

ANCIENT DNA

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DNA that has been recovered from archaeological and palaeontological remains makes it possible to go back in time and study the genetic relationships of extinct organisms to their contemporary relatives. This provides a new perspective on the evolution of organisms and DNA sequences. However, the field is fraught with technical pitfalls and needs stringent criteria to ensure the reliability of results, particularly when human remains are studied.

COPROLITES

Faecal material from humans and animals found at archaeological excavations.

HYDANTOINS

Oxidation products of the pyrimidine bases (cytosine and thymine).

Since the advent of techniques that allow DNA to be rapidly sequenced, evolutionary studies that rely on the comparison of DNA sequences from living organisms have become commonplace. However, contemporary DNA sequences provide only indirect evidence of the historical processes that have formed them over long periods of time. So, in a sense, the field of molecular evolution is 'time trapped'. The study of DNA from dead organisms offers a partial way out of this predicament. However, many technical pitfalls need to be avoided to allow the molecular evolutionist to go back in time and effectively 'catch evolution red handed'. In this review, we first discuss DNA decay and our current understanding of the limits to DNA preservation, before reviewing several technical issues of crucial importance for the retrieval of ancient DNA. Finally, we discuss some insights that have come from DNA sequences retrieved from ancient animals and hominids, as well as from the COPROLITES they left behind.

Post-mortem DNA decay

When an organism dies, its DNA normally becomes degraded by endogenous nucleases. Under fortunate circumstances, such as rapid desiccation, low temperatures or high salt concentrations, nucleases can themselves become destroyed or inactivated before all nucleic acids are reduced to mononucleotides. If this is the case, slower but still relentless processes start affecting the DNA (FIG. 1). For example, oxidation, as well as the direct and indirect effects of background radiation, will modify the nitrous bases and the sugar-phosphate backbone of the DNA. Furthermore, deamination, depurination and other hydrolytic processes will lead to destabilization and breaks in DNA molecules. All

these processes create problems for the retrieval of ancient DNA sequences. For example, a high proportion of cytosine and thymine residues in extracts of ancient tissues are oxidatively modified to HYDANTOINS, which block DNA polymerases and thus the PCR¹. Furthermore, deamination products of cytosine, for example, are common in ancient DNA and cause incorrect bases to be inserted during the PCR² (FIG. 1). After a long enough time, the cumulative effects of damage to the DNA will become so extensive that no useful molecules remain. Assuming physiological salt concentrations, neutral pH and a temperature of 15 °C, it would take about 100,000 years for hydrolytic damage to destroy all DNA that could reasonably be retrieved^{3,4}. Some environmental conditions, such as lower temperatures⁵, will extend this time limit, whereas other conditions will reduce it. However, to the best of our understanding, to consider amplification of DNA molecules older than one million years of age is overly optimistic.

Ancient DNA retrieval

For specimens of plants and animals that have been collected under controlled conditions and stored in museums in the past 200 years, the PCR technique has made the retrieval of DNA sequences almost routine. Starting with the study of kangaroo rats in the Mojave desert that were collected at the beginning of the century⁶, many studies have used museum collections to study populations over time^{7,8}, as well as the phylogenetic relationships of recently extinct species^{9–12}. However, for archaeological and palaeontological specimens, the retrieval of ancient DNA sequences is far from routine, as the researcher has to contend with the fact that very

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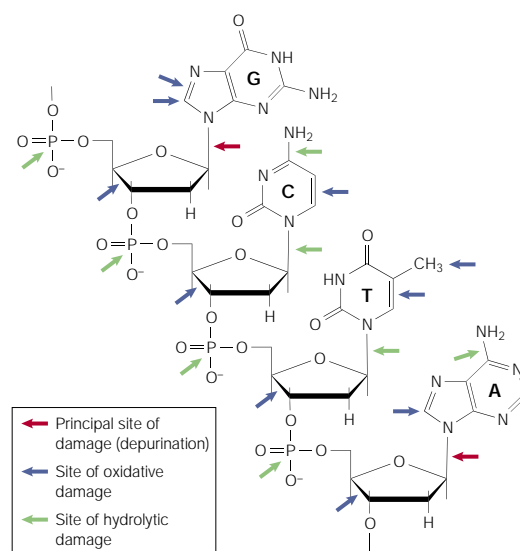


