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ANCIENT mtDNA SEQUENCES AND RADIOCARBON DATING OF HUMAN BONES FROM THE CHALCOLITHIC CAVES OF WADI EL-MAKKUKH

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ABSTRACT

DNA from fossil human bones can provide valuable information for understanding intra- and inter-population relationships. Using the DNA preserved inside crystal aggregates from human fossil bones containing relatively large amounts of collagen, we demonstrate the presence of reproducible mtDNA control region sequences. Radiocarbon dates from each bone show that the burial caves were used for up to 600 years during the Chalcolithic period (5^{th}-4^{th} millennium BP). A comparison of the ancient DNA sequences with modern mtDNA databases indicates that all samples can most likely be assigned to the R haplogroup sub-clades, which are common in West-Eurasia. In four cases more precise and confident haplogroup identifications could be achieved (H, U3a and H6). The H haplogroup is present in three out of the four assigned ancient samples. This haplogroup is prevalent today in West – Eurasia. The results reported here tend to genetically link this Chalcolithic group of individuals to the current West Eurasian populations.

Abbreviations: aDNA, ancient DNA; CRS, Cambridge Reference Sequence; mtDNA, mitochondrial DNA; HVS-I, Hypervariable segment 1.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. EF060054-EF060059, DQ020117).

KEYWORDS: aDNA, bone crystal aggregates, population genetics, collagen preservation, haplogroups, Chalcolithic Caves, Wadi el-Makkukh

INTRODUCTION

The southern Levant links the continents of Africa, Europe and Asia. This has been an attractive region for settlement since prehistoric times and served as a vital corridor of communication, migration, and trade. Phylogenetic analyses of modern human mitochondrial DNA (mtDNA) haplogroups in the Levant have facilitated reconstruction of human migrations through this corridor during prehistory (Richards et al., 1996). The objective of this study was to use ancient DNA to provide direct information on the genetics of individuals that once lived in this region, and in this way to learn more about possible past migrations.

The Wadi Makkukh site comprises 13 caves. The caves are located in the Judean Desert just north of the Dead Sea (Hirschfeld and Riklin, 2002). In three of the caves (nos. 1, 3 and 13) human skeletal remains were found, including at least 40 individuals. A single burial of a male still in articulation was found in Cave 13 (known as The Cave of the Warrior), together with grave goods that included textiles, a basket, wooden arrows and a wooden bowl (see reports in (Schick, 1998)). The latter have been dated to the Chalcolithic Period about 6,700 to 4,700 BP (Jull et al., 1998; Rowan and Golden, 2009). The other human bones were not found in articulation and there was no well defined stratigraphic organization. The presence of these organic artifacts indicates that preservation conditions are extremely good at this location.

Most of the human long bones (femurs) analysed from these caves contained relatively high contents of well preserved collagen. This is consistent with good preservation conditions for organic materials. One of the objectives of this study was to determine whether there is a correlation between the HCl insoluble collagen content of the bones and the preservation of the mitochondrial DNA, as this parameter could prove to be a useful pre-screening technique for identifying fossil bones with well preserved DNA. Other properties of fossil bones that are used for this purpose are racemization of aspartic acid in whole bone (Collins et al., 1999) and C/N ratios of collagen (Colson et al., 1997). We took advantage of another approach to extract better preserved DNA from fossil bones, namely to treat the bone powder with a strong oxidizing agent (NaOCl) and then extract only the organic material, including DNA, that was protected inside intergrown crystal aggregates (Salamon et al., 2005; Dissing et al., 2008). This approach also removes some authentic DNA and most of the contaminating DNA and inhibitors of the PCR reaction (Salamon et al., 2005; Malmström et al., 2007; Dissing et al., 2008). Using these two approaches, we were able to obtain 12 sequences of mtDNA control region from human femurs. Salamon et al. (2005) had previously shown that one femur from Wadi Makkukh did contain sequenceable DNA prepared in this manner.

The closed circular mtDNA molecule has several unique properties, such as high copy number, maternal inheritance, lack of recombination and high mutation rate (Pakendorf and Stoneking, 2005). These properties make it a powerful tool in population genetics and for understanding human history and evolution (Vigilant et al., 1991; Jorde et al., 1995) on the regional scale (Mountain et al., 1995; Pereira et al., 2005; Manwaring et al., 2006). The high copy number of mtDNA in a cell may improve its chance of survival in an aDNA sample. The mtDNA Control Region (CR) is a rapidly evolving non-coding region (Lopez et al., 1997) that includes the phylogenetically informative Hypervariable segment I (HVS-I, 16024-16383) and Hypervariable segment II (HVS-II, 57-372) regions (Stoneking, 2000). The process of mtDNA genotyping is usually based on the combination of HVS-I sequencing and the genotyping of specific coding region single nucleotide polymorphisms (SNPs). The phylogenetic information is obtained from the alignment of each sequence with the mtDNA Cambridge Reference Sequence (CRS). This provides a series of mutations called haplotypes. A group of similar haplotypes is named Haplogroup (Hg) and is best defined by a combination of coding region SNPs (Behar et al., 2007). The distribution of mtDNA haplogroups is well documented in different modern populations. This valuable demographic information can be used as a reference for ancient DNA (aDNA) studies.

