JASs *I*nvited *R*eviews
Journal of Anthropological Sciences
Vol. 84 (2006), pp. 65-83

Advances in ancient DNA studies

David Caramelli[1] & Giampietro Lago[2]

1) Department of Animal Biology and Genetics, Laboratory of Anthropology, via del Proconsolo 12, 50122 Firenze, Italy: e-mail david.caramelli@unifi.it

2) Carabinieri Scientific Investigation Department of Rome, Molecular Biology and Genetics Unit, Viale Tor di Quinto, 119, 00191 Roma, Italy

Summary – *Recent advances in DNA technology have made possible to recover DNA from archaeological and palaeontological remains allowing to the scientist to go back in time studying 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.*

Keywords – *Ancient DNA, molecular damage, new methodology, ancient nuclear DNA.*

Introduction

The first aDNA studies used bacterial cloning to amplify small sequences retrieved from skins of animal and human mummies, and revealed the inefficient reaction kinetics of this technique (Higuchi *et al*., 1984; Pääbo 1985, 1989). These studies demonstrated that the genetic material surviving in ancient specimens was often principally microbial or fungal in origin. Endogenous DNA was generally limited to very low concentrations of short, damaged fragments of multi-copy loci such as mitochondrial DNA (mtDNA). The invention of the polymerase chain reaction (PCR) made it possible to routinely amplify and study even single surviving molecules, allowing the number and range of aDNA studies to diversify rapidly (Pääbo 1989; Pääbo & Wilson 1988; Pääbo *et al*., 1989; Thomas, 1989). However, the enormous amplifying power of PCR also means that contamination from modern DNA becomes a major problem. Contamination is especially likely when previously amplified PCR products are present. False positives from intra-laboratory contamination remain a major problem in aDNA research. The large number of PCR cycles used to amplify aDNA means that it is actually quite difficult not to obtain positive (although not authentic) results. Many of the most extravagant aDNA reports have since been either disproved or effectively disregarded including early, spectacular claims of DNA sequences surviving for millions of years (Myr) in plants (Golenberg *et al*., 1990; Soltis *et al*., 1992, although see Kim *et al*., 2004), dinosaur bones (Woodward *et al*., 1994) and amber inclusions (Cano *et al.,* 1992a, b, 1993; DeSalle *et al*., 1992, 1993; Poinar *et al.,* 1993; DeSalle, 1994).

Despite this somewhat tarnished history, recent have improved standards, and aDNA is now emerging as a viable scientific discipline. A series of large scale studies have begun to reveal the true potential of aDNA to record the methods and processes of evolution, providing a unique way to test models and assumptions commonly used to reconstruct patterns of evolution, population genetics and palaeoecological change.

Molecular Damage

Within living cells, the integrity of DNA molecules is continually maintained by enzymatic repair processes (Lindahl, 1993). After the death of an organism, cellular compartments that normally sequester catabolic enzymes break down. As a

consequence, the DNA is rapidly degraded by enzymes such as lysosomal nucleases. In addition, the DNA molecule faces an onslaught of bacteria, fungi, and insects that feed on and degrade macromolecules (Eglinton & Logan, 1991). Under rare circumstances, such as when a tissue becomes rapidly desiccated after death or the DNA becomes adsorbed to a mineral matrix, it may escape enzymatic and microbial degradation. Even so slower but still relentless chemical processes start affecting the DNA. Many of these processes are similar or identical to those that affect the DNA in the living cell. However, after death they are not counter balanced by cellular repair processes and thus damage accumulates progressively until the DNA loses its integrity and decomposes, with an irreversible loss of nucleotide sequence information. PCR methods allow the occasional salvage of information from some rare samples in which the disintegration of DNA is not yet complete.

What happens in ancient samples?

The most obvious type of damage to DNA extracted from subfossil and fossil remains is its degradation to small average size, generally between 100 to 500 bp (Hofreiter *et al.*, 2001b; Pääbo, 1989). The reduction in size is due to both enzymatic processes that occur shortly after death and nonenzymatic hydrolytic cleavage of phosphodiester bonds in the phosphate-sugar backbone (Lindahl, 1993; Shapiro, 1981) that generate single-stranded nicks. The glycosidic bonds between nitrous bases and the sugar backbone are also subject to hydrolytic cleavage that results in abasic sites (Lindahl & Karlstro, 1973; Lindahl & Nyberg, 1972; Schaaper *et al.,* 1983). Once a nucleotide is released, the abasic site can undergo a chemical rearrangement that promotes occurrence of strand breakage at a rate similar to or slightly slower than base loss (Friedberg *et al.,* 1995; Shapiro, 1981). The extent of degradation by these processes depends upon the idiosyncrasies of preservation and can vary even among museum specimens of the same age. Sometimes, fragments as long as a few hundred base pairs (Cooper, 2001; Cooper, 1992; Haddrath & Baker, 2001) and sometimes even more than 1 kb (Lambert *et al.,* 2002) can be amplified. However, compared with contemporary DNA preparations from fresh tissues, ancient DNA is invariably of shorter length. The length of the DNA sequences that can be amplified by the PCR is limited not only by strand breaks but also by lesions that present blocks to the elongation of DNA strands by the *Taq* polymerase. Many such lesions are induced by free radicals such as peroxide radicals ($-O_2\cdot$), hydrogen peroxide (H_2O_2), and hydroxy radicals ($OH\cdot$), which are created by, among other causes, background radiation. Major sites of oxidative attack are the double bonds of both pyrimidines and purines, leading to ring fragmentation. In addition, the chemical bonds of the deoxyribose residues are susceptible to oxidation resulting in fragmentation of the sugar ring (Friedberg *et al.,* 1995; Lindahl, 1993). DNA extracted from fossil remains is susceptible to cleavage with an enzyme, endonuclease III, which is specific for oxidized pyrimidines (Pääbo, 1989). It has also been shown that paleontological specimens from a diverse range of environments and ages contain oxidized base residues (Höss *et al.,* 1996). Specifically, no DNA sequences could be amplified via PCR (Höss *et al.,* 1996) from samples with higher amounts of two oxidized pyrimidines 5-hydroxy-5-methylhydantoin (5-OH-5-MeHyd) and 5-hydroxyhydantoin (5-OH-Hyd), which block the *Taq* DNA polymerase. Another type of damage are cross-links, which also block the DNA polymerase and can even be observed directly by electron microscopy in ancient DNA preparations (Pääbo, 1989). By pyrolysis gas chromatography–mass spectroscopy, volatile components formed from Maillard products have been identified in ancient fecal remains (coprolites) (Poinar *et al.,* 1998). Maillard products are formed by condensation reactions between sugars and primary amino-groups in proteins and nucleic acids. Treatment with a reagent, N-phenacylthiazolium bromide (Vasan *et al.,* 1996), which breaks Maillard products, allows DNA sequences to be amplified from some ancient remains that otherwise are not amenable to amplification, for example 20,000-year-old ground sloth coprolites (Poinar *et al.,* 1998) and >40,000-year-old Neandertal bones (Krings *et al.,* 2000). In addition to fragmentation and DNA modifications that hinder the extension of DNA polymerases,

other known and unknown types of damage are common in ancient DNA. Some of these DNA modifications are problematic because although they allow the amplification of the template molecules, they cause incorrect bases to be incorporated during the PCR. The most common form of such modification is the hydrolytic loss of amino groups from the bases adenine, cytosine, 5-methylcytosine, and guanine, resulting in hypoxanthine, uracil, thymine, and xanthine, respectively (Friedberg *et al.,* 1995). The deamination products of cytosine (uracil), of 5-methylcytosine (thymine), and of adenine (hypoxantine) are of particular relevance for the amplification of ancient DNA since they cause incorrect bases (A instead of G, and C instead of T) to be inserted when new DNA strands are synthesized by a DNA polymerase.

Nucleotide Misincorporations in amplifications of ancient DNA

The occurrence of such modified bases is evident from the observation that when PCR products from ancient remains are cloned and the sequences of several clones compared, the number of differences contained among them is often larger than what is typically seen when modern DNA is amplified (Hansen *et al.,* 2001; Hofreiter *et al.,* 2001). Two types of evidence suggest that deamination of bases is a major cause. First, DNA extracted from ancient tissues is sensitive to uracil-DNA-glycosylase, an enzyme that removes uracil from DNA (Pääbo, 1989). Second, a large number of C to T and G to A changes are often observed in clones from ancient amplification products (Hansen *et al.,* 2001). In fact, even the two incorrect positions determined in the very first ancient DNA publication (Higuchi *et al.,* 1984) were of this type, one representing a C to T change and the second a G to A compared with the correct sequence (Pääbo & Wilson, 1988). This is consistent with the presence of deaminated C residues that are identical to uracil (U) residues and cause the incorporation of A residues rather than G residues by the *Taq* DNA polymerase (Hansen *et al.,* 2001). Such miscoding lesions in ancient DNA complicate the correct determination of ancient DNA sequences. To address this situation, it is necessary to distinguish between misincorporations induced by damage in the ancient DNA template and *Taq* DNA polymerase errors that occur in any PCR regardless of original DNA template quality. One way to do this is to perform multiple amplifications from DNA extracts containing just a few template molecules and clone the PCR products. Comparison of DNA sequences of multiple clones from such amplifications will reveal nucleotide differences that occur in all clones from one amplification but not in other amplifications from the same template preparation (Hofreiter *et al.,* 2001).

