

Palaeontology in a molecular world: the search for authentic ancient DNA

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Few recent events have focused such a blaze of publicity on palaeontology as the film by Stephen Spielberg of Michael Crichton's book *Jurassic Park*. The nub of the plot is that a molecular biologist manages to recover dinosaur blood from the guts of small biting insects fossilized in amber. From this he extracts dinosaur DNA and recreates living dinosaurs, which run amok. Although a work of fiction, the story brought together two disparate topics – palaeontology and molecular biology – in a way that led many people to believe that the resurrection of extinct species could soon be feasible.

Indeed, it seemed that science fiction was rapidly becoming science fact when in 1992, a year after the film was released, reports began to appear of DNA recovery from amber-encased insects 25–40 million years old^{1,2}. Shortly afterwards there was a report of DNA recovered from a 120-million-year-old amber weevil³. These and other reports of ancient DNA recovered from fossilized organisms^{5–12} suggested that DNA could survive over geological timescales and thus provide a unique opportunity for palaeontological investigation of evolutionary questions.

In the succeeding five years much work has been done in the search for ancient DNA in the fossil record, and we are a great deal wiser about what we can expect to be preserved in the geological record.

DNA decay and its survival potential

DNA is a chemically unstable molecule that decays spontaneously, mainly through hydrolysis and oxidation. Hydrolysis causes deamination of the nucleotide bases and cleavage of base-sugar (N-glycosyl) bonds, creating baseless sites. Deamination of cytosine to uracil and depurination (loss of the purines adenine and guanine) and are the two main types of hydrolytic damage¹³. Baseless sites weaken the DNA strand, causing strand breaks that fragment the DNA into smaller and smaller pieces. Oxidation leads to chemical modification of nucleotide bases and the eventual destruction of the ring structure of base and sugar residues in the DNA molecule¹⁴. DNA is also degraded by nonenzymatic methylation and a whole suite of biological enzymes.

In living organisms DNA undergoes constant repair to counteract this damage. After death, however, there is spontaneous degradation of the molecule, even if DNA can be shielded from the action of biological enzymes in a fully protected environment. The chances of unprotected DNA surviving over long periods are slight, unless special conditions exist for its preservation. Theoretical calculations suggest that DNA should not be able to survive for more than 10 000–100 000 years^{13,14}. Even if DNA does survive it is expected to

The survival of ancient DNA in specimens up to several thousands of years old is established. However, there have been several claims concerning the recovery of geologically ancient DNA from fossil material many millions of years old. The authenticity of these fossil DNA sequences is questionable on theoretical and empirical grounds, and the existence of authentic geologically ancient DNA remains to be proven.

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be highly fragmented and chemically modified^{15,16}.

Techniques for isolating and identifying ancient DNA

The study of ancient DNA is heavily dependent on a single technique – the polymerase chain reaction (PCR). PCR is a sensitive and powerful technique that, in theory, enables the rapid generation of many millions of copies of a particular target sequence of DNA. It is an essential tool in the study of ancient DNA because PCR enables us to amplify a specific DNA fragment from a few intact DNA molecules in the presence of

an excess of damaged molecules and other non-target DNA. However, the retrieval of authentic, unambiguous and reliable ancient DNA sequences using PCR can be problematic^{17,18}.

The sensitivity of PCR means that minute amounts of contaminating DNA may be preferentially amplified, especially when the ancient extract contains few or no endogenous DNA molecules. Contaminating DNA out-competes endogenous DNA during PCR because it is usually more recent in origin and, therefore, less damaged. If amplifications start from one or just a few DNA strands, much of the final product will contain incorrect nucleotide sequences^{18,19}, because of misincorporation of nucleotides where there are modified bases or baseless sites in the damaged ancient template and *Taq* polymerase errors occurring during the first few cycles of amplification. Chimeric DNA sequences may be produced via 'jumping' PCR when amplifications target DNA fragments that are longer than any template molecules present in the ancient extract or the template molecules are extensively damaged^{5,20}. These chimeric molecules may be derived from endogenous ancient template, contaminating DNA or a combination of both.

