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First ancient DNA sequences of the Late Pleistocene red deer (*Cervus elaphus*) from the Crimea, Ukraine

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ABSTRACT

The Emine-Bair-Khosar Cave (EBK), situated on the northern edge of the Lower Plateau of the Chatyrdag Massif (Crimean Mountains) is rich in palaeontological material accumulated over most of the Pleistocene. mtDNA was isolated from bones of three specimens of red deer (*Cervus elaphus*) dated to the late Pleistocene (MIS 3). These are the first ancient DNA sequences obtained for this species. The position of the three red deer individuals on the phylogeographic tree is based on mtDNA sequences of contemporary representatives of the Cervinae inhabiting the Northern Hemisphere. The results confirm the notion that the Crimean Peninsula was the north-easternmost refugium in Europe, and that during and after the Late Pleistocene it played a major role in recolonisation and dispersal of temperate species in the whole Eurasian continent.

1. Introduction

Massive climatic and environmental changes during the Pleistocene significantly influenced the distribution and the genetic diversity of plants and animals (Hofreiter and Stewart, 2009). The model of glacial refugia and habitat contraction to southern peninsulas in Europe as areas for the survival of temperate animal species during Pleistocene glaciations is, at present, widely accepted (Sommer and Nadachowski, 2006). Extensive palaeontological investigations carried out in recent years in Ukraine indicated that the Crimean Peninsula was one of the refugia in Eastern Europe (Markova, 2011).

The rich fossil collection was discovered in Emine-Bair-Khosar Cave (EBK) (Fig. 1), situated on the north edge of Lower Plateau of the Chatyrdag Massif (Crimean Mountains). EBK is one of the largest cavities in the area with a total length of 1460 m and a depth of 125 m. The cave entrance is a vertical shaft, which functioned over a long period of time (probably most of the Pleistocene) as a huge trap. The speleological and palaeontological investigations of EBK were started in the 1960's (Bachynsky and Dublyansky, 1963; Dublyansky and Lomaev, 1980) when nearly two hundred bones were collected from a small chamber near the main access passage. The bones belonged mainly to carnivores (*Canis lupus, Vulpes corsac, Ursus spelaeus, Panthera leo spelaea, Lynx lynx*) and some herbivores (*Equus* sp., *Cervus elaphus*). Further palaeontological studies started in 1999 and were especially intensive in 2002 and 2003 (Vremir and Ridush, 2002, 2005), when nine other sites were investigated inside the cave. The richest bone accumulations (sites Bb and Bc) yielded more than 5000 bones. At least 35 vertebrate species (mainly mammals, but also birds and reptiles) were recorded at various stratigraphic units (the taxonomic identification is still in progress). The vertebrate assemblages, the preservation and spatial distribution of the bone material, and the stratigraphical and micromineralogical data suggest peculiar taphofacies as well as a very complex sediment-entrapment process (Vremir and Ridush, 2005, 2006) (Fig. 2).

Excavations that started in 2005 and continued through 2008–2010 (Ridush and Proskurnyak, 2008) revealed another rich assemblage of animal bones at the Ba2 site. Deposits at this site are

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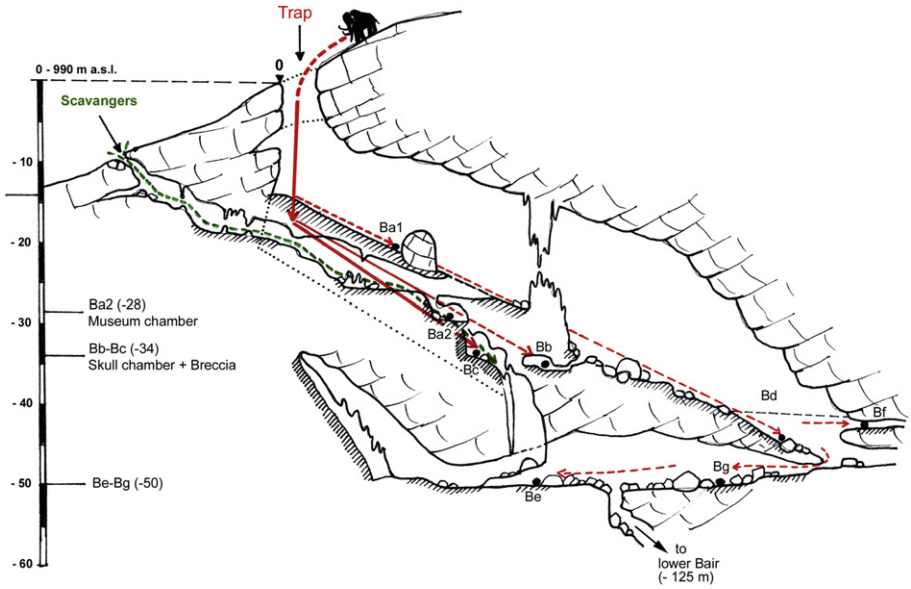

