

Can geologically ancient DNA be recovered from the fossil record?

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Palaeontology in the early 1990's was buzzing with excitement about the possibility of recovering ancient DNA from the fossil record. During the late 1980's molecular techniques for sequencing genes had advanced at an extraordinarily rapid pace, and had become so sensitive that 'dead' DNA was beginning to be recovered successfully from museum specimens and archaeological material that was a few hundred to a few thousand years old. Then, in 1990, a paper appeared claiming to have recovered DNA from fossil plant leaves 15-20 million years old (Golenberg *et al.*, 1990). Within a year the film of Michael Crichton's book *Jurassic Park* was released, and ancient DNA became the hottest topic around. The race was on to be the first to recover ancient DNA from the beautifully-preserved fossils that were entombed in amber.

In 1992 two independent laboratories, one in California and the other in New York, reported that they had recovered DNA from 25-30 million year old amber-entombed insects (Cano *et al.*, 1992a, b; De Salle *et al.*, 1990). Records kept tumbling when, shortly afterwards, the recovery of DNA from an 120 million year old amber beetle was reported (Cano *et al.*, 1993). Then a team working on Cretaceous bone reported that they had been able to sequence small lengths of ancient dinosaur DNA (Woodward *et al.*, 1994). Science fiction seemed to be rapidly turning into science fact, and palaeontology looked poised to enter an exciting new era of research.

However, the study of ancient DNA has not lived up to its early promise. Since 1994 additional claims for the recovery of DNA from geologically ancient material have appeared, but so have several papers which cast serious doubts on the reliability of the earlier work.

What Conditions favour the survival of DNA?

Deoxyribonucleic acid (DNA) is a very complex molecule which one would not expect to survive over long periods of time. In fact, even within living cells DNA is under constant repair (Lindahl, 1993a). Its survival in the fossil record therefore requires exceptional preservational conditions. DNA tends to be destroyed over time through two main processes, hydrolysis and oxidation, so that the presence of either water or oxygen pose a serious threat to its long-term survival. For DNA to survive, therefore, decay needs to be stopped rapidly at death and tissue must be protected in a dry and airless environment.

Calculations based on laboratory experiments suggest that DNA should not be able to survive for more than about 10,000

- 100,000 years, leading to some doubt whether DNA will ever be found in the geological record (e.g. Lindahl, 1993b). However, by the standards of most biological molecules DNA can be remarkably long lived and, given the right sort of environment, it is conceivable that DNA might be able to survive even longer.

How is ancient DNA recovered and identified?

The isolation and extraction of ancient DNA from fossils became feasible when molecular techniques became sufficiently sensitive to work from just a few starter molecules. This came about with the invention of the Polymerase Chain Reaction (PCR), a technique which allows short fragments of DNA to be copied rapidly and efficiently. DeSalle *et al.* (1993) provide a very readable introduction to the methods used by molecular palaeontologists.

Recovering ancient DNA is technically difficult because few molecules will have survived and these will almost certainly be degraded in structure. To increase the chances of success genes that are repeated several thousand times in the genome of each cell are generally targeted.

Because PCR is such an incredibly sensitive technique it is able to amplify DNA from only a few original molecules, so contamination by traces of modern DNA poses a very real problem. PCR may pick up and amplify small pieces of DNA in airborne fungal spores or from shed human cells in preference to even rarer and more degraded ancient DNA fragments. Consequently, extreme precautions need to be taken to prevent contamination from modern sources, and strict criteria are needed to recognise when modern contaminates may have been amplified.

Criteria for establishing the authenticity of the sequence

Three criteria need to be fulfilled before recovered DNA from a specimen can be considered authentic.

The extraction procedure must have followed stringent protocols to minimise the chances of contamination. An isolated laboratory dedicated to ancient DNA work is essential to keep fossil tissue physically isolated from any molecular work being carried out on extant species. This room and equipment must be kept scrupulously sterile. Access to the laboratory should be highly restricted and anyone entering the laboratory needs to don full protective clothing to avoid contaminating the room. Finally, there must be a full suite of negative controls so that



Trigona sp. - a stingless bee preserved in Holocene copal (plant resin) from East Africa. Courtesy of the photographic studio of The Natural History Museum, London

contaminants that do manage to intrude can be recognised. If PCR reactions which include all the reagents but lack fossil tissue prove positive, DNA must have been introduced from a source other than the fossil.

