Ancient fossil specimens of extinct species are genetically more distant to an outgroup than extant sister species are

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Keywords: Neanderthals, dinosaurs, mastodon, molecular clock hypothesis

Abstract

There exists a remarkable correlation between genetic distance and time of species divergence as inferred from fossil records. This observation has provoked the molecular clock hypothesis. However, data inconsistent with the hypothesis have steadily accumulated in recent years from studies on extant organisms. Here the published DNA and protein sequences from ancient fossil specimens were examined to see if they would support the molecular clock hypothesis. The hypothesis predicts that ancient specimens cannot be genetically more distant to an outgroup than extant sister species are. Also, two distinct ancient specimens cannot be genetically more distant than their extant sister species are. The findings here did not support these predictions. Neanderthals are more distant to chimpanzees and gorillas than modern humans are. Dinosaurs are more distant to frogs than extant birds are. Mastodons are more distant to opossums than other placental mammals are. The genetic distance between dinosaurs and mastodons is greater than that between extant birds and mammals. Therefore, while the molecular clock hypothesis is consistent with some data from extant organisms, it has yet to find support from ancient fossils.

Introduction:

Two equally valid data sets were evident from the early studies of protein homology across different species (Doolittle and Blombaeck, 1964; Fitch and Margoliash, 1967; Margoliash, 1963; Zuckerkandl and Pauling, 1962). The first compares a protein sequence from a recently evolved organism against those from organisms that appeared earlier in evolution. This comparison shows that humans are more related to monkeys, less to birds, still less to frogs, still less to fishes, still less to yeasts, and least to bacteria. The second set of data compares a protein sequence from an outgrop organism against those from organisms that appeared later in evolution. This comparison shows the genetic equidistance phenomenon where sister species are genetically equidistant to an outgroup. For example, species that originated from fishes but are distinct from fishes, such as frogs, reptiles, and humans, are all approximately equidistant to fishes in most genes.

Both sets of data are derived from the same sequence information and therefore each alone is sufficient to reveal any relationship between genetic distance and other properties of different species. One striking revelation of these two data sets was the remarkable correlation between genetic distance and time of species divergence as indicated by fossil records. This observation has provoked a novel hypothesis termed the molecular clock hypothesis (Kumar, 2005; Margoliash, 1963; Zuckerkandl and Pauling, 1962). The clock hypothesis was first informally proposed in 1962 based largely on the first data set (Zuckerkandl and Pauling, 1962). Margoliash made a formal statement of the molecular clock in 1963 after noticing the genetic equidistance phenomenon (Margoliash, 1963). “It appears that the number of residue differences between cytochrome C of any two species is mostly conditioned by the time elapsed since the lines of evolution leading to these two species originally diverged. If this is

correct, the cytochrome c of all mammals should be equally different from the cytochrome c of all birds. Since fish diverges from the main stem of vertebrate evolution earlier than ether birds or mammals, the cytochrome c of both mammals and birds should be equally different from the cytochrome c of fish. Similarly, all vertebrate cytochrome c should be equally different from the yeast protein."

The molecular clock hypothesis asserts that the rate of amino acid or nucleotide substitution is approximately constant per year over evolutionary time and among different species. Two different species are thought to gradually accumulate mutations over time since their most recent common ancestor. Their genetic distance in ancient times is thought to be smaller than their distance today.

The empirical observation of an apparently constant mutation rate per year has provoked the 'Neutral Theory'. But this theory is now widely acknowledged to be an incomplete explanation. For example, Ayala noted: "The theoretical foundation originally proposed for the clock, namely the neutrality theory of molecular evolution, is untenable. The vagaries of molecular rates of evolution have contributed much to invalidating the theory."(Ayala, 1999). Pulquerio and Nichols noted: "The 'Neutral Theory' is not a complete explanation, however. For example, it predicts a constant substitution rate per generation, whereas empirical evidence suggests something closer to a constant rate per year."

