Arxius de Miscel·lània Zoològica, 17 (2019): 209–218 ISSN: 1698–0476

mtDNA confirms the presence of *Moschus leucogaster* (Ruminantia, Moschidae) in Gaurishankar Conservation Area, Nepal

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Shrestha, B. B., Khatiwada, J. R., Thanet, D. R., 2019. mtDNA confirms the presence of *Moschus leucogaster* (Ruminantia, Moschidae) in Gaurishankar Conservation Area, Nepal. *Arxius de Miscel·lània Zoològica*, 17: 209–218, doi: https://doi.org/10.32800/amz.2019.17.0209

Abstract

mtDNA confirms the presence of Moschus leucogaster *(Ruminantia, Moschidae) in Gaurishankar Conservation Area, Nepal.* Musk deer (genus *Moschus*), an endangered mammal, is not only of great concern for its conservation, but it is also of great interest to understand its taxonomic and phylogenetic associations in Nepal. The aim of this study was to identify the taxonomic status of musk deer in Gaurishankar Conservation Area (GCA) using mitochondrial genomic data of cytochrome b (370 bps) through phylogenetic analysis of all the species of musk deer. The results showed that the species found in GCA is confirmed as Himalayan musk deer *Moschus leucogaster,* further expanding its distributional range in Nepal.

Key words: *Moschus leucogaster*, Phylogenetic analysis, Gaurishankar Conservation Area, Nepal

Resumen

El ADN mitocondrial confirma la presencia de Moschus leucogaster *(Ruminantia, Moschidae) en el Área de Conservación de Gaurishankar, Nepal.* El ciervo almizclero del Himalaya (género *Moschus*), un mamífero amenazado, no es solo objeto de gran preocupación por lo que respecta a su conservación sino también de gran interés para entender sus asociaciones taxonómicas y filogenéticas en Nepal. El objetivo de este estudio ha sido identificar el estatus taxonómico del ciervo almizclero del Himalaya en el Área de Conservación de Gaurishankar (GCA) utilizando datos genómicos mitocondriales del citocromo b (370 bps) mediante análisis filogenéticos de todas las especies de ciervo almizclero. Los resultados han mostrado que la especie hallada en GCA es el ciervo almizclero del Himalaya *Moschus leucogaster*, lo que amplía en mayor medida su área de distribución en Nepal.

Palabras clave: *Moschus leucogaster*, Análisis filogenético, Área de Conservación de Gaurishankar, Nepal

Resum

L'ADN mitocondrial confirma la presència de Moschus leucogaster *(Ruminantia, Moschidae) a l'Àrea de Conservació de Gaurishankar, Nepal.* El cérvol mesquer de ventre blanc (gènere *Moschus*), un mamífer amenaçat, no és només objecte de gran preocupació pel que fa a la conservació sinó també de gran interès per entendre les seves associacions taxonòmiques i filogenètiques al Nepal. L'objectiu d'aquest estudi ha estat identificar l'estatus taxonòmic del cérvol mesquer de ventre blanc a l'Àrea de Conservació de Gaurishankar (GCA) utilitzant dades genòmiques mitocondrials del citocrom b (370 bps) mitjançant anàlisis filogenètiques de totes les espècies de cérvol mesquer. Els resultats han mostrat que l'espècie trobada es el cérvol mesquer de ventre blanc *Moschus leucogaster*, la qual cosa amplia encara més la seva àrea de distribució al Nepal.

Paraules clau: *Moschus leucogaster*, Anàlisi filogenètica, Àrea de Conservació de Gaurishankar, Nepal

Received: 19/07/2019; Conditional acceptance: 11/10/2019; Final acceptance: 12/11/2019

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Introduction

With the advent of biotechnology, genetic information can be obtained through animals' degraded remains such as bones, dried skins, feces and fossils (Ramón-Laca et al., 2015; Silva et al., 2015). Genetic information can be accrued from DNA contained with these animal specimens. DNA test is the most popular method for identification of species. In this system, DNA sequence of mitochrondrial cytochrome b (cyt–b) gene has been widely used for species' identifications (Khatiwada et al., 2015). Cyt–b is probably the best–known mitochondrial gene with respect to function and structure of its protein product (Esposti et al., 1993). It is used as a valuable tool for the construction of the evolutionary relationship among population, species, and higher taxa (Harrison, 1989; Su et al., 1999) since it contains both slowly and rapidly evolving codon positions, as well as more conservative and more variable regions or domains (Meyer and Wilson, 1990; Irwin et al., 1991; Cantatore et al., 1994; Farias et al., 2001).

