Fine Characterization of the Iceman's mtDNA Haplogroup

F ,[1]* L E ,[1] L ,[1] I M ,[1] C O ,[1] D L [2]

[1] A A -A M /DNA A , D B M ,
C A ; C , I-62032 C , I
[2] A A M , D B E S ,
B , 40126 B , I

KE ORDS mummy; ancient DNA; mtDNA coding region

ABSTRACT Starting from specimens of the intestinal contents of the so-called Tyrolean Iceman or Ötzi (5,350–5,100 years before present), it was possible by polymerase chain reaction to amplify fragments of the human mitochondrial DNA (mtDNA) control region that correspond to the sequence found in 1994 at the Munich and Oxford laboratories and which had been attributed to the original DNA of the mummy. The particularly favorable condition of the specimens, showing very low contamination levels, made it easier to extend the analyses to the coding region, which had not previously been considered. The mtDNA of the European population is currently divided into nine (H, T, U, V, W, X, I, J, and K) main groups (haplogroups). The K haplogroup, in particular, is composed of two (K1 and K2) subclusters. The results demonstrate that the Iceman's mtDNA belongs to the K1 subcluster, yet it does not fit any of the three known branches (a, b, and c) into which the K1 subcluster is presently divided. In addition, some other sites, reported to be linked to environmental adaptation or pathologies, were investigated. Am J Phys Anthropol 000: 000–000, 2006.

The human mummy, found in the Alps on September 19, 1991 and popularly known as the Iceman, or Ötzi, has offered scientists a unique opportunity to investigate the life and health status of a Late Neolithic or Early Copper Age human. For this reason, through the years, the body and pieces of equipment found near it have undergone a number of scientific investigations (Spindler et al., 1995, 1996; Bortenschlager and Oeggl, 2000). In particular, Handt et al. (1994) examined the mitochondrial DNA (mtDNA) of the mummy. Initially, experiments performed in Munich led to the detection of many different sequences in the polymerase chain reaction (PCR) products from muscle, connective tissue, and bone specimens of the mummy's left hip, thus making it problematic to determine which of the sequences corresponded to the Iceman's original DNA. Subsequently, thanks to the application of decontamination protocols to two specimens and the use of very short amplification systems, the researchers (Handt et al., 1998) were able to identify a DNA fraction showing two differences (a C at position 16224 and a C at position 16311) from the reference sequence (Cambridge Reference Sequence, CRS). This sequence, also found in a bone sample which was independently analyzed in Oxford, was assumed to be the authentic one.

On September 25, 2000, the mummy was fully defrosted for the first time (Schiermeier and Stehle, 2000; Stone, 2000). On that occasion, several samples of the intestinal contents were collected under sterile conditions. Some specimens were utilized to reconstruct the composition of the man's last meals by DNA analysis (Rollo et al., 2002). In the course of the study, it was noted that, in addition to animal and higher plant DNA, a relatively large fraction of the DNA from the intestines was of human origin. The aim of the present research was to characterize the human DNA fraction.

MATERIALS AND METHODS

Sample collection and DNA extraction

Specimens of intestinal contents were collected on September 25, 2000 by Eduard Egarter Vigl, following the complete defrosting of the body kept, since 1998, at the South Tyrol's Museum of Archaeology (Bolzano, Italy).

described medium were added to each sample. The homogenates were collected in Eppendorf tubes, taking care to rinse the mortar and pestle with a further 350 μl of extraction medium, and then homogenates were extracted sequentially by using equal volumes of phenol, phenol/chloroform/isoamilic alcohol (25:24:1), and ether. The DNA fraction was precipitated from the final supernatant by centrifugation at 13,500 for 5 min after the addition of 1/10 volume of 2 M sodium acetate and 2.5 volumes of cold (−20°C) ethanol. The DNA precipitates were resuspended in 20 μl of sterile distilled water, and stored at −25°C until use.

DNA preparations from the colon and ileum were initially searched for animal, higher plants, and fungi, as reported in Rollo et al. (2002). The same samples were subsequently utilized for the present study.

All operations were carried out in a room dedicated to the manipulation of ancient DNA. The room is equipped with ultraviolet light and contains a bench microcentrifuge, a Speed-Vac concentrator, and positive-displacement pipettes. Strict cleaning criteria were routinely followed, including frequent treatment with bleach. Negative controls were performed throughout the procedure.

