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COMPLETE MITOCHONDRIAL GENOME SEQUENCE OF A HUNGARIAN RED DEER *(CERVUS ELAPHUS HIPPELAPHUS)* FROM HIGH-THROUGHPUT SEQUENCING DATA AND ITS PHYLOGENETIC POSITION WITHIN THE FAMILY CERVIDAE

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Recently, there has been considerable interest in genetic differentiation in the Cervidae family. A common tool used to determine genetic variation in different species, breeds and populations is mitochondrial DNA analysis, which can be used to estimate phylogenetic relationships among animal taxa and for molecular phylogenetic evolution analysis. With the development of sequencing technology, more and more mitochondrial sequences have been made available in public databases, including whole mitochondrial DNA sequences. These data have been used for phylogenetic analysis of animal species, and for studies of evolutionary processes.

We determined the complete mitochondrial genome of a Central European red deer, *Cervus elaphus hippelaphus*, from Hungary by a next generation sequencing technology. The mitochondrial genome is 16 354 bp in length and contains 13 protein-coding genes, two rRNA genes, 22 tRNA genes and a control region, all of which are arranged similar as in other vertebrates. We made phylogenetic analyses with the new sequence and 76 available mitochondrial sequences of Cervidae, using *Bos taurus* mitochondrial sequence as outgroup. We used 'neighbor joining' and 'maximum likelihood' methods on whole mitochondrial genome sequences; the consensus phylogenetic trees supported monophyly of the family Cervidae; it was divided into two subfamilies, Cervinae and Capreolinae, and five tribes, Cervini, Muntiacini, Alceini, Odocoileini, and Capreolini. The evolutionary structure of the family Cervidae can be reconstructed by phylogenetic analysis based on whole mitochondrial genomes; which method could be used broadly in phylogenetic evolutionary analysis of animal taxa.

Keywords: Cervidae – *Cervus elaphus hippelaphus* – NGS – mitochondrial DNA – phylogenetics

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INTRODUCTION

The family Cervidae, which includes 54 species of deer, constitutes the second most populous family of artiodactyls [7]. These species are distributed widely throughout the world, including the northern hemisphere as well as South America and Southeast Asia, and they have adapted to various climates and environments. As a result of those mentioned above, they vary widely in size, habitat, and behavior, which makes their classification difficult [3, 9, 22]. Red deer (*Cervus elaphus* L. 1758) is one of the most abundant and best known deer species. It is one of the most desirable and highly prized trophies (on the royal game for trophies) in Europe. Today it is the prime member of the megafauna in Hungary and according to record trophies stands in the center of wide attention, and has an increasing economic, cultural and ecological importance [1, 18, 29, 36, 38]. Being an important game species, the red deer has been extensively managed, introduced, restocked, and selectively hunted throughout its history and distribution area, and is farmed for meat and for antler products throughout the world [1, 18, 28, 33, 36, 38]. Thus, the species has been the target of genetic studies during the past few decades [3, 23, 28–29, 33, 36, 38].

The mitochondrial genome is a small and circular molecule consisting of 15–20 kb and is conserved in vertebrates [4, 21, 33]. It contains 37 genes, including 13 protein-coding genes, 22 transfer RNA genes, two rRNA genes, and the control region (D-loop). The excellent characteristics of the mitochondrial genome, such as the small size, its abundance in animal tissues, the strict orthology of encoded genes, and its uniparental inheritance make mitochondrial DNA (mtDNA) a reliable and easy-to-use marker for population genetics, reconstruction of phylogenetic relationships among vertebrates, and molecular evolution analysis [3–4, 16, 21, 28, 32–33, 37]. In animals, many phylogenetic trees have been reconstructed from the sequence of single genes or elements, for example, Cytb or the D-loop region. The information provided by single genes or genetic elements is poor, however, and such phylogenetic analyses cannot avoid bias [5, 9, 35].

Currently, mtDNA sequences are released quickly in public databases, and many mitochondrial genomes are available in GenBank, EMBL-Bank, and the DNA Databank of Japan. The high-throughput and low cost of next-generation sequencing (NGS) enables the efficient generation of large amounts of genome sequence data from which mtDNA sequences can be identified, thus, the release of mitochondrial genome data is expected to increase. In family Cervidae, mtDNA of approximately 34 species have been sequenced, and they are available for researchers, making phylogenetic analysis based on the whole mitochondrial genome feasible.

In this study, we determined and analyzed the complete mtDNA sequence of a Central European red deer from Hungary *(Cervus elaphus hippelaphus)* using NGS technology. We analyzed the phylogenetic relationships and estimated divergence times based on the complete mitochondrial genomes of family Cervidae retrieved from GenBank. The complete sequence adds to our knowledge of the genetics, the evolution and classification of cervids.

