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ANCIENT DNA: GENETIC ANALYSIS OF aDNA FROM SIXTEEN SKELETONS OF THE VEDROVICE COLLECTION

ABSTRACT: Bone and tooth samples from sixteen individuals of the Vedrovice skeletal collection were submitted to ancient DNA (aDNA) analyses of mitochondrial as well as nuclear DNA. Compared with other aDNA prehistoric samples analysed at the University of Mainz aDNA laboratories, the Vedrovice samples are generally not among the best preserved due to a low content of severely damaged DNA molecules. Only 37.5% of the individuals yielded consistent results reproducible from different extracts. It was possible to type mitochondrial DNA samples from three male and three female individuals. The resulting six different DNA sequences (haplotypes) were classified into 4 haplogroups: haplogroup K (represented by two individuals), haplogroup T2 (also represented by two individuals), haplogroup H and haplogroup J1c, each represented by one individual. All of these haplogroups have been identified amongst modern European populations, although the individual haplotypes are predominantly represented among today's Eastern-European populations. Two of the Vedrovice haplotypes are unique, and as yet not identified among the currently known modern lineages. Haplotype N1a, whose incidence among LBK individuals is relatively high elsewhere (Haak et al. *2005), was not recovered among the analysed individuals from Vedrovice.*

KEY WORDS: Ancient DNA (aDNA) – Mitochondrial DNA (mtDNA) – Sequences – Haplotype – Haplogroup

INTRODUCTION

Sixteen individuals from the Vedrovice skeletal collection were submitted to ancient DNA (aDNA) analysis to determine their genome. Mitochondrial and nuclear regions usually employed for population genetics were explored in order to identify differences between individuals. For all considered genetic markers, a great deal of previously collected data concerning both ancient and modern DNA, was available for comparison.

Two main issues have to be taken into consideration when performing aDNA-analysis. First, the success-rate of the analysis depends on the condition of preservation of the biological material. Specimens may not be able to undergo analysis by means of genetic techniques if kept in less than favourable environments for a long time, since their DNA may become severely damaged and thus extremely fragmented. For instance, strong acidic or extremely wet soils, high temperatures or a combination of different unfavourable conditions may damage DNA-molecules to the extent that residual fragments cannot be multiplied by polymerase-chain-reaction (PCR) and thus cannot be submitted to further aDNA-analysis.

Secondly, since PCR is a competitive chemical reaction, particular care must be used in avoiding the coeval or even preferential amplification of modern well preserved DNA from external sources. Contamination may occur during different phases of the analysis, and thus it is necessary to have a strategy to monitor, or even to reduce, the possible sources of contamination. At the ancient DNA facilities of the Institute of Anthropology, University of Mainz (Germany), procedures conducted before the amplification reaction are carried out under strictly "DNA-free" conditions in a pre-PCR laboratory which is physically separated

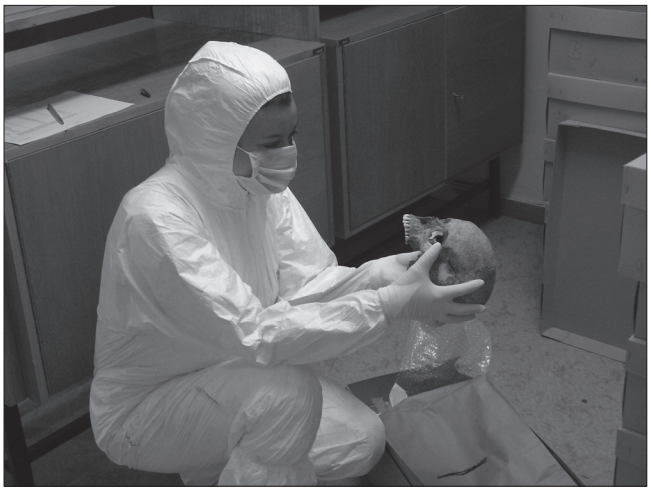

FIGURE 1. The author examining teeth for sampling at the Anthropos Institute of the Moravian Museum. Photo courtesy of Alena Lukes.

