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## ANCIENT MITOCHONDRIAL DNA POLYMORPHISMS FROM HUMAN NEOLITHIC SITES IN THE CARPATHIAN BASIN

**ABSTRACT:** *The Alföld Region of Hungary played a key role in the spread of Neolithic in Europe as being the main route towards Central and Northern Europe. From local excavations, nearly 500 skeletons have been uncovered so far from the Early-Middle Neolithic periods, providing an excellent base for the study of the first Central European farmers. Ancient DNA analysis of these human remains was needed to give reliable data on their genetic diversity and origin, because archaeological data were only accompanied by morphometrical investigations prior to our studies.*

*A primer walking strategy was used to map through the hypervariable region of the human mitochondrial DNA to identify any specific polymorphism containing sequence in the 7,000-8,000-year-old Neolithic remains. Amplifiable fragments were isolated and their sequences were determined via direct sequencing of PCR products of interest. The sizes of the amplification products were found from 80 to 455 bp, but this efficiency was only achievable when we applied a DNA purification step in the prePCR protocol. Direct DNA sequence analysis of PCR products of interest showed that HVR of the mitochondrial DNA contain point mutations just specifically bound to the 8,000-year-old aDNA, and the distinctive mutation patterns of nt16257 and nt16261 were repeatedly found, which is characteristic of the N9a mitochondrial haplogroup. This mtDNA lineage is rare among contemporary Europeans and more common in the East Asian samples, while according to our aDNA data, no polymorphisms of N haplogroups were found in more recent sites dating from the Middle Ages up to the present.*

*These data are in accordance with the knowledge of a heterogeneous genetic pattern of European Neolithic, but with local differences and novel genetic data. These polymorphisms were followed up throughout the Neolithic remains collection of the Hungarian Natural History Museum, and checked with amplification experiments using polymorphism specific primers. This analysis will be extended onto other Neolithic sites in Europe, or even outside Europe in order to determine the mtDNA polymorphism pattern of Neolithic people.*

**KEY WORDS:** *Neolithic – Ancient mitochondrial DNA – PCR – Single nucleotide polymorphism*

### INTRODUCTION

The Neolithic culture, the way of life of the first farmers in prehistoric times, is the key for understanding the human prehistory of Europe. It spread through Anatolia and the Balkans to all over Europe, and became dominant in major part of this area between the 10th and 5th millennia BC (Renfrew 1987). One of the main geographic routes of its

spread was in Central to Northern Europe and started from the region called the Great Hungarian Plain, which is the Alföld Region of Hungary (Ammerman, Cavalli-Sforza 1984).

The Early Neolithic Körös-Starčevo-Criş Culture (KSC) represents the first significant sustained Holocene occupation of the Great Hungarian Plain (Whittle 2004). Little information is known on the population size of humans living in the Carpathian Basin in that time, but



FIGURE 1. Examples of Neolithic remains used for aDNA studies from the collection of the Hungarian Natural History Museum (HNHM, Photo: Tamás Zeke and Zsuzsanna Guba). 1: right femur of a young female from the Starčevo site of Vörs-Máriaasszonyisziget (feature No. 52); 2: skull without mandible of the well-preserved male specimen from the Körös site of Szarvas 23, grave No. 20; 3: right femur of a male from the Körös site of Szakmár-Kisülés, grave No. 8 (note the thick compact bone tissue of diaphysis); 4: mandible and maxilla remains of the famous LPC/ALP Ecsegfalva 23A female specimen, providing the first partial mitochondrial aDNA sequence of Neolithic humans from Hungary; 5: restored skull without mandible of a male specimen from the fairly large LPC/ALP cemetery of Polgár-Ferencihát, grave No. 489; 6: nearly complete female skull from the LPC/ALP cemetery of Polgár-Kenderföldék, grave No. 436; 7: restored skull vault of Polgár-Folyás – Kásadombi dűlő, grave No. 111, representing a typical Middle Neolithic preservation state, which has provided amplifiable DNA as the previous skull; 8: robust male skull without mandible from Aszód-Papiföldék (Vác), grave No. 22, an example from the Late Neolithic collection of HNHM.

several localities can be found here. Their excavations in the Alföld territory resulted in a fairly large number of anthropological finds, too. Most of them are stored in either the Osteological Collection of the Hungarian Natural History Museum (HNHM), Budapest, Hungary or in the Osteological Collection of the Department of Anthropology, University of Szeged, Hungary. A few are deposited in local museum collections at various places. A detailed survey of Körös sites with anthropological remains was already published (Paluch 2004).

The KSC sites can be found in the southern part of the Great Hungarian Plain (Figure 3: Szarvas, Szakmár, Endrőd) and Transdanubia (Figure 3: Vörs), however, traces of their settlement activity were found in Transylvania and at the eastern edge of the Great Hungarian Plain as well.

KSC was followed by the Linear Pottery Culture, which colonised Central and Northern Europe. This culture developed in the Alföld, that is why it is named after this region – Alföld Linear Pottery Culture (ALP) (Whittle 1996). The successive ALP sites were concentrated in the northern part of the Alföld Region (Figure 3: Folyás, Füzesabony, Mezőkövesd, Polgár a. o.) but they overlapped with territories between the areas occupied by the above two subsequent cultures (Figure 3: Ecsegfalva a. o.).

Many of the Neolithic human remains stored in the HNHM present fairly good preservation (Figure 1). Many anthropometrical analyses were carried out which focused on the Neolithic finds (e. g. Guba *et al.* 1997). These former studies have shown that significant anthropological differences existed among the Early and Middle Neolithic

human specimens. The osteological traits bearing information on the lifestyle of peoples were also studied (Ubelaker *et al.* 2006). Further <sup>14</sup>C isotope analyses directly from human bones and isotope analyses of Neolithic human tooth remains are in progress to reveal the exact dating of findings, and are expected to provide more data on the lifestyle and demographic connections of the Early Neolithic population of Hungary as well. One of the recent isotope analyses showed that the Polgár-Ferencihát site is dated to be 7,474–7,068 years old (Raczky, pers. comm.).