The vast majority of such "consistent" substitutions are due to errors occurring in the first cycles of PCR, which is when the original DNA extracted from an ancient specimen serves as a template. By contrast, additional substitutions seen in single clones that also carry consistent substitutions will be due to misincorporations that occurred later during the PCR when molecules synthesized during previous PCR cycles serve as a template. Thus, if the frequencies of misincorporations between these two classes of substitutions are compared, the difference between substitutions induced by damage in the original template can be discerned from the inherent error rate of the PCR under the conditions that occur in the exact same PCR reaction. Differences seen between clones where no consistent substitutions occur are less informative, since they represent a mixture of misincorporations that occur when an ancient DNA molecule served as a template and misincorporations that are due to errors during later PCR cycles when newly synthesized molecules are the main source of templates. When consistent differences were compared with other differences in amplifications from the remains of 11 European cave bears that varied in age between 25,000 and >50,000 years, a remarkable difference in substitution patterns was seen (Hofreiter *et al.,* 2001). Among 48 consistent substitutions, all were C to T and G to A substitutions, whereas among the 23 substitutions that occurred in subsequent cycles of the PCR, only three changed a C to a T or a G to an A. Furthermore, in all cases when multiple consistent substitutions occurred in a single amplification, only C to T substitutions or only G to A substitutions were observed, i.e., in no cases were consistent C to T substitutions found

together with consistent G to A substitutions in one amplification. This suggests that these amplifications started from single DNA strands and that a single class of DNA damage is responsible. When templates from one strand were enriched by linear amplifications using a single primer prior to PCR, the substitution spectrum was largely or even completely due to incorporations of As at positions where the unmodified template carries a C. This type of misincorporation, which is eliminated when the template DNA is treated with uracil-DNA-glycosylase (Hofreiter *et al.,* 2001), is due to either deamination of cytosine residues to deoxyuridine residues in the DNA or, alternatively, deamination in conjunction with oxidation resulting in 5-hydroxyuridine residues. This type of miscoding lesion dominates quantitatively over other forms of miscoding lesions to such an extent that when C to T and G to A substitutions are disregarded, the error rate when ancient DNA templates are replicated does not differ from that when modern templates are replicated (Hofreiter *et al.,* 2001).

Our knowledge of damage in ancient DNA and of misincorporations caused by such damage is still limited. Further studies of larger numbers of specimens with a variety of techniques are therefore needed. In an analysis of large numbers of cloned human amplification products from ancient remains, Gilbert and coworkers (Gilbert *et al.,* 2003b) also found that C to T and G to A changes predominate. In addition, they observed an elevated frequency of T to C and A to G changes. They suggested that the latter changes were caused by deamination of adenine residues, producing hypoxanthine residues that cause cytosine residues to be incorporated by *Taq* DNA polymerase. From a chemical perspective, this is a likely scenario. However, the inference is based on the premise that miscoding lesions that cause a T to be read as a C will not occur. Contamination with human DNA is common, and known and unknown modifications and other unexpected effects may occur in ancient DNA. For example, Pusch & Bachmann claimed that most extracts from ancient remains induce mutations even in modern mitochondrial DNA added to the extracts and subsequently amplified by PCR (Pusch & *Bachmann*, 2004). These authors therefore posit the existence of some uncharacterized factor that makes the *Taq* DNA polymerase error prone. In addition, they claim that such errors tend to fall at positions known to vary among human mitochondrial control region sequences. This scenario of mutagenic DNA extracts is presented without a plausible mechanistic framework and is highly questionable. First, they are unable to reproduce their results using several extracts of ancient bones (Serre *et al.,* 2004b). Also, contaminating modern human DNAs often amplified from ancient remains fail to show a high frequency of misincorporations (Krings *et al.,* 1997). Nevertheless, this claim underscores the difficulty in excluding any particular misincoporation as "chemically impossible." The advantage of the approach in which consistent and non consistent changes are analyzed in the same clones is that misincorporations that occur when ancientDNA template molecules are replicated can be largely distinguished from those that occur when intact newly synthesized DNA molecules are replicated in one and the same PCR reaction. Thus, this approach takes into account any hitherto unknown DNA modification as well as factors that influence the DNA polymerase's fidelity.

It is clear that constant low temperatures play a central role in the longevity of aDNA molecules (Lindahl 1993; Hofreiter *et al.,* 2001b; Smith *et al.,* 2001; Willerslev *et al.,* 2004b). The oldest authenticated aDNA reports are all from permafrost settings, including greater than 50 kyr (thousand year)-old mammoth mtDNA (Höss *et al*., 1994), a greater than 65 kyr-old bison mtDNA (Gilbert *al.,* 2004a; Shapiro *al.,* 2004) and 300–400 kyr-old plant chloroplast DNA (cpDNA) and 400–600 kyr-old bacterial sequences (Willerslev *et al.*, 2003a, 2004a). In addition, Holocene and Pleistocene permafrost-preserved bones have permitted amplification products in the 900– 1000 bp size range (Barnes *et al.,* 2002; Lambert *et al.,* 2001). Other features, such as rapid desiccation and high salt concentrations, may also prolong DNA survival (Lindahl, 1993). However, kinetic calculations predict that small fragments of DNA (100–500 bp) will survive for no more than 10 kyr in temperate regions and for a maximum of 100 kyr at colder latitudes owing to hydrolytic damage (Poinar *et al,.* 1996; Smith *et al.*, 2001). Even under ideal conditions, amplifiable DNA is not thought to survive for longer than 1 Myr. The oldest DNA sequences may well exist in polar

icecaps, where constant temperatures of as low as -50°C and samples of more than 800 kyr old (e.g. Dom C, Antarctica) hold much promise. There have been several reports about ancient plant, microbial and viral DNA from ice core samples of up to 100 kyr old (Ma *et al.*, 1999; Priscu *et al.,* 1999; Willerslev *et al.,* 1999; DePriest *et al.,* 2000; Christner Christner *et al.,* 2001; Hansen & Willerslev, 2002), although authentication standards, such as the independent replication of results, have varied considerably.

The nightmare of contamination in ancient DNA studies

The criteria for authenticating aDNA results are continually evolving as new materials (e.g. microbial DNA and cultures) are studied (Handt *et al.,* 1994; Rollo & Marota, 1999; Austin *et al.*, 1997b; Cooper & Poinar 2001; Hofreiter *et al.,* 2001b; Willerslev *et al.,* 2004b). Although critical steps such as the cloning and independent replication of results have been widely accepted, reports are still being published in high-profile journals without these basic authentication procedures. It is a matter of concern that this includes studies of both ancient human and Neandertal sequences where contamination risks are pronounced (Adcock *et al.,* 2001; Serre *et al.,* 2004a). Significantly, this is also the case especially for all reports of DNA older than the theoretical limit of survival (0.1–1 Myr).

A major difficulty in detecting and preventing contamination is that the scale of the problems involved is not easily appreciated. For example, a successful PCR reaction can contain some 1012–1015 amplified molecules in a volume of less than 50ml, which is too large a concentration to comprehend effectively (Kwok & Higuchi, 1989). Air movement created when opening PCR tubes or transferring liquids will create and disperse microscopic aerosol droplets, which can easily contain over a million copies of the template per 0.005ml. As a consequence, PCR products can quickly become widely distributed across laboratory surfaces, corridors and through entire buildings via personnel movement and air-handling systems. Since one aerosol droplet can easily contain a thousand times the amount of amplifiable mtDNA found in 1 g of many ancient human specimens (105–106 copies; Handt *et al.,* 1996; Cooper *et al.,* 2001b), aDNA laboratories must be completely isolated both physically and logistically, preferably in buildings free from all molecular biological research. Furthermore, daily personnel movement should only proceed from ancient to modern laboratories, i.e. up the concentration gradient. Such simple precautions can prove as effective as high-tech positive air-pressure and UV irradiation systems, if rigorously enforced. Human and microbial DNA and cells are ubiquitous in all laboratory settings. It is prudent to assume that all laboratory reagents and tools are contaminated with human and microbial DNA when arriving from the manufacturer. Extensive cleaning of reagents (e.g. ultrafiltration) and tools is essential, with complete decontamination requiring prolonged exposure (e.g. UV irradiation (45W, 72 h), baking (more than 180 _C, 12 h), acid (2.5M HCl, 48 h) and/or sodium hypochlorite (50%, 48 h). Laboratory reagents and commercial equipment marked 'sterile' are not guaranteed to be free of either viable cells or nucleic acids. Similarly, autoclaving does not prevent the amplification of short DNA fragments (less than or equal to 150 bp), and often contaminates material with bacterial DNA. These issues are critically important in ancient human and microbial studies, but contamination often cannot be completely ruled out (Willerslev *et al.,* 2004b; M.T.P. Gilbert, unpublished data). The most intractable problems occur when the sample itself has been contaminated prior to analysis. This issue is of major significance with archaeological material, where specimens have been handled, and often washed, during excavation by a variety of individuals who may have DNA markers close to or even identical to the specimen DNA (Cooper, 1997; Serre *et al.,* 2004a). Similarly, the passive or active movement of cells in ancient microbial studies makes it difficult or impossible to completely exclude recolonization of ancient materials over time.

Criteria of authenticity

The first published criteria of authenticity (Pääbo, 1989) were limited to three points: (*a*) testing of control extracts should be performed in parallel with extracts from old specimens to detect

contamination introduced from reagents and solutions during the extraction procedure; (*b*) more than one extract should be prepared from each specimen and both should yield identical DNA sequences; (*c*) there should be an inverse correlation between amplification efficiency and size of the amplification product, reflecting the degradation and damage in the ancient DNA template. These criteria, although still useful, have been continuously extended (Cooper *et al.,* 1992; Handt *et al.,* 1994; Hofreiter *et al.,* 1989b; Lindahl, 1993) as novel aspects of contamination and misincorporations have become obvious. A substantial list of criteria now exists. Briefly, the rationales behind these are described as follows.