MATERIALS AND METHODS

DNA sequencing and assembly

Total genomic DNA was extracted from blood samples of a deer stag from Game Management Landscape Center of Kaposvár University (Bőszénfa, Hungary) using Duplica Prep Automated DNA/RNA Extraction System (EuroClone S.p.A., Italy). The blood was collected from a living animal performed by a trained veterinarian according to standard veterinary medical practice with a permission from the Hungarian Veterinary Chamber. Isolated DNA samples were sequenced on Illumina HiSeq 2000 platform according to the manufacturer's instructions. The mitochondrial genome sequence was assembled *de novo* from 2×100 bp paired-end reads using the MITObim (mitochondrial baiting and iterative mapping) pipeline [6]. In the first step, mirabait program from the MIRA version 4.0.2 package was used to select mitochondrial reads; for baiting we used the *Cervus elaphus* mitochondrion genome sequence (AB245427). In the second step we used the mitobait.pl script, which carried out three iterative steps to build the mitochondrial genome; the final sequence was trimmed at the ends manually. Sequence analysis was carried out using MEGA6 (Molecular Evolutionary Genetics Analysis version 6.0) [31], tRNA genes were identified by tRNAscan-SE [14]. The complete mtDNA sequence has been deposited into the GenBank (Accession No. KT290948).

Phylogenetic and evolution analysis of Cervidae

Mitochondrial genome sequences of family Cervidae were searched and downloaded from the National Center for Biotechnology Information website. Taxa names and accession numbers of complete mitochondrial genomes of Cervidae are listed in Table 1. The mitochondrial genome of *Bos taurus* was also downloaded for use as an outgroup. The complete nucleotide sequences of the H strand of 78 complete mitochondria were aligned using ClustalW2 [10] and manually adjusted in a few instances. The multiple sequence alignments were input into MEGA6 [31], and phylogenetic analyses were performed with two commonly used method of tree reconstruction. Neighbor-joining (NJ) analysis with the bootstrap test, including 1000 replicates, was performed using pairwise deletion for gaps/missing data [30]. Owing to the unavailability of data partitioning and the general time reversible model in MEGA6, however, the non-partitioned dataset and the best available Tamura–Nei model were used for the NJ reconstruction. Maximum likelihood (ML) analysis with the bootstrap test, with 1000 replicates, was performed using complete deletion for gaps/missing data, with the general time reversible model.

Divergence dates were estimated using a Bayesian relaxed molecular clock approach implemented in Phylo-Bayes 3.1 [11] using the ML topology of Fig. 3. Following previous research [5, 7, 37], we selected three time constraints for fossil calibration: the first was the oldest fossil of Cervidae (20 ± 2 mya), the second one was

Table 1
Species and accession numbers of mitochondrial genome sequences used in this study

Species	Accession number
Alces alces	JN632595
Alces alces cameloides	KP405229
Axis axis	JN632599
Axis porcinus	JN632600
Blastocerus dichotomus	JN632603
Capreolus capreolus 1	JN632610
Capreolus capreolus 2	KJ681480
Capreolus capreolus 3	KJ681481
Capreolus capreolus 4	KJ681482
Capreolus capreolus 5	KJ681483
Capreolus capreolus 6	KJ681484
Capreolus capreolus 7	KJ681485
Capreolus capreolus 8	KJ681486
Capreolus capreolus 9	KJ681487
Capreolus capreolus 10	KJ681488
Capreolus capreolus 11	KJ681489
Capreolus capreolus 12	KJ681490
Capreolus capreolus 13	KJ681491
Capreolus pygargus 1	KJ681492
Capreolus pygargus 2	KJ681493
Capreolus pygargus 3	KJ681494
Capreolus pygargus 4	KJ681495
Cervus elaphus	AB245427
Cervus elaphus alxaicus	KP172593
Cervus elaphus hippelaphus	KT290948
Cervus elaphus songaricus 1	KJ025072
Cervus elaphus songaricus 2	HQ191429
Cervus elaphus xanthopygus	GU457434
Cervus elaphus yarkandensis	GU457435
Cervus nippon centralis	AB211429
Cervus nippon hortulorum 1	HQ191428
Cervus nippon hortulorum 2	GU457433
Cervus nippon kopschi 1	HQ832482
Cervus nippon kopschi 2	JN389444
Cervus nippon sichuanicus	JN389443
Cervus nippon taiouanus	EF058308
Cervus nippon yakushimae	AB218689
Cervus nippon yesoensis	AB210267
Dama dama	JN632629

Table 1 (cont.)