MATERIALS AND METHODS

Samples

We studied 13 left femora from caves 1 and 3. Based on anatomical measurements the femora were taken from both females and males (Table 1). In cave no. 13, known as The Cave of the Warrior, an articulated skeleton was found. A femur and a humerus were analysed from this individual.

Table 1. Samples analysed in this study. Cave 1 and 3 samples are femurs, while the Cave 13 sample is a femur and a humerus from the same individual. Gender was determined based on anatomical measurements of the femur (Bass, 1995). WM25 femur was fragmented. The insoluble collagen content for each bone is shown. In this study ^{14}C dating was performed on bones from Caves 1 and 3 (RTT label). For Cave 13 ^{14}C dating was determined based on linen fabric that wrapped the articulated skeleton (Jull et al., 1998). Uncalibrated ^{14}C ages are reported in conventional radiocarbon years BP and calibrated dates were calculated based on Reimer et al. (2009) by means of the 2010 version OxCal v.4.1.5 of Bronk Ramsey. Calibrated ranges are given for ±1σ and ±2σ. For simplicity the total calibrated range is given. Samples are ordered by cave and by radiocarbon age. a) age obtained from linen fabric (607/75/1) by the NSF-AMS Laboratory, Tuscon (Jull et al., 1998), b) same linen sample as a) measured by decay counting at the Rehovot Laboratory (Jull et al., 1998).

Cave	Sample	Gender	Collagen content (wt.%)	Radiocarbon Lab #	^{14}C age ± 1σ year BP	Calibrated age ±1σ year BC	Calibrated age ±2σ year BC
1	WM10	female	4.9	RTT 4889	5655±40	4540-4450	4585-4365
	WM11	female	9.5	RTT 4890	5630±40	4505-4370	4540-4360
	WM30	male	10.7	RTT 4873	5330±35	4240-4065	4315-4045
3	WM28	male	8.0	RTT 4872	5595±40	4460-4365	4500-4345
	WM27	male	12.6	RTT 4434	5560±80	4490-4335	4585-4245
	WM25	?	8.5	RTT 5149	5475±40	4360-4265	4445-4240
	WM6	male	9.0	RTT 5147	5400±40	4330-4235	4345-4070
	WM1	male	5.5	RTT 5146	5360±40	4325-4070	4330-4050
	WM19	female	6.3	RTT 5148	5355±40	4315-4070	4325-4050
	WM2	male	7.2	RTT 4874	5300±40	4230-4050	4255-3995
	WM29	male	14.6	RTT 4435	5240±65	4230-3970	4255-3950
	WM3	male	3.0	Not analyzed			
	WM8	female	0.5				
13	CW	male	Femur – 10.0 Humerus – 10.0	AA 22235 RT 1946	5140±50 [a] 4925±50 [b]	4035-3810 3765-3650	4045-3795 3905-3635

Precautions regarding contamination

Dealing with human bones requires paying close attention to modern DNA contamination in the laboratory, as ancient DNA is low in quantity and quality. Even though the use of DNA from bone powder treated with the oxidant sodium hypochlorite (referred to as the aggregate fraction) should alleviate this problem, we followed references (Cooper and Poinar, 2000; Gilbert et al., 2005) and used the most stringent precautions for authentication of ancient DNA sequences and added several more. The precautions used were as follows:

1. Physical separation between the dedicated DNA extraction laboratory, the pre-PCR preparation area and the post PCR processing laboratory.

2. Sequences were obtained from samples with more than 5 weight % insoluble collagen.

3. The DNA was extracted from bone aggregates after treating the bone powder with sodium hypochlorite.

4. Two independent DNA extractions were carried out on independent parts of each bone. In one case, sample CW, it was possible to analyze two different bones from the same indi-

vidual. At least two amplifications were performed, one for each extract. Two negative controls for the extraction procedure and three negative controls for the PCR reaction were used. Positive controls were never used.

5. No re-amplification was carried out, to exclude contamination by PCR products.

6. The authentic sequence was determined from cloned sequences using different sets of primers.

7. Sequences were obtained for amplifications with >1000 copy number of target DNA, based on a calibration curve using the realtime PCR.

8. We did not observe amplifications of a human mtDNA 520bp fragment. Such large fragments are not usually present in aDNA extracts. Furthermore, no human mtDNA sequence of 132bp could be obtained from extracts from a cat bone. On the other hand, using specific primers the obtained sequences aligned well with previously published *Felis catus* sequence (Yoder et al., 2003).

9. Only one bone was processed at a time and by a single researcher (M.S.). Note that the HVS-I (16036-16498) sequence of M.S. is identical to the Cambridge Reference Sequence (CRS) and the HVS-II (30-429) contains the mutations A263G, 309.1C, 309.2C, 315.1C compared to the CRS.