Nevertheless, ancient DNA (aDNA) analysis of human remains is expected to provide reliable data on the genetic diversity and origin of one-time populations. Usually, aDNA investigations are based on the amplification of mitochondrial DNA (mtDNA). This small genome is more prone to give positive amplification, since it has a cell copy number higher than in nuclear genes. The quality and quantity of authentic DNA molecules in an ancient sample depends on many physical and chemical factors, which are not easy to predict even if the taphonomic history of a sample is known. There is a newly set up aDNA laboratory in accordance with the strict criteria required for

representing authentic aDNA results (Cooper *et al.* 2000). With the background of this dedicated laboratory in the Hungarian Natural History Museum, we aim at characterising the putative polymorphisms of the mitochondrial hypervariable region I by extracting ancient DNA of KSC and ALP Neolithic human remains.

**MATERIAL AND METHODS**

**Subjects**

The HNHM collection consists of nearly 500 skeletons, which are reliably dated by archaeology to the Early and Middle Neolithic Periods. 70 individual human remains from 15 sites belong to the KSC and 411 individual human remains from another 15 sites belong to the ALP Culture (*Table 1*).

Predominantly, the Early and Middle Neolithic human remains excavated in the territory of present-day Hungary were subjected to aDNA analysis. First, the findings of adults with overall good preservation, where the teeth and intact diaphyseal part of long bones were present as well,

FIGURE 2. Sequence comparison after direct sequencing of PCR products amplified from ancient mtDNA.

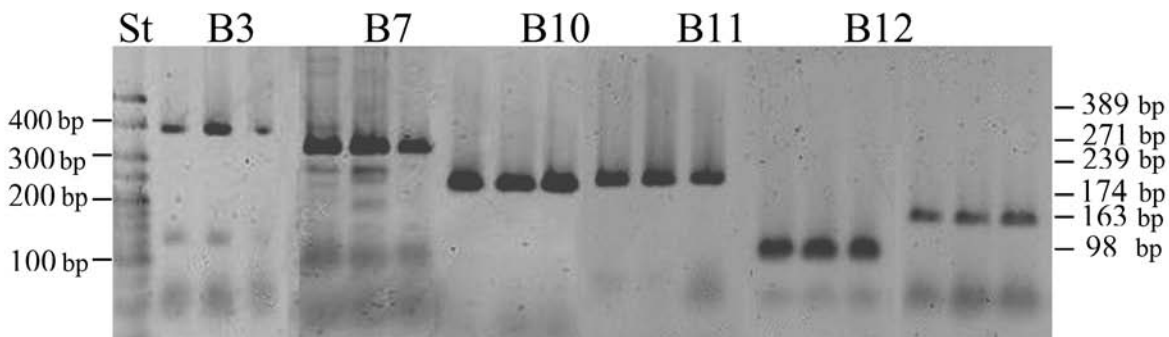


FIGURE 2A: Representation of PCR amplicons of Szarvas 23/20 DNA isolated for sequencing after being resolved in 2% agarose gel.

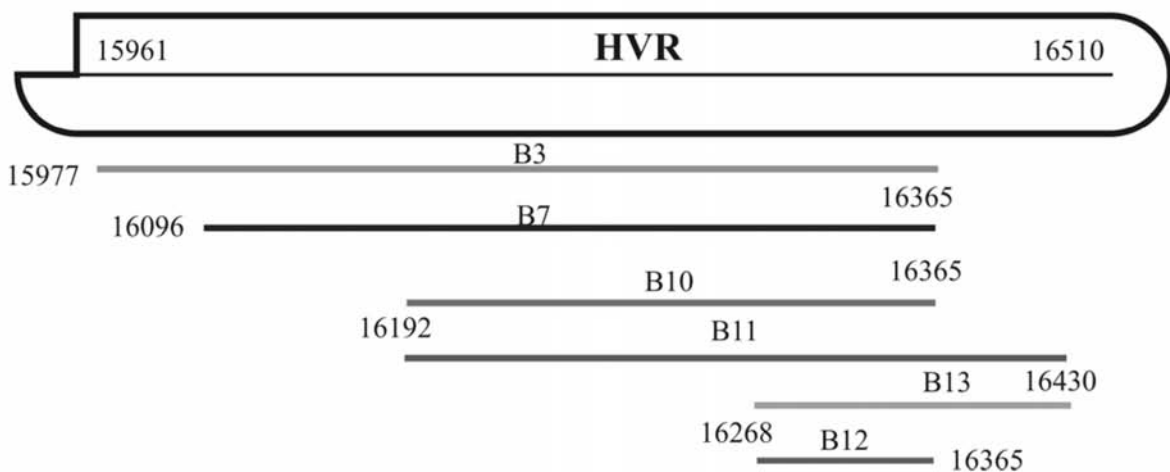


FIGURE 2B: PCR strategy showing the position of overlapping amplicons of HVR-I: B3, B7, B10, B11, B12, B13.

TABLE 1. Overview of Early-Middle Neolithic finds stored in the Hungarian Natural History Museum (for a detailed list of KSC human remains in the Carpathian Basin see Paluch 2004; the names of other important ALP localities with human remains in Hungary: Battonya-Gödrösök, Békés-Déül, Békés-Ludad, Békés-Povádzug, Berettyószentmárton-Morotva, Felsővadász-Várdomb, Füzesabony-Gubakút, Füzesgyarmat-Egyesúg, Garadna-elkerülö út, 2. lh, Gyoma-Özed – homokbánya, Kertészsziget-Akasztódomb – Galambos tábla, Kesznyéten-Lúci Abony, Hejökürt-Lidl logisztikai központ, Kisköre-Gát, Körösladány-Kengyelköz, Megyaszó, Mezőberény-Laposi kertek alja, Mezőcsát-Csemetekert, Mezőkövesd-Meleg-oldal, Mezőkövesd-Mocsolyás, Mezőkövesd-Nagy-fertö, Mezőzombor-Temetö, Miskolc-Hillebrand barlang, Onga, Rákóczifalva-Bivalytö, Sajószentpéter-Kövecses, Sajószentpéter-Vasúti örház, Tiszalök-Hajnalos, Tiszapüspöki-Karancs – Háromágdülö, Tiszavasvári-Keresztfal, Vadna, Zaránk, Zsáka-Vizesi tanya).

