CMLS, Cell. Mol. Life Sci. 59 (2002) 97–111
1420-682X/02/010097-15 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Review

Molecular paleontology

I. Marota* and F. Rollo

Laboratory of Molecular Archaeo-Anthropology/Ancient DNA, UNICAM, via F. Camerini 2, 62032 Camerino (Italy), Fax: + 39 0737 636216, e-mail: isolina@cambio.unicam.it

Received 5 April 2001; received after revision 5 July 2001; accepted 5 July 2001

Abstract. Molecular paleontology, i.e., the recovery of DNA from ancient human, animal, and plant remains is an innovative research field that has received progressively more attention from the scientific community since the 1980s. In the last decade, the field was punctuated by claims which aroused great interest but eventually turned out to be fakes – the most famous being the sequence of dinosaur DNA later shown to be of human origin. At present, the discipline is characterized by some certainties and many doubts. We know, for example, that we have reasonable chances to recover authentic DNA from a mammoth carcass, while our chances are negligible (or nonexistent) in the case of a dynastic mummy from Egypt. On the other hand, though we are developing convincing models of DNA decay in bone, we are not yet able to predict whether a certain paleontological or archeological site will yield material amenable to DNA analysis. This article reviews some of the most important and promising investigations using molecular paleontology approaches, such as studies on the conservation of DNA in human bone, the quest for ancient DNA in permafrost-frozen fauna, the Tyrolean iceman, and the Neandertals.

Key words. Ancient DNA; human bone; iceman; mammoth; neandertal; mummy.

Introduction

For the past two centuries, paleontologists and archeologists have collected remains of human, animal, and plant life from a more or less distant past. The study of these ancient remains was performed initially on the basis of the exterior shape of the specimens (morphology). Subsequently, if the preservation state of the samples made it possible, investigators deepened their quest to the level of cells and tissues and, eventually, molecules.

The new frontier of paleontology and archeology is represented by the study of DNA. This type of research is of particular interest because DNA is the repository of the genetic history of an organism: it thus represents a direct clue to evolution. The study of a DNA sequence from an old zoological specimen was presented for the first time in 1984 [1]. The researchers utilized an approximately 150-year-old fragment of desiccated muscle of an extinct equid, the quagga, to attempt the extraction of DNA. The attempt was successful. The DNA was then cloned into an appropriate vector (a phage DNA) to determine the nucleotide sequence of a cloned fragment of mitochondrial (mt)DNA. Subsequent phylogenetic analyses [2] showed that the quagga mtDNA sequence is very close to that for the Burchell zebra.

One year later, the scientific community was thrilled by the news that DNA from an ancient Egyptian mummy had been cloned and sequenced [3, 4]. Screening more than 20 mummies, Svante Pääbo discovered that the remains of a child living 2400 years ago contained what seemed to be well-preserved DNA. In this case too, the DNA could be cloned and sequenced. This led to the identification of two sequences of the so-called human Alu family. It is more than a historical curiosity that this result is no longer considered acceptable, because we have several indications that DNA is not preserved for millennia in Egyptian human or animal mummies (see below). We are, on the other hand, well aware that conta-

* Corresponding author.

mination is one of the major issues in ancient-DNA research, in particular when we are dealing with human remains.

Again in 1985, Rollo [5] showed that very short fragments of nucleic acid could be extracted from 3300-year-old cress seeds from the tomb of Architect Kha (Thebes, Egypt). The fragments were shown to be sensitive to ribonuclease treatment and capable of binding to modern plant genes coding for ribosomal DNA in a molecular hybridization assay.

The ancient-DNA field experienced an unprecedented boost with the development of the polymerase chain reaction (PCR) by Kary Mullis [6, 7]. PCR is an extremely powerful molecular technique that can make copies of a specific fragment of DNA in vitro using a DNA polymerase, a pair of oligonucleotide primers, and the four deoxynucleotides. With this technique, billions of copies of a single DNA molecule can be obtained within a few hours, overcoming a major problem of archeogenetic research, i.e., the extreme scarcity, due to degradation, of DNA in the specimens.

Over the years, thanks to the systematic application of PCR to the analysis of DNA isolated from archeological and paleontological specimens, the field has expanded considerably [8–11]. However, if used without very special care, PCR can give misleading results. For example, the enzymatic amplification of highly fragmented DNA produces chimeras [12].

