

# The Isolation and Identification of Neanderthal Mitochondrial DNA

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## INTRODUCTION

Over the past ten years the analysis of mitochondrial DNA (mtDNA) has become widespread when dealing with very low quantities of and/or highly degraded DNA. The advantages of mtDNA for this type of analysis have been well documented including its high copy number, which improves the chance of retrieving DNA, and its highly polymorphic, and therefore informative nature. In addition to forensic studies, mtDNA has been widely used in the study of ancient DNA (aDNA). In 1997 aDNA was reported to have been successfully extracted from a Neanderthal recovered from the Feldhofer Cave in Germany (1). This information was used to examine the origins of the modern European mtDNA pool. However, with only one Neanderthal sequence and no information about the molecular variation of Neanderthals, comparisons with modern human DNA could only be taken so far. Also, because of a number of unsubstantiated claims from the field of aDNA in previous years, some skepticism remained about the source of the "Neanderthal DNA" despite the stringent controls used. In the following article we discuss the second successful isolation of Neanderthal DNA along with the evolutionary significance of the data.

## ORIGINS OF MODERN EUROPEANS

There has been an extensive debate over the evolution of modern humans. The theory currently favored by the majority of people working in this field is that modern humans originated in Africa around 150,000–200,000YBP (years before the present). A subgroup of these modern humans then migrated out of Africa around 100,000YBP. Approximately 40,000YBP, the modern humans started to move into Europe where they would have encountered other hominids already in situ, namely the Neanderthals. The Neanderthals had been resident in Europe for ~200,000 years, but within ~10,000 years of the arrival of modern humans, the Neanderthals had disappeared. There are two mechanisms by which this may have occurred. The modern humans could have simply out-competed the Neanderthals, leading to their extinction, which is known as the replacement hypothesis. Alternatively there could have been breeding between modern humans and Neanderthals, thereby Neanderthal genes would have been absorbed into the modern human population. This question could be addressed in part by analyzing DNA from modern humans and Neanderthals, if Neanderthal DNA could be retrieved.

## ISOLATION OF ANCIENT DNA

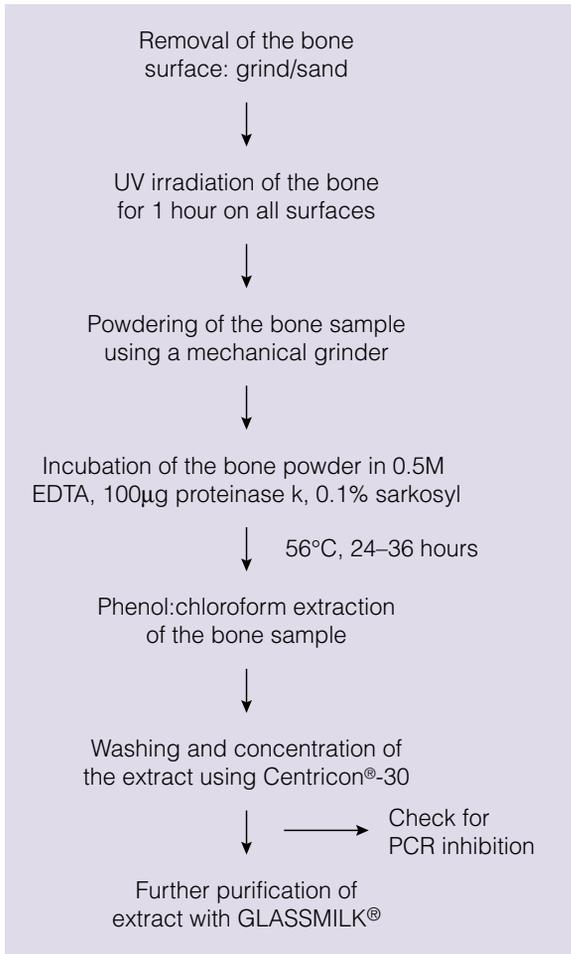
A rib sample of a Neanderthal infant was provided for molecular analysis. The infant had been discovered during the excavation of the Mezmaiskaya Cave (2), which lies at an altitude of 1,300 meters in the northern Caucasus in southern Russia. Prior to attempting to extract DNA, the bone's molecular preservation was assessed. The amount of collagen material in the sample and the composition of the collagen were measured (K. Liden) (3). The results suggested that the preservation was very good and therefore DNA extraction was initiated. The extraction method shown in Figure 1 was used. At all points in the procedure extreme care was taken not to contaminate the sample with modern DNA. When the DNA extract was tested for inhibitors of PCR, which are commonly co-purified from ancient samples, no significant inhibition was detected, therefore the extract did not have to undergo any additional purification steps.

