Genetic evidence indicates the occurrence of the Endangered Kashmir musk deer *Moschus cupreus* in Uttarakhand, India

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Abstract The Endangered Kashmir musk deer *Moschus cupreus* occurs in the western Himalayan region from Nepal to Afghanistan, but there is a lack of comprehensive and reliable information on its range. The region also harbours the Endangered Himalayan musk deer *Moschus leucogaster*, and this range overlap may have led to misidentification of the two musk deer species and errors in the delimitation of their ranges. Here, using genetic analysis of the mitochondrial DNA control region, we examined the phylogenetic relationship among musk deer samples from three regions in India: Ganderbal District in Jammu and Kashmir, and Kedarnath Wildlife Sanctuary and Nanda Devi Biosphere Reserve, both in Uttarakhand. The Bayesian phylogenetic analysis indicated a close genetic relationship between samples from Jammu and Kashmir, Kedarnath Wildlife Sanctuary and Nanda Devi Biosphere Reserve, validated by previously published sequences of Kashmir musk deer from Nepal. Our analyses confirmed the samples from Uttarakhand to be from the Kashmir musk deer, which was not previously known from this region. Therefore, we recommend further research in this area, to validate species identification and confirm the geographical distribution of the various species of musk deer. In addition, we recommend revision of the range of *M. cupreus* in the IUCN Red List assessment, to facilitate effective conservation and management of this Endangered species.

Keywords Control region, India, Kedarnath Wildlife Sanctuary, *Moschus cupreus*, *Moschus leucogaster*, mtDNA, musk deer, Nanda Devi Biosphere Reserve

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Introduction

Musk deer (genus *Moschus*, family Moschidae) are endemic to the Palearctic region, inhabiting fragmented areas of the Himalayan mountains, the Tibetan Plateau and the adjoining mountainous region in China and eastern Russia (Pan et al., 2015). They are solitary habitat specialists, mostly found in forests, alpine shrubland and above the treeline of alpine meadows. Seven species are recognized, of which five occur in the Himalayan range: the Kashmir *M. cupreus*, Alpine *M. chrysogaster*, Himalayan *M. leucogaster*, forest *M. berezovskii* and black *M. fuscus* musk deer (Grubb, 2005). Their populations are declining because of poaching for their musk pods, and habitat fragmentation and degradation. Because of unsustainable exploitation, all musk deer species have been included in the Appendices of CITES since 1979 (Zhou et al., 2004). Six musk deer species are categorized as Endangered on the IUCN Red List, and one as Vulnerable. In India, musk deer are included in Schedule I under the Wild Life (Protection) Act, 1972. Because of overlapping distribution ranges and morphological similarities, there is ambiguity regarding the taxonomy of musk deer species, hindering efficient conservation efforts (Pan et al., 2015).

The Kashmir musk deer is a little-studied species that occurs in Afghanistan (Ostrowski et al., 2016), Pakistan, India (Timmins & Duckworth, 2008) and Nepal (Singh et al., 2019). The historical distribution of musk deer species in the western Himalayan region has been primarily based on phenotypic characteristics such as external morphology and skull morphometry (Groves et al., 1995). Musk deer are cryptic, making species validation based solely on morphological traits unreliable (Grubb, 1982; Groves et al., 1995; Su et al., 2001). A recent study using molecular and camera-trap data reported a new record of Kashmir musk deer from Mustang in Nepal, west of the Annapurna Himalayas range (Singh et al., 2019), warranting an assessment of the range of the species. The Himalayan musk deer is morphologically similar to the Kashmir musk deer, with seasonally variable coat colours (Liu & Groves, 2014; Singh et al., 2019). Shukla et al. (2019) indicated the presence of different ecomorphs in musk deer species from Uttarakhand, India, suggesting the presence of distinct lineages whose validation warrants further investigation with molecular methods.

Advanced molecular tools for species identification and phylogenetic analysis have previously resolved phylogenetic

complexities in musk deer and aided species validation (Su et al., 2001; Pan et al., 2015). Genetic methods confirmed the presence of Himalayan musk deer, previously misidentified as Alpine musk deer, in Tibet (Guo et al., 2019). The mitochondrial DNA (mtDNA) control region has proven to be a robust marker for investigating intra-species genetic variation (Hu et al., 2006; Peng et al., 2009; Kumar et al., 2017; Gupta et al., 2018). Here, we describe novel genetic evidence for the presence of the Kashmir musk deer in the western Himalayan region of Uttarakhand, and assess its phylogenetic relationship with other musk deer species. Our findings provide insight into the range, evolutionary history and phylogeograpy of this enigmatic musk deer.

