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Ancient DNA-research

Ancient DNA research may be defined broadly as the study of DNA isolated from fossil plant or animal remains. The time frame is broad and it may include the study of living populations through the collection of hair, faeces or seeds, as well as the study of museum and archaeological specimens and fossil remains from the late Quaternary (the last 100 000 years) (Figure 1), but also fossils more than a million years old.

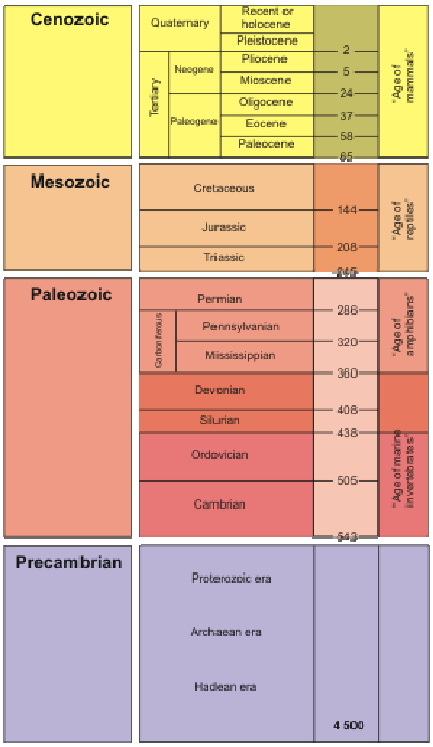

Figure 1. Geological time-scale
Redrawn from:
http://cronopio.geo.lsa.umich.edu/%7Ecrlb/

Ancient DNA research became feasible only recently, with the advent of techniques that allowed DNA to be rapidly amplified and sequenced. Today, reports of analyses of specimens from hundreds up to several thousand years old are almost commonplace. Nevertheless, as we shall see, molecular biologists need to avoid many technical pitfalls in order to go back in time and be sure of obtaining authentic results.

The history of ancient DNA began twenty years ago

The first ancient DNA report came in 1984, when DNA was extracted for the first time from an ancient specimen, the Quagga, a type of zebra that became extinct at the end of the 19th century [1] (Figure 2). One year later, in 1985, a second important report came. This time DNA had been extracted from a museum specimen of an ancient Egyptian mummy [2]. These ancient DNA sequences were exceptional because they were derived from 140- and 2 400-year-old specimens using cloning technologies (Figure 3) that require substantial amounts of DNA for sequencing. The report on the Egyptian mummy was also spectacular because the length of the DNA cloned was extraordinary (3 400 nucleotides), implying that long stretches of DNA could be preserved intact for more than 2 000 years.

Some years later, with the advent of the polymerase chain reaction technique (PCR) it became possible to amplify (reproduce) specific DNA sequences rapidly, starting from only few molecules and without the need of cloning technologies. Thus the requirement for a substantial source of initial DNA in the fossil sample was greatly reduced.

Some of the first studies reporting successful extraction and amplification of ancient DNA using PCR came from

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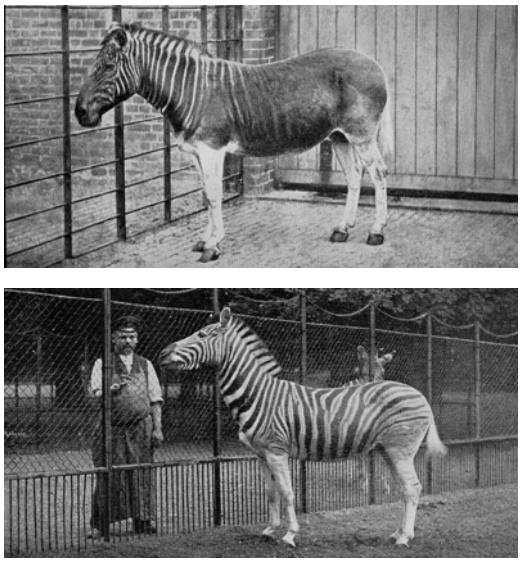

Figure 2. A picture of a quagga taken at London Zoo 1870 (it is one of only four photographs of a live quagga in existence)(top picture), and a picture of a Burchell's zebra (below).

