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Sequencing and Characterization of the Complete Mitochondrial Genome of Tufted Deer, *Elaphodus cephalophus* (Artiodactyla: Cervidae) and its Phylogenetic Position within the Family Cervidae

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LJ and WC conceived and planned the experiments. LZ, XB and ZZ performed the experiments, analyzed and interpreted the data. XB and HQ analyzed the sequence data. LJ and WC wrote the manuscript with input from all authors. All authors read and approved the manuscript.

Key words
***Elaphodus cephalophus*, Complete mitochondrial genome, Control region, Protein-coding genes, Molecular phylogeny**

ABSTRACT

The tufted deer (*Elaphodus cephalophus*) is a unique species in the genus *Elaphodus*. Currently, very little is known about its nuclear and mitochondrial genomes and their phylogenetic position within the family Cervidae. In this study, we have determined the complete sequence of the mitochondrial genome of the tufted deer, by using a long polymerase chain reaction (PCR) technique. The entire mtDNA sequence is 16,196 bp in length, which compared with previous studies is the least, containing 13 protein-coding genes, two ribosomal RNA genes, 22 transfer RNA genes, and a non-coding control region (CR, D-loop). The overall base composition included 33.37 % A, 29.33 % T, 23.94 % C, and 13.36 % G. According to 13 protein-coding genes and phylogenetic analysis, *Elaphodus* may have a sister relationship with another deer group *Muntiacus* and they belong to the monophyletic genus. This study depicted mitochondrial genome characteristics for *E. cephalophus* and deepened the understanding of the phylogenetic relationship of Cervidae.

INTRODUCTION

The tufted deer (*Elaphodus cephalophus*) is a unique species in subfamily Muntiacinae (muntjacs) belongs within the family Cervidae (Artiodactyla, Ruminantia). It is a native species to central and southwest China and northeastern Myanmar (Sheng *et al*., 1992; Wang, 2003; Grubb, 2005; Smith and Xie, 2008; Ohtaishi and Gao, 1990; Sun *et al*., 2016). Currently, the tufted deer is categorized as Near Threatened (NT) by the International Union for Conservation of Nature (Harris and Jiang, 2015). However, the information about *E. cephalophus* has been generally scarce due to difficulty for the population census, and therefore, there is little information on phylogeny and population genetics available for this species.

Understanding the evolutionary history, taxonomic status, genetic structure and diversity, and phylogeography enables effective conservation and management of endangered species (Osentoski *et al*., 2002; Zhang *et al*., 2007; Bu *et al*., 2014; Wada *et al*., 2007; Hassanin *et al*., 2012). Biosystematics provides evolutionary history, taxonomic status, and diversity through the genetic markers of species, thus clarifying the current evolutionary relationship (Hassanin *et al*., 2012). The traditional phylogenetic analysis of the family Cervidae is based on the evolutionary history

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Abbreviations
PCR, Polymerase chain reaction; ATP6 and ATP8, Subunits 6 and 8 of the F0 ATPase; Cytb, Cytochrome b; COXI-COXIII, Cytochrome c oxidase subunits 1-3; Ile, Isoleucine; Leu, Leucine; ND1-ND6 and ND4L, NADH dehydrogenase subunit 1-6 and4 L; 16S rRNA and 12S rRNA, Large and small subunit of ribosomal RNA genes; tRNAXXX, Genes encoding for transfer RNA molecules with corresponding amino acids denoted with a three-letter code and anticodon indicated in parentheses (XXX) when necessary; A+T rich region, Adenine+thymine- rich region.

and taxonomic status of the phylogenetic, biological, physiological, and fossil data, forming a modern deer classification system (Zhang and Zhang, 2012). However, due to the existence of many factors such as the environment, only the characteristic analysis can't truly reflect the phylogenetic relationship of the *E. cephalophus* and related species (Hassanin *et al*., 2012; Li *et al*., 2017). Therefore, it is necessary to use molecular markers (mtDNA) and genetic information to explore phylogenetic studies of the family Cervidae.

Previous phylogenetic analysis of mitochondrial (mt) DNA restriction fragment size polymorphism maps, mt 12S rRNA, 16S rRNA, the Cytb gene, and the control region of Muntiacinae species reveal that the *E. cephalophus* is a sister group of the genus *Muntiacus* in the Muntiacinae, and the *E. cephalophus* is genetically closer to muntjacs than the musk deer (Lan and Shi, 1994; Amato *et al*., 1999). The NADH4L and NADH4 gene phylogeny show that there is a rapid and parallel chromosomal number reduction in Muntiacinae and that *Elaphodus* and *Muntiacus* are basal split separated (Wang and Lan, 2000). Recent phylogenetic analysis of four markers (Cytb, COXII, aLAlb, and PRKCI) support monophyly of Muntiacinae (*Elaphodus* and *Muntiacus*) and estimates the origin of Muntiacinae at 7-8 million years ago (Gilbert *et al*., 2006). The complete mt genome provides a higher level of support than those based on individual or partial mt genes (Castro and Dowton, 2005; Krzywinski *et al*., 2006). Most taxonomic issues concern the genera of the family Cervidae: *Cervus*, *Rucervus,* and *Rusa* in the tribe Cervini, and *Odocoileus*, *Mazama,* and *Pudu* in the tribe Odocoileini. Furthermore, previous studies found that the taxonomic status of South American deer is more problematic and complex, and additional morphological and molecular data (including nuclear markers) are needed to understand the complex systematic relationships of the tribe Odocoileini (Duarte *et al*., 2008; Gilbert *et al*., 2006; Hassanin *et al*., 2012). The evolutionary structure of the family Cervidae can be reconstructed by phylogenetic analysis based on whole mitochondrial genomes; this method could be used broadly in phylogenetic evolutionary analysis of animal taxa (Azanza *et al*., 2013). Mt DNAs, especially those encoding protein genes, have been frequently utilized as a powerful tool for evolutionary studies of animals (Yang *et al*., 2012).

