1 **Complete mitogenome of Kashmir musk deer (*Moschus cupreus*) and its comparative**
2 **phylogenetic relationships**

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19 **Abstract**

20 The endangered Kashmir musk deer (*Moschus cupreus*) is native to the high altitudinal
 21 region of the Himalayas. In this study, we sequenced, annotated and characterized the
 22 complete mitogenome of *M. cupreus* to gain insight into the molecular phylogeny and
 23 evolution of musk deer. The mitogenome of *M. cupreus*, which is 16,354 bp long comprised
 24 13 protein-coding genes (PCGs), 22 transfer RNA genes (tRNAs), two ribosomal RNA genes
 25 (rRNAs) and non-coding control region. The *M. cupreus* mitogenome composition was
 26 highly A+T biased 68.42%, and exhibited a positive AT skew (0.082) and negative GC skew
 27 (0.307). The phylogenetic analysis suggested that KMD is the most primitive extant species
 28 in the genus *Moschus* whereas Alpine musk deer (*M. chrysogaster*) and Himalayan musk
 29 deer (*M. leucogaster*) are closely related. This result confirmed the placement of *M. cupreus*
 30 within the monotypic family Moschidae of musk deer. This study provides a better
 31 understanding of lineage identification and musk deer evolution for further research.

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33 **Keywords**

34 Moschidae; *Moschus cupreus*; musk; mitochondrial genome; phylogenetics

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36 Introduction

37 The Kashmir musk deer (*Moschus cupreus*) is a member of the genus *Moschus* in the
 38 monotypic family Moschidae (Grubb 1982). The species are endemic to the Palearctic region
 39 and mostly inhabit high altitudinal mountainous regions of the Himalayan ranges (Grubb
 40 1982). According to the IUCN database, the current population is in decline due to
 41 overexploitation, primarily caused by habitat destruction and illegal hunting of musk pod and
 42 skin in wildlife trade. The IUCN Red List includes *M. cupreus* in the ‘Endangered’ category
 43 and has listed in Appendix I under the Convention on International Trade in Endangered
 44 Species of Wild Fauna and Flora (CITES). In India, musk deer is categorized under the
 45 Schedule I of the Indian Wildlife Protection Act, 1972. Due to limited ecological and genetic
 46 evidence, the distribution range and evolutionary history of the species are still unclear (Pan
 47 et al., 2015). According to Grubb 2005, the distribution of *M. cupreus* is restricted to the
 48 Himalayan region of Kashmir, Pakistan and Afghanistan. However, a recent molecular study
 49 has provided a new distribution record of *M. cupreus* from Mustang, Nepal and west of
 50 Annapurna Himalayas range (Singh et al., 2019). The availability of complete mitogenomes
 51 will furnish molecular evidence that will resolve distribution, evolutionary as well as
 52 phylogenetic dilemmas. Out of the seven *Moschus* species, mitogenomes of *M. cupreus* and
 53 *M. fuscus* are still lacking. Due to maternal mode of inheritance, presence of conserved genes,
 54 rare recombination and high evolutionary rate, the mitogenomic data has been widely
 55 employed in phylogenetics and evolutionary genomics (Avise et al., 1985, Boore 1999).
 56 Hence, it warrants a comprehensive phylogenetic study of *M. cupreus* for clear species
 57 delimitation which would help in designing scientific strategies for conservation breeding
 58 musk deer.

59 Therefore, we sequenced and characterized for the first time, the complete mitogenome of *M.*
 60 *cupreus*. We further investigated the phylogenetic relationship of *M. cupreus* with other
 61 *Moschus* species.

62 Materials and methods

63 Two tissue samples of *M. cupreus* were used from the repository of Wildlife Forensic and
 64 Conservation Genetics (WFCG) Cell of our institute. These samples were forwarded by the
 65 State Forest Departments of Jammu & Kashmir and Uttarakhand. Genomic DNA (gDNA)

was extracted from the samples using the DNeasy Blood Tissue Kit (QIAGEN, Germany) in a final elution volume of 100 µl. The extracted DNA was checked by 0.8% agarose gel and thereafter diluted in final concentration in 40 ng/µl for further PCR amplification.