Contamination is by far the biggest problem of work with ancient DNA and can occur at many stages. The specimen containing the target DNA may be contaminated after death or during subsequent handling. Contamination may also occur during DNA extraction or the PCR via reagents, equipment and laboratory personnel. Contaminating DNA may be derived from a multitude of sources including other specimens²¹, microorganisms²², humans²³ or DNA generated in the laboratory by the PCR²⁴. It is most important, therefore, when working with ancient DNA to select appropriate specimens and tissues that are most likely to contain ancient DNA, to take measures to reduce the chances of contamination, and establish a methodology for the authentication of any putatively ancient DNA that is recovered (Box 1). Establishing the authenticity of ancient DNA sequences remains the biggest problem in the study of ancient DNA. The most important criterion for authenticity is that results should be reproducible^{17,20,25}.

Box 1. Requirements for the finding of authentic ancient DNA

1. Specimen selection

- Careful selection of specimens on the basis of evidence for good cellular and/or biomolecular preservation (e.g. histology¹⁹, amino acid racemization^{20,21}, or unusually good preservational conditions (e.g. mummification, low temperatures, absence of free water²¹).
- Where different tissues are available from the one specimen, choice of tissue samples that represent the best possible site for DNA preservation (e.g. bone or teeth rather than muscle or skin²¹).

2. Strict procedures to minimize contamination

- Choice of specimen (good preservation, undamaged) and tissue (intact, internal versus external) to obtain samples that are least likely to have been exposed to sources of contamination²¹.
- Careful preparation (surface sterilization or removing external surface) to eliminate surface contamination²¹.
- A dedicated laboratory to deal exclusively with ancient specimens. DNA extractions and setting up of the polymerase chain reaction (PCR). This laboratory should be physically isolated from those where related extant species are handled and subsequently manipulation of PCR DNA is carried out. It should be stocked with dedicated equipment, reagents and supplies¹⁷.
- Sterile laboratory conditions, including full protective clothing for laboratory staff, regular ultraviolet light irradiation and bleach treatment of benches, equipment and reagents where possible^{17,21,22}.
- Temporal separation of work on ancient DNA from that on modern DNA. Work on ancient specimens should precede work on modern relatives.
- Multiple negative controls to detect any contamination during DNA extraction and PCR set-up^{17,22,23}. Extractions and amplifications from one sample can be interspersed with those from another to monitor for cross-contamination.
- Careful choice of PCR primers that are as specific as possible to the group of organisms under study and that will not amplify DNA from obvious sources of contamination such as microorganisms and humans.

3. Authentication

- Reproducibility is essential. Putatively ancient DNA sequences must be reproducibly obtained from different extractions from the same sample, and from different tissue samples from different specimens^{17,22}. The ultimate test of authenticity of ancient DNA is independent replication in two separate laboratories¹⁵.
- DNA sequences should make phylogenetic sense^{17,22}.
- Extracted DNA should show certain characteristics expected of ancient DNA, particularly an inverse relationship between amplification efficiency and amplification length and a low copy number of target sequences in the extract^{17,22}.

Records of ancient DNA

Reports of ancient DNA, recovered from specimens ranging in age from recently extinct species less than 100 years old to insects in 120-million-year-old amber, have been accumulating over the past 10 years (Fig. 1). Some of them lie within the theoretical survival-time of DNA, and others greatly exceed it. The first reports created a wave of optimism that ancient DNA was to provide answers to previously unanswerable questions in evolutionary biology, archaeology and palaeontology.

Certainly, ancient DNA appears to have survived beyond its theoretical survival time in some specimens that are only a few hundreds or thousands of years old. Most of these records represent highly unusual preservation, where tissues have been protected from water or kept at low temperatures or both. For example, ancient DNA fragments have been successfully recovered from dried skins and archaeological bones of recently extinct animals such as the zebra-like quagga²⁴, the thylacine or Tasmanian wolf^{21,25}, and moas, the large flightless New Zealand birds²⁵. Ancient DNA fragments have also been recovered from 13 000-year-old bones of the giant ground sloth found in a cold cave deposit in southern Chile²⁶. The DNA extracted from all of these remains has been sequenced and shown to be useful in determining the phylogenetic relatedness of such species. The oldest authenticated records of ancient DNA come from woolly mammoths frozen in the permafrost of Siberia and estimated to be 50 000 or more years old^{21–24}.