from other parts of the Institute. Moreover, the post-PCR laboratories are situated in another building. Researchers cannot access the pre-PCR area without undergoing a systematic decontamination procedure consisting of a shower, hair wash, and change of clothes. Decontamination procedures are also applied to all materials entering the laboratories, including samples, chemicals and new equipment. Each surface, as well as equipment in the pre-PCR laboratory, is frequently cleaned with soap and bleach. Further procedures to safeguard against contamination include overnight UV-irradiation of the laboratory and UV-irradiation of water used in the laboratories. Finally, the genetic analysis includes the examination of markers that permit identification of contaminating molecules. Samples with different DNA-strains co-amplified in the same reaction-tube can be identified by cloning. All individuals working in the laboratories, as well as all archaeologists and anthropologists who had access to the samples are genetically characterized so that their data can be systematically compared with the obtained aDNA data. Through this process it is possible to determine whether ancient DNA samples, chemicals or laboratory environments were affected by contamination.

SAMPLES

One bone and one tooth sample were collected from each of the sixteen individuals selected for aDNA analysis during two separate sampling episodes, and transferred to our ancient DNA facilities (*Table 1*). Following protocols to limit the possibility of contamination during sampling, the samples were handled while wearing gloves, face mask and lab-overalls (*Figure 1*).

A selection of twenty-seven skeletons with variable conditions of preservation were proposed for aDNA analysis during the first sampling episode. Since the goal of this preliminary analysis was to demonstrate the presence of preserved ancient DNA in the biological material, only six of the best preserved skeletons were chosen for ancient DNA analysis. For the second set of samples, selection included 10 individuals identified as outstanding amongst the Vedrovice cemetery population during examination of paleopathology markers. In all instances, selection for aDNA analysis was dependent on the integrity of the bone and tooth samples, as well as their intact morphology (*Figure 2*). Generally, the skeletons of adult individuals are better preserved, and were thus preferred for the analysis. To recover more biological material, collection of molars was preferred.

TABLE 1. Collected samples.

Sampling	Burial	Anthropological information	Tooth sample	Bone sample
First sampling	22/75	Female, 35–45	VED 22a	VED 22b
	23/75	Male, 18–20	VED 23a	VED 23b
	59/78	Male, 25–30	VED 59a	VED 59b
	72/79	Female, 30–40	VED 72a	VED 72b
	77/79	Male, 40–45	VED 77a	VED 77b
	91/80	Female, 18–20	VED 91a	VED 91b
Second sampling	15/75	Male, 35–40	VED 15a	VED 15b
	48/77	Female, 18–20	VED 48a	VED 48b
	54/78	Male, 20–25	VED 54a	VED 54b
	73/79	Male, 20–25	VED 73a	VED 73b
	75/79	Female, 25–35	VED 75a	VED 75b
	79/79	Male, 25–35	VED 79a	VED 79b
	82/79	Male, 50+	VED 82a	VED 82b
	93a/80	Female, 18–25	VED 93a	VED 93b
	105/81	Child, 16–18	VED 105a	VED 105b
	107/82	Female, 18–20	VED 107a	VED 107b

FIGURE 2. Tooth sample photographed in the pre-PCR laboratory before milling.

To monitor possible contamination, samples of modern DNA were collected and analyzed from all persons (10 individuals) involved in the project, or who had recent access to the skeletal remains. In each phase of the work, the present-day samples were kept physically separated from the prehistoric samples, and amplified exclusively in the post-PCR laboratory.

SAMPLE PREPARATION

Once in the aDNA laboratory, the ancient samples were first submitted to standardized decontamination procedures consisting of 60 min UV-irradiation on each side (Haak *et al.* 2005). The samples were photographed before removing the entire surface with a dental drill (2–3 mm of dental enamel were removed using sandblast cleaning procedures – *Figure 3*) and again using UV-irradiation under the same conditions. Before the second UV-irradiation, the bone samples were cut into smaller pieces with diamond drills. The samples were then ground to fine powder using a mixer mill and stored until use at 4°C.

