**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 Lower Plateau of the Chatyrdag Massif (Crimean Mountains) is rich in palaeontological material, accumulated over most of the Pleistocene. The cave served as a natural trap for vertebrates whose skeletons are now very well preserved. Preliminary examination of the bone material showed that also genetic

material was preserved in most of it. We analysed mtDNA isolated from bones of three specimens of red deer (*Cervus elaphus*) dated for the late Pleistocene (MIS 3). These are the first ancient DNA sequences obtained for this species. We located the position of three red deer individuals on the phylogeographic tree based on the mtDNA sequences of contemporary representatives of the Cervinae inhabiting the Northern Hemisphere. Our 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.

Key words: *Cervus elaphus* phylogeography; ancient DNA; Pleistocene refugium;

## 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 mammal 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). It is one of the largest cavities in the area with a total length of 1.460 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 1960s (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*). Next 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 most numerous finds come from the Bc site ("Skull chamber"), where preserved partial and whole skeletons have accumulated very close to one another in several stages. Every stratigraphical unit presents particularities from the palaeontological and taphonomic point of view. The vertebrate assemblages, the preservation and

spatial distribution of the bone material, the stratigraphical (micromineralogical) and microambient data suggest a very peculiar taphofacies as well as very complex sediment-entrapment processes (Vremir and Ridush 2005, 2006).

New excavations started in 2005 and were continued in 2008-2010 (Ridush and Proskurnyak, 2008) revealed another rich assemblage of animal bones at the Ba2 site. Deposits at this site are formed by soil/loess material, transported into the cave from outdoors through the entrance pit, and by the limestone debris. Cave fill contains a lot of palaeontological remains (vertebrates and molluscs) and has visible subhorizontal stratification, making it possible to obtain a secular variation record and to apply magnetostratigraphical dating (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 general the most frequent in all distinguished layers are *Saiga tatarica borealis*, *Cervus elaphus* and *Bison priscus*. The other ungulate mammal species are represented by *Equus hidruntinus*/*hemionus*, *Megaloceros* sp., *Ceolodonta antiquitatis*, *Mammuthus primigenius*. Among carnivores the most frequent is *Vulpes* cf. *corsac* and some species of *Mustela*. Among lagomorphs *Lepus* is represented by two and *Ochotona* by one species. Small mammals are mainly represented by rodents *Microtus arvalis*, *Myodes glareolus*, *Arvicola*, *Apodemus* sp., *Allactaga* and *Lagurus lagurus*. Remains of insectivores (*Sorex*) and bats (Chiroptera) have also been found. Among birds *Pyrrhocorax graculus* and *Columba livia* remains are strongly dominant.

The section, 6 m deep, was studied at the Ba2 site where eight layers (A-H) were distinguished in the first 2.6 m. The uppermost part of the section (layers A-D) was probably deposited during the Holocene (Bondar, Ridush, 2009). Bones of *Cervus* from the layer G (2.0 – 2.6 m) were radiocarbon dated to 33,100 ± 400 BP (38,791 ± 1,526 cal BP) and from the deepest part (4.6 m and 4.7 m) of excavated profile for 42,000 ± 1,200 BP (53,020 ± 3,262 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). The fauna composition of EBK sites indicates that during the Late Pleistocene the Crimean Peninsula functioned as the European north-eastern most refugium for many mammal species of the temperate zone.

In this paper we present the results of analysis of DNA isolated from bones of 3 specimens of red deer (*Cervus elaphus*) excavated in EBK. The phylogeography of Cervinae was thoroughly studied (Polziehn and Strobeck, 2002; Ludt et al., 2004; Sommer et al., 2008; Sommer and Zachos, 2009; Skog et al., 2009), but species status of certain populations remains unclear. The studies on mitochondrial DNA of modern red deer populations (Polziehn and Strobeck, 2002; Mahmut et al., 2002; Ludt et al. 2004, Pitra et al. 2004) demonstrated that they can be divided into two separate groups of Western and Eastern red deer. 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) In this study we

investigate which of the red deer populations (Western or Eastern) inhabited Crimean Peninsula during Late Pleistocene and what was the role and place of this refugium in recolonization and dispersal processes of red deer populations.

## 2. Materials and methods

### 2.1. Materials

The studied specimens came from excavations conducted in 2007–2009 under supervision of Bogdan Ridush and in co-operation with the Palaeozoology Department, Zoological Institute, Wrocław University. The bones, excavated from the cave Emine-Bair-Khosar are kept at the Museum at the cave. Following identification and osteometric studies the material was subject to genetic studies. At the same time, samples for absolute dating were taken from the bones. Dating was performed at the Poznań Radiocarbon laboratory (Table 1).