Studies based on extant organisms have accumulated in recent years that often violate the hypothesis of a constant mutation rate (Ayala, 1999; Ho and Larson, 2006; Pulquerio and Nichols, 2007). The recent success in obtaining DNA and protein sequence information from ancient fossil specimens has created a novel opportunity to further test the molecular clock hypothesis. The hypothesis predicts that ancient specimens cannot be more distant to an outgroup than extant sister species are. Also,

two distinct ancient specimens cannot be more distant than their extant sister species are. However, the study here did not support these predictions.

Results and Discussion:

Neanderthals are more distant to chimpanzees than modern humans are

Neanderthals are a group of extinct hominids that inhabited Europe and western Asia from about 400,000 to 30,000 years ago. Analysis of molecular genetic variation in the mitochondrial and nuclear genomes of living human populations have generally supported the view that Neanderthals were completely replaced by modern humans without contributing any genes (Armour et al., 1996; Hammer et al., 1998; Stringer and Andrews, 1988; Tishkoff et al., 1996; Vigilant et al., 1991). However, these analysis rely on assumptions whose validity has been questioned (Templeton, 1992; Wolpoff, 1989). In contrast to the replacement model, the multiregional model that is largely based on fossil evidence suggests that Neanderthals were the ancestors of modern Europeans (Wolpoff et al., 2000).

Influenced by the molecular clock model of speciation, sequence analysis of Neanderthal DNAs are commonly thought to represent the best approach to resolve the issue of whether Neanderthals contributed some genes to modern humans. In 1997, a segment of the mitochondrial DNA (mtDNA) of Neanderthals was sequenced and was found to be distinct from modern humans (Krings et al., 1997). Subsequently, mtDNA sequences have been retrieved from eleven additional Neanderthal specimens. Although some of these sequences are extremely short, they are all more closely related to one another than to modern human mtDNAs (Orlando et al., 2006). This fact has been interpreted to mean that Neanderthals contributed no mtDNA to present-day humans. Recently, the Neanderthal nuclear genomic DNAs have been sequenced

(Green et al., 2006; Noonan et al., 2006). The data however suggests that there may be some degree of gene flow between modern humans and Neanderthals (Green et al., 2006).

The genetic equidistance phenomenon suggests that, if Neanderthals were alive today, they and living modern human would be equally distant to the outgroup chimpanzees. The molecular clock hypothesis predicts that ancient Neanderthals of 40,000-100,000 years ago cannot be more distant to chimpanzees than extant humans are. I first examined five published Neanderthal mitochondrial hyper-variable region I sequences that are longer than 300 nucleotides. By searching against all chimpanzee sequences (1000 sequences) in the NCBI database using the BLAST algorithm, the average similarity score between Neanderthal and chimpanzee is 235 for the most related and 63 for the least related (Table 1). For each Neanderthal sequence, I selected a closely related human sequence of equal length and used it to search the chimpanzee sequences. The average score between human and chimpanzee is 269 for the most related and 74 for the least related. I also determined the relationship with chimpanzees for five African tribes and six Australian DNAs of 8000-60000 years old (Table 1). All of these show higher similarities with chimpanzees than the Neanderthals do, although ancient Australian DNAs seem to be slightly more distant to chimpanzees than living humans do. So, modern human DNAs are more related to chimpanzees than the Neanderthals are.

I next studied the statistical significance of this finding. If the finding represents random noise of sequence comparisons, it should be easy to find some human sample sequences that show less identity with chimpanzees than Neanderthals do. I examined 11 Neanderthal hyper-variable region 1 sequences ranging in size from 31 bp to 379 bp. For each of these sequences, I identified a distinct modern human sequence of equal length that shows the highest identity with the Neanderthal sequence. These

Neanderthal-like sequences have the best chance of being equally related to chimpanzees as the Neanderthals are. They are all more distant to chimpanzees than the Cambridge Reference sequence of human mitochondrial DNA. They are in fact more distant to chimpanzees than other human sequences that are less similar to Neanderthals. For example, for the first reported Neanderthal sequence AF011222 (379 bp), the most closely related human sequence is AY957203 (94% identity) and the least related is AY210529 (90% identity). But AY957203 is more distant to chimpanzees than AY210529 is.