Locality	No. of individuals	Excavated by	Archaeological context
Balla Cave	1	Hillebrand	ALP
Csanytelek-Újhalastö	11	Hegedüs, Galánthai	ALP
Csongrád-Bokrospuszta	8	Hegedüs	ALP
Dévaványa-Barcéi Kishalom	1	Losits	Körös
Dévaványa-Katonaföldek II szelvény	1	Ecsedy	Körös
Ecsegfalva 23A	1	Whittle	ALP
Endröd (Gyomaendröd) 35	1	Makkai	ALP
Endröd 3/35	2	Makkay	Körös
Endröd 3/6 (Endröd-Kápolnahalom)	5	Makkay	Körös
Endröd 6/82	4	Makkay	Körös
Endröd-119	10	Makkay	Körös
Kisköre-Gát	4	Korek	ALP
M3/33	1	Raczky	ALP
Miskolc-Büdöspeszt	1	Kadic	ALP
Nagykörü-Tsz Gyümölcsös	1	Raczky	Körös
Polgár-Ferencihát M3/31	159	Raczky	ALP
Polgár-Folyás – Kásadombi dülö	1	Raczky	ALP
Polgár-Kenderföldek	147	Raczky	ALP
Polgár-Nagykasziba M3/12	4	Raczky	ALP
Polgár M3/29	58	Raczky	ALP
Szakmár-Kisülés	8	Bognár, Kutzián	Körös
Szarvas 8/23	18	Makkay	Körös
Szarvas 8/8	15	Krecsmarik	Körös
Szegvár-Táncsics utca 2/A	1	Hegedüs	Körös
Szentpéterszeg -Körtvélyes	1	Kalicz	Körös
Tiszaluc-Sarkadpuszta	8	Patay	ALP
Tiszavasvári-Deákalmi dülö	6	Istvánovics, Kurucz	ALP
Vaskút	1	Kutzián	Körös
Vörs, Máriaasszony -sziget	2	Kalicz, Bíró, Virág	Starčevo

were chosen to maximise the probability of aDNA survival. In the course of aDNA experimental work, these 8,000–7,000-year-old samples were always accompanied by the analysis of more recent samples for methodological reasons, that is to detect contamination and analyse finds of different taphonomic history.

### Sampling and DNA extraction

All prePCR modifications including sampling were carried out in a spatial and temporal separation from the postPCR modification in the laboratory of the Hungarian Natural History Museum, Budapest, which is dedicated to working with human aDNA. The laboratory rooms are fitted with a positive air pressure, overnight UV exposure, and the laboratory workers use suitable protective clothing and equipment to avoid modern DNA contamination.

Sampling was carried out in isolated places with no access but the preparator in a dedicated room (with no

previous human DNA work), with dedicated tools and pipettes, filter tips, protective clothes (full body suits, hairnets, filter-containing facemasks, and gloves were used). Frequent surface cleaning with detergent followed by 10% NaClO, overnight UV irradiation of surfaces and tools (tubes, pipette tips, pipettes, racks etc.) was carried out as well with no extra cleaning person. Molecular biology grade reagents (also DNAase-RNAase free, if applicable) were prepared in small aliquots, exposed to UV irradiation wherever possible.

In order to avoid intralaboratory DNA contamination, spatial separation between the pre- and postPCR experimental steps was applied and no more than one ancient sample was subjected to sampling and extraction procedures at the same time. Extractions were carried out in a laminar hood dedicated to this purpose, PCR setup was always made in a separated room in a DNA box. All prePCR experiments were carried out by Zsuzsanna Guba, whose

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1Stone Age Eulau16145 gaccacctgtagtacataaaaaacccaatccacatcaaaacccctccccatgcttacaagcaagtacagcaatcaaccttcaactatcac
2Ecsegfalva 23A 16145 ..a.....c.....c.....
3M98 t1 16145 a.....g.....
4mitCRS 16145 .....c.....
5Szakmár 8 16145 .....c.....
6Szarvas 23_20 16145 .....
7Derenburg 16145 ..a.....c.....
8Guinea 16145 ..t.....tc.....
9Philippines 16145 .....
10 NorthIndian 16145 .....

1Stone Age Eulau acatcaactgcaactccaaa-gccacccctcaccactaggatac 16278
2Ecsegfalva 23A .....t.....-.....a.... 16278
3M98 t1 .....-..... 16278
4mitCRS ..... 16278
5Szakmár 8 .....-..a..t..... 16278
6Szarvas 23_20 .....-..a..t..... 16278
7Derenburg .....t.....t..... 16278
8Guinea .....g.....-.....c..... 16278
9Philippines .....-..... 16278
10 NorthIndian .....a..... 16279

1Stone Age Eulau caacaaacctaccacccttaacagtagcatagtagtacaataagccatttacgtagcatagcacattacagtcaaatcccttctcgtcccca 16367
2Ecsegfalva 23A .....t..... 16367
3M98 t1 16264 ..... 16367
4mitCRS ..... 16367
5Szakmár 8 ..... 16367
6Szarvas 23_20 ..... 16367
7Derenburg ..... 16367
8Guinea .....c.....g..... 16367
9Philippines .....t.....c..... 16367
10 NorthIndian .....t..... 16368
    
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FIGURE 2C: Alignment of sequences of the direct sequenced PCR amplicons from Szarvas 23/20 and Szakmár Kisülés showing the location of polymorphisms found in our studies. The region of alignment corresponds to nt 16145-16367 in revised CRS with depicted polymorphisms. We used a Stone Age Eulau sequence as basis for comparison and the legends in the order of appearance are as follows: 1: Stone Age Eulau = Eulau sequence (90-5) from the Late Stone Age Corded Ware Culture, Germany (Haak *et al.* 2008); 2: Ecsegfalva 23A = sequence of Ecsegfalva 23A specimen (Haak *et al.* 2005); 3: M98 t1 = sequence of Mummy No. 98 Crypt of Vác, Hungary; 4: mitCRS = revised Cambridge Reference Sequence (Andrews *et al.* 1999); 5: Szakmár 8 = sequence of Szakmár-Kisülés grave 8 specimen from this study; 6: Szarvas 23\_20 = sequence of Szarvas 23 grave 20 specimen from this study; 7: Derenburg = sequence of a Neolithic LPC Derenburg specimen from Germany (Haak *et al.* 2005); 8: Guinea = recent New Guinean sequence (Tommaseo-Ponzetta *et al.* 2002); 9: Philippines = recent Philippine sequence (Tabbada *et al.* 2009); 10: NorthIndian = recent North Indian sequence (Darvishi *et al.* 2005).