One clue to successful archeomolecular investigation is knowledge of DNA chemistry, which can help us make useful inferences on the stability of the double helix through long time spans as a function of the environment. This, in turn, can strengthen (or challenge) the results of empirical research. The principal elements of DNA stability were unravelled in the 1970s.

DNA preservation and decay

The double helix possesses several points of weakness. Bases (purinic bases in particular) tend to be lost as a result of hydrolytic cleavage of the base-sugar bond (N-glycosidic bond). The baseless sites evolve further, causing strand breaks through a reaction of beta-elimination. With time, the whole mechanism leads to a progressive fragmentation of the helix into tiny fragments. Hydrolysis is also responsible for base deamination. Both temperature and pH of the medium strongly influence this reaction [13, 14].

In addition to hydrolysis, oxidative damage, caused by the direct interaction of ionizing radiation with the DNA, and that mediated by free radicals created from water molecules by ionizing radiation, will give rise to modification of the bases followed by the destruction of their ring structure. Other mechanisms, such as alkylation or UV irradiation, are unlikely to affect buried remains. Sugar residues are also subject to attack by oxygen, resulting finally in strand breakage [15, 16].

Generally, nucleic acids are hydrolyzed at substantial initial rates when introduced into waste water, seawater, freshwater, sediments, and soils. This is mainly due to the enzymatic activity of DNA-degrading microorganisms [17]. However, particulate constituents of soils and sediments such as quartz, feldspar, and clay minerals possess sorptive capacities for inorganic and organic material including DNA and proteins. In addition to minerals, organic compounds, such as humic acids, can also form complexes with DNA. Up to 10% of total organic phosphate in soil comes from DNA bound to humic acids. Indeed, some experimental observations indicate that the DNA half-life may be very long in sediments, especially if the DNA is inside dead cells.

From this premise we can predict that a DNA molecule will be very short lived in a warm environment rich in water, oxygen, and microorganisms. Conversely, the same molecule will have relatively high chances of surviving for years, decades, centuries, and even millennia if kept in a cold, dry, anoxic, and sterile environment.

Since the first report describing the identification of quagga DNA, results have accumulated, and at present we have a good deal of evidence showing that under certain circumstances, the original DNA can be reliably identified in animal and human remains dating up to and over 50,000 years before the present (BP). The chances of success are, however, dramatically different from sample to sample.

The ‘authentication’ of the paleomolecular result

There is a widespread opinion among specialists that no entirely convincing authentication can be performed on the sole basis of a nucleotide sequence retrieved from an ancient sample. Rather, sequence analysis should be the last link in a chain of assays. The specimen should initially be analyzed for cell and tissue preservation using histological techniques, then submitted to geochemical tests to verify whether the diagenetic state of the material is compatible with the survival of DNA. Once at least some of these assays have been completed, PCR amplification of the residual DNA can be initiated. The amplification product, in its turn, should be cloned, sequenced, and the sequences examined for phylogenetic consistency with the specimen.

A well-known diagenetic test is to evaluate the degree of racemization of some amino acids. The principle of this assay is rather simple, and the test has been used for a long time by geochemists and paleontologists. Recent work has shown that it can also be employed as a highly valuable tool in the ancient-DNA field [18].

With the exception of the optically inactive glycine (Gly), all amino acids used in proteins can exist in the form of two optical isomers (enantiomers), the D (dextro-rotatory) and the L (levo-rotatory). Of the two, only the L-enantiomer is used in protein biosynthesis. At the death of the cell, the protein L-amino acids turn into D-enantiomers until, after some time, the two forms are present in equal amounts (racemization). The rate of racemization varies from amino acid to amino acid. The process is influenced by temperature and by the presence of water and certain metal ions. The L-amino acids produced by living organisms are totally racemized (D/L = 1) in the geological environment within 10^5–10^6 years on the Earth's surface.

The rate, one of the fastest, of racemization of aspartic acid (Asp) is similar, over a wide temperature range, to that of DNA depurination [19–21]. We can thus use Asp racemization as an indicator of DNA degradation. In other words, we can use racemization values for Asp to predict whether or not a certain sample will contain the original DNA.

Other useful information can be obtained by checking a sample for amino acid racemization. Because the racemization of Asp is faster than that of other amino acids, such as alanine (Ala) and leucine (Leu), one would expect to find a higher D/L ratio for the first and lower ones for the latter if the amino acids are of the same age. In contrast, a D/L ratio for Ala and Leu higher than that for Asp may be taken as evidence of contamination by more recent amino acids.