Analysis of mitochondrial DNA has shown that the quagga (Equus burchelli quagga) was not a separate species of zebra, but a subspecies of Burchell's zebra (Equus burchelli).

The quagga formerly inhabited the southern regions of South Africa and, like other grazing mammals, had been ruthlessly hunted for meat and leather by South African farmers. They were seen by the farmers as competitors for the grazing of their livestock, mainly sheep and goats..

Photos from Brehm, A. Djurens Liv, 4:e Ed, Vol 2, Stockholm 1924.

fossil deposits located in northern Idaho, North America. The deposits, known as Clarkia beds, consisted of an exposed sequence of lake sediments dating from the early Miocene (between 17 and 20 million years ago). A report in 1990 described DNA sequences retrieved from the leaves of a Magnolia species from these beds [3]. The leaves found were apparently in an excellent state of preservation and showed a defined cellular structure. Remarkably, they even showed the presence of intact chloroplasts (organelles in the cell cytoplasm responsible for photosynthesis) in an extremely well preserved condition. It was from these organelles that it was possible to extract DNA. Two years later, an independent attempt also succeeded in extracting DNA from the Clarkia beds with samples of a Miocene *Taxodium* (bald cypress) [4]. This second success appeared to support the previous results from Magnolia leaves. However, both these pioneer studies were performed without PCR controls (Box 1) and later attempts to replicate the results failed, raising strong doubts as to the authenticity of these old DNA sequences. Nevertheless, the Magnolia study broke the million-year barrier and researchers at that time then turned attention to more "charismatic" creatures, such as dinosaurs and plants or animals preserved in amber. The culmination of these studies was the report in 1994 of a dinosaur mitochondrial DNA sequence from partially carbonised material from a coal deposit in Idaho [5].

Today there is a big controversy surrounding the authenticity of these million-year-old DNA sequences and it now appears probable that they are artefacts caused by contamination from modern DNA.

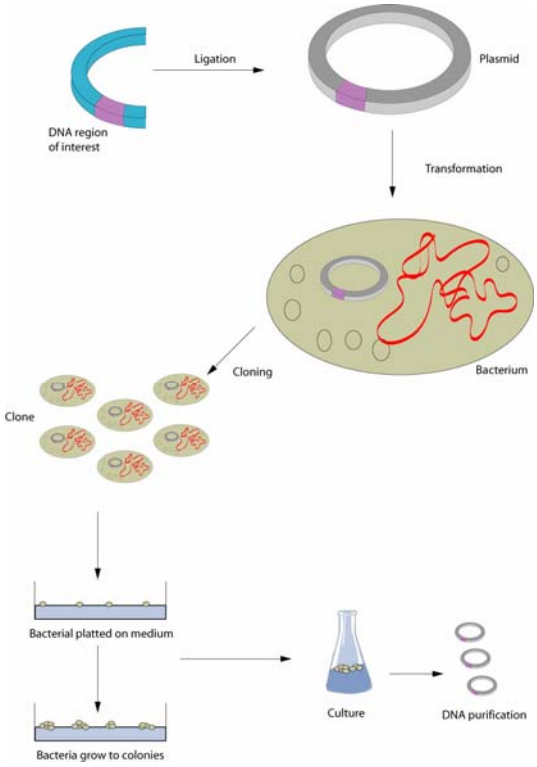

Figure 3. Cloning

- *Isolation of the DNA to be cloned.*
- *Binding of the DNA into the plasmid (ligation).*
- *Introduction of the plasmid into bacterial cells (transformation).*
- *Replication of the inserted plasmid along with the cell's chromosomes and to pass it along to its progeny.*
- *Selection of cells containing foreign DNA by screening for selectable markers (usually drug resistance).*
- *Purification of the plasmid DNA from the bacterial cells.*

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Box 1

Ancient DNA research presents extreme technical difficulties because of the minute amounts and degraded nature of surviving DNA and the exceptional risk of contamination. The need to authenticate results became clear when, in the mid-1990s, a series of high-profile studies were shown to be unrepeatable. For example, DNA reputed to come from a dinosaur [5] was actually contamination by a human mitochondrial gene. Over the succeeding years, the following criteria have been developed and put into practice by most of the scientists working with ancient DNA:

Physically isolated work area. To avoid contamination, it is essential that, prior to the PCR processes, all ancient DNA research is carried out in a dedicated, isolated environment.