The aligned sequences should make phylogenetic sense. Sufficient DNA needs to be recovered for its identity to be unambiguous. Ancient DNA recovered from a fossil insect, for example, ought to show the strongest similarity to sequences of its modern relatives. It is imperative that no modern relatives have been sequenced in the laboratory prior to work on the fossil material.

The results should be repeatable, either from different tissues of the same individual, or from different individuals being studied in independent laboratories.

● Records of ancient DNA from the recent past

There seems little doubt now that DNA can and has survived over a few hundreds to a few thousands or even tens of thousands of years under special conditions. Small fragments of DNA can be recovered from well-preserved archaeological specimens and recently extinct animals where the tissue from which DNA has been recovered has been isolated from water. Five modes of preservation exist:

(i) **Dried hides in museums.** One of the early successes came when DNA was recovered from the recently extinct quagga, a horse-like animal that roamed the plains of Africa until about 150 years ago. A dried hide in a museum collection proved to contain DNA (Higuchi *et al.*, 1984). Analysis of its DNA sequence showed that the quagga was most closely related to zebras.

Similar success was reported by Thomas *et al.* (1989) working on hides of the Thylacine or marsupial wolf. This carnivore was driven to extinction in the wild by farmers and hunters at about the turn of the century but survived until the 1920's in captivity. DNA was extracted and small fragments sequenced and again the phylogenetic position of the *Thylacine* was established on molecular grounds.

(ii) **Mummified tissues** Small DNA fragments have been recovered from Egyptian mummified human tissue between 2,000 and 2,500 years old (Pääbo, 1985).

(iii) **Dry, well-preserved bone and tissue** DNA fragments up to about 800 bases in length have been amplified from ancient human bone up to 5,500 years old (Hagelberg *et al.*, 1989; Horai *et al.*, 1989; Hänni *et al.*, 1990; Brown *et al.*, 1994; Hagelberg & Clegg, 1991; Hagelberg, 1994, 1996; Handt *et al.*, 1996). DNA has also been recovered from animal bones approximately 9,000 years old (Höss & Pääbo, 1993), from 4,300 year-old bones of the Moa, the extinct flightless New Zealand giant bird (Cooper *et al.*, 1992), and from the American mastodon (Yang *et al.*, 1996). Dried and mummified plant seeds have yielded amplifiable DNA up to about 4,000 years old (Rollo *et al.*, 1991). DNA sequences from 13,000 year-old bones of the recently extinct giant ground sloth, *Myodon darwini*, have also been recovered (Höss *et al.*, 1996). Identical PCR products were obtained from different specimens studied in two different laboratories, lending credence to their authenticity.

(iv) **Frozen Corpses** Humans preserved in ice, such as the 5,000 year-old Tyrolean ice-man, have yielded recoverable ancient DNA (Handt *et al.*, 1994). DNA has also been extracted from a frozen mammoth entombed in the permafrost of Siberia and

estimated to be at least 40,000 years old and possibly as much as 100,000 years old. Furthermore, the results have been replicated using bones and skin by other laboratories (Höss *et al.*, 1994; Taylor, 1996). This mode of preservation might seem strange since water is one of the main agents of destruction for DNA. However, by freezing water, it is effectively prevented from interacting with organic molecules.

(v) **Tar pit preservation** Ancient DNA has been recovered from a 14,000 year-old sabre-toothed tiger from La Brea tar pits in Los Angeles (Janczewski *et al.*, 1992). In this case it is the tar that presumably acted as a barrier to water, preventing the destruction of DNA in bone through hydrolytic attack.

● Geologically ancient DNA records

Three preservational settings have supposedly yielded geologically ancient DNA.

Miocene plant beds

The first record of geologically ancient DNA came from Miocene *Magnolia* leaves, 17-20 million years old, recovered from a lacustrine deposit in Idaho. These leaves were extremely well-preserved with intact cellular structure, in some cases showing intracellular organelles. They retained original coloration and the presence of flavonoids in these leaves suggesting limited exposure to oxygen (Golenberg *et al.*, 1991). The extracted and amplified sequences closely matched those known from extant *Magnolia* species.

