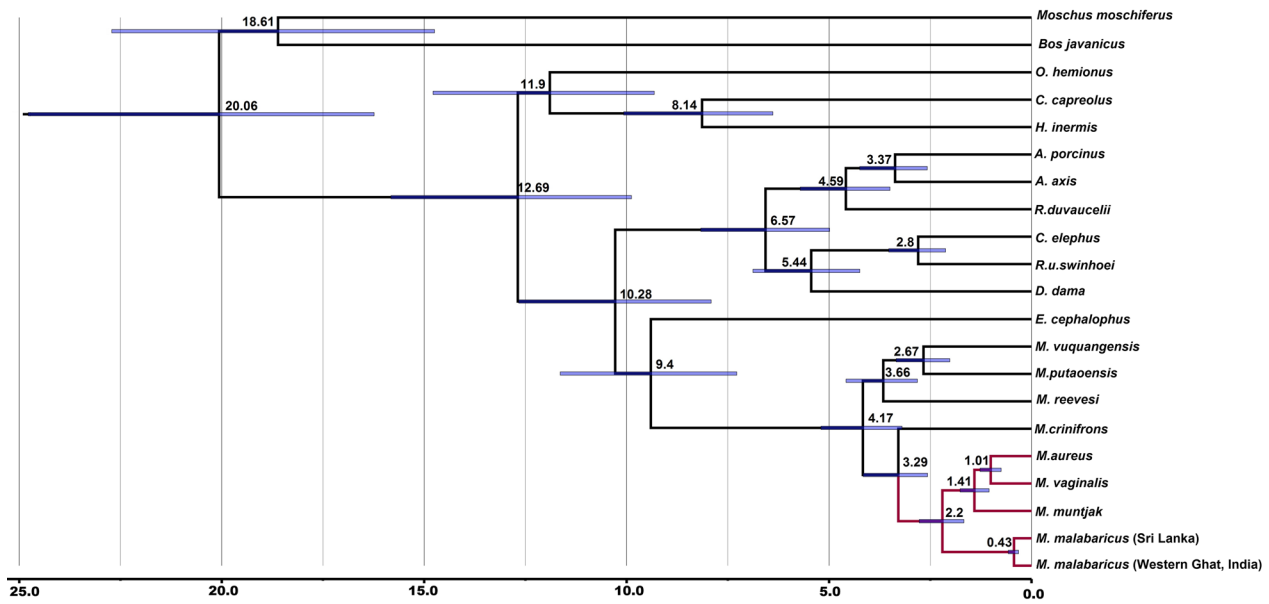

Fig. 3 Divergence time estimation based on maximum credibility tree using complete mitogenome generated from BEAST analysis

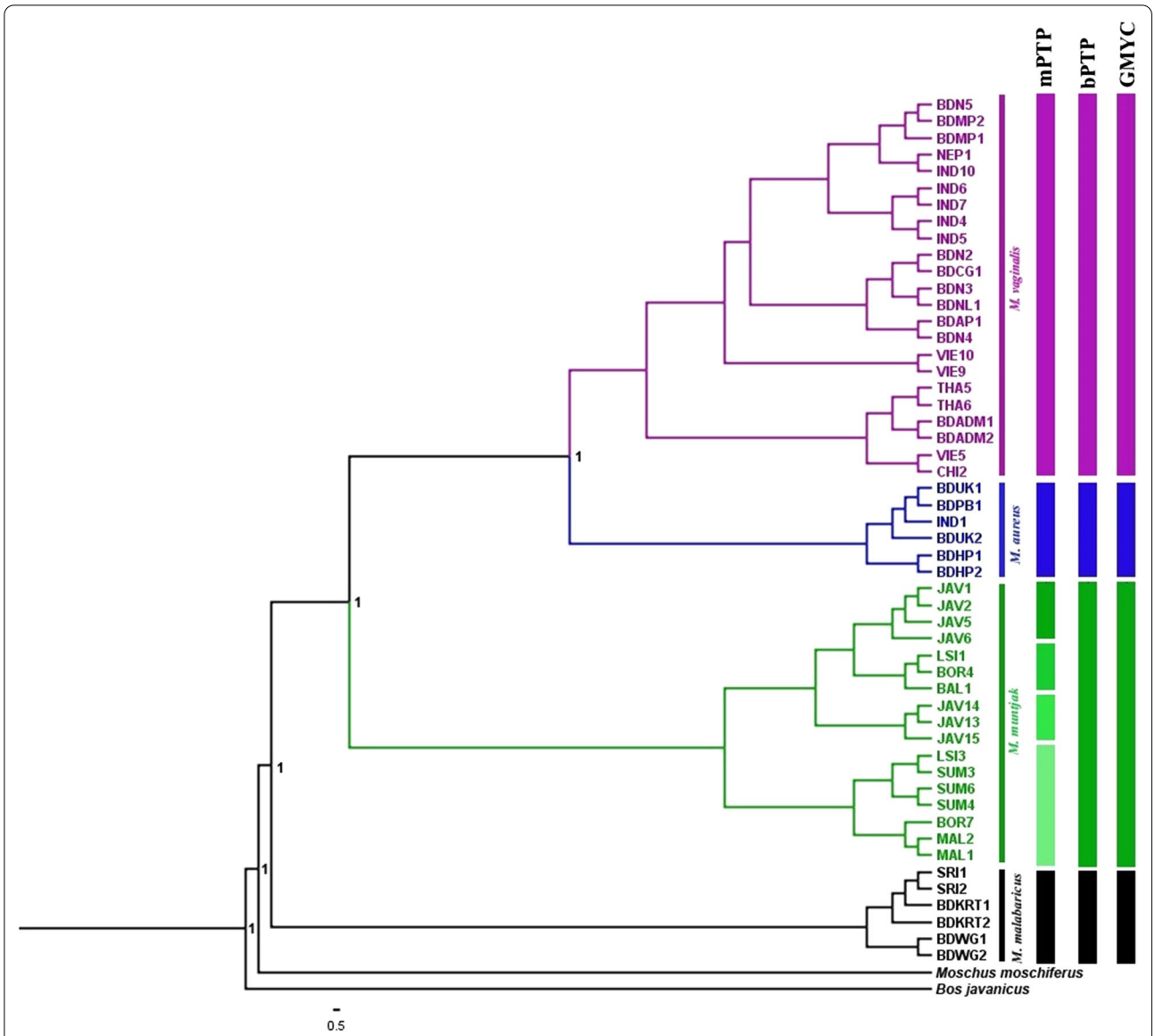

Fig. 4 Bayesian phylogeny based on complete mitochondrial genomes representing the result of three different molecular species delimitation methods. Multi-rate Poisson Tree Processes (mPTP), Bayesian Poisson Tree Processes (bPTP) and Generalized Mixed Yule-Coalescent (GMYC)