The bone samples were analysed one month after the tooth samples. Only samples which yielded the same DNA-sequences from independently analysed bone- and tooth-tissue can be considered as possible original sequences.

DNA EXTRACTION

For this, and each of the following steps, the samples were processed together with other ancient DNA samples from different sources, extraction blanks, and negative controls to monitor any source of contamination from the laboratory.

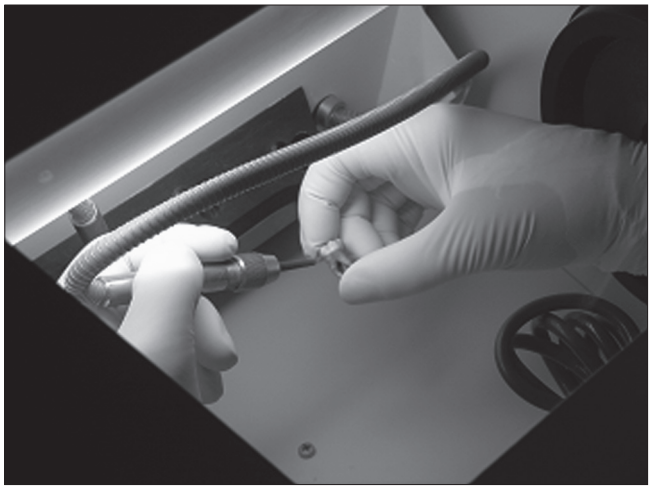

FIGURE 3. Sandblasting of a tooth in the pre-PCR laboratory. Photo courtesy of Guido Brandt.

Around 0.3 g powder of each sample were submitted to an extraction procedure routinely used for ancient DNA samples (Haak *et al.* 2005). This consists of a preliminary over-night incubation at 37 °C in a buffer solution containing enzymes and other chemical molecules (EDTA, soap), which isolates DNA from the cells. This is followed by a wash with phenol and chloroform, which separates (extracts) the water-solution containing DNA from all other organic molecules.

The extracts were then desalted and concentrated in apposite filter columns, portioned in small aliquots and stored at –20 °C.

DNA Amplification

Different aliquots (8 to 12 µl) of each extract were submitted to several DNA amplifications (PCR) for genetic typing. Amplification usually involved short DNA fragments, as might be expected of ancient DNA which is usually recovered fragmented. Extracts were amplified in different sets. If extraction blanks and negative controls amplified, the entire set was usually discarded.

Amplification of genomic DNA

Samples were analyzed using the Profiler Plus-Kit (Applied Biosystems) with a standardized protocol for ancient DNA (Bramanti *et al.* 2000). This allowed the detection of 9 STRs (short tandem repeats) carried on different chromosomes and the homologous gene Amelogenin, permitting the determination of biological sex. The length of the fragments varies between 100–300 bp (basepairs). The discrimination power of the complete series of amplified fragments is very high, and permits the establishment of likely maternal and paternal relationships between individuals.

Amplification of mitochondrial DNA

Regarding mitochondrial DNA (mtDNA), the complete HVR-I (hypervariable region I, np 15996–16409) of the non-coding region was analyzed by means of PCR. Four

