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Review Paper

Ancient DNA

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In the past two decades, ancient DNA research has progressed from the retrieval of small fragments of mitochondrial DNA from a few late Holocene specimens, to large-scale studies of ancient populations, phenotypically important nuclear loci, and even whole mitochondrial genome sequences of extinct species. However, the field is still regularly marred by erroneous reports, which underestimate the extent of contamination within laboratories and samples themselves. An improved understanding of these processes and the effects of damage on ancient DNA templates has started to provide a more robust basis for research. Recent methodological advances have included the characterization of Pleistocene mammal populations and discoveries of DNA preserved in ancient sediments. Increasingly, ancient genetic information is providing a unique means to test assumptions used in evolutionary and population genetics studies to reconstruct the past. Initial results have revealed surprisingly complex population histories, and indicate that modern phylogeographic studies may give misleading impressions about even the recent evolutionary past. With the advent and uptake of appropriate methodologies, ancient DNA is now positioned to become a powerful tool in biological research and is also evolving new and unexpected uses, such as in the search for extinct or extant life in the deep biosphere and on other planets.

Keywords: ancient DNA; palaeontology; palaeoecology; archaeology; population genetics; DNA damage and repair

1. INTRODUCTION

The evolutionary processes that generated modern species and populations are commonly inferred through the analysis of morphological and genetic markers, along with studies of abundance and distribution patterns (e.g. Avise 2000). However, the analysis of contemporary organisms can provide only indirect evidence of this history, and reconstructions remain tentative if they cannot be checked against the fossil record. While this has long been possible for morphological characters, ancient DNA (aDNA) methods are now providing a means to also record genetic changes in real time, at least over short geological time-scales.

The initial aDNA publications are just two decades old, and the methods and standards of this new field are still evolving rapidly. To review the current position and future directions of aDNA research, we discuss the colourful history of early publications, DNA degradation and long-term survival, contamination and authentication criteria, and the implications of recent advances.

2. HISTORY

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, and that 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 *et al.* 1989). However, the enormous amplifying power of PCR also created an increased sensitivity to contamination from modern DNA, and simultaneously, a major potential source of such contamination through the extraordinary concentrations of previously amplified PCR products. As a consequence, false positives resulting from intra-laboratory contamination remain a major problem in aDNA research. In fact, 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. Perhaps unsurprisingly, many of the most extravagant aDNA reports have since been either disproved or effectively disregarded. This includes 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.* 1992*a*,*b*, 1993; DeSalle *et al.* 1992, 1993; Poinar *et al.* 1993; DeSalle 1994). Some of these sequences originated from obvious human or microbial contamination (Zischler *et al.* 1995*a*; Gutierréz & Marín 1998), whereas it has not proved possible to repeat others independently (Sidow *et al.* 1991; Austin *et al.* 1997*a*,*b*). Many other claims remain in limbo, where a lack of appropriate methods or replication renders them effectively meaningless, e.g. human sequences from 'Mungo man' in Australia, or Cheddar Gorge in the UK

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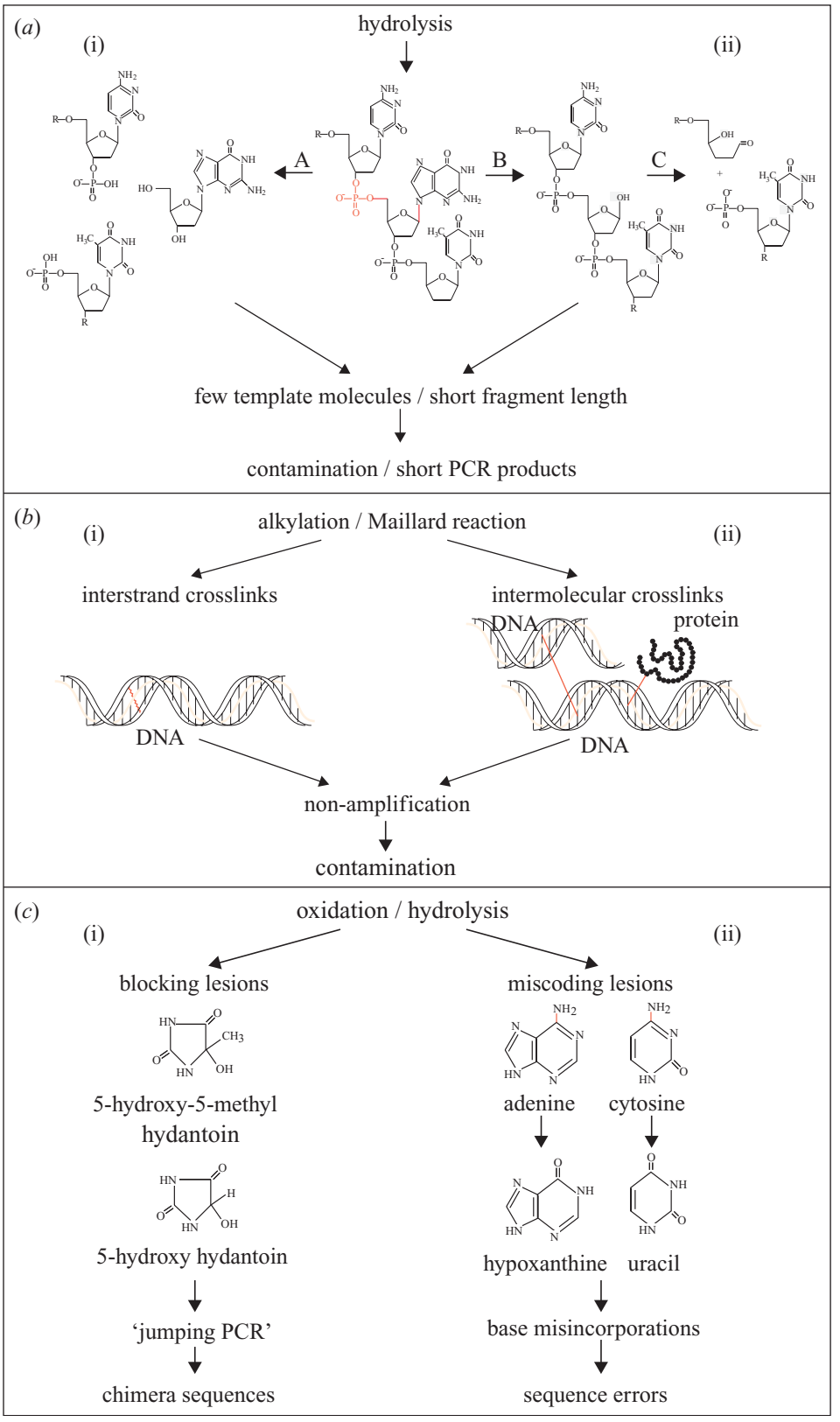

Figure 1. *Post-mortem* DNA modification in fossil remains, with the structures altered by damage shown in red. (*a*) Formation of strand breaks (single-stranded nicks) by hydrolytic damage. (i) Direct cleavage of the phosphodiester backbone (A). (ii) Depurination resulting in a baseless site (AP-site) (B) followed by breakage of the sugar backbone through β-elimination (C). Strand breaks are believed to be largely responsible for the short amplification length and the high rate of DNA loss in fossil remains. (*b*) Different types of crosslink formation. (i) Inter-strand crosslinks by alkylation. (ii) Intermolecular crosslinks by Maillard reaction. Crosslinks may prevent the amplification of endogenous template molecules, increasing the risk of contamination. (*c*) Oxidative and hydrolytic modification of bases resulting in (i) blocking lesions or (ii) miscoding lesions. Some oxidative damage results in lesions blocking the polymerase enzyme, and promoting chimeric sequences through 'jumping PCR'. Hydrolytic damage of bases may result in miscoding lesions, for example, deamination of cytosine and adenine to uracil and hypoxathine, respectively. These lesions will result in the incorporation of erroneous bases during amplification.

