Neandertal DNA and Modern Humans

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Abstract

The variation of mitochondrial DNA (mtDNA) between modern humans and Neandertal sequences lie outside the mtDNA sequence variation within modern humans. This variation has led several researchers to conclude that Neandertals did not contribute to modern human DNA and are a separate species that went extinct in Europe. It is feasible that DNA can be retrieved from specimens that died thousands of years ago, given the ideal preservation conditions and extraction protocols. However, DNA also decays as the organism decomposes. Spontaneous hydrolysis, oxidation, and nucleotide modifications are a few of the processes that cause DNA decay and likely interfere with reliably obtaining a mtDNA sequence that accurately reflects the Neandertal mtDNA sequence.

In addition to DNA decay, contamination of samples is also apparent in published Neandertal mtDNA sequences. A comparison of conserved sequence block 2 (CSB2) in hypervariable region II (HVRII) between Neandertal mtDNA and modern man, primates, and other mammals indicate that excess thymine in CSB2 of published Neandertal mtDNA is likely the result of contamination.

Introduction

A strong case has been made for the position that Neandertals were fully human, varying slightly, but not significantly, in skeletal structure from modern humans (Lubenow, 1992). The contrasting view that Neandertals are not fully human and never contributed to the modern human gene pool has received support from extracting and amplifying ancient DNA (aDNA) from Neandertal remains. Eleven Neandertal mitochondrial DNA (mtDNA) sequences have been published (Krings et al., 1997; Krings et al., 1999; Krings et al., 2000; Ovchinnikov et al., 2000; Schmitz et al., 2002; Lalueza-Fox et al., 2006; Caramelli et al., 2006; Orlando et al., 2006; Krause et al., 2007), and recently over one million base pairs of Neandertal nuclear DNA (nuDNA) were sequenced (Green et al., 2006; Noonan et al., 2006). These sequences claim to be evidence that Neandertals are distinctly different from modern humans and likely did not contribute to the modern human genome (Krings et al., 1997; Serre et al., 2004; Currat and Excoffier, 2004).

The average sequence difference between some of the published Neandertal mtDNA sequences and modern humans is about three times the number of average variation between modern humans. In fact, the putative mtDNA

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variation between modern humans and Neandertals lies completely outside the range of variation within modern humans (Krings et al., 1997). If currently obtained Neandertal mtDNA sequences accurately represent their original sequence, it would provide strong evidence that the two groups did not exchange DNA and they could be classified as different species or subspecies (*Homo sapiens* and *Homo sapiens neanderthalensis*). The relatedness between modern humans and Neandertals using mtDNA (or nuclear DNA) is dependent, however, on retrieving uncontaminated DNA from Neandertal bones and teeth that has been sufficiently preserved to prevent significant DNA decay.

Mitochondrial DNA and Contamination

mtDNA provides the best opportunity for acquiring templates to sequence ancient DNA. It is a 16,569 base pair, circular DNA strand divided into two functional regions—the control region (1120 base pairs, 16,024–16569 and 0–576) and the coding region (the remaining 15,450 base pairs), where proteins, transfer RNA, and ribosomal RNA are transcribed (Cutticchia, 1995). The control region is where transcription is regulated and is taxonomically significant, meaning there is enough variation in this region to distinguish sequences between species, members of different ethnic groups within a species, or even one human family from another. The two hypervariable regions, hypervariable region I, or HVRI (16,024–16,383), and hypervariable region II, or HVRII (57–372), in the control region are the sources of Neandertal mtDNA. Nine Neandertal mtDNA sequences are from HVRI and two sequences were obtained from HVRII.

An important factor in obtaining useful ancient DNA from mitochondria is the number of mtDNA molecules that can be extracted from a sample. Each sample from a living organism should have thousands of cells, 100-1000 mitochondria in each cell, and 5–10 copies of DNA in each mitochondrion, providing the >1000 mtDNA molecules required for efficient amplification (Krings et al., 1997). However, bone or tooth samples that provide mtDNA for sequencing will have far fewer intact cells, if any, and the DNA that is retrieved is likely bound to the hydroxyapatite of the bone or tooth. The loss of cells and DNA post-mortem damage emphasize the value of mtDNA with thousands of copies for sequencing targets in a living organism, and many successful aDNA sequences have started with under 100 templates (Hofreiter et al., 2001).

