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The thermal history of human fossils and the likelihood of successful DNA amplification

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Abstract

Recent success in the amplification of ancient DNA (aDNA) from fossil humans has led to calls for further tests to be carried out on similar material. However, there has been little systematic research on the survival of DNA in the fossil record, even though the environment of the fossil is known to be of paramount importance for the survival of biomolecules over archaeological and geological timescales. A better understanding of aDNA survival would enable research to focus on material with greater chances of successful amplification, thus preventing the unnecessary loss of material and valuable researcher time. We argue that the thermal history of a fossil is a key parameter for the survival of biomolecules. The thermal history of a number of northwest European Neanderthal cave sites is reconstructed here and they are ranked in terms of the relative likelihood of aDNA survival at the sites, under the assumption that DNA depurination is the principal mechanism of degradation. The claims of aDNA amplification from material found at Lake Mungo, Australia, are also considered in the light of the thermal history of this site.

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Keywords: Ancient DNA; Neanderthal; Modern Humans; Lake Mungo; Thermal History

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Introduction

The phylogenetic relationships between modern humans (*Homo sapiens*) and other species of *Homo* are a source of much debate (e.g. Mellars, 1999). A recent approach to collecting data to investigate the relationships is to isolate genetic material from fossil specimens (e.g. Krings et al., 1997; Ovchinnikov et al., 2000; Krings et al., 2000; Schmitz et al., 2002 for Neanderthals; Adcock et al., 2001 for early Australians), and compare this with modern human DNA. Initial successes have led to calls for further genetic sequences, from different geographic locations and age ranges (Höss, 2000; Adcock et al., 2001); however, the quality of aDNA studies in general, has recently been brought into question (Cooper & Poinar, 2000). The isolation of DNA from fossils is a destructive process, and when dealing with valuable material such as fossil humans it is important that a precautionary approach is taken. Assessment of the likelihood of a sample containing amplifiable DNA should be a prerequisite for such work, maximising returns from researcher time, funding, and most importantly, the valuable and irreplaceable resource of the fossils themselves.

It has been observed that DNA appears to survive best in cold dry environments such as permafrost, or high altitude caves, and biochemical studies suggest it is unlikely to survive for more than 100 000 years (Wayne et al., 1999). Yet, there has been little systematic work on the long-term survival of DNA in the fossil environment, and consequently assessments of the prospects of DNA survival remain anecdotal. The need to find further fossil human DNA sequences from wider geographical and temporal ranges is compelling. The proposed upper bound to survival of 100 000 years encompasses both the extinction of the Neanderthals, and the diversification of modern humans, but the oldest successful amplifications are from permafrost, not sites of human occupation.

While it is apparent that fossils from cold environments will have better biomolecular preservation than those from hot climates, and that younger fossils will be better preserved than older ones, the distinction between an old and cold fossil

and a young hot one is more difficult to assess. Attempts have been made to relate temperature dependent rates of DNA depurination to absolute copy numbers (Pääbo & Wilson, 1991; Marota et al., 2002), but they appear to have been over simplistic and as a result inaccurate (see discussion).

The preservation of biomolecules in the fossil environment is complex, especially in bone (Collins et al., 2002). In brief, bone degradation is considered to occur mainly by two processes; one rapid, mediated by microorganisms and fungi (Hackett, 1981; Bell et al., 1996), and the other, chemical degradation, which is a relatively slow process. For skeletal material to become part of the fossil record it is likely that microbial attack will have to be excluded (Trueman & Martill, 2002). If microbial taphonomy is inhibited, then the two major chemical pathways that will lead to DNA destruction are condensation (e.g. glycation of nucleobases, Pischetsrieder et al., 1999) and hydrolysis of the purine bases (Lindahl & Nyberg, 1972). The importance of cross-linking in the survival of DNA has not been investigated in detail so far (see Poinar, 1999). Principal factors that influence the rate of hydrolytic depurination are pH, amount of chemically available water and temperature. The first two factors are less significant in bone as bone itself exerts a substantial buffering effect between pH 4–9 (Bada & Shou, 1980), and the pore size distribution of bone encourages water retention (Hedges & Millard, 1995; Turner-Walker et al., in press). Deep burial will buffer temperature fluctuation, but only around an annual mean, and thus temperature is likely to play a substantial role in defining the envelope of DNA survival (Smith et al., 2001). Here we present a more detailed account of our assessment of the thermal history of fossil hominid sites from Northern Europe, and use this to rank sites according to their thermal age. We define thermal age as the time taken to produce a given degree of DNA degradation when temperature is held at a constant 10°C. The thermal age adjusts the chronological age of different sites according to their individual thermal histories, using the known temperature dependence of DNA depurination estimated in aqueous solution. A comparison is made between the DNA

depurination thermal ages of sites in NW Europe and Lake Mungo, a site in Australia where controversial claims have been made for the recovery of ancient DNA (Adcock et al., 2001; Cooper et al., 2001).