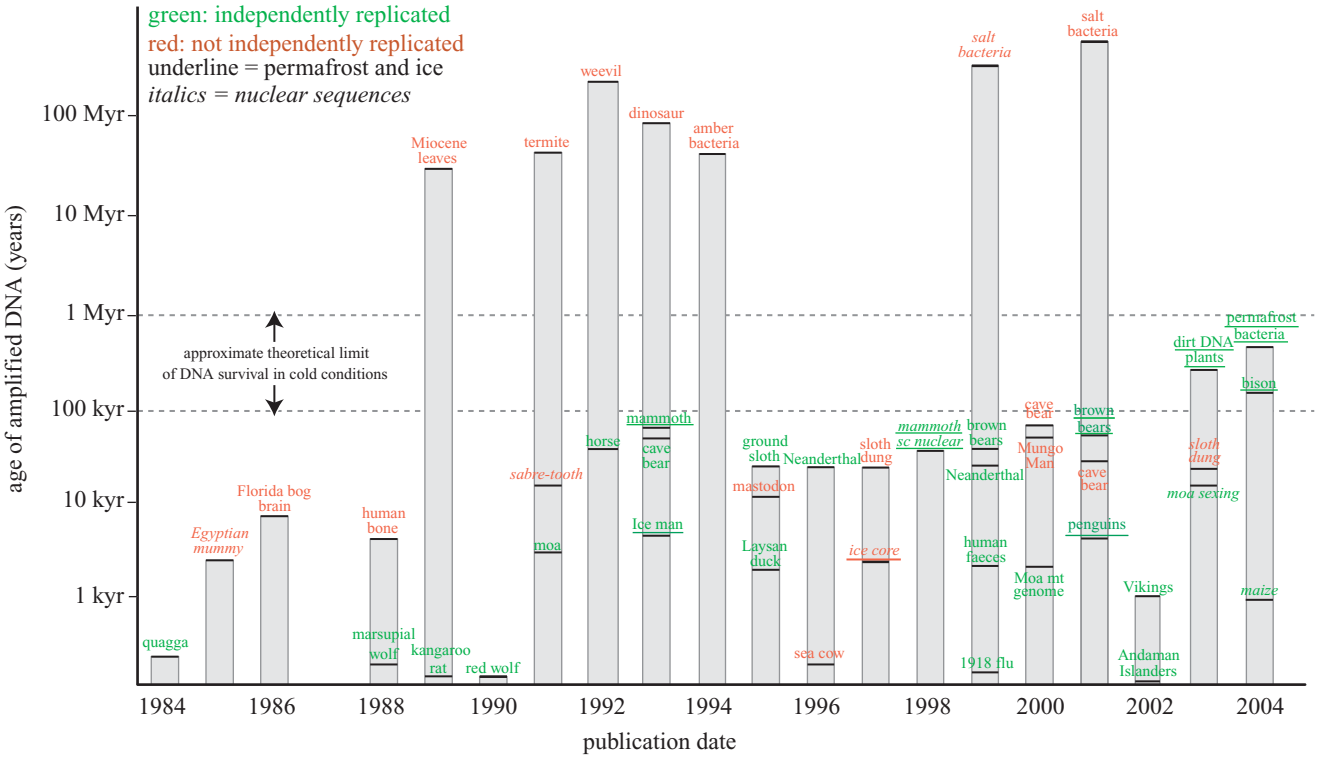

Figure 2. Key aDNA studies through time. Reports are shown with claimed age, date of publication, type of sequence and whether the results were authenticated by independent replication.

(Adcock *et al.* 2001; Cooper *et al.* 2001*a*) and the recovery of bacterial DNA and cells from amber and halite that are claimed to be many millions of years old (Cano & Borucki 1995; Vreeland *et al.* 2000; Fish *et al.* 2002; Willerslev *et al.* 2004*a*).

Despite this somewhat tarnished history, recent advances in knowledge about the tempo and mode of DNA template damage, sample contamination and biochemical diagenesis 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.

3. DNA DEGRADATION

(a) *Damage and repair*

The *post-mortem* instability of nucleic acids is central to the methodological problems inherent in aDNA research. In metabolically active tissues, damage to the DNA molecules is rapidly and efficiently repaired via a host of repair pathways (Lindahl 1993). By contrast, DNA damage accrues over time in inactive (dead or dormant) cells owing to factors such as spontaneous hydrolysis and oxidation, and consequently most ancient specimens do not contain any amplifiable endogenous DNA, while those that do possess only fragments in the 100–500 bp size range (Pääbo 1989; Handt *et al.* 1994; Höss *et al.* 1996). Early research showed that *post-mortem* DNA decay was characterized by strand breaks, baseless sites, miscoding lesions and crosslinks, and that these caused both sequencing artefacts and the preferential amplification of undamaged contaminant DNA (Pääbo 1989; Lindahl 1993; Höss *et al.* 1996; figure 1). However, the relative rates of various types of DNA damage and their mode of accumulation remain poorly characterized (Gilbert *et al.* 2003*a*,*b*).

aDNA amplification products are commonly characterized by sequencing artefacts, with a general bias toward CG→TA transitions and to a lesser extent AT→GC (Hansen *et al.* 2001; Hofreiter *et al.* 2001*a*; Gilbert *et al.* 2003*a*,*b*). The high number of CG→TA events appears to be owing to a high rate of hydrolytic deamination of cytosine (and its homologue 5-methyl cytosine) to uracil and thymine (figure 1*c*). The nomenclature is complicated by the complementary nature of DNA since an observed sequence change may have originated on either strand and so both possible paths are shown (e.g. C→T, or G→A on the opposing strand). However, *post-mortem* biochemical reaction pathways may be sufficiently limited that the original mutation event and strand can be identified, providing a means for sophisticated studies of *post-mortem* DNA decay and the detection of 'jumping PCR' artefacts (Gilbert *et al.* 2003*b*; figure 1*c*). A further important consideration is that miscoding lesions are not randomly distributed across the mtDNA genome as previously thought, but are concentrated in 'hot spots' where repeated hits occur. Intriguingly, the distribution of these regularly damaged sites in humans and bovids is very similar to those observed in regular evolutionary substitutions, meaning that aDNA damage can generate sequence artefacts that mimic expected evolutionary changes (Gilbert *et al.* 2003*a*; M. T. P. Gilbert, unpublished results). Recent studies of permafrost-preserved DNA indicate that inter-strand crosslinks, which prevent amplification, may accrue more quickly *post-mortem* than the single-stranded nicks that are largely responsible for fragmentation (figure 1*a*,*b*). Therefore, DNA sequences may be present in fossil remains long

after negative amplification results are obtained (Geigl 2002; Willerslev *et al.* 2004*a*).

Several methods have been used to overcome *post-mortem* modifications and increase the quantity and quality of amplifiable DNA templates and sequence reliability. Uracil-*N*-glycosylase (UNG) removes deamination products of cytosine and is an important means to test the origins of sequence variation (Hofreiter *et al.* 2001*a*; Gilbert *et al.* 2003*a*; figure 1*c*). *N*-phenacylthiazolium bromide (PTB) appears to break intermolecular cross-links caused by advanced glycosylation end products, although the exact mode of operation remains unclear (Poinar *et al.* 1998; figure 1*b*). Similarly, high fidelity polymerase enzymes such as *Pfu* and *Taq HiFi* minimize sequence error rates and increase amplification efficiency (Willerslev *et al.* 1999; Cooper *et al.* 2001*b*). However, the tempo and mode of *post-mortem* damage pathways remain too poorly understood to design effective *in vitro* enzymatic repair treatments for aDNA (Di Bernardo *et al.* 2002).

(b) *Long-term survival*

It is clear that constant low temperatures play a central role in the longevity of aDNA molecules (Lindahl 1993; Hofreiter *et al.* 2001*b*; Smith *et al.* 2001; Willerslev *et al.* 2004*b*). 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 *et al.* 2004*a*; Shapiro *et al.* 2004) and 300–400 kyr-old plant chloroplast DNA (cpDNA) and 400–600 kyr-old bacterial sequences (Willerslev *et al.* 2003*a*, 2004*a*; figure 2). 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 ice-caps, 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 *et al.* 2001; Hansen & Willerslev 2002), although authentication standards, such as the independent replication of results, have varied considerably.