Herein, in this study, the complete mitochondrial genome of the tufted deer, *E. cephalophus*, was analyzed, and its genome organization, gene arrangement, and characterization were described and the complete mtDNA and phylogenetic relationships of *Elaphodus* and Cervidae were rebuilt. These will be helpful to further facilitate future studies of species phylogeny and population genetics of *Elaphodus* and Cervidae species. The complete sequence will be a benefit to the study of the *E. cephalophus* and adds to our understanding of the systematic classification and evolutionary history of the cervids.

MATERIALS AND METHODS

Sample collection and genomic DNA extraction

The tufted deer, *E. cephalophus*, was originally collected from the experimental station of Tangjiahe Nature Reserve, Qingchuan County, Sichuan Province, China (104°45'29.37''E, 32°35'0.36''N, 1563 m above sea level), and identified according to key morphological characteristics. The 3 mL blood sample was preserved in tubes containing heparin and stored at -70 °C. The obtained genomic DNA (Sambrook and David, 2001; Ren *et al*., 2008) was used for further study after the quality of the genomic DNA was checked using 0.9% agarose gel and a spectrophotometer. Total genomic DNA was preserved at -20 degrees until it is used.

Primer design, PCR amplification, and sequencing

To amplify overlapping fragments that covered the whole mitogenome of *E. cephalophus* by normal PCR and long and accurate PCR (LA-PCR) methods. PCR amplification was performed using these primers with overlapped PCR fragments (Supplementary Table SI). The reaction volume amounted to 25 µl and contained 12.25 µl of sterilized distilled water, 2.5 µl 10× PCR reaction buffer, 2.5 µl of 25 mM $MgCl_2$, 2.5 µl dNTP mix, 1.25 µl of each primer (10 µM), 2.5 µl DNA template and 0.25 µl (1.25 U) of TaKaRa LA Taq polymerase or Taq polymerase (Takara, Japan), using the following conditions: 94 °C for 2.5 min (initial denaturation); 94 °C for 45 s (denaturation), 48-61 °C for 45s (annealing), 72°C for 1.5-3.0 min (extension) for 34 cycles; and a final extension at 72 °C for 8 min. The PCR products were resolved using electrophoresis with 1.0% agarose gel and extracted using the DNA Gel Extraction Kit (Qiagen, USA), and sent to Chengdu TsingKe Biological Technology Co., Ltd. (Chengdu, China), for sequencing from both directions with a primer-walking strategy to obtain complete mitochondrial genome sequence.

Sequence assembling and sequence analysis

The complete mitogenome of *E. cephalophus* was edited and assembled into contiguous, overlapping sequences using Genetyx 5.2 (Genetyx Corp., Japan). The primary DNA sequence data were characterized using BLAST searches at NCBI. Protein-coding genes, rRNA genes, and tRNA genes were initially identified by comparison with the mitochondrial genomes of related

species (*M. crinifrons* and *M. reevesi*). The nucleotide sequence and amino acid sequences of each of the PCGs were aligned using Clustal X 2.0 (Larkin *et al*., 2007). Then, the 13 mitochondrial protein-coding genes were defined from the presence of initiation and stop codons. The codon usage of the 13 protein-coding genes was determined using MEGA 7.0 (Kumar *et al*., 2016). The ends of the large and small subunit ribosomal RNA genes were assumed to be adjacent to the ends of their neighboring genes. Transfer RNAs genes were identified by their cloverleaf secondary structure using tRNAscan-SE 1.21 and ARWEN software (Lowe and Chan, 2016; Laslett and Canback, 2008), which gave Cove score cut-off values to obtain all tRNAs in the mitogenome. The putative replication origin (OL) and control region (CR, D-loop) were identified by sequence homology and from the proposed secondary structure. The complete mtDNA sequence of *E. cephalophus* reported in this article was deposited in GenBank under the accession number MN248532.

Nucleotide compositional skew analysis was carried out according to the formula: AT-skew = (A − T)/(A + T) and GC-skew= (G − C)/(G + C), respectively (Perna and Kocher, 1995). Gene overlap and intergenic-space sequences were hand-counted. The secondary structure of the putative O_L was analyzed with the program Mfold v.3.2 (http://mfold.bioinfo. rpi.edu/) with default settings (Zuker, 2003) and visualized using RNAViz (De Rijk and De Wachter, 1997).

Molecular phylogenetic analyses

To further clarify the phylogenetic relationships among Cervidae species, another 46 complete mitochondrial genomes of the following vertebrate species were retrieved from GenBank. Phylogenetic analyses were performed with the 13 complete mitochondrial genomes of the Cervidae species from GenBank (Supplementary Table SI). To determine the systematic status of *E. cephalophus*, all currently available and complete mitochondrial genomes of Cervidae were used in the phylogenetic analysis, with setting the *Giraffa camelopardalis* (AP003424), *Okapia johnstoni* (JN632674), and *Antilocapra americana* (NC_020679) as out-groups. The concatenated sequences of the 13 protein-coding genes of the complete mitochondrial genomes were used for phylogenetic analysis. The 13 mitochondrial protein-coding genes sequences were aligned by Clustal X 2.0 (Larkin *et al*., 2007) with the default settings.

Bayesian inference (BI) was performed using the MrBayes 3.2.7a program (Huelsenbeck and Ronquist, 2001). The confidence value was evaluated via bootstrap analysis with 100 replicates. Mr. Bayes was used to reconstruct the BI tree with the optimal evolutionary model GTR+I+G. Each search started from random trees, for 3×10^8 generations, each with four simultaneous Markov chains, sampling every 100 generations. Generations sampled before the chain reached stationary (burn-in) were discarded. After this, a majority-rule consensus tree was constructed showing relative occurrences of all nodes in the trees. Node support was assessed by the value of Bayesian posterior probabilities (BPP). Maximum Likelihood (ML) analysis was performed using PhyML v.3.0 (Guindon and Gascuel, 2003). The confidence level (Felsenstein, 1985) at each branch was evaluated by allowing four substitution rate categories and performing bootstrapping with 1000 replicates in ML analysis. The phylogenetic trees were viewed and edited by Figtree v1.4.3 (Rambaut, 2018).