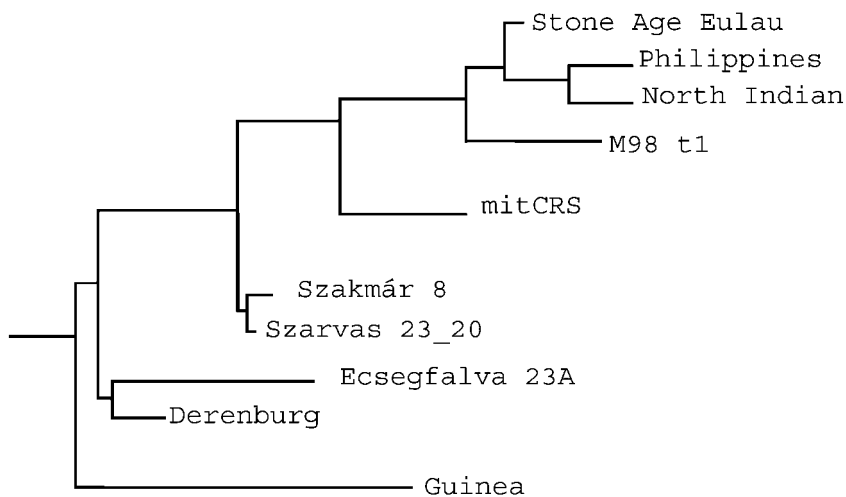


FIGURE 2D: Analysis of relationship by comparing each of the partial HVR-I sequences from alignment. The dendrogram was constructed with a distance-based tree-building method using the neighbour-joining algorithm using the Multi-Way multiple sequence alignment option in Clone Manager 7.0 and abbreviations as in Figure 2C.

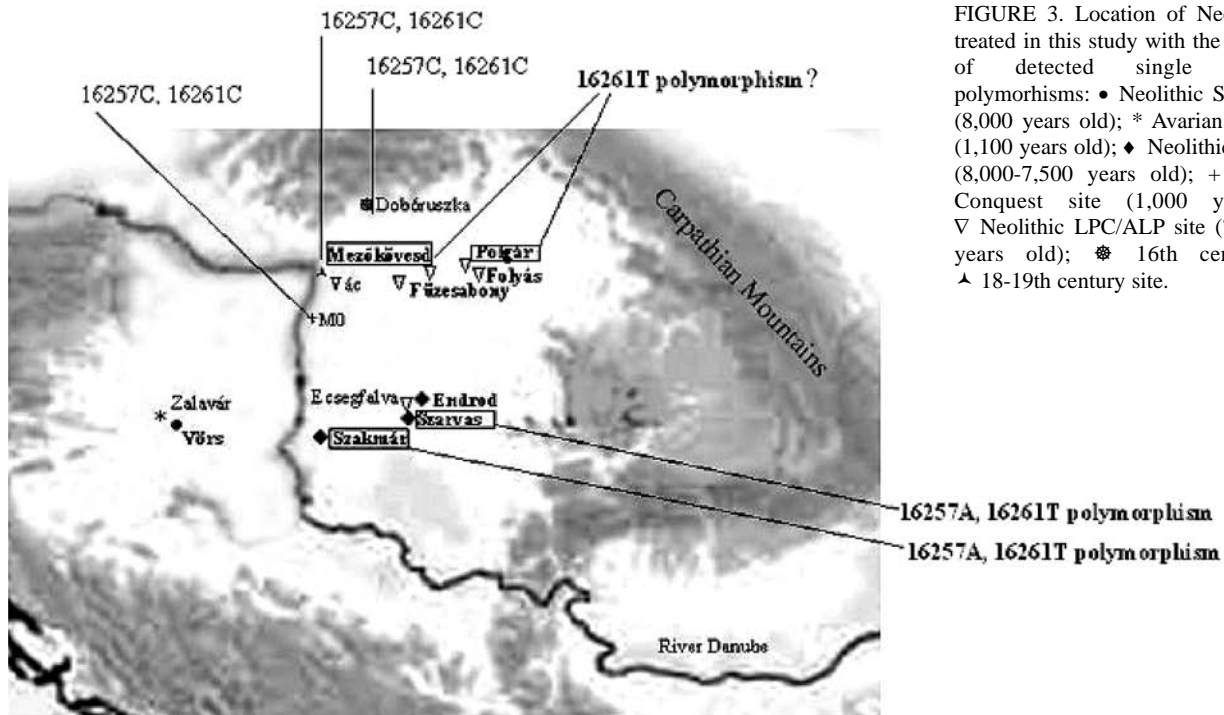


FIGURE 3. Location of Neolithic sites treated in this study with the distribution of detected single nucleotide polymorphisms: • Neolithic Starčevo site (8,000 years old); \* Avarian Period site (1,100 years old); ♦ Neolithic Körös site (8,000-7,500 years old); + Hungarian Conquest site (1,000 years old); ▽ Neolithic LPC/ALP site (7,500-7,000 years old); \* 16th century site; ▲ 18-19th century site.

mtDNA was typed, and its HVR-I region is identical to haplogroup H. To detect intralaboratory and intersample DNA contamination, we applied the extensive use of experimental blanks, one in each set of extraction, but at least after every fifth. If the extraction blank gave positive amplification result, the PCR was repeated with new batch of chemicals, and if it again contained amplifiable DNA, the extraction set was neglected and disposed. Every PCR setup was accompanied by more than one no template control (NTC) as well as by blanks to detect immediately any contaminated chemicals.

Teeth were soaked in commercial bleach, and then only radices were used for aDNA extraction. Tooth radices as well as diaphyseal long bone fragments (Figure 1: 1, 3) were pre-treated by scraping off their outer surfaces using a Microtool (MF Perfecta, type 9975-E, W&H Dentalwerk, Bürmoos GmbH, Austria). After removing external contaminants (surface grinding-removing and soaking in sodium hypo-chlorite followed by 10 minutes of UV exposure), 0.5–1.0 mg compact bone cubes and slices of tooth radices were cut and then pulverised in a spherical mineralogy mill (Mixer Mill, Retsch, Haan, Germany).

