Fine Characterization of the Iceman's mtDNA Haplogroup

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KEY WORDS 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). All operations were performed using sterile instrumentation inside the sterile facility annexed to the Iceman's cold storage room. DNA was isolated from three samples of the mummy's intestinal contents, corresponding to the ileum (68 mg), colon (58 mg), and rectum (111 mg), using the procedure and precautions previously described in Rollo et al. (2002). Specimens were resuspended in 350 μl of a medium containing 50 mM Na_2EDTA, 50 mM Tris-HCl (pH 8.0), 1% (weight/volume) sodium dodecyl sulphate (SDS), and 6% (volume/volume) water-saturated phenol. After inbibition (soaking), samples were left overnight at 4°C. The next morning, samples were transferred into sterile mortars and homogenized with pestles. During the milling phase, 350 μl of the above-

Grant sponsor: MIUR Project "Malattie e Regime di Vita nell'Italia Centro-Meridionale dei Secoli XIII–XIX, Fonti Biologiche e Storico-Letterarie."

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Received 22 March 2005; accepted 7 October 2005.

DOI 10.1002/ajpa.20384
Published online in Wiley InterScience (www.interscience.wiley.com).

TABLE 1. Oligonucleotide primers used for DNA amplifications

Mutation (region)	Primer sequence (5′ → 3′)[1]	Length of product (bp)	Annealing temperature (°C)
T16224C/T16311C (HVRI)	L16209: CCCCATGCTTACAAGCAAGT	162	55
	H16331: TTGACTGTAATGTGCTATGT		
A12308G (TRNL2)	L12257: CCCATGTCTAACAACATGG	123	49
	H12341: GGTTATAGTAGTGTGCATGG		
G9055A (ATP6)	L9027: CTAACATTACTGCAGGC	115	49
	H9105: TCAGTAGAATTAGAATTGTG		
T9698C/T9716C (COIII)	L9678: AATAGAAAACAACCGAAACC	104	49
	H9740: AGACTCGAAGTACTCTGAG		
T1189C (RNRI)	L1170: CACAGCTTAAAACTCAAAGG	81	51
	H1211: GATCGGGGTTTATCGATTAC		
A10978G (ND4)	L10928: TCACTGGATAAGTGGCGTTG	110	57
	H11000: CAACCTTTTCCTCCGACCC		
T12954C (ND5)	L12928: ATTTGCCTGCTGCTAG	96	59
	H12988: ACACTCCAACTCATGAGAGG		
G5913A (COI)	L5882: AGTCCAATGCTTCACTCAGC	93	57
	H5936: GTGTTCCAATGTCTTTGTGG		
C150T (HVRII)	L97: CGATAGCATTGCGAGACG	111	60
	H170: TTCGCCTGTAATATTGAACG		
G11719A (ND4L)	L11711: CGCAGTCATTCTCATAATC	106	54
	H11778: TTGAGAGAGGATTATGATGC		

[1] Light (L) and heavy (H) mtDNA strands.

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*g* 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 the High Pure PCR Product purification kit (Roche Molecular Biochemicals, Mannheim, Germany), and directly cloned using the pGEM-T Easy Vector System (Promega Corp., Madison, WI). Recombinant plasmids were isolated using a Miniprep kit (Applied Biosystems, Foster City, CA), and insert size and DNA concentration were assessed by gel electrophoresis. In the case of direct sequencing, PCR products were purified using the Qiaquick gel extraction kit (Qiagen, Germany). DNA sequences were obtained using an ABI-Prism 310 automated DNA sequencer and the BigDye Terminator Cycle Sequencing Ready Reaction kit (version 1.1, Applied Biosystems, Foster City, CA). Cycle sequencing products were purified by Centri-Sep spin columns (Princeton Separations, Adelphia, NJ).