Behaviour during and after excavation

Whenever possible, sample collection should be done at the original (archaeological) site with disposable gloves, face mask, head-dress gown, lab coat: All the instrument used for the collecting should be sterile. This minimizes the risk of the samples becoming contaminated by modem DNA and enables the immediate and appropriate storage of the samples. Storage of sample material should be as cool and dry as possible, preferably at -20°C. If samples are taken from museum collections where they generally have been stored at approximately room temperature, they should also be stored frozen, since it has been shown that periods at room temperature can cause further DNA damage. If electric or mechanical saws, forceps or scalpels are necessary for sampling, it is important to ensure proper cleaning of the blades between the sampling of two different individuals. This is best done by using successively concentrated soap or bleach, distilled water and a final wash in absolute ethanol in order to prevent cross-contaminations. It is also necessary to change the disposable gloves between different samples. If it cannot be excluded that contamination of the samples by members of the excavation team or by museum or any other personnel who have handled the material has occurred, it is recommended that a saliva sample from each person is taken as a control sample in order to enable genetic typing and the comparison of results. This is important in particular if only a single sample is going to be investigated. In cases where it is not possible to obtain saliva (or any other tissue) of a person suspected to be a potential contaminator, then some other material that is known to have been in contact with the person in question should be sampled and analyzed. Preferably the material should be of non-human origin. This sample is an important control and should not undergo the decontamination measures that are carried out for the sample that is the focus of interest.

1. Amplification products should be routinely cloned and multiple clones sequenced. This allows any heterogeneity in the amplification product to be unambiguously detected. It also allows the spectrum of errors to be estimated.

2. Blank extraction controls should be performed alongside extractions from ancient materials. Similarly, negative PCR controls should always be performed when ancient DNA templates are amplified. In fact, since contaminants present in laboratory reagents may be of so low a quantity that they are detected only sporadically in negative controls, several amplifications without any added template should be performed in each experiment. We find it useful to routinely do three such controls. A further concern is that some extracts of ancient remains contain substances, such as sugars and microbial DNA, that may serves as a "carrier" during the PCR, allowing a contaminant of low concentration to be amplified (Pääbo1990). Thus, a contaminant will become amplified when such an extract is added to the amplification but not in blank PCR controls, although it may be present there. To detect this effect, it is useful to add extracts from ancient species for which the primers used will not work to negative PCR controls to see if some amplification product appears.

3. Repeated amplifications from the same or different extracts from the same specimens are necessary for at least three reasons. First, they are useful to detect contamination of a particular extraction or amplification. Second, when very low numbers of template molecules exist in samples, extracts or aliquots of extracts may only sporadically contain DNA template molecules. Three extracts (Serre *et al.,* 2004a) may be a reasonable number of extraction attempts before a specimen of interest is abandoned as containing no useful DNA. Third, as discussed above, nucleotide misincorporations leading to consistent changes

can be detected only when multiple amplifications are performed.

4. Quantitation of the number of amplifiable DNA molecules present in an extract serves to determine if so few molecules initiate the PCR that consistent changes may occur. Note that PCR-based quantitation needs to be performed for each primer pair used since different primers may vary substantially in how efficiently they initiate amplifications. If a large number of molecules is present (>1000 may serve as a rule of thumb) (Handt *et al.,* 1996), and only one type of DNA sequence is expected, there is no need to perform several amplifications since consistent changes are extremely unlikely to occur. If fewer molecules are present, several amplifications are needed (criterion 1). The most economical way to proceed is to first perform two amplifications and sequence several clones from each. If a consistent difference between the two sets of sequences is observed (Hofreiter *et al.,* 2001), a third amplification is in general sufficient to determine which of the two sequence variants is reproducible, provided that what is studied are mitochondrial DNA sequences or other DNA sequences for which an individual is expected to carry only a single DNA type. If an autosomal sequence for which two alleles may exist is studied, the two amplifications should yield an approximately equal number of the two alleles if the amplification starts from many molecules. If it starts from few molecules, multiple successive amplifications are necessary to distinguish homozygous individuals from heterozygous individuals (Morin *et al.,* 2001; Navidi *et al.*, 1992). However, if the genotype of the individuals is not of interest, two to three amplifications will suffice (Greenwood *et al.,* 1999; Jaenicke-Despres *et al.,* 1992).

5. An inverse correlation between amplification efficiency and length of the amplification is a very simple indicator of the extent of degradation and blocking lesions present in an ancient DNA template. There are large differences in the length of amplifications that can be achieved from different specimens. Thus, whereas most ancient remains will not allow the amplifications of more than 100 or 200 base pairs of mitochondrial DNA (Pääbo, 1989), a few thousand-year-old remains of New Zealand flightless birds allow as much as about 500 bp of mitochondrial DNA to be retrieved in a single amplification (Cooper *et al.,* 1992; Cooper *et al.,* 2001), and amplifications up to 1.6 kb have been reported from permafrost remains (Lambert, 2002). In general, if shorter fragments are not more readily amplified than longer ones when compared with modern DNA sequences, it is an indication that the source of the DNA is likely be a modern contamination. If longer DNA sequences are determined by shorter overlapping segments, variable positions in the overlap or the primer site should ensure that the two sequences are indeed linked. Moreover the sequences should make phylogenetic sense; the i.e. do not appear to be a combination of different sequences, resulting from contamination of the samples studied by exogenous DNA.

6. Biochemical assays of macromolecular preservation serve two purposes. First, they support the claim that a specimen is well enough preserved to allow the preservation of DNA. Second, they may be used as rapid screening techniques to identify specimens that, according to their general state of preservation, may contain DNA. Several techniques have been suggested. Most widely used is the analysis of amino acids present in specimens (Poinar *et al.,* 1996), and the measures of amino acid preservation used have evolved as more experience has been gained. Thus, in our hands, the combination of total amount of amino acids, the composition of amino acids, and their extent of racemization is a useful proxy for DNA preservation in bones and teeth (Krings *et al.,* 1997; Poinar *et al.,* 1996; . Serre *et al.,* 2004a). Although the kinetics of racemization depend upon the position of the aspartic acid in the protein chain (Collins *et al.,* 1999), specimens that contain very little amino acids, a composition of amino acids that indicates that their macromolecules have been replaced by microorganisms, or where amino acids are extensively racemized are unlikely to contain endogenous DNA. Alternative methods include the estimation of the ratio of peptide fragments to single amino acids via mass spectrometry (Poinar & Stankiewicz, 1999), direct assessment of bone histology (Bailey *et al.,* 1996; Barnes *et al.,* 2000; Colson *et al.,* 1997; Jans *et al.,* 2004), determination of DNA damage via gas

chromatography/mass spectrometry (Höss *et al.,* 1996), measurement of porosity and density in bone (Nielsen-Marsh *et al.*, 2000), and transmission electron microscopy (Koon *et al.,* 2003). Large-scale studies of the correlation of each of these techniques with the preservation of unambiguously authentic ancient DNA would be very valuable.

7. DNA fragments derived from genomes of organelles such as the mitochondria (Bensasson *et al.,* 2001) are often present in the nuclear genome (Timmis *et al.,* 2004). Because mitochondrial DNA is the molecule of interest in most ancient DNA projects, such nuclear integrations may occasionally be amplified by PCR and be mistaken for the organellar DNA sequences. This is particularly likely to happen if the primers used differ from the organellar DNA sequence in the individual specimen but not from the version of the same sequence that exists as a nuclear insertion. Erroneous conclusions regarding intraspecific variation (Thalmann *et al.*, 2004) as well as species phylogenies (Vanderkuyl *et al.,* 1995) will then result. To prevent this problem, different primer sets can be used to amplify the same overlapping and variable sequences since it is very unlikely that two primer sets would both preferentially amplify a particular nuclear insertion (Krings *et al.,* 1997, Greenwood and Pääbo, 1999, Greenwood et al. 1999). However, in species where very large numbers of nuclear copies of mitochondrial DNA exist, multiple sequences may be obtained from all primer pairs, making the determination of mtDNA sequences impossible (Thalmann *et al.,* 2004).

8. A further criterion suggested early on when the seriousness of the contamination threat was realized (Allard *et al.,* 1995; Handt *et al.,* 1994a; Handt *et al.,* 1994b: Handt *et al.,* 1996; Hedges & Schweitzer, 1995; Henikoff, 1995; Richards *et al.,* 1995; Zischler *et al.,* 1995) is that crucial results should be reproduced in a second laboratory. This serves the same purpose as extraction and PCR controls in one laboratory (criterion 1), i.e., to detect a laboratory contaminant, for example a previous amplicon that exists in one laboratory. Replication in a second laboratory is thus an additional precaution to exclude the unlikely occurrence of a laboratory contaminant that fails to appear in blank extracts and negative PCR controls. This is warranted, in our opinion, when a novel and unexpected result of great consequence is obtained. In such cases, samples should preferably be sent independently from a museum or excavation directly to the two laboratories so that a potential laboratory contaminant cannot be transferred between laboratories.

9. Other criteria regarding especially the studies of human remains where contamination is more problematic; Evidence that similar DNA targets survive in associated faunal material is critical supporting evidence. Faunal remains also make good negative controls for human PCR amplifications.