Determining weight percent insoluble collagen in bone

Powdered bone (150 mg, particle size: <500μm) was dissolved in 4 ml of 1N HCl for 10 min with gentle shaking. The sample was centrifuged (12,000 *g* for 2 min) and the supernatant was discarded. This step was repeated one more time to assure complete dissolution. The insoluble fraction was washed three times with distilled water and eluted by centrifugation. The insoluble fraction was dried at 55°C overnight, weighed and collagen content was calculated as the weight percent (wt. %) of total bone powder.

FTIR Spectroscopy

Fourier Transform Infrared (FTIR) spectra (MIDAC Corp., Costa Mesa, CA, USA) were obtained by mixing about 0.1 mg of powdered sample with about 80 mg of KBr. Spectra were collected at 4 cm^{-1} resolution. FTIR spectra with absorptions at 1653 (amide I), 1539 (amide II) and 1457cm^{-1} (proline) in the appropriate proportions are indicative of the presence of well-preserved collagen (DeNiro and Weiner, 1988). The presence or absence of absorption peaks that are not those of collagen (such as a broad peak around 1100cm^{-1}) is an estimate of the quality of the collagen (Weiner et al., 1993, Weiner 2010).

Bone aggregates extraction

Following Salamon et al. (2005) bone aggregates were extracted from 200 mg of NaOCl-treated bone powder (4h), placed in a 15-ml sterile tube with 3 ml of 95% ethanol and sonicated for 1min. The sample was then vortexed and allowed to settle. The aggregates settled in 1 min.

DNA extraction and analyses

These procedures were performed following Salamon et al. (2005). Briefly 200 mg of aggregates were decalcified with 0.5mM EDTA (pH 8.0) and then incubated in proteinase K buffer for 20 min at 60°C and continued overnight at 37°C. The DNA was then extracted with 25:24:1 phenol:chloroform:isoamyl alcohol and n-butanol. The extracted DNA was purified and concentrated by using a YM30 unit (Millipore) to a final volume of 100 μl. To avoid cross-contamination only one sample was analysed each time. The state of DNA preservation was assessed by amplifying DNA fragments of increasing size using the same 5' primer. These fragments are integrated within HVS-I of the mtDNA genome. The primer sets used were: F16191 and R16301, R16356, R16410 or R16498 (Salamon et al. 2005). For the HVS-II region primer sets F30-R306 (5′-tcacgggagctctccatgca-3′, 5′-ggggtttggtggaaattttt-3′) and F152-R429 (5′-tattatttatcgcacctacg-3′, 5′-ctgttaaaagtgcataccgc-3′) were used. To avoid false negative results, we analyzed the DNA extracts for the presence of PCR inhibitors by spiking a lambda PCR (Amersham Biosciences, Piscataway, NJ) with aDNA extract. The magnitude of inhibition was

determined by a comparison of PCR products with and without aDNA extract. We also analyzed all extracts for modern human DNA contaminants by amplifying a fragment of 520 bp (primers F15998-R16498). The PCR products were cloned (pGEM-T Easy vector, Promega), and sequenced on an Applied Biosystems 3700 DNA analyzer machine.

Radiocarbon dating

The cleaning procedure of the bone collagen and the characterization of the recovered collagen followed the preparation procedure of (Yizhaq et al., 2005). All the samples were prepared as graphite at the Radiocarbon Laboratory at the Weizmann Institute (Label RTT) and measured using Accelerator Mass Spectrometry. Prior to the measurement, the weight percent purified collagen extracted for radiocarbon dating ranged between 2 and 7.7%. 300 to 600 mg of bone powder was used for radiocarbon dating. The quality of the collagen was checked again after extraction using infrared spectroscopy. All the IR spectra did not indicate the presence of other material except collagen. Radiocarbon dates were calibrated using the latest calibration curve (Reimer et al., 2009) by means of the software OxCal 4.1.5 Bronk Ramsey (2010) based on (Bronk Ramsey, 1995; Bronk Ramsey, 2001).

Haplogroup assignment and a comparison to modern populations

In this study, only partial CR sequences were available, therefore, haplogroup assignment could not be completely validated and each sequence was analyzed taking into account its given sequence range. In the first stage, we tried to identify specific HVS-I SNPs that are known to be indicators for specific haplogroups, based on Behar et al. (2007). We then tried to predict a haplogroup affiliation based on the fundamentals of the nearest neighbor method following Behar et al. (2007). The essence of this method is to search for matching haplotypes in a large, high quality, reference database that contains haplotypes with their suitable haplogroup affiliations. As a reference we used the largest standardized mtDNA database that is available, namely from the *Genographic Public Participation Project* (78,590 HVS-I genotypes). This public dataset contains mtDNA information of individuals (HVS-I haploype and haplogroup), but lacks their population affiliations (Behar 2007). Eventually, when a unique and highly reliable haplogroup was determined, we searched for its presence in modern population datasets from the literature. We define the HVS-I positions here to be between 16023-16569, similar to the Genographic study (Behar et al., 2007).