Figure 1 | DNA damage shown or likely to affect ancient DNA. A short segment of one strand of the DNA double helix is shown with the four common bases. Principal sites of damage are indicated by red arrows. Sites susceptible to hydrolytic attack are indicated by green arrows and those prone to oxidative damage by blue arrows. G, guanine; C, cytosine; T, thymine; A, adenine. (Modified with permission from REF. 4 © (1993) Macmillan Magazines Ltd.)

little and often no DNA survives in ancient tissues, whereas contemporary DNA is pervasive in the environment, both inside and outside the laboratory. Therefore, great precautions need to be taken to avoid the presence of extraneous DNA in the PCR. For example, the extraction and preparation of the PCR must be done in a laboratory that is rigorously separated from work involving modern DNA. Treatment of the laboratory equipment with bleach, UV irradiation of the entire facility, protective clothing and face shields are other routine precautions^{13,14}. In addition, several criteria of authenticity are essential for believing that DNA sequences are ancient (BOX 1). Despite these precautions, contamination is a continuous threat, if for no other reason than because the specimens themselves might be contaminated with modern DNA.

The greatest difficulties are encountered in the study of human remains. The reason is that human DNA is particularly prevalent in the environment of laboratories and museums, and cannot easily be distinguished from the DNA endogenous to ancient human remains. In fact, because human DNA sequences can readily be retrieved from ancient animal remains, we believe that many published studies that report ancient human DNA sequences are unreliable (BOX 2). Ancient animals pose less of a problem as the sequences retrieved are often testimony to their own authenticity if they are distinct from, yet related to, extant species in the same taxonomic group.

Multicopy DNA sequences are more likely to survive in ancient specimens, probably because they occur in larger numbers per cell. Therefore, most ancient DNA sequences that have been retrieved are

from mitochondria in mammals and from chloroplasts in plants. Only recently have the first short nuclear DNA sequences that are likely to be genuine been determined from PLEISTOCENE animals¹⁵, even if some earlier reports of nuclear sequences from human remains exist¹⁶. So, out of necessity, there is an almost complete reliance on mitochondrial DNA (mtDNA) sequences for phylogenetic studies of extinct animals. For species that are not very closely related to each other, this is not problematic because when enough time has passed between speciation events all parts of the genome are expected to show the same phylogeny. However, when closely related species, or population genetic questions, are studied, it is important to remember that the mtDNA represents a single genetic locus that might or might not reflect the overall history of the genome. Caution in the interpretations of the results is therefore necessary.

Assessment of preservation. Only a small proportion of old specimens that have been analysed contain DNA that can be amplified by PCR. Therefore, rapid screening methods are crucial to identify the large fraction of samples that are so badly preserved that there is no need to attempt DNA extractions. In particular, amino-acid analyses that take into account the total amount of amino acids preserved in a specimen, the amino-acid composition and the extent of RACEMIZATION of several amino acids, have in our hands proved to be a very useful proxy for DNA preservation¹⁷. Whenever any of these parameters exceed certain levels, DNA extractions have proved futile. In addition, amino-acid analyses can support the authenticity of DNA sequences retrieved from a specimen by showing that preservation conditions have been such that retrieval of macromolecules is conceivable (BOX 1). Another method that might serve the same purpose, but that has not yet been extensively shown to correlate empirically with the presence or absence of authentic ancient DNA sequences, is PYROLYSIS coupled with **gas chromatography and mass spectrometry (GC/MS)**¹⁸.