Contamination represents a major pitfall, especially when dealing with ancient human remains. In general, contamination is of two types. The first will affect all amplifications to the same extent. This contamination is due to contemporary DNA present in reagents used in tissue extractions or enzymatic amplification. It is generally easily detected by carrying out appropriate controls, such as amplifications from mock extractions and amplifications where no template DNA is added. Of greater concern is contamination of the second type, which affects individual extractions or amplifications. This may stem from handling archeological specimens prior to sampling, or from laboratory aerosols or amplification products. The main way to identify this second type of contamination is to repeat experiments from two or more independent samples from each individual studied.

In the case of ancient human and animal remains, the most common subject of investigation is perhaps mtDNA. The analysis of this molecule poses particularly serious problems due to the ease of sample contamination by modern mtDNA. Recent studies [22, 23] suggest that quantitative PCR can be employed to estimate mtDNA copy number in a mummified human tissue and that this estimate can be used as an additional validation criterion for ancient DNA.

A commonly experienced aspect of PCR is the low reproducibility of the product yield, even under the most controlled assay conditions. This variability may have various causes, including machine performance, reaction conditions, presence of inhibitors, and differences in sample preparation and purification of nucleic acids [24].

A reliable approach to molecular quantitation using PCR amplification is that based on coamplification of two similar templates (the target sequence and the reference template introduced at a known amount) of equal or similar length sharing the primer recognition sequences. During amplification, the two templates compete for the same primer set (competitive PCR), and consequently amplify at the same rate independently of the number of PCR cycles and of any predictable or unpredictable variables influencing the PCR amplification.

Jeremy Austin and coworkers [25] have established a methodology to deal with most specimens and archeomolecular investigations. In brief, this methodology recommends careful selection of specimens (on the basis of evidence for good cellular and/or biomolecular preservation), choosing tissue samples that represent the best possible site for DNA preservation, and careful sample preparation (e.g., surface sterilization) to eliminate surface contamination. The operations should be carried out in a laboratory exclusively used for the manipulation of ancient specimens. Work on ancient DNA should be temporally separated from that on modern DNA. Further, to detect any contamination, multiple negative controls should be performed during DNA extraction and PCR set up.

Finally, putatively ancient DNA sequences should be reproducibly obtained by submitting the same sample to multiple extractions. In addition, when the target DNA has a very low copy number and the experiment is particularly contamination prone, as in the case of the manipulation of very ancient human remains, the ultimate test of authenticity should be independent replication of the results in two separate laboratories.

This need for stringent 'criteria of authenticity' has been recently reiterated [26] by Alan Cooper and Hendrik Poinar (table 1).

Regarding the problem of contamination, one should take into account the results of a recent investigation performed on five well-preserved skeletal specimens from the western United States dating from 800 to 1600 AD [27]. These specimens yielded DNA samples with levels of contamination ranging from 0 to 100%, as determined by the presence or absence of New World-specific mitochondrial markers. The authors observed that no analytical indicator, including protein analysis, amino acid racemization, DNA quantitation, or inability to amplify large DNA fragments was able to predict the modern contamination present in the studied samples.

Table 1. Criteria of authenticity according to Cooper and Poinar [26].

Criterion	Explanation
Physically isolated work area	To avoid contamination, it is essential that, prior to the amplification stage, all ancient-DNA research is carried out in a dedicated, isolated environment. A building in which large amounts of the target DNA are routinely amplified is obviously undesirable.
Control amplifications	Multiple extractions and PCR controls must be performed to detect sporadic or low copy-number contamination, although carrier effects do limit their efficacy. Positive controls should be avoided as they provide a contamination risk.
Appropriate molecular behavior	PCR amplification strength should be inversely related to product size. Reproducible mitochondrial DNA should be obtainable if single-copy nuclear or pathogen DNA is detected. Deviations from these expectations should be justified. Sequences should make phylogenetic sense.
Reproducibility	Results should be repeatable from the same, and different, DNA extract of a specimen. Different overlapping primer pairs should be used to increase the chance of detecting mitochondrial gene insertions in the nucleus (numts) or contamination by a PCR product.
Cloning	Direct PCR sequences must be verified by cloning amplification products to determine the ratio of endogenous to exogenous sequences, damage-induced errors, and to detect the presence of numts. Overlapping fragments are desirable to confirm that sequence variation is authentic and not the product of errors introduced when PCR amplification starts from a small number of damaged templates.
Independent replication	Intra-laboratory contamination can only be discounted when separate samples of a specimen are extracted and sequenced in independent laboratories. This is particularly important with human remains or novel, unexpected results.
Biochemical preservation	Indirect evidence for DNA survival in a specimen can be provided by assessing the total amount, composition, and relative extent of diagenetic change in amino acids and other residues.
Quantitation	The copy number of the DNA target should be assessed using competitive PCR. When the number of starting templates is low (<1000), it may be impossible to exclude the possibility of sporadic contamination, especially for human DNA studies.
Associated remains	In studies of human remains where contamination is especially 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 amplification.