This claim was met with some scepticism, largely because the lacustrine sediments from which the fossil leaves were recovered were soaking wet and had probably been water-saturated ever since they were deposited. How could DNA have survived under such conditions when hydrolysis is the primary agent for its destruction? Nevertheless, shortly afterwards, a second laboratory claimed to have recovered DNA from the leaf of a different tree from these beds; this time from the Bald Cypress, *Taxodium* (Soltis *et al.*, 1992).

Golenberg *et al.*'s results, however, proved to be irreproducible. Sidow *et al.* (1991) tried to replicate the work in an independent laboratory. They found that the only DNA they could recover was bacterial and almost certainly Recent in origin. The published *Magnolia* and *Taxodium* sequences from these fossils remain unverified and the suspicion therefore remains that they represent modern DNA contaminants.

Even less compelling is the report by Manen *et al.* (1995) of higher plant and diatom DNA sequences from 8.5 million-year-old diatomite sediment accumulated in an anoxic volcanic lake. Again the preservation of fossils in this deposit seems highly promising for DNA preservation, with hair, skin and muscle well-preserved in mummified mammals and fossil insects showing their original coloration. Eleven out of 69 fossils were found to provide positive PCR amplifications, but, surprisingly, there was no correlation between the identity of the fossil plant and the DNA sequence obtained. Manen *et al.* (*op. cit.*) thought that they might be amplifying ancient DNA bound to the diatomite sediment, and indeed found that they could get DNA product from the sediment directly without including fossil tissue. This unfortunately greatly weakens their case and once again suggests that they are dealing with traces of modern contaminants.

Cretaceous dinosaur bones and eggs

In 1994 Woodward *et al.* claimed to have recovered fragments of DNA from dinosaur bones that were about 80 million years

old. Using stringent laboratory protocols to minimise the chances of contamination, they performed 2880 PCR extractions in total, from which just nine short DNA fragments (up to 170 bases long) were recovered. These proved difficult to match with any known sequence and Woodward speculated that the sequences might be degraded dinosaur sequences.

Once again the environment in which the bones had been preserved appeared inauspicious for DNA survival. The dinosaur bones came from a coal seam and had been deposited in water-laid sediment in a coastal deltaic environment. Furthermore the rank of the coal suggested that the sediments had been buried to a depth of 3 km and subjected to temperatures of between 90 and 95°C. Survival of DNA under those conditions seemed to many extremely improbable.

The source of the putative dinosaur sequences obtained became clear when four groups of workers independently found that they matched human pseudogene sequences (Hedges & Schweitzer, 1995; Henikoff, 1995; Allard *et al.*, 1995; Zischler *et al.*, 1995). Once again, despite the care taken to avoid contamination, the supposed fossil sequences proved to be modern in origin.

Another recent claim to have recovered ancient DNA from dinosaurs, this time from inclusions within a late Cretaceous dinosaur egg (An *et al.*, 1995), has proved to be equally short lived. An *et al.* reported successful amplification of DNA from six PCR attempts and, because they could find no identical sequence in living animals, they assumed that the sequences were ancient. Hong (1995), however, carried out a phylogenetic analysis on the putative dinosaur sequences and demonstrated that they were plant and/or invertebrate in origin and thus presumably modern contaminants.

Preservation in amber

By far the greatest number of claims for ancient DNA come from work on amber-entombed fossils. Amber is the hardened resin from plants, and small invertebrates occasionally become trapped and fossilised in this resin. Of all the methods of preservation this seems to have the most promise. The chances of DNA preservation in amber-entombed fossils were thought to be excellent because:

- (i) Amber entombs organisms rapidly and dehydrates their tissues, effectively mummifying them (Henwood, 1993). Amber is also impervious to water, protecting the enclosed fossil from any subsequent exposure to groundwater.
- (ii) Although there is diffusion of gas through amber (Beck, 1988), this is relatively slow and probably inhibits oxidation.
- (iii) The terpenoids that amber contains may act as a bactericide inhibiting microbial decay (Langenheim, 1990).
- (iv) Specimen preservation is often exquisite! TEM work on the mummified tissue reveals excellent cellular and even subcellular structural preservation (Poinar & Hess, 1982; Grimaldi *et al.*, 1994).
- (v) Amino Acids appear very well-preserved, suggesting limited racemisation has taken place in amber-entombed insects (Bada *et al.*, 1994).