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

| Sample symbol | EBK site | Layer | Sample description | AMS dating (Lab no.) | Calibrated age |
|---|---|---|---|---|---|
| J24 | EBH nr 1593 Ba2 D2(g); -240 | G | fragment of metatarsus | 33,100 ± 400 BP (Poz-35028) | 37,570 ± 781 BP |
| JM1 | EBH nr 2580 Ba2 C1 (b); - 320 | H | tooth ($M_1$) | 42,000 ± 1,200 BP (Poz-35027) | 45,641 ± 1,334 BP |
| J46 | EBH nr 2355 Ba2 C2(b); - 460 | H | fragment of metacarpus | > 47,000 BP (Poz-35026) | - |

### 2.2. DNA extraction, amplification and sequencing

Tooth and bone fragments were washed with bleach, rinsed with dd$H_2O$, UV irradiated for at least 20 minutes 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 (20mg/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 full *C. elaphus* cytochrome b sequence: (1140 bp): (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 reaction volume 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 ºC, followed by 45 cycles at 95 ºC for 30 s, 41ºC for 30 s, 72 ºC for 30 s and the final extension at 72 ºC for 7 min. PCR products were sequenced in ABI PRISM 3730xl 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 reaction volume 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 primer pairs show lower amplification efficiency in multiplex reaction thus concentrations of certain primers were increased (**Appendix A**). Amplification conditions consisted of a 12 min activation step at 95 ºC, followed by 25 cycles at 94 ºC for 30 s, 53ºC for 30 s, 72 ºC for 30 s and the final extension at 72 ºC for 10 min. Multiplex PCR products were used to prepare a library for pyrosequencing following the protocol of Stiller et al. (2009) and sequenced in Roche 454 sequencing platform.

The obtained sequences were deposited in GenBank under accession numbers: J24 HM596026, J46 HM596027 and JM1 HM596028.

**2.3. Sequence analysis and consensus calling**

Sequences obtained in singleplex PCR reactions were used to obtain consensus sequence 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).

In MrBayes we applied two strategies. In one we used one substitution model HKY+I+Γ for all cyt b positions, while in the second one (Fig. 1) we assumed three separate models for three codon positions: K2P+I (for the first codon positions), F81 (for the second codon positions), and GTR+Γ (for the third codon positions). 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 million or 20 million generations in the first and in the second strategy, respectively. After reaching convergence, we selected trees 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. In ML and NJ we used 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 maximum likelihood method, we also applied separate substitution models for three codon position: TN+Γ (for the first codon positions), R3 (for the second codon positions), and GTR+Γ (for the third codon positions), 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.