RESULTS

Genome organization, structure, content and base composition

As shown in Figure 1 and Table I, the mitochondrial genome of the tufted deer is a covalently closed circular double-stranded DNA molecule, which is a typical mammalian mitochondrial genome. The complete mitogenome of *E. cephalophus* is a closed-circular molecule of 16,196 bp in length, containing 13 protein-coding genes, 2 rRNA genes and 22 tRNA genes and non-coding regions (including the control region, CR), which the mitochondrial genome nucleotide number is the least one compared with previous studies in Cervidae. These complete mt genomes range from 16,196 to 16,505 bps in size (Supplementary Table SI). The details of gene location, overlap, and spacer sequences are presented in Figure 1 and Table I.

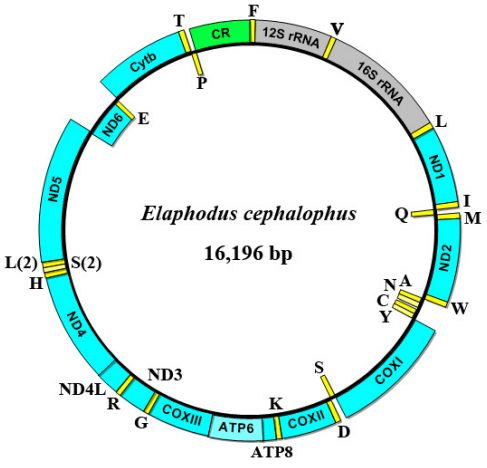

Fig. 1. Circular map of the complete mitochondrial genome of *Elaphodus cephalophus*. Genes encoded on the H-strand and L-strand are shown outside and inside the circular map of the mitogenome, respectively. 22 tRNA genes are named using single-letter amino acid abbreviations.

Table I. Organization of the mitochondrial genome of Elaphodus cephalophus.

Gene	Position		Sizes Nudeotide (bp)	Anti-codons (tRNA)	Start	Stop*	Intergenic † nudeotide	Strand
	From	To						
tRNA-Phe	1	69	69	GAA			0	H
12S ribosomal RNA	70	1026	957				0	H
tRNA-Val	1027	1093	67	TAC			0	H
16S ribosomal RNA	1094	2660	1567				0	H
tRNA-Leu	2661	2735	75	TAA			2	H
ND1	2738	3694	957		ATG	TAA	-1	H
tRNA-Ile	3694	3762	69	GAT			-3	H
tRNA-Gln	3760	3831	72	TTG			2	L
tRNA-Met	3834	3902	69	CAT			0	H
ND2	3903	4946	1044		ATA	TAG	-2	H
tRNA-Trp	4945	5012	68	TCA			1	H
tRNA-Ala	5014	5082	69	TGC			1	L
tRNA-Asn	5084	5156	73	GTT			0	L
rep-origin	5557	5188	32				0	H
tRNA-Cys	5189	5255	67	GCA			0	L
tRNA-Tyr	5256	5324	69	GTA			1	L
COXI	5326	6870	1545		ATG	TAA	-3	H
tRNA-Ser	6868	6936	69	TGA			8	L
tRNA-Asp	6945	7013	69	GTC			0	H
COXII	7014	7697	684		ATG	TAA	3	H
tRNA-Lys	7701	7768	68	TTT			1	H
ATP8	7770	7970	201		ATG	TAG	-40	H
ATP6	7931	8611	681		ATG	TAA	-1	H
COXIII	8611	9395	785		ATG	TA-	-1	H
tRNA-Gly	9395	9464	70	TCC			0	H
ND3	9465	9811	347		ATA	TA-	1	H
tRNA-Arg	9812	9880	69	TCG			-1	H
ND4L	9881	10177	297		GTG	TAA	-7	H
ND4	10171	11548	1378		ATG	T--	0	H
tRNA-His	11549	11617	69	GTG			0	H
tRNA-Ser	11618	11677	60	GCT			1	H
tRNA-Leu	11679	11748	70	TAG			0	H
ND5	11749	13569	1821		ATA	TAA	-17	H
ND6	13553	14080	528		ATG	TAA	0	L
tRNA-Glu	14081	14149	69	TTC			4	L
Cytb	14154	15296	1143		ATG	TAA	0	H
tRNA-Thr	15297	15366	70	TGT			-1	H
tRNA-Pro	15366	15431	67	TGG			0	L
Control region	15432	16196	765				0	H

*T-- or TA- represent incomplete stop codons; †Numbers correspond to the nucleotides separating adjacent genes, negative numbers indicate overlapping nucleotides. ‡H and L indicate genes transcribed on the heavy and light strands, respectively.

Also, as in other vertebrates, most of the mitogenome genes were encoded on the H-strand, except ND6 and eight tRNA genes (tRNA-Gln, tRNA-Ala, tRNA-Asn, tRNA-Cys, tRNA-Tyr, tRNA-Ser, tRNA-Glu, and tRNA-Pro), which were encoded on the L-strand. The overall base composition values of the mtDNA were 33.37%, 23.94%, 13.36%, and 29.33% of A, C, G, and T, respectively, with an A+T content of 62.70%, which is similar to that of *M. reevesi*, *M. muntjak* and *M. crinifrons* (Table I).

As for intergenic spacers and overlap region, 25 nucleotides are dispersing in eleven intergenic spacers from 1 to 8 bp and contiguous genes overlapping at eleven boundaries by a total of 77 bases from 1 to 40 bp in the whole mtDNA of *E. cephalophus.* The longest intergenic spacer sequence is 8 nucleotides located between tRNA-Leu2 and tRNA-Phe, and overlap (40 bp) existed between genes tRNA-His and tRNA-Ser2 (Table I).

Protein-coding genes

The protein-coding genes which are related to the initiation and termination of genes of the *E. cephalophus* are identical to those of other *Muntiacus* species (Shi *et al*., 2003; Zhang *et al*., 2004). All of the 13 mitochondrial PCGs found in other animals are also presented in *E. cephalophus*, including *ATP6, ATP8, COXI, COXII, COXIII, ND1, ND2, ND3, ND4, ND4L, ND5, ND6* and *Cytb* genes. The total size of these PCGs is 11,406 bp, accounting for 70.42% of the entire mitogenome sequence.