Pulverised samples in extraction buffer were as follows: 0.1 M EDTA, pH 8.5 (Sigma-Aldrich) instead of 0.5 M; 0.5% N-lauryl sarcosine (Sigma-Aldrich); 10 mg/ml proteinase K stock prepared so that it was diluted 50-fold (Fermentas) in a reaction volume of 3 ml in DNA-free water (Fermentas) to a percentage of 0.2 mg/ml. Extractions were incubated at 37°C for 12–36 hours, but with the same timing for parallel experiments. The basic timing was 36 hours, but shorter incubations for 20 hours were also applied and worked well when an extra 10% of proteinase K was added after 12 hours. Repeated experiments were also carried out at 12-hour incubation just to shortcut the preparations, but

sometimes they failed and they were finally eliminated. After proteinase K treatment, DNA was extracted by a phenol-chloroform method (Burger *et al.* 2004) using Phenol/Chloroform/Isoamylalcohol (25/24/1; pH 7.5–8.0 from Sigma-Aldrich) and Molecular biology grade Chloroform (Sigma-Aldrich). Aqueous phase was to be washed with DNA-free water (Fermentas) and concentrated on Microcon mini-columns (Millipore, MA, USA) concentrators following the manufacturer's instruction. A new purification technique, additional to concentrating through Microcon, has been developed for extracted aDNA, so that it had to be further purified with E.Z.N.A cycle pure kit (Omega Biotek, GA, USA) before amplification and subsequent analysis, where the final elution was made with 50 µl DNA-free water (Fermentas).

#### PCR amplification of partial HVR I fragments of human mtDNA

Ancient DNA supposed to be well fragmented after extraction. In order to eliminate this problem, different amplicon sizes from different regions of the HVR were applied to avoid the negative effect of this template integrity problem in PCR. We called this primer walking strategy, because the forward primer of the next amplicon overlaps well with the reverse primer of the upstream amplicon (Figure 2B, positions of the primers correspond to the 3' and 5' nucleotides numbered after rCRS) (Andrews *et al.* 1999). Primers were manufactured by MWG Biotech, Germany. More than 30 different primers were used for PCR and sequencing (data not shown) according to the primer map given in Figure 2B. These overlapping primer pairs were designed to the HVR region of human mtDNA using the revised Cambridge Reference Sequence (Andrews *et al.* 1999) so that all the PCR reactions should run under the

same annealing temperature and no touch-down approach was required. The cycle conditions (Epp5333000018 Mastercycler, Eppendorf, Hamburg, Germany), as usual, consisted of an initial denaturation at 94°C for 3 min, 40 cycles of 94°C for 35 sec, 53°C for 35 sec, 72°C for 35 sec, followed by a final adenylation at 60°C for 10 min.

Amplification reactions were set up using 2x Fermentas MasterMix (Fermentas) in a final volume of 20 µl with 0.25 µM primers and 2 µl of the purified extract. One extraction provided enough DNA for only 20 PCR reactions maximum. PCR inhibition was ruled out by using a novel extra purification technique with the help of E.Z.N.A Cycle Pure Kit, and this will be detailed in a methodological paper in preparation.

### DNA sequence analysis

PCR products were resolved on 2.0% agarose gel (Fermentas) alongside a Hyper Ladder V from Bioline, London, UK, which made possible to judge the amount of amplicons from the gel. The DNA bands corresponding to the amplicon sizes expected were cut and purified from the gel by using E.Z.N.A gel extraction Kit (Omega Biotek). 10–20 ng of the DNA were subjected to a single sequencing reaction. One purified PCR product provided enough DNA for 2–10 sequencing reactions, seriously depending on the efficiency of PCR. The number of possible sequencing reactions was determined by gel photos with an easy densitometry. Cloning step was eliminated to get rid of the vain effort to subclone and work with ambiguous fragments and contamination. Direct sequencing of the agarose gel purified PCR products after air drying was carried out by MWG Biotech sequencing services (Germany). PCR products were sequenced on both strands at least in doublets and all the ambiguous results were eliminated. Determined sequences were analysed in a polymorphism screen and their similarities were analysed with multi-way alignments using the default parameters in CloneManager Suit 7.0. This procedure does exhaustive pair-wise global alignments of all sequences and progressive assembly of alignments using the neighbour-joining phylogeny.

## RESULTS

The oldest Neolithic specimens that were successfully subjected to aDNA analysis were the 8,000-year-old remains at Vörs-Máriaasszony sziget, feature No. 52, Starčevo Culture as well as 4 specimens from 4 different localities representing the Körös Culture, ca. 7,500 years old: Szarvas 23, grave 20; Endrőd 6, grave 1; Szarvas 8, grave 2/5; Szakmár-Kisülés, grave 8. We managed to work on another 4 individuals from the ca. 7,000-year-old ALP: Folyás-Kásadombi dűlő, grave 111; Füzesabony-Gubakút, grave 9; Mezőkövesd-Mocsolyás, grave 25; Polgár-Ferencihát, grave 489 (Figure 1, Table 1). These were accompanied by the younger samples of Zalavár-Útontúli temető, grave 23 (ca. 1,100 BP); M0-elkerülő, grave 3 (ca. 1,000 BP), the medieval remains found in the crypt of Dobóruszka (from

the 16th century), and one ca. 150-year-old naturally mummified specimen of the Dominican Crypt of Vác.

The thick compact layer of whitish colour of the Szakmár and Vörs femurs gave a good impression of the bone remains with good DNA preservation. Teeth in intact position sitting in the mandible or maxilla were also preferably chosen for analysis, as shown in Szarvas and Polgár skulls, however, more fragmented skull remains (e.g. Ecsegfalva, Folyás) contained amplifiable mtDNA, too. We noticed that the degree of calcification – as estimated from the time needed for pulverisation – positively correlates with the likelihood of DNA preservation. We started a systematic sampling of the Early and Middle Neolithic remains to find the remains with DNA survival, and subjected them to our standard ancient DNA extraction and amplification processes.

When working with ancient DNA, we faced the challenges of amplification of low copy number DNA templates. To get a reliable sequence data of an aDNA sample, we stated that the independent reproducibility of the experiments is also a criterion. Long bone fragments and tooth samples have been used in the experiments published in this paper, so that extractions were repeated until reliable sequence for each efficient extraction was achieved. The number of necessary repetitions varied by sample, and a lot of very badly preserved remains were ruled out. The total number of sequencing reactions reached 162 and the sequencing efficiency of the PCR products was as follows: 75% if we counted only the identical sequences and those with single nucleotide polymorphisms only, eliminating those with gaps and sequences containing non-matching parts and also obviously non-human artefacts, whose origin is still unclear, but occurs only when low amounts of PCR products are achievable. In some rare cases, faint bands produced only artefacts after sequencing, so that later only obviously efficient PCR reactions were used for subsequent experiments.