Each copy of mtDNA provides a target for primers (short oligonucleotides) that initiate the polymerase chain reaction (PCR) in amplifying enough mtDNA for sequencing. It is assumed that enough undamaged Neandertal mtDNA molecules are still present to provide a template for the PCR. Theoretically, several PCR amplifications with extracted template mtDNA provide sufficient sequences to derive a consensus sequence and accurately represent the original Neandertal mtDNA sequence. The more template mtDNA that is available at the start of the amplification process, the less likely the PCR product will be contaminated by extraneous sources of DNA.

Contaminating DNA can block the PCR amplification process, incorporate foreign DNA bases into targeted DNA, or result in the amplification of only nontargeted DNA (Pusch and Bachmann, 2004). Contamination is a constant obstacle for obtaining authentic ancient DNA sequences, and the evidence for contamination has been observed in ancient mtDNA samples as well as ancient nuclear DNA (Wall and Kim, 2007). Valid research requires adequate experimental protocols to prevent the incorporation of contaminated DNA (Cooper and Poinar, 2000; Gilbert et al., 2005; Willerslev and Cooper, 2005). These requirements include a facility reserved only for ancient DNA research, the independent verification of the putative sequence in another laboratory, treatment with enzymes to remove damaged bases, and a sufficient number of clones to derive a consensus sequence (Cooper and Poinar, 2000; Gilbert et al., 2005; Bower et al., 2005; Willerslev and Cooper, 2005).

DNA Decay

Precautions can be taken to minimize contamination in aDNA samples, but the problem of DNA decay poses more difficult problems to obtain authentic ancient mtDNA sequences. As soon as an organism dies, DNA begins to degrade, and the repair mechanisms that maintain DNA sequence fidelity in living systems no longer function. Spontaneous hydrolysis and oxidation result in double-strand breaks, abasic sites, and nucleotide modifications or miscoding lesions (Lindahl, 1993). Double-strand breaks and abasic sites (most commonly depurination) can prevent PCR amplification, while nucleotide modifications can be incorporated into the amplified PCR product, mimicking the expected evolutionary changes in the putative DNA sequence.

Spontaneous hydrolysis results in approximately 2,000–10,000 depurination events in each human cell per day. This is due to the instability of the N-glycosyl bond between a purine (adenine and guanine) and the 2' carbon of the deoxyribose sugar (Lindahl, 1993). In living systems, endonucleases rapidly initiate repair processes to maintain the integrity of DNA (Lindahl, 1993). However, after the organism dies, depurination results in small fragments of DNA, which are more difficult to amplify than intact DNA from a living organism. Kinetic calculations predict that amplifiable fragments less than 400 base pairs will survive no longer than 10,000 years

at temperate conditions (Poinar et al., 1996). As the temperature decreases, the rate of DNA decay also decreases, making samples in permafrost the best candidates for aDNA sequencing (Smith et al., 2001; Willerslev and Cooper, 2005).

All of the environmental factors that affect DNA decay are not completely understood, but higher temperatures and increased moisture appear to be two of the more significant factors that accelerate DNA decay. Whether or not aDNA sequences are damaged or accurately represent the original sequence of the living organism, the retrieval of any DNA from ancient organisms is evidence for a *recent existence* of less than 1 million years for permafrost specimens (mammoths and bacteria) and less than 10,000 years for temperate specimens, including Neandertals (Poinar et al., 1996; Smith et al., 2001; Willerslev and Cooper, 2005).

The most common result of decay in DNA is the deamination of cytosine resulting in a base change to uracil (Hansen et al., 2001; Hofreiter et al., 2001). This change is easily defined by the chemical nomenclature of the two bases. Cytosine is 2–oxy–4–amino pyrimidine and uracil is 2–oxy–4–oxy pyrimidine. Post–mortem damage from cytosine deamination can accumulate fairly quickly in the context of aDNA, considering that the half–life of a cytosine residue is about 200 years under human physiological conditions (37°C and 7.4 pH) (Lindahl and Nyberg, 1974). Cytosine deamination occurs an estimated 100–500 times a day in a living cell (Lindahl, 1993), where accompanying repair mechanisms are able to correct the damage. Uracil is a base not normally incorporated into the DNA sequence of any organism; consequently many organisms, including humans, have an enzyme, uracil–N–glycosylase (UNG), to remove deaminated cytosine (uracil) when it is incorporated into DNA. When an organism dies, spontaneous cytosine deamination can occur through a hydrolysis reaction that removes the amine group converting cytosine to uracil (Lindahl, 1993). Without UNG to repair the damaged base, any postmortem sequencing reaction will identify the deaminated cytosine as uracil and pair it with adenine on the complementary strand. In mtDNA, if the deaminated cytosine is on the heavy strand (H strand), an adenine will be incorporated on the complementary light strand (L strand) in place of the original guanine.