The most common start codon was ATG, which accounts for 69.23% of the 9 start codons, followed by ATA (23.08 %). Nine protein-coding genes (*ND1, ND4, ND6, ATP6, ATP8, COXI, COXII ,COXIII* and *Cytb*) are initiated with ATG as start codon except for ND2, ND3, ND5 with ATA and ND4L with GTG. Remarkably, among the species of Cervidae, usage of GTG as the start codon in the ND4L gene is less typical and merely observed in *E. cephalophus*, *Cervus nippon* and *R. tarandus*, while ATG is used as the start codon in other deer. The GTG could directly initiate translation in mammalian cells with a lower level of protein expression (Hwang *et al*., 2005). The most common stop codon was TAA, which accounts for 61.54% of the total stop codons. The recognizable complete stop codon TAA or TAG formed the stop codon for ten PCGs (*ND1, ND2, COXI, COXII, ATP8, ATP6, ND4L, ND5, Cytb* and *ND6*), with the other three PCGs (COIII, ND3, and ND4) exhibiting incomplete stop codons with a single T-- or TA- residue. However, it is common for termination codons to be truncated (to T- or TA-) in metazoan mitochondrial genomes and a reasonable interpretation for this is that mRNA polyadenylation forms the complete UAA stop codon (Boore, 2001). The absence of a complete stop codon in the *COXIII, ND3* and *ND4* genes is a common characteristic of mammalian mitochondrial genomes. *ATP6* and *ATP8* shared 40 nucleotides, *ND4* and *ND5* shared 17 nucleotides, and *ND4* and *ND4L* shared 7 nucleotides. In addition, protein-coding genes and *tRNA* genes share one to three nucleotides. In particular, the size of the *Cytb* gene of either the tufted deer or *Rangifer tarandus* (1143bp) is 3bp longer than that of *Cytb* gene (1140bp) reported in most other Cervidae mt genomes. The *E. cephalophus* have a subterminal AAA followed by the stop codon TAA, while *R. tarandus* also has a possible subterminal GGA followed by the stop codon TAA (Randi *et al*., 1998).

On the H-strand, the frequencies of nucleotides were A>T=C>G at the first codon position, T>C>A>G at the second codon position, and A>T>C>G at the third codon position. While, on the L-strand, the frequencies of nucleotides were G>T>A>C at the first codon position, T>G>A>C at the second codon position, and T>G>A>C at the third codon position (Supplementary Table SII). At the third codon position, the least frequent nucleotide was G on the H-strand and C on the L-strand, probably reflecting the mutation pattern of the mitochondrial genome, as nucleotides at the third codon position are under the least selective pressure.

Codon usage in *E. cephalophus* mitochondrial protein-coding genes is shown in Supplementary Table SIII and Supplementary Figure S2. A total of 3,802 codons constitute the 13 PCGs and CUA (6.42%), AUU (6.23%), AUA (5.55%), ACA (4.26%), UUU (3.87%) and UUA (3.87%) are the five most frequently used codons. Whereas CAG, UGU, CGU, GCG and CGG codons are rarely represented, all of these only accounted for 0.79%. Leu (L), Phe (F), Ile (I), Thr (T) and Met (M) are the most abundant amino acids in the 13 PCGs of *E. cephalophus* mitochondrial genome (Supplementary Table SIII and Supplementary Figure S1).

Transfer and ribosomal RNA genes

The 22 tRNA genes were identified in the mitochondrial genome of the *E. cephalophus* and their predicted secondary clover-leaf structures. These tRNA genes are scattered throughout the mitogenome and the size of the tRNA genes ranges from 60 bp for tRNA-Ser to 75 bp for tRNA-Leu. The base use of tRNA is shown in Table I. The eight genes (*tRNA-Gln, tRNA-Ala, tRNA-Asn, tRNA-Cys, tRNA-Tyr, tRNA-Ser, tRNA-Glu* and *tRNA-Pro* genes) are encoded by light chain (L), and the rest of the tRNA genes are encoded by heavy chain (H). Under normal conditions, the mitochondrial genome of vertebrates has the highest conservativeness of *tRNA-Met, tRNA-His* and *tRNA-Leu* sequences, and *tRNA-Ser* has the greatest variability, and the homology is only 54% to 64%. All the

predicted tRNAs display a typical clover-leaf secondary structure, except for tRNA-Ser (AGY) that lacks the DHC loop, which exists in other animal mitochondrial genomes. The potential second structures of the tRNA are quite similar among *M. muntjak*, *M. crinifrons* and *M. reevesi*. Besides, a total of 38 non-Watson-Crick base pairs were observed in 22 tRNA genes andthere are thirty-eight G-U wobble pairs, which form a weak hydrogen bond in the tRNAs (Supplementary Fig. S3). The remaining atypical ten pairs (one U-C, two U-U, four A-C, two A-A, one A-G) unmatched were observed in tRNA-Phe (receptor arm, one A-A), tRNA-Met (receptor arm, one A-G and TΨC arm, two U-U), tRNA-Arg (anticodon arm, one A-C), tRNA-Asp (receptor arm, one A-C and TΨC arm, one A-C), tRNA-Arg (receptor arm, one U-C), tRNA-Ser (AGN) (anticodon arm, one A-A and one A-C) respectively.

The smaller (12S rRNA) and larger (16S rRNA) ribosomal subunits were identified on the H-strand in the *E. cephalophus* mitogenome, which are located between *tRNA-Phe* and *tRNA-Leu* (UUR) genes, and separated by the *tRNA-Val* gene. The length of 12S rRNA is 957 bp and the length of 16S rRNA is 1,567 bp. The composition of nucleotides of 12S rRNA is 22.78% T (U), 22.68 % C, 36.68 % A and 17.87 % G. And the composition of nucleotides of 16S rRNA is 25.34% T(U), 19.78 % C, 37.72 % A and 17.17 % G.