Quantitation. Estimations of the number of ancient template DNA molecules from which the PCR starts are another useful way to ensure the quality of old DNA sequences. If amplifications start from very few or even single DNA strands, then errors in the first cycles will become incorporated into all molecules in the final PCR products. Such errors can be numerous if the amount of damage in the DNA is large. Furthermore, if amplifications are carried out from, for example, a MICROSATELLITE locus on an autosome, then one of the two alleles will fail to be amplified if the amplification starts from a single molecule, causing the individual to be erroneously typed as a homozygote. Finally, contamination can be extremely hard to exclude if the genuine template is so rare that amplification is possible only in some of the reactions. Therefore, a determination of the number of template molecules from which a PCR starts is extremely useful and can be achieved by using COMPETITIVE PCR¹⁹, and more recently, by using REAL-TIME QUANTITATIVE PCR²⁰. If

PLEISTOCENE
The geological time period from two million to 10,000 years ago.

RACEMIZATION
The change in the three-dimensional structure of amino acids from one form to a mirror image over time.

PYROLYSIS GC/MS
An analysis in which macromolecules are decomposed by heat, and the products analysed by gas chromatography followed by mass spectrometry.

MICROSATELLITES
A class of repetitive DNA that is made up of repeats that are 2–8 nucleotides in length. They can be highly polymorphic and are frequently used as molecular markers in population genetics studies.

COMPETITIVE AND REAL-TIME QUANTITATIVE PCR
A PCR analysis in which the approximate number of molecules that initiate the reaction is measured either by competition with a template of known concentration or by accumulation of product throughout the PCR.

Box 1 | Authenticity criteria to determine ancient DNA sequences

Biochemical assay for macromolecular preservation

Amino-acid analysis should show that the state of preservation of the specimen is compatible with DNA preservation.

Extraction controls and PCR controls

Mock extractions and PCRs without template DNA are elementary controls that should be carried out.

Inverse correlation between amplicon length and amplification efficiency

Generally, amplification of only short DNA pieces is possible.

Quantitation of numbers of template molecules

If the number of DNA molecules that initiate the PCR is less than ~1,000, at least three independent amplifications need to be analysed, and the products need to be cloned and several clones should be sequenced.

Exclusion of nuclear insertions of mitochondrial DNA

Pieces of mitochondrial DNA (mtDNA) exist in the nuclear genome and represent a potential source of false results. Sometimes they can be detected by the observation of several mtDNA sequences from an extract, or by finding the same sequence in another closely related species. If overlapping amplifications using different primers detect the same sequence over an area that is highly variable, it is unlikely that the sequence derives from a nuclear insertion^{18,32}.

Amplification from a second extract

The reproduction of the results from a second, independent extract should show that the result is reliable.

Reproduction in a second laboratory

When a new or unexpected result is obtained, reproduction in a second laboratory, preferably from a sample that has been independently sent from the museum to the second laboratory, ensures that a laboratory-specific contamination is not the source of the sequence determined.

a PCR starts from 1,000 or more molecules, then an experiment does not need to be repeated to verify that nucleotide misincorporations do not influence the results¹⁹. When fewer molecules initiate a reaction, several repetitions become necessary. In addition, the cloning of the PCR product and sequencing of several clones will then be essential to detect problems (FIG. 2). For example, if several different sequences occur in a mitochondrial PCR product it indicates the presence of contamination or nucleotide misincorporations in early cycles of the PCR.

Antediluvian DNA. DNA sequences that are older than about one million years were termed 'antediluvian' in an insightful and timely correspondence to *Nature* in 1993 (REF. 21). All such sequences have since either proven impossible to reproduce or have been shown to derive from an identifiable source of contamination. So, the DNA sequences from **Miocene** (5–24 million years before present) inclusions in amber cannot be reproduced^{22,23}, and such inclusions have been shown to be highly modified by **DIAGENETIC** processes²⁴. DNA sequences from Miocene plant compression fossils have similarly been impossible to reproduce²⁵ and DNA sequences retrieved from a **CRETACEOUS** dinosaur bone have been shown to be derived from an insertion of mtDNA in the human genome²⁶. So, barring some unimaginable technical advance, **DIACHRONICAL** studies of DNA sequences are

confined to the past one million years, and more probably to the past 100,000 years.