DNA preservation and decay in bone

Bone is by far the most abundant type of remains available for ancient-DNA analysis. For this reason, understanding the processes through which archeological bone loses its DNA is significant both to make informed sample choices and to authenticate results.

After burial, bones undergo diagenesis, caused by a variety of physical, chemical, and biological processes. The action of physical factors such as pressure and temperature on bone decomposition is a recognized phenomenon. These factors give rise to microcracks resulting, in their turn, in the loss of bone splinters and, ultimately, in bone fracture. Causes can vary: soil or sediment pressure, temperature, but also, in the case of subsurface finds, presence of building works, the transit of vehicles, or even stamping. Chemical decomposition, however, is a more complicated process that depends essentially on soil pH, drainage, and redox potential. One of the most obvious decomposition phenomena seen in histological sections of archeological bone is focal destruction or 'tunnelling', a loss of structural integrity which can destroy the original structure of the haversian system [28]. These tunnels are presumed to be of microbial and/or fungal origin [29, 30].

The preservation of the original DNA in archeological bone has been the subject of several investigations aimed at establishing general rules. Noreen Tuross [31] determined the amount of DNA in ancient bone specimens, the age of which ranged from 5000 BC to 1929 AD. The DNA was extracted from decalcified bone using a perfusion protocol followed by a variety of purification techniques, and its concentration was determined by ethidium bromide staining. Yields ranged from 3.4 µg/g of bone to below detectable limits. In this analysis, however, no attempt was made to discriminate between residual human DNA and the DNA of soil microorganisms which might have entered the bone.

Robert Hedges and coworkers [32] studied the distribution of histological preservation of bone over time. The changes they observed were not correlated with the date of burial, but rather with the environment in which the bones were preserved. This suggests that the causes of the changes do not operate gradually and is consistent with the idea of microbiological attack being more vigorous during the early stages of burial. Histological preservation seems to be influenced more by the burial environment, for example the hydrology: waterlogged environments appear to preserve the integrity of bones, probably by preventing their attack by aerobic microorganisms.

Examination of a sample of 72 animal and human bones for histological preservation, nitrogen content, and the presence of amplifiable mitochondrial DNA [33] showed that the success rate in amplifying mitochondrial DNA from ancient bones was correlated with the preservation of the structure rather than the nitrogen content of the bone. Discriminant analysis suggested that histology is the best discriminant variable and that survival of DNA is not correlated with the age of the sample in the range studied (200–12,000 years BP).

A first attempt to correlate the conservation/degradation of human DNA with the absence/presence of bacterial DNA in archeological bone was performed in our laboratory [34, 35]. DNA was extracted from 47 bone samples belonging to three Etruscan burial sites dating from the seventh to third centuries BC, and submitted to PCR amplification using primer pairs directed to Y repeat, X-Y amelogenin gene homologs, and 16s ribosomal RNA gene sequences. The data were compared with each other. Most of the DNA preparations were found to contain a bacterial component, although the presence of bacterial DNA did not seem to appreciably influence the sex test.

Figure 1 illustrates some of the modifications that, presumably, take place in the bone matrix in the course of diagenesis: protein degradation, amino acid racemization, and nucleic acid degradation. In addition, the figure shows how the penetration of exogenous components of microbial origin into the bone matrix can alter the diagenetic parameters.

