# Miocene DNA sequences — a dream come true?

A chloroplast DNA sequence amplified by the polymerase chain reaction from a 16 million year old leaf raises the hope of directly following molecular change on an evolutionary time scale.

The advent of the polymerase chain reaction (PCR) is transforming many aspects of molecular biology. An example of this is the field of 'molecular archaeology' — the retrieval of DNA sequences from ancient tissues — which owes its very existence to this technique [1]. The ability of the PCR to amplify a few intact DNA molecules that are extracted from old tissues has for the first time made it possible to go back in time and study old DNA sequences. Indeed, the PCR can even recreate longer sequences from short fragments in the test tube [2]. Thus, we can now dream about catching molecular evolution red-handed. So far, dreams about this sort of time travel have come true only for short trips into the past. Until recently, the oldest DNA sequences determined were 40 000-year-old mammoth sequences (R.G. Higuchi, unpublished results).

But last year, Golenberg *et al.* [3] published work that seems to surpass our wildest dreams. They report the extraction and amplification of a chloroplast DNA sequence from a fossil leaf that is about 16 million years old! The leaf in question comes from Clarkia, Idaho. At that site, copious amounts of plant remains exist in clay sediments that were deposited in the shallow waters of a lake that existed in Northern Idaho in the mid Miocene [4] (Fig.1). When these leaves are excavated from the water-soaked clay, in some instances they retain a greenish hue which quickly changes to brownish black on exposure to the ambient milieu. Chloroplasts, starch grains, cell walls and occasional mitochondria have been identified in leaves from Clarkia [5]. Biochemically, these fossils are similarly well preserved. In particular, the glycosidic bond has been shown to survive in a variety of compounds, including flavonoids and starch [6].

Golenberg *et al.* took the intelligent approach of extracting the nucleic acids at the excavation site within minutes of exposure of the fossils. Back in the laboratory, they analysed the extracts by gel electrophoresis and found that about ten per cent of the extracts contained what seemed to be high molecular weight DNA. These extracts were used to amplify a segment of the chloroplast *rbcL* gene, for which this group has collected sequences from a large number of species. One of the extracts used was prepared from a leaf identified as the extinct species *Magnolia latahensis*. After 30 cycles of PCR from this extract, the 790-base-pair (bp) fragment of the *rbcL* gene was visible in an electrophoretic gel stained with ethidium. This is in stark contrast to amplifications of mitochondrial DNA from a few thousand-year-old dried tissues, where it is barely possible to visualize fragments that are 100–150 bp long after 40 cycles of PCR. When analysed with tree-building methods, the sequence of the amplified fragment grouped weakly within the Magnoliaceae family, being approximately equidistant between one Magnolia and one Liriodendron sequence. On the basis of the phylogenetic inference and the fact that the *M. latahensis* sequence is not identical to any other known *rbcL* sequence the authors conclude that this is the first Miocene DNA sequence ever determined.

**Fig.1.** Fossil leaves excavated at Clarkia in August 1990 by S.B., A.C.W. and colleagues.

If this finding can be verified and reproduced, this work will undoubtedly be regarded as an epoch-making contribution to evolutionary biology. If it is possible to compare DNA sequences over a geological time scale, our dreams about time-travel will be fantasies no more. We will be able not only to address taxonomic questions involving long-since extinct plant taxa, but also to assess evolutionary rates directly, provided that we can identify ancestral and descendant species and populations. This will probably be possible as, if the Clarkia site yields Miocene sequences, then other sites where environmental conditions have been comparable must exist. A series of such sites would make possible the eventual identification of evolutionary intermediates and thus the direct timing of the molecular clock.

Nevertheless, there are reasons to be only cautiously enthusiastic at this point. One of the reasons is the fact that

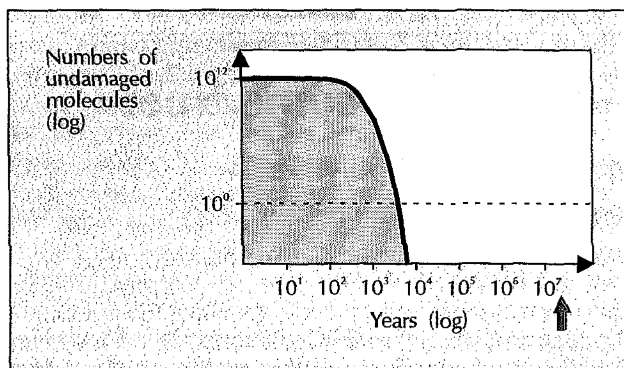


**Fig. 2.** Survival of 800-bp DNA segments in aqueous solution. The number of segments that remain unaffected by depurination at 15°C and pH 7 is plotted against time, starting with $10^{12}$ segments. After about 5 000 years the last molecule is depurinated. The red arrow indicates the approximate age of the Clarkia fossils (16 million years).

the Clarkia deposit is saturated with water and that it is thought that water has been present in the deposit since the Miocene. Because the rate of spontaneous depurination of DNA in aqueous solution is well studied [7], the rate at which a DNA segment of certain length is expected to survive can be calculated. Figure 2 shows the result of such a calculation where the numbers of 800-bp molecules that remain undamaged are plotted against time, starting with $10^{12}$ molecules, which may be the approximate number of chloroplast genomes in one gram of leaf tissue. At pH 7 and 15°C, the last 800-bp fragment will be depurinated after about 5 000 years. At higher or lower pH the rate would be faster, whereas at 5°C the reaction would be two to four times slower. As baseless sites rapidly lead to strand breaks [8], it seems that the probability of any 800-bp DNA molecule surviving 16 million years in the presence of water is excluded. But this is a theoretical consideration and the empirical results will have to take precedence if proven right. One possible explanation for the apparent contradiction between theory and observations is that pressure could force the water out of the chloroplasts and leave some chloroplast DNA molecules trapped in a lipid environment with very little water present. This could have two effects. First, there may be too little water present in these 'lipid cages' to keep the purines and C-1 hydroxyls of the sugar residues hydrated. This would drastically slow down depurination. Second, as the hydrolysis products could not escape, they may accumulate in concentrations high enough to impede further depurination. Finally, if the DNA molecules become immobilized due to compression, the C–N bond may be limited in its ability to stretch and bend, which may also slow down depurination. Experiments aimed at determining the water content of the Clarkia fossils would be very worthwhile.

Another source of concern are recent preliminary results from material that was excavated last autumn at Clarkia in collaboration with Dr Jack Smiley (University of Idaho) and Dr Michael Clegg (University of California, Riverside). In extracts prepared from the newly excavated leaves, we could verify the observation that about one extract in ten contains high molecular weight DNA. But this DNA is of eubacterial rather than plant origin (Arend Sidow and S.P., unpublished results). So far, we have been unable to amplify chloroplast sequences from these extracts or others where no DNA can be seen using ethidium bromide-stained gels. However, the success rate is not likely to be high and work on the plant remains from Clarkia as well as other similar sites has only just begun. By the concerted efforts of workers from different fields we will soon know if, and to what extent in the future, molecular biology will turn into a subdiscipline of palaeontology.

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Svante Pääbo, Lehrstuhl für Allgemeine Biologie, Institut für Zoologie, Universität München, Luisenstrasse 14, W-8000 Munich 2, Germany. Allan C. Wilson, Division of Biochemistry and Molecular Biology, University of California, Berkeley, California 94720, USA.



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