Control amplifications. Multiple extraction and PCR controls must be performed to detect sporadic or low-copy number contamination. All contaminated results should be reported.

Appropriate molecular behaviour. PCR amplification strength should be inversely related to product size (large PCR products should be more difficult to obtain). Deviations from these expectations should be explained. Sequences should make phylogenetic sense.

Reproducibility. Results should be repeatable from the same, and different, DNA extracts of a specimen.

Cloning. PCR sequences must be verified by cloning amplified products to determine the ratio of endogenous (originating from the fossil) to exogenous (originating from other DNA – contamination) sequences as well as errors made by the PCR induced by the damage in the ancient DNA

Independent replication. Contamination in the laboratory can only be discounted when separate samples of a specimen are extracted and sequenced in independent laboratories. This is particularly important with human remains or novel, unexpected results.

Biochemical preservation. Indirect evidence for DNA survival in a specimen can be provided by assessing the total amount, composition, and relative extent of change in other macromolecules present in the fossil remain.

Quantitation. The copy number of the DNA target should be assessed using competitive Real Time-PCR (a recently developed variation of PCR designed for quantitative purposes where the machines can display and monitor the progress in terms of number of molecules in each reaction as the reaction proceeds) When the number of starting templates is low (< 1 000), it may be impossible to exclude the possibility of sporadic contamination, especially for human DNA studies.

Associated remains. In studies of human remains where contamination is especially problematic, evidence that similar DNA targets survive in associated faunal material is critical supporting evidence..

From Cooper och Poinar 2000 [13].