muntjak and *M. malabaricus* (0.039). A parallel pattern was observed in microsatellite analysis where the observed genetic differentiation between the *M. aureus* and the *M. vaginalis* was 0.062, and *M. malabaricus* was 0.105, whereas *M. vaginalis* to *M. malabaricus* was 0.080 (Table 2). We also calculated genetic differentiation with other *Muntiacus* species and recovered a comparatively low level of genetic differentiation between red muntjac lineages with *M. criniforns* (0.056 to 0.057) than with other species such as *M. reevesi* (0.065 to 0.068), *M. vuquangensis* (0.060 to 0.068) and *M. putaoensis* (0.067 to 0.069). The spatial genetic analysis revealed a significant correlation between the pairwise genetic distances among geographical subsets and geographical distances (Mantel test statistic, $rM = 0.513$; $P = 0.0009$, Fig. 7). This pattern of isolation by distance (IBD) was strongly influenced by the genetic differentiation and major geographical distance between the red muntjac lineages.

Discussion

The study brings together the extensive analyses of complete mitochondrial and microsatellite loci variation to understand the Quaternary climatic fluctuations and geological events on the probable influence on the

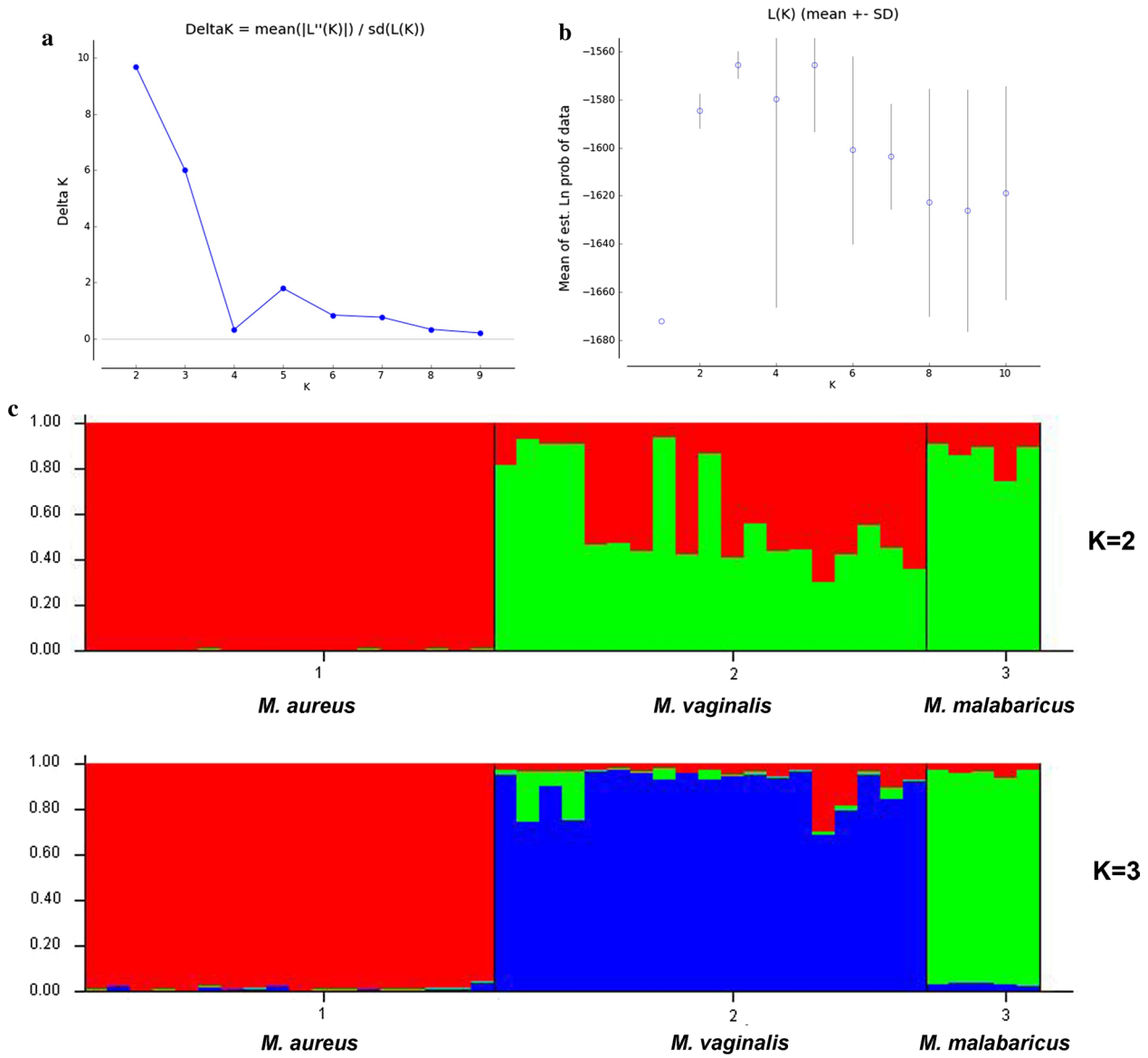

Fig. 5 Results of genetic clusters inferred from Structure 2.3.4. **a** Delta K values with respect to K **b** Mean of estimates Ln probability of data with respect to K. **c** Barplot indicating the genetic structure at K = 2 and K = 3, showing the population assignments for each individuals of *M. aureus*, *M. vaginalis* and *M. malabaricus*, respectively. The Y axis is depicting the proportion derived from each cluster