Species	Accession number
Dama mesopotamica	JN632630
Elaphodus cephalophus	DQ873526
Elaphurus davidianus 1	JN632632
Elaphurus davidianus 2	JN399997
Hippocamelus antisensis	JN632646
Hydropotes inermis 1	JN632649
Hydropotes inermis 2	EU315254
Hydropotes inermis argyropus 1	JX254914
Hydropotes inermis argyropus 2	KP203884
Mazama americana 1	JN632656
Mazama americana 2	JN632657
Mazama gouazoubira	JN632658
Mazama nemorivaga 1	JN632659
Mazama nemorivaga 2	JN632660
Mazama rufina	JN632661
Muntiacus crinifrons	AY239042
Muntiacus muntjak	AY225986
Muntiacus reevesi micrurus	EF035447
Muntiacus vuquangensis	FJ705435
Odocoileus hemionus	JN632670
Odocoileus virginianus 1	JN632671
Odocoileus virginianus 2	JN632672
Odocoileus virginianus 3	JN632673
Odocoileus virginianus 4	HQ332445
Ozotoceros bezoarticus	JN632681
Przewalskium albirostris 1	JN632690
Przewalskium albirostris 2	HM049636
Pudu mephistophiles	JN632691
Pudu puda	JN632692
Rangifer tarandus	AB245426
Rangifer tarandus phylarcus	KM506758
Rucervus duvaucelii	JN632696
Rucervus eldi 1	JN632697
Rucervus eldi 2	HM138200
Rusa alfredi	JN632698
Rusa timorensis	JN632699
Rusa unicolor swinhoei 1	EF035448
Rusa unicolor swinhoei 2	DQ989636
Bos taurus	HM045018

the split between Cervini and Muntiacini (9 ± 1 mya), and the third one was the oldest fossil of tribe Odocoileini (5 ± 1 mya). Using such constraints involves the assumption that the age of the oldest fossil attributed to a node is a good approximation of the minimum age of the node. As recommended by [11], the analyses were conducted using the CAT-GTR + Gamma 4 model and a log-normal auto correlated clock relaxation model, calculations were conducted 30,000 cycles sampling posterior rates and dates every 10 cycles. Posterior estimates of divergence dates were then computed from the last 2500 samples accounting for the initial burn-in period.

RESULTS

Mitochondrial genome sequence of Cervus elaphus hippelaphus

The complete mtDNA of the Central European red deer is 16 354 bp in length, including 22 transfer RNA (tRNA) genes, two ribosomal RNA (rRNA) genes, 13 protein-coding genes and a noncoding region (Table 2 and Fig. 1). The orientation and gene organization are identical to other species of cervids [8, 12–13, 25–27, 33, 35], and to the typical organization in mammals. Most of the mitochondrial genes are encoded on the heavy (H) strand, except the ND6 gene and nine tRNA genes (Fig. 1). The nucleotide composition of the H strand is 33.3% of A, 24.4% of C, 13.5% of G and 28.8% of T.

The protein-coding genes of the red deer are identical to those of other deer [8, 12–13, 25–27, 33, 35]. The total length of the 13 protein-coding genes is 11,426 nucleotides. The longest one is the ND5 gene (1821 nucleotides), whereas the shortest is the ATP8 gene (201 nucleotides). Ten out of the 13 protein-encoding genes have an ATG start codon, except the ND2, ND3 and ND5 genes, which have an ATA start codon. Ten of the genes have a complete stop codon, TAA or TAG, while three of them appears to end with an incomplete stop codon (single T or TA).

Twenty-two transfer RNA genes were identified in the mtDNA of red deer. The total length of the 22 tRNA genes is 1523 nucleotides, and ranges from 66 ($tRNA^{Pro}$) to 75 nucleotides ($tRNA^{Leu}$). All tRNA genes have the cloverleaf secondary structure, except $tRNA^{Ser}$ that does not have the ''DHU'' arm. Like in other vertebrates, there are two ribosomal RNA genes in the red deer mitochondrion. The lengths of the 12S rRNA and 16S rRNA are 955 and 1567 nucleotides, respectively, and they are located between $tRNA^{Phe}$ and $tRNA^{Leu}$, separated by $tRNA^{Val}$.