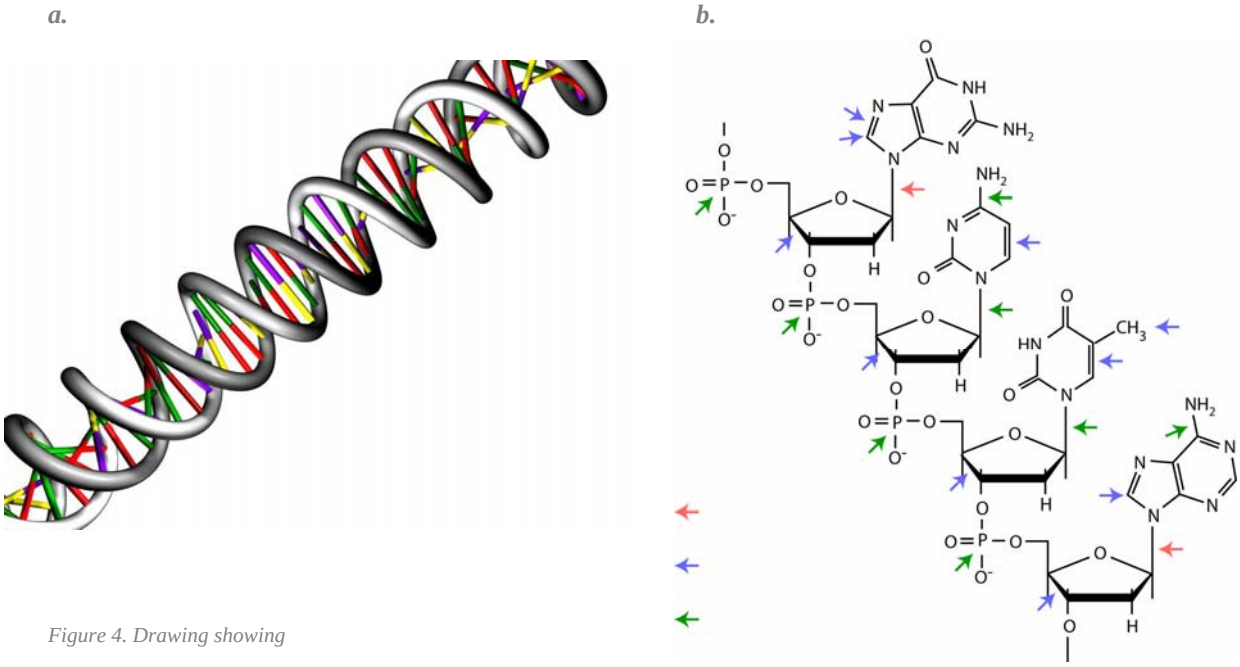

Figure 4. Drawing showing

a. part of the two strands of the DNA double helix
b. a short segment of one strand of the DNA double helix is shown with the four common bases and points of attack. Red arrows indicate principal sites of damage, green arrows those susceptible to hydrolytic attack and blue arrows those prone to oxidative damage.
Redrawn after: Hofreiter et al. 2001[14]

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How DNA molecules degrade

One of the main areas of scepticism for the authenticity of the million-year-old DNA sequences lies in the ability of strands of DNA to remain intact for such long periods. It is argued that when an organism dies, endogenous (internal) enzymes called nucleases, normally present in the cell, will degrade DNA molecules (Figure 4). In addition, these molecules have to face attack by various types of fungi and bacteria that are present in the surroundings and which usually feed on and degrade macromolecules. Under favourable circumstances, however, such as rapid desiccation, high salt concentration or low temperatures, the nucleases can themselves be inactivated before all DNA molecules present in the dead organism are reduced to mononucleotides. Nonetheless, even in these favourable circumstances, slower processes like oxidation and hydrolytic processes will start affecting DNA, eventually leading to its disappearance. If, for instance, organisms are preserved under a long time in oxygen-rich environments, oxidation will modify the nitrous bases and the sugar-phosphate backbone of the DNA. Similarly, in the presence of water, deamination, depurination and other hydrolytic processes will lead to destabilization and break DNA strands. The final result is that the long DNA molecules are reduced to small average size (between 100 and 500 nucleotides long) and, after a long enough time, the cumulative effects of damage will become so extensive that no intact molecule remains in the tissues, representing an irreversible loss of nucleotide information. PCR has made possible the occasional recovery of the last pieces of information present in the samples where DNA decay is not yet complete by reproducing millions of copies of the few short molecules remaining and allowing them to be readily sequenced.

It has been calculated that in normal conditions (physiological salt concentrations, neutral pH and a temperature around 15 C˚) it would take approximately 100 000 years for hydrolytic damage to completely destroy all DNA molecules present in the cells of a fossil organism. Environments with low temperatures and dry conditions can extend this time limit by up to four or five times, and even more in exceptional cases, while environments with opposite conditions will reduce it. Therefore, it is generally assumed that the retrieval of DNA from sequences older than 100 000 years will be difficult, and, in most cases, impossible to achieve.

What is achievable with ancient DNA?

Even within the limited time period of 100 000 years or so, the evolutionary questions that can be addressed are far reaching and DNA information from specimens of late Quaternary age have recently provided new insights on many evolutionary processes in many higher organisms, such as plants and animals, but also in viruses and bacteria.

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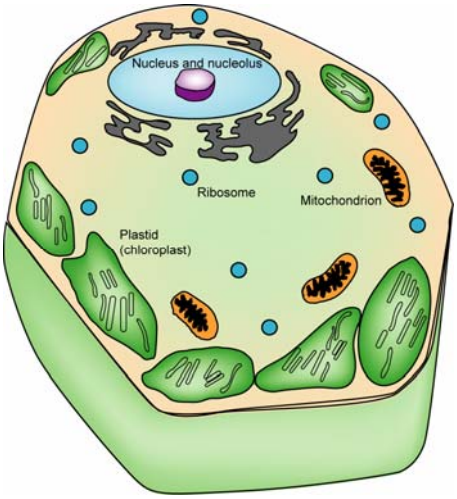

Figure 5. Simplified plant cell (an animal cell would lack the plastids) with some internal structures.

Usually, the nature of the evolutionary question being asked determines which genes to target in a genetic study. Different genes in the same organism evolve at different rates, therefore, if the evolutionary study involves the examination of genetic change across tens of thousands of years, then a gene that evolves slowly is appropriate. On the other hand, shorter time-scale studies will require a gene with a more rapid rate of evolution.