RESULTS

DNA isolated from three (ileum, colon, and rectum) specimens of the mummy's intestinal contents was PCR-amplified, using a DNA primer pair (L16209/H16331) designed to bind to the light (L) and heavy (H) strands of a 162-bp-long portion of the first hypervariable region (HVRI) of human mtDNA (Table 1). The sequences were aligned with the putative Iceman's sequence and with the revised version of the CRS (Anderson et al., 1981). In the case of the ileum (I) and rectum (DS) samples, all sequences (10 and 17, respectively) contain the C mutation at the 16224 and 16311 positions (Fig. 1). In the case of the colon (CR) sample, 2 out of 10 sequences are identical to the CRS sequence, while the others correspond to the putative Iceman's sequence. In addition, the sequences contain several other substitutions, possibly due to PCR errors and postmortem damage (Gilbert et al., 2003). The complete list of mutations present in the 35 sequences containing the 16224 and 16311 mutation with respect to their consensus is given in Table 2.

To better define the Iceman's mtDNA position, we considered a set of mutations located in the coding region (Table 1).

In this region, on the basis of the shared transition at the 9698 position, haplogroup K forms a sister clade with haplogroup U8 (Finnilä et al., 2001). Moreover, Quintana-Murci et al. (2004) identified a subset of haplogroup U8

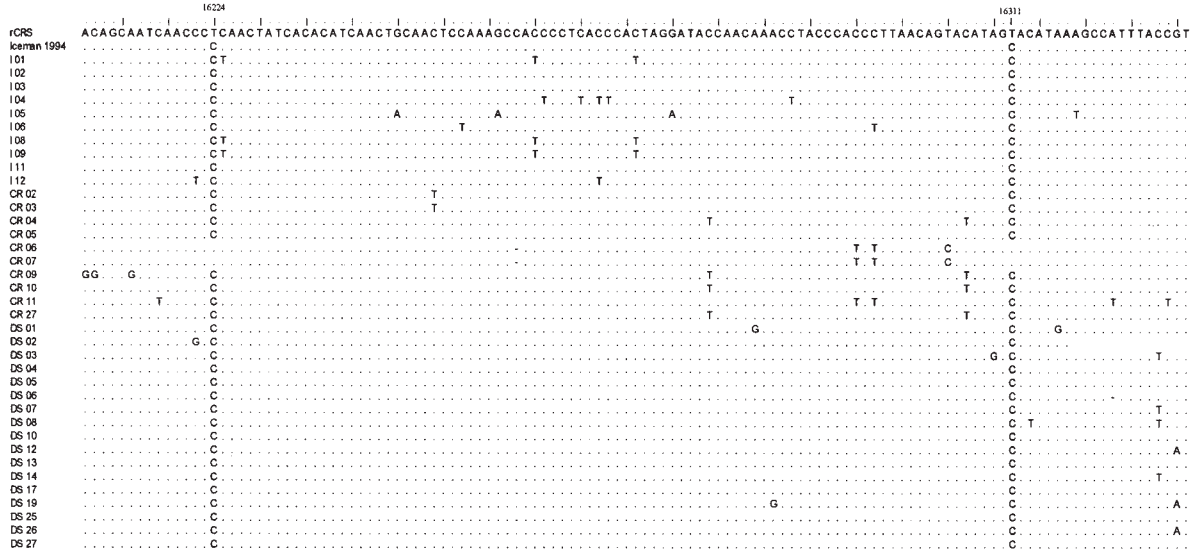


Fig. 1. Alignment of 37 HVRI sequences from Iceman's ileum (I), colon (CR), and rectum (DS), with revised CRS sequence and Iceman's sequence found by Handt et al. (1994).