Deamination of adenine to hypoxanthine and guanine to xanthine also occurs, but at less than 2–3% of the rate of cytosine deamination (Lindahl, 1993), making them less likely to cause major changes in aDNA sequences. Cytosine to thymine (C→T) and guanine to adenine (G→A) transitions are classified as Type 2 transitions in the context of aDNA sequencing, representing the deamination of cytosine to uracil (Hansen et al., 2001). Type 1 transitions represent the possible deamination of adenine→ hypoxanthine, which results in an A→G and T→C transition on complementary DNA strands (Table I).

Obviously, scientists who sequence aDNA are aware of the problems that deamination can cause; one of the required protocols is to treat the samples with UNG to remove deaminated cytosine residues that could produce erroneous sequences from PCR amplification. UNG is believed to remove all deaminated cytosines eliminating Type 2 transitions from DNA (Hofreiter et al., 2001). However, treatment with UNG before amplification is not without problems. The removal of deaminated cytosine leaves an abasic site, creating strand nicks that can prevent amplification of the aDNA strand (Hofreiter et al., 2001). This is particularly worrisome when there might only be 100 templates available for amplification. Reducing the number of templates increases the risk of incorporating contaminating extraneous DNA into the targeted sequence, or completely sequencing contaminants (Pusch and Bachmann, 2004).

Evidence exists that cytosine deamination is not the only source of Type 2 transitions in aDNA. Gilbert et al. (2007) and Hoss et al. (1996) both found that UNG treatment left behind half of the C→T Type 2 transitions that were identified from damaged sequences in controlled experiments. Presumably, the C→T transition resulted from the decay of guanine to adenine on the complementary strand. Gilbert et al. (2003; 2007) noticed this, at first speculating that UNG did not remove all deaminated cytosines and was successful mainly on longer templates. Apparently, these C→T transitions might occur from an (as yet) unidentified degradation of guanine to adenine and be as frequent as cytosine deamination in nonliving samples (Gilbert et al., 2007). The C→T transition would result from sequencing the complementary strand opposite the G→A deamination event. This observation has prompted Gilbert et al. (2007) to observe that most of the knowledge

Table I. Type 1 and Type 2 Transitions. DNA decay will result in transitions on both strands when sequenced. Cytosine deamination is the most common form DNA damage in living systems.

Transition Type	H Strand	L Strand	Composite Change
Type 1	T→C	A→G	TA→CG
Type 2	C→T	G→A	CG→TA

about DNA damage comes from living systems and not from aDNA, leaving the possibility that post-mortem DNA is damaged from processes that are not yet understood (Gilbert et al., 2007).

One possible explanation for post-mortem conversion of G→A is the co-extraction of divalent metal ions that have been shown to damage DNA (Pusch and Bachmann, 2004). Concentrations of some divalent metal ions increase several times (up to 5,000 times) in aDNA when compared to contemporary samples, raising the possibility of mutagenic effects when aDNA is extracted and amplified through PCR (Velasco-Vazquez et al., 1997). This is particularly true of manganese. The G→A transition frequently occurs at site 1138 of the human *FGFR3* gene in the presence of 0.25mM $MnCl_2$. The same mutation occurs when human template DNA is spiked with aDNA, indicating that aDNA is the source for the mutagenic manganese (Pusch et al., 2004). It also explains why independent sequencing of putative aDNA samples would incorporate the same Type 2 transitions; the source of the mutagenesis (manganese) is in the bone or tooth material supplying the aDNA. The possibility that mutagenic effects from divalent metal ions cause sequence divergence between Neandertals and modern humans casts serious doubt on aDNA sequences being validated by independent laboratories.