We were highly interested in the quality and fragmentation level of the aDNA fragments in each sample. With our set of overlapping primers, we were able to test which size of amplicons can be amplified in our extracts. It came out, as expected, that shorter amplicons could be amplified better, which is often seen as a sign of authenticity of aDNA (data not shown). A new purification technique was developed for extracting aDNA which follows the step of concentrating through Microcon to avoid PCR inhibition: the concentrated extracts were further purified with E.Z.N.A Cycle Pure kit (Omega Biotek) before amplification and subsequent analysis. This technique additional to the usual one (Haak *et al.* 2005) is required for efficient PCR in older Neolithic samples. Usually, efficient amplification of aDNA was achieved if this purification step was applied in the prePCR protocol (data not shown). This phenomenon may have resulted from the effective removal of PCR inhibitors with the Cycle Pure kit.

More recent samples were also subjected to the same analytical processes to trace the intersample and intralaboratory DNA contamination. Good DNA survival is represented by the 150-year-old Vác mummy No. 98 samples of high-quality DNA preservation (Guba, Pap

2007), but poor preservation of ancient DNA was shown up for several remains in this bone collection so that reliable sequences from these, even with huge effort, were not achievable (data not shown). However, some extracts appeared more suitable for the ancient DNA extraction by showing up constant reproducibility. For the best-analysed Neolithic remains such as Szarvas 23/20, both tooth and bone extracts worked well for PCR analysis (*Figure 2A*). Several samples from extractions of bones and teeth from these remains were subjected to amplification by our primer pair collection. With the use of our primer walking strategy, PCR products ranging from 64 base pairs to 455 base pairs could be amplified. Overlapping PCR amplicons (*Figure 2B*) from these preparations were used for sequencing. All PCR fragments were sequenced on both strands in doublets and only those without ambiguous base pairs in the sequence were used for construction of the individual HVR of mtDNA sequences of the Neolithic samples, which is summarised in the alignment in *Figure 2C*. There was no ambiguity experienced after aligning all sequences derived from the individual sequenced fragments and three single nucleotide changes were repeatedly found as aligned and compared to the revised Cambridge Reference Sequence (rCRS) (Andrews *et al.* 1999). These polymorphisms were as follows: 16223C→T, 16257C→A, 16261C→T (*Figure 2C*). The first one was also found in the Ecsefalva Neolithic mtDNA (Haak *et al.* 2005), but the later two proved to be novel for Neolithic.

A multi-way alignment has been performed on the confirmed sequences acquired in our laboratory (*Figure 2D*) and this was supplemented with other published European Neolithic sequences: two Linear Pottery Culture sequences, Ecsefalva 23A from Hungary and Derenburg 1 from Germany (Haak *et al.* 2005), and one Eulau sequence from the Late Stone Age Corded Ware Culture, Germany (Haak *et al.* 2008). To compare them with the recent sequences, besides the rCRS (Andrews *et al.* 1999) three non-European partial HVR sequences (Tommaseo-Ponzetta *et al.* 2002, Tabbada *et al.* 2009, Darvishi *et al.* 2005) were also used as outgroups. Only the overlapping partial sequences were considered (16145–16367). A dendrogram (*Figure 2D*) was elaborated suggesting the pattern of relatedness of all of the sequences aligned. The dendrogram is constructed with a distance-based tree-building method using the neighbour-joining algorithm. The amount of dissimilarity (distance) between two aligned sequences is used to derive the tree. Although this is only a rather experimental approach on sequences from an extended time period, the grouping of Körös sequences (Szarvas 23/20, Szakmár-Kisülés 8), and that of ALP sequences (Deb1b and Ecsefalva 23A) is obvious, and they seem to be rather far from the present-day European common rCRS sequence. Now our survey is on its way to extend the efficiency of this comparative analysis and apply the statistical level.

The two Körös individuals bearing the polymorphisms at nt 16257 and 16261 come from two different localities (Szarvas and Szakmár) whereas the nearby Ecsefalva 23A specimen from the subsequent culture has another

polymorphism pattern (Haak *et al.* 2005). Mezőkövesd and Polgár individuals from a more northern location of ALP culture probably have the nt 16261 polymorphism as well (see below and *Figure 3*). Medieval and more recent specimens did not show these polymorphisms. Variation in the human mitochondrial genome is usually described through haplogroup assignments, i.e. polymorphisms in HVR to rCRS give haplotypes which can be assigned to matrilineal lineages called haplogroups. The nt 16257 and 16261 correspond to the N9a haplogroup according to Genebase mtDNA Haplogroup Reference Guide version 2.6 (www.genebase.com). The nt 16261 alone in Mezőkövesd and Polgár individuals is not enough for estimating their haplogroup as it occurs in more than one haplogroup due to probably multiple independent mutations of the site.

Upstream primers with base pair substitution for all the four nucleotides at their 3' end, specific for Szarvas 23/20 polymorphism detected as nt16261C→T, were designed to separate modern and Neolithic DNA in PCR. These forward primers differ in the 3' end nucleotide, and their products are named M2C, M2G, M2A, M2T. For the polymorphism nt16257C→A two upstream primers were also designed in a similar manner, which are specific of modern and Neolithic DNA respectively, and their produced fragments are called M1C, M1A. Amplification reaction using the polymorphism-specific M1A and M2T primers and Szarvas 23/20 aDNA extract as template, gave positive amplicons, while the rCRS sequence identical primers M1C and M2C, on the contrary, gave no positive amplification results (*Figure 4*). G and A specific primers as controls showed up with no amplification, as expected. Among the newly analysed aDNA samples, Szakmár showed a clear polymorphic pattern (*Figure 4*), and it was verified by sequencing its different PCR products. Further PCR analyses of Neolithic remains such as Mezőkövesd 25 and Polgár 489 showed that one of the polymorphisms occurs in these samples as well, and their sequencing is in progress. The same polymorphisms were also verified in aDNA from other remains of the Szarvas site (data not shown).

This way we were able to detect polymorphic Neolithic DNA specifically from the remains, separating them from modern DNA and even from contaminating DNA. This will allow us to choose among the SNP bearing Neolithic extracts prior to sequencing as well as to avoid subsequent failure and work only with useful aDNA.