Musk deer (genus *Moschus,* Linnaeus, 1758) are widely distributed in the sub–alpine and alpine vegetation (2,500 to 4,500 m) of the Himalayan region of Nepal (Kattel, 1992). All the species of musk deer belongs to order Cetartiodactyla of family Moschidae (IUCN, 2013). Although many morphological studies have been conducted on the taxonomy of this group, there are still controversies regarding the number of its species and sub–species and the phylogenetic relationship among them (Groves et al., 1995). Seven species within the genus *Moschus* are recognized in the world. They include Anhui musk deer (*M. anhuiensis* Wang et al., 1982), Kashmir musk deer (*M. cupreus* Grubb, 1982), Siberian musk deer (*M. moschiferus* Linnaeus, 1758), black musk deer (*M. fuscus* Li, 1981), Himalayan musk deer (*M. leucogaster* Hodgson, 1839), forest musk deer (*M. berezovskii* Flerov, 1929) and Alpine musk deer (*M. chrysogaster* Hodgson, 1839) (IUCN, 2013). Three species of musk deer ie *M. chrysogaster*, *M. leucogaster* and *M. fuscus* are said to be found in Nepal (Jnawali et al., 2011; Timmins and Duckworth, 2015; Wang and Harris, 2015; Harris, 2016) but their

exact distribution and status are still dubious, and they all were considered as one species: *M. chrysogaster* before their taxonomic separation (Jnawali et al., 2011). Although all the three species of musk deer found in Nepal are categorized as Endangered by IUCN (IUCN, 2013), only *M. chrysogaster* is enlisted as a protected mammal by the National Park and Wildlife Conservation Act 1973 due to lack of documentation on the confirmation of species of musk deer (Jnawali et al., 2011). There are only a few studies on taxonomic and phylogenetic analysis of musk deer in the context of Nepal (Singh et al., 2019). The Gaurishankar Conservation Area (GCA) is the newly established conservation area where molecular studies confirming the species presence are crucial for its conservation and management in the future. In this study, we aimed to determine the taxonomic status of musk deer in the GCA using mitochondrial genomic data of cytochrome b through phylogenetic analysis of all the species of musk deer.

Material and methods

Study area

The GCA is located in central Nepal, encompassing Ramechhap, Dolakha and Sindhupalchok districts. It has an area of 2,179 km^2 (fig. 1). It was declared the 'Conservation Area' in January 2010, and its management was entrusted for twenty years to the National Trust for Nature Conservation (NTNC) in July 2010 (DNPWC, 2011). It is located in the high mountain physiographic region of Nepal and consists of 35.38 % forestland, 9.76 % shrubland and 8.79 % grassland. It has 16 major vegetation types and great faunal diversity that includes 34 species of mammals, 16 species of fishes, 10 species of amphibians, 8 species of lizards, 14 species of snakes and 235 species of birds (DNPWC, 2013). Besides musk deer (*Moschus* spp.), endangered species found in the conservation area are snow leopard (*Panthera uncia*), clouded leopard (*Neofelis lupus*), leopard cat (*Felis benghalensis*), red panda (*Ailurus fulgens*), wolf (*Canis lupus*), and Chinese pangolin (*Manis pentadactyla*) (DNPWC, 2013; Shrestha and Meng, 2014).

Our study included the surrounding areas of Risan Gumbo Himal from Hum danda to Gumbo danda at Lapche area of Lamabagar VDC of Dolakha district which encompasses an elevation range of 3,500 to 4,200 m and lies between 28º 6' 7" and 28º 7' 3" N latitude and 86º 9' 59" and 86º 10' 52" E longitude. It has four types of vegetation: Betula forest mostly dominated by *Betula utilis*; mixed forest having a mixed species of *Betula utilis, Abies spectabilis, Sorbus* spp.*, Rhododendron campanulatum, Salix* spp. and *Juniperus indica*; rhododendron forest mostly dominated by *Rhododendron campanulatum;* and alpine scrub mostly dominated by shrubby rhododendron species i.e., *Rhododendron lepidotum, Rhododendron ciliatum* and *Rhododendron anthopogan.*

Pellet sample collection

We opportunistically collected 39 fecal pellet samples (faeces) from the Lapche area of GCA (fig. 1) between 2013 and 2014. These samples were preserved in 95 % ethanol for further molecular analysis. For each pellet sample, the sampling date, the location and geographical coordinates were recorded. The sample pellets were used for laboratory analysis for species' identification.