TABLE 2. List of mutations in 35 HVRI sequences (showing 16224 and 16311 substitutions) from Iceman's intestines compared to consensus

Mutation	Nucleotide	Number of mutations
A→G or T→C	16210; 16215; 16283; 16285; 16309; 16316	6
C→G or G→C	16211; 16222	2
C→T or G→A	16218; 16222; 16225; 16248; 16251; 16259; 16260; 16264; 16266; 16267; 16270; 16278; 16287; 16294; 16296; 16306; 16313; 16327; 16328	37
G→A or C→T	16244; 16255; 16274; 16328	6
A→T or T→A	16318; 16322	2
A-deletion or T-deletion	16322	1

(U8b), characterized by the K diagnostic marker 9052*Hae*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).

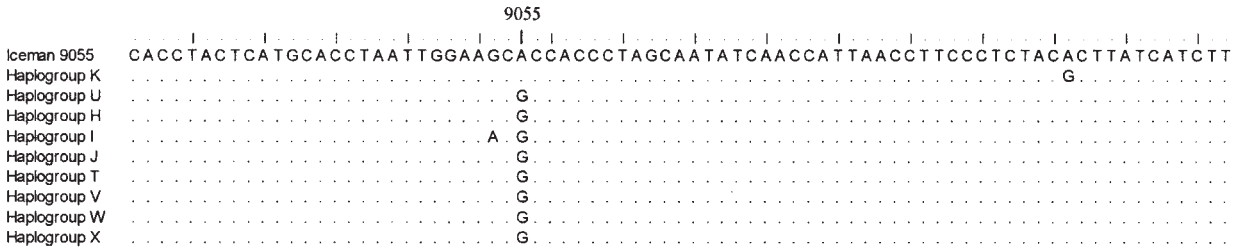
A more detailed characterization of the haplogroup may be obtained by considering the different branches (K1a, K1b, and K1c) into which the K1 subcluster divides. The K1a branch is identified by the specific polymorphisms 10978, 12954 (Herrnstadt et al., 2002), and 497 (Palanichamy et al., 2004), but 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%) is present in the Ötztal area (Austria), north of the site where the mummy was found, as previously reported by Handt et al. (1994). Haplogroup-K frequency, however, is also high (20%) in the Ladin populations which live on the southern slopes of the eastern Alps (Vernesi et al., 2002). Other populations from northern Italy (Veneto), Switzerland, Austria, and Germany exhibit a standard European frequency ranging from 3–8.3% (Mogentale-Profizi et al., 2001; Dimo-Simonin et al., 2000; Pult et al., 1994; Brandstatter et al., 2003; Hofmann et al., 1997; Lutz et al., 1998; Baasner et al., 1998; Baasner and Madea, 2000; Pfeiffer et al., 2001; Poetsch et al., 2003).

In addition, to investigate K1-haplogroup distribution, we examined, on the Human Mitochondrial Genome Database (http://www.genpat.uu.se/mtDB, kept by Max Ingman, Uppsala University, Uppsala, Sweden), 92 K-haplogroup sequences, determined by the 9055A mutation. These sequences are selected from 1,504 complete sequences and from 560 worldwide coding regions from positions 577–16002. When we consider the K1 muta-

a

b



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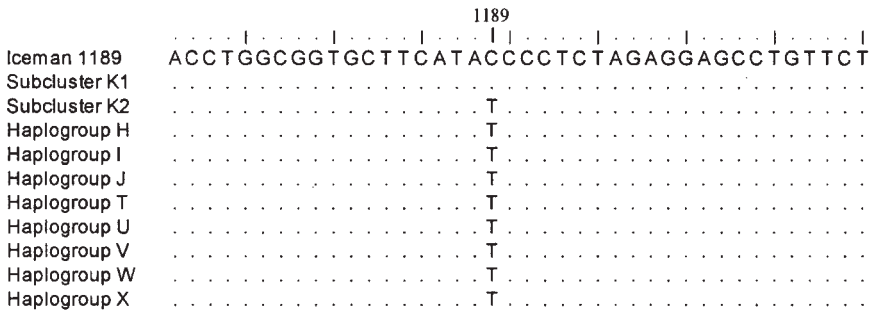

