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Notes and Comments

The Unresolved Location of Ötzi's mtDNA Within Haplogroup K

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A recent study by Rollo et al. (2006) attempted to determine, at a fine level of molecular resolution, the phylogenetic position of the Iceman's mtDNA by genotyping a set of conservative coding region markers. The re-examination confirmed the control region signature mutations T16224C/T16311C characteristic of haplogroup K reported earlier in the study by Handt et al. (1994). It should be noted, however, that while Handt et al. reported a 354 base stretch of HVS1, in the Rollo et al. study only 122 nucleotides have been sequenced. The basal K haplotype within this short sequence is common throughout Europe, so the individual identification cannot be considered as irrefutable evidence to argue that the consensus sequence was obtained from endogenous DNA.

Rollo et al. suggested that some of the cloned control region sequences (presumed to be contamination) match the Cambridge Reference Sequence (CRS), but a far more parsimonious explanation is that the DNA derives from an individual with European haplogroup T affiliation, identified by the motif 16294T/16296T/16304C in clones CR05 and CR06. Interestingly, this population of templates appears to have formed a chimera in clone CR11, likely as a result of jumping PCR due to damaged templates blocking the extension of the polymerase. The presence of damaged templates in very low copy number is confirmed by the fact that just five molecules appear to be responsible for half of the clones from the control region, judged by the repetitive motifs of damage that are present.

The uncertainty over the origin of the DNA proposed as a contaminant (i.e. the possible haplogroup T) leaves open the possibility that other extractions and amplifications have been compromised. Here it would have been helpful to know the mtDNA profile of the workers associated with this study, in particular if anyone was haplogroup K. A quantitative PCR to assess the total copy number of the mtDNA templates present would have further assisted the reader to judge the utility of the control region sequences. Even if there were some form of additional support for them, it is not advisable to rely upon direct sequencing results for coding region SNPs reported as matching the CRS. This is not to say that the results are necessarily wrong, just that there are alternative explanations for them, which need to be eliminated.

A second area that would benefit from a more informed approach is the phylogenetic hypothesis that Ötzi's mtDNA does not lie within any of the three known clades of K1, which is not fully tested. The authors have constructed a phylogenetic tree that combines data from two published sources (Hernstadt et al., 2002; Palanichamy et al., 2004). This tree, unfortunately, confuses the definition of the major branch K1a with one of its minor subsets, identified by three mutations (C497T, T12954C, and A10978G). This is an unfortunate oversight as the most recent paper, cited by Rollo et al., clearly demonstrates that the mutation at np 497 is solely responsible for defining K1a (Fig. 1).

As K1a is the most prevalent of the three branches (80% of all K), and is particularly frequent in the Mediterranean area, this is a non-trivial omission. In fact, the data provided by Rollo et al. only securely excludes membership of $\sim$7% of haplogroup K sub-branches, covered by the single informative coding region SNP reported at np 5913 (K1b) and K1a1b1a—defined by T12954C and A10978G (Behar et al., 2006). There is also a possible case for excluding their consensus sequence from K1c on the basis of the HVS2 motif, but as the authors themselves point out, such assignments are unreliable. This is particularly relevant in the present case, as the mutations at nps 00146 and 00152 occur recurrently, six and eight times respectively on the haplogroup K background (Behar et al., 2006).

If Ötzi's mtDNA really does belong to an as yet undefined branch of K1, this would indeed provide the most powerful argument that the DNA extracted is really endogenous, regardless of the control region motif. However, Rollo et al. have not achieved their claim of placing the Iceman in this unique phylogenetic position. The data produced, even if it is authentic and reliable, do not assign the mtDNA sequences beyond the vast majority of a rather common West Eurasian haplogroup. With K1a representing more than 80% of the entire haplogroup, the lack of resolution offered by the control region motif highlights the acute problems of authenticity associated with European archaeological samples being genotyped in a European laboratory.

Due to the decision not to test np 497, in this particular case, the question of an adequate methodology for working with ancient human DNA becomes secondary. Nevertheless, in any future work extensive cloning of *all* the results should be a pre-requisite in order to provide evidence of potential sequence variation at key coding region SNPs. This data, if provided together with details of copy number and potential contaminants, would facilitate a more detailed assessment of the preservation of

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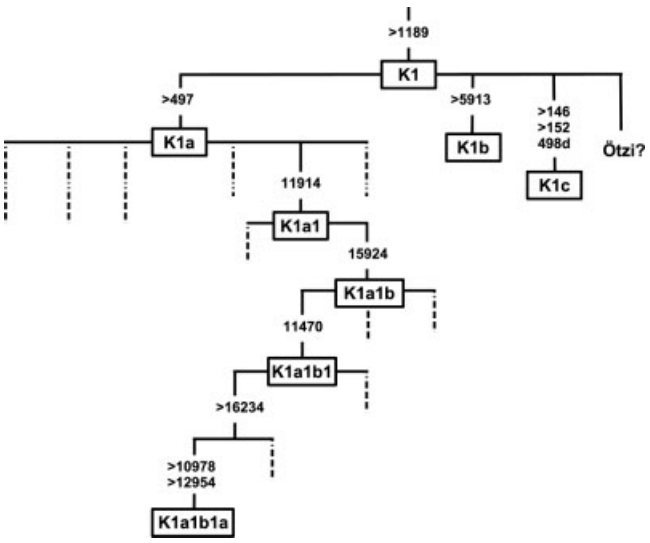