4. CONTAMINATION AND AUTHENTICATION OF RESULTS

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.* 1997*b*; Cooper & Poinar 2001; Hofreiter *et al.* 2001*b*; Willerslev *et al.* 2004*b*). 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.* 2004*a*). Significantly, this is also the case especially for all reports of DNA older than the theoretical limit of survival (0.1–1 Myr; figure 2). Table 1 and figure 3 list the rigorous criteria and null hypotheses, in methodological order, required to authenticate aDNA results. However, it is important to note that even when all of these criteria have been passed the results may potentially still not be authentic.

(a) *Contamination*

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 10^{12}–10^{15} amplified molecules in a volume of less than 50 µl, 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 0.005 µl. 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 (10^5–10^6 copies; Handt *et al.* 1996; Cooper *et al.* 2001*b*), 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 (45 W, 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.* 2004*b*; 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.* 2004*a*). Similarly, the passive or active movement of cells in ancient microbial studies makes

Table 1. The analysis of nucleic acids (and cultures) from fossil remains should address the following criteria (see § 4 for details). (Appropriate laboratory facilities and controls, independent replication and cloning of amplification products are essential and should not be compromised (after Handt *et al.* 1994; Cooper & Poinar 2001; Hofreiter *et al.* 2001*b*; Gilbert *et al.* 2003*a*,*b*; Willerslev *et al.* 2004*b*).

criteria of authenticity
physically isolated pre-PCR facility nightly UV irradiation of surfaces, isolated ventilation, flow hoods or glove boxes, daily cleaning with bleach and movement only from pre-PCR to post-PCR buildings/areas
blank controls extraction and PCR controls should be performed at a ratio of at least 1 : 5 and 1 : 1 with samples, respectively; similar ratios are required for culturing experiments
independent reproducibility a subset of results should be verified by independent replication in another laboratory to rule out intra-laboratory contamination; critical for key results
cloning and sequencing a subset of PCR products should be cloned to assess damage, contamination, and to detect nuclear insertions (numts); repeat extractions and amplifications should also be assessed
time-dependent pattern of damage and diversity provides strong support for antiquity in microbial studies
decontamination of reagents and specimens bleach, acid, ultra-filtration, baking and UV irradiation of reagents and tools should be routine; particularly important in ancient human and microbial studies. The surface of samples should be removed.
DNA from associated remains can provide strong circumstantial support in ancient human and microbial studies
appropriate molecular behaviour an inverse relationship between amplification length and strength support authenticity; expected copy number relationship (e.g. between mtDNA, chloroplast DNA (cpDNA) and nuclear DNA (nuDNA)
uracil-N-glycosylase (UNG) treatment UNG removes deamination products of cytosine, and is particularly important in studies where results involve few substitution differences
quantification of starting templates particularly important in studies where interpretation is based on few substitution differences, or few specimens
biochemical preservation measures of diagenesis can indicate whether DNA survival is likely

it difficult or impossible to completely exclude recolonization of ancient materials over time.

(b) *Detecting contamination*

Over the past two decades a simple set of criteria has been developed to detect laboratory-based contamination (Pääbo 1989; Cooper & Poinar 2001). This includes blank DNA extraction and PCR controls, which need to be performed at high ratios in aDNA experiments (e.g. 1 : 5 and 1 : 1 with normal extracts, respectively). It is important to note that negative blank controls may easily conceal low-level laboratory contamination owing to poorly understood carrier effects (Cooper 1993; Handt *et al.* 1994). This commonly overlooked problem and the high risk of PCR-based contamination demonstrate why independent replication in a separate laboratory is so crucial (Cooper & Poinar 2001; Willerslev *et al.* 2004*b*). In general, the use of positive controls should be avoided owing to the contamination risk that they pose.

The independent replication of results by another laboratory is currently the strongest argument against laboratory-based contamination (whether from tools, reagents, researchers or PCR products) because it is highly unlikely that the same erroneous sequence will be independently obtained twice. The routine independent replication of results also maintains the highest standards of experimental practice in participating laboratories, and this psychological factor should not be undervalued in such contamination-prone research. In our opinion, the history of erroneous aDNA publications illustrates the need for all ongoing work to feature the independent replication of a subset of samples (e.g. 10%, or all key results), even when unsurprising results are obtained. Importantly, it is necessary not just to perform, but also to clearly demonstrate that the research is of the highest possible standards.

Sample contamination is often more difficult to detect than laboratory-based contamination, and is a pervasive problem in studies of ancient human material (Serre *et al.* 2004*b*). As many museum specimens have been handled for decades, contaminant molecules may also appear ancient and exhibit appropriate molecular behaviour, i.e. an inverse relationship between amplification efficiency and fragment length (Handt *et al.* 1994). For archaeological remains, reproducible amplification of the same sequence by independent laboratories using different parts of a specimen, for example, tooth and femur, would provide good support. Standard tests, such as phylogenetic sense (Cooper & Poinar 2001; Hofreiter *et al.* 2001*b*), can

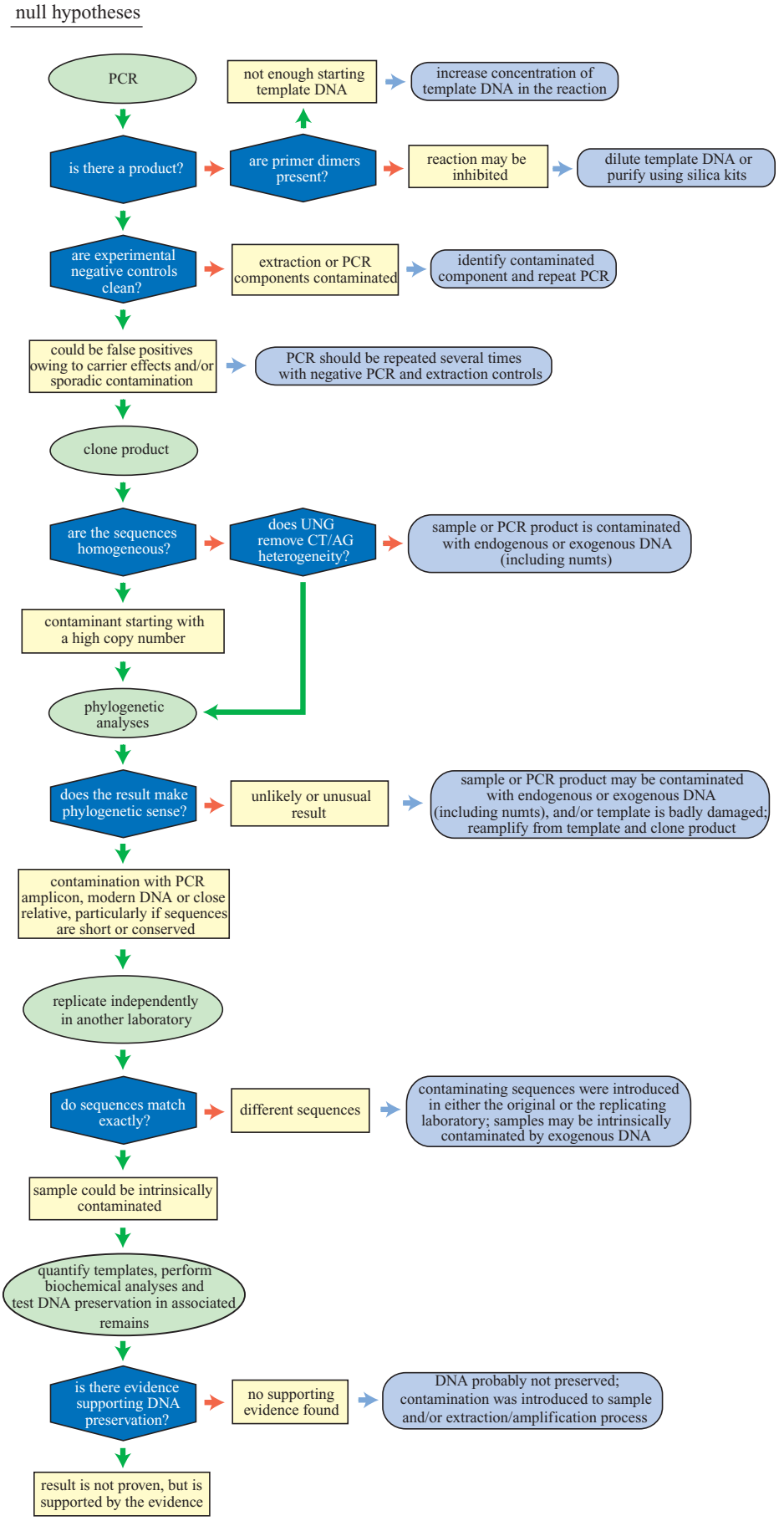

Figure 3. (*Caption opposite*.)