Applications of ancient DNA retrieval

Extinct animals. For the retrieval of DNA sequences from non-human remains that are up to 50,000 to perhaps 100,000 years old, the past decade has seen much progress (FIG. 3). Several studies have revealed the phylogenetic relationships of extinct animals, generally on the basis of mtDNA. For example, the mtDNA of the **marsupial wolf** of Australia has been shown to be related to other Australian marsupials rather than to carnivorous marsupials in South America. This means that several of the morphological features that marsupial wolves share with South-American marsupial carnivores have evolved independently on the two continents^{9,10,12}. By contrast, the mtDNA of the extinct **moas** of New Zealand have been shown to be related to flightless birds in Australia rather than to the extant kiwis in New Zealand²⁷. This indicates that New Zealand was colonized twice by flightless ostrich-like birds. Recently, the retrieval of mtDNA from ancient remains has been taken to a new level through the determination of the entire mtDNA genomes of ~16,500 base pairs from two moa species²⁸.

It is even becoming possible to start addressing population genetic questions in the Late **Pleistocene**, the time before and during the last Ice Age. So, the mtDNA types of Alaskan brown bears, which are today found in distinct geographical regions, have been shown to have coexisted in the same region ~30,000 years ago²⁹. This has consequences for the received wisdom among molecular conservation geneticists who often regard the sharing of related mtDNA sequences in a group of animals as indicative of long-term population separation, defining 'conservation units' that need to be managed as separate groups³⁰. The fact that the bear mtDNA types occurred together in one and the same area before the last Ice Age shows that much of the current distribution of mtDNA types might be due to more recent phenomena, such as the random loss of mtDNA lineages in small populations during the last glacial maximum. So, mtDNA might not always be a good metric for what constitutes a 'conservation unit'.

In another recent project, 191 contemporary horses were studied and were found to be very diverse with respect to mtDNA sequences. The same mtDNA sequences were then determined from eight horses that were 12,000–28,000 years old and preserved in Alaskan **PERMAFROST**, as well as from eight horses from Estonia and Sweden that were 1,000–2,000 years old. Some of the mtDNA sequences of the Pleistocene horses, as well as all of the mtDNA sequences from horses from archaeological sites in Northern Europe, were found to fall within the variation of modern horses³¹. So, the mtDNA sequence diversity is neither a result of an acceleration of the evolutionary rate of horse mtDNA nor of a very recent introduction of wild horse mtDNAs into the domestic gene pool. Instead, much of the mtDNA of wild horses has entered modern horses early during domestication.

DIAGENESIS

All physical, chemical and biological changes undergone by any material from the time of its initial deposition in the environment.

CRETACEOUS

The geological time period from 144 to 65 million years ago.

DIACHRONICAL

A continuous process over time rather than a process at one time point (synchronical).

PERMAFROST

A layer below the surface soil that never thaws in subarctic regions.



Figure 2 | Alignment of eight mitochondrial DNA clones sequenced from a single amplification from a 26,000-year-old cave-bear bone. The consensus sequence was confirmed by sequencing additional amplification products. The high prevalence of C/G → T/A changes is probably due to deamination of C residues in the ancient DNA templates. Note that direct sequencing of the PCR product would lead to ambiguous results at least at two positions (arrows) (M.H. *et al.*, unpublished results).

NEANDERTHAL
A hominid form, morphologically distinct from contemporary humans, that existed until ~30,000 years ago in Europe and western Asia.