Methods

The thermal regime of a fossil is governed by two major factors, the mean temperature and the variation about this mean, both of which will vary over time due to climatic changes. Thus, to reconstruct the thermal history of a fossil, data must be obtained for both the modern day temperature of the site and the palaeotemperature.

For a fossil buried in open ground the thermal regime of the fossil is assumed to be the same as that of the surrounding soil. It has been demonstrated that good estimates ($\pm 1^\circ\text{C}$) of mean soil temperature can be taken from mean air temperature (MAT) data obtained from local weather stations (Kusada & Achenbach, 1965; Toy et al., 1978), although the thermal regime (i.e. extent of seasonal fluctuation in the soil) is difficult to predict. The amount of seasonal fluctuation in the burial environment, and hence the *effective* MAT for a chemical reaction (see Wehmiller et al., 2000) will be controlled by many factors including; burial depth, the fluctuation in the climate, the thermal properties of the burial environment (Krarti et al., 1995) and local effects (e.g. vegetation or snow cover). Consequently, assessment of the thermal history of open sites is difficult as such information is not usually available nor can it be readily extrapolated back into the past.

In the cave environment the MAT is generally considered a good approximation of local deep cave air temperatures (Bogli, 1980). Deep cave temperatures are also known to be quite static and prone to little seasonal variation, also humidity in caves is high, and not prone to large fluctuations. For a fossil buried in a cave sediment, any fluctuations around the mean value will be dampened further by the cave sediment. It should be considered however that there is more fluctuation at the cave entrance in both temperature and humidity.

We have attempted to reconstruct the thermal history of Northwest European Neanderthal cave sites, and that of the early anatomically modern human site of Lake Mungo in Australia, by combining both modern day temperature data, and palaeoclimatic evidence.

Thermal model for Northwest European Neanderthal cave sites

Modern day mean annual temperatures of the Neanderthal cave sites listed (Table 1) have been estimated from weather station data nearest to each locality. For simplicity, and due to a lack of quantitative palaeoclimate data, the Holocene is considered to be a stable climatic period with constant temperature back to 10.4 kyr BP. Temperatures for this period were estimated from sequences of continuous data (years-decades) from a weather station in the locality of each Neanderthal site (Baker et al., 1994). Only mean temperatures are used in this model, as there is assumed to be no variation about the mean for the burial environment.

Altitudes of the cave sites in metres have been estimated from GTOPO30 (Gesch et al., 1999) using a weighted average of the four nearest values to the location unless the correct altitude is known (italics, Table 1). For eleven sites where the altitude is known, the best fit relationship between GTOPO30 estimates and true altitude is given by

$$\text{altitude estimate} = 1.07 \times \text{known altitude} + 10.42 \text{ m.}$$

The correlation is highly significant, $R^2 = 0.977$, $n = 11$.

A thermal lapse rate with altitude of $6^\circ\text{C}/\text{km}$ has been used to account for differences in altitude between the cave site and the nearest weather station.

Pleistocene thermal histories of the sites have been reconstructed by applying one of four different regional models, (i) northern France and the Benelux countries, (ii) Germany, (iii) Poland (based on data in Aalbersberg & Litt, 1998; Huijzer & Vandenberghe 1998; Coope et al., 1998; Witte et al., 1998) and (iv) Southern France (Fig. 1). The thermal history of southern France has

Table 1
Ranking of European Neanderthal Cave sites based upon thermal age

Site	Age kyr	Palaeoclimate Model	Altitude (m)	MAT°C Holocene	Thermal Age _(kyr@10°C)	Thermal Age _(kyr@10°C) at				
						10 kyr	32 kyr	50 kyr	74 kyr	130 kyr
Ochoz	NA	P	524	6.9		5	11	13	17	32
Khulna	NA	P	514	7		6	11	13	17	33
Sipka	NA	P	432	7.1		6	11	14	17	33
Spy	NA	NF	179	8.9		8	13	16	19	34
Subalyuk	NA	P	416	8.2		7	13	16	19	38
Engis	NA	NF	172	9.3		9	14	16	20	35
Fond de Foret	NA	NF	183	9.5		9	14	17	20	37
La Naulette	NA	NF	168	9.6		9	15	17	20	37
Soulabe las Maretas	NA	SF	754	8.9		8	13	16	21	38
Neanderthal	50	G	128	10.1	19	10	16	19	22	43
Saint Brelade	NA	NF	10	11.1		12	19	21	25	47
Arcy sur Cure	32	SF	125	11.4	21	13	21	26	34	61
La Chaise (B.-Delauney)	NA	SF	393	10.4		11	18	22	28	50
Malarnaud	NA	SF	475	10.5		11	18	22	28	51
Rigabe	NA	SF	400	11.4		13	21	26	34	61
Regourdou	NA	SF	196	11.6		14	22	27	35	63
Rochelot	NA	SF	141	11.9		14	23	29	37	67
Montgaudier	NA	SF	128	12		15	24	29	38	68
Caminero	NA	SF	131	12.1		15	24	30	39	70
Fontchevade	NA	SF	120	12.1		15	24	30	39	70
Petit Puymoyen	NA	SF	131	12.1		15	24	30	39	70
Chateauneuf sur C	NA	SF	102	12.2		15	25	31	39	71