However, it is well established that many ancient specimens are recalcitrant to DNA extraction or subsequent enzymatic manipulation of extracted DNA, or are so poorly preserved that the amount of undamaged endogenous DNA is too small to be of any real use^{18,20}. The survival of ancient DNA appears to be influenced less by the age of a specimen than by the environmental conditions under which it was preserved^{15,16}.

There is solid evidence that ancient DNA can possibly survive as long as 100 000 years under unusual conditions of preservation. Beyond this, does ancient DNA survive into the geological past? We know that there are theoretical reasons why such DNA survival is not expected, but there have been several claims. Each requires careful examination.

Miocene plant fossils

Chloroplast DNA sequences have been recovered from plant fossils obtained from two Miocene lake deposits, the first at the Clarkia site in northern Idaho, USA, and the second at Ardèche in France.

At the Clarkia site, DNA was retrieved by two independent groups from well preserved *Magnolia* leaves⁶ and *Taxodium* specimens⁷ 17–20 million years old. Superficially, these specimens seemed potential sites for the preservation of geologically ancient DNA. Ultrastructural studies of the *Magnolia* leaves showed they were well preserved with intact cellular structure, including, in many cases, intracellular organelles such as chloroplasts. The extracted and amplified sequences were phylogenetically related to extant *Magnolia* and *Taxodium* sequences, respectively, suggesting that authentic ancient DNA had been recovered.

However, the leaves were taken from wet sediments that had been continuously waterlogged since deposition. Because water is a primary agent for the degradation of DNA it is difficult to reconcile the conditions of the deposits with the large DNA fragments that appeared to be present in the leaves. The biomolecular preservation reported in leaves from the Clarkia site is not consistent with the apparent preservation of DNA. Biopolymers, including polysaccharides and proteins, are not preserved²⁵, and amino acids are extensively racemized²⁶. Racemization of certain amino acids from the L- to D-enantiomers occurs at a similar rate to the depurination of DNA. The extent of amino acid racemization in an ancient sample can therefore be used to assess the potential level of DNA depurination and hence whether the sample contains endogenous DNA.

Other workers have tried to replicate the work on *Magnolia* in independent laboratories²⁷. High-molecular-weight DNA could be recovered from about 10% of the specimens examined. However, no plant DNA was detected; the only DNA recovered was bacterial, and almost certainly Recent in origin. Failure to amplify plant DNA from a large number of Miocene plant fossils from the Clarkia site, including *Magnolia* and *Taxodium* specimens¹⁷, has cast further doubt on the reproducibility, and therefore authenticity, of the DNA sequences in the two initial reports.

At the Ardèche site, fossil material was preserved in a diatomite deposit 8.0–8.5 million years old. Chloroplast DNA sequences were retrieved from 11 well preserved leaf fossils¹¹. Silica, which is a major component of the diatomite sediment, binds DNA and was thought to have protected it from degradation. However, there was no correlation between the identity of the fossil plant and the DNA sequences obtained. Ten of the 11 fossil DNA sequences showed greater similarity to sequences from entirely unrelated species of plants than to those of modern relatives. Higher-plant DNA was also amplified from extracts of the sediment itself.

Together, these results suggest that the amplified DNA was not derived from the fossil specimens.

Cretaceous 'dinosaur' bone

In 1994, Woodward and co-workers published a report⁶ in which they claimed to have directly sequenced DNA from a large bone that was about 80 million years old. The size and age of the bone led them to speculate that it was a dinosaur bone. The PCR was attempted 2880 times on extracts from two bone fragments, yielding just one short (170 base-pair) fragments of a mitochondrial DNA gene. These fragments proved difficult to match with any known sequence but the sequences appeared to be intermediate between those found in reptiles and mammals. This led to speculation that the sequences might be degraded dinosaur DNA.