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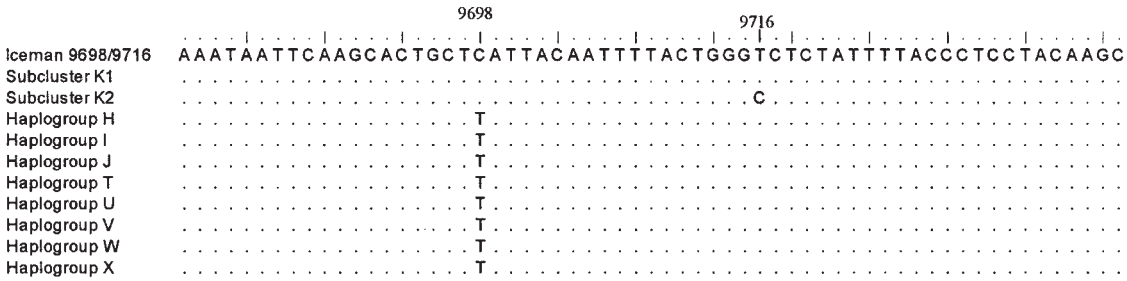

Fig. 2. Characterization of UK superhaplogroup (**a**), of K haplogroup (**b**), and of K1 (**c**) and K2 (**d**) subclusters.