Of the 11 independent sequences of hyper-variable region I, 9 showed more similarity between modern humans and chimpanzees than between Neanderthals and chimpanzees and 2 (both of 31 bp in length) showed equal similarity (Table 2). The observation of 9 cases of more similarity between humans and chimpanzees against 0 case of more similarity between Neanderthals and chimpanzees is highly significant by Chi square test (Chi-square = 6, $P<0.025$, one degree of freedom). In addition, the two published hyper-variable region II sequences of Neanderthals are also more distant to chimpanzees than modern humans are. The data suggests that significantly more human samples than Neanderthals show higher similarity with chimpanzees. A randomly chosen human mitochondrial DNA is almost certain to be more related to chimpanzees than a randomly chosen Neanderthal mitochondrial DNA is. Therefore, modern humans are significantly more related to chimpanzees than the Neanderthals are.

Another way to test the statistical significance of this finding is to randomly select a number of human sequences and see how many of them are more or less related to chimpanzees than a randomly selected Neanderthal sequence is. If humans and Neanderthals are equally related to chimpanzees, then the number of human sequences that are more related to chimpanzees than the Neanderthals are should be similar to the number of human sequences that are less related to chimpanzees than the

Neanderthals are. I randomly selected 20 human mitochondrial sequences from Genbank and determined whether their similarity to chimpanzees is greater or less than the similarity between the Neanderthal sequence AF011222 (379bp) and chimpanzees. The Neanderthal sequence AF011222 was used to BLAST or retrieve all the human mitochondrial sequences from Genbank (about 14000 sequences) that show significant similarity with the Neanderthal sequence, ranging in identity between 94% and 90%. From this list of 14000 human sequences ordered by their degree of similarity with the Neanderthal sequence, every 700 th sequence was selected for analysis and a total of 20 was selected. All 20 sequences showed greater similarity with chimpanzees than the similarity between the Neanderthal sequence AF011222 and chimpanzees. This is highly significant by Chi square test (Chi-square = 13.33, $P<0.001$, one degree of freedom).

To confirm this result using only independently verified or conserved Neanderthal sequences, I next identified a region of 186 bp that is completely shared by 4 independent Neanderthal samples, DQ859014 (303 bp, sample from Spain), AF011222 (379bp, sample from Feldhofer, Germany), AY149291 (357 bp, sample from Feldhofer 2, Germany), and AF282971 (357 bp, sample from Vindija Cave, Croatia). This conserved 186 bp region is from position 118 to 303 of DQ859014. I next analyzed the corresponding 186 bp region from the 20 randomly selected human sequences that were used in the above analysis. All of these human sequences show more similarity between human and chimpanzee than between Neanderthal and chimpanzees ($P<0.001$). The Neanderthal 186 bp region is also more distant to chimpanzees than the human Cambridge Reference sequence. Thus, even when I only used conserved Neanderthal sequences that have been verified by independent sequencing analysis of 4 independent samples from 3 vastly separate locations, I still found that Neanderthals are significantly more distant to chimpanzees than modern humans are. The analysis

suggests that the greater distance between Neanderthals and chimpanzees as observed here is not a result of sequencing errors or artifacts.

Neanderthals are more distant to gorillas than modern humans and chimpanzees are

I next examined whether Neanderthals are also more distant to gorillas than chimpanzees and modern humans are. If Neanderthals were alive today, they would be equidistant to gorillas as chimpanzees and humans are. If the molecular clock hypothesis is true, ancient Neanderthals cannot be more distant to gorillas than extant chimpanzees or modern humans are.