As at the Clarkia site, the environment of preservation seems hostile to DNA. The bone came from a coal seam and had been deposited in a coastal deltaic environment. The rank of the coal suggested that the sediments had been buried to a depth of 3 km and subjected to temperatures of 90–95°C. Poor preservation of amino acids in the bones is consistent with this unfavourable environment and suggests that endogenous DNA was unlikely to survive intact³⁶. Recent re-analyses of the putatively ancient sequences by several different groups showed them to be mammalian in origin^{38–40} and almost certainly derived from human pseudogene sequences^{41–45}, that is, segments of mitochondrial DNA that have become incorporated into the human nuclear genome. Stringent precautions were taken to minimize contamination, but it appears that the supposed fossil sequences were modern in origin.

DNA from amber-entombed insects

By far the greatest number of claims for ancient DNA are based on work with amber-entombed fossils: stingless bees (*Proplebeia dominicana*)^{1,2}, termites (*Mastotermes electrodominicus*)^{3,9}, wood gnats (*Valessegnya disjuncta*)¹⁰, a plant (*Hymenaea protera*)¹² and bacteria⁸ from Oligocene Dominican amber 25–35 million years old, and a weevil (*Libanorhinus succinus*)⁴ from Cretaceous Lebanese amber, 120–135 million years old. Furthermore, the authors of these reports apparently achieved a success rate of more than 90% in recovering ancient DNA.

If geologically ancient DNA is to be found anywhere it must surely be in fossils preserved in amber. Amber entombs specimens completely, after which they rapidly dehydrate⁴², so that the tissue is effectively mummified. The terpenoids that are the major constituent of the resin may inhibit microbial decay⁴⁴. The morphological preservation of some amber-entombed specimens is exquisite, even at subcellular level^{43,45}. Biochemical preservation also seems to be exceptional – levels of amino acid racemization in amber preserved insects are comparable to those from much younger archaeological specimens, such as the woolly mammoth, that have yielded authentic ancient DNA³⁶. This suggests that DNA may be similarly well preserved in amber.

The DNA sequences retrieved from all amber-preserved organisms meet several criteria of authenticity. Most importantly, the fossil sequences make phylogenetic sense, and DNA has been recovered from a variety of different organisms. However, the extraction and amplification of fossil DNA sequences from amber-preserved organisms has yet to be reproduced in independent laboratories. Three groups have tried and failed to find any evidence of authentic ancient DNA in more than 40 insects preserved in Dominican and Baltic amber. Attempts to extract ancient DNA from two Dominican amber beetles⁴⁶ and more than 30 different insects in Baltic amber⁴⁷ yielded only contaminating sequences