demographic pattern and population genetic structure among the red muntjac groups. Previous phylogeographic studies of red muntjac showed a clear division between Northern and Southern lineages, indicating physical barriers to gene flow resulting from extremely dry climatic conditions caused by global ice advances [14]. As the taxonomy of muntjacs is considerably fragile and still debated [5], ongoing research aims to resolve the phylogenetic complexities to elucidate the exact number of lineages. The population genetic studies on red muntjacs will act as definitive tools to understand the lineage diversification, genetic structuring, and diversity, resulting in developing appropriate conservation and management strategies for this enigmatic species. Previously, Martins et al. (2017) reported three mitochondrial lineages: The Srilankan red muntjac (*M. malabaricus*), Northern red muntjac (*M. vaginalis*) and Southern red muntjac (*M. muntjak*) [14]. The present study on genetic structure and differentiation among red muntjac populations exhibited four genetically distinct lineages from its geographical distribution range in South and Southeast Asia. Structure, DAPC and FCA analyses supported

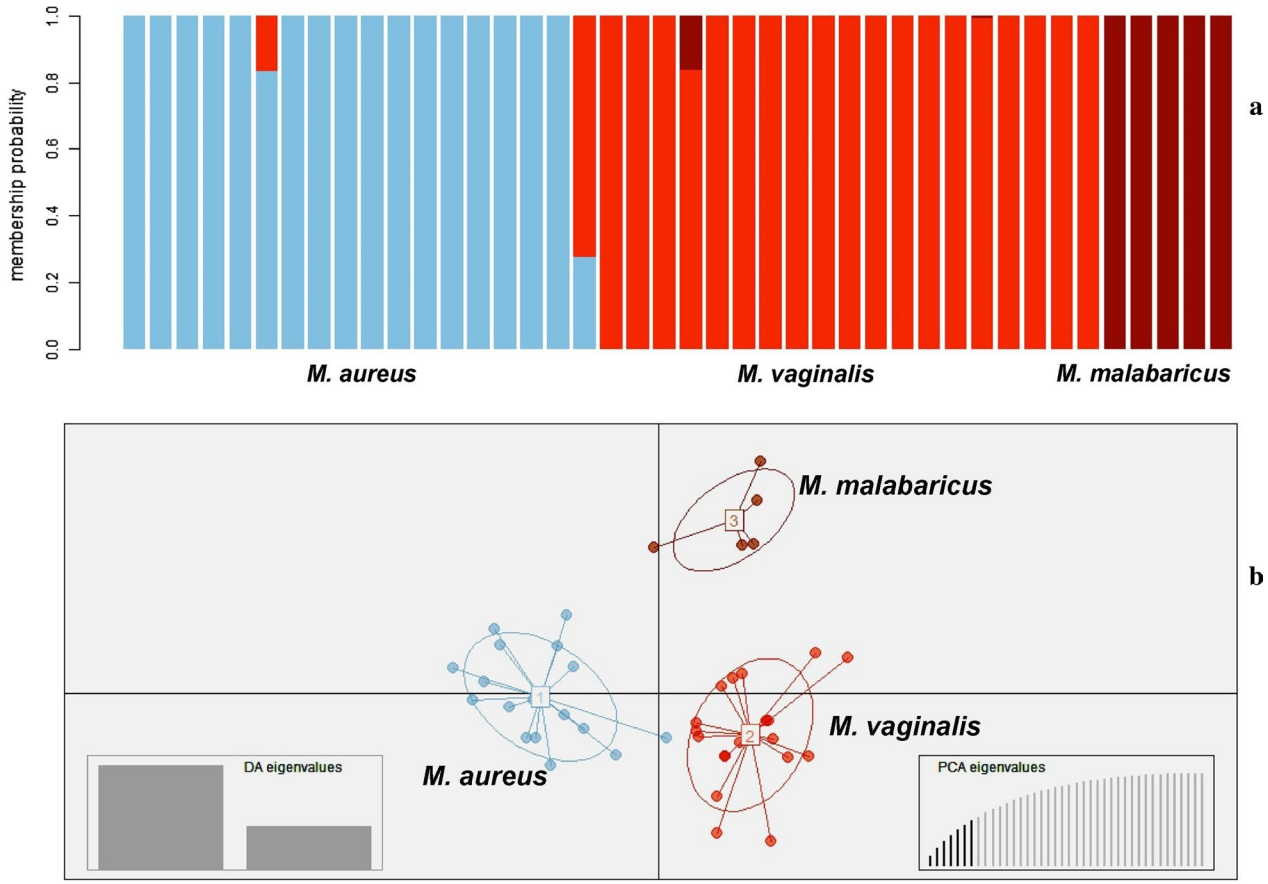

Fig. 6 The bar plot results show genetic clustering implemented in Discriminant Analysis of Principal Components (DAPC) (**a**). Each column along the X-axis represents one red muntjac individual. The Y-axis represents the assignment of the membership probability of each individual. Scatterplots of DAPC using a hierarchical islands model and shown by different colors and inertia ellipses (**b**). The DA and PCA eigenvalues of the analyses are displayed in the inset

Table 2 Genetic differentiation among red muntjac and other Muntiacus species

Species	1	2	3	4	5	6	7	8
M. vaginalis	–	**0.062**	**0.080**					
M. aureus	0.0208	–	**0.105**					
M. malabaricus	0.0396	0.0401	–					
M. muntjak	0.0269	0.0264	0.0399	–				
M. criniforns	0.0557	0.0572	0.0562	0.0571	–			
M. reevesi	0.0668	0.0657	0.0682	0.0685	0.0651	–		
M. vuquangensis	0.0679	0.0683	0.0672	0.0681	0.0665	0.0606	–	
M. putaoensis	0.0693	0.0690	0.0676	0.0695	0.0692	0.0624	0.0486	–