In another population study, DNA sequences from **cave bears** — extinct bears that existed in Europe and Western Asia until ~10,000 years ago — have been studied from several caves in Europe. When their mtDNAs are compared to those of contemporary brown bears,

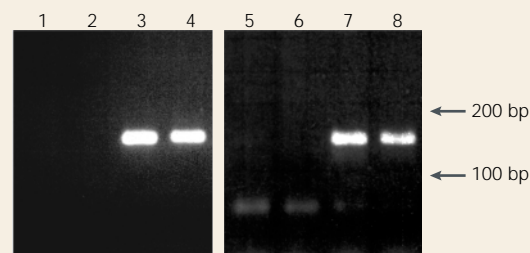
the cave bears are found to have diverged from the brown bears long before divergence of mtDNA sequences in the two species³². The diversity among cave bears was less than among modern brown bears, which is probably related to the fact that they inhabited a smaller area than their contemporary relatives.

These and other studies in progress show that we can now begin to reconstruct the population history of extinct animals, going back in time to well before the last Ice Age. The permafrost in Siberia and North America will certainly prove a particularly rich archive of genetic variation, because preservation of DNA is clearly better in cold environments^{5,17,18}, and because permafrost deposits contain huge numbers of preserved individuals.

Neanderthals. NEANDERTHALS were a hominid form — more closely related to contemporary humans than to chimpanzees — that inhabited Europe and Western Asia until shortly after 30,000 years ago. When a mtDNA sequence was determined from the Neanderthal-type specimen found in 1856 in the Neander Valley in western Germany, it proved not to be directly related to the mtDNA of modern Europeans³³. Instead, the lineage leading to the **Neanderthal** mtDNA diverged ~500,000 years ago³⁴, whereas the common ancestor of the mtDNAs of all living humans lived about 170,000 years ago³⁵. Even if the absolute dates change if more is learnt about the rates and mode of evolution of the mtDNA, this shows that the

Box 2 | Ancient human DNA sequences?

Remains of Late Pleistocene humans have been found in Upper Cave, Zhokouchien, China⁵⁰. Two well-preserved, ~30,000-year-old teeth from this site were subjected to amino-acid analysis. The extent of racemization of aspartic acid was 0.11 and 0.10, respectively; that is, ratios below the value of, for example, the Neanderthal-type specimen (0.12) from which endogenous DNA has been amplified³³. Part of each tooth was ground to powder and extracted, following published protocols³³. A mock extraction was processed alongside the samples. DNA was amplified using primers for the mitochondrial DNA (mtDNA) 16S rRNA gene, as described⁶⁵. Both teeth yielded amplification products (lanes 3 and 4), whereas the mock extract and PCR controls were negative (lanes 1 and 2). A second extraction was analysed from each tooth with the same result (lanes 5–8). The amplification products were sequenced and shown to be human. Next, a piece of hypervariable region I of the mtDNA control region was amplified twice from one of the teeth (using primers H16209 and L16303). Direct sequencing, using the primer H16209, revealed a reproducible 93-base-pair (bp) sequence that differed at one position from over 6,000 contemporary mtDNA sequences at present in the database. When the experiment was repeated, the same sequence was obtained. At this point, most studies of human remains would be published, often in a prestigious journal, making the claim that these DNA sequences stem from the human teeth (for example, REFS 36,51–55).



However, when the two control region amplification products were cloned and 20 clones from each were sequenced, a total of 20 different human sequences were found among the clones. This result is surprising in view of the fact that only one mtDNA sequence would be expected from a tooth. A further fact that proves that all the above results are due to contamination — the teeth that were analysed came from cave bears found in Upper Cave rather than from humans! We facetiously neglected to indicate this above to illustrate the fact that had these been human teeth, failure to adhere to all the criteria in BOX 1 would undoubtedly result in the publication of false results. Sadly, the problem of contamination, as well as the criteria in BOX 1, is often ignored in published work on ancient human DNA sequences. It is particularly noteworthy that direct sequencing in this case failed to indicate the complex mixture of sequences present.