RESULTS

The bones in these caves (except for the skeleton in Cave 13) were not articulated and were not excavated in a well defined stratigraphic order. We therefore only analysed the left femora in order to ensure that each bone is from a different individual. Furthermore, as both Chalcolithic and Roman artifacts were present in these caves, all the bones that produced DNA sequences were radiocarbon dated. The dates prove that they are all from the Chalcolithic (Table 1 and Figure 1). Radiocarbon dates of the linen fabric that wrapped the articulated skeleton from Cave 13 showed that it is also from the Chalcolithic (Jull et al., 1998) and we assume that the bones of this individual are of the same age. Table 1 also shows the insoluble collagen contents of each of the bones analysed and the sex identifications.

As contamination is always a serious problem when analyzing human ancient DNA, we took many precautions (see Materials and Methods). The strategy used to sequence the mtDNA involves analyzing successively longer fragments starting with the same forward primer. We noted a positive correlation between the longest reproducible PCR fragment that could be obtained from each bone and the insoluble collagen content of the bone (Figure 2). This shows that the insoluble collagen content of the whole bone is a good indication of DNA preservation in the aggregates.

Figure 3 shows the mtDNA sequences obtained. Four femora produced fragments of 327bp, one produced fragments of 239bp and in the remainder only shorter fragments could be

sequenced. We note that all the blanks in these experiments showed no evidence of contamination. In order to exclude the presence of modern human contamination in our system, we also analysed these samples for the presence of a 520 bp fragment from the human HVS-I region. Such a fragment is greater than the length expected from ancient human DNA samples. No such fragment was obtained.

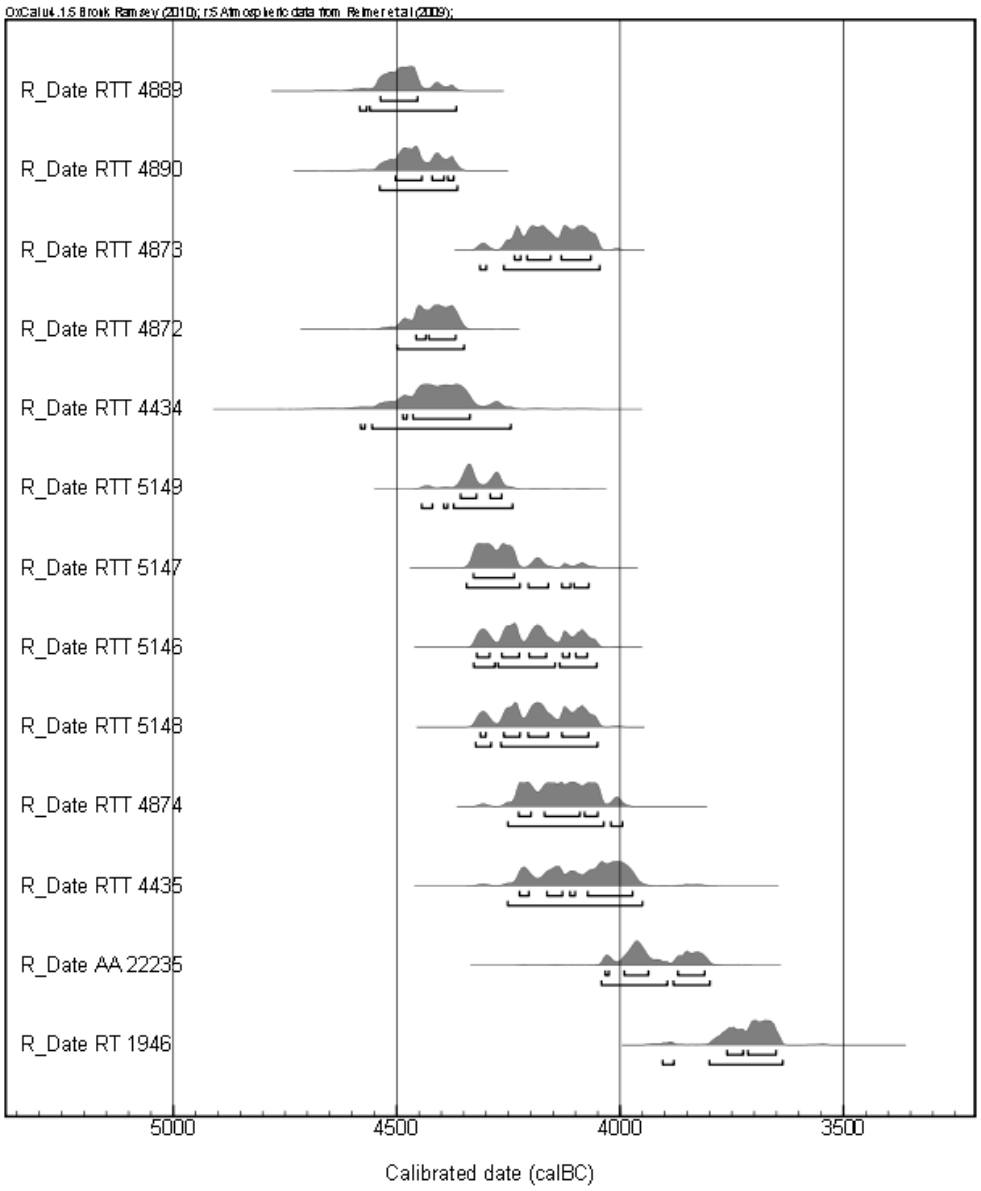


Figure 1: Probability distribution of the calibrated radiocarbon calibrated ranges for the samples in Table 1. The dates are ordered by cave and by radiocarbon age. Note that the one articulated skeleton found, is also the youngest specimen dated. This is consistent with this skeleton remaining in articulation.