Like in most vertebrates, the light strand replication origin (OL) is between $tRNA^{Asn}$ and $tRNA^{Cys}$, and consists of 32 nucleotides. The non-coding control region, 920 nucleotides in length, is located between $tRNA^{Pro}$ and $tRNA^{Phe}$. The red deer mtDNA control region contains conserved sequence blocks (CSB-F, CSB-E, CSB-D, CSB-C, CSB-B, CSB-1 and CSB-2), and all four termination-associated sequences (TAS-1 to TAS-4) (Fig. 2.). These sequences are highly similar to other species of Cervidae [2, 12–13, 25–27, 33]. The conserved sequence blocks, and the termination-

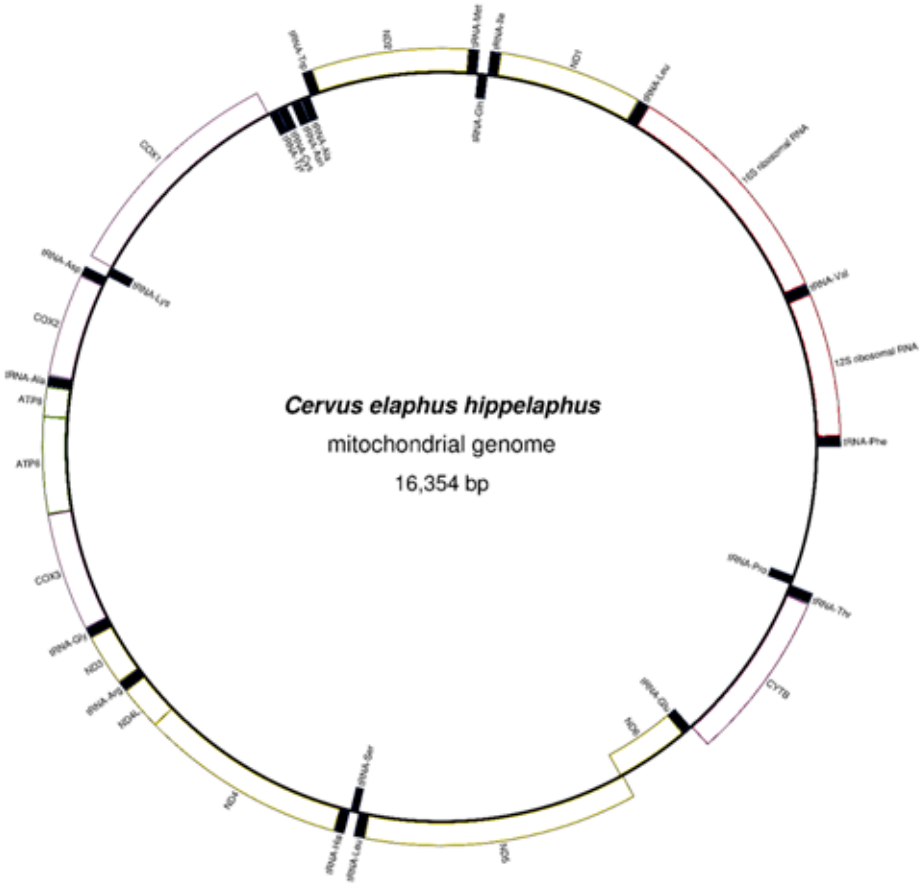

Fig. 1. The map of the Hungarian red deer mitochondrial genome

associated sequences can be used for fine-scale population studies, since they are amongst the most rapidly evolving segments in the animal mtDNA [19, 24, 28].

Mitogenomic phylogeny of the family Cervidae

The NJ and ML phylogenetic trees, which are based on mitochondrial genome sequences, have similar topology, as shown in Figure 3. All supra-generic taxa described in previous studies [7, 34–35, 37] are highly supported, i.e. the subfamily Cervinae with two tribes Cervini and Muntiacini, and the subfamily Capreolinae with three tribes Alceini, Odocoileini and Capreolini. The monophyly of Cervidae was well supported, with a bootstrap value of 100% in NJ and ML. The intertribal rela-