Non-coding sequences

Like most other Cervidae species, the putative origin of L-strand replication (OL) is located in the cluster of five tRNA genes (WANCY region) between *tRNA-Asn* and *tRNA-Cys*, which consists of 32 nucleotides (5'-CTTCTCCCGCCGCGAAGAAAAAAGGCGGGAG-3') (Table I). Secondary structures with a 5'-GCGGG-3' motif at OL are known to act as initiation signals for L-strand replication in most vertebrates (Zardoya and Meyer, 1996; Hurst *et al*., 1999; Wong and Clayton, 1985). The motif 5'-GCGGG-3' is at the base of the stem, which is highly conserved in Cervidae andthis area has an ability to form a stable stem-loop secondary structure consisting of 11bp in the stem and 12-14 bp in the loop from Cervidae species (Supplementary Fig. S4). The stem sequence of O_L was highly conserved among vertebrate mtDNAs, whereas the loop sequence was more variable (Supplementary Fig. S4).

The base mutation rate is the highest in the D-loop region, and the length variation is the largest. The control region is located between the *tRNA-Pro* gene and the *tRNA-Phe* gene. The size of the control region (also known as the D-loop region) is 765bp in length, similar to that of *Mazama rufina* (JN632661, 778bp) and *Pudu mephistophiles* (NC_020739, 796 bp). The A+T content of this region is 61.05%, which is lower than that of protein-coding genes (62.90%). The Cervidae mtDNA control region harbors the extended termination-associated sequence (ETAS) domain, the central conserved domain, and the conserved sequence block (CSB) domain (Sbisa *et al*., 1997), i.e. CSB-F, CSB-E, CSB-D, CSB-C, CSB-B, CSB-1, CSB-2 and all four termination associated sequences (TAS-1 to TAS-4) (Fig. 2).

Phylogenetic reconstructions

In this study, the concatenated 13 PCGs data of the mitogenome sequences contained 11,376 nucleotide positions, including 6,556 conserved sites, 4,820 variable sites, and 4,067 potentially parsimony-informative sites. Phylogenetic trees were reconstructed using BI and ML analyses based on the nucleotide dataset. Using the 13 PCG sequences to concatenate may achieve a more complete analysis. BI and ML methods consistently support similar tree topologies by high node-supporting values. So far, combined with the 46 mitochondrial genome sequences collected in the GenBank database, we reconstructed the phylogenetic relationships between *E. cephalophus* and other Cervidae species using the nucleotide sequence of 13 PCGs.

Based on our phylogenetic results using complete mitochondrial genomes, the family Cervidae is divided into two subfamilies, Cervinae and Capreolinae. The two subfamilies contain five tribes, Cervini, Muntiacini, Odocoileini, Alceini, and Capreolini, which are well determined by the phylogenetic trees of the family Cervidae (Fig. 3). Five genera of the family Cervidae are found to be para- or polyphyletic: *Cervus*, *Mazama*, *Pudu*, *Rucervus,* and *Rusa* (Fig. 3). While seven genera were found to be monophyletic: *Dama*, *Axis*, *Elaphodus*, *Hydropotes*, *Capreolus*, *Odocoileus,* and *Muntiacus*. Several robust relationships between genera of Cervidae were detected in this study. Within Odocoileini, South American deer falls into two divergent clades: the Clade 1 includes *Pudu mephistophiles*, two species of *Odocoileus*, *O. virginianus*, *O. hemionus* and two species of *Mazama*, *M. americana*, *M. rufina* (BPP=1.00; BP=1000); the Clade 2 contains *Pudu puda*, *Ozotoceros bezoarticus*, *Hippocamelus antisensis*, and two species of *Mazama*, *M. nemorivaga* and *M. gouazoubira* (BPP=1.00; BP=1000). Consequently, the monophyly of two genera of Odocoileini is questioned by our phylogenetic tree: *Mazama* and *Pudu*. In addition, the genus *Mazamaa* and *Pudu* are found to be paraphyletic because *M. americana* appears as a sister-group of two species of *Odocoileus*, and *M. rufina* and *P. mephistophiles* formed sister species again. Then, *P. puda* was in the original position of Clade 2 (Fig. 3). Within Cervini, three genera are found to be poly- or paraphyletic: (1) Cervus, as *C. albirostris* is the sister-group