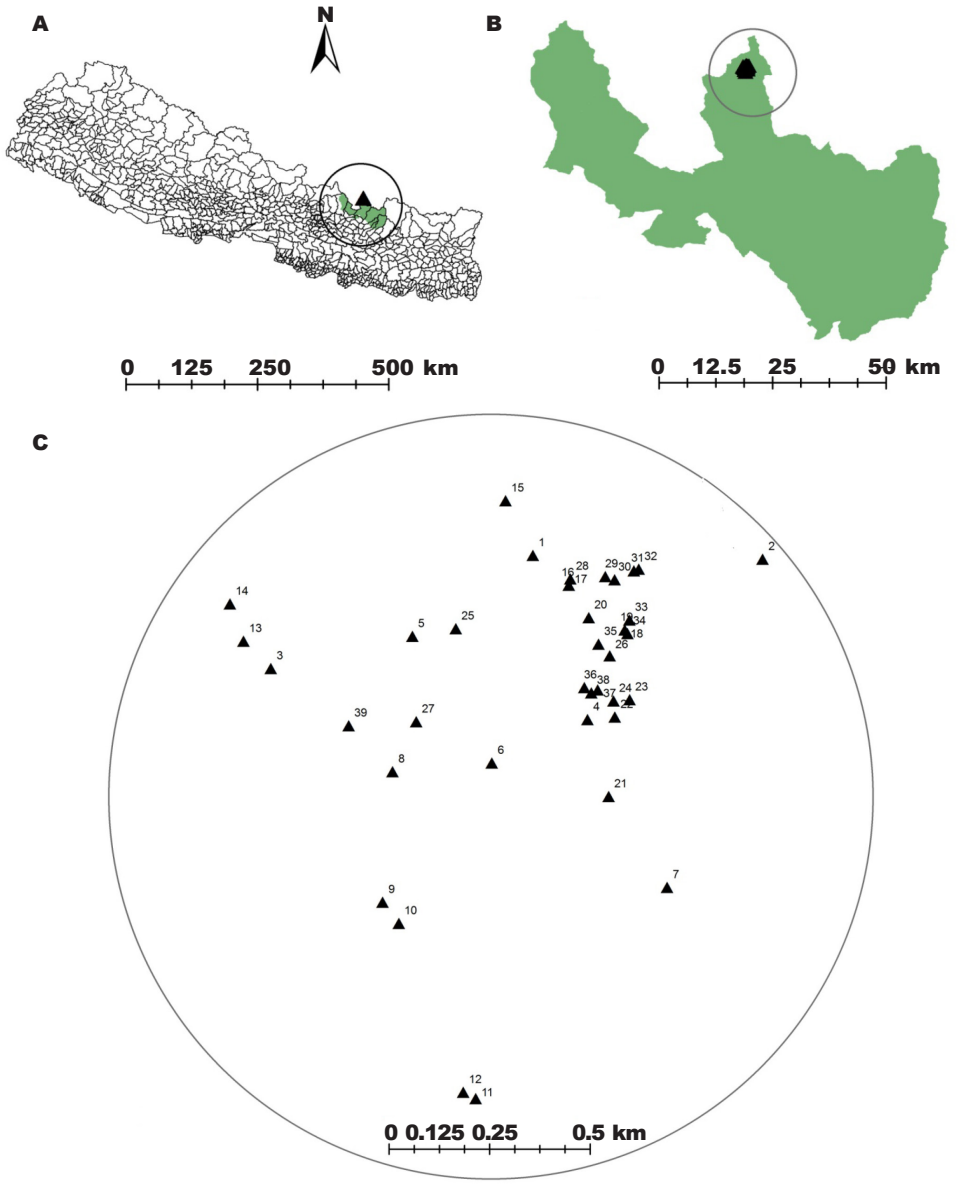

Fig. 1. Map showing the study area: A, location of Gaurishankar Conservation Area in Nepal; B, location of Lapche of Dolakha District where the samples were collected; C, location of the 39 sampling sites in the Lapche area of GCA. (The triangles indicate the fresh latrines from where samples were collected.)