Figure 3 | **Some extinct organisms from which DNA sequences have been determined.** From bottom left to top right: quagga⁵⁶, marsupial wolf^{9,10,12}, sabre-toothed cat⁵⁷, moa²⁷, mammoth⁵⁸⁻⁶², cave bear⁶³, blue antelope⁶⁴, giant ground sloth⁶⁵, Aurochs⁶⁶, mastodon⁶⁰, New Zealand coot⁶⁷, South Island piopio¹¹, Steller's sea cow⁶¹, Neanderthal^{33,34,39,40}, *Aptornis defossa*⁶⁸, Shasta ground sloth^{46,48}, pig-footed bandicoot⁶⁹, moa-nalo⁷⁰ and *Myotragus balearicus*⁷¹.

Neanderthals went extinct without contributing mtDNA to contemporary humans. So, the view of modern human origins that assumes a recent African origin, and little genetic contribution to the current gene pool from archaic humans elsewhere is correct for mtDNA. It does not, of course, exclude the fact that mixing between modern humans, which arrived in Europe from Africa ~40,000 years ago, and Neanderthals took place, but there is no evidence of such a mixture from the molecular studies. If it becomes possible in the future to study mtDNAs from many early modern humans, the occurrence of Neanderthal mtDNA types among them would prove that mixing did occur. Recently, a report of mtDNA sequences from skeletons of early modern humans from Australia revealed a sequence that tends to fall outside the variation of modern humans³⁶. This could represent a variant that was present in modern humans at the time, or alternatively a mtDNA contribution of archaic humans to the modern human gene pool that was subsequently lost by drift. However, contamination with contemporary human DNA makes the study of human remains extremely difficult (BOX 2), and the study in question did not fulfil the criteria that in our experience are necessary

COALESCENCE
The joining of genetic lineages to common ancestors when they are traced backwards in time.

to exclude (to the greatest extent possible) that the results are due to contamination(s) (BOX 1). This might be a particular concern in this case, because the mtDNA in question shows similarity to a nuclear insertion sequence that has previously been shown to represent a contamination in ancient DNA studies²⁶. So, further work, and perhaps even new technical approaches, are necessary before any definitive statements about the mtDNA gene pool of early modern humans can be made.

In principle, another way to estimate the extent of any putative mixing between modern humans and Neanderthals would be to study nuclear genes from the Neanderthals themselves. However, even if we ignore the technical problems involved with this, it would not be very informative as we already know that many nuclear genes have COALESCENCES that pre-date the putative separation of Neanderthals and anatomically modern humans ~500,000 years ago. We can therefore reasonably assume that the amount of variation at most nuclear loci in Neanderthals falls within the amount of variation found among humans today³⁷. So, the overall genetic relationship of Neanderthals and modern humans was not very distant and might in general have resembled that of subspecies of present-day apes more than that of different species^{34,38}. However, the divergence of the mtDNA pools indicates a temporal separation that could be verified by studying, for example, Y-chromosomal sequences, should this become possible in the future.

The recent determination of mtDNA sequences of a Neanderthal from the Caucasus (~30,000 years old³⁹) and of another from Croatia (over 42,000 years old⁴⁰) (FIG. 4) points to the exciting possibility of studying aspects of the population history of this archaic group of hominids. For example, humans differ from the great apes in having much less DNA sequence variation both in mtDNA and in nuclear DNA³⁸. Furthermore, the distribution of mtDNA sequence variation in contemporary humans indicates that they expanded from a smaller population ~50,000 years ago⁴¹, whereas there is little or no evidence for an expansion in the great apes. It would be extremely interesting to investigate mtDNA variation among Neanderthals to address the questions of whether they were similar to humans in having much variation and to obtain an indication of whether an

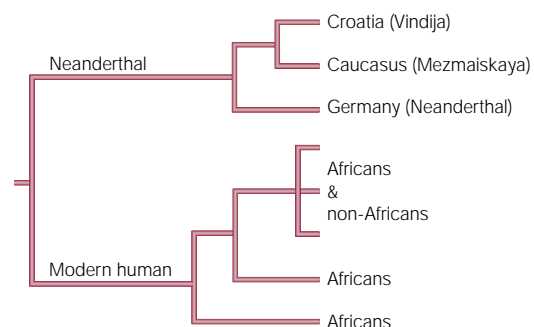


Figure 4 | **Schematic phylogenetic tree.** This relates the mitochondrial DNAs of modern humans and also three Neanderthals from Croatia⁴⁰, Caucasus³⁹ and Germany³³.