Figure 3. The sequential series of null hypotheses necessary for authenticating aDNA sequences, starting from the PCR reaction. Green ovals show the procedures, blue boxes represent an evaluation of results. Yellow boxes are considerations that need to be taken into account, and grey boxes are suggestions for additional procedures. Green and red arrows indicate a positive and negative result, respectively. Blue arrows are suggestions for further action.

be weak in such situations (Cooper 1997) and are generally not useful in studies of ancient microbes where novel sequences are the norm, owing to the lack of knowledge about extant microbial diversity (Willerslev *et al*. 2004*b*).

Quantification of template copy number by real time PCR is useful when a single sequence is amplified (Handt *et al.* 1996), but less so when 'universal' primers are used to obtain sequences from a variety of taxa (e.g. many microbial or plant studies). Results obtained from amplifications starting from less than 1000 template molecules have proved difficult to reproduce and can be biased by damage-induced misincorporation of bases (Handt *et al.* 1996; Gilbert *et al.* 2003*a*). Quantification also does not reveal whether some or all of the starting molecules are contaminants.

(c) *Nuclear insertions*

Insertions of mtDNA and cpDNA sequences in nuclear chromosomes are of special concern in aDNA studies. It has not been possible to purify organellular DNA from fossil remains, and nuclear insertions (numts) are generally not easily distinguishable from damaged or even authentic mtDNA sequences. The chances of detecting numts can be maximized by amplifying overlapping fragments and cloning PCR products. If mistaken for *bona fide* organelle sequences, numts can seriously confound phylogenetic and population genetic studies. For instance, there is an example of a reported dinosaur DNA sequence that was later found to be a human numt (Zischler *et al.* 1995*a*). This problem is intensified as all parts of the human mtDNA are found in the nuclear genome, sometimes as fragments up to 14 654 bp in length, and numbering more than 40 copies (Mourier *et al.* 2001). Numts have been reported from mammoth (Greenwood *et al.* 1999), moa (Cooper *et al*. 2001*b*) and rhino (Orlando *et al.* 2003) and should be taken into account when amplifying mtDNA and cpDNA sequences (figure 3; table 1). If fully characterized, numts may also be used as 'molecular fossils' to improve phylogenetic resolution (Zischler *et al.* 1995*b*).

(d) *Predicting DNA survival*

The exaggerated risk of false positive results in aDNA studies has stimulated attempts to predict DNA survival in fossil remains prior to genetic analysis. For example, the extent of amino acid racemization of the L to D-enantiomers of aspartic amino residues has been related to DNA depurination (Poinar *et al.* 1996; figure 1*a*). Flash pyrolysis with gas chromatography/mass spectrometry (GC/MS) suggests that samples containing amplifiable DNA sequences have abundant pyrolysis products assigned to 2,5-diketopiperazines of proline-containing dipeptides (Poinar & Stankiewicz 1999). Studies of 'thermal age', i.e. the number of years required for a certain degree of DNA degradation (by depurination) at a constant temperature of 10 °C, indicate that sites with thermal ages of below 17 kyr may contain specimens with amplifiable DNA (Smith *et al.* 2001).

Although non-PCR-based methods of addressing DNA survival are needed, especially when dealing with ancient human DNA and micro-organisms, they are currently not reliable. Recent reports imply that depurination kinetics will not be similar to racemization in all tissues (Collins *et al.* 2002). For example, the presence of aspartyl and asparagine residues (Geiger & Clarke 1987), actual amino acid sequence and the rate of denaturation (Collins *et al.* 1999) all influence aspartic acid racemization without affecting depurination. Furthermore, the exact thershold values for amino acid racemization associated with DNA survival continues to evolve (Poinar *et al.* 1996; Schmitz *et al.* 2002; Serre *et al.* 2004*a*), and the original Neanderthal-DNA-containing specimen (Krings *et al.* 1997) falls outside this guideline, confirming that the method has limitations. Additionally, depurination is not the only significant form of damage determining the survival of amplifiable DNA sequences. Recent studies have shown that intermolecular and inter-strand cross-links may be more significant (Poinar *et al.* 1998; Willerslev *et al.* 2004*a*; figure 1*b*). Additionally, thermal age prediction has not proved useful in dry conditions (Poinar *et al.* 2003), although the development of approaches to include rate kinetics of damage processes other than hydrolytic deamination promise to make the method useful for predicting DNA survival across a range of sites. Lastly, there is evidence to suggest that micro-environments may offer very different preservation potentials within samples, limiting the predictive power of these methods. As a consequence, simple methods such as the amplification of DNA from associated remains (e.g. plants, animals) offer an alternative means to predict the survival of DNA in ancient human and microbial specimens.

5. ANCIENT ANIMAL AND PLANT GENETICS

(a) *Population genetics*

aDNA holds tremendous potential for the study of ancient animal and plant populations. Studies on brown bears, penguins, cave bears, horses, dogs and bison (Leonard *et al.* 2002; Loreille *et al.* 2001; Vila *et al.* 2001; Barnes *et al.* 2002; Hofreiter *et al.* 2002, 2004; Leonard *et al.* 2002; Ritchie *et al.* 2004; Shapiro *et al.* 2004) have shown that aDNA can reveal population movements and local extinctions back into the Late Pleistocene. Such studies have considerable power to examine the effects of climate change (e.g. around the Last Glacial Maximum) and to test theories and develop methods used in population genetics and palaeobiology. For example, aDNA studies of Beringian brown bears revealed surprisingly large amounts of haplogroup extinction and replacement during the Late Pleistocene and Holocene, and very little interchange of females between populations (Barnes *et al.* 2002). Similarly, recent large-scale studies of North American and Eurasian bison mtDNA sequences reveal how fluctuations in genetic diversity over the past 150 kyr are closely linked to climate change (Shapiro *et al.* 2004). Interestingly, the initial decline in steppe bison genetic diversity started more than 20 kyr earlier than signs of significant early human presence in North America and therefore cannot be linked to human overkill as previously suggested (Martin 1984).

Studies of ancient populations have repeatedly shown that modern patterns of distribution and diversity are often owing to relatively recent events, and can give very misleading views of past evolutionary processes (Cooper *et al.* 1996; Barnes *et al.* 2002; Goldstein & DeSalle 2003; Shapiro *et al.* 2004). This can have major conservation implications, such as the legal ability to reintroduce extirpated taxa (e.g. Laysan duck, *Anas laysanensis*), or the apparent creation of new conservation units or species when formerly clinal distributions are disrupted (e.g. northeastern beach tiger beetle, *Cicindela dorsalis dorsalis*). Conversely, studies of several ancient populations of large vertebrates have shown extensive gene flow between taxa recognized as different species or subspecies, suggesting that the morphological plasticity in some fossil groups may have been considerably underestimated (Bunce *et al.* 2003; Huynen *et al.* 2003; Shapiro *et al.* 2004).