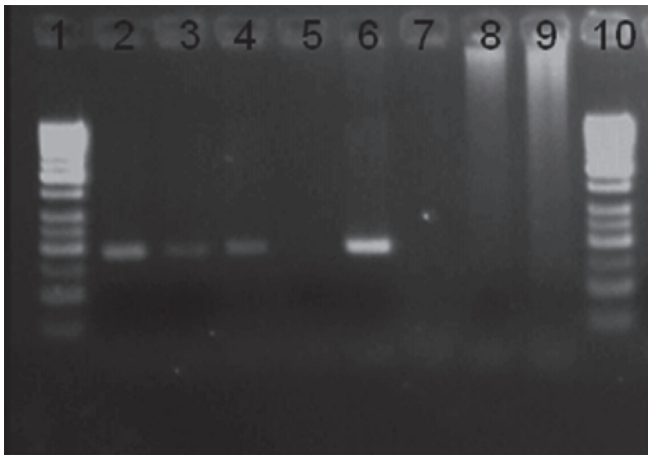

FIGURE 4. Image of an electrophoretic run. Aliquots of amplification products are submitted to an electric field and photographed. The intensity of the DNA-bands is proportional to the quantity of amplified molecules. Lines 1 and 10: length ladder. 2: VED 22a; 3: VED 91a; 4: VED 77a; 5: VED 72a; 6: BAL 4; 7 and 8: extractions blanks; 9: PCR negative control.

partially overlapping fragments, whose maximal length is 145 bp, were amplified. These reactions are routinely employed in our laboratories for very degraded and old specimens, and entail standardized protocols (Haak *et al.* 2005).

To better clarify the phylogenesis of the sequences, in some cases we also genotyped the HVR-II, and investigated the coding region by means of RFLP (restriction fragment length polymorphism) analysis. For this, we amplified a short fragment and looked for the presence of a single informative mutation in a specific position by using cutting enzymes. All this information assists in the classification of mitochondrial DNA-sequences that share specific mutations in a "family group", generally defined "haplogroup".

Cloning

All the mtDNA-samples that yielded a reaction were submitted to cloning procedures (Haak *et al.* 2005) and were also partially direct sequenced. Cloning is recommended to discriminate among different sources of DNA (for instance, original and contaminating molecules), which can be co-amplified in the same reaction tube. Each PCR-product is inserted using a vector in a bacterial culture. Five-eight selected clones from each PCR-product potentially containing DNA molecules of different origins were then sequenced.

Sequencing

The sequencing products were analysed by capillary electrophoresis on an ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems). Further sequence analyses were carried out using scientific programs for the alignment of the DNA sequences, compared with a reference sequence (CRS, Cambridge reference sequence; Anderson *et al.* 1981, Andrews *et al.* 1999).

ANCIENT SAMPLE RESULTS

Nuclear DNA

Despite good morphology, none of the samples successfully amplified nuclear DNA. The length of single STR markers usually allows the amplification of ancient and damaged DNA (Bramanti *et al.* 2000), except when molecules are exceedingly fragmented. This negative result is not unexpected considering that only 15% of prehistoric samples studied at the aDNA-laboratories of Mainz yield nuclear DNA amplifications, most of which come from different latitudes and colder climates than Vedrovice, or even from cold caves (Burger *et al.* 2007). The unsuccessful amplification of nuclear DNA may also be interpreted as an indication of probable absence of intact recent contamination in our samples. The possible presence of co-extracted inhibitors that hindered the PCR is discussed in the following section.

Mitochondrial DNA

Because of the higher number of mitochondria in a cell, the probability of recovering DNA in an ancient DNA analysis is always higher for mitochondrial DNA than for nuclear DNA. Nevertheless, the general degree of preservation of analyzed mitochondrial DNA molecules in the samples is very poor. *Figure 4* shows a typical pattern of amplification of the Vedrovice samples, compared with a well preserved specimen from a different collection (line 6). The signal of the amplification-products from Vedrovice (lines 2–5) on the agarose gel is very weak, which may reasonably explain the difficulties encountered in subsequent cloning of the sequences. The successfully amplified sequences are generally characterised by the typical ancient DNA damage pattern with artefacts due to post-mortem substitutions of single nucleotides. Moreover, the presence of only one damaged molecule in all the clones indicates that in some cases both the tooth and bone powders contain very few molecules of mtDNA which started the PCR-reaction (Hofreiter *et al.* 2001). Despite the very rigorous adherence to protocols and decontamination methods used in our laboratory, some sporadic contamination is also recognizable among the clones, as is usual for human ancient DNA.