Figure 5 | 20,000-year-old ground sloth coprolite. DNA sequences from both the defecator and its diet have been determined from this sample⁴⁶.

expansion took place, and if so, when. Although three Neanderthal mtDNA sequences are obviously too few to gauge their diversity, it is interesting to note that the variation seen so far falls in the middle of the human distribution, whereas variation of the all the great ape species is much larger⁴⁰.

Coprolites. Faecal material is commonly found at excavations of human settlements and of caves that are frequented by animals. Because contemporary SCATS have proved a useful source of DNA from both the defecator and its diet^{42–44}, it was frustrating that well-preserved Late Pleistocene coprolites (FIG. 5) had failed to yield DNA. However, pyrolysis GC/MS studies showed that coprolites, as well as other ancient remains, contain large amounts of crosslinks between reducing sugars and amino groups, presumably in proteins as well as in DNA. Therefore, it was a logical step to attempt to remove such crosslinks from coprolite extracts when PTB (*N*-phenacyl thiazolium bromide) — a reagent that breaks sugar crosslinks — became available⁴⁵. This made the amplification of coprolite DNA from cave sites in the southwestern United States possible⁴⁶. Many of these caves contain layers of faeces that span tens of thousands of years⁴⁷. PTB is also helpful in extractions from some Pleistocene bones⁴⁰.

SCATS
Faecal material left behind
by animals.

Using coprolite deposits, it is possible to follow the change in diet of defecating animals through time. For example, the analysis of faecal DNA from **ground sloths** has shown that pine forests in southern Nevada were present ~28,500 years ago. However, sloths living in the same cave almost 20,000 years later, at the end of the last glaciation and shortly before their extinction, were found to have a diet more reminiscent of the current flora in the region⁴⁸. Recently, DNA has also been retrieved from faeces obtained from three 2,000-year-old Native Americans⁴⁹. Eight different plants were shown to have formed part of the diet of these individuals. One striking observation was the presence of meat from large mammals, such as pronghorn antelope and bighorn sheep, in these samples — a dietary component that is not possible to detect by a morphological analysis of the faeces. So, molecular analyses of coprolites are likely to prove a rich source of information about the genetics and dietary habits of ancient animals and humans.

Conclusions

Studies of ancient DNA from museum collections and Late Pleistocene animals have provided several new insights into the evolutionary history of both species and populations, and the determination of Neanderthal DNA sequences has provided a molecular perspective on the genetic relationship of current humans with an extinct hominid form. A particularly exciting development is the analysis of DNA from ancient coprolites because it yields information about the diet and thus the behaviour of ancient organisms. However, technical pitfalls make the field liable to dubious results unless many precautions and experimental controls are implemented. This is particularly true for human remains. Future developments that remove or repair chemical damage in the ancient DNA, as well as use the presence of such damage to verify that DNA sequences are indeed ancient, would be welcome additions to the field.

Links

FURTHER INFORMATION **gas chromatography and mass spectrometry** | **Miocene** | **Pleistocene** | **quagga** | **marsupial wolf** | **sabre-toothed cat** | **moa** | **mammoth** | **cave bear** | **blue antelope** | **giant ground sloth** | **mastodon** | **Steller's sea cow** | **Neanderthal** | **bandicoots** | ***Myotragus balearicus***

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Acknowledgements

We thank Qin Zhan-Xiang for providing the cave bear samples; and the Max Planck Society, the Deutsche Forschungsgemeinschaft and the Bundesministerium für Bildung und Forschung for financial support.