PCR amplification and sequencing:

The complete mtDNA genome was amplified and sequenced by using 21 different sets of primers (Hassanin et al., 2009). In addition to the available set of primers, 3 fragments did not successfully amplify, therefore, we designed novel primers to cover the gaps (Supplementary file: ST1). DNA amplifications were performed in 20 µl reaction volume containing 1 × PCR buffer (50mM KCl, 10mM Tris-HCl, and pH 8.3), 1.5mM MgCl₂, 0.2mM of each dNTPs, 3 pmol of each primer, 5U of DreamTaq DNA Polymerase and 1 µl (~30 ng) of template DNA. The PCR protocol was set as follows: 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 undertaken at 72 °C for 10 min. PCR amplification was confirmed by electrophoresis and visualized under UV transilluminator. To remove unwanted residual primers, the PCR products were then treated with Exonuclease-I and Shrimp Alkaline Phosphatase (Thermo Scientific Inc.) at 37°C for 20 min and at 85°C for 15 min. The purified fragments were sequenced directly in an Applied Biosystems Genetic Analyzer, ABI 3500 XL in both directions.

Mitogenome alignment and annotation

The complete mitogenome of *M. cupreus* was generated by aligning the overlapping fragments of DNA sequences using Sequencher® version 5.4.6 (Gene Codes Corporation, Ann Arbor, MI, USA). Annotation of mitogenome was performed by using the Mitos Web Server (Bernt et al., 2013). The complete mtDNA of 16,354 bp was obtained and the gene map of *M. cupreus* was generated using CGView Server (Grant et al., 2008). MEGA X (Kumar et al., 2018) was implemented to calculate the base composition in the mtDNA genetic code. Bias in nucleotide composition among the complete mitogenome of *M. cupreus* was estimated using skew analysis where: AT skew = (A-T)/(A+T) and GC skew = (G-C)/(G+C) (Perna et al., 1995). The Relative Synonymous Codon Usage (RSCU) values of 13 protein-coding genes (PCGs) of *M. cupreus* were calculated using MEGA X (Kumar et al., 2018). The intergenic spacer and overlapping regions interspersed between genes of complete mitogenome were estimated manually.

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100 **Phylogenetic analysis and genetic differentiation**

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102 The phylogenetic analysis was performed using the whole mitogenome of *M. cupreus*
103 sequenced along with available GenBank database of *Moschus* species: Alpine musk deer (*M.*
104 *chrysogaster* KP684123 and KC425457), Himalayan musk deer (*M. leucogaster* NC042604
105 and MG602790), Siberian musk deer (*M. moschiferus* FJ469675), Anhui musk deer (*M.*
106 *anhuiensis* KP684124 and KC013352) and Forest musk deer (*M. berezovskii* EU043465). To
107 get a better insight into the phylogeny of musk deer, we also included eight mitogenomes
108 representing Cervini, Bovini, and Boselaphini from GenBank. Besides, one sequence of
109 *Moschiola indica* (KY290452) was chosen as an outgroup. A bayesian consensus tree was
110 constructed in BEAST version 1.7 (Drummond et al., 2012). Bayesian inference analysis was
111 finalized by using Monte Carlo Markov Chain (MCMC) chain method for 10 million
112 generations and sampling one tree at every 1000 generations using a burn-in of 5000
113 generations. FigTree v1.4.0 (<http://tree.bio.ed.ac.uk/software/figtree/>) was used to visualize
114 the phylogenetic tree. We estimated the similarity of complete mitogenomes among musk
115 deer species using MatGAT (Campanella et al., 2003). Additionally, we calculated gene-
116 specific mean pairwise genetic distance within the musk deer species to elucidate
117 differentiation among the 13 PCGs using MEGAX (Kumar et al., 2018) implementing
118 Tamura 3-Parameter Model (T92 + G).