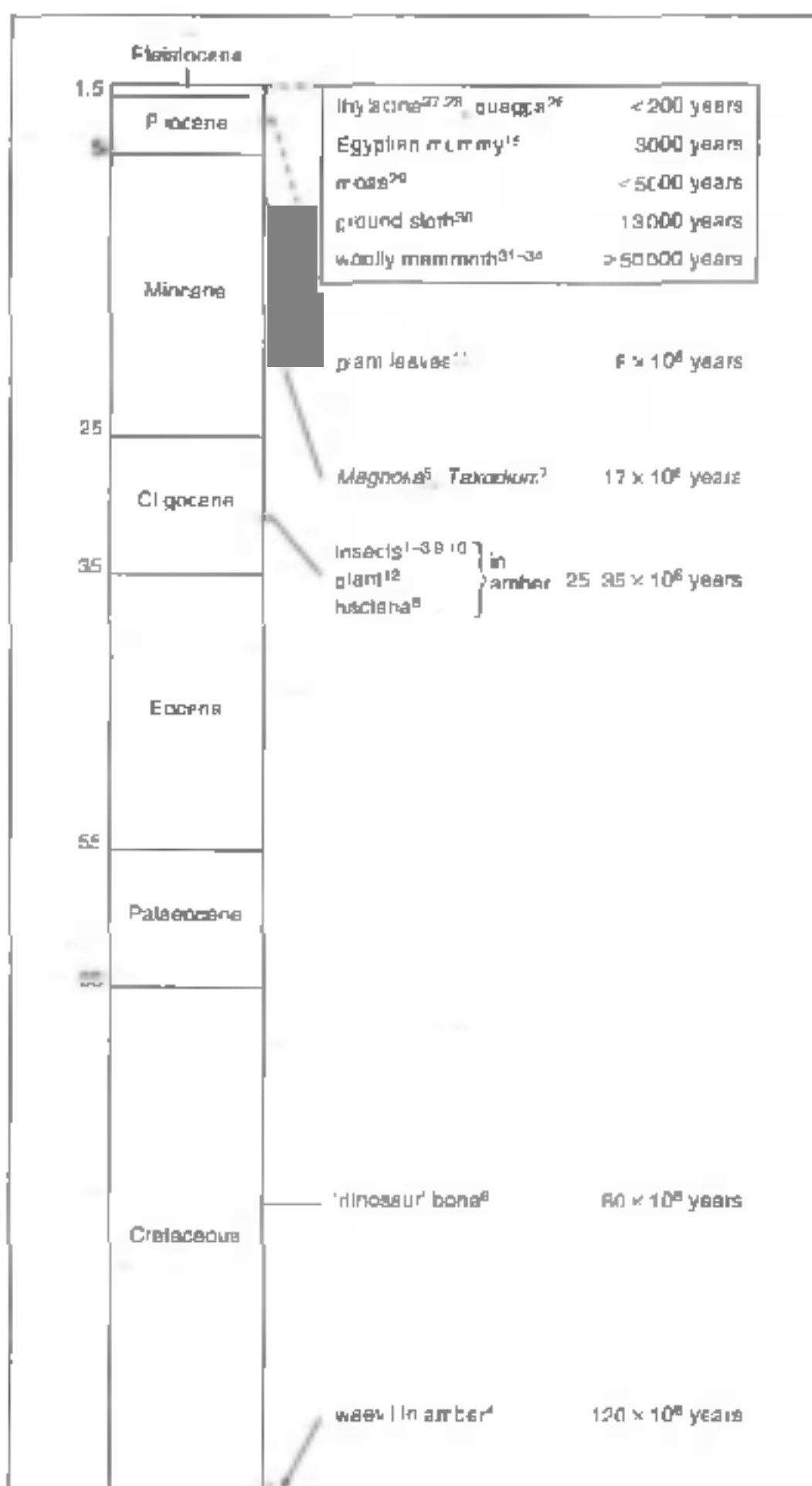


Fig. 1. Geological timescale (in millions of years) with some of the more important reports of ancient DNA recovery. Note that most records lie in the past 50 000 years at the very top of the timescale, which is within the theoretical lifespan of DNA.

from an extant grasshopper and humans and fungi, respectively. The third attempt⁴⁸ involved ten specimens of the stingless bee, *Proplebeia dominicana*, the first amber-preserved insect from which fossil DNA sequences were claimed. Additionally, two flies from Dominican amber and three specimens of a second genus of bee from East African copal less than two million years old were examined. Once again, only contaminating DNA sequences of vertebrate and fungal origin were recovered from these fossils. The lack of reproducibility of DNA sequences from amber-preserved insects, particularly the previously studied Dominican amber bee, casts serious doubt on the authenticity of earlier claims.

Conclusion

Although no amount of negative evidence can disprove the existence of geologically ancient DNA, the failure of all

claims to meet one or more criteria of authenticity shows that it is highly unlikely that geologically ancient DNA survives in any fossil material so far studied. Even if geologically ancient DNA exists in a small fraction of exceptionally well preserved fossils, it is debatable whether it will have a significant impact in the field of evolutionary biology because of its extreme rarity and probable highly degraded state. Although the initial optimism that palaeontological research would be advanced by the study of geologically ancient DNA seems to have been unfounded, studies of 'dead' DNA from much younger material continue to hold promise for research in archaeology, population genetics, and evolutionary and conservation biology.

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