Fig. 1. Mapa que muestra el área de estudio: A, localización del Área de Conservación de Gaurishankar en Nepal; B, localizavión de Lapche, en el distrito de Dolakha donde se recolectaron las muestras; C, localización de los 39 puntos de muestreo en el área de Lapche de GCA. (Los triángulos indican los puntos donde se recogieron muestras fecales.)

DNA extraction, PCR amplification and sequencing

A dry pellet from each sample was cut into smaller pieces using sterile scissors and tweezers and further processed for DNA extraction as recommended in the protocol by Qiagen QIAamp DNA Stool kit (Qiagen, Valencia, CA, USA). Polymerase chain reaction (PCR) was used to amplify the mitochondrial genes cytochrome b (cyt–b). The primers and PCR conditions were used as described by Pan et al. (2015). PCR products were visualized in 1.5 % agarose gel and positive PCR products were sequenced following bi–directional sequencing from a ABI 3100 automated sequencer.

Sequence analysis

All available nucleotide sequences of the cyt–b gene of *Moschus* species were downloaded from the NCBI GenBank database and the data source made available by Pan et al. (2015) and Singh et al. (2019) (table 1). The sequences of *Alces alces americana, Ovis aries* and *Tragulus kanchil* were also downloaded to be used as outgroups. Only 10 of the 39 pellet samples yielded a complete sequence. All nucleotide sequences were assembled by SeqMan and visually checked to determine the accuracy of the variables site identified by the program. All the sequences were then aligned with ClustalW in BIOEDIT Version 7.1.9 (Thompson et al., 1994) using the default settings. All the newly determined sequences were deposited in GenBank under accession numbers (MN720942–MN720951). Phylogenetic analysis was conducted using the maximum likelihood (ML) estimation. The maximum likelihood analysis was conducted in MEGA7 with 1,000 bootstraps (Kumar et al., 2016).

Results and discussion

Phylogenetic relationship of musk deer from GCA

The aligned dataset of cyt–b sequence contained 370 bps including 69 variable sites and 51 parsimony informative sites (excluding outgroups). The phylogenetic relationships strongly support genus *Moschus* as a monophyletic clade with higher bootstrap supports (bootstrap = 99). Molecular data analysis suggested that the population of musk deer in Lapche, GCA, were genetically similar with *M. leucogaster* from western Nepal and Qinhai, Tibet, China and were nested together in a ML tree (fig. 2). The uncorrected genetic divergence of the cyt–b gene sequences among the *M. leucogaster* population of Lapche, GCA, Manang, Nepal and Tibet, China ranged from 0.00 to 0.3 % (table 2) whereas relatively low genetic divergences between *M. leucogaster* and its closest relatives *M. chrysogaster* and *M. fuscus* were 1.4 % and 1.7 % respectively.

The pellet samples used in this study confirmed the presence of Himalayan musk deer *M. leucogaster* in the GCA. Although three species of musk deer (*Moschus fuscus, Moschus chrysogaster* and *Moschus leucogaster*) are said to be distributed in Nepal (Timmins and Duckworth, 2015; Wang and Harris, 2015; Harris, 2016), most studies conducted in central and eastern Nepal regarding distribution, habitat ecology, latrines, associated plant composition and diversity, and gastro–intestinal parasites of musk deer have considered musk deer species as *M. chrysogaster* (Aryal et al., 2010; Aryal and Subedi, 2011; Subedi et al., 2012; Shrestha and Moe, 2015; Achhami et al., 2016). The potential misidentification of musk deer species is due to their secretive behaviour and similar morphological characteristics (Groves et al., 1995; Su et al., 2000; Guha et al., 2007). As musk deer are shy and nocturnal it is difficult to detect them in daytime (Green, 1986). Additionally, if they are seen in daytime they hide in the shrub understory, and even if they are encountered in forest and open areas they are visible only for a few seconds (Singh et al., 2019). Furthermore, it is not easy to identify the species of musk deer from their physical appearance and morphological

Table 1. GenBank accession number (N) of specimens used in the phylogenetic analysis.

Tabla 1. Número de entreda en GenBank (N) de los especímenes utilizados en el análisis filogenético.