I first made sure that my method of analysis using mitochondrial DNA is able to reveal or confirm that humans and chimpanzees are indeed equidistant to gorillas. I randomly selected 30 hyper-variable region I sequences from a list of 1000 chimpanzee sequences from Genbank that were retrieved by BLAST using the Neanderthal DQ859014 (303 bp) sequence (every 33 th sequence from this list was selected). Each of these sequences was used to identify a most closely related human sequence. Next, each chimpanzee sequence and its corresponding human sequence of equal length were used to obtain a best BLAST similarity score with gorilla non-nuclear mitochondrial sequences in the Genbank. These scores were used to determine whether the distance between a chimpanzee sequence and gorillas is greater or not than that between a corresponding human sequence and gorillas. Among the 30 comparisons, 13 showed greater similarity between chimpanzees and gorillas than between humans and gorillas, 11 showed less, and 6 showed equally similarity ($P>>0.05$). The result therefore confirms the well-established genetic equidistance phenomenon where chimpanzees and humans are equidistant to gorillas.

I next examined all available Neanderthal mitochondrial sequences that are long enough to be informative for my analysis (longer than 30 bp of homology with gorillas). These included 6 hyper-variable region I and 2 hyper-variable region II sequences. For each Neanderthal sequence, a closely related chimpanzee sequence of equal length was selected. Each Neanderthal sequence and its corresponding chimpanzee sequence were used to obtain a best BLAST similarity score with gorilla non-nuclear mitochondrial sequences in the Genbank. All 8 comparisons showed greater similarity between chimpanzees and gorillas than between Neanderthals and gorillas ($P < 0.025$ by chi square test, one degree of freedom). This result is significantly different from the result of equidistance of humans and chimpanzees ($P< 0.025$).

Therefore, while my analysis showed that humans and chimpanzees are equidistant to gorillas, it also showed that ancient Neanderthals are significantly more distant to gorillas than chimpanzees are. It suggests that the difference between Neanderthals and chimpanzees in their distance to gorillas is outside the variation range that is allowed by the genetic equidistance phenomenon. By the same standard that is used to justify the conclusion that humans share the same distance to gorillas as chimpanzees, Neanderthals clearly do not share the same distance to gorillas as chimpanzees. This result further supports the conclusion that Neanderthals are more distant to an outgroup than extant sister species are.

Since most of the Neanderthal mitochondrial DNAs have been carefully studied to be authentic, DNA damage is unlikely the cause for the higher distance between Neanderthals and chimpanzees. The fact that several independent Neanderthal DNA specimens share the same mutations also makes it highly unlikely that the Neanderthal specific mutations are a random result of DNA damage. Indeed, these Neanderthal DNA sequences have been previously used to justify the claim that Neanderthals did not contribute to human DNA pools (Krings et al., 1997). Obviously such claims would be

baseless if the Neanderthal DNA sequences were to contain significant amount of sequencing errors or artifacts.

I also analyzed the Neanderthal nuclear DNA data reported recently (Green et al., 2006; Noonan et al., 2006). These data also show that Neanderthal DNAs are more distant to chimpanzees than modern humans are. However, since these data contain large amounts of sequencing errors and artifacts that have not been eliminated by multiple independent analysis of multiple independent samples, they are not very informative for the analysis here. Future studies will be needed to uncover truly authentic nuclear DNA sequences that can be used to substantiate the conclusion drawn here from analysis of authentic mitochondrial DNA sequences.