The pairwise F_{ST} values based on the complete mitogenome and microsatellite loci (in bold) are shown above and above the diagonal, respectively

the result with three genetic clusters of red muntjac from India. The species delimitation methods supported taxonomic resolution findings among *Muntjacs*, corroborating the divergence between *M. vaginalis* and *M. aureus* as distinct taxonomic units while grouping the *M. malabaricus* from the Western Ghats and Sri Lanka within a single taxonomic unit. We detected clear genetic signatures of the Northwestern and Western Ghats lineages of red muntjac, whereas few alleles of these lineages were observed in the Indian mainland red muntjac

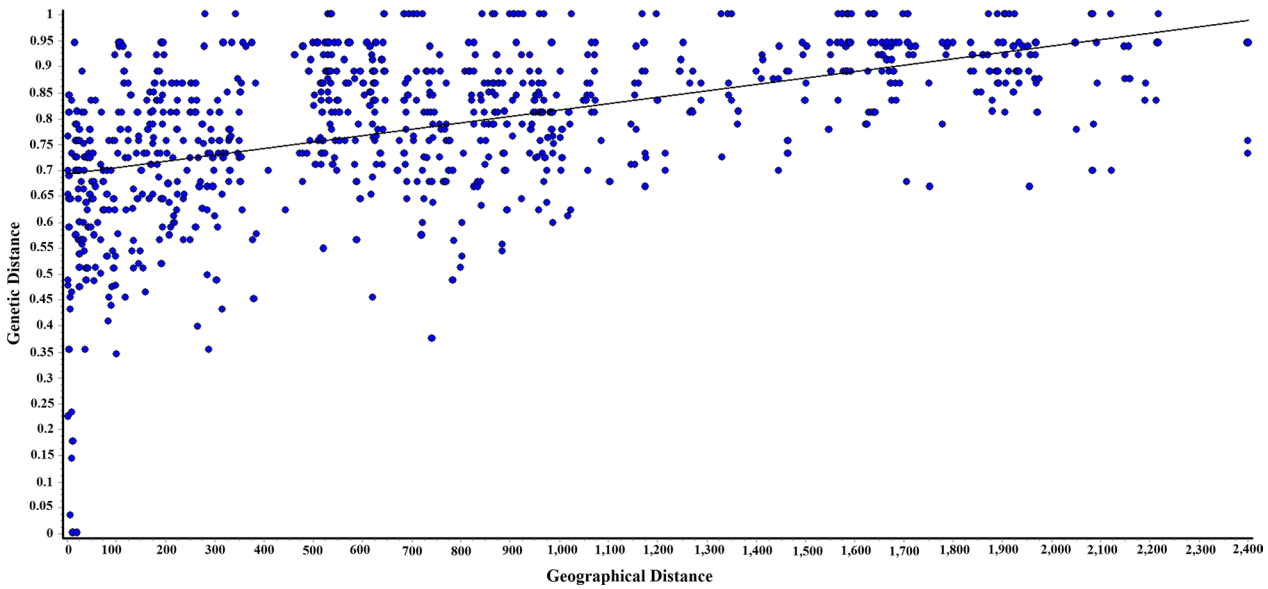

Fig. 7 Correlation of genetic and geographical distance in kilometer between red muntjac population using microsatellite data (Mantel test statistic, $rM = 0.513$; $P = 0.0009$)

indicating historical gene flow, while the current populations have undergone well-defined structuring and differentiation. Congruent to their distribution pattern, all red muntjac populations exhibited high haplotypes and microsatellite diversity with significant divergence among them. In addition, comparatively lower *Ar* and H_E were observed in *M. aureus*, which could be attributed to a limited distribution range. The mitogenomic data estimated the split between the Northwestern and Mainland lineages to late Pleistocene ~ 1.01 Mya ($CI_{95\%}$: 0.75–1.27), indicating the most recent split among red muntjacs. The Himalayan red muntjac inhabits the foothills of the Himalaya in the Northwestern part of the Indian states of Uttarakhand, Himachal Pradesh, and Punjab. The Northwest lineage was suspected to be distributed in Northwestern and Central India and Myanmar [5]. The presence of distinct genetic lineage from the Northwestern Indian region was also speculated by Martins et al. based on a single sequence (*IND1*) from Himachal Pradesh Province that formed a distinct placement [14]. However, due to the unavailability of sufficient sample size, explicit depiction of the lineage could not be done. Hence, we confirmed the previously suspected hypothesis and provided molecular evidence for the Northwestern lineage from India.

The rise of anthropogenic activities in the late Quaternary was a key factor that changed the global biodiversity pattern [21]. Qinghai-Tibetan Plateau (QTP) upliftment played a significant role in faunal and floral diversification and evolution in Himalayan ranges [25]. The upliftment of the Himalayas followed by the Plio-Pleistocene glaciation led to the evolution of high altitudinal elements shaping biogeographic evolution in the Indian Himalayan region [18]. The Himalayan region enabled allopatric speciation through geographic isolation and adaptive diversification across altitudinal gradients [26, 27]. This diversification was also driven by pre-adapted lineages immigrating and undergoing subsequent speciation [28, 29]. Long-term isolation in fragmented habitats restricted gene flow and caused genetic divergence [30–32] that contributed to the evolution of genetically distinct lineages [33]. It was followed by rapid civilization in the Indo-Gangetic plain of North India, causing extensive destruction of natural habitat, altering plants and animals' ecological and distributional patterns [18].

In India, the Western Ghats lineage was genetically more diverse than the Mainland, Northwestern and Sundaland populations. The phylogenetic analysis indicated that the Srilankan red muntjac (*M. malabaricus*) is the oldest red muntjac lineage showing genetic similarity with India's Western Ghats population. The genetic divergence result suggested that the Srilankan red muntjac split from the mainland lineage during Pleistocene ~ 2.2 Mya when the climatic condition was quite complex in India [14]. Despite the present biogeographic separation between Southern India and Sri Lanka, both populations are genetically similar at the mitogenomic level. A similar genetic relationship was observed in *Paradoxurus* (Palm civets), where Southern India and Sri Lanka clustered with each other [22].