An unknown factor in this model is represented by the relative length of the microbe-free and microbe-present periods. For example, one can suppose that the action of soil microorganisms starts soon after burial and contributes to accelerate endogenous protein and DNA degradation. Alternatively, one can suppose that most of the endogenous proteins and DNA degrade relatively soon due to chemical factors (hydratation, pH, redox potential) and only later do soil microorganisms colonize the bone matrix.

To test these issues, we analyzed a sample of 32 human femurs historically dated to 200 years BP for their state of collagen polymerization, aspartic acid racemization, presence of human mitochondrial DNA, presence of bacterial DNA, and presence of lower-eukaryote DNA (fungi, yeasts, protozoans). In addition, to identify the eukaryotic microorganisms associated with the bones, we prepared and screened an amplicon library of 18s ribosomal RNA genes [36].

The results of the analysis indicated that endogenous DNA vanished before significant microbial colonization could take place. The bones are archeologically recent and diagenetic parameters such as collagen preservation and the aspartic acid DL ratio were in most cases consistent with the absolute age of the specimens. Nevertheless, when the bones were checked for the presence of endogenous DNA remnants, only a minor portion of the samples (16%) produced amplification signals. The analysis of microbial DNA, on the other hand, was consistent with the hypothesis that the attack of the environmental microflora on the bone had just started. This is witnessed by the fact that only less than one-third (31%) of the specimens showed the presence of bacteria, and less than one-fifth (16%) that of eukaryotic microorganisms.

We can further point out that, rather unexpectedly, no known saprophytic fungus was found. This suggests that

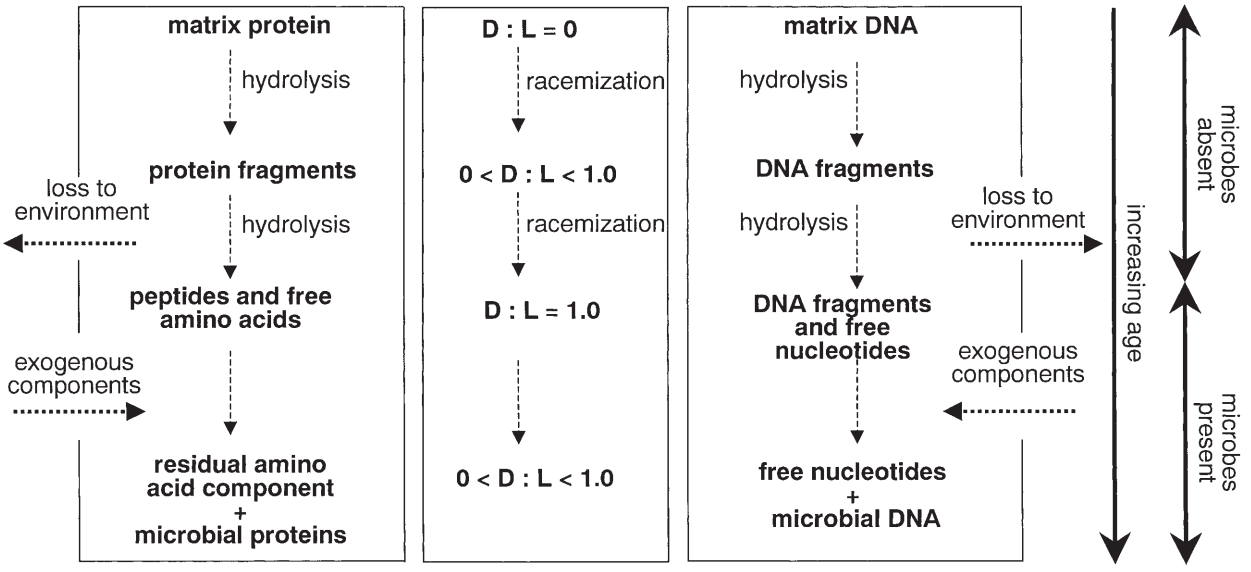

Figure 1. Model of the molecular diagenesis of bone, showing the decay of proteins, DNA, and their constituents [36]. A similar model can also be applied to the diagenesis of mummified soft tissue.

fungi may not be among the first colonizers of bone. On the other hand, the finding of a variety of flagellates and lower metazoans is consistent with the geopedological situation of the area where the bones lay (a plain crossed by rivers and ditches).

The DNA of the Pharaos: a false myth?