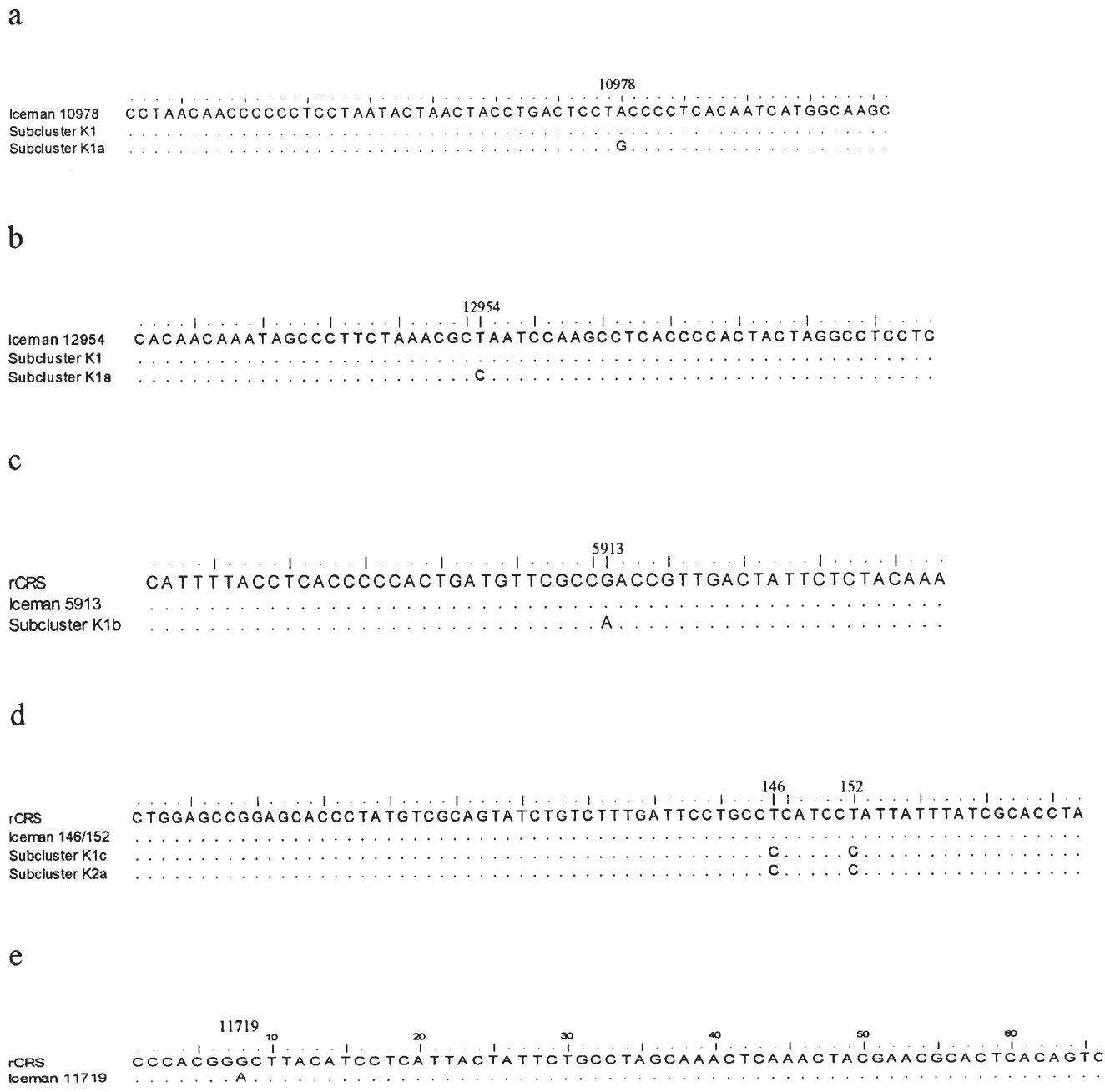

Fig. 3. Characterization of K1a (**a, b**), K1b (**c**), and K1c (**d**) subclusters, and of 11719 site (**e**), associated with reduced sperm mobility.

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 men with reduced sperm mobility. We already showed that the Iceman's mtDNA contains the 9055 transition that identifies the K haplogroup. A further analysis of the 11719 position, using the primer pair L11711/H11778 (Fig. 3e), demonstrates that it also contains the 11719 transition.

DISCUSSION

Screening of the three (ileum, colon, and rectum) L16209/H16331 libraries shows that human mitochondrial DNA, the sequence of which corresponds to the one previously indicated as the putative Iceman's sequence (Handt et al., 1994), can be isolated in an almost uncontaminated form from the mummy's intestines. This result can be explained with the consideration that the

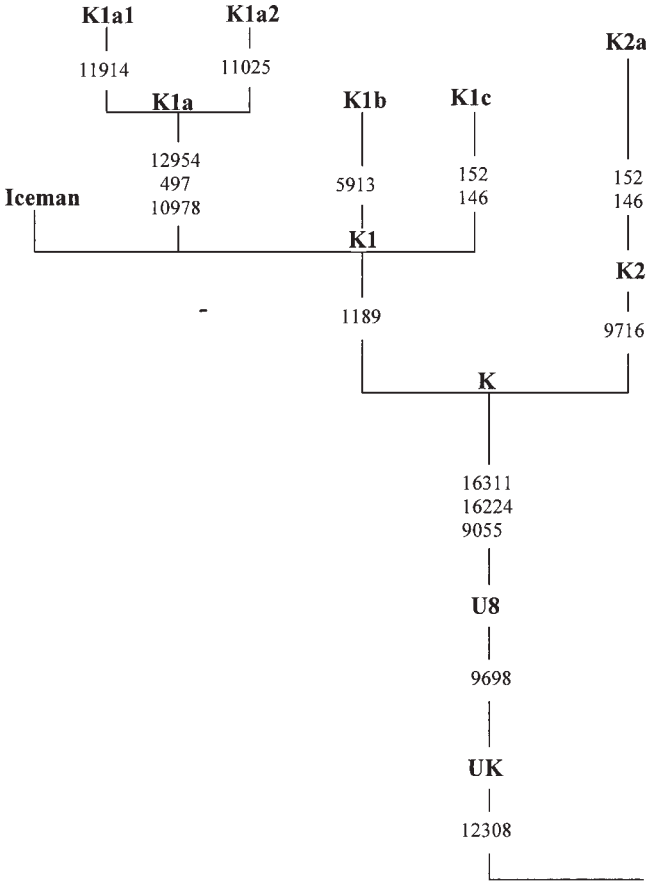

Fig. 4. Phylogenetic tree of mtDNA K haplogroup and subclusters, showing Iceman's lineage.