We then analysed the HVS-II region of the mitochondrial DNA in two of the bones that gave the Cambridge Reference Sequence (CRS) in the HVS-I region (WM28 and CW). Note that MS, the person who performed the laboratory analyses, also has the CRS sequence in this region. In both samples, sequences that differ from the CRS were obtained (Figure 4). These sequences are therefore different from the HVS-II sequence of MS, thus excluding contamination in the laboratory from MS. Furthermore, if modern DNA contamination had occurred, we would have expected the longer DNA fragments to have the CRS sequence and not only the shorter fragments. We would also not have expected there to be a correlation between in-

soluble collagen content of the bones and fragment length. We are therefore of the opinion that the sequences obtained are not from laboratory contamination. Damage to the DNA is another serious source of confusion in aDNA studies. In our study we frequently obtained reproducible sequences, implying relatively little sequence variation in the extract. A few of the clones (6%) did however contain variations that did not reproduce. We regard sequence reproducibility as an important criterion of authenticity.

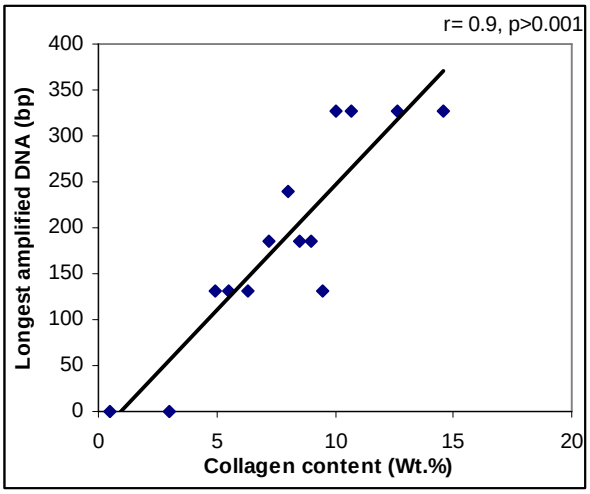


Figure 2: Correlation between the amount of insoluble collagen content and the longest length of mtDNA PCR product obtained.

The mtDNA sequencing of the bones from 12 individuals (out of the 14 tested) produced at least five distinct haplotypes that excluded maternal relationships between them (Figs. 3 and 4). In four cases we could confidently assign them to specific haplogroups (H, H, U3a, H6). It was however impossible to assign a specific haplogroup for eight of the samples that contained the CRS (Wm1, Wm2, Wm6, Wm10, Wm11, Wm19, Wm25) or 16362C (Wm29) in the range of the sequenced region, since too many possibilities exist for Hg assignments.

Sample CW that was also sequenced for HVS-II (CRS at: HVS-I 16210-16498 and 263G 309.1C 309.2C 309.3C 315.1C at: HVS-II 30-429) did not contain any known haplogroup predicting motif, and based on matching HVS-I haplotypes alone too many haplogroup assignments are possible. We then searched for matching jointed haplotypes of HVS-I and HVS-II in the literature and in our laboratory databases (unpublished data), and found only two perfect matches that belong to H haplogroup. The 309 C repeat is a hyper-mutable nucleotide string that may frequently change in its length. We therefore also searched for haplotypes that did not contain the rare mutation 309.3C. Most of the matching sequences (19/20) also belonged to haplogroup H. Therefore, the sample CW is most likely assigned to H haplogroup, or its sub-clades.

Sample Wm28, that was also sequenced for HVS-II (CRS at: HVS-I 16210-16410 and 263G 309.1C at: HVS-II 30-429), did not contain any known haplogroup predicting motif, and based on matching HVS-I haplotypes alone, too many haplogroup assignments are possible. In addition, we could not find any matching haplotypes for the mutations of HVS-I and HVS-II in the literature and in our laboratory database. In the next search, we alternately disregarded the mutable positions of C repeat strings, 309.2C and 315.1C. We then found 10 similar haplotypes that were all assigned to haplogroup H. Sample Wm28 can therefore most likely be assigned to H haplogroup, or its sub-clades.

Sample Wm27 (16343G 16390A at: HVS-I 16210-16498) contains the mutation 16343G which is a characteristic of haplogroup U3. We found 48 matching HVS-I sequences in the Genographic database. They were composed of seven distinct sequences and all of them belong to the U3 haplogroup. In addition, the mutation 16390A is typical of haplogroup U3a (Achilli et al., 2005). Sample Wm27 was therefore confidently assigned to U3a haplogroup.