Fig. 1. Scheme of the EBK cave (Vremir and Ridush, 2005).

formed from soil/loess material transported into the cave from the outside through the entrance pit and by limestone debris. The cave fill contains numerous palaeontological remains (vertebrates and molluscs) and has visible subhorizontal stratification (Bondar and Ridush, 2009). The fauna consists of more than 30 species of large and small mammals as well as birds and lower vertebrates. In the 6 m deep Ba2 section, eight layers were distinguished in its upper 2.6 m. The uppermost part of the section was probably deposited during the Holocene (Bondar and Ridush, 2009). Bones of *Cervus* from the layer 2.0–2.6 m deep were radiocarbon dated to 33,100 ± 400 BP (38,791 ± 1526 cal BP) and those from the deepest part (4.6 m and 4.7 m of excavated profile) were dated to 42,000 ± 1200 BP (53,020 ± 3262 cal BP) and >46,000 BP, respectively. Radiocarbon dating locates the Ba2 EBK assemblage in Middle (Bryansk-Dunayevo/Insterstadial) Valdaian age (the early part of MIS 3).

This paper presents the results of the analysis of DNA isolated from bones of three specimens of red deer (*C. elaphus*) excavated from EBK. The phylogeography of Cervinae has been thoroughly studied (Polziehn and Strobeck, 2002; Ludt et al., 2004; Sommer et al., 2008; Sommer and Zachos, 2009; Skog et al., 2009), but the species status of certain populations remains unclear. The studies on mitochondrial DNA of contemporary red deer populations demonstrated that they can be divided into two separate groups, the Western and Eastern red deer (Polziehn and Strobeck, 2002; Mahmut et al., 2002; Ludt et al., 2004; Pitra et al., 2004). It has been shown that during cold periods, European red deer populations were restricted to refugial areas, mainly Iberian Peninsula, Apennine Peninsula, Balkan Peninsula and Carpathians (Sommer and Zachos, 2009; Skog et al., 2009). This study investigates which of the red deer populations (Western or Eastern) inhabited the Crimean Peninsula during the Late Pleistocene and what the role of this refugium in recolonization and dispersal processes of red deer populations was.

2. Materials and methods

2.1. Materials

The bones excavated in 2007–2009 from Emine-Bair-Khosar were kept at the local museum. Following identification and osteometric studies, the material samples were taken for C^{14} dating and for DNA analysis. Dating of materials was performed at the Poznań Radiocarbon laboratory and is shown in Table 1.

2.2. DNA extraction, amplification and sequencing

Tooth and bone fragments were washed with bleach, rinsed with ddH_2O, UV irradiated for at least 20 min on each side and pulverized in a cryogenic mill (Spex CentriPrep). Up to 500 mg of bone powder was incubated overnight at 40 °C in 1.6 ml of extraction buffer (0.5 M EDTA, 0.7 mg of proteinase K (20 mg/ml) (Bioline), 0.1 M DTT, 50 mM PTB, 0.5% N-Lauryl sarcosine salt) with constant agitation. After incubation, the supernatant was subjected to phenol:chloroform:isoamyl alcohol (25:24:1, v:v:v) DNA extraction followed by extraction by chloroform and isopropanol precipitation. The DNA precipitate was resuspended in 60 μl of TE.