Neandertal DNA

Neandertal DNA Decay

A decrease in the Neandertal to modern human Type 1: Type 2 transitions in mtDNA, compared to a modern human to modern human ratio, is an expected result of the deamination of cytosine to uracil or damage to guanine that would result in a transition to adenine. To determine the difference in Type1: Type 2 transitions, a reliable ancestral sequence for both modern humans and Neandertals is required as a baseline. The Eve1.0 Consensus Sequence (Carter, 2007) provides this baseline for a comparative study between Neandertal and modern human mtDNA. Not only does the Eve1.0 provide a baseline, but the evidence that all humans descended from one woman (Cann et al., 1987) and the overwhelming statistical consensus sequence from Eve1.0 (Carter, 2007) provides confidence that Eve1.0 is reasonably comparable to the first human mtDNA sequence. Even in an evolutionary scenario, Eve1.0 would predate the Neandertal specimens by more than 100,000 years, again making it a feasible source as a baseline sequence. In HVRI, the Eve1.0 consensus sequence is also identical to the revised Cambridge Reference Sequence (Anderson et al., 1981).

Therefore, the Eve1.0 consensus sequence was used as a baseline sequence to determine if the mtDNA Type 1: Type 2 transitions from Neandertals and modern humans are significantly different from the ratio within modern human sequences. Figure 1 shows a sequence alignment for the HVRI from nine Neandertals and Eve1.0. The alignment was made using ClustalX 2.0.7, employing the suggested pairwise and multiple alignment parameters for nucleic acids (Hall, 2004). Type 1 and Type 2 transitions and transversions were selected visually. Chi-square analysis (Moore, 2000) was used to evaluate the significance of differences in the Type 1: Type 2 transition of mtDNA between Neandertals and modern humans and the Type 1: Type 2 transitions within modern humans. The alignment identifies 40 transition sites between the nine Neandertal HVRI sequences and the Eve1.0 HVRI. Of these 40 sites, 16 are Type 1 transitions and 24 are Type 2 transitions. The number of transitions for HVRI in modern humans was determined from over 4300 partial and full modern human mtDNA sequences available on the National Center for Biotechnology Information (NCBI) Web site.

From these sequences (Ruiz-Pesini et al., 2007) 232 transitions were identified in the HVRI. Of these, 128 are Type 1 transitions and 104 are Type 2 transitions (data not shown). The frequency (44.8%) of Type 2 transitions is in agreement with the G+C content for Eve1.0 HVRI (46.03%) and gives the expected results for the number of Type 1 to Type 2 transitions found among modern humans. A chi-square test for the difference between Type 1: Type 2 transitions in Neandertal: modern human, and modern human: modern human gave values of $X^2 = 3.7$, with a P value = <0.10 and much closer to 0.05. A P value of 0.05 or less is arbitrarily chosen as the level for "significance" in a chi-square test (Moore, 2000). The P value given for the Neandertal: modern humans Type 1: Type 2 transitions, is barely outside of the 0.05 standard but cannot be dismissed as being insignificant. The P value of 0.05 denotes that the events tested might occur by chance less than 5% of the time. The P value for the Type 1: Type 2 transitions between Neandertal and modern humans are slightly above this 5% level of chance. This indicates that the results are most likely affected by some other factor than chance. In this case, the other factor suggested is DNA damage.

The differences in the Neandertal and modern human Type 1 to Type 2 transitions clearly points to some factor affecting this ratio other than random chance or a small sample size. A good explanation for the increase in Type 2 transitions is the activity of DNA decay through the deamination of cytosine to uracil, damage from high concentrations of divalent metal ions such as manganese or an as yet unidentified decay process of guanine to adenine and not simply sequence variation between Neandertals and modern humans.