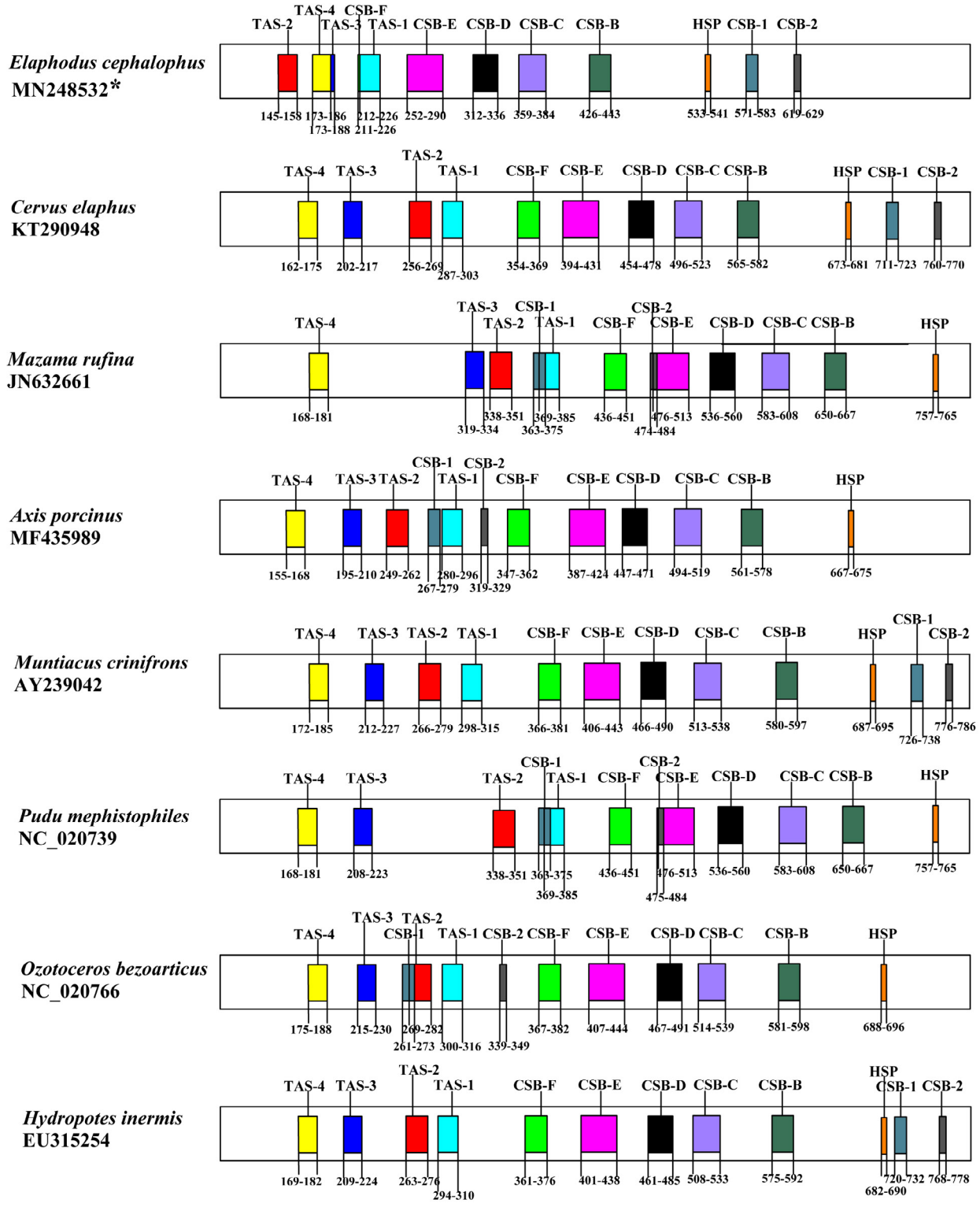

Fig. 2. Sequences and the conserved elements of the control region of the *Elaphodus cephalophus*. Primary sequence features (TAS-1, TAS-2, TAS-3, TAS-4, and CSB-B, CSB-C, CSB-D, CSB-E, CSB-F, and Origin of H replication, and CSB-1, CSB-2) has been marked and the numbers marked at both ends represent the position of the feature sequence.

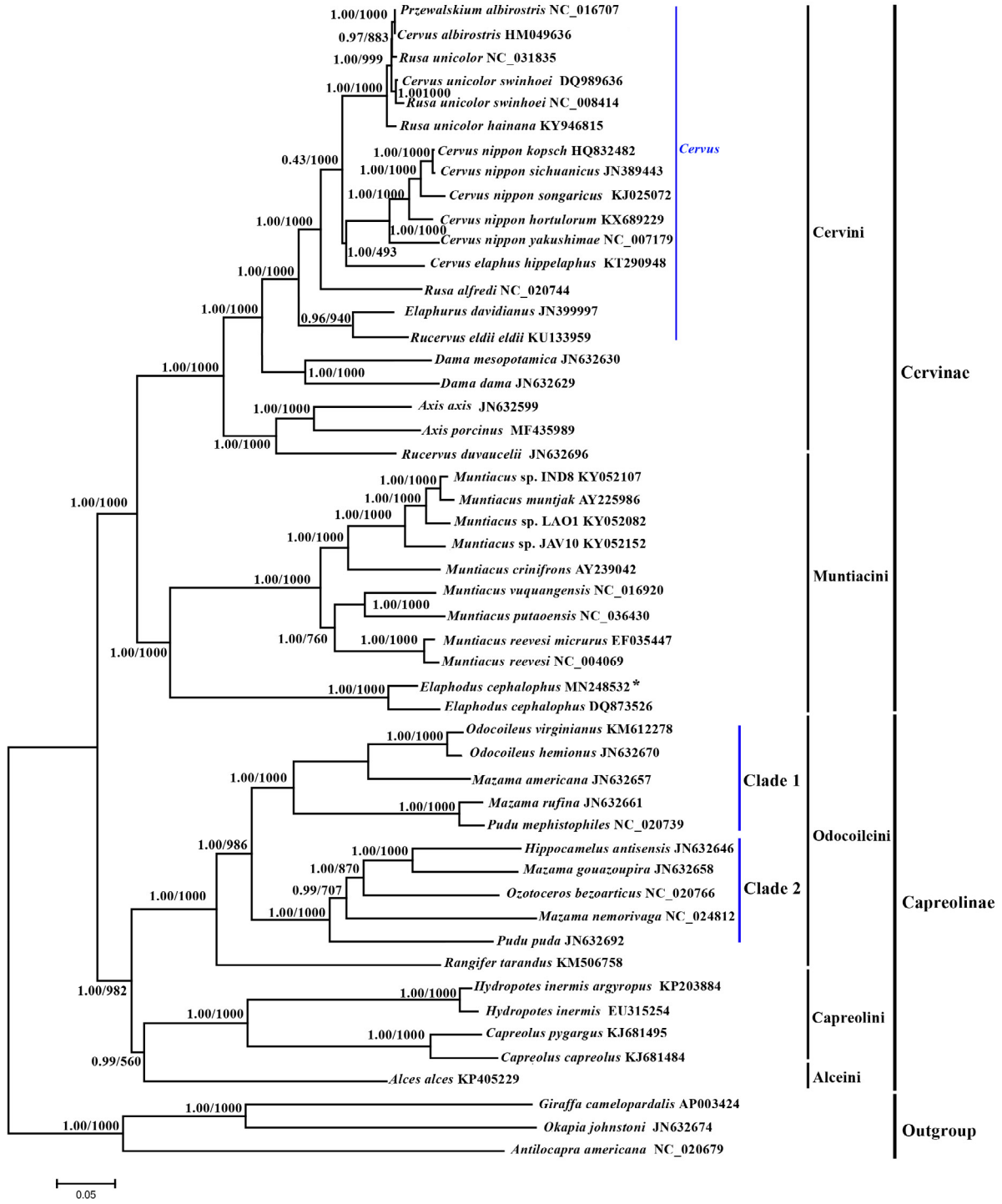

Fig. 3. The phylogenetic analyses investigated using Maximum likelihood (ML) and Bayesian inferences (BI) analysis indicated evolutionary relationships among 46 mitochondrial genome sequences based on the tandem nucleotide alignments of 13 protein-coding genes. The tree topologies produced by ML and BI analyses were equivalent. Bayesian posterior probability with 2×10^8 generations and bootstrap support values for ML analyses with 1000 replicates are shown on the nodes. *Giraffa camelopardalis* (AP003424), *Okapia johnstoni* (JN632674) and *Antilocapra americana* (NC_020679) were selected as an outgroup. Several classification suggestions are shown in blue at the right of the taxa.