Two approaches were used to obtain *C. elaphus* cytochrome *b* (cyt *b*) sequence: (1) Twelve PCR primer pairs (Set 1, Appendix A) were designed using Primer3 (v. 0.4.0) software. Primer pairs were screened for potential secondary structure using the AutoDimer and Fast PCR software. Amplifications were performed in singleplex reactions for all three samples in a 25 μl volume reaction containing 2 μl mock or ancient DNA extracts, 0.2 μM forward and reverse primers, 1 μl of BSA (5 mg/ml) and 12.5 μl AmpliTaq Gold PCR Master Mix (Applied Biosystems) in a C1000 Bio-Rad thermal cycler. Amplification conditions consisted of a 12 min activation step at 95 °C, followed by 45 cycles at 95 °C for 30 s, 41 °C for 30 s, and 72 °C for 30 s and a final extension at 72 °C for 7 min. PCR products were sequenced in a ABI PRISM 3730 × l DNA sequencer. (2) Multiplex PCR reaction and pyrosequencing protocol designed by Stiller et al. (2009) was applied. A second set (Set 2, Appendix A) of twelve primer pairs was designed. Amplification was performed in a 25 μl volume reaction containing 2 μl mock or ancient DNA extracts, 0.16–0.32 μM forward and reverse primers and 1.5 μl AmpliTaq Gold PCR Master Mix (Applied Biosystems) in a C1000 Bio-Rad thermal cycler. Some of the primer pairs showed lower amplification efficiency in multiplex reactions, thus concentrations of certain primers were increased (Appendix A). Amplification conditions consisted of a 12 min activation step at 95 °C, followed by 25 cycles at 94 °C for 30 s, 53 °C for 30 s, and 72 °C for 30 s and