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 European HVRI and HVRII sequences (Richards et al., 1998, 2000; Macaulay et al., 1999), and 8–10% (Allard et al., 2002; Herrnstadt et al., 2002; Brandstatter et al., 2003) of the coding region SNPs. Most of the haplogroups and subclusters are now well-established in the literature (Ingman et al., 2000; Helgason et al., 2000; Finnilä et al., 2001; Maca-Meyer et al., 2001; Herrnstadt et al., 2002; Mishmar et al., 2003; Coble et al., 2004; Palanichamy et al., 2004), and detailed phylogenies of mtDNA lineages have been obtained.

This finding that haplogroup-K frequency is high (20%) in the Ladin populations which live on the southern slopes of the eastern Alps, in addition to those from the Ötztal area (31%), is interesting in the light of isotopic analyses carried out on the tooth enamel and bone of the Iceman, indicating that he spent both his childhood and adult years in the region south of the main Alpine ridge (Müller et al., 2003). However, it may be worth mentioning that, in the DNA analysis, the sample sizes of these two comparative populations are rather small (20 and 16 individuals, respectively). The percentage values cannot therefore be considered statistically conclusive, and all the more so as most of the K types in the two populations do not match the Iceman.

Coskun et al. (2003) associated some mutations of the mtDNA control region with the longevity and cold adaptation of the individual. In particular, a C→T transition at the 150 position was associated with longevity. The fact that the Iceman's DNA does not show the mutation is consistent with the observation that this is present in several African-, Asian-, and European-specific haplogroups, but apparently not in the K haplogroup (Coskun et al., 2003). Other mtDNA mutations were associated with pathologies that affect tissues and cells that require very large energy supplies (Tatuch et al., 1992; Trounce et al., 1994). Substitutions 9055A and 11719A, in particular, were reported as being frequent in men with reduced sperm mobility (Holyoake et al., 2001).

While the idea that Ötzi suffered from sterility is intriguing, for possible social implications and, in particular, as a clue to the so-called "disaster" into which he seems to have incurred (Spindler, 1994), the presence of the two substitutions (9055 and 11719) in the mummy's DNA cannot be taken as evidence that the Iceman actually suffered from this kind of pathology, as the 11719 position is characterized by rather high variability (39% A and 61% G), and mutations at this position are also associated with haplogroups (H, V, and T) other than K (Coble et al., 2004).

CONCLUSIONS

Ancient human DNA studies are problematic because of the extreme risk of contamination of samples and laboratories with modern human material, and it is critically important that a number of criteria be followed (Cooper and Poinar, 2000). The finding, at 10 years' distance from the work of Handt et al. (1994), that the mtDNA fragments previously indicated as belonging to original genetic material of the Iceman can be retrieved in an almost uncontaminated form from the internal organs of the mummy makes a very strong point in favor of their authenticity; all the more so, as paleoecologically consistent animal and higher plant DNA, less prone to contamination than human DNA, were retrieved from the same intestinal samples (Rollo et al., 2002). In this sense, Ötzi's mitochondrial DNA is likely to provide the most convincing case of "authentication" of an ancient human DNA specimen in the literature. Finally, it is also probably worth noting that the present results may help establish molecular standards for the preservation of the body and future investigations (Egarter Vigl, 2003).

ACKNOWLEDGMENTS

The authors are indebted to Eduard Egarter Vigl of the Regional Hospital of Bolzano (Bolzano, Italy), and to

the South Tyrol Museum of Archaeology (Bolzano, Italy), for providing the mummy specimens.

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