The combination of ancient sequences and coalescent methods has considerable power to reconstruct detailed demographic histories, test models of population genetics and reveal much novel information about microevolutionary processes. These methods can even recover demographic data for taxa that have been through recent population bottlenecks, which would normally remove genetic signals (Shapiro *et al.* 2004). Such methods also provide an opportunity to directly estimate evolutionary rates of nucleotide substitution and directly date phylogenetic events without using an external palaeontological calibration for a molecular clock (Lambert *et al.* 2001; Ritchie *et al.* 2004; Shapiro *et al.* 2004). The results show that palaeontologically calibrated rate estimates are often significantly slower than those calibrated from aDNA population studies and may reflect differences in sequence substitution processes operating at short and long timeframes.

(b) *Nuclear DNA*

Sequences from multicopy loci are easier to recover from fossil remains than single-copy sequences, probably owing to the presence of larger numbers per cell. Consequently, there has been an almost complete reliance on mtDNA and cpDNA sequences in aDNA research. However, these markers may not reflect the overall history of the genome when closely related species or population genetic questions are addressed (Hofreiter *et al.* 2001*b*). Recent results show that multi- and single-copy nuclear DNA (nuDNA) sequences can also be obtained from fossil remains, such as Pleistocene sloth coprolites from Nevada (Poinar *et al.* 2003). This study used single-copy nuDNA sequences to resolve the phylogenetic position of the extinct Shasta ground sloth (*Nothrotheriops shastensis*), and demonstrated that Pleistocene DNA survival was also possible in specimens preserved in warm arid climates and not just permafrost-preserved fossils (Greenwood *et al.* 1999, 2001).

Plant aDNA sequences have been retrieved from samples such as seeds (Rollo & Amici 1991; O'Donoghue *et al.* 1996; Brown 1999; Banerjee & Brown 2002) and ancient maize cobs (Jaenicke-Després *et al.* 2003). Interestingly, the maize study recorded how variants were selected at three nuDNA genes that effect architecture, storage protein synthesis and starch production. The results show that these genes were largely homogenized as early as 4.4 kyr ago by Mexican farmers, although one of the loci was not fixed until *ca.* 2 kyr ago. Encouragingly, this study indicates that aDNA studies of selection will become increasingly possible as phenotypically important loci are identified by genome projects.

(c) *Organellular genomics*

The majority of aDNA research has relied on relatively short sequences (*ca.* 1 kb) obtained through the amplification of numerous small overlapping fragments (*ca.* 100–500 bp). However, the field was recently brought into the genome era when the sequences of two complete mtDNA genomes (*ca.* 16.5 kb) were determined for extinct species of New Zealand moa (Cooper *et al.* 2001*b*). Partial mitochondrial genome sequences of two other moa were also generated (Haddrath & Baker 2001), demonstrating that it is possible to reconstruct long genetic sequences from fossil remains using numerous short amplifications. Such large amounts of sequence data are desirable as they permit detailed phylogenetic analyses and statistically precise estimates of molecular divergence dates.

It is important to note that even if it becomes possible to reconstruct ancient nuDNA sequences, it would still be impossible to bring extinct organisms back to life. Among many other requirements, the cloning of complex organisms needs a complete and undamaged nuDNA genome, packaged correctly, and a compatible maternal host. The characteristic damage and extensive fragmentation of aDNA make this unfeasible.

(d) *Dirty DNA*

The discovery that diverse vertebrate mtDNA and plant cpDNA sequences may be preserved in permafrost and cave sediments has opened the way for aDNA analyses of biological change at the ecosystem level (Hofreiter *et al.* 2003; Willerslev *et al.* 2003*a*).

It has been possible to recover DNA from small samples (*ca.* 2 g) of sediments up to 300–400 kyr old, including sequences of herbs, shrubs, trees, mosses, and megafauna such as mammoth, bison and horse (Willerslev *et al.* 2003*a*). Sedimentary DNA offers the potential to link fauna and flora directly in space and time over broad areas, and could provide an important complement to pollen records which are complicated by variations in influx rates, long-distance dispersal and vegetative plant growth (Guthrie 1990; Anderson *et al.* 1994). Angiosperm pollen generally does not contain cpDNA (Shi-yi 1997), so the majority of the plant cpDNA sequences in sediment must originate from seeds and/or somatic tissues such as fine rootlets (Willerslev *et al.* 2003*a*). The origin of the animal DNA sequences in sediments seems to be waste products and potentially nails and skin cells. (Lydolph *et al.* 2004). Although the potential of sedimentary DNA is remarkable, important concerns include the effects of water movement on DNA mobility in non-frozen areas, and possible biases in the deposition and survival rates of different taxa. Encouragingly, free DNA molecules in the soil have been shown to bind tightly to the edges of humic acids, clays, feldspar and quartz, which limits their movement and degradation by endonucleases (Lorenz & Wackernagel 1994).

6. 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.* 2005*a*).

Several reports show that despite rigorous protocols (Cooper & Poinar 2001; Hofreiter *et al.* 2001*b*), 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.* 2001*b*). 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.* 2005*b*). 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.* 2004*a*).

Many excavated archaeological remains appear to contain DNA from multiple individuals (Gilbert *et al.* 2003*a*,*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.* 2001*b*) were followed. However, because the resulting sequences were indistinguishable from modern Europeans, sample contamination must remain the null hypothesis. By contrast, Serre *et al.* (2004*a*) 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.* 2004*a*). 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) 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.* 2004*b*). Until recently there seemed 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.* 2003*a*,*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 (table 1). 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.

7. THE MICROBIAL PROBLEM

Many studies have reported the survival of ancient microbial DNA from archaeological remains (e.g. Reid *et al.* 1999; Rollo *et al.* 1995, 2002; Rollo & Marota 1999; Cano *et al.* 2000; Raoult *et al.* 2000; Rothschild *et al.* 2001; Zink *et al.* 2001; Fletcher *et al.* 2003) but unfortunately very few have used independent replication to authenticate results. As a consequence there is some doubt over the validity of many studies, especially when research has been done in microbiology departments where the microbial DNA sequences might be expected as a local contaminant. It is a matter of concern that many of the ancient microbial sequences are similar or identical to modern strains, or come from environments unlikely to preserve DNA (Gilbert *et al.* 2005*a*). This is unfortunate, as the rapid evolutionary rates of many pathogens provide a unique opportunity to use phylogenetic analyses to verify the sequences as ancient, i.e. ancestral to modern diversity. Where independent replication has been attempted, such as studies of *Yersinia pestis* sequences in European plague victims (Raoult *et al.* 2000), the results have been negative (Gilbert *et al.* 2004*b*).

Reports of viable microbial cells and DNA from materials hundreds of millions of years old, such as fossil plant and animal remains, deep sediments, amber and halite (e.g. Kennedy *et al.* 1994; Cano & Borucki 1995; Shi *et al.* 1997; Vorobyova *et al.* 1997; Vreeland *et al.* 2000; Fish *et al.* 2002; Vreeland & Rosenzweig 2002) also seem surprising. By contrast, a recent study of bacterial DNA under ideal deep-frozen conditions suggests a maximum survival time for amplifiable bacterial DNA of between 400 kyr and 1.5 Myr (Willerslev *et al.* 2004*a*), which is close to theoretical predictions (figure 2).

Claims regarding ancient micro-organisms are extremely difficult to authenticate because a novel sequence cannot be used as a criterion for authentication since only *ca*. 1–5% of the potential modern contaminants (i.e. extant microbial diversity) are thought to be known (Ward *et al.* 1992). Furthermore, like human DNA, microbial contamination is everywhere (Willerslev *et al.* 2004*b*) and recent results suggest that contemporary microbes have a global distribution and that endemics are rare, i.e. everything is everywhere (e.g. Fenchel *et al.* 1997; Finlay 2002). As a consequence, the independent replication of results will be a less rigorous criterion of authenticity in ancient microbial studies, and clear time-dependent patterns of diversity and DNA degradation may be needed to demonstrate authenticity (Willerslev *et al.* 2004*b*).