PCR amplification and sequencing

DNA amplifications were performed in 50 μl of a reaction medium of the following composition: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 2.5 enzyme units Taq polymerase (Ampli Taq Gold, Perkin Elmer, Palo Alto, CA), 200 mM each dNTP, 300 ng each primer, and 1 μl of DNA preparation (we tested serial dilutions from 1/10 to 1/100). The reaction mixture was pretreated with DNAse (2 enzyme units for 30 min at room temperature) to eliminate contaminant DNA. The DNAse was subsequently inactivated at 95°C for 15 min. The thermal profile (40 cycles) was set as follows: 1 min at 94°C, 30 sec at the relevant annealing temperature, and 1 min at 72°C, with a final extension of 10 min at 72°C.

The list of oligonucleotide primer-pairs utilized and the corresponding annealing temperatures are given in Table 1. Amplification products were checked by electrophoresis on 2.5% (weight/volume) agarose, purified using

(U8b), characterized by the K diagnostic marker 9052 II, so this finding strengthens their relationship. More recently, Palanichamy et al. (2004) identified six subhaplogroups (K1a, K1a1, K1a2, K1b, K1c, and K2a).

The alignment of the Iceman's ileum DNA sequence, obtained by PCR amplification using the L12257/H12341 (TRNL2) primer-pair and by direct sequencing, with the corresponding sequence of the K, U, H, I, J, T, V, W, and X haplogroups (Fig. 2a), shows that the mummy sequence belongs to the UK superhaplogroup.

To further discriminate between the U and K haplogroups, we PCR-amplified a 115-bp-long portion of the coding region (ATP6) encompassing the 9055 position by the use of the L9027/H9105 primer-pair. The result (Fig. 2b) shows that the Iceman's DNA contains an A substitution and thus confirms its belonging to the K haplogroup. The K cluster is divided into the two K1 and K2 subclusters by the 1189 (Rieder et al., 1998; Finnilä et al., 2001) and 9716 specific polymorphisms (Herrnstadt et al., 2002), respectively. We analyzed portions of the coding region (locations RNR1 and COIII), using the primer-pairs L1170/H1211 and L9678/H9740, respectively. The results show (Fig. 2c,d) that the Iceman belongs to the K1 subcluster. In addition, amplification using the L9678/H9740 primer-pairs allowed us to further confirm the K haplogroup by showing the 9698 transition (Fig. 2d).

A more detailed characterization of the haplogroup may be obtained by considering the different branches (K1a, K1b, and K1c) into which the K1 subcluster divides. The K1a branch is identified by the specific polymorphisms 10978, 12954 (Herrnstadt et al., 2002), and 497 (Palanichamy et al., 2004), but the K1b branch only by mutation 5913 (Palanichamy et al., 2004), and the K1c branch by the two mutations 152 and 146 (Palanichamy et al., 2004). The analysis of the Iceman's DNA using L10928/H11000 (ND4), L12928/H12988 (ND5), L5882/H5936 (COI), and L97/H170 (HVRII) shows (Fig. 3a–e) that the Iceman's DNA does not fit the K1a, K1b, or K1c branch. It rather seems to represent a previously unknown branch of the K1 subcluster (Fig. 4). This lineage is therefore categorized as haplogroup K1*.

To investigate K-haplogroup frequency distribution in the contiguous geographical regions of the Alps, we compared 2,676 HVRI region sequences (http://www.hvrbase.org/, Handt et al., 1998). The highest frequency (31%)