In some cases we were not able to amplify some of the overlapping fragments – mostly the largest ones (145 bp). The presence of salts and other material in the samples which can inhibit the amplification is usually avoided by the use of purification columns on the extracts, although the survival of some inhibitors to the procedure often cannot be excluded. Nevertheless, the presence of inhibitors can be excluded for the Vedrovice samples since the same DNA-extracts yielded reproducible results in other amplifications.

Despite the encountered difficulties, six out of sixteen individuals gave consistent reproducible results from independent extractions. The amplifications could therefore be genetically typed for mitochondrial DNA. Results for each analyzed individual are listed below, separated by sex. In each instance, the list of consistent mutations (haplotype)

is given in comparison to the CRS. The haplogroup is indicated in brackets, with additional information regarding the sample, haplotype and haplogroup provided in the text below. A critical approach and stringent criteria was used for typing. Results presented in square brackets are not considered further.

MALES

Individual 15/75
16126c 16294t 16296t 16304c (Haplogroup T2)
Both tooth and bone extracts showed 4 consistent mutations. The DNA looks damaged and old, thus probably original. Some sporadic contamination was identified after cloning. The identified sequence, 16126c 16294t 16296t 16304c, is widespread in Europe, particularly in the north-east. This sequence can be classified as haplogroup T2, similar to individual 23/75. Five other prehistoric samples (out of 150) analyzed in our laboratories, also share the same lineage as Individual 15/75. Haplogroup T2 is thought to have been introduced into Europe in the Lower Upper Palaeolithic, and at the same time as haplogroup K which is also represented in the Vedrovice cemetery population (Individuals 22/75 and 91/80), as well as the H-16304 founder type (Individual 59/78). In contrast, the root types of H, U4, I and HV are associated with the Middle Upper Palaeolithic, and the root type of U is associated with the Early Upper Palaeolithic (Richards *et al.* 2000).

Individual 23/75
16126c 16294t 16304c (Haplogroup T2)
By submitting tooth and bone tissue to independent extractions, HVR-I sequences belonging to mitochondrial DNA haplogroup T2 were reproducibly amplified and cloned. The number of substitutions is consistent with the presumable degree of degradation of the molecules. The haplotype is represented in the modern European and Russian population, although the frequency cannot be calculated at this time. One of our lab-collaborators shares the same haplotype, together with two from at least 120 other prehistoric samples analyzed in our laboratories at the same time as the Vedrovice collection. The probability that our collaborator, who is working on animal DNA and has had no access to the samples, contaminated the 3 individuals is in our opinion very low because of the adopted precautions (adequate lab-cloths, complete replication of the analysis at different times and in different sets, multiple blank samples and so on), but cannot be completely excluded (true of most ancient DNA samples). For additional discussion of haplogroup T2, see Individual 15/75.

Individual 54/78
[16093c? (H?)]
Unfortunately only two fragments out of four could be examined due to a lack of amplification. The mutation 16093c could not be reproduced, and thus it was not possible to type this individual.

Individual 59/78
16304c 16400t (H)
The produced clones show a noticeable degree of degradation, probably attributable to post-mortem substitutions. The mutation 16304c was observed in both of the extracts associated with mutation 16400t. This haplotype was not identified in our database, nor in the literature, but it is noteworthy that the majority of published HVR-I sequences do not include the last part of the sequence. Three complete sequences from the databanks that share the 16400t mutation do not present 16304c. One of the project anthropologists also carries the mutation 16304c (only), but his access to the samples was very limited. Investigation of the coding region (– 7028 *Alu I*) helped to classify the sequence of individual 59/78 to the haplogroup H. Together with T, T2 and K, the H-16304 founder type is considered to have been introduced into Europe during the Lower Upper Palaeolithic (Richards *et al.* 2000).

Individual 73/79
Multiple sequences were found among the clones. Thus, this individual could not be genetically typed.