In higher organisms there is a choice of three genomes to target: mitochondrial DNA (mtDNA), plastid DNA (cpDNA) and nuclear DNA (nDNA). Mitochondria and plastids (or chloroplasts) are organelles found in the cytoplasmic compartment of the cells (Figure 5) that possess their own DNA information in form of multiple circular DNA molecules. Mitochondria are responsible for carrying out aerobic respiration and are present in both plant and animal cells, while plastids are specific to plant cells and control of processes such us food manufacture, storage and photosynthesis. The DNA of these two organelles have been extensively used in ancient DNA studies because it is found in several hundred copies per cell, which increases dramatically the chances of survival in ancient specimens. In addition, both DNA types are inherited uniparentally (directly and unchanged in their DNA sequence from one parent to the progeny) thus rendering relatively simple the interpretation of evolutionary data. However, in plants, mtDNA has the disadvantage of evolving too slowly. In some regions the mutation rate can be 100 times slower than in animal mtDNA. Thus mtDNA extracted from relatively young fossil plant tissues, such as seeds from archaeological deposits dating back to no more than 2000 years, will not indicate any appreciable change when related organisms are compared. For this reason, cpDNA, with a higher rate of evolution, has usually been preferred for ancient DNA studies in plants. Finally, nDNA, present in the nucleus in the form of chromosomes, has a much higher rate of evolution than organelle DNA and is therefore very useful in discriminating between closely related species even in short time-scale evolutionary studies. However, with nuclear sequences there is an increased risk of false results during PCR amplification because nuclear genes are present only in one copy in each cell. For this reason, genes from the nuclear genome are rarely studied in ancient specimens.

Animal studies

Recently, many studies of ancient DNA molecules retrieved from non-human remains have made it possible to go back in time to approximately 100 000 years ago, and study the phylogenetic relationships of extinct animals with modern relatives. For example, the mtDNA of two extinct Moa species of New Zealand shows that they are more closely related to modern flightless birds

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in Australia (emus and cassowaries) than they are to the living kiwis in New Zealand [6]. This implies that flightless birds colonised New Zealand at least twice.

Similarly it has been shown that mtDNA of the extinct marsupial wolf of Australia is more closely related to other Australian marsupials than it is to South American carnivorous marsupials [7]. This has important implications, because it indicates that the morphological features that the marsupial wolves share with the South American marsupials have evolved independently on two different continents (convergent evolution).

It is even now possible to address genetic questions at the population level using ancient DNA. Sequences of mtDNA from cave bears (an extinct species of bear that lived in Europe and in Western Asia until 10 000 years ago) have been studied from remains in several European caves [8]. When their DNA was compared to that of the two main lineages of living brown bears defined in Europe, the cave bears were found to have diverged around 1.2 million years ago, long before the brown bears appeared. Moreover, the diversity within cave bears was less than within brown bears, suggesting that they inhabited smaller areas than their contemporary relatives.

Another example of these types of studies comes from 6 000-years-old DNA from Adélie penguins in the Antarctic, which made it possible to determine the rate at which the mtDNA of these birds evolved [9]. The large colonies in which these penguins live have existed for thousands of years, with the same bird species and their descendents returning to the same site year after year. As a result, there are layers of bones in the colonies that have been kept frozen for thousands of years. Fossil bones were collected from various layers, and dated, and blood samples were gathered from living birds at the same site. From these samples, a short mtDNA sequence was analysed and the changes between ancient and modern samples were counted. From that the rate of evolutionary change that occurred in specific regions of the mtDNA was obtained.