The Western Ghats and Sri Lankan lineages' common origin might be due to the historical connectivity between these two landscapes. Thereafter, changes in sea levels may have led to isolation causing local endemism [22, 34, 35]. In India, the discontinuity of Western Ghats with mainland population could be due to unfavorable habitat conditions that culminated in isolated patches forming the refugia population inhabiting Western Ghats biodiversity hotspot [36]. The barrier formed by India's central dry zone restricted the gene flow between the Western Ghats and the rest of the Indian population of red muntjac. The restriction in species distribution is probably due to pronounced climate fluctuation in the last glacial maxima that caused the contraction of the rainforest, with forest-dwelling species restricted to the available habitats in high elevation areas [21, 22].

The mainland red muntjac (*M. vaginalis*) is distributed in a larger landscape in India (i.e., Chhattisgarh, Odisha, West Bengal, North East India, and Andaman & Nicobar Islands) and Indo-Chinese region (Vietnam, China and Thailand). The Andaman & Nicobar Islands individuals were genetically closer to the population of Indo-Chinese red muntjac. Unsuitable conditions limited the mainland population to small humid forest patches. Interglaciation led to the emergence of forests in drier areas that enabled former distributions to reoccupy and extend the red muntjac range. The Southern red muntjac inhabited the Sudanic region (Java, Sumatra, Bali and Borneo) and the lineage diversification has been described by Martins et al. [14]. The major transition zone between the Indochinese and Sundaic zoogeographic subregion is the Isthmus of Kra located on the Malay/Thai Peninsula that might act as a possible barrier preventing gene flow between populations [20, 37]. It is also speculated that rather than geophysical barrier, repeated rapid sea-level changes may have resulted in species' isolation [37]. A similar diversification pattern has also been observed in South and Southeast Asian species, like rodents, masked palm civet, common palm civet and *Macaca* spp., where the formation of the contemporary phylogeographic and genetic structure occurred during the same period [22, 38–41]. The Southern red muntjac split around 1.4 Mya from the Indochinese Mainland population. This divergence estimation is congruent with the lineages diversification in Amphibians [42]; Birds [43]; Mammals [37]; bats [44] and Leopard cat [45] that occurred in the Indochinese and Sundaic region. Faunal diversification between the Indochinese and Sundaic regions might have resulted from fluctuation in the Indian summer monsoon [46] and sea level rise [37, 47].

Conclusions

The genus *Muntiacus* is a useful model for identifying cryptic lineages and studying South and Southeast Asian biogeography. This study highlights the need for taxonomic revision within the red muntjac group and confirmed that the Himalayan red muntjac (*M. aureus*) has recently separated from *M. vaginalis*. We suggest the Himalayan red muntjac lineage to be managed as a distinct evolutionary significant unit (ESUs). There is also a need to reassess the conservation status in the IUCN Red List. Identifying concrete geographic limits of red muntjacs should be supplemented with rigorous and robust sampling in South and Southeast Asia. Hence, we suggest undertaking research based on mitochondrial and microsatellite markers to address red muntjacs' unclear status across their entire distribution range. The study contributes to understanding the large-scale drivers of species and provides insight into the linkage of environmental changes with distribution and niche dynamics. Based on these estimates, we can understand the species' speciation events in South and Southeast Asia impacted by biogeographical changes during the Late Pleistocene.

Methods

Sample collection and DNA extraction

We used 42 archival samples of northern red muntjac from different regions of India, including the Northwestern region (NWI = 18), Mainland (localities of north and central) (MLI = 14), northeastern (NEI = 3), Western Ghats (SI = 5) and Andaman & Nicobar Islands (AI = 2) (Additional file 1: Table S2, Table S4 and Fig. 8). Genomic DNA was extracted from tissue and hair samples using modified DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) protocol, whereas, for antlers and bone samples, we followed Gu-HCl-based silica binding method [48]. These samples were collected from dead animals by the local Forest Department from India's known localities and sent to Wildlife Forensic and Conservation Genetic Cell, Wildlife Institute of India, Dehradun. Since the samples were collected from the dead animals, the Animal Ethics Committee approval was not required. The authors confirm that all experiments were performed following the relevant guidelines and regulations.

PCR amplification of complete mitogenome and sequencing

Polymerase chain reactions (PCRs) amplifications were carried out in 20 µl volumes containing 10–20 ng of extracted genomic DNA containing, 1 × PCR buffer (Applied Biosystems), 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 5 pmol of each primer, and 5 units of Taq DNA polymerase (Thermo Scientific). We performed PCR