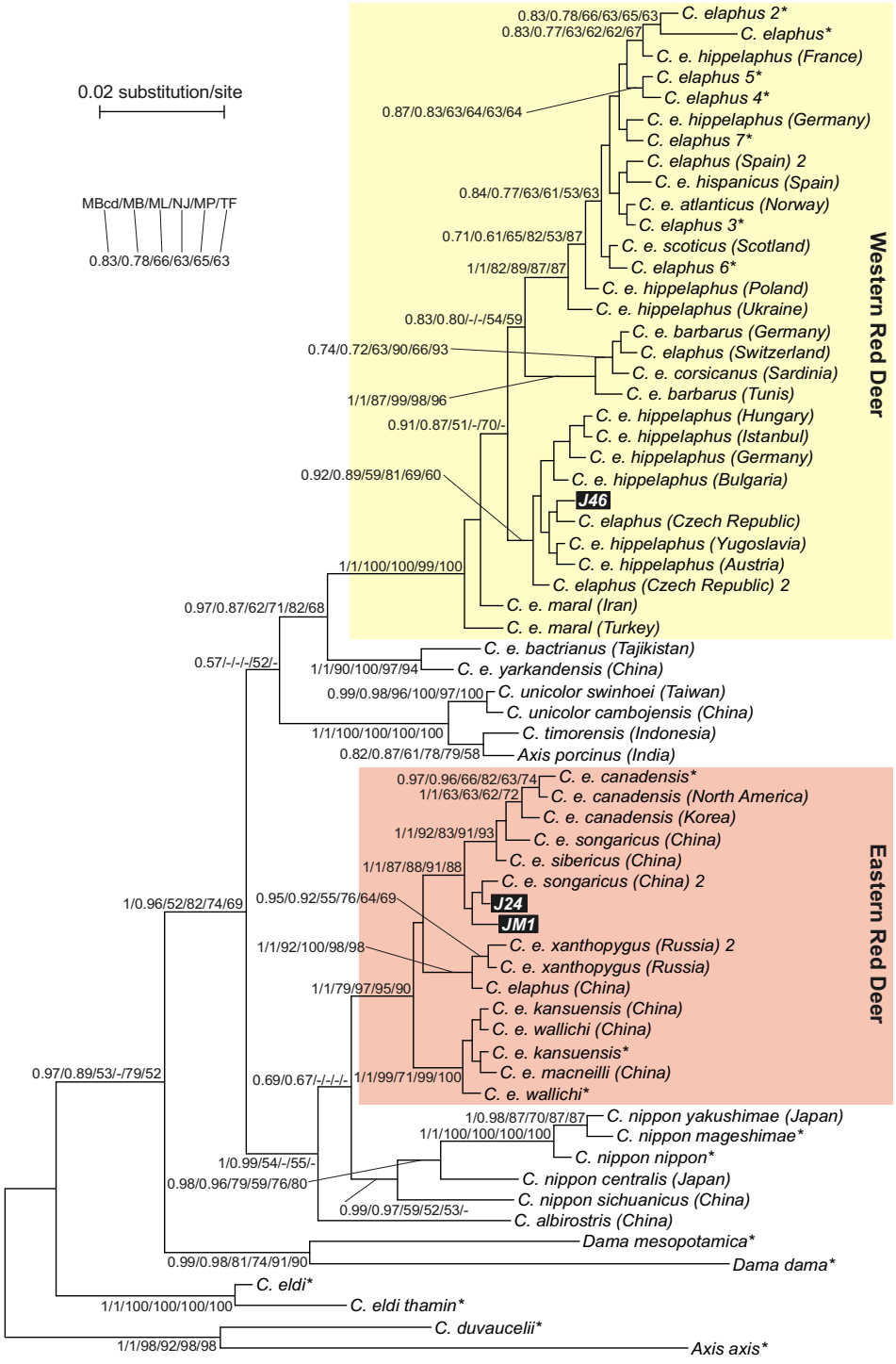

Fig. 2. Red deer phylogeny obtained for cytochrome b in MrBayes under separate models for three codon positions. Accession number of *Cervus* cyt b sequences obtained from the NCBI data bank: *C.elaphus* EU878391, *C. elaphus 2* AB001612, *C. elaphus 3* EF139145, *C. elaphus 4* EF139146, *C. elaphus 5* EU004023, *C. elaphus 6* EU004020, *C. elaphus 7* DQ524848, *C. elaphus (Czech Republic)* DQ524848, *C. elaphus (Czech Republic) 2* DQ524847, *Axis axis* AY607040; *C. duvaucelii* AY607041; *Dama dama* AJ000022; *Dama mesopotamica* AY607034; *C. e. scoticus* AB021099; *C. unicolor swinhoei* DQ989636; *C. e. wallichi* FJ611889; *C. e. kansuensis* AB021098; *C. e. canadensis (Korea)* EF139147; *C. e. canadensis* AB021096; *C. nippon centralis* AB211429; *C. n. nippon* AB021093; *C. nippon mageshimae* AB021092; *C. nippon yakushimae* AB218689; *C. eldi thamin* AY607037; *C. eldi hainanus* AY157735. The remaining sequences were taken from Ludt et al. (2004). Origin of specimens is written in brackets. Unknown origin is marked by asterisk. Sequences of specimens studied in this work are marked with black squares. Numbers at nodes, in the order shown, correspond to: posterior probabilities estimated in MrBayes under separate models for three codon positions (MBcd) and one model for all positions (MB), bootstrap support values calculated in PAUP by maximum likelihood (ML), neighbor joining (NJ) and maximum parsimony (MP), as well as in TreeFinder (TF). Values of the posterior probabilities and bootstrap percentages lower or equal to 0.50 and 50%, respectively, were omitted or indicated by a dash "-". Low support values for some internal branches are also not shown.

a final extension at 72 °C for 10 min. Multiplex PCR products were used to prepare a library for pyrosequencing following the protocol of Stiller et al. (2009) and sequenced using a Roche 454 sequencing platform. The obtained sequences were deposited in GenBank under accession numbers J24 HM596026, J46 HM596027 and JM1 HM596028.

Table 1
Cervus elaphus samples from Emine-Bair-Khosar Cave.

Sample symbol	EBK site	Sample description	AMS dating (Lab no.)	Calibrated age
J24	EBH nr 1593 Ba2 D2(g); −240	fragment of metatarsus	33,100 ± 400 BP (Poz-35028)	37,570 ± 781 BP
JM1	EBH nr 2580 Ba2 C1 (b); −320	tooth (M_1)	42,000 ± 1200 BP (Poz-35027)	45,641 ± 1334 BP
J46	EBH nr 2355 Ba2 C2(b); −460	fragment of metacarpus	>47,000 BP (Poz-35026)	–

2.3. Sequence analysis and consensus calling

Sequences obtained in singleplex PCR reactions were used to obtain consensus sequences for samples J46, J24 and JM1. Each PCR fragment was obtained in at least two independent PCR reactions and sequenced. Sequences of samples J24 and JM1 were additionally confirmed by pyrosequencing of PCR fragments obtained in multiplex reaction. Consensus sequences were called for each sample using SeqMan Pro software (DNAStar Lasergene).