Despite these problems, nearly all of the geologically ancient microbial claims have not been checked by either the analysis of DNA preservation states, or more disconcertingly, by independent replication. For example, reports of bacterial DNA and viable cells surviving for hundreds of millions of years in halite were published in *Nature* (Vreeland *et al.* 2000; Fish *et al.* 2002) without verification of the result by real-time PCR, DNA damage data or independent replication. Several papers have commented on the exceptional similarity of these microbial DNA sequences to contemporary ones (Graur & Pupko 2001; Nickle *et al.* 2002) including gram-negative proteobacteria with fragile cell walls and no known adaptations to long-term DNA preservation. It is also notable that members of nearly all major microbial groups have been recovered from multi-million-year-old materials, despite possessing huge differences in hardiness and resistance to DNA degradation (Kennedy *et al.* 1994), strongly suggesting that contamination may be involved.

8. MARS: A POSSIBLE NEW RESEARCH AREA

During the coming decades, the search for evidence of extant or extinct life on other planets, especially Mars, will be a central focus of scientific research. Martian samples will provide an opportunity to test ideas such as Panspermia, which suggests that life may have travelled between planets. While contentious (Willerslev *et al.* 2004*b*), it is clear that cells such as bacterial endospores can survive considerable amounts of time in space (at least 6 years; Horneck *et al.* 1994) and the heat and pressure developed during meteorite escape and entry (Nicholson *et al.* 2000).

Any nucleic acids present on Mars have high chances of long-term survival owing to the extremely low temperatures found in areas such as the Martian permafrost and polar icecaps, which are believed to be millions or billions of years old. Calculations have shown that the Martian icecaps are likely to be in the $-50\,^{\circ}\mathrm{C}$ to $-110\,^{\circ}\mathrm{C}$ temperature range (Fisher 2000), which would theoretically allow a 100 bp bacterial DNA fragment to survive depurination for between *ca*. 3.4×10^{9} and 3.1×10^{21} years (Willerslev *et al.* 2004*b*), which is well beyond what can be expected on Earth. Of course, any microbial-like DNA sequences obtained from Martian samples will face the same problems of authentication as those from fossil remains on Earth, and may indeed represent contamination carried by spacecraft. Approaches similar to those suggested for authenticating ancient bacterial claims (table 1) may be a starting point, but it will be extremely difficult because even the strictest protocols for authentication cannot completely rule out contamination, and novel sequences would not necessarily imply an endogenous origin.

For any form of new exploration, whether extraterrestrial, deep sea or icecap, it is critical that the severe contamination problems revealed by previous aDNA studies are taken into account when designing instruments and methods for the collection and analysis of nucleic acids (Hansen *et al.* 2002). In this way, the hard lessons learned over the past two decades will be useful for a completely different set of environments, and potentially even more valuable specimens.

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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.

Anderson, P. M., Bartlein, P. J. & Brubaker, L. B. 1994 An early Wisconsin to present history of tundra vegetation in north-western Alaska (U.S.A.). *Quatern. Res.* **41**, 306–315.

Austin, J. J., Ross, A. J., Smith, A. B., Fortey, R. A. & Thomas, R. H. 1997*a* Problems of reproducibility—does geologically ancient DNA survive in amber-preserved insects? *Proc. R. Soc. Lond.* B **264**, 467–474. (doi:10.1098/rspb.1997.0067)

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

Avise, J. C. 2000 *Phylogeography: the history and formation of species*. Cambridge, MA: Harvard University Press.

Banerjee, M. & Brown, T. A. 2002 Preservation of nuclear but not chloroplast DNA in archaeological assemblages of charred wheat grains. *Ancient Biomol.* **4**, 59–63.

Barnes, I., Matheus, P., Shapiro, B., Jensen, D. & Cooper, A. 2002 Dynamics of Pleistocene population extinctions in Beringian brown bears. *Science* **295**, 2267–2270.

Brown, T. A. 1999 How ancient DNA may help in understanding the origin and spread of agriculture. *Phil. Trans. R. Soc. Lond.* B **354**, 89–98. (doi:10.1098/rstb.1999.0362)

Brown, P., Sutikna, T., Morwood, M. J., Soejono, R. P., Jatmiko, Wayhu Saptomo, E. & Rokus Awe Due 2004 A new small-bodied hominin from the Late Pleistocene of Flores, Indonesia, *Nature* **431**, 1055–1061. (doi:10.1038/nature02999)

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

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

Cano, R. J., Poinar, H. N. & Poinar Jr, G. O. 1992*a* 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. 1992*b* 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., Tiefenbrunner, F., Ubaldi, M., Del Cueto, C., Luciani, S., Cox, T., Orkand, P., Kunzel, K. H. & Rollo, F. 2000 Sequence analysis of bacterial DNA in the colon and stomach of the Tyrolean Iceman. *Am. J. Phys. Anthropol.* **112**, 297–309.

Caramelli, D. (and 10 others) 2003 Evidence for a genetic discontinuity between Neandertals and 24,000-year-old anatomically modern Europeans. *Proc. Natl Acad. Sci. USA* **100**, 6593–6597.

Christner, B. C., Mosley-Thompson, E., Thompson, L. G. & Reeve, J. N. 2001 Isolation of bacteria and 16S rDNAs from Lake Vostok accretion ice. *Environ. Microbiol.* **3**, 570–577.

Collins, M. J., Waite, E. R. & van Duin, A. C. T. 1999 Predicting protein decomposition: the case of aspartic-acid racemization kinetics. *Phil. Trans. R. Soc. Lond.* B **354**, 51–64. (doi:10.1098/rstb.1999.0359)

Collins, M. J., Nielsen-Marsh, C., Hiller, J., Smith, C. I., Roberts, J. P., Prigodich, R. V., Wess, T. J., Csapo, J., Millard, A. R. & Turner Walker, G. 2002 The survival of organic matter in bone. *Archaeometry* **44**, 383–394.

Cooper, A. 1993 DNA from museum specimens. In *Ancient DNA* (ed. B. Herrmann & S. Hummel), pp. 149–165. New York: Springer.

Cooper, A. 1997 Ancient DNA: how do you really know when you have it? *Am. J. Hum. Genet.* **60**, 1001–1002.

Cooper, A. & Poinar, H. N. 2001 Ancient DNA: do it right or not at all. *Science* **18**, 289.

Cooper, A., Rhymer, J. D., James, H. F., Olson, S. L., McIntosh, C. E., Sorenson, M. D. & Fleischer, R. C. 1996 Ancient DNA and island endemics. *Nature* **381**, 484.

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

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

Cooper, A., Drummond, A. J. & Willerslev, E. 2004 Would the real Neandertal please stand up? *Curr. Biol.* **14**, R463–R464.

DePriest, P. T., Ivanova, N. V., Fahselt, D., Alstrup, V. & Gargas, A. 2000 Sequences of psychrophilic fungi amplified from glacier-preserved ascolichens. *Can. J. Bot.* **78**, 1450–1459.

DeSalle, R. 1994 Implications of ancient DNA for phylogenetic studies. *Experientia* **50**, 543–550.

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.

Di Bernardo, G., Del Gaudio, S., Cammarota, M., Galderisi, U., Cascino, A. & Cipollaro, M. 2002 Enzymatic repair of selected cross-linked homoduplex molecules enhances nuclear gene rescue from Pompeii and Herculaneum remains. *Nucleic Acids Res.* **30**, e16.

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.

Fenchel, T., Esteban, G. F. & Finlay, B. J. 1997 Local versus global diversity of microorganisms: cryptic diversity of ciliated Protozoa. *Oikos* **80**, 220–225.

Finlay, J. F. 2002 Global dispersal of free-living microbial eukaryote species. *Science* **296**, 1061–1063.

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.

Fisher, D. A. 2000 Internal layers in an 'accublation' ice cap: a test for flow. *Icarus* **144**, 289–294.

Fletcher, H. A., Donoghue, H. D., Holton, J., Pap, I. & Spigelman, M. 2003 Widespread occurrence of *Mycobacterium tuberculosis* DNA from 18th-19th century Hungarians. *Am. J. Phys. Anthropol.* **120**, 144–152.