Methods

The samples used in this study originated from Kedarnath Wildlife Sanctuary and Nanda Devi Biosphere Reserve, both of which are located in Uttarakhand state, India. The 975 km² Kedarnath Wildlife Sanctuary is rich in biodiversity and one of the largest protected areas in the western Himalaya, covering altitudes of 1,025–3,662 m. To the east of Kedarnath Wildlife Sanctuary, the Valley of Flowers National Park forms a part of the Nanda Devi Biosphere Reserve (6,407 km², altitude 1,800–7,817 m). The Ganderbal District of Jammu and Kashmir is a hilly and semi-hilly area of 1,045 km², at altitudes of 1,650–3,000 m.

Samples and DNA extraction

We used a total of eight tissue samples of musk deer: six from Kedarnath Wildlife Sanctuary, one from Nanda Devi Biosphere Reserve, and one from Ganderbal, Jammu and Kashmir (Fig. 1, Supplementary Table 1). Seven of these samples (MDUK1–MDUK5, MDUK18 and MDJK1) were sent by the State Forest Departments to the Wildlife Forensic laboratory of the Wildlife Institute of India, and sample MDUK19 was collected in Nanda Devi Biosphere Reserve by a team from the Wildlife Institute of India. In addition, we included 12 musk pods sent by the Forest Department from the Chamoli district of the Kedarnath Wildlife Division, to assess the phylogenetic relationship between these samples and the samples from Uttarakhand and Jammu and Kashmir. We extracted genomic DNA (gDNA) from the samples using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) protocol.

PCR amplification and sequencing

We performed the PCR in 20 µl reaction volumes containing 10–20 ng of extracted gDNA. The PCR master mix contained: 1 × PCR buffer (Applied Biosystems, Thermo Fisher Scientific, Waltham, USA), 2.0 mM $MgCl_2$, 0.2 mM of each dNTP, 2 pmol of each primer, and 5U of Taq DNA polymerase. We successfully amplified 485-bp-long portions of mtDNA control region using the primers Cerv.tPro: 5′-CCACYATC AACACCCAAAGC-3′ and CervCRH: 5′-GCCCTGAARA AAGAACCAGATG-3′ (Balakrishnan et al., 2003). The PCR conditions were: an initial denaturation for 5 minutes at 95 °C, followed by 35 cycles at 95 °C for 45 s, 55 °C for 45 s and 72 °C for 45 s, with a final extension of 72 °C for 15 minutes. The efficiency and reliability of PCR were monitored using positive and negative control reactions. The PCR products were electrophoresed on 2% agarose gel and visualized under UV light. Positive amplicons were treated with Exonuclease-I and USB Shrimp alkaine phosphatase (Affymetrix, Inc., Santa Clara, USA) for 15 min each at 37 °C and 80 °C, respectively, to remove any reaction residues. The purified fragments were sequenced directly in an Applied Biosystems Genetic Analyzer 3500XL from both primers, and set using a BigDye 3.1 kit (Applied Biosystems, Thermo Fisher Scientific, Waltham, USA).

Data analysis

We sequenced the generated amplicons from both directions of targeted mtDNA fragments and edited them with *SEQUENCHER 4.9* (Gene Codes Corporation, Ann Arbor, USA). The sequences were aligned and visually inspected using the *CLUSTAL X 1.8* multiple alignment programme (Thompson et al., 1997). We validated the species of all eight samples with known geographical origin (MDUK1–MDUK5, MDUK18, MDUK19 and MDJK1) by comparison with published musk deer sequences: *M. moschiferus* (n = 2), *M. chrysogaster* (n = 3), *M. anhuiensis* (n = 1), *M. leucogaster* (n = 20), *M. berezovskii* (n = 24) and *M. cupreus* (n = 5) from GenBank (National Center for Biotechnology Information, Bethesda, USA; Supplementary Table 2). Because sequences of *M. cupreus* from Afghanistan were not available in GenBank, we validated *M. cupreus* using sequences from Nepal (Singh et al., 2019). Thereafter, the sequences of seized musk pods (n = 12) from Uttarakhand were further validated with data from samples of known geographical origin, and with sequences from Singh et al. (2019). All sequences were further included for phylogenetic analysis.