of *Przewalskium* (BPP = 1.00; BP = 1000) with *C. elaphus* and *C. nippon* at the outside; (2) *Rusa*, as *R. alfredi* is the sister-group of a large clade uniting the three other *Rusa* species with all species of *Cervus* and *Przewalskium* (BP=1000; PP =1.00). (3) *Rucervuseldii* and *Elaphurus davidianus* form sister groups, while another species (*R. duvaucelii*) of this genus is associated with the genus *Axis*, *A. porcinus* and *A. axis*. In Capreolini, *Hydropotes* and *Capreolus* congregate on the same branch to form sister genera. While in Muntiacini, *E. cephalophus* was clustered into one group with all species from the genus *Muntiacus* with a high bootstrap support value (PP=1.00; BP=1000). Phylogenetic analysis also supports monophyly of genus *Elaphodus* and *Muntiacus*, the genus *Elaphodus* possesses a relatively closer relationship with the genus *Muntiacus* than with other genera at the basal position in Cervinae (PP=1.00; BP=1000). In the genus of *Muntiacus*, *M. reevesi* has a sister relationship with the group of *M. vuquangensis* and *M. putaoensis* (BP=760; PP =1.00). And the closest relationships are *M. muntjak* and *M. crinifrons* from Figure 3.

DISCUSSION

Characteristics analysis of the mitochondrial genomes

The *E. cephalophus* mitogenome possessed the same gene order with those of *Muntiacus muntjak*, *M. putaoensis*, *M. reevesi*, *Cervus albirostris* and *Elaphurus davidianus* (Hill *et al*., 2017; Zhao *et al*., 2018). In comparison with the *Elaphurus* and other *Muntiacus* species, the mitogenome of *E. cephalophus* is slightly shorter than that of the *E. davidianus* and other *Muntiacus* species, which had diverged later than tufted deer from the Muntiacinae subfamily. As consistent with the results of Rose (Rose, 2008), the tufted deer had experienced a relatively long period of independent evolution. And phylogenetic analyses support a sister relationship with muntjacs (Pang *et al*., 2008). The length and base composition of complete mitogenomes among *Cervidae* species mainly depended on the size of non-coding control region (D-loop) in this region (Hassanin *et al*., 2012; Hill *et al*., 2017). Also, to the D-loop region, the mitochondrial genome of *E. cephalophus* is similar to that of the published species in gene arrangement and base composition (Hassanin *et al*., 2012; Zhao *et al*., 2018). In this study, D-loop region possessed a shorter sequence (765 bp) and has no tandem repeat units which is consistent with most deer species (Pang *et al*., 2008; Hassanin *et al*., 2012; Zhao *et al*., 2018), however, the sequence submitted by Hill *et al*. (2017) had one longer sequence (1081 bp) and one tandem repeat unit type (5.2 repeat units of 40 bp), and resulting in a large change in the length of the region. Therefore, the analysis of *E. cephalophus* shows that there is a large genetic variation and length change in this region.

D-loop features

Noncoding region (D-loop) sequences are highly similar to other species of cervid species (Wada *et al*., 2010; Frank *et al*., 2016; Shao *et al*., 2016). The TAS-1 motif functionally correlates with the termination of the D-loop. In the 5′-end region, the putative point of arrest of the D-loop synthesis is different among the species of the Cervidae species. The termination region of DNA synthesis (ACCCC motif) is present in the control region of *E. cephalophus*, *Mazama nemorivaga*, *Cervus dama*, *Dama*, *Alces alces*, *Ozotoceros bezoarticus* and *Odocoileus heminous*, while the GCCCC motif exists in *Muntiacus* species, *Rusa*, *R. tarandus, Hydropotes inermis, Mazama gouazoupira*, *C. elaphus, C. nippon, Capreolus*, *Przewalskium albirostris*, *Rucervus eldii eldii* and *Elaphurus davidianus*. In addition, *Rucervus duvaucelii* and *Mazama americana* possess the TCCCC motif. In the 3′-end domain of the control region, the putative initiation site for H-strand replication (AGCATCCC) is highly conserved in the Cervidae species. The *E. cephalophu* have many of the conserved sequence blocks (CSB-F, CSB-E, CSB-D, CSB-C, CSB-B, and CSB 1 and CSB 2), which display high similarities in the Cervidae species (Douzery and Randi, 1997). The motifs, CSB-F, CSB-E, CSB-D, CSB-C, and CSB-B might provide some information for examining the structure and function relationships of the D-loop (Cui *et al*., 2009; Zhong *et al*., 2018). Besides, CSB-1 and CSB-2 motifs were also identified, which may be associated with positioning RNA polymerase for both priming replication and transcription (Shadel and Clayton, 1997; Zhong *et al*., 2018). These motifs differ in order in some species (Fig. 2). The 3′-end of CSB-1 is more conservative in the Cervidae, containing a similar G(C/T)(A/G)CAT motif. A CSB-1-like motif is observed in some *Odocoileus* and *Mazama* species (Douzery and Randi, 1997). Previous studies have shown that most mammals possess the two other separated conserved blocks, CSB-2 and CSB-3, situated in the middle of the 3’-end region. CSB-2 is present in the *E. cephalophu* and other Cervidae species, but CSB-3 is absent in *E. cephalophu*, *Odocoileus* and *Muntiacus*. Besides, CSB-3 is often absent in some large mammals, *Balaena mysticetus*, *Ovis aries*, *Cephalorinchus commersonii*, *Physeter macrocephalus*, and *Ornithorhynchus anatinus* (Douzery and Randi, 1997; Sbisa *et al*., 1997). The present Cervidae species obviously possess a single fused CSB 2+3, i.e. *Mazama* and *Cervus* (Ambriz-Morales *et al*., 2016; Yang *et al*., 2012; Wada *et al*., 2010) and significant repeat regions were not observed in the D-loop region.