Sample Wm30 (16311C 16362C 16482G at: HVS-I 16210-16498) contains the mutation 16482G which is a characteristic of H6 haplogroup (Roostalu et al., 2007). In the search for matching haplotypes among the Genographic database 15 HVS-I sequences were found (consisting of two distinctive sequences). All of them belong to H haplogroup or its sub-clades. For estimating the frequency of 16482G in H haplogroup, we searched for this mutation in this database sub-set. Among the samples that contain this mutation, 99.2% (n=365) belong to H haplogroup or its sub-clades. Matching haplotypes of H6 haplogroup, that contain the mu-

tation 16311C were found recently and named as haplogroup H6a1a1 (Behar, 2008). Sample Wm30 was therefore confidently assigned to H6 and most likely belong to H6a1a1 haplogroup.

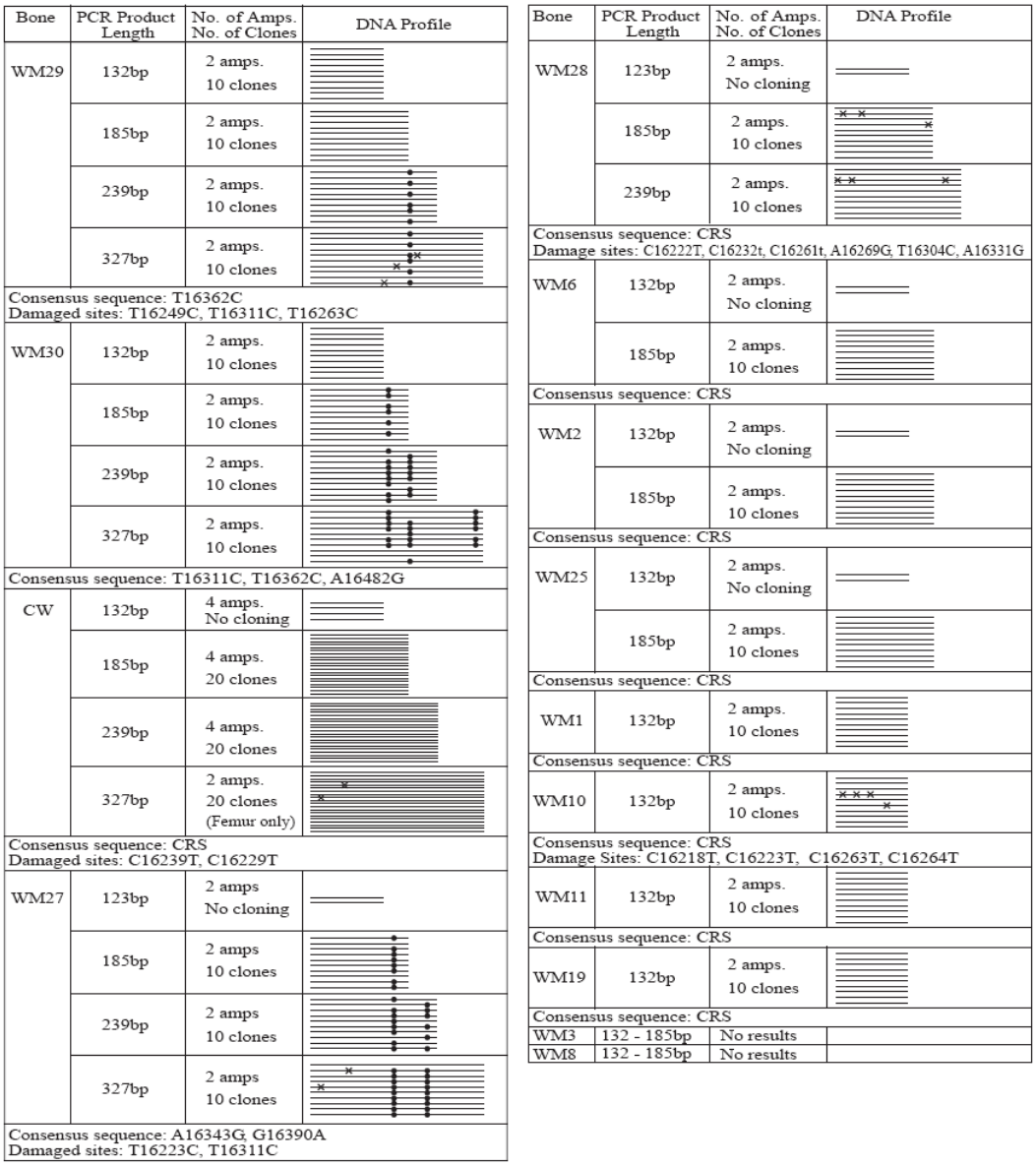


Figure 3: Scheme showing HVS-I sequences of increasing length with dots representing variations from the CRS (● -constant variant, O - sporadic variant). Total number of amplifications (amps.) and cloned sequences are shown. The position and type of each variation are listed. Most of the sequences obtained from amplifications of different fragment lengths and from different extracts are identical. An empty box means no PCR product. The first 5 clones originated from the first amplification and the second five from the second amplification. In the case of sample CW the first 10 clones originated from the femur and the second 10 from the humerus.