Table 1 (continued)

Site	Age kyr	Palaeoclimate Model	Altitude (m)	MAT°C Holocene	Thermal Age (kyr@10°C)	Thermal Age (kyr@10°C) at				
						10 kyr	32 kyr	50 kyr	74 kyr	130 kyr
Hortus	NA	SF	390	12.2		15	25	31	39	71
Marillac	NA	SF	106	12.2		15	25	31	39	71
Rene Simard	NA	SF	103	12.2		15	25	31	39	71
Montmaurin	NA	SF	355	12.3		15	25	31	40	72
Quinzano	NA	SF	71	12.5		16	26	32	42	75
Orgnac	NA	SF	320	12.7		17	27	34	43	78
Pech de l'Aze	NA	SF	169	12.7		17	27	34	43	78
La Masque	NA	SF	150	12.8		17	28	34	44	80
La Chapelle aux Saints	NA	SF	149	12.9		17	28	35	45	81
Combe Grenal	NA	SF	108	13.1		18	29	36	47	84
Lezetxiki	NA	SF	266	13.3		19	31	38	49	88
Monsempron	NA	SF	71	13.3		19	31	38	49	88
Roc de Marsal	NA	SF	75	13.3		19	31	38	49	88
Grotta del Principe	NA	SF	12	14.7		24	40	49	64	115
La Chaise (Abri Suard)	130	SF	393	10.4	50	11	18	22	28	50
Caverna delle Fate	NA	SF	280	15.2		26	44	54	70	126
Lazaret	NA	SF	25	16.1		31	52	64	83	149
Mezmaiskaya (constant temp)	32	Holocene	1310	5.7	14	4	14	N/A	N/A	N/A
Mezmaiskaya (with cooling)	32	Holocene, 0°C Pliostocene	1310	5.7	7	4	7	N/A	N/A	N/A

NB. DNA has been successfully amplified from material from Feldhofer (Kriings et al., 1997, Schmitz et al., 2002), the later study dating material to 40 kyr BP in which case the thermal age of the material is $\sim 17_{\text{kyr@10}^\circ\text{C}}$ and Mezmaiskaya (Ovchinnikov et al., 2000); an unsuccessful attempt has been made from material from La Chaise Abri Suard (Cooper et al., 1997).