Species	Locality	N
M. leucogaster	Lapche, GCA, Nepal	MN720942
M. leucogaster	Lapche, GCA, Nepal	MN720943
M. leucogaster	Lapche, GCA, Nepal	MN720944
M. leucogaster	Lapche, GCA, Nepal	MN720945
M. leucogaster	Lapche, GCA, Nepal	MN720946
M. leucogaster	Lapche, GCA, Nepal	MN720947
M. leucogaster	Lapche, GCA, Nepal	MN720948
M. leucogaster	Lapche, GCA, Nepal	MN720949
M. leucogaster	Lapche, GCA, Nepal	MN720950
M. leucogaster	Lapche, GCA, Nepal	MN720951
M. leucogaster	Humde, Manang, ACA Nepal	MK363276
M. leucogaster	Chame, Manang, ACA, Nepal	MK363279
M. cupreus	Marpha, Mustang, ACA, Nepal	MK363288
M. cupreus	Nuristan, Afghanistan	MK363292
M. leucogaster	Tibet, China	AF026889
M. anhuiensis	Huoshan, Anhui Province, China	NC020017
M. anhuiensis	Yuexi, Anhui Province, China	KP684124
M. berezovskii	Maerkang, Sichuan Province, China	EU043465.
M. berezovskii	China	NC012694
M. moschiferus	China	JN632662
M. moschiferus	China	NC013753
M. chrysogaster	Xinglong Mountain, Gansu Province, China	KC425457
M. chrysogaster	Qinghai Lake, Qinghai Province, China	KP684123
M. fuscus	Bijiang, Yunnan Province, China	AF026888
O. aries	–	NC001941
T. kanchil	–	JN632709
A. americana	–	M98484

characteristics (Groves et al., 1995; Singh et al., 2019). Singh et al. (2019) confirmed the presence of Himalayan musk deer *M. leucogaster* in Manang and Kaski district. Based on their study, we can predict that the distribution of *M. leucogaster* may extend up to central and eastern parts of Nepal. The GCA lies in the central part of Nepal close to Kaski and Manang district. This study confirms the presence of *Moschus leucogaster* in central Nepal and further supports the possible distribution of this species in Central and Eastern Nepal, including Langtang National Park, Makalu Barun National Park, Sagarmatha National Park and Kanchanjunga Conservation Area, and adjoining areas of Central and Eastern Nepal.