## CONCLUSIONS

The way of Neolithisation in Europe, i.e. the way how the agriculture and animal husbandry based Neolithic way of life followed the hunter-gatherer way of life in Europe, has been for decades already one of the most researched fields in archaeology and anthropology. Systematic archaeological excavations still reveal new Neolithic sites worldwide as well as in Hungary (e.g. Ecsefalva site, excavated by Whittle) providing new material for research. However, the re-examination of material excavated even a hundred years



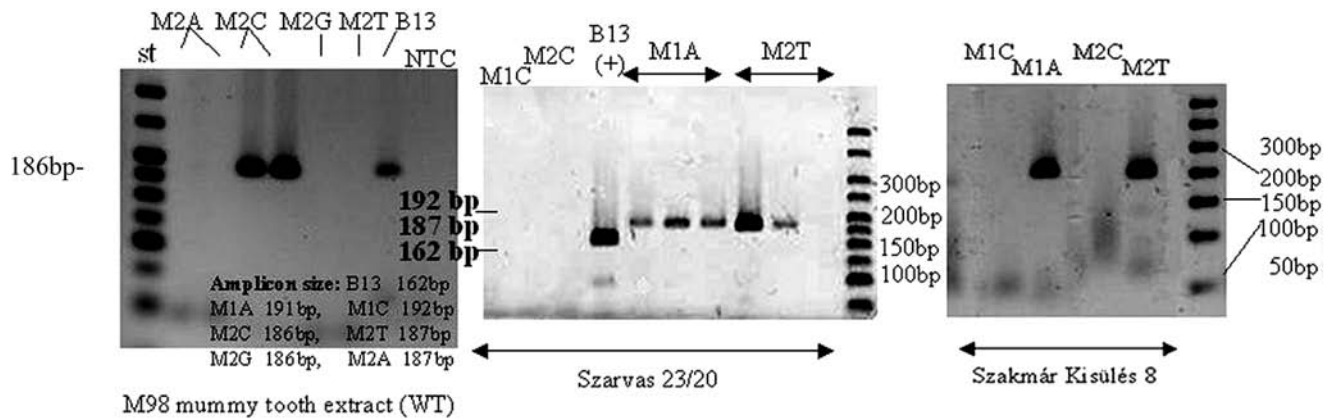


FIGURE 4. PCR detection of polymorphisms in Neolithic samples by base pair substitution specific primers designed for the identified SNPs from Szarvas 23/20. PCR products resolved in 2 % agarose gels. M1 Polymorphism 1 at nt 16257, M2 Polymorphism 2 at nt 16261, and fragments are depicted as A, C, G, T specific or according to their primer 3' end. All primers and amplicons from the same sample were identical except the 3' nucleotide, and this is given above the amplicons. B13 were applied as a positive control (cf. Figure 2). M98 t1 of Mummy No. 98 Crypt of Vác, Szarvas 23 grave 20 and Szakmár-Kisülés grave 8 DNA were used as templates in this study as given below the gel photos.

ago with new scientific approaches (e.g. biomolecular and isotope analyses) bears a great significance, too. The Great Hungarian Plain was an important place in the spread of Neolithic, so that the Early Neolithic findings from here might as well reveal key data of this process.

The Early Neolithic Körös-Starčevo-Criş Culture (KSC) represents the first significant sustained Holocene occupation of the Great Hungarian Plain. The main importance of the KSC was the transmission of the Neolithic way of life into the Carpathian Basin, connecting this area to agriculturists of Anatolia and the Balkans. The “classic” Neolithic way of life might have reached their ecological limit here, being quite close to the region of Fertile Crescent where the Neolithic developed (Sümegei, Kertész 2001). Traces of human environmental modification associated with this type of agricultural activities can only be found from a period ca. 1,000 years later in the northern part of the Great Hungarian Plain. So it seems that the expansion of farming came to a halt in the eastern and southern Carpathian Basin for up to 1,000 years (Hertelendi *et al.* 1998). The progress might have been stopped by an assumed Mesolithic population (Makkay 1996), but it can be also explained by ecological reasons (Sümegei, Kertész 2001). During this time farming techniques are thought to have adapted to Central European ecological and climatic conditions, leading to the transition from the Mesolithic to Neolithic in other areas of the Carpathian Basin as well. A new Neolithic culture, the Alföld Linear Pottery Culture (ALP) evolved north of the Great Hungarian Plain. There are opinions deriving it from the Körös Culture (e.g. Makkay 1996), but others suggest that local hunter-gatherers have adopted the Neolithic way of life without any major demographic impact (Kertész 2002). The Körös influence, whether demic or cultural, has led to the development of the European Neolithic Linear Pottery Culture, which colonised

the Western and Northern Europe. The earliest food-producing communities on the North European Plain were those of the Linear Pottery Culture between 5,400 and 5,000 BC (Whittle 2004). The radiocarbon dates show a very rapid spread of this culture, covering the distance between northern Hungary and northern Germany within no more than 200 years.

Newly, ancient DNA analysis of mitochondrial DNA of European ALP people was published (Haak *et al.* 2005) as well as aDNA analysis of South-European Neolithic Impressed Ware people (Sampietro *et al.* 2007), and later it was extended on Mesolithic human finds as well (Bramanti *et al.* 2009). In the Central-European studies, the predominant mtDNA types found in the ancient samples are also found in modern Europeans, but at considerably different frequencies, suggesting that the diversity observed today cannot be explained by simple demographic processes and direct lineaging. Only one ALP remain from the territory of present-day Hungary has been involved in these studies (Ecségfalva 23A specimen). The other LPC human specimens subjected to aDNA analysis presented the later and more northern appearance of LPC in Europe. However, there is a significant number of Neolithic findings from Hungary, which are known to have excellent DNA preservation, making even the analysis of nuclear DNA possible (Burger *et al.* 2007). The number of samples analysed so far is low, but already now it is obvious that a simple demographic model cannot be drawn between the first farmers and the recent Europeans as it was suggested by the demic diffusion model. Population demographic simulations refer to other significant population exchange/movements, because the present mtDNA haplotype diversity differs from the Neolithic one. Haplotypes which are rare today in Europe were likely more common among the Neolithic Europeans, such as N1a

(Haak *et al.* 2005) and N9a (this study). Today, the N9a haplogroup is mainly found in Asia, and is more common in Asia than in Europe, although it occurs in Europe at a rather low frequency (Malyarchuk *et al.* 2006). This N9a haplogroup has not been found in the Neolithic Central- and South-European samples analysed earlier (Haak *et al.* 2005, Sampietro *et al.* 2007). Although we do not have statistical distribution data yet, it seems that the newly analysed remains from this study show up a reproducible amount of N9a haplogroup mutations from specimens.