PCR was performed with two Neanderthal-specific primer pairs that generated 256bp and 232bp products. After 40 cycles, enough product was produced to allow sequencing. In addition to direct sequencing, the PCR products were subcloned into a plasmid vector to allow further



Skull of the Neanderthal discovered during the excavation of the Mezmaiskaya Cave.

3187CA12\_0A



**Figure 1. Flow diagram showing the isolation of DNA from the bone samples.** No PCR inhibition was detected with the Neanderthal bone-extracted sample after the Centricon®-30 purification stage; therefore, the final cleanup step of using GLASSMILK® (GENECLEAN®, Bio101) was not performed.

sequence analysis. The sequence results from both direct sequencing and subcloned PCR products were compiled to identify the endogenous sequence, identifying changes in the sequence that had been introduced by the *Taq* polymerase enzyme during amplification.

An important control for any ancient DNA work is to be able to duplicate the results in another laboratory. At the outset of this work, half the rib sample was sent to the Archaeological Research Laboratory of Stockholm University where, in addition to collagen analysis and radiocarbon dating, DNA analysis was also performed (A. Götheström). Analysis was more problematic at Stockholm due to the presence of several subclones that appeared to have DNA from a mixture of sources. However, sequence identical to that obtained from the Glasgow laboratory was identified.

**ANALYSIS OF RECOVERED DNA**

In total we generated 345bp of mitochondrial sequence. The next step was to ask whether this was actually ancient DNA, and if it was, how does

it compare to modern human DNA and previously isolated Neanderthal DNA. This case was especially important, as we had generated relatively long PCR products from an ancient non-permafrost sample. Radiocarbon dating of the sample dated it to ~ 29,000YBP (K. Liden); therefore, products greater than 100–150bp were not expected.

**COMPARISON TO MODERN HUMANS**

The first part of the analysis was to compare our aDNA to the Cambridge Reference Sequence (CRS) (4). The two sequences were aligned and any base substitutions, insertions or deletions were recorded. Figure 2 shows one of the cloned PCR products in comparison to a modern human mtDNA identical to the CRS’s hypervariable region I showing one insertion and one substitution: in total there were 22 substitutions and one insertion. When comparing any two modern Europeans, the average number of base substitutions over the equivalent region of mitochondrial sequence is  $5.28 \pm 2.24$ ; therefore, the level of variation between the sequence obtained from the Neanderthal rib and the CRS was extremely high. This high level of differentiation was seen in a larger scale comparison to 300 modern European sequences with an average number of substitutions of  $25.45 \pm 3.27$ . Similar results were obtained when the sequence was compared to modern Asians and sub-Saharan Africans:  $23.27 \pm 4.06$  and  $23.09 \pm 2.86$ , respectively. Based on these results, it appeared that the DNA extracted from the rib was not closely related to any modern mtDNA.

**COMPARISON TO NEANDERTHAL DNA**

When we compared our aDNA to the previously existing aDNA sample (1), the two Neanderthal sequences differed by 12 substitutions and were more closely related to each other than they were to modern human mitochondrial DNA. Since then, one further Neanderthal mtDNA sequence analysis has been reported from a specimen discovered in the Vindija Cave, Croatia (5). The Vindija Neanderthal differs from the Mezmaiskaya and Feldhofer Neanderthals by only 6 substitutions over the same region, and all three share the insertion at position 16,263. A more rigorous phylogenetic analysis was undertaken using the computer program PAUP (6). When the Neanderthal sequences were compared to modern human sequences, the Neanderthals formed a group distinct from all modern humans (Figure 3).