What is achievable

Given that the chemical properties of DNA probably restrict the survival of any molecules to this side of a million years even in favourable environments where low temperatures and dry conditions slow the rate of chemical processes that degrade DNA (Höss *et al.,* 1996; Smith *et al.,* 2001; Smith *et al.* 2003*;* Willerslev, 2004), what has the study of ancient DNA achieved to date and what can be expected in the future? Below, we outline some broad areas where ancient DNA sequences have yielded novel insights and where further progress can be expected.

The Phylogenies

An obvious avenue of research opened up by ancient DNA sequences is the ability to relate extinct species with extant species via molecular phylogenies. Australian marsupial wolves (Krajewsk *et al.*, 1997; Krajewsk *et al.*, 1992; Thomas *et al.,* 1989), New Zealand moas (Cooper *et al.,* 1992; Cooper *et al.,* 2001; Haddrath *et al.,* 2001), American ground sloths (Greenwood *et al.,* 2001; Höss *et al.,* 1996) and endemic Hawaiian geese (Paxinos *et al.*, 2002) wolly mammoth (Capelli *et al* 2006) are examples of about 50 extinct animal species for which this has been done. In fact, many natural history museums, realizing that their collections represent genetic repositories, have established guidelines for removal of samples for molecular analyses and even installed molecular laboratories to work on their collections (Suarez & Tsutsui, 2004). DNA sequences that occur in many

hundred copies per cell, such as mitochondrial DNA and chloroplast DNA, are more often retrievable from ancient specimens than are nuclear DNA sequences that occur only once per haploid genome. Therefore, phylogenies cannot usually be estimated from several genetic loci. This limits the ability to resolve phylogenies of species that either diverged recently in time or so rapidly that different parts of the genome have different phylogenies. However, there are encouraging indications that this limitation can sometimes be overcome. For example, nuclear DNA sequences have been determined from several Pleistocene animals (Greenwood *et al.,* 1999; . Poinar *et al*., 2003) and from plants preserved in dry environments (Goloubinoff *et al.,* 1993; Jaenicke-Despres *et al.,* 2003). Recently, sex determination of moa samples using nuclear DNA sequences has revealed that several moa forms previously regarded as different species based on their morphology were, in fact, male and female birds of the same species (Bunce *et al.,* 2003; Huynen *et al*., 2003). Consequently, the number of moa species has been reduced from 11 to 9.

Population Genetics

The preservation of many individuals from a single locality, either in the form of museum specimens collected by earlier generations of naturalists or retrieved by archaeologists at a single site, provides the opportunity to track changes in the population over time. The first example of this was a study of three populations of kangaroo rats in California that were collected by zoologists in the first half of the past century. When present-day populations sampled at the identical localities were compared with the museum specimens (Thomas *et al.,* 1990), spatial stability of mitochondrial lineages was demonstrated - a situation that may be typical of undisturbed habitats. This stability, however, is not always the case. A recent study in the Chicago area demonstrated that mitochondrial lineages of mice have been replaced over the last 150 years, probably due to human influence (Pergams *et al.,* 2003). Other species for which population history has been followed over time are rabbits (Hardy *et al.,* 1995), pocket gophers (Hadly *et al.,* 1998), black-footed ferrets (Wisely *et al.,* 2002), sea otters (Larson *et al.,* 2002), otters (Pertoldi *et al.,* 2001), grizzlies (Miller & Waits, 2003), red squirrels (Hale *et al.,* 2001), canids (Verginelli *et al* 2005) penguins (Lambert *et al,* 2002; Ritchie *et al*., 2004) and Cattle (Beja Pereira *et al.,* 2006). A landmark study used analysis of late Pleistocene brown bears to radically alter the view of bear population dynamics in Alaska (Barnes *et al.*, 2002). Whereas mitochondrial brown bear lineages today are neatly distributed in different geographical areas of the world, this study showed that the same mitochondrial lineages coexisted in a single area about 35,000 years ago. This has potentially great implications for conservation genetics as it is often argued that mitochondrial lineages that are spatially separated today have been separated for much longer time periods and may represent "subspecies" adapted to different environments. As a consequence, it is often suggested that they should be managed separately and not allowed to mix in captivity or through enhancement of wild stocks. For bears, ancient bear DNA sequences have proved that contemporary samples do not reproduce long-term patterns. In the future, direct testing of the phylogeographic patterns of additional species will, it is hoped, clarify whether they are recent effects of random genetic drift in small populations or represent long-term separation of populations.

Ancient human DNA: a contentious issue

While animal and plant aDNA studies have seen rapid progress, contamination issues have undermined promises that such research would also revolutionize bioarchaeology (Herrmann & Hummel 1996). Early successful studies using material from hot environments such as Egypt (Pääbo, 1985) and Florida (Hauswirth *et al*., 1994) are now recognized as probable contaminants. For example, the Egyptian mummy sequence was a very large (3.4 kb) fragment of nuDNA, which is highly unusual, and was recovered from a region where temperatures make DNA survival very unlikely (Marota *et al.,* 2002; Gilbert *et al.,* 2005a). Several reports show that despite rigorous protocols (Cooper & Poinar 2001; Hofreiter *et al*., 2001b), modern human contamination is widespread in amplification products from ancient extracts (Richards *et al.,* 1995;

Handt *et al*., 1996; Krings *et al.,* 1997; Kolman & Tuross 2000; Hofreiter *et al.,* 2001b). It also appears impossible to clean contemporary human DNA from human bones and teeth despite extensive treatment with UV irradiation and bleach (Gilbert *et al.,* 2005b). It appears that the porosity of bone and dentine in teeth are the main entry routes for DNA generated from sweat, skin fragments and exhaled cells, reinforcing the urgent need for disposable gloves and face-masks during excavation and handling of archaeological specimens. Interestingly, hair may be a more reliable source for ancient human DNA studies, as it appears less susceptible to contamination than bone and teeth (Gilbert *et al*., 2004a).

Recently Salamon *et al*. (2005) have demonstrated that relatively well preserved DNA is occluded within clusters of intergrown bone crystals that are resistant to disaggregation by the strong oxidant NaOCl. They obtained reproducible authentic sequences from both modern and ancient animal bones, including humans, from DNA extracts of crystal aggregates. The treatment with NaOCl also according to the authors, minimizes the possibility of modern DNA contamination. With this technique they demonstrated the presence of a privileged niche within fossil bone, which contains DNA in a better state of preservation than the DNA present in the total bone. This approach to extracting relatively well preserved DNA from bones could significantly improves the chances of obtaining authentic ancient DNA sequences, especially from human bones.

Many excavated archaeological remains appear to contain DNA from multiple individuals (Gilbert *et al.,* 2003a,b), raising the issue of how to authenticate ancient human DNA when 'unique' sequences, such as the Neanderthal (Krings *et al*., 1997) or distinct modern human groups like the Andaman Islanders (Endicott *et al*., 2003), are not reproducibly obtained. A good example is the analysis of Italian Cro-Magnon specimens (Caramelli *et al.,* 2003), where comprehensive protocols of authentication (Cooper & Poinar 2001; Hofreiter *et al*., 2001b) were followed. However, because the resulting sequences were indistinguishable from modern Europeans, sample contamination must remain the null hypothesis. By contrast, Serre *et al*. (2004a) assume that it is impossible to authenticate any modern human sequence obtained from archaeological specimens and instead confirm the absence of Neanderthal-specific mtDNA sequences from five European early modern human (EMH) specimens. Since coalescence theory indicates that the (inferred) modern human mtDNA sequences of the five EMH specimens are unlikely to exactly match the 5–7 ancestral lineages of modern populations, this effectively doubles the number of modern human mtDNA lineages known to exist in the Late Pleistocene. This value was used with population genetic models to calculate that the maximum Neanderthal genetic contribution to EMH is likely to have been less than 25% (Serre *et al*., 2004a). Although not independently replicated, this study demonstrates how aDNA can dramatically increase the resolving power of population genetics studies (Cooper *et al.,* 2004). The retrieval of putative Neanderthal mtDNA sequences (Krings *et al.,* 1997, 1999, 2000; Ovchinnikov *et al.,* 2000; Schmitz *et al,.* 2002, Lalueza Fox . *et al,* 2005, Orlando *et al* 2006, Caramelli *et al.,* 2006, Lalueza Fox *et al*., 2006) is the major highlight in ancient human DNA studies because it allowed direct testing of hypotheses about the origin of the modern human gene pool. Importantly, recent suggestions that ancient sequences such as the Neanderthal results might be due to PCR artefacts (Pusch & Bachmann, 2004) appear unjustified, and may result from poor experimental design and methodology (Serre *et al*., 2004b). Until recently there seemed to be little hope for obtaining DNA sequences from other extinct hominids (e.g. *Homo erectus*). However, the recent discovery of the 'Hobbit'-sized *Homo floresiensis* on the island of Flores (Indonesia) dated to be just 18 kyr old (Brown *et al*., 2004; Morwood *et al.,* 2004) will potentially allow for DNA characterization. Otherwise, advances in protein sequencing techniques and the stability of certain proteins (Nielsen Marsh *et al.,* 2002) may also provide a means for such comparisons, although the limited phylogenetic utility of short amino acid sequences constrains the resolving power possible. A final complicating issue in ancient human mtDNA studies is the authentication of haplogroup designations. Mutational hot spots may generate

erroneous, but potentially, credible results when PCR reactions are initiated from small numbers of DNA molecules (Gilbert *et al.*, 2003a,b). This is exacerbated in studies of human control region sequences, where haplogroup identifications are routinely categorized using fewer than five site changes, and in extreme cases (e.g. some European groups) by as few as one. In such situations, the use of real-time PCR and UNG treatment is highly advisable. A far more reliable approach is to characterize multiple variable positions around the mitochondrial genome to define a haplotype, as shown by Maca-Meyer *et al.,* (2004) in a study of the colonization of the Canary Islands.