Molecular phylogenetic analysis in Cervidae

In recent ten years, some researchers have investigated and analyzed the related species of the deer family, with morphological data (Geist, 1998), partial mitochondrial sequences (Ludt *et al*., 2004; Wang and Lan, 2000; Pitra *et al*., 2004; Lister *et al*., 2005; Pang *et al*., 2008), a few nuclear genes (Cronin *et al*., 1996; Gatesy *et al*., 1996) or the combination of the latter two (Gilbert *et al*., 2006), and mitochondrial genome (Hassanin *et al*., 2012; Frank *et al*., 2016). The use of protein-coding gene sequences of the mitogenome has become an informative strategy for deducing and reconstructing phylogenetic relationships (Pang *et al*., 2008; Hassanin *et al*., 2012; Yang *et al*., 2012; Zhang and Zhang, 2012; Frank *et al*., 2016). Within the Cervidae, from our phylogenetic results with complete mitogenomes, the family is divided into two subfamilies, Cervinae and Capreolinae, which is consistent with previous findings (Frank *et al*., 2016; Zhang and Zhang, 2012; Pitra *et al*., 2004; Gilbert *et al*., 2006; Hassanin *et al*., 2012; Wang and Yang, 2013). All intergeneric classification described in previous studies (Gilbert *et al*., 2006; Hassanin *et al*., 2012; Hassanin and Douzery, 1999a, b; Frank *et al*., 2016) are well supported, i.e., the subfamily Cervinae with two tribes, the tribes Cervini and Muntiacini, and the subfamily Capreolinae with three tribes, the tribes Capreolini, Alceini, and Odocoileini. The intertribal relationships within Cervinae are robust from the phylogenetic tree, while in Capreolinae most intertribal relationships are not robust, indicating rapid diversifications and variations. The evolutionary tree showed not only the phylogeny of the whole family but also the monophyly of tribes, such as Cervini and Muntiacini, and so on (Fig. 3). These results are similar to previous studies (Hassanin *et al*., 2012; Frank *et al*., 2016).

As proposed by Gilbert *et al*. (2006), these species should be classified into the genus *Cervus*: *Elaphurus davidianus*, *Przewalskium albirostris*, *Rucervus eldii,* and all species of *Rusa*, which were consistent with the results of previous studies (Hassanin *et al*., 2012; Frank *et al*., 2016). This is in agreement with trees constructed earlier by others using various molecular data (Wang and Lan, 2000; Gilbert *et al*., 2006) and morphological data (Geist, 1998). Our phylogenetic results also support this classification (Fig. 3). However, this classification is very complex. Because the data and samples used are different, the results obtained are also different (Pang *et al*., 2008; Hassanin *et al*., 2012; Zhang and Zhang, 2012; Martins *et al*., 2017). Some taxonomic issues concern the genera of the family Cervidae (Fig. 3): *Cervus*, *Rusa*, and *Rucervus* in the tribe Cervini, and *Odocoileus*, *Mazama*, and *Pudu* in the tribe Odocoileini. From phylogenetic tree, *Elaphodus* and *Muntiacus* belong to a monophyletic genus which is in agreement with trees constructed earlier by others (Wang and Lan, 2000; Gilbert *et al*., 2006). Within Cervini, three genera are found to be paraphyletic: (i) *Cervus*, as *Cu. nicolor swinhoei*, *C. albirostris* and *R. unicolor* are the sister-group of *Przewalskium* with *R. unicolor hainana* at the outside which is not consistent with the results of previous studies (Hassanin *et al*., 2012); (ii) *Rucervus*, as *Ru. duvauceli* is related to *Axis*, whereas *Ru. eldi* is allied to *Elaphurus;* (iii) *Rusa*, as *R. alfredi* is the sister-group of a large clade uniting the two other *Rusa* species with all species of *Cervus* and *Przewalskium* which is similar to the findings of Hassanin *et al*. (2012). Within Odocoileini, South American deer falls into two divergent clades (Fig. 3). Clade 1 groups *Odocoileus*, *Pudu mephistophiles* and two other species of *Mazama*, *M. americana* and *M. rufina*. The monophyly of two genera of Odocoileini is therefore questioned by our data: Mazama and *Pudu*; the second one (Clade 2) includes Hippocamelus, Ozotoceros, Pudu puda, and two species of Mazama, *M. gouazoubira* and *M. nemorivaga.* Our phylogeny supports previous findings (Hassanin *et al*., 2012). Thus, the evolution of the Cervidae species is complicated, and some phylogenetic relationships are still confusing. Furthermore, some relationships within this group are unclear, systematic taxonomy, molecular (mitochondrial and nuclear genes), and morphological data are needed from a wide geographical area in order to clarify the complex systematic status and evolutionary history within the Cervidae.

CONCLUSIONS

In this study, we describe the complete mitogenome which is 16,196 bp in length of the endangered species *E. cephalophus*. The overall base composition included 33.37 % A, 29.33 % T, 23.94 % C, and 13.36 % G. According to 13 protein-coding genes and phylogenetic analysis, *Elaphodus* may have a sister relationship with another deer group *Muntiacus* and they belong to the monophyletic genus. The genus *Elaphodus* possesses a relatively closer relationship with the genus *Muntiacus* than with other genera at the basal position in Cervinae. In the genus of *Muntiacus*, *M. reevesi* has a sister relationship with the group of *M. vuquangensis* and *M. putaoensis*. In addition, the complete miotgenome of *E. cephalophus* provides useful information for further understanding of the phylogenetic classification and evolution of Cervidae species and the genetic structure of *E. cephalophus.*

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Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20210912140931

Statement of conflict of interest

The authors have declared no conflict of interest.

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