Individual 77/79
[16298c (V)]
It was not possible to obtain amplified DNA from both of the extracts belonging to individual 77/79. Only the tooth yielded results and showed a single consistent mutation, 16298c. This haplotype may belong to the haplogroup V. This haplogroup is thought to have been introduced into western Europe about 15–12,000 years ago, and is therefore considered to be a "Neolithic" haplogroup. It is widespread in western Europe, and its frequency is particularly noticeable among modern Basque and Saami populations, probably due to drift effects. The same sequence has also been identified in one other LBK individual from Germany (Haak *et al.* 2005). Unfortunately, although the DNA appears to be old, since the results could not be reproduced, this individual was excluded from further consideration.

Individual 79/79
Only the bone extract yielded amplification product. The DNA does not appear damaged, and may therefore be the product of recent contamination. Thus, no haplotype was defined.

Individual 82/79
The DNA indicates multiple sequences, one of which can be identified as contaminating DNA from one of the anthropologists (number 2 in *Table 2*) associated with this project. Thus, no lineage was determined.

FEMALES

Individual 22/75

16093c 16224c 16261t 16290t 16311c (Haplogroup K)

Both the tooth and bone extracts, although obtained at different times, yielded the indicated sequence. Some contaminating sequences are present among the clones, but the rest of DNA looks damaged, and thus likely old. None of the project participants share the same haplotype. This haplotype has not yet been recovered in modern populations (database of 33,000 individuals; K. Tambets, pers. comm.). Nevertheless, close HVR-I derivatives are present, one with 16093c 16224c 16261t 16311c, but without 16290t from a Syrian Komi sample (Bermisheva *et al.* 2002), a population living in Volga-Ural region of eastern Europe. Based on unpublished data, it seems that this infrequently occurring lineage is distributed predominantly in modern eastern Europe, and mostly among Slavic-speaking populations. In addition, another haplotype with HVR-I derivatives 16224c 16261t 16311c is again typed among eastern European Slavic-speakers (K. Tambets, pers. comm.), but is also found among Ethiopians (Kivisild *et al.* 2004). Finally, another haplotype with 16093c 16224c 16290t 16311c, but without 16261t, is reported by Pereira *et al.* (2000) in southern Portugal. By investigating the coding region of the mitochondrial DNA (+12308 *Hinf I*), it was possible to associate Individual 22/75 with haplogroup K. Living individuals are widespread in Eurasia, the Near East, and northern Africa. In western Europe, haplogroup K accounts for nearly 7% of the total modern data (Richards *et al.* 1998). The estimated age of this haplogroup is 16,000 years, and the widespread geographic distribution suggests a post-glacial expansion as a plausible explanation (Richards *et al.* 1998).

Individual 48/77

16069t 16126c (J1c)

Two consistent mutations were identified among the clones, 16069t and 16126c, classified into haplogroup J1c. This haplogroup was also identified in: one LBK individual (Haak *et al.* 2005) and two additional Neolithic samples from Catalonia (Sampietro *et al*. 2007). Presently, this haplogroup is widely distributed in Eurasia. According to Richards *et al.* (2000) the root types of J and T1, are the two major founders associated with the Neolithic.

Individual 72/79

[…16224c 16311c… (K)]

Although analyses were repeated several times on both tooth and bone samples, the frequent lack of amplification made the investigation of this sample very difficult. The extracts appeared to contain little or no residual DNA; sometimes only one damaged molecule started the polymerase reaction. Unfortunately, two fragments of the HVR-I region could not be amplified at all. The two consistent mutations 16224c and 16311c make phylogenetic sense and would belong to haplogroup K. The analysis of the HVR-II region could not firmly exclude contamination from one of the anthropologists on the project, who bears the same HVR-I mutations (individual 10 in *Table 2*).

Individual 75/79

The partially analyzable clones showed multiple sequences, thus the haplotype of this individual could not be defined.