Neanderthal studies

Neanderthals were hominids that inhabited Europe and western Asia from around 300 000 years ago until disappearing from the fossil record shortly after 30 000 years ago. Using fossils and cultural evidence, palaeontologists have long argued for a substantial genetic contribution of Neanderthals to the modern human populations that arrived more recently, making Neanderthals progenitors of modern Europeans. However, reinterpretation of the fossil data suggests that modern humans in fact replaced Neanderthals when they arrived in Europe around 40 000 years ago. Ancient DNA analysis confirmed this hypothesis. An mtDNA sequence was determined from the Neanderthal-type specimen

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found in 1856 in the Neander Valley in Western Germany [10]. It proved not to be directly related to the mtDNA of modern humans. Instead the lineage leading to the Neanderthal DNA diverged circa 500 000 years ago, whereas the common ancestor of all living modern humans lived about 170 000 years ago. Results from this ancient DNA study showed that the Neanderthals went extinct without contributing DNA to modern humans. So, the view of modern human origin that assumes a recent African origin and little genetic contribution from archaic humans (the so-called 'out of Africa' theory) is probably correct, at least as far as mtDNA evidence is concerned. This does not exclude the possibility that mixing between our ancestors and Neanderthals took place when modern humans arrived in Europe, but there is no evidence for this, at the moment, from mtDNA molecular studies.

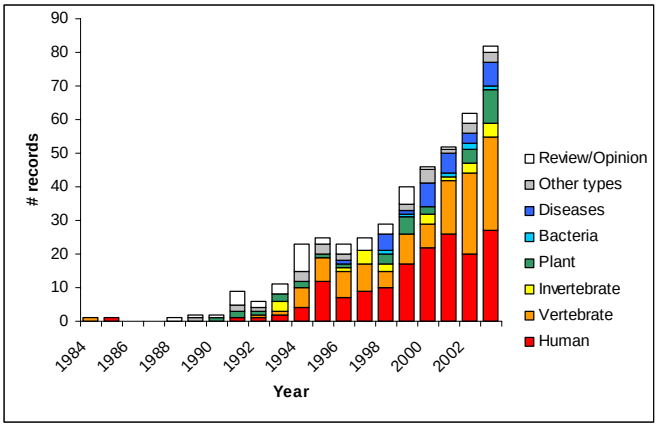

Figure 6. Number of papers published between 1983 and 2004 retrieved from a public reference data bank (Institute for Scientific Information (ISI) Web of Science) using "ancient DNA" as search term, categorized according to the study organisms.
Från Gugerli et al. [15]

Unfortunately, contamination with modern human DNA makes studies on ancient human remains extremely risky and difficult to perform. If, for instance, some Neanderthals carried mitochondrial sequences similar to those of modern humans, such sequences might be erroneously regarded as modern contaminants.

Plant studies

There are few examples of ancient DNA studies in the published literature from the plant fossil record (Figure 6). In part this is due to the exceptional environments required to preserve DNA but also because the retrieval of well preserved fossilised hard tissues such as wood are rare compared to the retrieval of bones in animals. Or it simply because extinct animals like dinosaurs, mammoths and the Neanderthals, are more charismatic organisms than plants. Nevertheless, in the last twenty years several plant remains containing ancient DNA have been obtained from herbarium collections, archaeological remains, lake sediments, peat, caves, fossil deposits, coprolites and amber.

The first reports came in the 1990s when DNA sequences of Magnolia leaves, earlier cited, dating back millions of years were published.

In successive years these earlier reports were followed by a number of studies on DNA extracted from plant tissues incorporated in amber (Figure 7). Amber is a fossilized plant resin, mainly produced by conifers, and is an important preservation medium for both plant and animal remains. When the resin is formed it is sticky, and any animal or plant fragment (small flowers, leaves, seeds, small insects) that fall into it is incorporated. The process is rapid, and effectively the fragments become encased in a dehydrated state. Most

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common inclusions are insects (86.7%) and arachnids (11.6%), while animals of other classes occur only in 1.7% and plants in 0.4% of cases. Fossil leaves contained in amber and dated from the Oligocene (between 24 and 34 millions years ago) have been recovered from a number of localities in Europe and it has been possible to extract DNA from a plant species contained in fossil amber from the La Toca mines in the Dominican republic. Unfortunately, as explained above, based on the principle that DNA cannot survive for millions of years, the majority of these results have been questioned and it may be that they are artefacts due to contamination from modern DNA.

Figure 7. Organisms in amber. Photo Elisabeth Strömberg.