Table 2
Genes of the red deer's mitochondrial genome

Name	Site	Length	Intergenic nucleotide[a]	Strand	Start codon	Stop codon	Anti-codon
$tRNA^{Phe}$	1–69	69		H			GAA
12S rRNA	70–1024	955	0	H			
$tRNA^{Val}$	1025–1091	67	0	H			TAC
16S rRNA	1092–2658	1567	0	H			
$tRNA^{Leu}$	2664–2738	75	5	H			TAA
ND1	2741–3697	957	2	H	ATG	TAA	
$tRNA^{Ile}$	3697–3765	69	–1	H			GAT
$tRNA^{Gln}$	3763–3834	72	–3	L			TTG
$tRNA^{Met}$	3837–3905	69	2	H			CAT
ND2	3906–4949	1044	0	H	ATA	TAG	
$tRNA^{Trp}$	4948–5015	68	–2	H			TCA
$tRNA^{Ala}$	5018–5086	69	2	L			TGC
$tRNA^{Asn}$	5088–5160	73	1	L			GTT
$tRNA^{Cys}$	5193–5260	68	32	L			GCA
$tRNA^{Tyr}$	5261–5329	69	0	L			GTA
COX1	5331–6875	1545	1	H	ATG	TAA	
$tRNA^{Lys}$	6873–6941	69	–3	L			CTT
$tRNA^{Asp}$	6949–7016	68	7	H			GTC
COX2	7018–7701	684	1	H	ATG	TAA	
$tRNA^{Ala}$	7705–7772	68	3	H			AGC
ATP8	7774–7974	201	1	H	ATG	TAA	
ATP6	7935–8615	681	–40	H	ATG	TAA	
COX3	8615–9414	800	–1	H	ATG	TA	
$tRNA^{Gly}$	9399–9467	69	–16	H			TCC
ND3	9468–9814	347	0	H	ATA	TA	
$tRNA^{Arg}$	9815–9883	69	0	H			TCG
ND4L	9884–10,180	297	0	H	ATG	TAA	
ND4	10,174–11,551	1378	–7	H	ATG	T	
$tRNA^{His}$	11,552–11,620	69	0	H			GTG
$tRNA^{Ser}$	11,621–11,680	60	0	L			
$tRNA^{Leu}$	11,682–11,751	70	1	H			TAG
ND5	11,752–13,572	1821	0	H	ATA	TAA	
ND6	13,556–14,083	528	–17	L	ATG	TAA	
$tRNA^{Glu}$	14,084–14,152	69	0	L			TTC
CYTB	14,157–15,299	1143	4	H	ATG	TAA	
$tRNA^{Thr}$	15,300–15,369	70	0	H			TGT
$tRNA^{Pro}$	15,369–15,434	66	–1	L			TGG

[a]Negative numbers indicate that adjacent genes overlap.

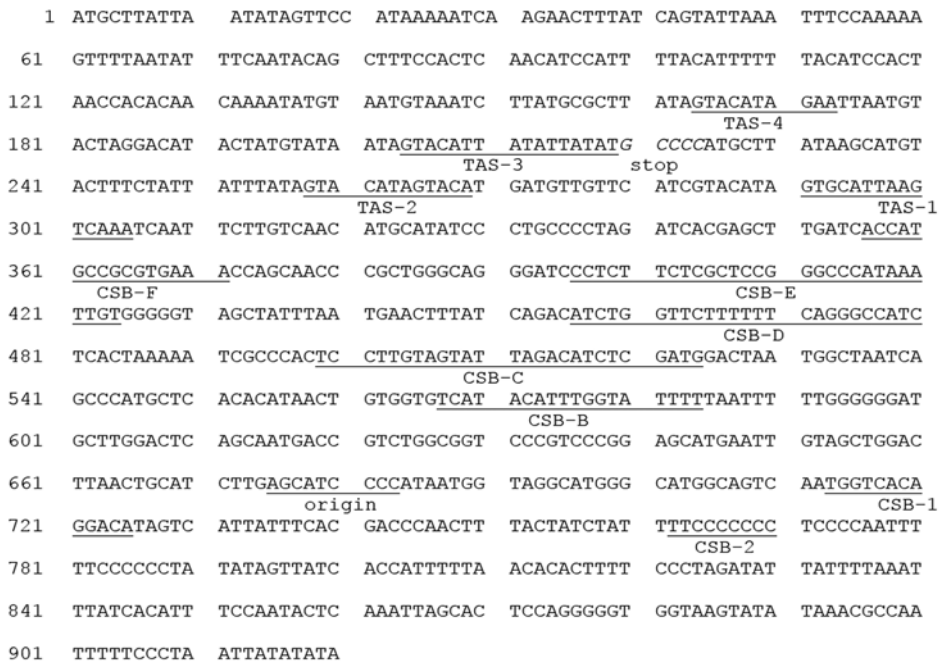

Fig. 2. Sequences and the conserved elements of the control region of the red deer. Primary sequence features (TAS-1, TAS-2, TAS-3, TAS-4, CSB-B, CSB-C, CSB-D, CSB-E, CSB-F, origin of H replication, CSB-1, CSB-2) are underlined and stop (putative point of arrest of the D-loop synthesis) is marked in italics

tionships within Cervinae are robust, but in Capreolinae most intertribal relationships are not robust, suggesting rapid diversifications. The phylogenetic tree showed not only the phylogeny of the whole family but also the monophyly of tribes, such as Muntiacini. The phylogenetic tree suggested classification information within species, for example, the division of *Cervus elaphus* into two clades: a western clade with *C. e. hippelaphus* and *C. e. yarkandensis*, and an eastern clade with *C. e. xanthopygus*, *C. e. alxaicus* and *C. e. songaricus*, which were Asian ecotypes [2, 7, 15, 20, 37].