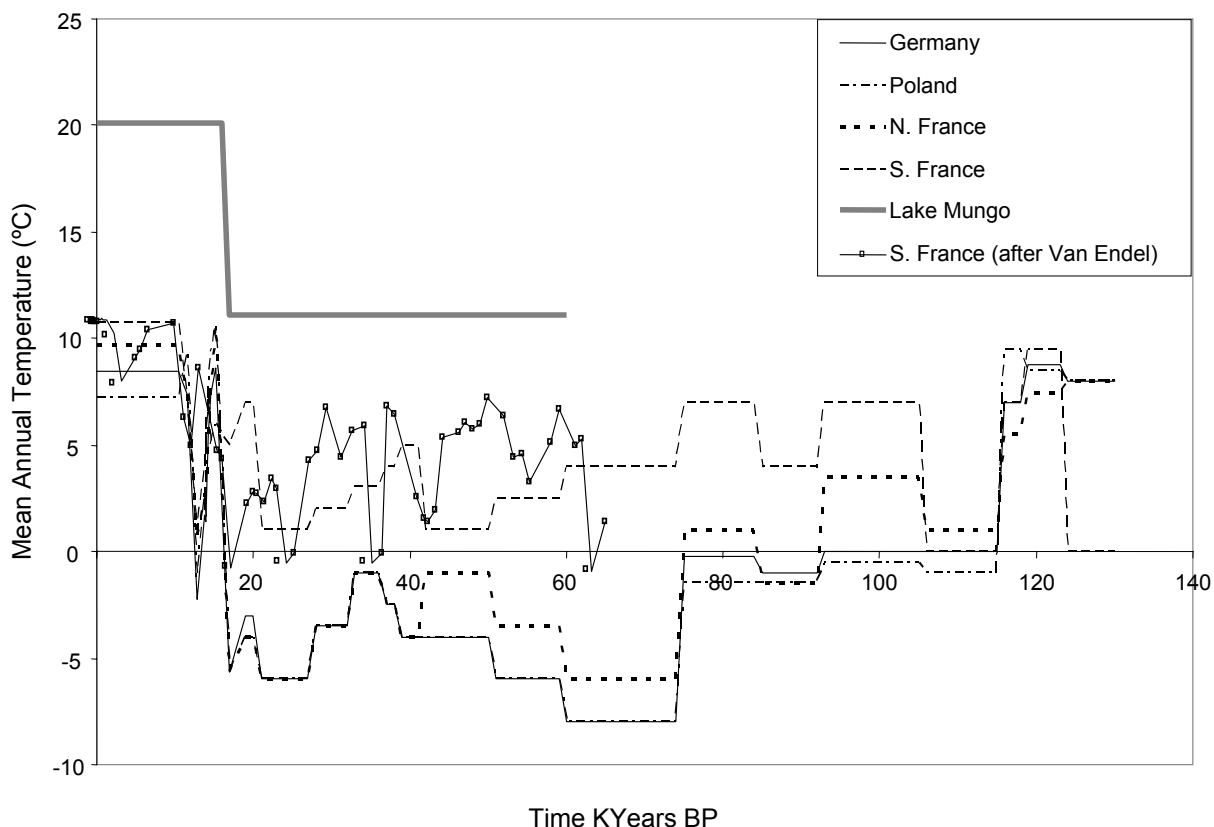


Fig. 1. Regional palaeoclimate models for Northwest Europe and Lake Mungo Australia. Holocene (0–10.4 kyrBP) temperatures are the average mean air temperature taken from the ISLSCP data set (Meeason et al., 1995; Sellers et al., 1995) for Germany, Poland, S. France and N. France. S. France alternative based on Van Andel, 1997, and Lake Mungo on Ambrose, 1984 and Miller et al., 1997.

been reconstructed using quantitative data from Guiot et al. (1993), compiled using methods similar to those of the other studies. In cases where palaeoclimatic data are incomplete, palaeotemperature values have been assigned based on similar climatic periods for the same region, or values in similar regions for the same period.

The average difference between modern mean annual temperature taken from the International Satellite Land Surface Climatology Project (ISLSCP 1) data set (Meeason et al., 1995; Sellers et al., 1995) and that of the reconstructed mean annual temperature for the last glacial maximum (LGM) has been calculated for each region. To assign each site a temperature for the last glacial maximum, the average LGM/Holocene difference is subtracted from the Holocene temperature for

each site. All other differences between Holocene and palaeotemperatures are calculated relative to this LGM/Holocene difference. Thus each site has a palaeotemperature curve based on its current (Holocene) temperature, and its regional palaeoclimatic curve. There is an implicit assumption in this approach; that current differences in temperature caused by geographic location etc. are constant with time. In part this assumption is true, as the main differences in temperature between sites (especially within one region) will be due to differences in altitude, these differences are maintained in this model (although this does assume that the thermal lapse rate remains constant). Conversely the palaeoclimatic evidence suggests that the temperature gradient across Europe, currently north-south, has fluctuated in the past, in both direction

and inclination, and local effects such as vegetation cover and local weather conditions will have changed. These factors will affect both inter- and intra-regional variation, and are not accounted for in the model. The reconstructions also ignore the possibility that geothermal heating may have elevated cave temperatures during periods of reduced precipitation in the Pleistocene.

An alternative climate model for southern France has been used, for comparative purposes, based on the climate curve produced by Van Andel (1997), essentially a reconstruction based on palaeoclimatic data from Les Echets (Guiot et al., 1989). This model is not confined to the chronology of the data above, but only covers the last 60 kyr. This model has been used to compare how thermal ages differ using alternative palaeoclimate models.

During periods of glaciation it is likely that cave deposits underwent periods of freezing. The relationship between air temperature and permafrost surface temperature is difficult to predict due to the zero curtain effect (Zhang et al., 1997). However, the rate of depurination in frozen samples is thought to be weakly temperature sensitive (Osborne & Phillips, 2000) so this problem is somewhat circumvented. For the purposes of modelling we have taken a nominal effective temperature of 0°C for fossils in permafrost, i.e. where the model predicts a temperature of 0°C or less.

The thermal age of Mezmaiskaya has also been reconstructed, although we lack quantitative palaeoclimatic data for this region. We have therefore used two reconstructions for comparison: one using a constant Holocene temperature throughout, the other using 0°C for the first 22 kyr of deposition and the Holocene temperature for the last 10 kyr.

Thermal model for Lake Mungo

The thermal history of Lake Mungo has been reconstructed using measured sediment temperatures from Lake Mungo (Ambrose, 1984) and reconstructed palaeotemperatures inferred from isoleucine epimerisation rates from ratite eggshells (Miller et al., 1997). The data for burials at 1.5 m at stations 1 and 2 (Ambrose, 1984) were used to calculate the effective burial temperatures.