2.4. Phylogenetic analyses

Phylogenetic relationships of ancient and modern *Cervus* specimens were estimated by six approaches using three programs: MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003), PAUP* 4.0b (Swofford, 1998), and TreeFinder (Jobb et al., 2004).

With MrBayes, two strategies were applied. The first used one substitution model HKY + I + Γ for all cyt b positions, while the second one (Fig. 1) assumed three separate models for three codon positions: K2P + I (for the first codon position), F81 (for the second codon position), and GTR + Γ (for the third codon position). The models were selected according to the MrAIC 1.4.4. program (Nylander, 2004) based on Phyml 3.0 (Guindon and Gascuel, 2003), which includes models implemented in MrBayes. Two independent runs starting from random trees, using 4 Markov chains, were carried out. Trees were sampled every 100 generations of 10 or 20 million generations, in the first and in the second strategy, respectively. After reaching convergence,trees were selected in the stationary phase from the last 7 or 5.5 million generations, respectively.

Trees in PAUP were constructed by maximum likelihood (ML), neighbor joining (NJ), and maximum parsimony (MP) methods. ML and NJ used the TPM3uf + I + Γ model as proposed by Phyml-based jModeltest 0.1.1 (Posada, 2008). Bootstrap MP, final ML and MP trees were searched from 10 starting trees obtained by stepwise addition with random-addition sequence. TBR branch-swapping algorithm was applied on starting tree in the tree search and bootstrap procedures for PAUP ML and MP methods.

In TreeFinder using the maximum likelihood method, separate substitution models were also applied for three codon position: TN + Γ (for the first codon position), R3 (for the second codon position), and GTR + Γ (for the third codon position), as suggested by this program's Propose Model module.

The non-parametric bootstrap analyses were performed on 1000 replicates for each of PAUP and TreeFinder methods. In all analyses among-site rate variation was modelled on a gamma distribution with five category rates.

3. Results and discussion

The phylogeny and taxonomy of the family Cervidae is relatively well known due to several molecular studies which were published in recent years (Polziehn and Strobeck, 2002; Pitra et al., 2004; Ludt et al., 2004; Sommer et al., 2008; Skog et al., 2009). The red deer (*C. elaphus*) belongs to the subfamily Cervinae, which according to the fossil record and geographic distribution originated in Central Asia and dispersed to Europe and North America (Di Stefano and Petronio, 2002; Ludt et al., 2004). In all of the papers quoted above, the role played by the Pleistocene refugia for the present pattern of distribution of the red deer populations in Europe is stressed. Out of three European lineages established on the basis of D-loop sequences, the western and eastern groups are linked to an Iberian and Balkan refugium, respectively, while the third one is associated with Sardinian or African refugia (Skog et al., 2009; Niedziałkowska et al., 2011).

All prior molecular studies of Cervidae were conducted on contemporary material, and the fossil samples served only to validate the dates of divergence of particular genera obtained by molecular dating (e.g. Gilbert et al., 2006). An attempt was made to isolate DNA from the red deer specimens found in the Crimean EBK cave, dated for 33,100 ± 400 BP (sample J24), 42,000 ± 1200 BP (JM1) and >47,000 BP (J46). For all samples, the sequence for 732 bp of cyt *b* gene was obtained, which, in red deer phylogenetics, is the most commonly used marker.

In the case of the first two specimens, sequences were obtained by pyrosequencing of DNA fragments amplified in the multiplex PCR reaction. In the case of the J46 sample, analysis was unable to obtain fragments of good quality suitable for pyrosequencing, so 12 separate amplicons were sequenced, covering the cyt b sequence.

Cyt *b* sequences of all three Pleistocene red deer specimens were included in the phylogenetic tree (Fig. 1), constructed for over 60 Cervinae species whose cyt *b* sequences were obtained from GenBank. The phylogenetic tree obtained in this study is similar to those obtained by Ludt et al. (2004), and consists of two clearly separated and well-supported clades of Western and Eastern Red Deer. The sequence obtained for the oldest specimen (J46, >47,000 BP) is located in the clade of Western Red Deer together with contemporary specimens from southern and eastern Europe, while the two younger ones (J24, 33,100 ± 400 BP and JM1, 42,000 ± 1200 BP) belong to the Eastern Red Deer clade together with specimens from the Far East. Two younger specimens from Crimea are located on the phylogenetic tree close to *C. e. songaricus* from China, which, according to Ludt et al. (2004), belongs to the North-Asia/America red deer population, one of the three populations within the Eastern clade. The oldest specimen is located close to *C. e. hippelaphus* from former Yugoslavia, Bulgaria and Hungary, and clearly belongs to the Balkan population of the Western clade. According to Skog et al. (2009), this population belongs to the haplogroup C, one of the three haplogroups found in Europe.