Other unusual preservation media for younger plant remains (up to 40 000 years) are middens founds in the arid areas of southwestern North America, the Middle East and South Africa. These middens, which provide shelter for animals, consist of plant material that they collected and which became encased in crystallized urine. The crystallized urine, combined with the extremely dry environment, provides an exceptional preservation medium. Extraction of plant material from these middens is simple and attempts have been made to extract DNA and other ancient macromolecules from this material, with encouraging results. Permafrost (permanently frozen subsoil) is also a good site for preserving DNA because it is continuously cold. Recent extractions of DNA from organic material in Siberian permafrost sediments have generated a list of DNA from 19 different taxa of angiosperms, gymnosperms and mosses, as well as DNA from eight kinds of animals, some of them extinct [11]. The material was searched only for bacterial DNA, but it was soon appreciated that it was possible to recover fragments of DNA from plant and animal organisms in peat samples that had no sign at all of macrofossils. The important result of this study was that the Siberian peat yielded what is now believed to be the oldest reliable ancient DNA from plants (circa 400 000 years old). This permafrost plant DNA indicates that the area called Beringia, which spanned eastern Siberia and western Alaska, may have been a vegetation-rich steppe rather than sparse polar tundra. The results also suggested that grasses declined from 36% to 3% after about 11 000 years, consistent with the notion that climate change played a big role in the mass extinction of mammoths, ground sloth and other large North American mammals occurring in that period. However, there are other explanations of the same data.

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Finally, a key area of research where ancient plant DNA studies have proved to be particularly promising is the study of plant domestication. There have been several successful reports of ancient DNA isolated from archaeological remains of cultivated plants. Archaeological sites are in fact rich sources of relatively well-preserved and well-dated fossil remains usually not older than a few thousand years. These studies have made important contribution to understanding the origin and the spread of agriculture or the appearance of domesticated plants and how these have evolved. Evidence has been obtained that ancient species of maize (*Zea mays*) circa 4500 years old, were as diverse as modern species and that there were a number of genetic lineages contributing to modern maize from throughout the wild *Zea mays* population [12], disproving the previously held view (based only on archaeological records) that maize domestication was a single isolated event.

How reliable are ancient DNA sequences?

Many areas of concern with the amplification of ancient DNA are associated with problems with PCR amplification and the probability of contamination of short fragments of DNA. With questions arising from the inability to reproduce the million-year-old results from the early 1990's, and scepticism about the long-term preservation of DNA molecules, it is not surprising that much of the research potential of ancient DNA has not yet been realized and that the number of studies is still relatively low.

Nevertheless, in studies of more recent, but still ancient DNA, has proved to be extremely useful and, as we have seen, the study of ancient DNA from museum collections, archaeological remains and late Quaternary specimens have provided new insights in the evolutionary history of many organisms as well as attracting much attention and many practitioners. Unexplored opportunities for ancient DNA studies are still many, not only in plants and animals but also in other organisms, such as bacteria and viruses.

Currently, the retrieval of ancient DNA sequences is relatively simple for specimens of plants and animals that have been collected under controlled conditions and stored in museums over the past 200 years. But for older archaeological and palaeontological specimens (up to 100 000 years) these studies are far from trouble-free. If strict criteria for authentication and experimental controls are not implemented, many technical pitfalls make this exiting field of research predisposed to questionable results. The power of PCR is such that, even when all necessary precautions have been followed, erroneous results are common. This is particularly true for example, with human studies.

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Few degraded DNA molecules survive in ancient tissues, whereas DNA from modern individuals is pervasive in all environments, both inside and outside the laboratory. As a consequence, it is necessary to pay close attention in order to avoid the presence of extraneous DNA in the PCR. Extraction and preparation of PCR should be done in specific laboratories devoted to ancient DNA studies and be physically separated from buildings involving work with modern DNA. In addition a number of routine precautions must be followed, like treatment of the equipment with bleach, UV irradiation, the use of protective clothing and face shields (particularly when working with human remains). Criteria of authenticity have been recently published suggesting how ancient DNA research should be carried out [13] (Box 1).

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16. A paleobotanists dream in amber
http://www.ambergallery.com/

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http://www.dnalc.org/Shockwave/pcranwhole.html

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