Effective DNA depurination temperature (T_{eff}) is the constant temperature equivalent which causes the net equivalent depurination that will have been accrued by the DNA over one year, taking into account seasonal temperature fluctuation and using a temperature dependent reaction having an activation energy of E_a 127 kJ mol⁻¹ (Lindahl & Nyberg, 1972). Total annual reaction was calculated using a sinusoidal wave model using the mean annual temperature and variation about this mean for modern temperatures and palaeotemperatures (see Appendix B for an example of the calculation of thermal age). The palaeoclimate of Southern Australia is markedly different to that of Northwest Europe. A model based upon amino acid epimerisation data indicates a 9°C warming after 16 kyr BP with no other significant excursions (Miller et al., 1997). We have therefore used a simple palaeoclimatic model using the present day effective temperatures calculated from sediment temperatures for the period 16–0 kyr BP, which was preceded by a period 9°C cooler than the present arithmetic mean annual temperature, from 60 kyr BP–16 kyr BP (Fig. 1).

In addition we have calculated the thermal age at constant mean temperature, to account for dampening effects assuming that the burial was at great depth for most of its history.

Rates of biomolecular deterioration

The amount of DNA depurination at each site has been calculated as the integration of the site's palaeoclimatic curve, the age of the fossils at the site, and the activation energy (E_a) of the depurination reaction (Lindahl & Nyberg, 1972). To enable comparison, sites are ranked using 'thermal age', where the calculated rates are normalised to what the rate would be at 10°C (see Appendix B). Sites that have multiple occupancy or have not been accurately dated, a number of alternative thermal ages are given corresponding to different actual periods of burial.

Results and discussion

The results of this analysis can be seen in Table 1. The Feldhofer cave site from which DNA

has been amplified is ranked as ten of thirty-nine, with a thermal age of $\sim 19_{\text{kyr}@10^\circ\text{C}}$. If our estimate of the Holocene temperature is 1°C in error at this site, the thermal age is approximately $3_{\text{kyr}@10^\circ\text{C}}$ ($\sim 16\%$) different. The sequence of ages given for each site reveals an important feature of the thermal history. For example, at Feldhofer, if a fossil is considered to be 50 kyr, 45% of the thermal age is accumulated over the first 40 kyr of deposition, and 55% of the thermal age is accumulated during the last 10 kyr. Recent radiocarbon determinations have directly re-dated the fossil humans at Feldhofer to about 40 kyr (Schmitz et al., 2002), this re-dating to 40 kyr will reduce the thermal age by only $2_{\text{kyr}@10^\circ\text{C}}$. Furthermore, if a fossil was deposited 74 kyr BP at Feldhofer, the thermal age would only be $\sim 3_{\text{kyr}@10^\circ\text{C}}$ older than a fossil deposited at 50 kyr BP. This reveals the importance of considering the entire thermal history of a fossil, and the effect of the warming during the Holocene. This also indicates that the temperature at which the fossil is held during the Holocene is of key importance to biomolecular survival, and that the absolute age of the fossil may be of negligible importance if the fossil is kept very cold. Using the alternative climate model based on Van Andel (1997) a southern French site with a current estimated temperature of 12.2°C (e.g. Marillac) will have its thermal age reduced by approximately $1_{\text{kyr}@10^\circ\text{C}}$ at 50 kyr, and by $2_{\text{kyr}@10^\circ\text{C}}$ at 74 kyr (3–5%). This demonstrates some of the error involved in estimating thermal age, and highlights the importance of the climate model.

The thermal age of Mezmaiskaya (also a site where aDNA has been successfully amplified; Ovchinnikov et al., 2000) is likely to have been overestimated here, where we have not modelled any cooling during the Pleistocene, but it is still lower than that of Feldhofer cave. This is probably due to the altitude of the site (1.3 km), keeping the site cold in comparison with others.

If published data of aDNA amplification success and failure is compared with the age of the material, there is no apparent pattern of survival, however, if it is compared to the thermal age of the material, then it becomes apparent that only thermally young material is likely to yield amplifiable aDNA (Fig. 2). Most successes come from either

permafrost deposits or young material (<5 kyrs) from temperate regions (mainly Europe). Thus, one might expect the limit of DNA survival to be defined by the thermally oldest material from this area, i.e. Feldhofer (Krings et al., 1997) and Scladina (Lorreille et al., 2001), particularly when considering the technical difficulties of these studies. When the thermal age of Lake Mungo is compared to other studies, the thermal age is far greater than any other reported successes.

Pääbo & Wilson (1991) used a theoretical approach, based upon the rate of depurination of DNA at 15°C and pH of 7.0 in physiological solution (Lindahl & Nyberg, 1972), to suggest that it would take approximately 5 ka to destroy the last amplifiable 800 bp of chloroplast DNA (cpDNA) fragment in 1 g of leaf tissue given a starting content of 10^{12} cpDNA g^{-1} . Marota et al. (2002) estimated the survival limit of cpDNA in papyri at 35°C to be ~ 800 years, using a reaction rate for depurination at 15°C based upon the values calculated by Pääbo & Wilson (1991), but instead of using the published activation energy (127 kJ m^{-1} , Lindahl & Nyberg, 1972) supposed a trebling of the rate of reaction per 10°C temperature increase (i.e. 76 kJ m^{-1}).