Dinosaurs are more distant to frogs than extant birds are

Next, I analyzed the recently published peptide sequences of collagen fragments derived from a 68-million-year-old dinosaur (*Tyrannosaurus rex*, MOR 1125) (Asara and Scheweitzer, 2008; Asara et al., 2007b). Of the seven peptide sequences of *T. rex* available for analysis, six are not informative as they are clearly identical to some extant species and therefore must be equally distant to an outgroup as the extant species are. However, one 15 amino acid (aa) peptide, GAPGPQGPAGAPGPK, appears to be unique to *T. rex* (Asara et al., 2007b). The more authentic version of this peptide was subsequently found to be GAPGPQGPSGAPGPK (Asara et al., 2007a; Asara and Scheweitzer, 2008). This *T. rex* peptide is most similar (14/15 identity) to collagen α1t2 (COL2A1) from many species including chicken (*G. gallus*), suggesting that it is derived from α1t2 of *T. rex.* It is also similarly related to newt α1t1, which has led Asara and Schwitzer to assign it as α1t1 (Asara and Scheweitzer, 2008). But that may be

mistaken. The α1t2 assignment here is more consistent with the fact that *T. rex* is closer to bird than to amphibians.

My analysis of this 15 aa α1t2 peptide shows that all extant species examined (3 amphibians, 1 bird, 6 mammals) that are descended from fish are all identical to each other at a region of 13 aa corresponding to residue 3 to 15 of the *T. rex* peptide. This 13 aa region is therefore 100% conserved among extant amphibians, birds, and mammals. In contrast, the *T. rex* α1t2 peptide is 12/13 identical to amphibians. Since the change of one amino acid occurred at a 100% conserved region among all extant fish descendants, it cannot be an insignificant neutral mutation. So, dinosaurs are significantly more distant to amphibians than birds are.

I next compared full-length collagen α1t2 (~ 1400 aa) of frogs, chickens, and humans to confirm that chickens and humans are roughly equidistant to frogs at the collagen α1t2 locus. Indeed, for the α1t2 full-length protein frogs are 88% identical to chickens and 86% identical to humans. Thus, the α1t2 protein is a valid and consistent indicator of the genetic equidistance phenomenon.

Mastodons are more distant to opossums than extant mammals are

I also analyzed the 4 collagen peptide sequences of a 160,000- to 600,000-year-old mastodon (Asara et al., 2007b). All 4 are unique to mastodon and are not identical to any sequence in the database. The 9 aa pepdide GSEGPQGTR is derived from a region in α1t1. This region of α1t1 is 100% or 9/9 identical among all extant mammals examined, including opossum (*Monodelphis domestica*), cow, dog, rat, mouse, and human. In contrast, the mastodon sequence is 8/9 identical to opossum, indicating that mastodon is more distant to opossum than its extant sister placental mammal species.

The 15 aa peptide GAPGPQGPGGAPGPK is derived from the same region of α1t2 as the above discussed *T. rex* peptide (Asara et al., 2007b). The authentic version of this peptide was later found to be GAPGPQGPGGSPGPK (ASARA ET AL., 2007A). Its closest match, among all known sequences, is in fact the *T. rex* sequence (13/15 identical). As discussed for *T. rex*, a 13 aa region of this α1t2 peptide is 100% or 13/13 identical among all extant mammals examined. In contrast, the mastodon sequence is 10/13 identical to opossum, again indicating that mastodon is more distant to opossum than its extant sister placental mammal species.

The 19 aa peptide EGAPGSEGAPGRDGAIGPK is derived from α1t1. It is 14/19 identical to opossum. In contrast, all placental mammals examined, including human, cow, dog, rat, mouse, are 17/19 identical to opossum. The identities among all mammals are all equal or higher than 17/19. The 33 aa peptide GLTGPIGPPGPAGAPGDKGEGGPSGPAGPTGAR is derived from α1t1. In this case, mastodons and other placental mammals (rat, cow, dog, and mouse) are equally related (29/33) to opossums. So, the combined results from the 4 peptides suggest that both α1t1 and α1t2 of mastodons are more distant to opossums than extant sister species are.