DISCUSSION

We report here sequences of mtDNA control region from 12 individuals from the Chalcolithic period. These sequences were obtained from DNA preserved in crystal aggregates. We note a good correlation between the insoluble collagen contents and the DNA fragment lengths. In 4 cases a reliable haplogroup assignment could be made that shows that these individuals are related to modern West Eurasian populations.

This study shows that the HCl insoluble collagen content of fossil bones can be used as a rapid and easy pre-screening method to identify bones more likely to have well preserved

DNA in their aggregate fraction. The choice of the best preserved bone samples available is important for successful aDNA analysis. Other methods (amino acid racemization, C/N ratio) that monitor collagen diagenesis have been used to predict the state of preservation of DNA in bones (Colson et al., 1997; Bada et al., 1999; Collins et al., 1999; Poinar and Stankiewicz, 1999). Collins et al. (1999) noted that racemization is not a reliable a proxy for DNA preservation since denaturation of collagen is not always consistent with DNA depurination. In a study of 34 fossil cattle bones from 4 archaeological sites, no correlation was observed between the D/L ratio of aspartic acid and mtDNA preservation (Buckley et al., 2008). The use of the C/N ratio is problematic, as it is not known whether the calculated value is based on a loss of nitrogen from collagen molecules or a gain of carbon (Tuross, 2002). Götherstrom et al. (2002) determined the state of DNA preservation based on the presence or absence of PCR products of an expected size in a gel. In this study we use reproducible cloned sequences to determine the state of preservation of ancient DNA. Using collagen from fossil bones for radiocarbon dating, Hedges and van Klinken (Hedges and van Klinken, 1992) characterized bone containing less than 2% collagen as poorly preserved and not suitable for analysis (Hedges and van Klinken, 1992; van Klinken, 1999). This value is similar to the cut-off we found in this study for DNA analysis. The cut-off may vary between different geographical regions. We therefore propose that selection of bones for aDNA analysis could first involve a pre-screening step using the insoluble collagen content in order to identify the best preserved bones for aDNA analysis. A similar approach proved to be effective when choosing fossil bones most suitable for radiocarbon dating (Yizhaq et al., 2005). A similar proposal was made by Buckley et al (Buckley et al., 2008).

Most ancient DNA studies of human population assemblages are composed of just several individuals sometimes obtained from different periods of time in a restricted geographic area (Colson et al., 1997; Lalueza-Fox et al., 2004; Ricaut et al., 2004; Sampietro et al., 2006). In a study of 22 skeletons from Mycenae, Greece, authentic mtDNA was identified in 4 individuals, two of whom were identified as brother and sister (Bouwman et al., 2008). In a site in Laconia, Greece, 8 bones out of 20 from Middle Bronze graves yielded authentic mtDNA (Chilvers et al., 2008).

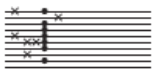
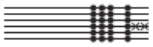
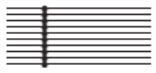
Bone	PCR Product Length	No. of Amps. No. of Clones	DNA Profile
CW	277 bp	2 amps. 10 clones	
	278 bp	2 amps. 6 clones	
Consensus sequence: A263G, 309.1C, 309.2C, 309.3C, 315.1C Damaged sites: C114T, C147T, C227T, C273T, C320T, C323T			
WM28	277 bp	2 amps. 10 clones	
	278 bp	2 amps. 10 clones	
Consensus sequence: A263G, 309.1C			

Figure 4: Scheme showing two short overlapping fragments of the HVS-II region. Dots represent variations from the CRS (•-constant variant, x-sporadic variant). Total number of amplifications (amps.) and cloned sequences are shown. The position and type of each variation are listed. Most of the sequences obtained from amplifications of different fragment lengths and from different extracts are identical.

The 3 caves in Wadi Makkukh contained bones from at least 40 individuals. The question therefore arises as to whether these individuals were buried in the cave together or over a fairly long period of time. Based on the calibrated radiocarbon results the maximum period of time that the cave was used as a burial site is around 600 years, namely from the middle to late Chalcolithic period in this region. These caves could therefore conceivably contain individuals from one genetically related family, or that unrelated individuals were buried there. Our mtDNA information contains at least five distinctive sequences, therefore it can be concluded that not all of the individuals are of the same maternal lineage.

HVS-I data alone often do not contain sufficient information for confident assignment of haplogroup affiliation. In our study it was possible to assign a reliable haplogroup affiliation for only 4 of the 12 samples (haplogroups: H, H, U3a, H6).