We calculated the number of haplotypes in the dataset (from the eight samples of known provenance, 12 seized musk pods and *M. cupreus* sequences from Nepal) using *DnaSP 5.0* (Librado & Rozas, 2009). The Bayesian phylogenetic tree among all the mtDNA control region sequences of musk deer was constructed using *BEAUti and the BEAST 1.7* (Drummond et al., 2012). We used a sequence from the Indian mouse deer *Moschiola indica* (NC037993) as the outgroup. We deployed the best-fit nucleotide substitution model Hasegawa–Kishono–Yano (HKY) + G + I to obtain the best tree topology in phylogenetic analysis. The

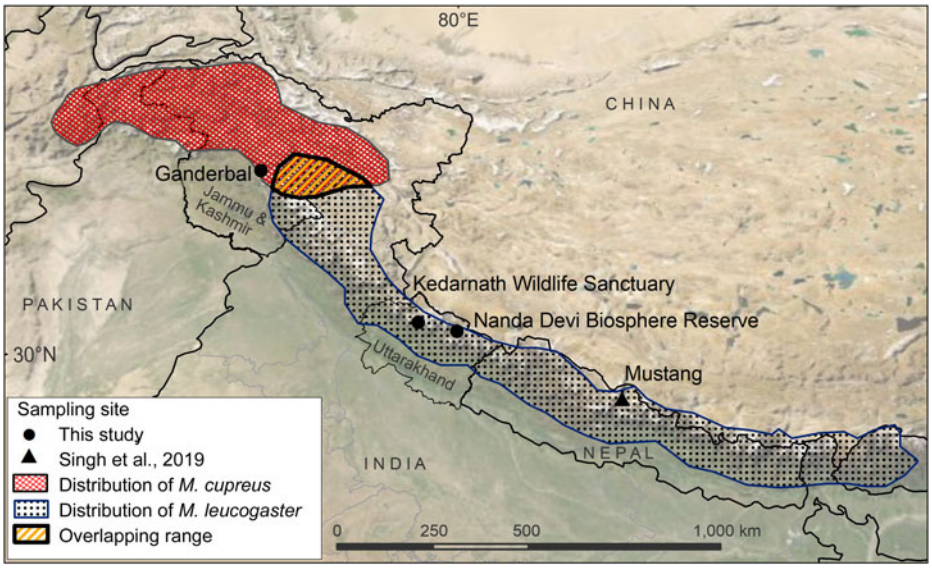

FIG. 1 Sampling locations from this study and Sing et al. (2019), and distribution ranges of Kashmir musk deer *Moschus cupreus* and Himalayan musk deer *Moschus leucogaster* according to the IUCN Red List assessments (Timmins & Duckworth, 2015a,b).

Markov chain Monte Carlo process was run for 10 million generations with a random starting tree and we sampled one tree every 1,000 generations. We discarded the first 25% of generations as burn-in. Convergence of values and effective sample size was assessed in *Tracer 1.7* (Rambaut et al., 2018). The resulting phylogenetic trees were visualized in *FigTree 1.4.0* (Rambaut & Drummond, 2012). The spatial distribution of haplotypes was generated using a median-joining network in *PopART* (Leigh & Bryant, 2015). The evolutionary divergence among sequence pairs between musk deer groups was estimated with a p-distance model, including substitution transition and transversion calculated in *MEGA X* (Kumar et al., 2018); p-distance is the proportion of nucleotide sites by which two compared sequences differ.

Results

In the Bayesian phylogenetic analysis, the seven samples from Uttarakhand (MDUK1–MDUK5, MDUK18, MDUK19) clustered into a clade with a high posterior probability (PP) value (PP ~ 0.99) with *M. cupreus* sequences from Jammu and Kashmir (MDJK1) and published sequences from Nepal, whereas *M. moschiferus* formed the basal clade (PP ~ 0.75; Fig. 2). All seized musk pods (MDUK6–MDUK17) showed 98.5–100% homology with the *M. cupreus* sequences in GenBank and also clustered within the clade of *M. cupreus* (Supplementary Fig. 1). All 25 sequences of musk deer from this study and from Singh et al. (2019) were grouped into eight haplotypes (Supplementary Table 3). Haplotype 1 was common in samples from both Nepal and Uttarakhand, representing three and seven sequences, respectively. Five unique haplotypes (Haplotypes 3–7) were only present in the samples from Uttarakhand, whereas Haplotypes 2 and 8 were unique to samples from Nepal and Jammu and Kashmir, respectively. The Bayesian Inference tree topology indicated that *M. cupreus* and *M. moschiferus* had evolved earlier than *M. chrysogaster*, *M. anhuiensis*, *M. leucogaster* and *M. berezovskii* (Supplementary Fig. 2). We have submitted the newly identified haplotypes of *M. cupreus* to GenBank (Accession numbers MT822697–MT822703). The sequences of six musk deer species were grouped into 38 haplotypes (Supplementary Fig. 3). The median-joining network showed weak haplotype clustering within *M. berezovskii* and *M. chrysogaster*, whereas strong structuring was observed in *M. cupreus* and *M. leucogaster*.