Fossils in amber have been entombed rapidly in a sterilising medium, isolated from water and under reduced oxygen diffusion. If ancient DNA is to be found anywhere it is surely going to be here.

In the last few years there have been a number of reported recoveries of ancient DNA from amber-entombed insects,

plants and bacteria (Cano *et al.*, 1992a, b, 1993, 1994; DeSalle *et al.*, 1992; Poinar *et al.*, 1993; DeSalle, 1994), all but one from 25-35 million-year-old Dominican amber. However, none of these reports has ever been replicated for the same organism in an independent laboratory and the one reported successful replication in the same laboratory remains anecdotal (Schweitzer & Cano, 1994). Although all workers have taken strenuous precautions to minimise the risks of contamination, until an independent laboratory can replicate the results there will always remain some doubt as to the authenticity of the DNA recovered.

The supposed recovery of geologically ancient DNA from bacteria in amber, or the revival and culturing of ancient bacteria that have lain dormant for millions of years (Cano *et al.*, 1994; Cano & Borucki, 1995) were quickly criticised (Priest, 1995; Beckenbach, 1995) or discounted (Hammamoto & Horikoshi, 1994). There is simply no way of authenticating that the bacterial products recovered represented ancient DNA. The validation of ancient DNA claims therefore rests on proving that insect DNA can be recovered from fossil insects by other laboratories.

Over the last three years several laboratories have tried and failed to amplify authentic ancient DNA from amber-entombed insects (Howland & Hewitt, 1994; Pawlowski *et al.*, 1996; Austin *et al.*, 1997). These studies are extensive (more than 45 specimens from three different amber deposits) using five different extraction techniques, all known to work effectively in recovering much younger 'dead' DNA.

Austin *et al.* (1997) made an extensive study of the stingless bee *Proplebeia* from 25-35 million-year-old Dominican amber. This was the first species that ancient DNA was supposedly recovered from (Cano *et al.*, 1992) and for which subsequent successful replication has been reported (Schweitzer & Cano, 1994). Out of 156 PCR attempts made on tissue extracts seven gave positive PCR amplifications with blank controls. However, on cloning, these proved to be different from one another and non-insect in origin, closest either to fungal or higher vertebrate sequences. Thus, despite the blank controls, it is apparent that the traces of DNA recovered came from modern contaminants. Austin *et al.* (1997) also attempted to extract DNA from smaller-bodied scuttle-flies (Phoridae) from Dominican amber, but met with an equal lack of success. Nor could they recover verifiable insect DNA from the very much younger stingless bees of West African plant resins just a few hundred to a few thousand years old.

This lack of replicability does not by itself disprove the existence of ancient DNA in amber-entombed insects, but does put a giant question-mark over previous studies. It is hard to avoid the conclusion that amber simply does not appear to preserve ancient DNA even over relatively short intervals of time. Clearly, superb anatomical preservation in a fossil is no guarantee of molecular preservation.

Prospects

● Six years on, the optimism and enthusiasm surrounding the field of geologically ancient DNA has largely vanished: the emperor's new clothes have been found to be wanting! Whereas there seems to be reasonable grounds for believing that DNA may be recoverable from dead animals and plants a few hundred to a few tens of thousand years old, the three highly publicised claims for survival into the geological record have all been seriously undermined by their lack of reproducibility. DNA needs exceptional conditions for its preservation, conditions which remove water from the tissue and inhibit oxidation. Only amber-entombment appeared to provide an environment

favourable for the long-term survival of DNA, but recent work has failed to prove that geologically ancient DNA is preserved in amber. The oldest records of DNA that have been authenticated therefore come from woolly mammoths preserved as frozen corpses. Thus, although ancient DNA can be used to tackle questions on an archaeological time-scale it is looking less and less likely that it can survive on a geological time-scale.

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