Ancient DNA versus Forensics

Forensic genetics in principle focuses on modern DNA taking into consideration both the nuclear and the mitochondrial genomes. Normally, forensic analysis is not faced with the severe problems of degradation and biochemical modification that conditions ancient DNA studies. The universal and steady phenomenon of profound DNA degradation is such an inexorable limitation that ancient DNA analyses have almost universally focused on mitochondrial sequences which are much more plentiful. Today, the leap over this hurdle into the dimension of nuclear DNA analysis is somewhat overdue. The analysis of ancient nuclear DNA will open striking new horizons. However, severe fragmentation of the DNA molecule can also occurs in some forensic specimens. This degradation is a point in common with ancient genomes, and makes experience learned in ancient DNA studies applicable to forensic samples. This apparently superficial analogy has turned into a fundamental issue as both disciplines make wide use of PCR technology to overcome the problem of expanding the 'surviving' integral template-molecule. In fact, the two fields need to deal with a number of experimental asperities: (a) the DNA molecules retrieved and to be copied are often degraded down to 100-300 bp or less; (b) the amount of molecules per gram of specimen may be critically low (down to thousands and less) (e) very typical failures in molecular amplification are met in both fields (essentially caused by biochemical modifications and/or the presence of PCR inhibitors); (d) authenticity of results is often jeopardized by a typical competition between the authentic sample (the one the investigator has an interest into) and a foreign genome (whatever else DNA, in the broadest sense) (e) very strict measures are needed to assure that the procedures amply the genome of interest, with tight controls placed at various steps of the procedure, in order to prevent contamination; (f) there is a steady search for setting general criteria of authentication whose compliance can confer plausibility to the final results. The general criteria conferring authenticity and the methodology to adopt for ensuring credible results are probably the benchmark on which to test whether ancient DNA studies and forensics really have something to swap (Capelli *et al.,* 2003).

The future of ancient DNA

The very small amount of DNA in fossil samples, the decay of the molecules over time and contamination with DNA from other organisms have proven to be considerable hurdles. As a result, most knowledge about molecular evolution comes from the analysis of mitochondrial and plastid DNA, simply because it is more abundant and easier to analyse. But the analysis of ancient DNA is about to enter a new era. Two recently developed techniques—multiplex PCR and a new genomic sequencing technology—allow the recovery of meaningful sequence data from nuclear rather than just mitochondrial or chloroplast DNA. Many of the field's leading researchers are already thinking about how to use this new technology.

Multiplexing ancient DNA

Because DNA degrades with time even when preserved under ideal conditions, sequence amplification is compromised by deletions or substitutions that either cause the process to fail or result in mistakes that can lead to false conclusions. The probability of such errors is proportional to the length of the strand; although longer sequences contain more information, in practice researchers have had to make do with overlapping shorter ones, and then painstakingly piece them together. More importantly, the process is usually constrained by the small amount of template DNA in the sample. For this reason, no DNA sequences longer than

about 1,000 base pairs (bp) have been recovered, even from widely studied Pleistocene mammalian species such as mammoths, ground sloths and cave bears. The breakthrough in ancient DNA sequencing came in 2005, when Michael Hofreiter from the Max Planck Institute for Evolutionary Anthropology in Leipzig, Germany, and colleagues described in *Nature* their new multiplexing technique for reconstructing a longer DNA sequence from several small molecules (Krause *et al.*, 2005). They demonstrated this by reconstructing the entire mitochondrial genome of the Pleistocene woolly mammoth, *Mammuthus primigenius*, comprising 16,770 bp, from about 200 mg of bone. In essence, multiplexing is a two-stage PCR. It uses multiple primer pairs in one PCR reaction to target subsequences within the complete DNA sample. In the case of the woolly mammoth mitochondrial DNA (mtDNA), 46 such primer pairs were chosen that marked overlapping DNA sequence fragments and spanned the entire mtDNA genome (Fig. 1). To cut out the overlaps and generate coherent products that could then be amplified in the second PCR stage, the researchers divided the primer pairs into two sets, each comprising alternate pairs. Each of these two sets was amplified in a multiplex PCR, requiring only as much ancient DNA template as would be used normally for a short target sequence. Having obtained the two amplified sequences, which together spanned the whole mtDNA genome, the samples were divided into 46 parts and used as templates for a secondary PCR to amplify each of the 46 products separately.

Ancient DNA goes nuclear

Only two days after the Krause *Nature* publication, *Science* released a report on the large-scale sequencing of nuclear and mitochondrial DNA from the jawbone of a 27,000-year-old Siberian mammoth (Poinar *et al.*, 2005). The team, headed by Hendrik Poinar, and Stephan Schuster from, used a new genome sequencer developed by Stanford University and 454 Life Sciences (Branford, CT, USA). The technology circumvents the need to clone DNA samples before sequencing. Instead, the DNA is broken into small fragments and encapsulated in a lipid bubble, thereby allowing their multiplication in isolation. Using this new tool, the researchers were able to sequence 28 million bp from the ancient fossil, 13 million of which were from the mammoth itself. Until now, scientists have usually relied on mtDNA to construct phylogenetic trees. As any sample will typically have 1,000 times more mtDNA than nuclear DNA (nuDNA)— because each animal cell has many mitochondria—this makes it easier to derive meaningful sequence information. However, although nuDNA is less abundant, it seems less prone to degradation and damage over time, so the chance of recovering longer intact strands may actually be better. DNA damage is lower in nuclear DNA than in mitochondrial DNA, maybe because nuclear DNA is better protected by proteins.

Although it may never be possible to recreate extinct organisms from their DNA, the new multiplexing technique could enable scientists to expand their study of phenotypes by investigating properties such as skin colour or behavioural traits. Among the many important questions that could be answered is whether the Neanderthals - the last human species to become extinct - were able to

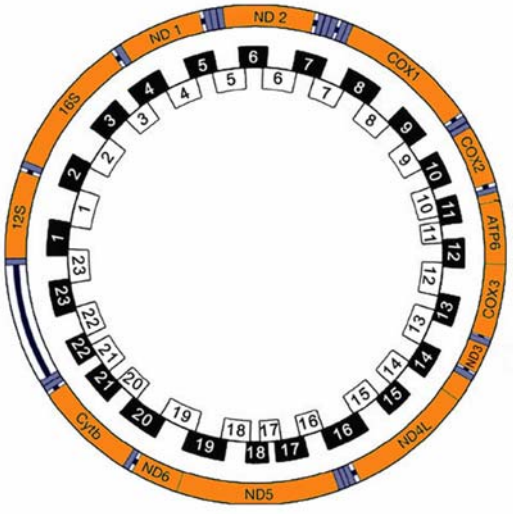

Fig. 1 - Circular genome (orange), showing the positions of the control region and the genes encoding 22 transfer RNAs (grey boxes), 2 ribosomal RNAs and 13 proteins. The positions of the 46 amplification products used are depicted in black (first set) and white (second set) (Krause* et al.*, 2005, modified).

speak and, if so, how well. To do this, various groups hope to target the *FOX2P* gene, which is believed to confer the ability to speak in humans. This research could help to resolve the debate over why the Neanderthals became extinct around 30,000 years ago, as one theory claims that modern humans gained the upper hand linguistically.

On May 2006 the first nuclear DNA sequences from a Neanderthal (*Homo neanderthalensis*) have been reported by Svante Pääbo, a palaeogeneticist at the Max Planck Institute for Evolutionary Anthropology in Leipzig, Germany. He began his Neanderthal Genome Project about two years ago and his team have probed 60 Neanderthal specimens from museums for hints that the DNA might have survived millennia of degradation. Two of the specimens showed promise, and on 12 May Pääbo's team reported at the Biology of Genomes meeting at New York's Cold Spring Harbor Laboratory that they had managed to sequence around a million base pairs of nuclear DNA — around 0.03% of the genome — from one of them. This is a 45,000-year-old male specimen found in Vindija Cave outside Zagreb, Croatia. Typically, DNA to be sequenced must be cloned in bacteria to produce large enough amounts for study. But because the Neanderthal DNA had broken down into tiny fragments, Pääbo and his colleagues used a same sequencing technique used to analyze the Mithocondrial mammuth: the Pyro-sequencing. They are now analysing the results to work out how the different fragments fit together so that they can be compared with the modern human genome sequence. One finding so far is that the Neanderthal Y chromosome is substantially more different from human and chimp Y chromosomes than are other chromosomes. This suggests that little interbreeding occurred, at least among the more recent Neanderthal species. Edward Rubin, director of the Joint Genome Institute in Walnut Creek, California, works with Pääbo. The two are also working to sequence Neanderthal DNA by the traditional method. James Noonan, a postdoc in Rubin's lab, reported at the Cold Spring Harbor meeting that preliminary analysis of the 75,000 base pairs sequenced so far shows that Neanderthals diverged from the lineage that led to modern humans about 315,000 years ago - around the time that had been thought. *Homo sapiens* is known to have evolved at least 200,000 years ago (McDougall *et al.,* 2005).

The more extensive nuclear DNA sequences should pin down the timing of the split more precisely, and comparing genes for particular traits could help researchers work out which characteristics were shared by Neanderthals, and when such traits arose. Such comparisons could also confirm whether Neanderthals did contribute isolated genes to the human lineage. For example, John Hardy, a geneticist at the National Human Genome Research Institute in Bethesda, Maryland, has hypothesized that Neanderthals may have contributed a gene that is linked to several neurodegenerative diseases, because it is found in people of European ancestry, where the Neanderthals lived. Proving that theory would require finding this version of the gene in the Neanderthal genome.