In accordance with the detected drift in the European gene pool proved for the Neolithic Age (Haak *et al.* 2005), Hungarian Neolithic sequences are separated by polymorphisms from the recent findings of populations living in the same area. Whether or not this fact is statistically significant and refers to a large population are still questions, and we will concentrate on this topic with a larger number of samples in the future. If it should be the case, our results would also confirm that people of the Neolithic cultures in Europe might have not been the direct ancestors of populations of historical times, and migration movements of populations would have always exerted strong influence on the genetic structure of humans in Europe.

Basing on the PCR results presented here, we can conclude that these Neolithic finds can be reproducibly distinguished from the more recent ones on the basis of their polymorphisms, what is more, different Neolithic sites show polymorphisms, which are not common in modern mtDNA pool from the same region. This analysis will be extended on other Neolithic sites in Europe, or even outside Europe in order to determine the mtDNA polymorphism pattern of Neolithic people. With growing number of analysed authentic sequences, a better estimation of genetic diversity of the Early Neolithic can be approached and statistically analysed in the Carpathian Basin.

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## REFERENCES

- AMMERMAN A., CAVALLI-SFORZA L. L., 1984: *The Neolithic Transition and the Genetics of Populations in Europe*. Princeton University Press, Princeton.
- ANDREWS R. M., KUBACKA I., CHINNERY P. F., LIGHTOWLERS R. N., TURNBULL D. M., HOWELL N., 1999: Reanalysis and revision of the Cambridge reference sequence for human mitochondrial DNA. *Nature Genetics* 23: 147.
- BRAMANTI B., THOMAS M. G., HAAK W., UNTERLAENDER M., JORES P., TAMBETS K., ANTANAITIS-JACOBS I., HAIDLE M. N., JANKAUSKAS R., KIND C. J., LUETH F., TERBERGER T., HILLER J., MATSUMURA S., FORSTER P., BURGER J., 2009: Genetic discontinuity between local hunter-gatherers and Central Europe's first farmers. *Science* 326: 137–140.
- BURGER J., KIRCHNER M., BRAMANTI B., HAAK W., THOMAS M. G., 2007: Absence of the lactase-persistence-associated allele in early Neolithic Europeans. *Proc. Natl. Acad. Sci. U.S.A.* 104: 3736–3741.
- COOPER A., POINAR H. N., 2000: Ancient DNA: do it right or not at all. *Science* 289: 1139.
- DARVISHI K., SHARMA S., BHAT A. K., RAI E., BAMEZAI R. N. K., 2007: Mitochondrial DNA G10398A polymorphism imparts maternal Haplogroup N a risk for breast and esophageal cancer. *Cancer Letters* 249: 249–255.
- GUBA ZS., PAP I., 2007: Initial analysis of DNA from mummified and Neolithic human remains. *Antropológiai Közlemények* 48: 153–160.
- GUBA ZS., SZATHMÁRY L., ALMÁSI L., 1997: Craniology of Neolithic in Hungary. *Papers on Anthropology* VII: 90–104.
- HAAK W., BRANDT G., DE JONG H. N., MEYER C., GANSLMEIER R., HEYD V., HAWKESWORTH C., PIKE A. W. G., MELLER H., KURT W., ALT K.W., 2008: Ancient DNA, Strontium isotopes, and osteological analyses shed light on social and kinship organization of the Later Stone Age. *Proc. Natl. Acad. Sci. U.S.A.* 47: 18226–18231.
- HAAK W., FORSTER P., BRAMANTI B., MATSUMURA S., BRANDT G., TÄNZER M., VILLEMS R., RENFREW C., GRONENBORN D., ALT K. W., BURGER J., 2005: Ancient DNA from the first European farmers in 7500-year-old Neolithic sites. *Science* 310: 1016–1018.
- HERTELENDI E., SVINGOR É., RACZKY P., HORVÁTH F., FUTÓ I., BARTOSIEWITZ L., 1998: Duration of tell settlements at four prehistoric sites in Hungary. *Radiocarbon* 40: 659–667.
- KERTÉSZ R., 2002: Mesolithic hunter-gatherers in the northwestern part of the Great Hungarian Plain. *Praehistoria* 3: 281–304.
- MAKKAY J., 1996: Theories about the origin, the distribution and the end of the Körös culture. In: L. Tóth (Ed.): *At the Fringes of Three Worlds: Hunter-Gatherers and Farmers in the Middle Tisza Valley*. Pp. 35–49. Szolnok.
- MALYARCHUK B. A., VANECEK T., PERKOVA M. A., DERENKO M. V., SIP M., 2006: Mitochondrial DNA variability in the Czech population with application to the ethnic history of Slavs. *Human Biology* 78: 681–696.
- PALUCH T., 2004: A Körös–Starčevo kultúra temetkezései. [Die Bestattungen der Körös–Starčevo-Kultur.] *Jósa András Múzeum Évkönyve* 46: 23–51.
- SAMPIETRO M. L., LAO O., CARAMELLI D., LARI M., POU R., MARTI M., BERTRANPETIT J., LALUEZA-FOX C., 2007:

- Palaeogenetic evidence supports a dual model of Neolithic spreading into Europe. *Proceedings of the Royal Society B: Biological Sciences* 274: 2161–2167.
- RENFREW C., 1987: *Archaeology and Language*. Jonathan Cape, London.
- SÜMEGI P., KERTÉSZ R., 2001: Palaeogeographic characteristic of the Carpathian Basin – an ecological trap during the early Neolithic? In: R. Kertész, J. Makkay (Eds.): *From the Mesolithic to the Neolithic*. Pp. 405–416. Budapest.
- TABBADA K. A., TREJAUT J., LOO J. H., CHEN Y. M., LIN M., MIRAZON-LAHR M., KIVISILD T., DE UNGRIA M. C., 2009: Philippine mitochondrial DNA diversity: a populated viaduct between Taiwan and Indonesia? *Molecular Biology and Evolution* 27: 21–31.
- TOMMASEO-PONZETTA M., ATTIMONELLI M., DE ROBERTIS M., TANZARIELLO F., SACCONI C., 2002: Mitochondrial DNA variability of West New Guinea populations. *Amer. J. of Phys. Anthropol.* 117: 49–67.
- UBELAKER D. H., PAP I., GRAVER S., 2006: Morbidity and mortality in the Neolithic of northeastern Hungary. *Anthropologie* 44: 241–257.
- WHITTLE A., 1996: *Europe in the Neolithic*. Cambridge University Press, Cambridge.
- WHITTLE A., 2004: Connections in the Körös culture world: Exchange as an organising principle. *Antaeus* 27: 17–26.

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