It is known that currently in Crimea only the Western red deer is present (Ludt et al., 2004). It is therefore interesting that between 37,570 and 45,641 BP, Crimea was occupied by representatives of the Eastern red deer, while somewhere before 50,000 BP, the representative of Western group was discovered. Such population replacement can only be explained in connection with Pleistocene climate changes. It seems plausible that the Crimean Peninsula served as an additional refugium during the early stages of Valday glaciation (Würm, Weichselian glaciation), or at least at the beginning of the Bryansk-Dunayevo interstadial (Interstadial

WII/WIII) for the red deer populations close to the Balkan group (haplogroup C in Skog et al., 2009), which today is found in eastern and southern Europe, south of the Carpathian-Alpine arch (Ludt et al., 2004; Sommer et al., 2008). On the other hand, the end of the interstadial period was the time of invasion of cold-loving forms in more open habitats (*Saiga tatarica*, *Allactaga* sp., *Lagurus lagurus*), which were associated with cold steppe fauna (mammoth steppe) of central-eastern Asia. The similarity of J24 and JM1 specimens to recent specimens of *C. elaphus songaricus* from Tien Shan, China and *C. elaphus sibiricus* from China, Mongolia, indicates that red deer could also belong to this wave of "cold steppe" fauna.

The similarity between ancient Crimean populations of red deer and its extant populations from Central Asia may also indicate that the environments existing at that time in the Crimea and Central Europe were similar to that at present in some parts of Central Asia. The similarity of environments finds its confirmation in the studies of Horsák et al. (2010), who found that the Altai mollusc habitats and communities were much similar to those known from the last glaciation of Central Europe. These authors suggest that the Altai landscape is the recent analogue of the environment of the full-glacial period of Central Europe. Animal species occurring there may be relics of the glacial faunas. It can be hypothesized that the faunas inhabiting Europe during cold episodes (glaciations) originated from Central Asia. During interglacial and interstadial periods they retreated to these areas to be replaced by European forms, which had survived in the refugia. Because of its location on the migration route from Central Asia, its environmental conditions and the fact that during the Pleistocene it often became an isolated island, the Crimea was no doubt an important refugium, where many species could survive in order to spread again to both central-eastern Europe and Asia. During the period of occurrence of eastern genotypes of the red deer in the Crimean Mts, which was correlated with interstadial periods (47,000–41,000 BP; 38,000–36,000), the mountains held plant communities of forest-steppe character, with a high proportion of deciduous trees. Boreal forest steppe vegetation occurred during periods of harsher climate (41,000–38,000 BP), which in the next cold period (28,000–27,000 BP) were replaced by grasslands (Gerasimenko, 2010). Analogous plant communities exist at present in the Altai. Horsák et al. (2010) suggest that this area serves as a refugium for both plant and animal communities that existed in Europe during glaciations.

At present, the Crimea is inhabited by populations of *C. elaphus* which are close to "A" haplogroup from central and western Europe (Ludt et al., 2004), suggesting that during the Holocene some barriers existed that prevented invasion of the Crimea by "C" haplogroup and favoured migrations of forms from Central Europe. Further studies on ancient DNA from Quaternary bone remains from the Crimea and the rest of Ukraine should make it possible to test these hypotheses and expand the knowledge of vertebrate phylogeography in Eurasia.

4. Conclusions

The analysis of the cyt *b* sequences of three red deer specimens from Crimea, dated for the Late Pleistocene (MIS 3), revealed that one of them is related to European, while the other two are related to contemporary specimens from China. This result sheds new light on the directions of the red deer migrations during the Pleistocene and strongly suggests that during the Late Pleistocene, the Crimea served as a refugium for red deer and other mammal species.