119 **Results and discussion**

120 **Mitogenome organization**

121 We obtained a 16,354 bp long circular mitochondrial genome of Kashmir Musk deer (*M.*
122 *cupreus*) which has been submitted in the GenBank (MTXXXXXX and MTXXXXXX). It
123 showed the typical mammalian mitogenomic arrangement with 22 tRNA genes, 13 protein-
124 coding genes (PCGs), two rRNA genes and a non-coding hypervariable control region (Fig.
125 1). The mitogenome arrangement and gene distribution of *M. cupreus* showed similarity with
126 other *Moschus* species (Jang et al., 2010; Guo et al., 2018). The total nucleotide composition
127 in *M. cupreus* was: A (33.9%), T (28.8%), C (24.4%) and G (12.9%) (Table 2). Apart from
128 ND6 gene, the other eight tRNA genes (tRNAGln, tRNAAla, tRNAAsn, tRNACys,
129 tRNATyr, tRNASer, tRNAGlu, tRNAPro) were also encoded on the light strand (L-strand)
130 whereas remaining genes were encoded on the heavy strand (H-strand). Six pairs of
131 overlapping regions in the mitogenome were observed among tRNA-Val / 16S rRNA, tRNA-
132 Ile / tRNA-Gln, COI / tRNA-Ser, ATP8 / ATP6, ND4L / ND4 and tRNA-Thr / tRNA-Pro.

The overlapping region ranges from 1 to 33 bp. The smallest overlapping region was found between tRNA-Thr / tRNA-Pro (-1 bp) whereas the longest was between ATP8 / ATP6 (-33 bp). Between tRNA-Pro and tRNA-Phe, a hypervariable control region was positioned. Furthermore, 22 intergenic spacers were found in between the mitochondrial regions which range from 1 to 32 bp length, while the longest space was found between tRNA-Asn and tRNA-Cys (Table 1). To identify nucleotide composition in the complete mitogenome of *M. cupreus*, we examined the values of AT-skew, GC-skew, and AT%. The resultant value of AT-skew was positive whereas the value of GC-skew was negative. The A+T and G+C content in the whole mitogenome was found to be AT biased with 62.5% and 37.5% respectively (Table 2).

Protein coding genes (PCGs)

The total length of the 13 PCGs was estimated at 11,404 bp in *M. cupreus* mitogenome that included 37 bp overlapping fragments constituting 69.50% of the mitogenome (Table 2). The nucleotide composition of PCGs was A = 33% T = 29.5%, G = 11.6 and C=26. We found a higher abundance of AT% than GC%. The *M. cupreus* PCGs comprised twelve majority strand or H-strand genes (NADH dehydrogenases: ND1, ND2, ND3, ND4, ND5, and ND4L; three cytochrome c oxidases: COI, COII, and COIII; two ATPases: ATP6 and ATP8, and one cytochrome b: Cyt *b* gene) and one minority strand or L-strand gene (NADH dehydrogenase: ND6 gene) (Table 1). To understand the nucleotide distribution in PCGs, we estimated base skewness resulting in AT and GC skew of 0.056 and -0.383 receptively in PCGs. This suggests that in *M. cupreus*, an abundance of A is significantly higher than that of T while the abundance of C is significantly higher than that of G (Table 2). All the 13 PCGs started with ATN codon (ATG or ATA or ATT), congruent with other musk deer species. Excluding the stop codons, the relative synonymous codon usage (RSCU) for the 13 PCGs of *M. cupreus* comprised 3801 codons (Fig. 2).

Ribosomal RNA and transfer RNA genes

In the *M. cupreus* mitogenome, two ribosomal RNA and twenty-two tRNA genes were recognized. The 12S rRNA was 955 bp long while 16S rRNA was 1572 bp long. The 12S rRNA gene was situated between tRNA-Phe and tRNA-Val, and likewise, the 16S rRNA gene was situated between tRNA-Val and tRNA-Leu. The total AT content of the two ribosomal rRNA was 61.5%, which was consistent with other species of musk deer (Table 3). The estimated average values of AT and GC skews in rRNA was found to be 0.211 and

–0.098 receptively (Table 2). 22 tRNA genes were distributed with variable lengths from tRNA-Ser (60 bp) to (tRNA-Leu (75 bp). The overall AT and GC content in 1511 bp long tRNA was 64.5% and 35.5% respectively. The resultant value of AT and GC skew was 0.026 and 0.086, receptively (Table 2). Out of the 22 tRNAs, fourteen genes were located on H-strand while the other eight were on L-strand (Table 1).