Individual 91/80

16093c 16224c 16311c + 152c / 309.1c (K)

Both the tooth and the bone sample yielded the same result: the HVR-I sequence 16093c 16224c 16311c, belonging to haplogroup K. Haplogroup K was also associated with Individual 22/75. This haplotype is widespread at low to moderate frequency in both Near Eastern and European populations. In some cases it seems that a unique molecule bearing substitutions started the amplification reaction in our extracts, supporting the hypothesis that the DNA is old and damaged. Nevertheless, one of the anthropologists on the project also bears the same haplotype (*Table 2*). Fortunately, analysis of the mitochondrial region HVR-II excluded this individual as the potential contaminant.

TABLE 2. Sequences recovered from the modern DNA of Vedrovice project collaborators (anthropologists). Single mutations are indicated –16000 referring to the CRS. Hg: Haplogroup.

Individual	HVR-I (–16,000)	HVR-II	Hg
1.	126c 163g 186t 189c 294t		T1
2.	256t 270t 301t 399g	73g 195c 263g 309.1c 315.1c	U5a
3.	311c	152c 263g 309.1c 315.1c	H/U?
4.	189c 356c	263g 315.1c	H?
5.	304c	261g 309.1c 315.1c	H-16304?
6.	153a 189c 298c	72c 93g 263g 309.1c 315.1c	pre-V
7.	CRS		H?
8.	192t 256t 270t 291t 399g		U5a1
9.	111t 192t 256t 270t 291t 399g	73g 263g 315.1c	U5
10.	093c 224c 311c	73g 195c 263g 315.1c	K

Individual 93a/80
[16192t 16270t? 16286? 16287? (U5a1?)]
Unfortunately, this individual could not be completely analyzed due to a lack of amplification from the bone extract. Only the first mutation was reproduced and this is not enough to classify the individual to a haplogroup. If the determination was correct, this should be a very interesting example of U5a1, one of the oldest branches of U in Europe.

Individual 107/82
[16311c (H, HV, R)]*
A consistent mutation, 16311c, was observed in the clones from both extracts. The DNA appears damaged and therefore presumably old. Nevertheless, Anthropologist 3 on the project (*Table 2*) shows the same mutation, a likely source of contamination. The samples were not considered further.

NON-ADULTS

Individual 105/81
Several lineages were identified among the clones from both bone and tooth extracts. No haplotype could be inferred.

MODERN SAMPLES

Results of genetic analyses of the modern samples are presented anonymously (*Table 2*).

CONCLUSION

Eight males, seven females and one non-adult from the Vedrovice cemetery population were analysed by means of aDNA techniques. The analyses yielded 6 (3 male and 3 female individuals) reproducible sequences, which are widespread at different frequencies throughout Europe. The sole exception was female 22/75 (possibly also male 59/78) who bears a unique haplotype. Compared with other aDNA analyses of prehistoric samples carried out at the University of Mainz aDNA laboratories, the 37.5% amplification success rate is low. It is therefore possible to conclude the Vedrovice samples are generally not among the genetically best preserved ones: the DNA content is poor and the few molecules appear to be severely damaged.

Concerning the represented haplogroups, we observed a prevalence of T2 and K sequences, whose founders are proposed to have been introduced into Europe during the Lower Upper Palaeolithic (Richards *et al.* 2000). These two haplogroups were also identified in an earlier analysis of an LBK sample from north-central Europe (Haak *et al.* 2005) with a frequency of 20.8% (±16.28; CI 95%) and 16.7% (±14.9; CI 95%) respectively. The low number of analysed individuals permits no further statistical consideration of the Vedrovice samples. The haplogroup N1a, whose frequency among other LBK populations was relatively high (25%±18.3%; CI 95%; Haak *et al.* 2005), was not identified among the Vedrovice reproducible sequences. However, the presence of N1a cannot be fully excluded due to stochastic sampling errors. In this regard, it is interesting to note that none of the specific mutations for haplogroup N1a were observed, not even among clones that could not be reproduced.

Concerning the pattern of distribution of the identified sequences, it is interesting to note their present-day distribution throughout eastern Europe. This is particularly true for female 22/75 for whom an eastern haplotype origin was suggested on the basis of modern data.

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