Geiger, T. & Clarke, S. 1987 Deamidation, isomerization, and racemization at asparaginyl and aspartyl residues in peptides. Succinimide-linked reactions that contribute to protein degradation. *J. Biol. Chem.* **262**, 785–794.

Geigl, E.-M. 2002 On the circumstances surrounding the preservation and analysis of very old DNA. *Archaeometry* **44**, 337–342.

Gilbert, M. T. P., Willerslev, E., Hansen, A. J., Barnes, I., Rudbeck, L., Lynnerup, N. & Cooper, A. 2003*a* Distribution patterns of post-mortem damage in human mitochondrial DNA. *Am. J. Hum. Genet.* **72**, 32–47.

Gilbert, M. T. P., Hansen, A. J., Willerslev, E., Barnes, I., Rudbeck, L., Lynnerup, N. & Cooper, A. 2003*b* Characterisation of genetic miscoding lesions caused by post-mortem damage. *Am. J. Hum. Genet.* **72**, 48–61.

Gilbert, M. T. P. (and 10 others) 2004*a* 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. 2004*b* 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., Barnes, I., Collins, M. J., Smith, C., Eklund, J., Goudsmit, J., Poinar, H. & Cooper, A. 2005*a* News and comments: the long-term survival of ancient DNA in Egypt: response to Zink and Nerlich. *Am. J. Phys. Anthropol.* (In the press.)

Gilbert, M. T. P. (and 14 others) 2005*b* Biochemical and physical correlates of DNA contamination in archaeological bones and teeth. *J. Arch. Sci.* (In the press.)

Goldstein, P. E. & DeSalle, R. 2003 Calibrating phylogenetic species formation in a threatened species using DNA from historical specimens. *Mol. Ecol.* **12**, 1993–1998.

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.

Graur, D. & Pupko, T. 2001 The Permian bacterium that isn't. *Mol. Biol. Evol.* **18**, 1143–1146.

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., Lee, F., Capelli, C., DeSalle, R., Tikhonov, A., Marx, P. A. & MacPhee, R. D. E. 2001 Evolution of endogenous retrovirus-like elements of the woolly mammoth (*Mammuthus primigenius*) and its relatives. *Mol. Biol. Evol.* **18**, 840–847.

Guthrie, R. D. 1990 *Frozen Fauna of the mammoth steppe: the story of blue babe*. The University of Chicago Press.

Gutiérrez, G. & Marín, 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. Lond.* B **268**, 939–945. (doi:10.1098/rspb.2001.1587)

Handt, O., Höss, M., Krings, M. & Pääbo, S. 1994 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**, 376–386.

Hansen, A. J. & Willerslev, E. 2002 Perspectives for DNA studies on polar ice cores. In *The Patagonian icefields: a unique natural laboratory for environmental and climate change studies* (ed. G. Casassa, *et al.*), pp. 17–29. Kluwer Academic/Plenum Publishers.

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

Hansen, A. J., Willerslev, E., Mørk, S., Hedegaard, M. M., Rønn, R. & Jeffares, D. C. 2002 JAWS: just add water system—a device for detection of nucleic acids in the Martian ice caps. In *Proc. 2nd Eur. Workshop on Exo/Astrobiology Graz, Austria (ESA SP-518,* November 2002) (ed. H. Lacoste), pp. 309–311. ESA Publication Division, ESTEC.

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

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

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, S., von Haeseler, A. & Pääbo, S. 2001*a* DNA sequences from multiple amplifications reveal artifacts induced by cytosine deamination in ancient DNA. *Nucleic Acids Res.* **29**, 4793–4799.

Hofreiter, M., Serre, D., Poinar, H. N., Kuch, M. & Pääbo, S. 2001*b* Ancient DNA. *Nature Rev. Genet.* **2**, 353–360.

Hofreiter, M. (and 12 others) 2002 Ancient DNA analyses reveal high mitochondrial DNA sequence diversity and parallel morphological evolution of late Pleistocene cave bears. *Mol. Biol. Evol.* **19**, 1244–1250.

Hofreiter, M., Mead, J. I., Martin, P. & Poinar, N. H. 2003 Molecular caving. *Curr. Biol.* **13**, 693–695.

Hofreiter, M., Rabeder, G., Jaenicke-Després, V., Withalm, G., Nagel, D., Paunovic, M., Jambresiç, G. & Pääbo, S. 2004 Evidence for reproductive isolation between cave bear populations. *Curr. Biol.* **14**, 40–43.

Horneck, G., Bücker, H. & Reitz, G. 1994 Long-term survival of bacterial spores in space. *Adv. Space Res.* **14**, 41–45.

Höss, M., Pääbo, S. & Vereshchagin, N. K. 1994 Mammoth DNA sequences. *Nature* **370**, 333.

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

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

Jaenicke-Després, V., Buckler, E., Smith, B. D., Gilbert, M. T. P., Cooper, A., Doebley, J. & Pääbo, S. 2003 Early allelic selection in maize as revealed by ancient DNA. *Science* **302**, 1206–1208.

Kennedy, M. J., Reader, S. L. & Swierczynski, L. M. 1994 Preservation records of microorganisms: evidence of the tenacity of life. *Microbiology* **140**, 2513–2529.

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.

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.

Krings, M., Geisert, H., Schmitz, R., Krainitzki, H. & Pääbo, S. 1999 DNA sequence of the mitochondrial hypervariable region II from the Neanderthal type specimen. *Proc. Natl Acad. Sci. USA* **96**, 5581–5585.

Krings, M., Capelli, C., Tschentscher, F., Geisert, H., Meyer, S., von Haeseler, A., Grossschmidt, K., Possnert, G., Paunovic, M. & Pääbo, S. 2000 A view of Neandertal genetic diversity. *Nature Genet.* **26**, 144–146.

Kwok, S. & Higuchi, R. 1989 Avoiding false positives with PCR. *Nature* **339**, 237–238.

Lambert, D. M., Ritchie, P. A., Millar, C. D., Holland, B. J., Drummond, A. & Baroni, C. 2001 Rates of evolution in ancient DNA from Adélie penguins. *Science* **295**, 2270–2273.

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

Leonard, J. A., Wayne, R. K., Wheeler, J., Valadez, R., Guillén, S. & Vilà, C. 2002 Ancient DNA evidence for old world origin of new world dogs. *Science* **298**, 1613–1616.

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

Loreille, O., Orlando, L., Patou-Mathis, M., Philippe, M., Taberlet, P. & Hänni, C. 2001 Ancient DNA analysis reveals divergence of the cave bear, *Ursus spelaeus*, and brown bear, *Ursus arctos*, lineages. *Curr. Biol.* **11**, 200–203.

Lorenz, M. G. & Wackernagel, W. 1994 Bacterial transfer by natural genetic transformation in the environment. *Microbiol. Rev.* **58**, 563–602.

Lydolph, M. C., Jacobsen, J., Arctander, P., Gilbert, M. T. P., Gilicinsky, D. A., Hansen, A. J., Willerslev, E. & Lange, L. 2004 Beringian paleoecology inferred from permafrost-preserved fungi DNA. *Appl. Environ. Microbiol.* (In the press.)

Ma, L.-J., Rogers, S. O., Catranis, C. M. & Starmer, W. T. 1999 Detection and characterization of ancient fungi entrapped in glacial ice. *Mycologia* **92**, 286–295.

Maca-Meyer, N., Arnay, M., Rando, J. C., Flores, C., Gonzaléz, 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.

Martin, P. S. 1984 Prehistoric overkill: the global model. In *Quaternary extinctions* (ed. P. S. Martin & R. G. Klein), pp. 345–403. Tucson, AZ: University of Arizona Press.

Morwood, M. J. (and 13 others) 2004 Archaeology and age of a new hominin from Flores in eastern Indonesia. *Nature* **431**, 1087–1091.

Mourier, T., Hansen, A. J., Willerslev, E. & Arctander, P. 2001 The human genome project reveals a continuous transfer of evenly distributed large mitochondrial fragments to the nucleus. *Mol. Biol. Evol.* **18**, 1833–1837.