Mitochondrial control region sequence

The CR region was positioned between tRNA^{Pro} and tRNA^{Phe} which was 925 bp long. The AT content was higher than the GC content with the base composition of CR region as 32.2% A, 32.3% T, 13.8% G and 21.6% C. The skew value of AT and GC for CR was 0.002 and –0.220, respectively (Table 2). The CR sequence of *M. cupreus* was found to be smaller as compared to the previously reported Himalayan Musk deer (*M. leucogaster*) sequence of 1001 bp with a 77 bp long insertion (Guo et al., 2018). The insertions and deletions (INDEL) have been studied in many mammalian species such as *Rucervus eldii*: 78 bp (Balakrishnan et al., 2003), *Homo sapiens*: 9 bp (Thangaraj et al., 2005), *Rusa unicolor*: 40 bp (Gupta et al., 2015), and *Rucervus duvaucelii*: 94 bp (Kumar et al., 2017).

Phylogenetic relationship and genetic distance

The phylogenetic relationship was estimated based on the complete mitogenomes of *Moschus* species with other species of Cervini, Bovini, and Boselaphini. The result indicated all *Moschus* species clustering in a monophyletic clade distinct from other species of Cervini, Bovini, and Boselaphini (Fig. 3). Previously, *M. moschiferus* was reported as the most primitive species in genus *Moschus* (Vislobokova et al., 2009) while the study by Pan et al., 2015 suggested that the ancestral lineage of the present musk deer was likely to be distributed in the Tibet Plateau margin or adjacent mountains around the Sichuan Basin. Our phylogenetic tree indicated that *M. cupreus* was the basal clade in *Moschus*. This indicated *M. cupreus* to be the most primitive ancestral species in the genus *Moschus*.

We calculated the genetic relatedness of *M. cupreus* with other musk deer species based on 13 PCGs, 2 rRNA and Control region (Fig. 4). The overall genetic similarity of *M. cupreus* indicated high genetic similarity (average 92%) with other musk deer species. We also calculated gene-wise genetic differentiation where results indicated varied genetic differentiation in all 13 PCG genes of musk deer (Fig. 5).

Conclusion

Previously, *M. cupreus* has been described as a subspecies of *M. chrysogaster* (Timmins et al., 2015). Based on morphological similarity, *M. cupreus* was also confused with *M. leucogaster*. Thereafter, Groves et al., 1995 speculated that it might be a separate species, which was subsequently confirmed by Grubb 2005. Evolutionary history and phylogenetic position of *M. cupreus* in the genus *Moschus* was still uncertain due to limited genetic information. The novel complete mitogenome of Kashmir Musk deer (*M. cupreus*) will aid in accurate species identification and form baseline information for further exploration into *Moschus* and related species. Availability of large scale mitogenomic data will also help enforcement agencies in molecular tracking of seized wildlife items from musk deer apart from accurately guiding in-situ and ex-situ management strategies to efficiently conserve musk deer. It will also help in wildlife forensics for the identification of musk deer body parts (musk pod, canine and meat) recovered in the illegal wildlife trade.

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277 **Table 1: Organization of the mitochondrial genome of *M. cupreus***