All 12 samples most probably fall into R haplogroup sub-clades, which are characteristic of the West-Eurasia region, and encompass 90-95% of the European population (Kivisild, 2000). We also note that all 12 samples contain the SNP 16223C that is typical of R haplogroup sub-clades, and they might belong to any of its sub-clades, namely haplogroups such as H, U, T, J, K, V etc.(Finnila et al., 2001). It was previously shown that only 2.5% of the pre-haplogroup-R mtDNA genomes have the SNP 16223C, and only 1.1% of R haplogroup sub-clades have the SNP 16223T (Behar et al., 2007). The assignment of our samples into the R haplogroup sub-clades is consistent with the current world distribution of these haplogroups in modern West Eurasian populations. The common haplogroups of West-Eurasia are sorted into several major lineages. Most of them are sub-clades of the major R haplogroup: H, J, U, K, T, V (encompassing 90-95% of the European lineages), and others are outside this branch: I, W, and X (Kivisild, 2000). These haplogroups incorporate about 95% of the mtDNA variation in European populations (Torroni et al., 1996; Richards et al., 2000; Simoni et al., 2000; Torroni et al., 2001; Richards et al., 2002). Most of the prevalent haplogroups of Europe are most likely of Near Eastern origin (Achilli et al., 2004). The haplogroups are thought to have formed tens of thousands of years ago, and correspond to early human migrations and are usually associated to specific geographic regions (Behar et al., 2007).

Sample CW and Wm28 were most likely assigned to haplogroup H. This haplogroup is the most common haplogroup in West-Eurasia; its frequency in Europe is over 40%. In the Near East, the Caucasus and Central Asia this haplogroup frequency is about 10-30%. The CRS sequence is the prevalent haplotype (25%) of haplogroup H (Roostalu et al., 2007). Haplogroup H divergence time in Europe was 20,000-30,000 BP (Kivisild, 2000). It is thus interesting that H haplogroup was prominent in this group of individuals from the Chalcolithic as well. (In our study 3 of 4 haplogroup assigned samples belong to H haplogroup). Since it is so widespread in West Eurasia, it is impossible to locate this common haplotype in a specific modern population. For final H haplogroup validation, it is necessary to genotype the SNPs 2706A or 7028C. H haplogroup can be further divided into its sub-clades (such H1, H2, H3, H4 etc.) (Roostalu et al., 2007).

Sample Wm27 was confidently assigned to haplogroup U3 and it most likely belongs to haplogroup U3a. The frequency of haplogroup U3 in Europe is 1% and in the Near East is 6% (Richard 2007). Few matching haplotypes of U3a haplogroup were found in different modern populations that are present all over West-Eurasia (Iran, Syria, Lebanon, Morocco, Ukraine, Russia, Poland, Germany, Iceland), and 3 samples were found in Jewish communities from Poland, Romania and Turkey (Behar 2008). The estimated coalescence time of haplogroup U3 is about 16–27,000 BP in the Near East and about 12–27,000 BP in Europe (Richards et al., 2000). We therefore conclude that the U3 haplogroup was already present in the West Eurasian gene pool around 6,000 BP, and probably within its sub-branch, U3a hg, as well. For final haplogroup validation of this sample, it will be necessary to genotype the SNPs: 14139G or 15454C for Hg U3 and 2294G or 4703C for U3a Hg (Achilli 2005).

Sample Wm30 was confidently assigned to haplogroup H6 and it most likely belongs to haplogroup H6a1a1. Haplogroup H6 is largely restricted to the Near East and the South Caucasus and is found in very low frequencies all over Europe (Roostalu et al., 2007). It is most frequent in Central and Inner Asia (Loogväli 2004). Its sub-clade, haplogroup H6a1, is one of the oldest clades in the Near East with a coalescence time of 20,200±10,900 BP, but in Europe it has an extremely young expansion age of 1800±1300 BP (Roostalu 2007). Remarkably, H6a haplotypes that contain the mutation 16311C were detected recently in 12 individuals from different Jewish communities from Turkey (5 samples), Morocco (3 samples), Poland, France, Tunisia and Algeria. The mtDNA of the H6a samples from Turkish Jews was completely sequenced and assigned as haplogroup H6a1a1, with coalescence time of 1293 BP (Behar, 2008). Although position 16311 is relatively mutable and therefore could appear independently in distinct haplotypes (Stoneking, 2000), its pres-

ence in separated and distant communities of the same ethnicity may indicate a common ancestry. Only deeper clade analysis of complete mtDNA sequences of this haplotype can confirm this possibility. For final haplogroup validation of this sample, it is necessary to genotype the SNPs: 2706A and 7028C for H haplogroup, 3915A or 4727G for H6a haplogroup (Loogvali 2004), and 7325G or 11253C for H6a1a1 haplogroup (Behar 2008).

CONCLUSIONS

The information that is embedded in aDNA samples may provide novel insights regarding demographic history, migrations, introgressions and genetic connections between ancient and modern populations. This information can also validate accepted hypotheses based only on modern data. In this study of human bones from around 6000 BP, we found 12 mtDNA sequences that most probably belong to R haplogroup sub-clades that are typical of West Eurasia. We also found a high frequency of H haplogroup, which is the most prevalent haplogroup today West Eurasia. The results reported here thus tend to genetically link this Chalcolithic group of individuals to modern West Eurasian populations.

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