The mean pairwise genetic distance analysis indicated that *M. cupreus* from Nepal were genetically similar to the Jammu and Kashmir and Uttarakhand populations, with low sequence divergences estimated between the groups (1%) and within the species group (0.8%). Among the musk deer species, *M. cupreus* was closest to *M. moschiferus* (8.8–9.0%) followed by *M. leucogaster* (10%), whereas the maximum genetic difference was observed with *M. berezovskii* (10.9%) (Table 1). High intra-species divergences were observed in *M. chrysogaster* (6.3%) and *M. berezovskii* (4.7%). We observed weak genetic clustering within *M. berezovskii*, forming two separate clades. Moreover, the complete mitogenome sequence (JQ608470) of *M. chrysogaster* clustered within the *M. berezovskii* clade, raising concerns over its validity (Supplementary Figs 2 & 3). The high divergence in *M. berezovskii* requires research to facilitate lineage confirmation.

Discussion

Our study indicates the presence of *M. cupreus* in Jammu and Kashmir and Uttarakhand, India. The analysis was supported by strong phylogenetic affinities with recently identified lineages of *M. cupreus* from Mustang, Nepal (Grubb, 2005; Singh et al., 2019). It was also corroborated by

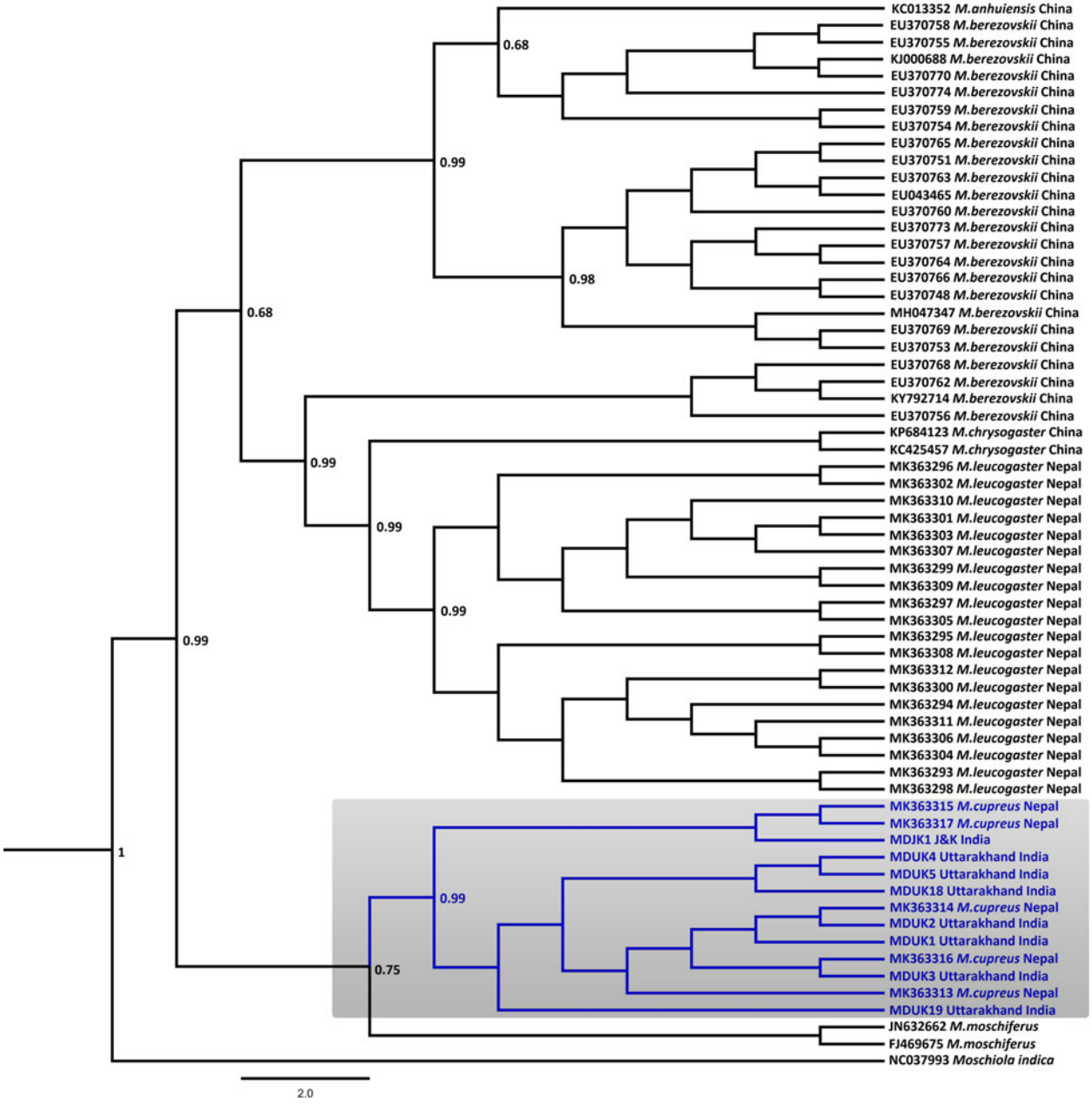

FIG. 2 Bayesian (Markov chain Monte Carlo) consensus tree of musk deer based on the mtDNA control region. Posterior probability values are provided at their respective nodes. The Indian mouse deer *Moschiola indica* (NC037993) was used as the outgroup. The clade of the Kashmir musk deer *M. cupreus* is shaded. The scale bar represents substitutions of nucleotides.

TABLE 1 Genetic p-distance of the mtDNA control region of the genus *Moschus* are represented below the diagonal and standard error values are shown above the diagonal.

	Species	1	2	3	4	5	6	7
1	*M. cupreus* (Nepal)		0.003	0.012	0.013	0.013	0.014	0.012
2	*M. cupreus* (India)	0.010		0.012	0.013	0.013	0.014	0.013
3	*M. moschiferus*	0.088	0.090		0.013	0.012	0.013	0.012
4	*M. leucogaster*	0.100	0.101	0.094		0.009	0.013	0.011
5	*M. chrysogaster*	0.103	0.102	0.095	0.047		0.012	0.008
6	*M. anhuiensis*	0.107	0.112	0.100	0.090	0.063		0.007
7	*M. berezovskii*	0.109	0.111	0.098	0.093	0.071	0.046	

predictions based on mapping of climate refugia and habitat suitability that suggested the occurrence of *M. cupreus* in the Himalayan belt stretching from central Nepal to north-west India (including Uttarakhand and Himachal Pradesh), through the Kashmir region to Afghanistan (Singh et al., 2020).