Gene	Positions			Codon			Strand	Space/overlap [#]
	Start	End	Size	Start	Stop*	Anti-codon		
tRNA-Phe	1	68	68	-	-	GAA	H	0
12S ribosomal RNA	69	1023	955	-	-	-	H	0
tRNA-Val	1024	1090	67	-	-	TAC	H	0
16S ribosomal RNA	1089	2660	1572	-	-	-	H	-2
tRNA-Leu	2662	2736	75	-	-	TAA	H	+1
ND1	2740	3690	951	ATG	-AA	-	H	+3
tRNA-Ile	3696	3764	69	-	-	GAT	H	+5
tRNA-Gln	3762	3833	72	-	-	TTG	L	-3
tRNA-Met	3836	3904	69	-	-	CAT	H	+2
ND2	3905	4942	1038	ATA	TA-	-	H	0
tRNA-Trp	4947	5013	67	-	-	TCA	H	+4
tRNA-Ala	5015	5083	69	-	-	TGC	L	+1
tRNA-Asn	5085	5157	73	-	-	GTT	L	+1
tRNA-Cys	5190	5255	66	-	-	GCA	L	+32
tRNA-Tyr	5256	5323	68	-	-	GTA	L	0
COX1	5325	6869	1545	ATG	TAA	-	H	+1
tRNA-Ser	6867	6935	69	-	-	TGA	L	-3
tRNA-Asp	6943	7010	68	-	-	GTC	H	+7
COX2	7012	7692	681	ATG	TA-	-	H	+1
tRNA-Lys	7699	7766	68	-	-	TTT	H	+6
ATP8	7768	7962	195	ATG	T--	-	H	+1
ATP6	7929	8603	675	ATG	TAA	-	H	-33
COX3	8609	9391	783	ATG	T--	-	H	+5
tRNA-Gly	9393	9461	69	-	-	TCC	H	+1
ND3	9462	9806	345	ATA	T--	-	H	0
tRNA-Arg	9809	9877	69	-	-	TCG	H	+2
ND4L	9878	10171	294	ATG	T--	-	H	0
ND4	10168	11535	1368	ATG	T--	-	H	-4
tRNA-His	11546	11615	70	-	-	GTG	H	+10
tRNA-Ser	11616	11675	60	-	-	GCT	H	0
tRNA-Leu	11677	11746	70	-	-	TAG	H	+1
ND5	11747	13549	1803	ATT	TA-	-	H	0
ND6	13557	14075	519	ATA	T--	-	L	+7
tRNA-Glu	14077	14147	71	-	-	TTC	L	+1
CYTB	14152	15285	1134	ATG	T--	-	H	+4
tRNA-Thr	15295	15364	70	-	-	TGT	H	+9
tRNA-Pro	15364	15429	66	-	-	TGG	L	-1
control region	15430	16354	925	-	-	-	H	0

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Table 2: Nucleotide composition and Skewness in the complete mitogenome of *M. cupreus*

	Size (bp)	A%	T%	AT skew	G%	C%	GC skew
Whole mitogenome	16354	33.9	28.8	0.082	12.9	24.4	-0.307
PCGs	11404	33.0	29.4	0.056	11.6	26.0	-0.383
tRNAs	1511	33.1	31.4	0.026	19.3	16.2	0.086
rRNAs	2527	37.6	24.5	0.211	17.1	20.8	-0.098
Control region	925	32.3	32.3	-0.002	13.8	21.6	-0.220

Table 3: Nucleotide composition indices in different regions of mitogenomes of *Moschus* species

Species	Accession number	Whole Mitogenome		Protein Coding Genes (PCGs)		Ribosomal RNA (rrnl)		Control region	
		Length (bp)	AT (%)	Length (bp)	AT (%)	Length (bp)	AT (%)	Length (bp)	AT (%)
<i>M. cupreus</i>	MTXXXXXX	16354	62.5	11404.0	62.3	2527.0	61.9	925	64.5
<i>M. chrysogaster</i>	KP684123	16354	61.9	11404.0	61.6	2528.0	61.5	924	63.2
<i>M. leucogaster</i>	NC042604	16431	62.0	11404.0	61.6	2528.0	61.6	1001	63.9
<i>M. anhuiensis</i>	KP684124	16352	62.0	11404.0	61.8	2527.0	61.2	923	62.5
<i>M. berezovskii</i>	EU043465	16354	62.1	11404.0	62.0	2528.0	61.2	924	62.8
<i>M. moschiferus</i>	FJ469675	16351	62.2	11401.0	61.9	2528.0	61.5	923	63.7

Figure 4: Graph showing the similarity of *M. cupreus* with other musk deer species.

Figure 5: Comparative pairwise genetic distance in the protein-coding gene of musk deer species