To further confirm that extant mammals are indeed equidistant to opossums at the α1t1 and α1t2 loci, I analyzed the sequence identity of full-length α1t1 and α1t2 between opossums and cows and between opossums and humans. For the full-length α1t2 protein, opossums are 95% identical to cows and 95% identical to humans. For the full-length α1t1 protein, opossums are 90% identical to cows and 90% identical to humans. Thus, both α1t1 and α1t2 proteins are valid and consistent indicators of the genetic equidistance phenomenon.

The genetic distance between dinosaurs and mastodons is greater than that between extant birds and mammals

The molecular clock hypothesis predicts that ancient birds and ancient mammals should be closer than extant birds and mammals are. The 15 aa peptide, GAPGPQGPSGAPGPK, of collagen α1t2 of dinosaurs offers an opportunity to test this prediction since equivalent sequences of this peptide is available for the ancient mammal mastodon and for many extant birds and mammals as described above. For a 13 region corresponding to residue 3 to 15 of the *T. rex*. peptide, all extant birds and mammals are 100% identical. However, the *T. rex*. sequence is 11/13 or 85% identical to mastodon. So the genetic distance between dinosaurs and mastodons is greater than between extant birds and mammals.

Summary

This study shows that DNA and protein sequences of ancient fossil specimens are not consistent with the molecular clock hypothesis. Although it remains a possibility for some ancient sequences to be non-authentic, it seems unlikely at least in the case of Neanderthals where some of the sequences have been verified multiple times using multiple independent specimens. Data inconsistent with the constant mutation rate hypothesis have so far come only from studies on extant organisms. The study here represents the first time that ancient fossil sequences were found to be inconsistent with the molecular clock hypothesis.

Acknowledgments:

I thank Dr. John Hawks for critical reading of the manuscript. This work was supported by NIH (RO1 CA 105347).

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Table 1. Similarity scores between Neanderthals (N) and chimpanzees (C) and between modern humans (MH) and chimpanzees. Each Neanderthal sequence is represented by its length; for example N357 represents the Neanderthal sequence that is 357 nt in length (AF282971). For each sequence comparison, the highest scores and the lowest scores are shown.

	N vs. C	MH vs. C
N357	234-72	262-72
N379	234-56	309-80
N378	246-58	278-80
N301	218-62	240-64
N345	244-66	256-72
Average	235-63	269-74
African		
Mandenka		272-74
Kikuyu		262-72
Fulbe		254-68
Kanuri		206-62
Tuareg		270-72
Average		253-70
Ancient Australian		
LM3		232-62
KS1		252-64
KS13		256-64
KS16		230-64
KS7		244-64
KS8		264-48
Average		246-61

Table 2. Significantly more modern human samples than Neanderthal samples show more similarity with chimpanzees. Each Neanderthal sample sequence has a sequence name indicating its length in base pairs. Most of these have Genbank accession numbers as listed. For samples of very short sequence of 31 bp, no Genbank accession numbers are available and the sequences were retrieved from the original publication (Serre et al., 2004). For each Neanderthal sample, a closely related modern human sequence sample with accession number listed was selected for analysis. These Neanderthal-like human sequences are in fact less similar to chimpanzees than other human sequences are. For each sample sequence, the similarity between modern humans (MH) and chimpanzees (C) could fall into one of three categories: higher, equal, or lower than that between Neanderthals (N) and chimpanzees, which is indicated by the '+' sign under each category.

			Similarity		
Seq. Name	Neanderthal	M. Human	MH-C>N-C	MH-C=N-C	MH-C<N-C
N357	AF282971	AY210562	+		
N379	AF011222	AY957203	+		
N357.2	AY149291	AF346985	+		
N123	DQ464008	AF243467	+		
N378	DQ836132	AY232956	+		
N303	DQ859014	AY390323	+		
N345	AF254446	AY632956	+		
N31-Vi80		EF187311	+		
N31-Vi77		DQ359679	+		
N31-Engis 2		DQ359638		+	
N31-La Chapelle-aux-Saints		EF187295		+	
Total			9	2	0 (P<0.025)