We have used a slightly modified approach to calculate the survival of the last fragment, using the original Lindahl & Nyberg (1972) kinetics (see Appendices). For the original conditions described by Pääbo & Wilson (1991) our estimate for the median survival time of the last surviving 800 bp fragment, from an original 10^{12} copies is an order of magnitude lower, 359 years (and the first and 99th percentiles of survival time are estimated at 334 and 413 years respectively). The corresponding estimate for 35°C (Marota et al., 2002), is 11.5 years (10.7 and 13.2; 1st and 99th percentiles, respectively). Assuming the copy number of mitochondrial DNA (mtDNA) in fresh bone to be about $2.5 \times 10^{11} \text{ g}^{-1}$ (Table 2) we estimate the survival of a 105 bp fragment in 0.4g of bone (the fragment and sample size used by Krings et al., 1997, to successfully amplify aDNA from the Neanderthal type specimen) at 10°C and pH 7.4 to be 15 ka (14 ka and 17 ka; 1st and 99th percentiles), extending to ~ 107 ka at 0°C (100 ka and 124 ka, 1st and 99th percentiles).

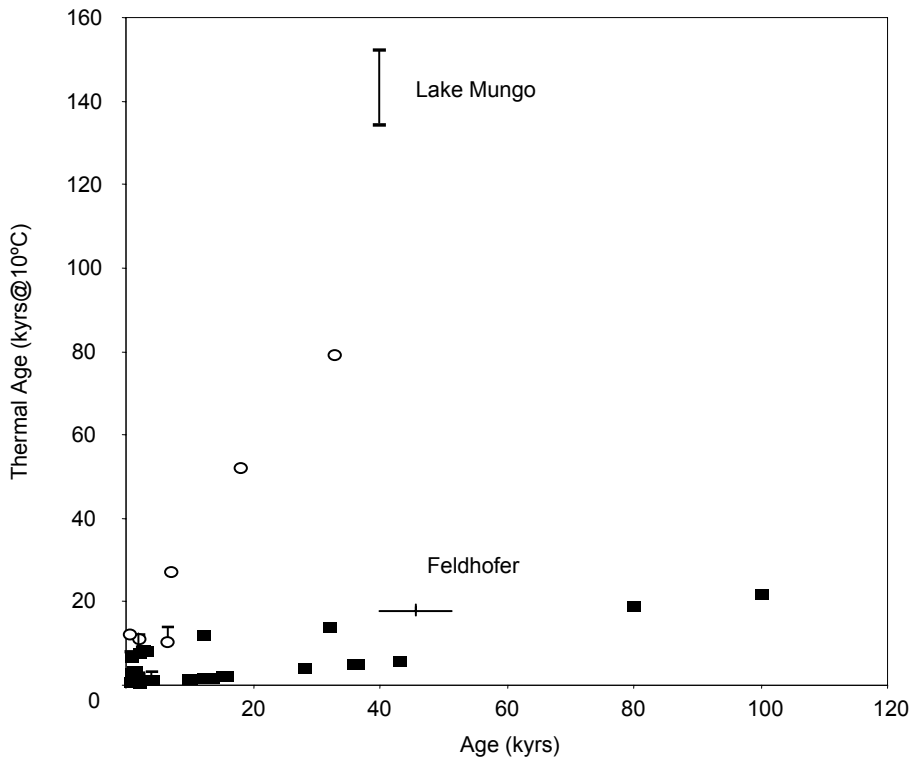


Fig. 2. Comparison of thermal age (@10°C) and fossil age for reports of DNA amplification success and failure. Full squares represent successful DNA amplification and open circles failures. Feldhofer is represented as a range reflecting the uncertainty of the age of the site. The range of Thermal Age at lake Mungo represents uncertainty in the thermal model to use due to possible different burial histories. (Data taken from Adcock et al., 2001; Bailey et al., 1996; Colson et al., 1997; Cooper et al., 1996; Cooper et al., 1997; Fleischer et al., 2000; Hoss et al., 1994; Krings et al., 1997; Leonard et al., 2000; Leonard et al., 2002; Loreille et al., 2001; Ovchinnikov et al., 2000; Stone & Stoneking, 1999; Vilà et al., 2001).