Fig. 1. Phylogenetic tree of the main branches of mtDNA haplogroup K1. The mutations typed by Rollo et al. are indicated by an arrow, which can only securely exclude membership of K1b. Although the transition at np 497 defining all of K1a was known to the authors, they did not type it. Consequently, Ötzi could lie almost anywhere within K1a, representing ~80% of the entire haplogroup. Membership of K1c is also possible as the hypervariable markers 00146 and 00152 occur multiple times in parallel on the haplogroup K background. On this evidence, further work is needed to achieve the authors' stated aims of resolving the position of the Iceman's mtDNA within haplogroup K.

DNA and patterns of damage, and, in turn, the authenticity of the results.

In conclusion, to extend ancient mtDNA analysis beyond the control region is to be encouraged, but a more informed and extensive genotyping strategy is required and needs to be coupled with far more stringent tests of authenticity to stand up to the level of scrutiny required for working with ancient human DNA. When the most important results match the CRS, these may have to go beyond anything currently practiced in studies of ancient DNA and forensics to convince the skeptics that the sequences recovered are indeed, genuine. But, ultimately, it does not matter how authentic your results are if the phylogenetic markers tested are not able to support the conclusions reached.

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The Resolved Location of Ötzi's mtDNA Within Haplogroup K: A Reply to Endicott et al.

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In their commentary on our recent paper (Rollo et al., 2006) Endicott and colleagues raised several issues concerning both the authenticity of the human mtDNA

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sequences found and the location of the Iceman's mtDNA within haplogroup K. They recognize that our results are not necessarily wrong, just that there are alternative explanations for them, which need to be eliminated through the use of a more informed approach.

The first observation concerns the length of HVS1 stretch sequenced, 122 bp in our paper and 354 bp in that of Handt et al. (1994), and the fact that the signature mutations T16224C/T16311C within this sequence define the K basal haplotype which is common throughout Europe. The comment by Endicott et al. is that the individual identification cannot be considered as irrefutable evidence that the sequence was obtained from endogenous DNA.

Endicott et al. seem to have misunderstood the aim of our experiment. The test was designed in order to check whether we could obtain results comparable to those of Handt et al. (1994) despite the fact that we were using different groups of specimens.

The identification of the two signature mutations, a result that one could achieve simply by sequencing the above quoted 122 bp stretch, confirmed that this was the case and thus, implicitly, that the results of the two investigations were corroborating each other according to the 6th criterion of authenticity (independent replication). This is one of the nine criteria: i) isolation of work areas; ii) negative control extractions and amplifications; iii) appropriate molecular behavior; iv) reproducibility; v) cloning of products; vi) independent replication; vii) biochemical preservation; viii) quantification; ix) associated remains composing the guidelines proposed by Cooper and Poinar (2000). These guidelines are designed to ensure the quality of ancient DNA data and conclusions (Gilbert et al., 2005).

Endicott et al., further suggest that two clones from the colon (CR) library (CR05 and CR06), indicated by us as possible contaminants on the basis of their similarity to the Cambridge Reference Sequence (CRS), are likely to derive from an individual with European haplogroup T affiliation. This observation is interesting in speculative terms but does not alter the results nor the conclusions. The same can be said about the identification of one chimeric clone (CR11).

It is well known that the proportion of chimeric clones can be as high as 30% even in libraries of modern PCR amplified DNA (Wang and Wang, 1996), thus the finding of one chimeric clone in three libraries of damaged DNA (2.7%) cannot be a cause of real apprehension.

Finally, rather obscure remains the statement by Endicott et al. that the uncertainty over the origin of the DNA proposed as a contaminant leaves open the possibility that other extractions and amplifications have been compromised. This conclusion does not appear justified, all the more so as we discarded the CR specimen in the subsequent analysis of the coding region.

Endicott et al. focus on the sequencing of the control region (despite the fact that, taken alone, in no way can examination of the control region conclusively demonstrate the endogenous origin of the DNA, irrespective of the length of the stretch sequenced) and miss an authentication test which marks a substantial advancement in respect to the Handt et al. (1994) paper.

This is the analysis of associated plant and animal remains (criterion ix).

The principle of the test is that if there are plant or animal remains associated to the human ones one should also be able to obtain DNA from them. As plant and animal DNA is much less contamination-prone than human DNA, its finding would offer a strong argument in favour of the authenticity.