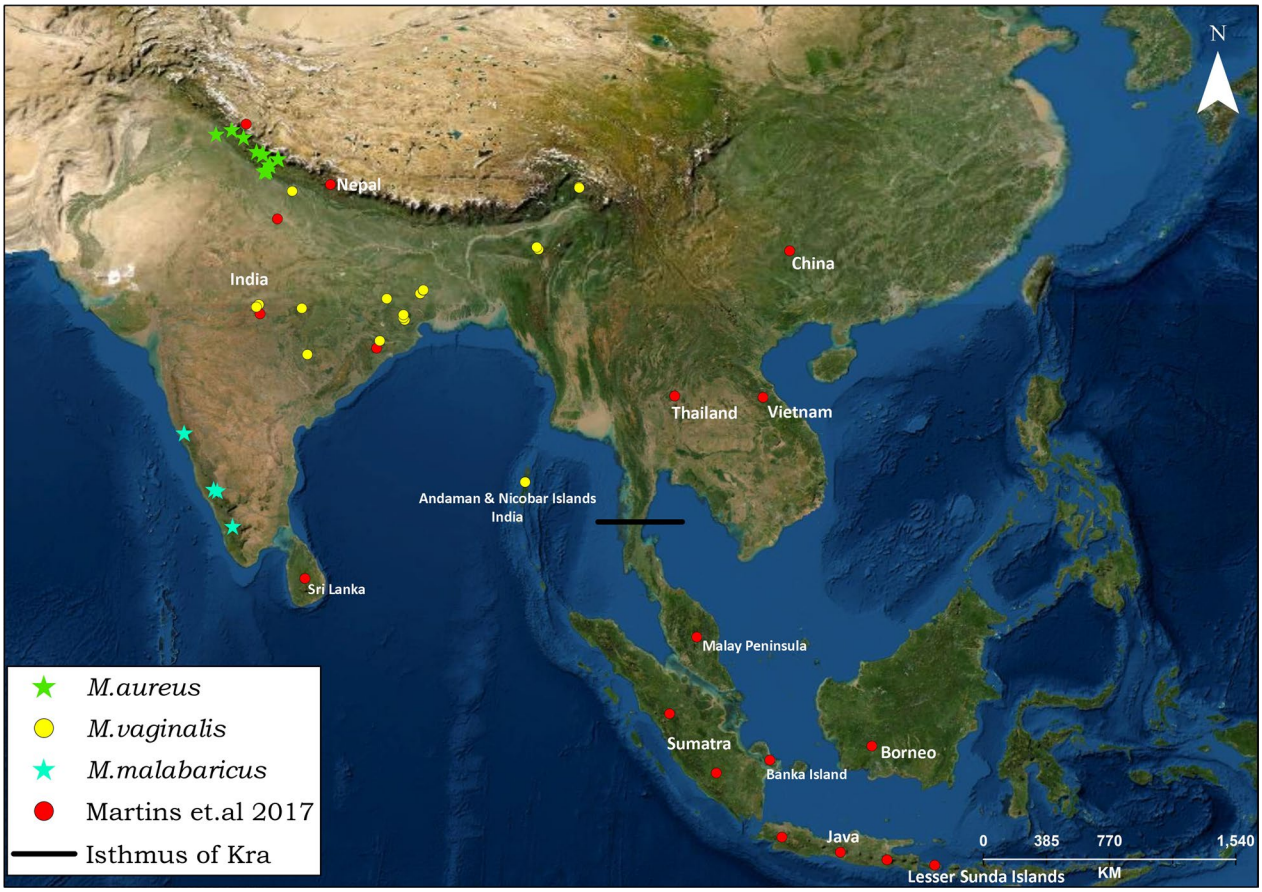

Fig. 8 Map depicting the sampling sites of red muntjacs. Yellow dots represent samples collected for the present study; red dots indicate the sampling location used in Martins et al. 2017. The horizontal black line indicates the position of the Isthmus of Kra

amplification using 23 overlapping fragments of complete mtDNA (Additional file 1: Table S3) [49]. Besides, we included the fragments of complete Cytochrome *b* [50] and Cytochrome c oxidase subunit-I gene [51] to increase the overlapping. PCR cycles for DNA amplification were 95 °C for 5 min; followed by 35 cycles of 95 °C for 40 s (denaturation), 54–56 °C (annealing) for 40 s, 72 °C for 50 s (extension) and a final extension of 72 °C for 15 min. The efficiency and reliability of reactions were monitored using controls. The PCR products were electrophoresed on 2% agarose gel and visualized under UV light in the presence of Ethidium bromide dye. The amplified PCR products were treated with Exonuclease-I and Shrimp alkaine phosphatase (USB, Cleveland, OH) for 15 min each at 37 °C and 80 °C, respectively, to remove any residual primer. The purified amplicons were then sequenced bidirectionally using BigDye Terminator 3.1 on an automated Genetic Analyzer 3500XL (Applied Biosystems, Carlsbad, CA, USA).

Microsatellite loci amplification and genotyping

Nine cross-species microsatellite loci: INRA001 [52], Ca18 [53], BM6506 [54], RT1, RT27, NVHRT48 [55], CelJP27 [56], and T156, T193 [57] were selected for population genetic analysis of red muntjac. Three sets of multiplex panels were carefully assembled based on molecular size and labeled fluorescent dyes of loci. To avoid ambiguity and amplification errors, for each sample, three multiplex PCR were carried out in 10 µl reaction volumes containing 5 µl of QIAGEN Multiplex PCR Buffer Mix (QIAGEN Inc.), 0.2 µM labeled forward primer (Applied Biosystems), 0.2 µM unlabeled reverse primer, and 20–100 ng of the template DNA. PCR cycles for loci amplification were 95 °C for 15 min; followed by 35 cycles of 95 °C for 45 s (denaturation), 55 °C (annealing) for 1 min, 72 °C for 1 min (extension) and a final extension of 60 °C for 30 min. The reliability of reactions was monitored using positive and negative controls. Alleles were resolved in an ABI 3500XL Genetic Analyzer (Applied Biosystems) using the LIZ 500 Size Standard

(Applied Biosystems) and analyzed using GeneMaker ver 2.7.4 [58].

Data analysis

A total of 16 complete mitogenome sequences of red muntjac from five different localities of India were generated (Additional file 1: Table S2 and GenBank accession No. MT671398-MT671408; MT758349-MT758353). Sequences obtained from forward and reverse direction were edited and assembled using SEQUENCHER® version 4.9 (Gene Codes Corporation, Ann Arbor, MI, USA). The annotation of complete mitogenome was done using Mitos WebServer [59] and MitoFish [60]. Careful manual annotation was also conducted by sequence alignment with their related homologs sequences or species for ensuring the gene boundaries. Additionally, 36 sequences of northern red muntjac (*M. vaginalis*) (n = 17); southern red muntjac (*M. muntjak*) (n = 17) and Sri Lankan (*M. malabaricus*) (n = 2) muntjac were included from GenBank to cover wide distribution ranges (Additional file 1: Table S1, Fig. 1). The dataset of 52 sequences of red muntjac was aligned using the CLUSTAL X 1.8 multiple alignment programs [61] and alignments were checked by visual inspection. DnaSP v 5 [62] was used to estimate the haplotype (h) and nucleotide (π) diversity. The neutrality statistical approaches Tajima's D [63] and Fu's *Fs* [64] were used to investigate the demographic history of each population using Arlequin ver 3.5 [65].