Our thermal age limit of $\sim 19_{\text{kyr}@10^\circ\text{C}}$ is thus beyond what may be estimated from the copy number calculation, 15 kyr. Copy number estimates of 105 bp fragments from the Feldhofer bones however, are approximately $2.5\text{--}3.75 \times 10^3$ copies remaining per gram of bone (Krings et al., 1997). Using the same parameters, this would suggest that the rate would need to be approximately 4.9 times slower than the rate at pH 7.4 at 10°C. The rate of depurination at pH 7.88 is approximately twice as slow as that at pH 7.4, and is a more realistic pH for reactions in bone. Furthermore, Lindahl (1993) reports a two-fold reduction in depurination rate when adsorbed to apatite. Marguet & Forterre (1998) report that rates of depurination are retarded for both double stranded and single stranded DNA in the presence

of chloride salts, due to their direct interaction with purine nucleotides; thus elevated salt concentrations may also reduce the predicted rates of DNA deterioration. Differences in absolute rates will not affect the relative rankings of the sites given in Table 1, but would compromise predictions of the remaining copy numbers (c.f. Pääbo & Wilson, 1991); absolute rates of DNA deterioration are therefore not estimated for the Neanderthal sites.

Given the technical difficulty of amplifications from the Neanderthal material and the variable state of bone preservation, we would suggest that in the absence of convincing screening methods, (see Collins et al., 1999) $19_{\text{kyr}@10^\circ\text{C}}$ is a sensible thermal limit. Note, however, that this does not mean that all sites with thermal ages lower than

Table 2
Estimation of mtDNA content in compact bone

	value	Comment
1 Volume occupied by osteocyte lacunae	1.5% of bone	HgIP of human bone (NB slightly higher values are seen in bird bone)
2 Volume occupied by osteocyte lacunae	1.7–2.8%	http://www.orl.med.umich.edu/orl/archgroup/a6.htm
3 Size of individual osteocyte lacunae μm	11.7–17.4 length 4.8–6.6 width 3–3.4 height	http://www.orl.med.umich.edu/orl/archgroup/a6.htm
4 Hence volume of osteocyte	$200 \mu\text{m}^3$	
5 Volume of osteocyte occupied by mitochondria	20%	in liver cells values of 15–25% reported
6 Skeletal Density of modern bone gcm^{-3}	1.4–1.7	increases ultimately to 3.0 due to loss of collagen and water
7 Hence numbers of osteocytes per gram modern bone	5×10^7	assuming a density of 1.47gcm^{-3}
8 No of mitochondria per cell	1000	Ranges from 800–1500 depending upon values given in (5)
9 Hence no of copies of mitochondria per gram	5×10^{10}	
10 No of copies of mtDNA per mitochondria	5	literature values range from 2–20
11 No of copies of mtDNA per gram of bone	2.5×10^{11}	estimate from 3×10^{10} – 1×10^{12} using the extremes given in (5) and (10)

HgIP, Mercury Intrusion Porosimetry.

Table 3
Modelled thermal ages of Lake Mungo fossils if buried at 1.5 m

Station	1	1	2	2
Year	82/83	83/84	82/83	83/84
Mean Soil at 1.5 m depth Temperature*	20.1	19.1	20.6	19.8
Soil Temperature at 1.5 m depth Amplitude*	2.5	1.7	7	5.9
Effective temperature after 16 kyr BP	20.4	19.2	22.5	21.2
Effective Temperature before 16 kyr BP	11.1	10.1	11.6	10.8
Thermal age (kyr@10C) if 60 kyr	162	133	216	176
Thermal age (kyr@10C) if 40 kyr	137	112	189	152
Thermal age (kyr@10C) if 60 kyr (no amplitude)	157	131	172	148
Thermal age (kyr@10C) if 40 kyr (no amplitude)	132	110	145	125

*Data taken from Ambrose (1984).

Feldhofer *will* contain amplifiable aDNA, as other factors may accelerate deterioration (Ovchinnikov et al., 2001; Collins et al., 2002). Furthermore, by amplification of shorter fragments it is possible to extend the temporal range further. Thus reports of the successful amplification of an 88 bp product from a cave bear 80 kyr–100 kyr at Scladina Cave (Loreille et al., 2001) with a thermal age of $\sim 20_{\text{kyr@10}^\circ\text{C}}$ are consistent with the results from Feldhofer (considering the actual rate of DNA degradation at Feldhofer is slower than we predict).

Using data from Ambrose (1984), a number of thermal models for the Lake Mungo site can be established (Table 3) based upon differences in temperature recorded at different times and different depths. Although the range of calculated ‘thermal ages’ for a 60 kyr fossil is large (131–216 $_{\text{kyr@10}^\circ\text{C}}$), they are an order of magnitude higher than that of Feldhofer cave. If Feldhofer cave lies close to the technical limit of amplification at 19 $_{\text{kyr@10}^\circ\text{C}}$ it would seem highly unlikely that there would be amplifiable DNA in the Lake Mungo fossils (especially as the longer fragment lengths of 153 to 189 bp targeted by Adcock et al. (2001) will decay 1.4 and 1.7 times more rapidly than a comparable 105 bp product). If the site is only 40 kyr old as has been suggested (Gillespie & Roberts, 2000; Bowler et al., 2003; but compare Grün et al., 2000), the thermal age is reduced by at most 16%, still much higher than that of Feldhofer. The extreme thermal age at the site, the poor state of preservation of the material, acknowledged as being too fragmentary to sex