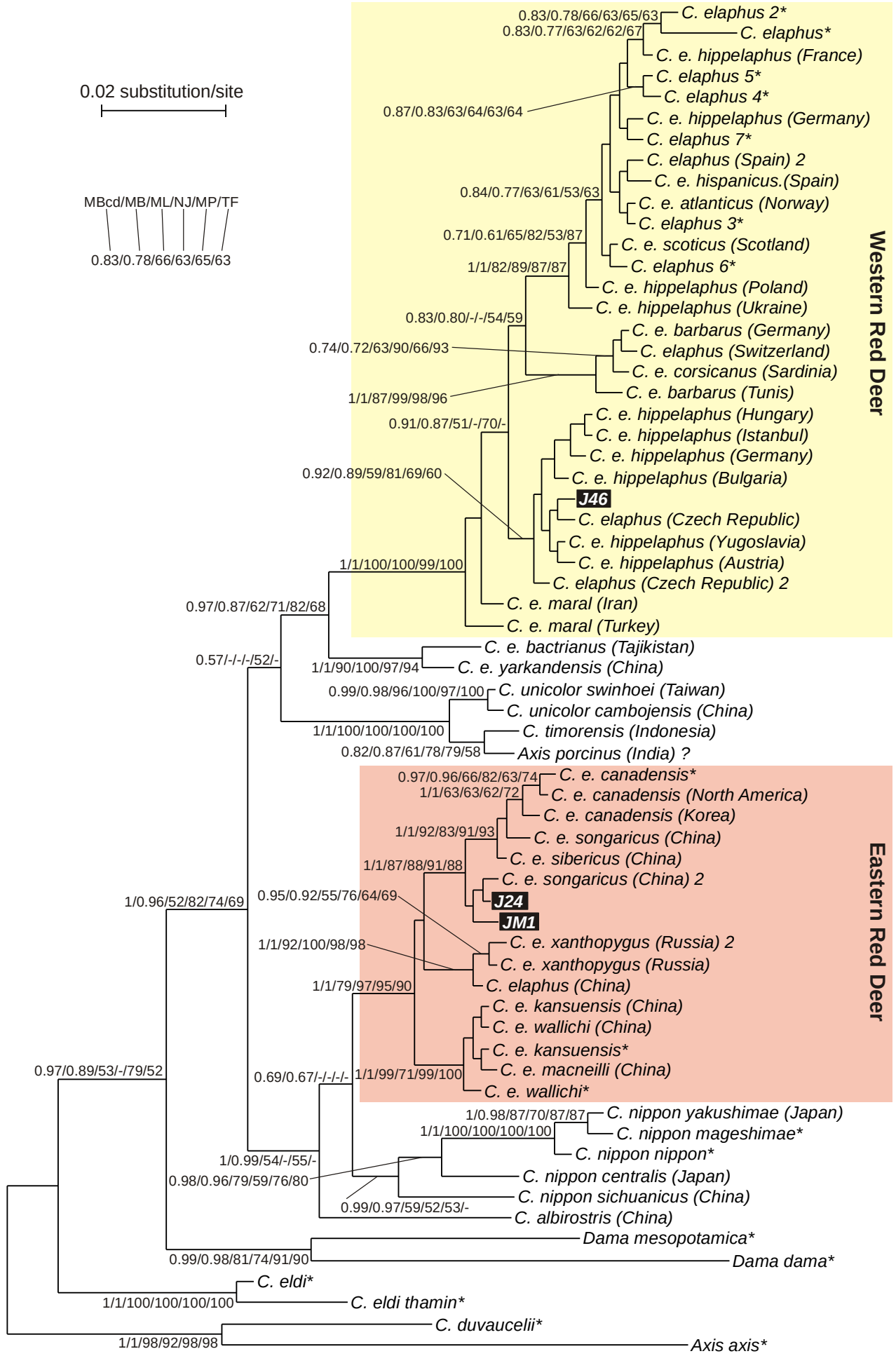


Figure 1. Red deer phylogeny obtained for cytochrome b in MrBayes under separate models for three codon positions..

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.

## 3. Results and discussion

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 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 the D-loop sequences, the western and eastern are linked to an Iberian and Balkan refugium, respectively, while the third one is associated with Sardinia or Africa refugia (Skog et al., 2009; Zachos and Hartl, 2011, Niedziałkowska et al., 2011).

All prior molecular studies of Cervidae were conducted on contemporary material, the fossil samples served only to validate the dates of divergence of particular genera obtained by molecular dating (e.g. Gilbert et al., 2006). We have attempted to isolate DNA from the red deer specimens found in the Crimean EBK cave, dated for 33,100±400 BP (sample J24), 42,000±1,200 BP (JM1) and >49,000 BP (J46). For all samples we managed to obtain the sequences of 732 nt of cytochrome b (cyt b) gene which in the 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 we were unable to obtain fragments of good quality suitable for pyrosequencing and we sequenced 12 separate amplicons, covering the appropriate cyt b sequence.

Cyt b sequences of three Pleistocene red deer specimens were included into the phylogenetic tree (Fig. 1), constructed of over 60 Cervinae species, whose cyt b sequences were obtained from GenBank. Most of these sequences were deposited in the database by Ludt et al. (2004). For many of these samples their geographic origin is known. 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. It can be seen that the sequence obtained for the oldest specimen (J46, >49,000 BP) locates in the clade of Western Red Deer, together with contemporary specimens from southern and eastern Europe while two younger ones (J24, 33,100±400 BP and JM1, 42,000±1,200 BP) belong to the Eastern Red Deer clade together with specimens from the Far East. Two younger specimens from Crimea locate on the phylogenetic tree close to *C. e. songaricus* from China, which, according to Ludt et al. (2004), belong to the North-Asia/America red deer population, one of the three populations within the Eastern clade. The oldest specimen locates 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 of 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: *Cervus 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 (Tian Shan, China, Mongolia) may also indicate that 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 in the Altai mollusc habitats and communities 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 facts that during the Pleistocene it often became an isolated island, the Crimea no doubt was an important refugium where many species could survive to spread again in 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), which in the next cool period (28 000 – 27 000) were replaced by grasslands (Gerasimenko 2010). Analogous plant communities exist till today in the Altai. Horsák et al. (2010) suggest that the area constitutes a refugium for both plant and animal communities which 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 other two to contemporary specimens from China. This result sheds new light on the directions of the red deer migrations during Pleistocene and strongly suggests that during the Late Pleistocene the Crimea served as a refugium for the red deer and other mammal species.

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