Acknowledgements

We thank Roscoe Stanyon for comments that improved the manuscripts and Lucio Milani for his revision.

References

Adcock G. J., Dennis E. S., Easteal S., Huttley G. A., Jermin L. S., Peacock W. J. & Thorne A. 2001 - Mitochondrial DNA sequences in ancient Australians: implications for modern human origins. *Proc. Natl. Acad. Sci. USA*, 98: 537–542.

Allard M.W., Young D., Huyen Y. 1995. Detecting dinosaur DNA. *Science,* 268: 1192.

Austin J.J., Ross A.J., Smith A.B., Fortey R.A. & Thomas R.H. 1997a. Problems of reproducibility—does geologically ancient DNA survive in amber-preserved insects? *Proc. R. Soc. London Ser. B,* 264: 467–474

Austin J.J., Smith A.B. & Thomas R.H. 1997b. Palaeontology in a molecular world: the search for authentic ancient DNA. *Trends Ecol. Evol.*, 12: 303–306.

Bailey J.F., Richards M.B., Macaulay V.A., Colson I.B., James I.T., *et al.* 1996. Ancient DNA suggests a recent expansion of European cattle from a diverse wild progenitor species. *Proc. R. Soc. London Ser. B,* 263:1467–1473

Barnes I., Matheus P., Shapiro B., Jensen D. &

Cooper A. 2002. Dynamics of Pleistocene population extinctions in Beringian brown bears. *Science,* 295:2267–2270.

Barnes I., Young J.P.W. & Dobney K.M. 2000. DNA-based identification of goose species from two archaeological sites in Lincolnshire. *J. Archaeolog. Sci.,* 27:91– 100.

Beja-Pereira A., Caramelli D., Laluesa Fox C., *et al.* 2006 – The origin of European cattle: Evidence form modern and ancient DNA. *Proc. Natl Acad. Sci. USA,* 10: 8113-8118.

Bensasson D., Zhang D.X., Hartl D.L., Hewitt G.M. 2001. Mitochondrial pseudogenes: evolution's misplaced witnesses. *Trends Ecol. Evol.,* 16:314–321.

Brown P., Sutikna T., Morwood M. J., Soejono R. P., Jatmiko Wayhu Saptomo E. & Rokus Awe D. 2004. A new small-bodied hominin from the Late Pleistocene of Flores, Indonesia, *Nature,* 431: 1055–1061.

Bunce M., Worthy T.H., Ford T., Hoppitt W., Willerslev E., *et al.* 2003. Extreme reversed sexual size dimorphism in the extinct New Zealand moa Dinornis. *Nature,* 425:172–175.

Cano R.J., Poinar H.N. & Poinar Jr.G O. 1992a. Isolation and partial characterisation of DNA from the bee *Proplebeia dominicana* (Apidae: Hymenoptera) in 25-40 million year old amber. *Med. Sci. Res.,* 20: 249–251.

Cano R.J., Poinar, H.N., Roublik, D.W. & Poinar Jr,G.O. 1992b. Enzymatic amplification and nucleotide sequencing of portions of the 18S rRNA gene of the bee *Proplebeia dominicana* (Apidae: Hymenoptera) isolated from 25-40 million year old Dominican amber. *Med. Sci. Res.*, 20: 619–622.

Cano R.J., Poinar, H.N., Pieniezak N.S. & Poinar Jr,G.O. 1993. Enzymatic amplification and nucleotide sequencing of DNA from 120–135 million year old weevil. *Nature,* 363: 536–538.

Cano R.J. & Borucki, M.K. 1995. Revival and identification of bacterial spores in 25-million-year-old to 40-millionyear- old Dominican amber. *Science,* 268: 1060–1064.

Capelli C., Tschentscher F & Pascali V.L. 2003. "Ancient" protocols for the crime scene? Similarities and differences between forensic genetics and ancient DNA analysis. *Forensic Sci Int.*, 131:59-64.

Capelli C., MacPhee R.D., Roca A.L., Brisighelli F., Georgiadis N., O'Brien S.J. & Greenwood A.D. 2006. A nuclear DNA phylogeny of the woolly mammoth (*Mammuthus primigenius*). *Mol. Phylogenet. Evol.*, 40:620-627.

Caramelli D., Lalueza-Fox C., Vernesi C., Lari M., Casoli A., Mallegni F., Chiarelli B., Dupanloup I., Bertranpetit J., Barbujani G., Bertorelle G. 2003. Evidence for a genetic discontinuity between Neandertals and 24,000-year-old anatomically modern Europeans. *Proc. Natl. Acad. Sci. USA*, 100: 6593–6597.

Caramelli D., Laluesa Fox C., Condemi S., Longo L., Milani L., Manfredini A., *et al.* 2006. A Highly divergent DNA sequence in a Neandertal individual from Italy. *Curr Biol.,* 16:R630-632.

Collins M.J., Waite E.R. & van Duin A.C.T. 1999. Predicting protein decomposition: the case of aspartic-acid racemization kinetics. *Philos. Trans. R. Soc. London Ser. B,* 354:51–64.

Colson I.B., Richards M.B., Bailey J.F., Sykes B.C. & Hedges R.E.M. 1997. DNA analysis of seven human skeletons excavated from the terp ofWijnaldum. *J. Archaeol. Sci.,* 24:911–917.

Cooper A., Rambaut A., Macaulay V., Willerslev E., Hansen A.J. & Stringer C. 2001a. Human origins and ancient human DNA. *Science,* 292:1655–1656.

Cooper A., Lalueza-Fox C., Anderson S., Rambaut A., Austin J. & Ward R. 2001. Complete mitochondrial genome sequences of two extinct moas clarify ratite evolution. *Nature,* 409:704–707.

Cooper A., Mourer-Chauvire C., Chambers G.K., von Haeseler A., Wilson A.C. & Pääbo S. 1992. Independent origins of New Zealand moas and kiwis. *Proc. Natl. Acad. Sci., USA,* 89:8741–8744.

Cooper A. & Poinar H.N. 2000. Ancient DNA: Do it right or not at all. *Science,* 289:1139.

DeSalle R., Gatesy J., Wheeler W. & Grimaldi D. 1992. DNA Sequences from a fossil termite in Oligo-Miocene amber and their phylogenetic implications. *Science,* 257: 1933–1936.

DeSalle R., Barcia M. & Wray C. 1993. PCR jumping in clones of 30-million-year-old DNA fragments from amber preserved

termites (Mastotermes electrodominicus). *Experientia,* 49: 906–909.
DeSalle R. 1994. Implications of ancient DNA for phylogenetic studies. *Experientia,* 50: 543–550.
Eglinton G. & Logan G.A. 1991. Molecular preservation. *Philos. Trans. R. Soc. London Ser. B,* 333: 315–327 (see also discussion, pp. 27–28).
Endicott P., Gilbert M.T.P., Stringer C., Lalueza-Fox C., Willerslev E., Hansen A.J. & Cooper A. 2003. The genetic origins of the Andaman Islanders. *Am. J. Hum. Genet.*, 72: 178–184.
Fish S.A., Shepherd T.J., McGenity T.J. & Grant W.D. 2002. Recovery of 16S ribosomal RNA gene fragments from ancient halite. *Nature,* 417: 432–436.
Friedberg E.C., Walker G.C., Siede W., 1995. *DNA Repair and Mutagenesis*. ASM Press, Washington D.C.
Gilbert M.T.P., Hansen A.J., Willerslev E., Rudbeck L., Barnes I., *et al*. 2003b. Characterization of genetic miscoding lesions caused by postmortem damage. *Am. J. Hum. Genet.,* 72:48–61.
Gilbert M.T.P., Willerslev E., Hansen A.J., Barnes I., Rudbeck L., Lynnerup N. & Cooper A. 2003a. Distribution patterns of post-mortem damage in human mitochondrial DNA. *Am. J. Hum. Genet*., 72: 32–47.
Gilbert M.T.P., Barnes I., Collins M.J., Smith C., Eklund J., Goudsmit J., Poinar H. & Cooper A. 2005a. News and comments: the long-term survival of ancient DNA in Egypt: response to Zink and Nerlich. *Am. J. Phys. Anthropol.,* 128:110-114.
Gilbert M.T.P., Wilson A.S., Bunce M., Hansen A.J., Willerslev E. *et al.* 2004a. Ancient mitochondrial DNA from hair. *Curr. Biol.*, 14: R463–R464.
Gilbert M.T.P., Cuccui J., White W., Lynnerup N., Titball R.W., Cooper A. & Prentice M. B. 2004b. Absence of *Yersinia pestis*-specific DNA in human teeth from five European excavations of putative plague victims. *Microbiology,* 150: 341–354.
Gilbert M.T.P. 2005b. Biochemical and physical correlates of DNA contamination in archaeological bones and teeth. *J. Archeol. Sci.*, in press.
Goloubinoff P., Pääbo S., Wilson A.C. 1993. Evolution of maize inferred from sequence diversity of an adh2 gene segment from archaeological specimens. *Proc. Natl. Acad. Sci. USA,* 90:1997–2001.
Golenberg E.M., Giannassi D.E., Clegg M.T., Smiley C.J., Durbin M., Henderson D. & Zurawski G. 1990. Chloroplast DNA from a Miocene Magnolia species. *Nature,* 344: 656–658.
Greenwood A.D., Castresana J., Feldmaier Fuchs G. & Pääbo S. 2001. A molecular phylogeny of two extinct sloths. *Mol. Phylogenet. Evol.,* 18:94–103.
Greenwood A.D., Capelli C., Possnert G. & Pääbo S. 1999. Nuclear DNA sequences from Late Pleistocene megafauna. *Mol. Biol. Evol.,* 16:1466–1473.
Greenwood A.D. & Pääbo S. 1999. Nuclear insertion sequences of mitochondrial DNA predominate in hair but not in blood of elephants. *Mol. Ecol.,* 8:133-137.
Gutierrez G. & Marin A. 1998. The most ancient DNA recovered from an amber-preserved specimen may not be as ancient as it seems. *Mol. Biol. Evol.*, 15: 926–929.
Haddrath O. & Baker A.J. 2001. Complete mitochondrial DNA genome sequences of extinct birds: ratite phylogenetics and the vicariance biogeography hypothesis. *Proc. R Soc. London Ser. B,* 268: 939–945.
Hadly E.A., Kohn M.H., Leonard J.A. & Wayne R.K. 1998. A genetic record of population isolation in pocket gophers during Holocene climatic change. *Proc. Natl. Acad. Sci. USA,* 95:6893–6896.
Hale M.L., Lurz P.W., Shirley M.D., Rushton S., Fuller R.M. & Wolff K. 2001. Impact of landscape management on the genetic structure of red squirrel populations. *Science,* 293:2246–2248.
Handt O., H¨oss M., Krings M. & Pääbo S. 1994a. Ancient DNA: methodological challenges. *Experientia,* 50:524–529.
Handt O., Krings M., Ward R.H. & Pääbo S. 1996. The retrieval of ancient human DNA sequences. *Am. J. Hum. Genet.,* 59:368–376.
Handt O., Richards M., Trommsdorf M., Kilger