Acknowledgments

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Appendix A

A1. Singleplex PCR primers Set 1

Symbol	Sequence	Amplicon size (bp)	Final concentration [μM]	Primer's localization (nucleotide positions in mitochondrial genome, bp)
1F	ATGACCAATCCGAAAAA	174	0.2	14162–14180
1RN	ATCAGATGTATAGTGTATC		0.2	14317–14335
2FN	CTACAAATCCTCACAGGCC	172	0.2	14288–14306
2RN	CGTCCTACATGTATAAACAGA		0.2	14440–14460
3F	GATACACTATACATCTGAT	247	0.2	14317–14335
3R	TGGTAGGACATATCCTAC		0.2	14546–14563
4F	GACGTGAAACATCGGAGTAG	164	0.2	14494–14513
4RN	CTCAGATCCATTCGACTAGG		0.2	14638–14657
5F	TCTCCCATTTATCATCGC	162	0.2	14712–14730
5R	GAGTACTAGAAGTAAGATG		0.2	14854–14872
6F	AATCCCCTTTCATCCTTAT	170	0.2	14812–14830
6R	GTATCATTCAGGTTTAATA		0.2	14962–14980
7F	ACTCAACACACCCCCTCAT	140	0.2	14944–14962
7R	TGAAGAAGAGGCATGAGAATT		0.2	15064–15084
8F	TAGTCTCATCCATCCTAATC	184	0.2	15042–15061
8R	GATGCTAGTTGTCCAATAA		0.2	15207–15225
9F	TAGTAGCAGACCTATTA	146	0.2	15144–15160
9R	GGAGGTTGTTTTCGAT		0.2	15275–15290
10F	CAATCACCAGCACAATCG	146	0.2	15261–15278
10R	GGTGTTGATAGTGGGG		0.2	15392–15407
R1FCytb	CGATACATACATGCCAACGG	191	0.2	14399–14418
R1RCytb	GCTCCTCAGAATGATATTTG		0.2	14570–14589
R2FCytb	CAAATATCATTCTGAGGAGC	168	0.2	14570–14589
R2RCytb	GAGTGCTGCGATAATAAATGG		0.2	14717–14737

A2. Multiplex PCR primers Set 2.

Symbol	Sequence	Amplicon size (bp)	Final concentration [μM]	Primer's localization (nucleotide positions in mitochondrial genome, bp)
CERV1F	GAAAAACCATCGTTGTCATTCA	154	0.16	14125–14146
CERV1R	GACTCCTAGTAATGAGCCGAAA		0.16	14257–14278
CERV2F	CTCCCAGCCCCATCAAATA	163	0.16	14222–14240
CERV2R	AATTGACATCTCGACAGATATGG		0.16	14362–14384
CERV3F	GCGATACACTATACATCTGATACAA	158	0.16	14315–14339
CERV3R	TAGTACAGGCCTCGCCCTAC		0.16	14453–14472
CERV4F	CGGGGCATCAATATTTTTCA	163	0.16	14416–14435
CERV4R	TGATATTTGTCCTCATGGTAGG		0.16	14557–14578
CERV5FN	TACAGTTATAGCCACAGCATTCG	163	0.16	14524–14546
CERV5RN	TAGGGTTGCTTTGTCTACTGAAAAG		0.16	14662–14686
CERV6F	TGGGACAAACCTAGTCGAATG	158	0.16	14629–14649
CERV6R	TTGGGTTATTAGATCCTGTTTCG		0.16	14764–14786
CERV7F	GCAGCACTCGCTATAGTACAC	150	0.22	14729–14749
CERV7R	TAAGAAGAGTACAAGAAGTAAGATG		0.22	14854–14878
CERV8F	CAAAATCCCCTTTCATCCTT	164	0.16	14809–14828
CERV8R	CAGGTTTAATATGAGGGGGTGT		0.16	14951–14972
CERV9FN	TGGAGATCCAGATAACTACACC	170	0.32	14911–14932
CERV9RN	GAAGAGGCATGAGAATTAAG		0.32	15061–15080
CERV10FN	CCAACAAACTAGGAGGAGTCT	165	0.16	15015–15035
CERV10RN	CTCCGATTCATGTAAGTGTTAGT		0.16	15157–15179
CERV11F	GACCATTCAGTCAATGCCTATTC	159	0.16	15114–15136
CERV11R	TGCTGGTGATTGGTATGAGG		0.16	15253–15272
CERV12F	TTGGACAACTAGCATCTGTCT	147	0.16	15210–15231
CERV12R	TGCTCTCCTTTTCTGGTTTACA		0.16	15335–15356

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