**CONCLUSION**

Analysis of the aDNA sequences showed two things. First, the DNA recovered from the Mezmaiskaya Neanderthal was very similar to the Feldhofer Neanderthal and has subsequently been shown to be similar to the Vindija Neanderthal. Therefore, it can be concluded with a high degree of confidence that Neanderthal DNA has been recovered and that this is not some kind of peculiar contamination. Second, the Neanderthal DNA is significantly different from modern human mtDNA, forming a distinct group.

These results indicate that Neanderthals contained a distinct type of mtDNA. While it is not possible to know whether Neanderthals and modern humans did interbreed, based on the Neanderthal and modern humans analyzed to date, it is possible to conclude that Neanderthals did not pass any of their mtDNA on into the modern European mtDNA pool. Further analysis of Neanderthal DNA will provide information on the molecular diversity of the Neanderthals.

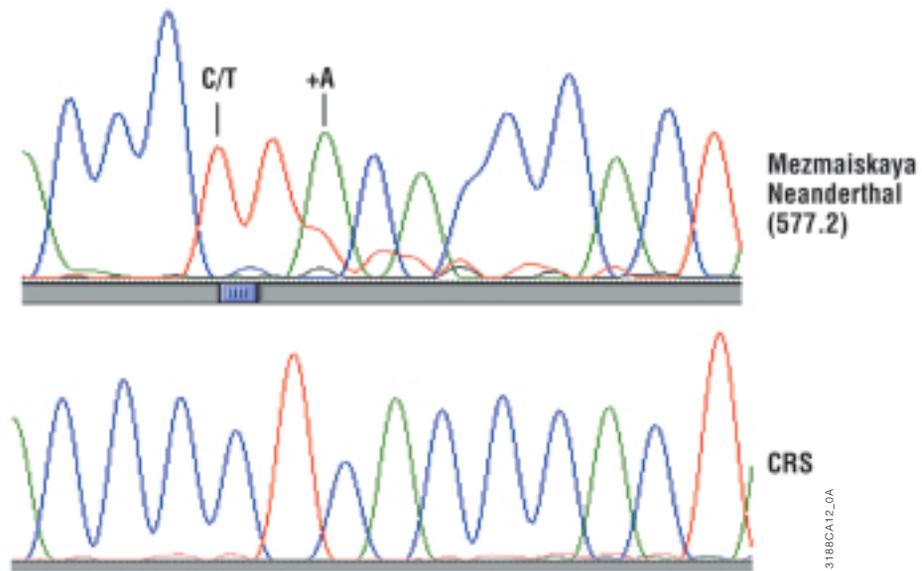


Figure 2. A fragment of the mtDNA HVR1 Sequence of the Mezmaiskaya Neanderthal (577.2) in comparison to a modern human sequence identical to the HV1 of the Cambridge Reference Sequence (CRS). The insertion of an A at position 16,263 and the C to T substitution at position 16,262 are shown.

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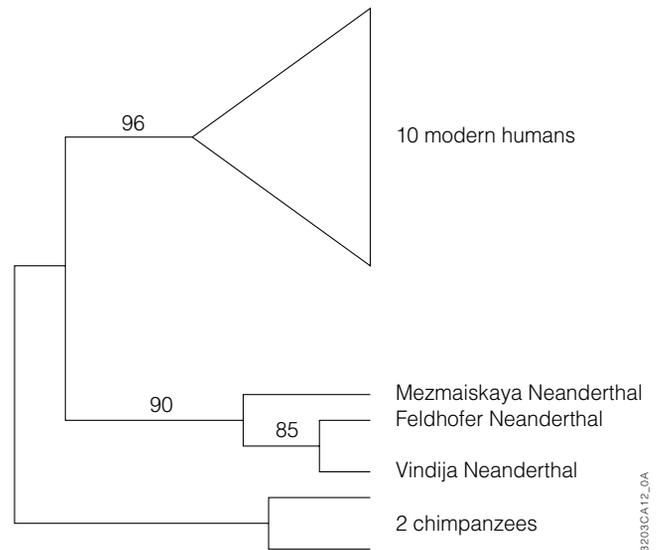


Figure 3. Phylogenetic analysis of the Neanderthal DNA. This diagram shows the results of a maximum parsimony search with the three Neanderthal sequences analyzed to date along with 10 modern humans and two chimpanzee sequences that were used as an outgroup. The numbers refer to the bootstrap frequencies (%).

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