Nicholson, W. L., Munakata, N., Horneck, G., Melosh, H. J. & Setlow, P. 2000 Resistance of bacterial endospores to extreme terrestrial and extraterrestrial environments. *Mol. Bio. Rev.* **64**, 548–572.

Nickle, D. C., Learn, G. H., Rain, M. W., Mullins, J. I. & Mittler, J. E. 2002 Curiously modern DNA for a '250 million-year-old' bacterium. *J. Mol. Evol.* **54**, 134–137.

Nielsen-Marsh, C. M., Ostrom, P. H., Gandhi, H., Shapiro, B., Cooper, A., Hauschka, P. V. & Collins, M. J. 2002 Sequence preservation of osteocalcin protein and mitochon-

drial DNA in bison bones older than 55 ka. *Geology* **30**, 1099–1102.

O'Donoghue, K., Clapham, A., Evershed, R. P. & Brown, T. A. 1996 Remarkable preservation of biomolecules in ancient radish seeds. *Proc. R. Soc. Lond.* B **263**, 541–547.

Orlando, L., Leonard, J. A., Thenot, A., Laudet, V., Guerin, G. & Hänni, C. 2003 Ancient DNA analysis reveals woolly rhino evolutionary relationships. *Mol. Phylogenet. Evol.* **28**, 76–90.

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

Pääbo, S. 1985 Molecular cloning of ancient Egyptian mummy DNA. *Nature* **314**, 644–645.

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. 1988 Polymerase chain reaction reveals cloning artefacts. *Nature* **334**, 387–388.

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

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

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

Poinar, H. N., Höss, M., Bada, J. L. & Pääbo, S. 1996 Amino acid racemization and the preservation of ancient DNA. *Science* **272**, 864–866.

Poinar, H. N., Hofreiter, M., Spaulding, G. S., Martin, P. S., Stankiewicz, A. B., Bland, H., Evershed, R. P., Possnert, G. & Pääbo, S. 1998 Molecular coproscopy: dung and diet of the extinct ground sloth *Nothrotheriops shastensis*. *Science* **281**, 402–406.

Poinar, H. N., Kuch, M., McDonald, G., Martin, P. & Pääbo, S. 2003 Nuclear gene sequences from a Late Pleistocene sloth coprolite. *Curr. Biol.* **13**, 1150–1152.

Priscu, J. C. (and 11 others) 1999 Geomicrobiology of subglacial ice above Lake Vostok, Antarctica. *Science* **286**, 2141–2144.

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

Raoult, D., Aboudharam, G., Crubezy, E., Larrouy, G., Ludes, B. & Drancourt, M. 2000 Molecular identification by 'suicide PCR' of *Yersinia pestis* as the agent of medieval black death. *Proc. Natl Acad. Sci. USA* **97**, 12 800–12 803.

Reid, A. H., Fanning, T. G., Hultin, J. V. & Taubenberger, J. K. 1999 Origin and evolution of the 1918 'Spanish' influenza virus hemagglutinin gene. *Proc. Natl Acad. Sci. USA* **96**, 1651–1656.

Richards, M., Sykes, B. & Hedges, R. 1995 Authenticating DNA extracted from ancient skeletal remains. *J. Archeol. 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 Adélie penguin lineages in Antarctica. *Mol. Biol. Evol.* **21**, 240–248.

Rollo, F. S. & Amici, A. 1991 Nucleic acids in mummified plant seeds: biochemistry and molecular genetics of pre-Columbian maize. *Genet. Res.* **58**, 193–201.

Rollo, F. & Marota, I. 1999 How microbial ancient DNA, found in association with human remains, can be interpreted. *Phil. Trans. R. Soc. Lond.* B **354**, 111–119. (doi:10.1098/rstb.1999.0364)

Rollo, F., Sassaroli, S. & Ubaldi, M. 1995 Molecular phylogeny of the fungi of the iceman's grass clothing. *Curr. Genet.* **28**, 289–297.

Rollo, F., Ubaldi, M., Ermini, L. & Marota, I. 2002 Ötzi's last meals: DNA analysis of the intestinal content of the Neolithic glacier mummy from the Alps. *Proc. Natl Acad. Sci. USA* **99**, 12 594–12 599.

Rothschild, B. M., Martin, L. D., Lev, G., Bercovier, H., Bar-Gal, G. K., Greenblatt, C., Donoghue, H., Spigelman, M. & Brittain, D. 2001 *Mycobacterium tuberculosis* complex DNA from an extinct bison dated 17,000 years before the present. *Clin. Infect. Dis.* **33**, 305–311.

Schmitz, R. W., Serre, D., Bonani, G., Feine, S., Hillgruber, F., Krainitzki, H., Pääbo, 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**, 13 342–13 347.

Serre, D., Langaney, A., Chech, M., Teschler-Nicola, M., Paunovic, M., Mennecier, P., Hofreiter, M., Possnert, G. & Pääbo, S. 2004*a* No evidence of Neandertal mtDNA contribution to early modern humans. *PLOS Biol.* **2**, 313–317.

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

Shapiro, B. (and 25 others) 2004 The impact of large-scale climate change on genetic diversity in large mammal populations. *Science*. (In the press.)

Shi, T., Reeves, R. H., Gilichinsky, D. A. & Friedmann, E. I. 1997 Characterization of viable bacteria in Siberian permafrost by 16S rDNA sequencing. *Microbiol. Ecol.* **33**, 169–179.

Shi-yi, H. 1997 A cytological study of plasmid inheritance in angiosperms. *Acta Botanica Sinica* **391**, 363–371.

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

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* **10**, 771–772.

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

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

Vila, C., Leonard, J. A., Gotherstrom, A., Marklund, S., Sandberg, K., Liden, K., Wayne, R. K. & Ellegren, H. 2001 Widespread origins of domestic horse lineages. *Science* **291**, 474–477.

Vorobyova, E., Soina, V., Gorlenko, M., Minkovskaya, N., Zalinova, N., Mamukelashvili, A., Gilichinsky, D. A., Rivkina, E. & Vishnivetskaya, T. 1997 The deep cold biosphere: facts and hypotheses. *FEMS Microbiol. Rev.* **20**, 277–290.

Vreeland, R. H. & Rosenzweig, W. D. 2002 The question of uniqueness of ancient bacteria. *J. Ind. Microbiol. Biotechnol.* **28**, 32–41.

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.

Ward, D. M., Bateson, M. M., Weller, R. & Ruff-Roberts, A. L. 1992 Ribosomal RNA analysis of microorganisms as they occur in nature. *Adv. Microbial Ecol.* **12**, 219–286.

Willerslev, E., Hansen, A. J., Christensen, B., Steffensen, J. P. & Arctander, P. 1999 Diversity of Holocene life forms in fossil glacier ice. *Proc. Natl Acad. Sci. USA* **96**, 8017–8021.

Willerslev, E., Hansen, A. J., Brand, T. B., Binladen, J., Gilbert, M. T. P., Shapiro, B., Bunce, M., Wiuf, C., Gilichinsky, D. A. & Cooper, A. 2003*a* Diverse plant and

animal DNA from Holocene and Pleistocene sedimentary records. *Science* **300**, 792–795.

Willerslev, E., Hansen, A. J., Rønn, R. & Nielsen, O. J. 2003*b* Panspermia—true or false? *Lancet* **362**, 406.

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

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

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

Zink, A., Haas, C. J., Reischl, U., Szeimies, U. & Nerlich, A. G. 2001 Molecular analysis of skeletal tuberculosis in an ancient Egyptian population. *J. Med. Microbiol.* **50**, 355–366.

Zischler, H., Höss, M., Handt, O., von Haeseler, A., van der Kuyl, A. C., Goudsmit, J. & Pääbo, S. 1995*a* Detecting dinosaur DNA. *Science* **268**, 1192–1193.

Zischler, H., Geisert, H., von Haeseler, A. & Pääbo, S. 2003*a* A nuclear 'fossil' of the mitochondrial D-loop and the origin of modern humans. *Nature* **378**, 489–492.