The genetic evidence presented here highlights the need for extensive sampling of musk deer species across their range, to facilitate reliable identification of species and to update information on their distribution. Uttarakhand's western Himalayan region is believed to harbour *M. leucogaster* (Timmins & Duckworth, 2015b) as well as *M. cupreus* (Timmins & Duckworth, 2015a). However, all samples analysed in this study were of *M. cupreus*, which raises the possibility that *M. cupreus* may have been misidentified as *M. leucogaster* because of morphological similarities between the two species. This warrants a comprehensive reassessment of the distribution ranges of *M. cupreus* and *M. leucogaster* in the western Himalayas, to enable effective management of these threatened musk deer species. All musk deer populations are decreasing, primarily because of poaching for musk pods, but also as a result of habitat degradation and hunting for meat consumption. Reliable information on each species' distribution will help guide enforcement agencies such as local forest departments and management authorities to formulate appropriate strategies for in situ and ex situ conservation.

In addition, a range-wide population assessment will help identify poaching hotspots and combat wildlife trafficking. Therefore, we recommend extensive field sampling, including the recording of ecological data and photographic evidence, to clarify the distribution limits of the various musk deer species in the Indian subcontinent. All musk deer species should be treated as distinct evolutionary significant units, requiring long-term monitoring and evidence-based management. Reliable information about the distribution of cryptic species such as musk deer is crucial for implementing effective laws to protect and manage them.

We provide the first evidence of *M. cupreus* from Kedarnath Wildlife Sanctuary and Nanda Devi Biosphere Reserve in Uttarakhand, India, based on genetic analysis of the mtDNA control region. Our findings provide new information on the species' geographical distribution and will aid in formulating effective conservation strategies for this Endangered species. We recommend revising the distribution range of *M. cupreus* in the IUCN Red List record for this species, to support evidence-based management of musk deer in the region. Future research should include a comprehensive ecological and molecular assessment, with high throughput sequencing and microsatellite markers, and molecular tracking of confiscated items in the wildlife trade. A collaborative study across all range countries of musk deer species is vital for a comprehensive population and distribution assessment of these threatened species.

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Author contributions Conceptualization of study: SKG, AK, BS; data analysis: AK, BS, SS, KBG; writing: all authors; revision: AK, SKG.

Conflicts of interest None.

Ethical standards This research abided by the *Oryx* guidelines on ethical standards. All tissue samples used in this study were collected and forwarded by the State Forest Department of Uttarakhand and Jammu and Kashmir to the Wildlife Institute of India. All samples were collected from dead animals; approval from an institutional animal ethics committee was thus not required.

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