The ninth criterion is applied only seldom by ancient human DNA specialists and this is, presumably, for two reasons: 1) there may be no convenient plant or animal

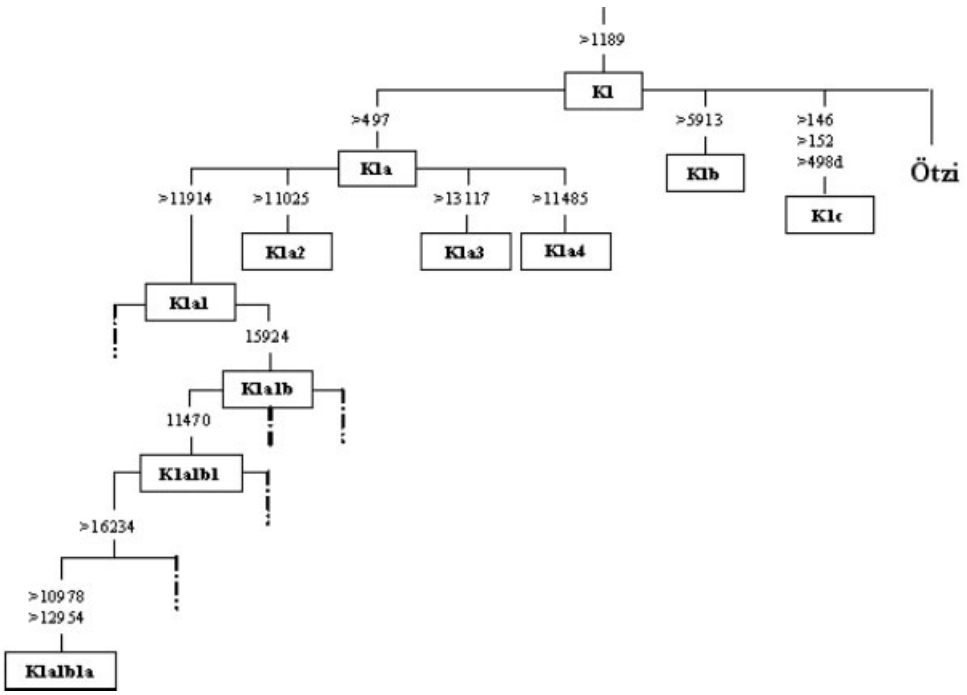

Fig. 1. Updated phylogenetic tree of the main branches of mtDNA haplogroup K1. The mutations typed are indicated by an arrow. The new results demonstrate the exclusion of Ötzi's mtDNA from K1a, K1b, and K1c.

remains in association with the human remains; 2) it is very demanding as it requires carrying out a parallel investigation on different genetic systems which can be even more laborious that the primary study.

In the case of Ötzi, the analysis of human DNA has been preceded by a meticulous screening of plant and animal DNA libraries obtained from samples of the intestinal content (Rollo et al., 2002). In particular, it has been shown that two types of mammal (red deer and alpine ibex) mtDNA are present in the intestines, in addition to human mtDNA. Given this finding, and the fact that the same intestinal specimens were used for the subsequent study, the most parsimonious explanation of the results is that the endogenous mtDNA of the mummy is also preserved.

Surprisingly, Endicott et al. seem unaware of this point. Conversely, our position on the debate over the authentication of ancient DNA results is to treat with caution any ancient European DNA identification claims, irrespective of the methodological sophistication displayed in the analysis of the human sequences, if they lack the control of the associated animal or plant DNA.

A second group of comments refers to the location of the Iceman's DNA within haplogroup K and, in particular, to the role of the C497T mutation in the definition of the K1a subcluster. We admit that the exclusion of this site from our analysis was an unfortunate oversight.

However, as our study of the mummy's mtDNA is still in progress, with the long-term aim of determining the whole sequence, we have examined the C497T site, together with a number of other sites, in the time elapsed since the publication of our paper. The results are shown in Figure 1.

Actually, there is no C497T mutation in the Iceman's mtDNA. As a precaution against the fact that the region where the 497 site is located may be subjected to hotspots, we have also checked other positions which characterize the K1a clusters (K1a1, K1a2, K1a3, and K1a4) and are located in the coding region (Behar et al., 2006). This test has further confirmed that the Iceman's mtDNA cannot belong to the K1a subhaplogroup.

As for the exclusion of our consensus sequence from the K1c subcluster, we have checked the 498 deletion (Behar et al., 2006) and found that it is not present in the mummy's DNA. It may be worth mentioning that the examination of the 497 and 498 positions has been repeated three times using different primer pairs.

These results were preliminarily presented and discussed at the international meeting on Haploid markers of DNA in evolutionary genetics and forensic held in Ancona, Italy, on May 26th, 2006. We are grateful to Endicott et al. for giving us the opportunity to discuss our work also in this journal. The present results show conclusively that the Iceman's mtDNA belongs to the K1 haplogroup, yet it does not fit any of the three known branches into which the K1 cluster is divided.

According to Endicott et al. if Ötzi's mtDNA really does belong to an as yet undefined branch of K1 this would indeed provide the most powerful argument that the DNA extracted is really endogenous. Though we are reluctant to share the peremptoriness of the Endicott and colleagues' statement, we are nevertheless tempted to admit that indeed this might be the case.

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