Neandertal DNA Contamination

Without an original, living Neandertal mtDNA sequence, it would seem

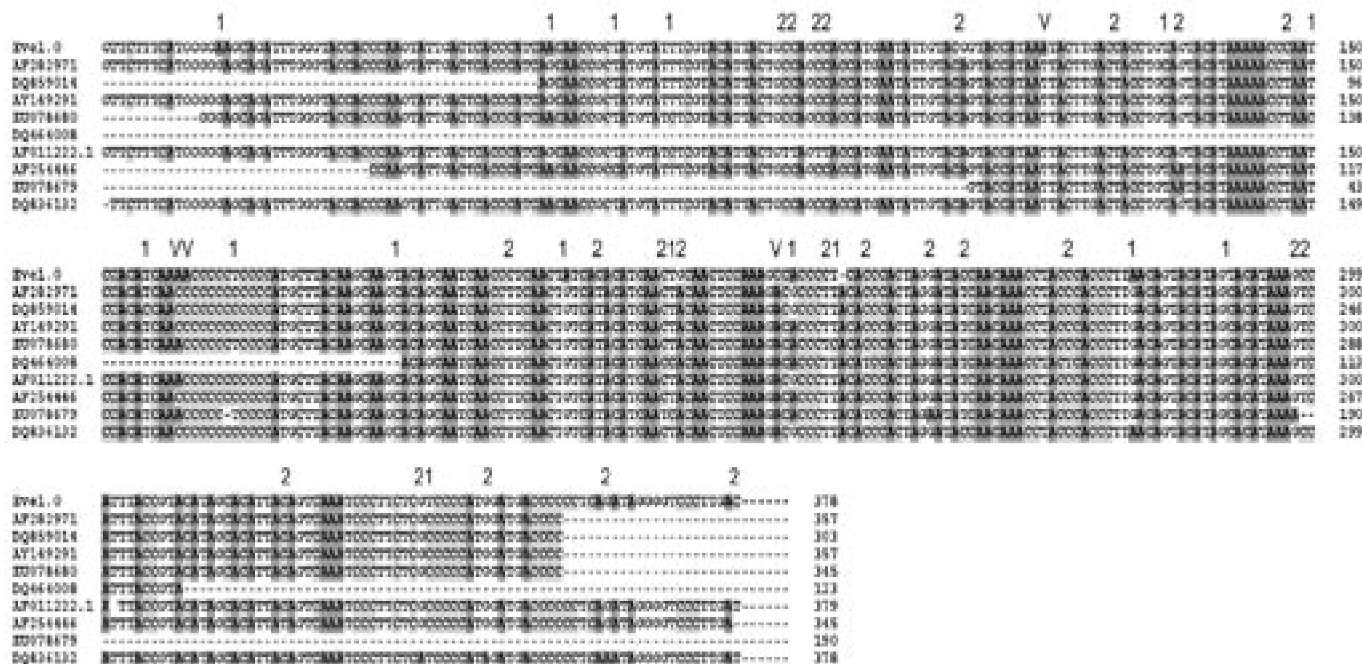

Figure 1. ClustalX alignment for nine Neandertals and Eve1.0 hypervariable region I (HVRI). Eve 1.0 position one in the alignment is position 16023 in HVRI. Type 1 transitions are labeled "1," Type transitions are labeled "2" and transversions are labeled as "V." Type 1 and Type 2 transitions were determined by comparing base changes from Eve 1.0 to the Neandertal sequences.

difficult to identify contamination or artifacts in any of the putative Neandertal sequences extracted from bones. Sequence differences between Neandertals and modern humans could be real differences and not the result of contamination. However, comparisons between Neandertal mtDNA and conserved sequences in other vertebrates, including man, can help identify problems within a putative ancient DNA sequence by identifying base changes or indels (insertions and deletions) that are not present in any of the other conserved sequences. (Here, conserved sequences can be defined as "similar" sequences and not necessarily because they were conserved through evolution from one kind of organism to another.)

To be identified as contaminants, sequence divergence from a conserved region would have to be so irregular from all known conserved sequences that its presence would likely interfere with normal transcription and replication, or the sequence could be shown to be identical to a known sequence from another organism (e.g., a fungus), or another part of the organism's genome (e.g., a pseudogene). Typically, searches for conserved sequences require comparisons of protein-coding genes. Fortunately, the arrangement of the mitochondrial genome in all vertebrates is essentially identical and there are conserved sequence elements in the mtDNA control region. Several of these conserved sequences are found in the HVRII for which there are two Neandertal mtDNA sequences. Within all vertebrate HVRII regions are three highly conserved sequences called conserved sequence blocks (CSBs). In humans the three sequences are located at sites 213–235 (CSB1), 299–315 (CSB2), and 346–363 (CSB3) (Ruiz-Pesini et al., 2007), and all three of these are included in the two Neandertal HVRII sequences. Of these conserved sequences, CSB2 is the most informative for the identification of contamination in Neandertal sequences. CSB2 is an important part of the transcription machinery in mitochondria. It has been identified as a transcription termination element, and mutations in this region can impair or abolish transcription termination (depending on the degree of divergence from the optimal CSB2 sequence) (Pham et al., 2006).