A total of nine polymorphic loci were used to analyze the 42 red muntjac samples for population genetic studies (Additional file 2: Table S5). The number of loci with genotyping error due to null alleles was assessed using MICROCHECKER 2.2.3 [66]. The CERVUS ver 3.0.6 program [67] was used to estimate the polymorphic information content (PIC), the number of alleles per locus, the observed (H_o) and, expected (H_E) heterozygosity. The *Ar* and mean inbreeding coefficient (F_{IS}) [68] was estimated using FSTAT ver 2.9.3 [69]. All the loci were checked for HWE in GenAlEx v6.5 [70].

Phylogeography and population genetic structure

The phylogenetic analysis was conducted in BEAST ver 1.7 [71]. Two outgroup species, *Bos javanicus* (JN632606) and *Moschus moschiferus* (FJ469675) were used to root the phylogenetic tree and the resulting tree was visualized with FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/). The spatial distribution of haplotypes was visualized by MJ network created using the PopART software [72]. The genetic distance between lineages was calculated based on the Tamura-3 parameter with a discrete Gamma distribution (TN92 + G) with the lowest BIC score value implemented in MEGA X [73].

To estimate divergence times of red muntjac clades, we inferred genealogies using a strick clock in BEAST ver 1.7 [71]. We performed the dating estimates using 16 sequences downloaded from NCBI, including the species of Cervidae, Muntiacini, Bovidae and Moschidae, i.e., the Chital (*Axis axis*, JN632599), Swamp deer (*Rucervus duvaucelii*, NC020743), Red deer (*Cervus elaphus*, AB245427), European Roe deer (*Capreolus capreolus*, KJ681491), Fallow deer (*Dama dama*, JN632629), Water deer (*Hydropotes inermis*, NC011821), Mule deer (*Odocoileus hemionus*, JN632670), Hog deer (*Axis porcinus,* MH443786), Formosan sambar (*Rusa unicolor swinhoei*, DQ989636), Tufted deer (*Elaphodus cephalophus,* DQ873526), Chinese muntjac (*Muntiacus reevesi,* NC008491), Giant muntjac (*Muntiacus vuquangensis,* FJ705435), Putao muntjac (*Muntiacus puhoatensis,* MF737190), Black muntjac (*Muntiacus crinifrons,* NC004577), Banteng (*Bos javanicus*, FJ997262), and Musk deer (*Moschus moschiferus*, FJ469675).

Divergence times of phylogenetic clades were calibrated using minimum age of fossil record at two points: one point was calibrated at 18 Mya (normal distribution prior, SD = 2) as the TMRCA (time to the most recent common ancestor) for the split between Bovidae and Moschidae, while the other was set to 17.2 Mya (normal distribution prior, SD = 2) for the split between Cervidae and Bovidae + Moschidae [74, 75]. We used a Yule-type speciation model and the HKY + I + G substitution rate model for tree reconstruction. We conducted two independent analyses, using MCMC lengths of 10 million generations, logging every 1000 generations. All the runs were evaluated in Tracer v. 1.6. The first 10% per run was discarded as burn-in. Maximum credibility trees were obtained with TreeAnnotator (implemented in BEAST ver 1.7 Package) The final phylogenetic tree was visualized in FigTree v.1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

To determine the most likely number of the genetic cluster within India, we performed a Bayesian clustering implemented in STRUCTURE 2.3.4 [76]. We analyzed our data using the admixture model and allele frequencies were assumed to be independent with a burn-in of 10,000 followed by 100,000 MCMC (Markov chain Monte Carlo) replications. Ten independent runs were carried out for each cluster set (K) from 1 to 10. We also used the ΔK metric to determine the statistically most support number of clusters (K) in web interface STRUCTURE HARVESTER [77]. Further, DAPC method was also implemented to identify the number of genetic clusters of the population using the ADEGENET package in R [78]. DAPC is a multivariate and model-free approach that maximizes the genetic differentiation between groups with unknown prior

clusters, thus improving populations' discrimination without requiring the population to be in HWE. FCA was also performed using the GENETIX 4.05 software package [79]. CONVERT 1.31 [80] was used to convert the required input file format. The pairwise F_{ST} values among the populations were calculated using GenAlEx ver6.5 [70]. The correlation between the pairwise genetic and geographic distances was performed to detect the pattern of isolation by distance between the disjointed areas, according to Mantel's test implemented in Alleles in Space 1.0 [81].

Species delimitation analyses

Species delimitation tests were conducted to validate taxonomic units of *Muntjacs* based on phylogenetic trees derived from whole mitogenome sequences. The analyses were performed using three different approaches: I- Multi-rate Poisson Tree Processes (mPTP) [82]; II- Bayesian Poisson Tree Processes (bPTP) [82]; and III- Generalized Mixed Yule-Coalescent (GMYC) [83].

The mPTP and bPTP analyses were performed on the phylogenetic tree using web server https://mptp.h-its.org and https://species.h-its.org, respectively. Specified parameters for MCMC, thinning, burn-in and seed value were kept as per default settings. The mPTP model employs a fast approach to estimate the maximum likelihood delimitation from an inferred phylogenetic tree, while the bPTP model adds Bayesian support values to the delimited species inferred from the phylogenetic tree. The GMYC model employed the phylogenetic tree after time calibration using HKY + I + G substitution rate model. It delimits species based on likelihood approach fitting branching models within and among species to reconstructed phylogenetic tree. The branch lengths were estimated under a relaxed log-normal clock algorithm in BEAST ver 1.7 [71]. We used MCMC lengths of 10 million generations, logging every 1000 generations. All the runs were evaluated in Tracer v. 1.6. The first 10% per run was discarded as burn-in. Maximum credibility trees were obtained with TreeAnnotator ver1.7 [71]. Single threshold of GMYC model was performed using web server https://species.h-its.org and the output tree is visualized in Tree view [84].