Estimation of divergence times in family Cervidae

Estimated divergence times of family Cervidae are shown in Figure 4. According to the phylogenetic tree, the origin of family Cervidae was estimated to be a mean value of 10.69 mya, indicating that the true divergence times of family Cervidae were later than the fossil evidence suggested. The subfamilies Capreolinae and Cervinae became divided at 9.77 and 8.92 mya, respectively, whereas the five tribes, Cervini, Muntiacini, Alceini, Odocoileini, and Capreolini, were divided at different times. The

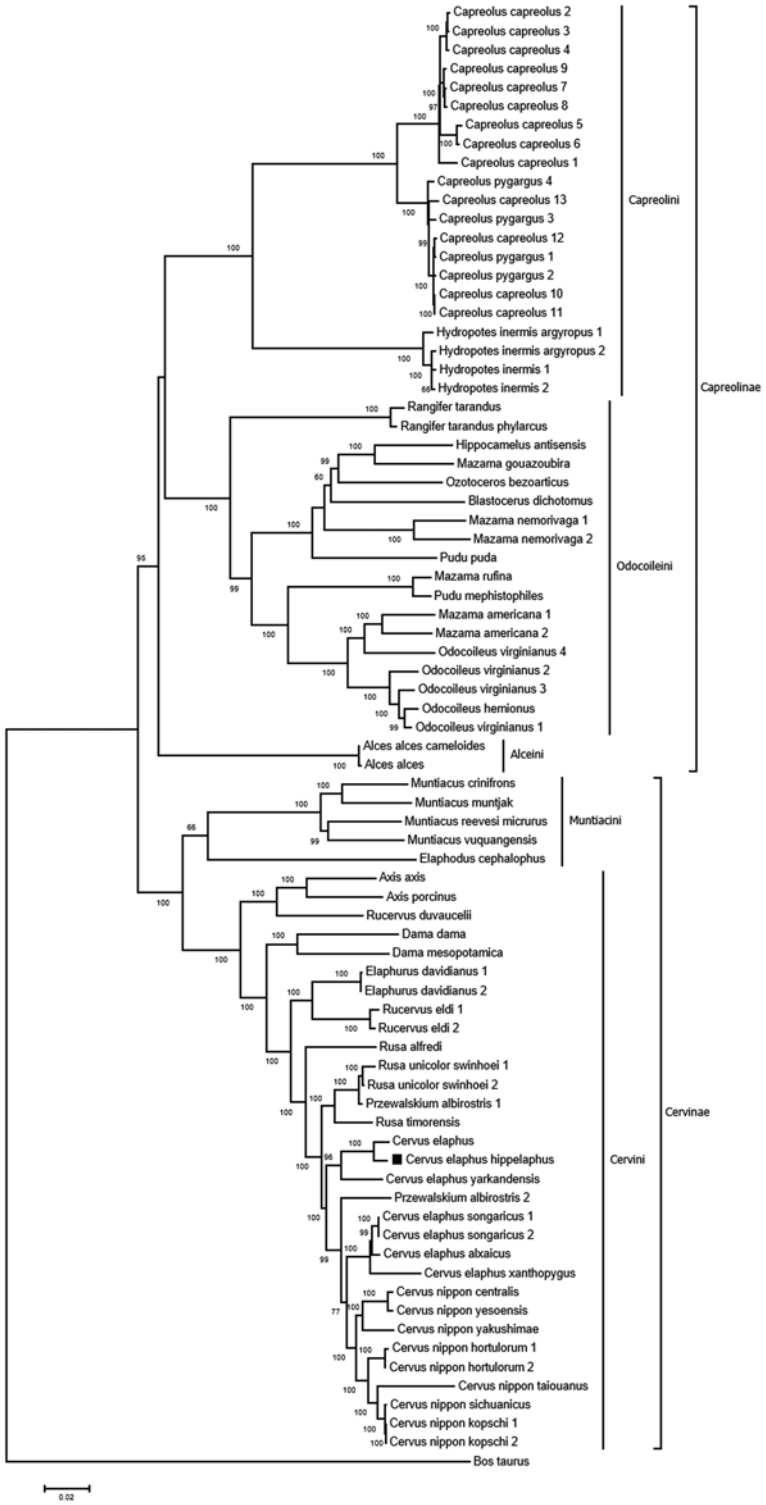

Fig. 3. Maximum Likelihood phylogenetic tree of the relationships among family Cervidae based on whole mitochondrial genomes, *Bos taurus* served as outgroup. Number on branches or nodes indicates the bootstrap values estimated by 1000 bootstrap replications (%), values lower than 60 are not shown. Scale bar represent branch length (substitution per site). The black square indicates the Hungarian red deer