To identify any unusual elements in the Neandertal HVRII sequences, a comparison was made with Eve1.0 and several primates. The identification of CSB2 for 21 *Simiiformes* primates was made by comparing all three human conserved sequence blocks, and their relative position to each other, with each primate mtDNA sequence available

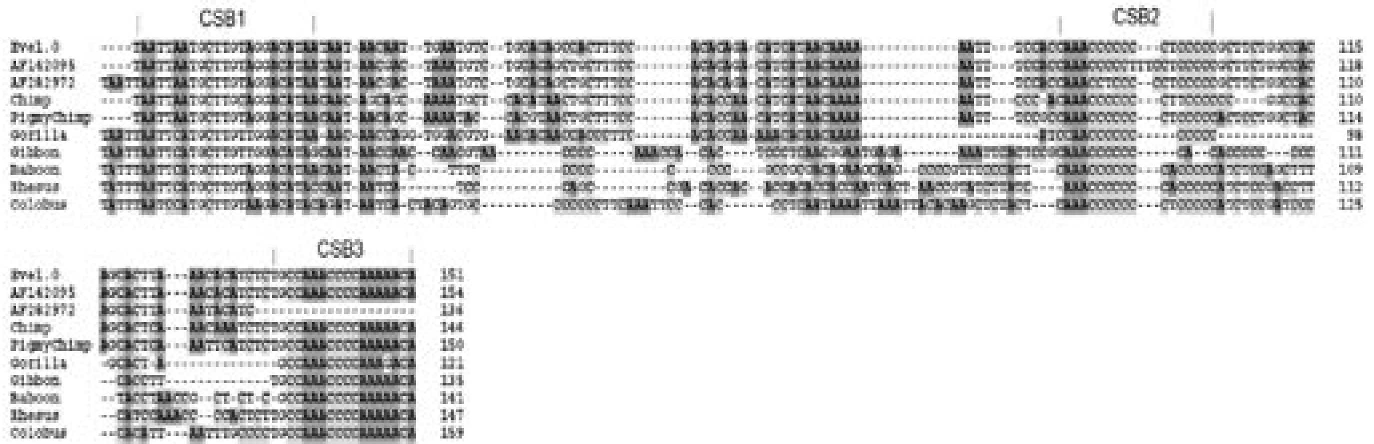

Figure 2. ClustalX alignment of HVRII with all three conserved sequence blocks (CSBs) for Eve 1.0, two Neandertals (AF142095 and AF282972), and seven representative primates from the NCBI database. AF28972 ends before CSB3. Gorilla has a long intervening sequence between CSB2 and CSB3 not found in other primates. This region (Gorilla 16194–16339) was deleted to shorten the alignment. The homology of all three CSBs is obvious as well as the thymine insertion in Neandertal AF142095.

at NCBI. A ClustalX alignment of all three conserved sequence blocks, two Neandertals, Eve1.0, and seven of the 21 primates was made for comparison (Figure 2). The ClustalX alignment was used with the same parameters as in Figure 1. The alignment was manually adjusted to bring the three conserved sequence blocks for each primate into alignment. The conserved elements of CSB2 are obvious from the alignment, as are also the two Neandertal insertions. In the alignment, Neandertal AF142095 has a thymine trimer inserted into the middle of what is called the poly-cytosine tract (polyC 309). A thymine trimer has not been identified for any of the 4300 modern human sequences (Ruiz-Pesini et al., 2007; Carter, 2007), and none of the 21 primates have this insertion. A thymine insertion is present between sites 304-305 in Neandertal AF282972. This insertion is also not present in any of the primates in the alignment and is not listed among any of the modern human polymorphisms (Ruiz-Pesini et al., 2007; Carter, 2007). This is strong evidence that the thymine trimer in AF142095 and the thymine insertion in AF282972 are artifacts and not part of the original Neandertal sequence. The addition of all 21 primate sequences does not remove the Neandertal insertions, nor does an alignment including the entire HVRII region with conserved sequence blocks 1–3 (rCRS 213–363), as no primate sequence has a thymine trimer in this region (CSB2) of mtDNA.

Removing the thymine insertions in both Neandertals produces a sequence that is identical to Eve1.0 and the rCRS. Neandertal AF142095 CSB1 and 3 are both identical to Eve1.0, strengthening the argument that the original Neandertal CSB2 sequence also should be identical to modern humans. It is possible that the single thymine insertion in AF282972 would not severely interfere with CSB2 function, but it is difficult to suggest that the thymine trimer in AF142095 would not interfere with CSB2 function regardless of its location in any primate. The absence of this type of insertion in 4300 modern human sequences and the aligned primates suggests that the thymine trimer is lethal to human mitochondria and is not a unique feature of Neandertal CSB2.