reliably (Brown, 2000; Thorne & Curnoe, 2000), and having ‘negligible organic preservation’ (Gillespie & Roberts, 2000 p. 727) indicate that DNA preservation at the site as a whole is likely to be poor. This coupled with the concerns raised over the technical aspects of the Lake Mungo sequences (Cooper et al., 2001) suggest that the reports of amplification of aDNA from Lake Mungo should be treated with caution until the work can be better substantiated, e.g., repeated in another laboratory. If DNA can be extracted from fossils with thermal ages as high as that of Lake Mungo the potential for finding aDNA in other samples, particularly many of the Neanderthal sites, are greatly increased.

Conclusion

The application of molecular techniques to fossil materials is an expanding field, and can provide invaluable data on the relationships between modern humans and their fossil relatives. At present only a few authentic sequences of fossil hominid DNA have been reported. Without a better understanding of the survival of DNA in the fossil record, valuable fossils may be damaged and destroyed and much time wasted in the search for more aDNA sequences. The thermal history of fossils is a useful indicator for the likelihood of survival of biomolecules in the fossil record. If DNA depurination is considered to be the key mechanism of DNA degradation in fossil bone material (excluding the role of micro-organisms,

which may be a prerequisite for long term survival, Trueman & Martill, 2002), then the thermal history of the site will be a key parameter in defining the state of DNA preservation. The analysis of the thermal history of northwest European cave sites from which Neanderthal remains have been recovered, and subsequent conversion to a relative amount of DNA degradation, indicates that few other northwest European Neanderthal cave sites are likely to yield amplifiable DNA using present techniques. Furthermore when the relative amount of DNA degradation at Lake Mungo is compared with that of northwest European cave sites, we would predict that the likelihood of amplifying authentic endogenous DNA from fossils from this site is very low. If this is true, then the authenticity of this sequence must be brought further into doubt (Cooper et al., 2001).

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Appendices

Appendix A: Calculation of the probability of the last amplifiable fragment remaining with time

The calculation is based upon the assumptions that random scission occurs and that all fragments are equally vulnerable to degradation.

Let T_0 be the time at which the last amplifiable fragment is lost. Then the probability that the loss of the last amplifiable fragment occurs at or before time t is given by

$$P(T_0 \leq t) = [1 - \exp(-l_{bp}kt)]^N$$

where N is the initial copy number

l_{bp} is the length of fragment in base pairs
 k is the rate of reaction

The p th percentile, t_p , of the distribution of T_0 is, therefore, given by

$$t_p = -\frac{1}{l_{bp}k} \ln \left[1 - \left(\frac{p}{100} \right)^{\frac{1}{N}} \right]$$

Appendix B: Calculation of DNA thermal age

The thermal age of a fossil is calculated as in the following example, for a 16 kyrBP fossil bone from Lake Mungo (Station 1, 1.5 m depth), using temperature data for the year 1982/1983 (Ambrose 1984).

The modern day effective temperature T_{eff} is calculated using a simple sinusoidal model of soil temperature variation throughout the year, based upon soil temperatures, with mean 20.1°C and amplitude 2.5°C

Changes in rate of reaction are calculated using the formula

$$k = Ae^{-E_a/RT}$$

The rate of depurination ($k \text{ yr}^{-1}$) in bone is estimated at pH 7.4 from data given in Lindahl & Nyberg (1972), an activation energy, $E_a = 127 \text{ kJ mol}^{-1}$ and a pre-exponential constant, $A = 1.45 \times 10^{11} \text{ s}^{-1}$, which is slightly lower than that used by Pääbo & Wilson (1991; $A = 3.26 \times 10^{11} \text{ s}^{-1}$ at pH 7.0), have been used here, and $R = 8.314 \text{ J mol}^{-1} \text{ K}^{-1}$ and T is the temperature (in Kelvin) of the reaction.

As the rate of rate of depurination is exponentially related to the reciprocal of temperature the amplitude of the sine wave is an important factor. The average rate of depurination is calculated for 1 year (k_{avg}) accounting for the exponential increase in rate. This rate of reaction is approximately equivalent to the sample being held at a constant 20.4°C, the *effective DNA depurination* temperature. This procedure is reproduced for each temperature in the thermal history of a fossil.

The relative amount of DNA depurination at each site has been normalised to the equivalent amount of damage to the DNA as if the sample were held at a constant 10°C, and reported as the thermal age. Therefore, in the above example, for the last 16 kyr of burial the rate of reaction at

20.4°C is approximately 6.71 times faster than that at 10°C, i.e., every year at this temperature is equivalent to 6.71 years at 10°C. For this period the sample accumulates $16 \text{ kyr} \times 6.71 \text{ thermal years} = 107_{\text{kyr@10}^\circ\text{C}}$.

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