C., Simanainen J., *et al.* 1994b. Molecular genetic analyses of the Tyrolean Ice Man. *Science,* 264:1775–1778.

Hansen A., Willerslev E., Wiuf C., Mourier T. & Arctander P. 2001. Statistical evidence for miscoding lesions in ancient DNA templates. *Mol. Biol. Evol.,* 18:262– 265.

Hardy C., Callou C., Vigne J.D., Casane D., Dennebouy N. *et al.* 1995. Rabbit mitochondrial DNA diversity from prehistoric to modern times. *J. Mol. Evol.,* 40:227–237.

Hauswirth W.W., Dickel C.D., Rowol D. J. & Hauswirth M.A. 1994. Inter- and intra-population studies of ancient humans. *Experientia,* 50, 585–591.

Hedges S.B. & Schweitzer M.H. 1995. Detecting dinosaur DNA. *Science,* 268: 1191–1192

Henikoff S. 1995. Detecting dinosaur DNA. *Science,* 268:1192.

Herrmann B. & Hummel S. 1996. *Ancient DNA.* Springer, New York.

Higuchi R., Bowman B., Freiberger M., Ryder O.A. & Wilson A.C. 1984. DNA sequences from the quagga, an extinct member of the horse family. *Nature,* 312: 282– 284.

Hofreiter M., Jaenicke V., Serre D., Haeseler A.V. & Pääbo S. 2001. DNA sequences from multiple amplifications reveal artefacts induced by cytosine deamination in ancient DNA. *Nucleic Acids Res.,* 29: 4793–4799.

Hofreiter M., Serre D., Poinar H.N., Kuch M. & Pääbo S. 2001b. Ancient DNA. *Nat. Rev. Genet.,* 2:353.

Höss M., Jaruga P., Zastawny T.H., Dizdaroglu M. & Pääbo S. 1996. DNA damage and DNA sequence retrieval from ancient tissues. *Nucl. Acids Res.,* 24:1304–1307.

Hoss M., Dilling A., Currant A. & Pääbo S. 1996. Molecular phylogeny of the extinct ground sloth *Mylodon darwinii*. *Proc. Natl. Acad. Sci. USA,* 93:181–185.

Huynen L., Millar C.D., Scofield R.P. & Lambert D.M. 2003. Nuclear DNA sequences detect species limits in ancient moa. *Nature,* 425:175–178.

Jaenicke-Despres V., Buckler E.S, Smith B.D., Gilbert M.T.P., Cooper A., *et al.* 2003. Early allelic selection in maize as revealed by ancient DNA. *Science,* 302:1206–1208.

Jans M.M.E., Nielsen-Marsh C.M., Smith C.I., Collins M.J. & Kars H. 2004. Characterisation of microbial attack on archaeological bone. *J. Archaeol. Sci.,* 31:87–95.

Kim S., Soltis D. E., Soltis P. S. & Sue Y. 2004. DNA sequences from Miocene fossils: an ndhF sequence of *Magnolia latahensis* (Magnoliaceae) and an rbcL sequence of *Persea pseudocarolinensis* (Lauraceae*). Am. J. Bot.,* 91: 615–620.

Kolman C. & Tuross N. 2000. Ancient DNA analysis of human populations. *Am. J. Phys. Anthropol.,* 111: 5–23.

Koon H.E.C., Nicholson R.A. & Collins M.J. 2003. A practical approach to the identification of low temperature heated bone using TEM. *J. Archaeolog. Sci.,* 13: 1393–1399.

Krajewski C., Buckley L. & Westerman M. 1997. DNA phylogeny of the marsupial wolf resolved. *Proc. R. Soc. London Ser. B,* 264: 911–917.

Krajewski C., Driskell A.C., Baverstock P.R. & Braun M.J. 1992. Phylogenetic relationships of the thylacine (Mammalia: Thylacinidae) among dasyuroid marsupials: evidence from cytochrome *b* DNA sequences. *Proc. R. Soc. London Ser. B,* 250:19–27.

Krause J., Dear PH, Pollack J.L., Slatkin M., Spriggs H., Barnes I., Lister A.M., Ebersberger I., Paabo S. & Hofreiter M. 2006. Multiplex amplification of the mammoth mitochondrial genome and the evolution of Elephantidae. *Nature* 439:724-727.

Krings M., Capelli C., Tschentscher F., Geisert H., Meyer S., *et al.* 2000. A view of Neandertal genetic diversity. *Nat. Genet.,* 26:144–146.

Krings M., Stone A., Schmitz R.W., Krainitzki H., Stoneking M. & Pääbo S. 1997. Neandertal DNA sequences and the origin of modern humans. *Cell,* 90:19–30.

Laluesa Fox C., Sampietro L.M., Caramelli D., Puder Y. & Lari M. 2005. Neandertal evolutionary genetics: mitochondrial DNA data from the iberian peninsula. *Mol. Biol. Evol.,* 22:1077-1081.

Lalueza Fox C., Krause J., Caramelli D., Catalano G., Milani L., *et al.* 2006. Low genetic diversity suggests a population continuity of Iberian and other European Neanderthal. *in press.*

Lambert D.M., Ritchie P.A., Millar C.D., Holland B., Drummond A.J. & Baroni C. 2002. Rates of evolution in ancient DNA from Adelie penguins. *Science,* 295:2270–2273.

Larson S., Jameson R., Etnier M., Fleming M. & Bentzen P. 2002. Loss of genetic diversity in sea otters (*Enhydra lutris*) associated with the fur trade of the 18th and 19th centuries. *Mol Ecol.,* 11: 1899–1903.

Leonard J.A., Wayne R.K. & Cooper A. 2000. Population genetics of Ice Age brown bears. *Proc. Natl. Acad. Sci. USA,* 97:1651–1654.

Lindahl T. 1993. Instability and decay of the primary structure of DNA. *Nature* 362: 709–715.

Lindahl T. & Karlstro O. 1973. Heatinduced depyrimidination of deoxyribonucleic acid in neutral solution. *Biochemistry,* 12: 5151–5154.

Lindahl T. 1993. Recovery of antediluvian DNA. *Nature,* 365: 700.

Lindahl T. & Nyberg B. 1972. Rate of depurination of native deoxyribonucleic acid. *Biochemistry,* 11: 3610.

Maca-Meyer N., Arnay M., Rando J. C., Flores C., Gonzale´z A. M., Cabrera V. M. & Larruga J. M. 2004. Ancient mtDNA analysis and the origin of the Guanches. *Eur. J. Hum. Genet*., 12: 155–162.

Marota I., Basile C., Ubaldi M. & Rollo, F. D. 2002. DNA decay rate in papyri and human remains from Egyptian archaeological sites. *Am. J. Phys. Anthropol*., 117: 310–318.

Miller C.R. & Waits L.P. 2003. The history of effective population size and genetic diversity in the Yellowstone grizzly (*Ursus arctos*): implications for conservation. *Proc. Natl. Acad. Sci. USA,* 100: 4334–4339.

Morin P.A., Chambers K.E., Boesch C. & Vigilant L. 2001. Quantitative polymerase chain reaction analysis of DNA from non-invasive samples for accurate microsatellite genotyping of wild chimpanzees (*Pan troglodytes verus*). *Mol. Ecol.,* 10: 1835–1844.

Morwood M.J., Soejono R.P., Roberts R.G., Sutikna T., Turney C.S. *et al.* 2004. Archaeology and age of a new hominin from Flores in eastern Indonesia. *Nature,* 431: 1087–1091.

Navidi W., Arnheim N. & Waterman M.S. 1992. Amultiple-tubes approach for accurate genotyping of very small DNA samples by using PCR - statistical considerations. *Am. J. Hum. Genet.,* 50:347–359.

Nielsen-Marsh C.M., Hedges R.E.M., Mann T. & Collins M.J. 2000. A preliminary investigation of the application of differential scanning calorimetry to the study of collagen degradation in archaeological bone. *Thermochim. Acta,* 365:129–139.