Neandertal Thymine Trimer and DNA Decay

A realignment of CSB2 provides another possible scenario to explain the existence of the thymine trimer in Neandertal AF142095 (Krings et al., 1999). The thymine trimer in AF142095 might be a sequence length polymorphism in CSB2 similar to that observed in modern humans (Figure 3). Although CSB2 is highly conserved in humans and primates, this research, and that of others (Santos et al., 2005; Carter, 2007), show that a sequence length polymorphism designated at site 309 is the most variable in the entire human mitochondrial genome (Table II). This poly-cytosine tract has four different sequence length polymorphisms identified in humans (Carter, 2007) with anywhere from 7–10 cytosines. The poly-cytosine tract ranges from sites 303 to 309 and many humans are heteroplasmic in this region, having mtDNA with two different lengths in their cells.

The addition of cytosine to this region has not been associated with any specific disease (Ruiz-Pesini et al., 2007) or disorder, and the variations here are

Eve1.0	CAAACCCCC - - - CCTCCCCCGCT
PolyC309.1	CAAACCCCC - - CCCTCCCCCGCT
PolyC309.2	CAAACCCCC - CCCCTCCCCCGCT
PolyC309.3	CAAACCCCCCCCCCTCCCCCGCT
AF142095	CAAACCCCCT T TCCTCCCCCGCT

Figure 3. A manual alignment for conserved sequence block 2 of Eve1.0, three human variants PolyC309.1, PolyC309.2, PolyC309.3, and Neandertal AF142095. Position 1 is Eve1.0 or rCRS position 299 in the mitochondrial DNA control region.

quite common, indicating the addition of cytosine has little if any effect on the functioning of CSB2. In Figure 3, an alignment between a modern human sequence with 10 cytosines in the poly-cytosine tract (polyC309.3) and AF142095 can be made with no gaps. Could the original Neandertal sequence have 10 bases in this tract with the thymine trimer substituting for the three extra cytosines that rarely appear in human poly-cytosine tracts? Probably not, since neither man nor primates have three thymine bases in the poly-cytosine tract. A unique sequence divergence that is this dramatic in a conserved region is an indication of contamination or decay and not a species-specific characteristic. If the thymine trimer is not an insertion from a contaminating source of DNA, it likely resulted from post-mortem deamination in an individual with 10 cytosines in the poly-cytosine tract.

Summary

Advances in the extraction and amplification of ancient DNA from Neandertals has provided a glimpse into the genomes of ancient humans. However, documented problems with contamination and DNA decay (Pusch et al., 2004; Wall and Kim, 2007) continue to plague efforts to show phylogenetic relationships between Neandertals and modern humans (in a Biblical sense) from aDNA sources. In this study, the high number of Type 2 transitions in Neandertal mtDNA HVRI sequences indicates that decay processes, such as the deamination of cytosine or guanine, are incorporated onto putative Neandertal mtDNA HVRI sequences.

The novel thymine trimer present in CSB2 of Neandertal AF142095 (Krings et al., 1999) is not found in any other primate and is not one of several possible variations in any of the 4300 human HVRII sequences, indicating that it is an artifact from DNA contamination or the product of DNA decay. Removal of the CSB2 thymine trimer in Neandertal AF142095 makes this Neandertal identical to humans in all three HVRII conserved sequence blocks and is evidence that the original Neandertal mtDNA was not significantly different than modern humans. In spite of the rigorous controls instituted to eliminate damaged and contaminated aDNA sequences, evidence continues to mount that the current methodology is inadequate to eliminate all possible sources of DNA decay and contamination. Because of these problems, conclusions made about the relationship of Neandertals to modern humans cannot be fully assessed using currently available Neandertal DNA sequences, and the problems described in this paper will need to be assessed with every new ancient DNA sequence that becomes available.

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Table II. Frequency of sequence length polymorphisms and heteroplasmy in human conserved sequence block 2 polyC 309.

Study	No. Sequences	309C	309.1CC 309.2CCC	309.3CCCC
Present[a]	37	0.46	0.54	None
Carter 2007[b]	827	0.52	0.48	0.001
Ruiz-Pesini 2007[c]	4300	present	present	present
Santos 2005[a,d]	48	0.46	0.54	N/A

[a] Frequency of heteroplasmic families
[b] Frequency for 309.1CC is 0.37, and 309.2CCC is 0.11
[c] Only the presence of these polymorphisms are given
[d] Individual sequence length polymorphisms are not identified

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