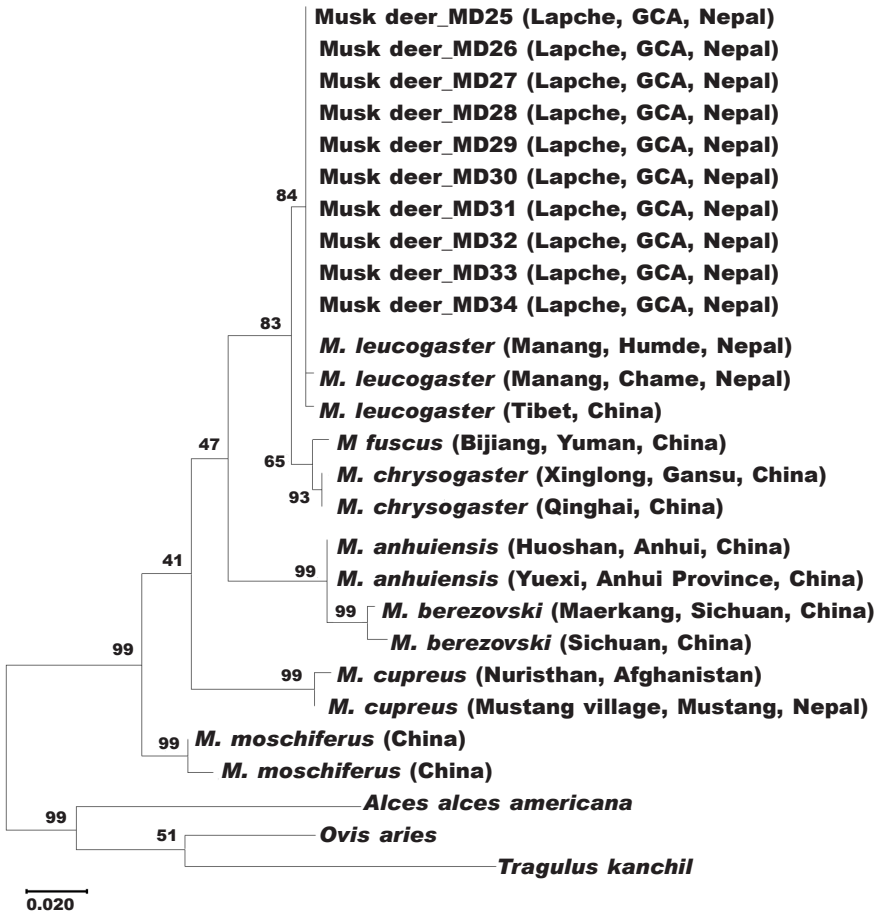

Fig. 2. Maximum likelihood tree estimated based on mtDNA cyt–b sequences. Values on branches of the tree show the bootstrap support value for maximum likelihood. *A. americana, O. aries* and *T. kanchil* were selected as outgroups. Sample names correspond to those given in table 1.

Fig. 2. Árbol de máxima probabilidad estimada basado en secuencias de ADN mitocondrial (cyt–b). Los valores de las ramas del árbol muestran el valor de soporte bootstrap para la máxima probabilidad. A. americana, O. aries *y* T. kanchil *se seleccionaron como exogrupos. Los nombres de las muestras corresponden a los indicados en la tabla 1.*

Table 2. Genetic uncorrected p–distance of the cyt–b sequences of the genus *Moschus* used in this study. Only two samples were used for this analysis as there was no genetic variation among 10 samples from Lapche.

Tabla 2. Distancia genética no corregida (p–distance) de las secuencias cyt–b del género Moschus *utilizadas en este estudio. Utilizamos únicamente dos muestras para este análisis dado que no existieron variaciones genéticas entre las 10 muestras de Lapche.*

	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	*M. leucogaster* – Lapche	–																		
2	*M. leucogaster* – Lapche	0.000																		
3	*M. leucogaster* – Manang	0.000	0.000																	
4	*M. leucogaster* – Manang	0.000	0.000	0.000																
5	*M. cupreus* – Mustang	0.082	0.082	0.082	0.082															
6	*M. cupreus* – Afghanistan	0.078	0.078	0.078	0.078	0.004														
7	*M. berezovskii*	0.082	0.082	0.082	0.082	0.122	0.118													
8	*M. berezovskii*	0.086	0.086	0.086	0.086	0.126	0.121	0.007												
9	*M. anhuiensis*	0.061	0.061	0.061	0.061	0.099	0.095	0.018	0.022											
10	*M. anhuiensis*	0.061	0.061	0.061	0.061	0.099	0.095	0.018	0.022	0.000										
11	*M. moschiferus*	0.072	0.072	0.072	0.072	0.086	0.082	0.094	0.098	0.073	0.073									
12	*M. moschiferus*	0.080	0.080	0.080	0.080	0.094	0.090	0.094	0.098	0.073	0.073	0.007								
13	*M. chrysogatser*	0.007	0.007	0.007	0.007	0.074	0.070	0.090	0.094	0.069	0.069	0.072	0.080							
14	*M. chrysogaster*	0.007	0.007	0.007	0.007	0.074	0.070	0.090	0.094	0.069	0.069	0.072	0.080	0.000						
15	*M. fuscus*	0.011	0.011	0.011	0.011	0.078	0.074	0.086	0.090	0.065	0.065	0.068	0.076	0.011	0.011					
16	*M. leucogaster* – Tibet	0.004	0.004	0.004	0.004	0.086	0.082	0.086	0.090	0.065	0.065	0.076	0.085	0.011	0.011	0.007				
17	*O. aries*	0.156	0.156	0.156	0.156	0.198	0.192	0.193	0.192	0.176	0.176	0.174	0.179	0.156	0.156	0.170	0.160			
18	*T. kanchil*	0.209	0.209	0.209	0.209	0.242	0.237	0.242	0.249	0.244	0.244	0.207	0.218	0.220	0.220	0.214	0.214	0.203		
19	*A. americana*	0.186	0.186	0.186	0.186	0.205	0.200	0.209	0.202	0.207	0.207	0.180	0.185	0.176	0.176	0.181	0.191	0.177	0.229	

Acknowledgements

This study was financially supported by Mohamed bin Zayed Species Conservation Fund, UAE (Project no. 13257839) and Idea Wild. We would like to thank Department of National Parks and Wildlife Conservation, Nepal and National Trust for Nature conservation for granting research permission in Gaurishankar Conservation Area. Dr. Janak Raj Khatiwada is supported by Chinese Academy of Sciences President's International Fellowship Initiative (2018PB0016) postdoctoral fellowship.

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