Orlando L, Darlu P, Toussaint M, Bonjean D, Otte M & Hanni C. 2006. Revisiting Neandertal diversity with a 100,000 year old mtDNA sequence. *Curr. Biol.*, 16: R400-R402.

Ovchinnikov I., Gotherstrom A., Romanova G., Kharitonov V., Lide´n K. & Goodwin, W. 2000. Molecular analysis of Neanderthal DNA from the northern Caucasus. *Nature,* 404: 490–493.

Pääbo S. 1990. Amplifying ancient DNA. In M.A. Innis, D.H. Gelfand, J.J. Sninsky & T.J. White (eds): *PCR-Protocols and Applications - A Laboratory Manual*, pp. 159– 66. Academic Press, San Diego.

Pääbo S. & Wilson A.C. 1988. Polymerase chain reaction reveals cloning artefacts. *Nature,* 334: 387–388.

Pääbo S. 1989. Ancient DNA: extraction, characterization, molecular cloning and enzymatic amplification. *Proc. Natl. Acad. Sci. USA,* 86:1939–1943.

Pääbo S. & Wilson A.C. Miocene DNA sequences a dream come true. *Curr. Biol.*, 1: 45-46.

Pääbo S., Higuchi R. G. & Wilson A. C. 1989. Ancient DNA and the polymerase chain reaction. *J. Biol. Chem*., 264: 9709–9712.

Paxinos E.E., James H.F., Olson S.L., Sorenson M.D., Jackson J. & Fleischer R.C. 2002. MtDNAfrom fossils reveals a radiation of Hawaiian geese recently derived from the Canada goose (*Branta canadensis*). *Proc. Natl. Acad. Sci. USA,* 99: 1399–1404.

Pergams O.R.W., Barnes W.M. & Nyberg D. 2003. Rapid change in mouse mitochondrial DNA.

Nature, 423: 397.

Pertoldi C., Hansen M.M., Loeschcke V., Madsen A.B., Jacobsen L. & Baagoe H. 2001. Genetic consequences of population decline in the European otter (*Lutra lutra*): an assessment of microsatelliteDNAvariation in Danish otters from 1883 to 1993. *Proc. R. Soc. London Ser. B,* 268: 1775 - 1781.

Poinar H. N., Poinar Jr G. O. & Cano R. J. 1993. DNA from an extinct plant. *Nature,* 363: 677.

Poinar H., Hoss M., Bada J.L. & Pääbo S., 1996. Aminoacid racemisation and the preservation of ancient DNA. *Science,* 272: 864-866.

Poinar H., Kuch M., McDonald G., Martin P. & Pääbo S. 2003. Nuclear gene sequences from a Late Pleistocene sloth coprolite. *Curr. Biol.,* 12: 1150–1152.

Poinar H.N., Hofreiter M., Spaulding W.G., Martin P.S., Stankiewicz B.A., *et al.* 1998. Molecular coproscopy: dung and diet of the extinct ground sloth *Nothrotheriops shastensis*. *Science,* 281: 402–406.

Poinar H.N. & Stankiewicz B.A. 1999. Protein preservation and DNA retrieval from ancient tissues. *Proc. Natl. Acad. Sci. USA,* 96: 8426–8431.

Pusch C.M. & Bachmann L. 2004. Spiking of contemporary human template DNA with ancient DNA extracts induces mutations under PCR and generates nonauthentic mitochondrial sequences. *Mol. Biol. Evol.,* 21: 957–964.

Richards M.B., Sykes B.C. & Hedges R.E.M. 1995. Authenticating DNA extracted from ancient skeletal remains. *J. Archaeol. Sci.,* 22:291-299.

Ritchie P.A., Millar C.D., Gibb G.C., Baroni C. & Lambert D.M. 2004. Ancient DNA enables timing of the Pleistocene origin and Holocene expansion of two Adelie penguin lineages in Antarctica. *Mol. Biol. Evol.,* 21: 240–248.

Salamon M, Tuross N, Arensburg B & Weiner S. 2005- Relatively well preserved DNA is present in the crystal aggregates of fossil bones. *Proc. Natl. Acad. Sci. U S A.*, 102: 13783-13788.

Schaaper R.M., Kunkel T.A. & Loeb L.A. 1983. Infidelity of DNA-synthesis associatedwith bypass of apurinic sites. *Proc.Natl. Acad. Sci. USA,* 80: 487–491.

Serre D., Langaney A., Chech M., Teschler- Nicola M., Paunovic M., *et al.* 2004a. No evidence of Neandertal mtDNA contribution to early modern humans. *PLoS Biol.,* 2: 313–317.

Serre D., Hofreiter M. & Pääbo S. 2004b. Mutations induced by ancient DNA extracts? *Mol. Biol. Evol.,* 21: 1463–1467.

Shapiro R. 1981. Damage to DNA caused by hydrolysis. In E. Seeberg, K. Kleppe (ed): *Chromosome Damage and Repair*, pp. 3–12. Plenum, New York.

Sidow A., Wilson A. C. & Paabo S. 1991. Bacterial DNA in *Clarkia* fossils. *Phil. Trans. R. Soc. Lond. B,* 333: 429–433.

Soltis P. S., Soltis D. E. & Smiley C. J. 1992. An rbcL sequence from a Miocene Taxodium (bald cypress). *Proc. Natl Acad. Sci. USA*, 89: 449–451.

Smith C.I., Chamberlain A.T., Riley M.S., Cooper A., Stringer C.B. & Collins M.J. 2001. Neanderthal DNA. Not just old but old and cold? *Nature,* 410:771–772.

Smith C.I., Chamberlain A.T., Riley M.S., Stringer C. & Collins M.J. 2003. The thermal history of human fossils and the likelihood of successful DNA amplification. *J. Hum. Evol.,* 45: 203–217.

Schmitz R. W., Serre D., Bonani G., Feine S., Hillgruber F., Krainitzki H., Pa¨a¨bo S. & Smith F. H. 2002. The Neandertal type-site revisited: interdisciplinary investigations of skeletal remains from the Neander Valley, Germany. *Proc. Natl Acad. Sci. USA*, 99: 13342–13347.

Suarez A.V. & Tsutsui N.D. 2004. The value of museum collections for research and society. *BioScience,* 54: 66–74.

Thalmann O., Hebler J., Poinar H.N., Pääbo S. & Vigilant L. 2004. Unreliable mtDNA data due to nuclear insertions: a cautionary tale from analysis of humans and other great apes. *Mol. Ecol.,* 13: 321–335.

Thomas R.H., Schaffner W., Wilson A.C. & Pääbo S. 1989. DNA phylogeny of the extinct marsupial wolf. *Nature,* 340: 465–467.

Thomas W.K., Pääbo S., Villablanca F.X. & Wilson A.C. 1990. Spatial and temporal continuity of kangaroo rat populations shown by sequencing

mitochondrial DNA from museum specimens. *J. Mol. Evol.,* 31:101–112.

Timmis J.N., Ayliffe M.A., Huang C.Y. & Martin W. 2004. Endosymbiotic gene transfer: Organelle genomes forge eukaryotic chromosomes. *Nat. Rev. Genet.,* 5:123–35

Vanderkuyl A.C., Kuiken C.L., Dekker J.T., Perizonius W.R.K. & Goudsmit J. 1995. Nuclear counterparts of the cytoplasmic mitochondrial 12S ribosomal-RNA gene—a problem of ancient DNA and molecular phylogenies. *J. Mol. Evol.,* 40: 652–657.

Vasan S., Zhang X., Kapurniotu A., Bernhagen J., Teichberg S., *et al.* 1996. An agent cleaving glucose-derived protein crosslinks in vitro and in vivo. *Nature,* 382: 275–278.

Verginelli F., Capelli C., Coia V., Musiani M., Falchetti M., Ottini L., Palmirotta R., Tagliacozzo A., De Grossi Mazzorin I. & Mariani-Costantini R. 2005. Mitochondrial DNA from prehistoric canids highlights relationships between dogs and South-East European wolves. *Mol Biol Evol.*, 22: 2541-2551.

Vreeland R.H., Rosenzweig W.D. & Powers D.W. 2000. Isolation of a 250 million-year-old halotolerant bacterium from a primary salt crystal. *Nature*, 407: 897–900.

Willerslev E., Hansen A.J., Binladen J., Brand T.B., Gilbert M.T.P., *et al.* 2003. Diverse plant and animal genetic records from Holocene and Pleistocene sediments. *Science,* 300: 791–795.

Willerslev E., Hansen A.J. & Poinar H.N. 2004. Isolation of nucleic acids and cultures from fossil ice and permafrost. *Trends Ecol. Evol.,* 19: 141–147.

Wisely S.M., Buskirk S.W., Fleming M.A., McDonald D.B., Ostrander E.A. 2002. Genetic diversity and fitness in black-footed ferrets before and during a bottleneck. *J. Hered.,* 93: 231–237.

Willerslev E., Hansen A. J., Brand T. B., Rønn R., Barnes I., Wiuf C., Gilichinsky D. A., Mitchell D. & Cooper A. 2004a. Long-term persistence of bacterial DNA. *Curr. Biol.*, 14: R9–R10.

Woodward S. R., Weyand N. J. & Bunell M. 1994. DNA sequence from Cretaceous period bone fragments. *Science*, 266: 1229–1232.

Zischler H, Hoss M, Handt O, von Haeseler A, van der Kuyl AC & Goudsmit J. 1995. Detecting dinosaur DNA. *Science,* 268: 1192–1193.