Abbreviations

IUCN: International Union for Conservation of Nature; MJ: Median-Joining; TMRCA: Time to a most recent common ancestor; PIC: Polymorphic information content; *Ho*: Observed heterozygosity; H_E: Expected heterozygosity; DAPC: Discriminant analysis of principal components; FCA: Factor correlation analysis; IBD: Isolation by distance; QTP: Qinghai-Tibetan Plateau; PCR: Polymerase chain reaction; mPTP: Multi-rate poisson tree processes; bPTP: Bayesian poisson tree processes; GMYC: Generalized mixed Yule-Coalescent.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12862-021-01780-2.

Additional file 1. Additional tables and figure.

Additional file 2: Table S5. Microsatellite genotype data of Indian red muntjac samples.

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Authors' contributions

SKG conceived the ideas; BS and AK generated the data and produced the DNA sequences; BS, AK and SKG analyzed the data; BS and AK led the writing; SKG and VPU supervised the study and writing of this paper. All authors read and approved the final manuscript.

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Availability of data and materials

Sequence data are available through NCBI GenBank (Accession No. MT671398-MT671408; MT758349-MT758353).

Declarations

Ethics approval and consent to participate

This study was conducted using the dead remains of the animals and no live animal was captured, hence Institutional Animal Ethics approval was not required.

Consent for publication

Not Applicable (All the information/images are owned by the authors).

Competing interests

The authors declare that they have no competing interests.

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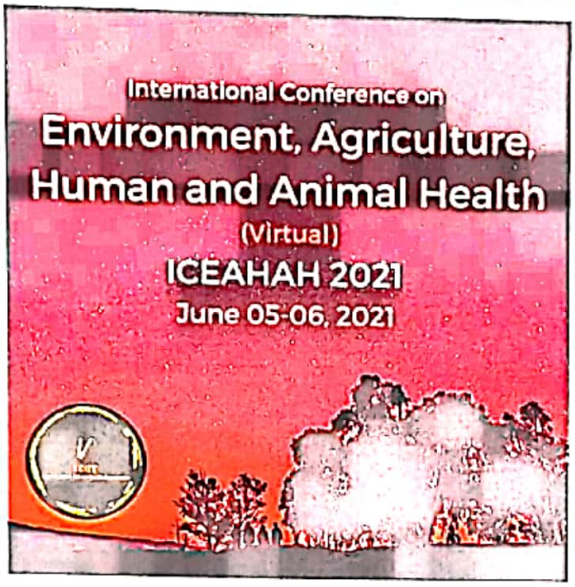

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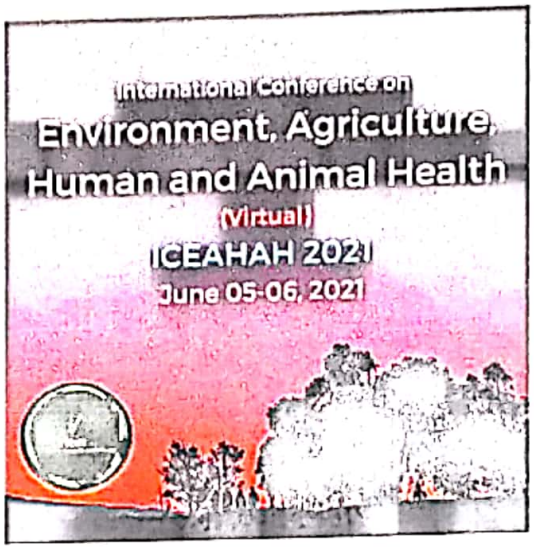

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Phylogeography and Phylogenetics of red muntjacs

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Molecular evidence of genetically distinct lineage of red muntjac from Uttarakhand, India

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Abstract:

Introduction

Identifying and understanding the factors that shaped population genetic structure and lineage diversification in continuous landscapes results of biogeographic boundaries has been a challenging goal in ecology and biogeography. Because of its wide distribution ranges and covers large vegetation types in South and Southeast Asia, the red muntjac represents an excellent model to test phylogeographic hypothesis, how many lineages of red muntjac are existing in India? Here, we investigated the phylogeographic pattern of red muntjac from Uttarakhand region and compared it with other known lineages, with the aim to address the genetic structure of red muntjac from North-West India and provide recommendations for a taxonomic revision.

Material and Methods

We investigated the phylogeographic pattern and population genetic structure by assaying variations at complete mitogenome. We sequenced 16 complete mitogenome of Indian red muntjac and also included 36 previously published sequences to cover wide geographical distribution range. Phylogenetic relationship, median-joining network and divergence dates were estimated using complete mitogenome.

Results

Our analysis revealed that Uttrakhand region along with Himachal Pradesh and Punjab hold distinct lineages of red muntjac. The newly identified lineages of Himalayan red muntjac found in North western part of India which is previously described based on their morphological differences. This study also highlights that presently India holds three mitochondrial lineages of red muntjac whereas four were identified from Indian subcontinent: Himalayan red muntjac (*M. (m.) aureus*), Northern red muntjac (*M. vaginalis*), Sri-Lankan+Western Ghat India (*M. malabaricus*) and Southern red muntjac (*M. muntjak*) from Sundaland. Estimates of the divergence timing indicate that the North-West lineages and Northern lineage split during the late Pleistocene approximately 0.83 Myr ($CI_{95\%}$:0.53–1.26), which is the younger lineages whereas *M. malabaricus* is the most primitive lineage among all the red muntjac.

Conclusion

The pronounced climate fluctuation during Quaternary period regarded as the most important factors influencing current distribution of muntjac species to restrict themselves in north-west areas. Our finding provided evidence of new lineages within red muntjac group

from Uttarakhand India, and it should be managed as evolutionary significant unit (ESUs). There is a need of taxonomic revision for Himalayan red muntjac for its conservation status under IUCN Redlist.

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