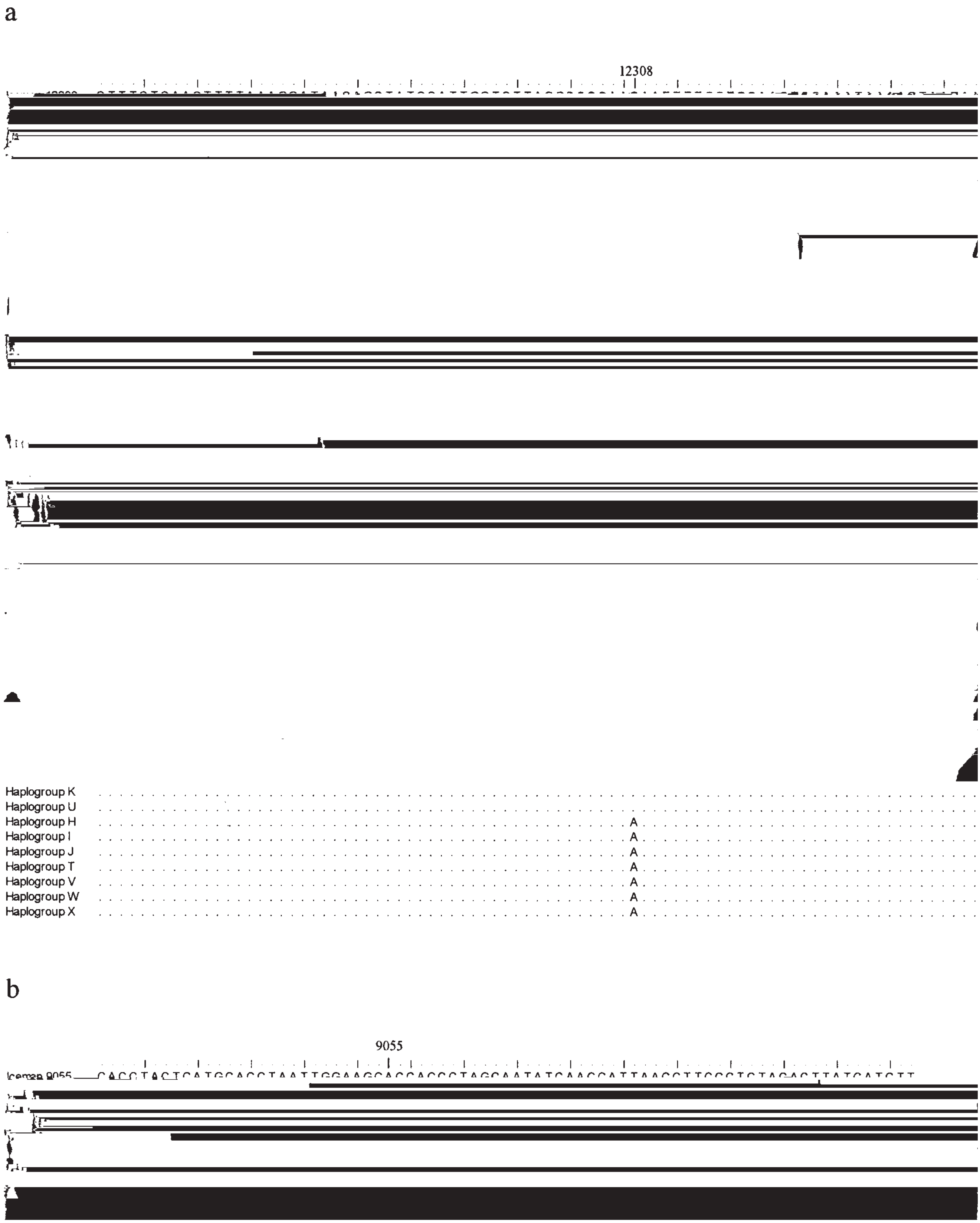

Fig. 2. Characterization of UK superhaplogroup (**a**), of K haplogroup (**b**), and of K1 (**c**) and K2 (**d**) subclusters.

tional pattern observed in Ötzi (16224C, 16311C, 9055A, 1189C, 146T, and 152T), we find 16 non-K1a sequences and one K1b sequence. Among the 16 non-K1a sequences, all with the 16320C mutation, seven present 16093C and nine present 16093T, confirming the inconsistency of these HVSI mutational sites to discriminate between the K1 and K2 subclusters, as pointed out by Palanichamy et al. (2004). Thirteen sequences (5.4%) are found in individuals from Europe (Coble et al., 2004), and three (1.6%) from Finland (Moilanen et al., 2003).

Holyoake et al. (2001) suggested that nucleotide substitutions 9055A and 11719A are particularly frequent in intestinal contents are better protected from contamination than other possible specimens.

The analysis of the coding region shows that the Iceman's mtDNA corresponds to the K haplogroup. In the past, the mutation sites 16224C and 16311C in the control region were used to identify the K haplogroup (Torroni et al., 1996; Macaulay et al., 1999). More recently, Helgason et al. (2001), using a phylogenetic network of HVS1 sequences from populations in the North Atlantic region, identified the additional mutation sites 16093TC and 16320CT. These two sites were used to characterize, respectively, the K2 and K1 subclusters, while the 16291CT and 16319GA mutations further defined the K2 (K2a and K2b) subcluster. However, on the basis of a recent study combining all published mitochondrial complete sequences sampled from western Eurasia, Palanichamy et al. (2004) suggested that the mutations of the D-loop region should not be trusted as diagnostic markers.

Haplogroup K accounts for between 6–7% of the total the South Tyrol Museum of Archaeology (Bolzano, Italy), for providing the mummy specimens.

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