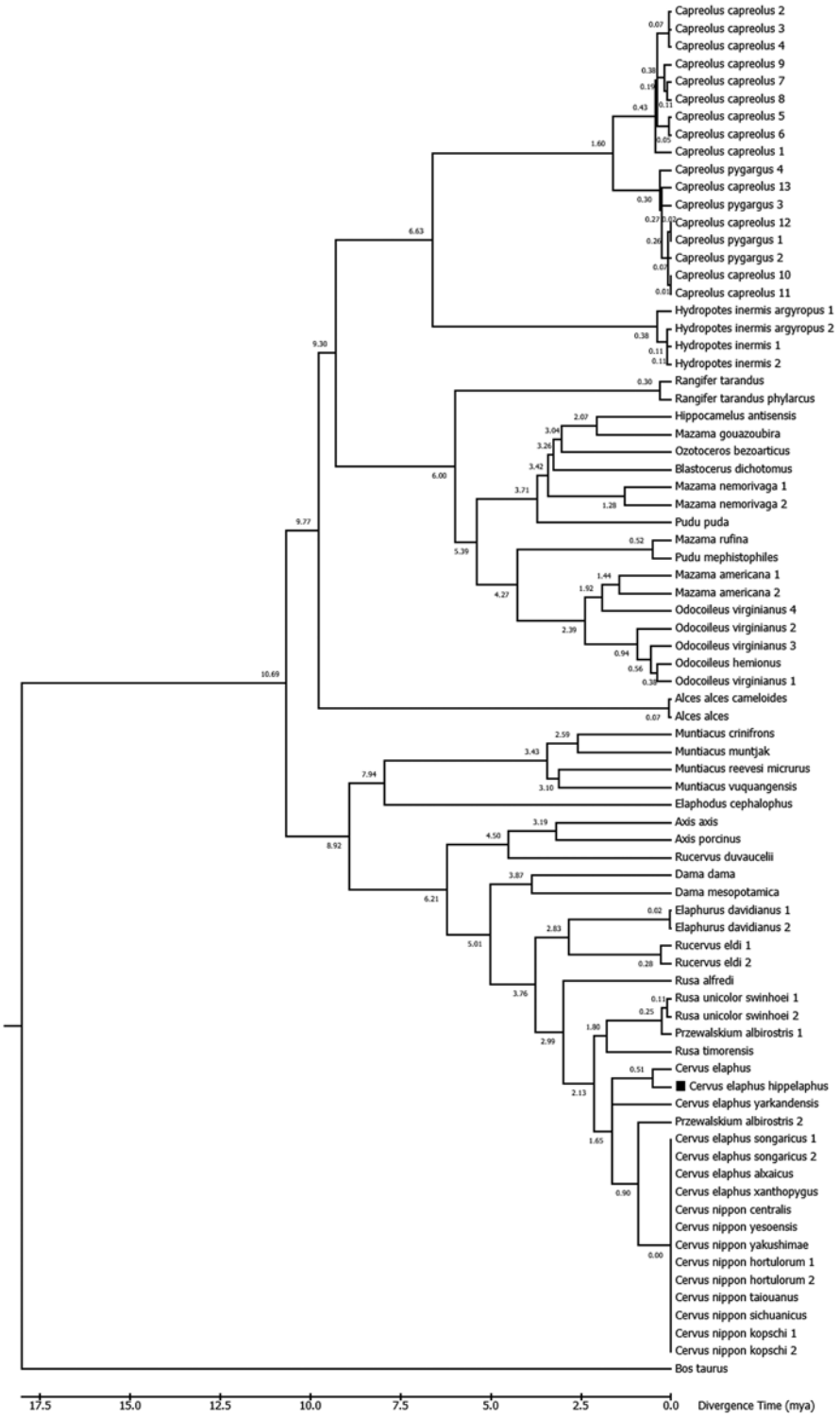

Fig. 4. Phylogenetic tree of family Cervidae and outgroup *Bos taurus* based on the whole mitochondria genomes, divergence times were estimated by Bayesian method, and they were shown on the labels of nodes. The black square indicates the Hungarian red deer

divergence times of some species of Cervidae were very small; e.g. the divergence time between *Cervus elaphus songaricus*, *Cervus elaphus alxaicus*, *Cervus elaphus xanthopygus*, and *Cervus nippon* subspecies was lower than 0.01 mya, which suggested that they may not belong to two separate species but may belong to the varieties of *C. nippon*.

DISCUSSION

During the last two decades, extensive efforts have been made to investigate phylogenetic relationships of Cervidae, and also in Cetartiodactyla [e.g. 3, 5, 7, 9, 17, 20, 22, 32, 35]. However, relationships within this group remain unclear. It is possible that different molecular markers have a different evolutionary rate; even if the same markers, the substitution rate varies among taxa. Thus, a single gene or a short DNA sequence applying to phylogeny reconstruction is very likely to produce an incorrect tree topology for a systematic bias and/or long-branch attraction. The complete mitochondrial genome provides a higher level of support for molecular systematics than those based on individual or partial mitochondrial genes [5, 9, 21]. In the present study, we determined the complete mitochondrial genome of a Central European red deer, *Cervus elaphus hippelaphus*, from NGS data. The protein-coding genes of the red deer are identical to those of other deer species [8, 12–13, 21, 25–27, 33, 35], and have a methionine start codon, ATG or ATA, and mainly also complete stop codons, TAA or TAG. It is common for termination codons to be truncated, to T or TA, in metazoan mitochondrial genomes, such codons are presumably completed as TAA by post-transcriptional polyadenylation [21, 33]. The size and orientation of tRNA and rRNA genes are similar to those of other cervids [8, 12–13, 21, 25–27, 33, 35]. The lack of the ''DHU'' arm in $tRNA^{Ser}$ has been reported in other species of Cervidae [8, 21, 25–27, 35]. The non-coding control region contains conserved sequence blocks, and termination-associated sequences; and can be used for fine-scale population studies, since it is amongst the most rapidly evolving segments in the animal mtDNA [17, 19, 24, 28]. The high-throughput approach for sequencing was useful for obtaining the whole mitochondrial genome of red deer, so this method could be used broadly among animal taxa.

Based on our phylogenetic results using whole mitochondrial genomes, family Cervidae is divided into two subfamilies, Cervinae and Capreolinae, which are also called subfamilies Plesiometacarpalia and Telemetacarpalia [5, 7, 22, 34, 37]. The two subfamilies contain five tribes, and four of these, Cervini, Muntiacini, Odocoileinae, and Capreolini, are well determined by the phylogenetic trees of family Cervidae. Hungarian red deer joined to the semi-domesticated red deer of New Zealand with a bootstrap value of 100%, and clustered to tarim red deer (*C. e. yarkandensis*) forming a western clade of red deer. Other *Cervus elaphus* subspecies, which were Asian ecotypes, formed a second, eastern clade; which is consistent with previous reports [2, 7, 12, 15, 20, 37].

The relationships among sika deer inferred from our phylogenetic and evolutionary analyses were inconsistent with subspecies designation, but similar to previous mitochondrial phylogenies [12, 15, 35]. The divergence times (less than 0.01 mya) suggest that *Cervus elaphus songaricus*, *Cervus elaphus alxaicus*, *Cervus elaphus xanthopygus* are more closely related to *Cervus nippon* than to other *Cervus elaphus* subspecies. The close relationship of these two groups, as described previously, could be the result of the evolution of the genus *Cervus* in Asia [5, 7, 9, 15, 22, 35, 36], or maybe it could be attributed to anthropogenic influences as seen in European deer [18, 20, 28–29, 33, 36].

The mtDNA distances between several species studied here are smaller than those generally obtained between subspecies (<2%), which raises the possibility of species misidentification or imperfect taxonomy (species synonymy). In some cases, the sequences described may have been misidentified. For example in the case of *Capreolus* species, from which some are described as belonging to *Capreolus pygargus* [17], but deposited in GenBank as *Capreolus capreolus*. A second example of possible species assignation error concerns *Mazama rufina* and *Pudu mephistophiles*, which share very similar mtDNA genomes (98.6%). Sequences were obtained from pieces of skin collected in Colombia, where the same name can be used to designate both species, and in addition, both species live in the same habitat, and exhibit the same color pattern [7]. The placement of two isolates of *Przewalskium albirostris* also suggest species misidentification as they lay on different branches. The placement as the sister species to a *C. nippon* and *C. elaphus* clade seems the appropriate one as suggested by [22]. The other isolate may be attributed to a misidentified specimen of *Rusa* spp.

The determined divergence times of the family Cervidae are somewhat higher, than dates estimated from previous molecular phylogenetic analysis [5, 22, 37], which suggested that family Cervidae was present at 7.7–9.6 mya. These time estimates, and also our estimate indicate that the true divergence times within family Cervidae were later than the fossil evidence suggested. The evolution of Cervidae is complex, and relationships within this group are unclear, due to possible species misidentification and imperfect taxonomy.

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