The first successful DNA extraction from archeological human remains in the history of molecular archeoanthropology was performed using tissue samples from an Egyptian mummy [4]. Since then, however, doubts that the environmental conditions of Egypt are suitable for the conservation of DNA for long time-spans have been raised to the point that Cooper and Wayne [37] in a recent review commented thus: 'Ironically, one of the initial ancient DNA studies, the cloning of a 3.4 kilobase pair piece of phylogenetically uninformative nuclear DNA from a 2,400-year-old Egyptian mummy appears likely to be a contaminant in hindsight, both because of its size and nature'.

van der Kuyl and colleagues [38, 39] examined six left femurs and a right humerus from different monkey species (possibly baboons, African green monkeys, or Barbary macaques), approximately 2000–2400 years old. The primate bones (actually remnants of mummified bodies) had been collected at the North Saqqara Baboon Galleries (Saqqara, Egypt). Two DNA preparations were obtained from each of the specimens, and amplified using oligonucleotide primers designed for a 104-bp fragment of the mitochondrial 12s rRNA gene. From a total of 14 samples, 3 were repeatedly negative for the mt DNA PCR, 4 showed weak bands on the gel, while the rest gave clearly positive results. All extraction controls were negative. However, upon sequencing, many of the clones were found to be derived from human contamination. Contaminating sequences other than human were also found. Clones homologous to a published bird 12s rRNA sequence originated from two bones. This second contamination probably occurred in the Galleries. An explanation was that the niche where the two bones were kept is located near to a shaft which allows pigeons free access. Pigeons were also seen to nest in the Galleries niches.

Finally, a total of ten sequences were obtained, which could be aligned with mitochondrial 12s rRNA, but none of these sequences appeared monkey-like. Later, they were shown to be copies of the nuclear counterparts of the cytoplasmic mitochondrial 12s rRNA of humans.

A comparative analysis of the amino acid racemization and DNA persistence was performed [18] on one baboon bone sample and five human femurs from Egypt. The first specimen was dated to 2300 years BP while the other five were dated to 4500 years BP. When nucleic acid fractions obtained from the bones were tested using mitochondrial primers designed to amplify very short DNA fragments, no specific amplification product was observed. The racemization tests, on the other hand, gave consistent indications that the specimens had undergone extensive diagenetic modification.

The refractoriness to PCR amplification of the nucleic acids extracted from simian bones of the Ptolemaic epoch was reiterated in further research [40] which identified the presence of a high proportion of modified bases in the specimens, as assessed by gas chromatography/mass spectrometry (GC/MS).

More recently [41], in a study of 132 mummies and skeletons of late pre-Dynastic and early Pharaonic age, only 2 samples yielded reproducible sequences when cloned amplification products derived from independent extracts were compared.

A thorough search for residual human DNA has been recently conducted in our laboratory on two Egyptian mummies (unpublished results). The first comes from a recent archeological exploration at the necropolis of Saqqara and has been dated to the early Ptolemaic period. The second is the so-called Narni (Narni, Italy) mummy. This is a female body dated to the reign of Ptolemy III (284–221 BC). DNA extraction and PCR amplification were carried out following the strictest precautions to avoid contamination. In neither case did we obtain amplification of human mitochondrial DNA even when using oligonucleotide primers designed to bind to very short (90-bp) fragments.

Egyptian writing sheets (papyri) were made with strips from the stem (caulis) of *Cyperus papyrus*. We extracted DNA from samples of modern papyri varying in age from 0 to 100 years BP and from ancient specimens from Egypt, with an age-span from 1300 to 3200 years BP. The copy number of the plant chloroplast DNA in the sheets was determined using a competitive PCR system designed on the basis of a short (90-bp) tract of the chloroplast ribulose bisphosphate carboxylase large subunit (rbcL) gene sequence. The results allowed us to establish that the DNA half-life in papyri is about 19–24 years. This figure means that the last DNA fragments will vanish within no more than 500–700 years from when the sheets are manufactured (fig. 2).

In a parallel investigation, we checked the archeological specimens for the presence of residual DNA and determined the extent of racemization of Asp in both modern and ancient specimens. The results confirmed the complete loss of authentic DNA even in the least ancient (eighth century AD) papyri. On the other hand, when the regression for Asp racemization rates in papyri was compared with that for human and animal remains from Egyptian archaeological sites, they were, quite surprisingly, found to be virtually identical. This study provides an indirect argument against the reliability of claims about the recovery of authentic DNA from Egyptian

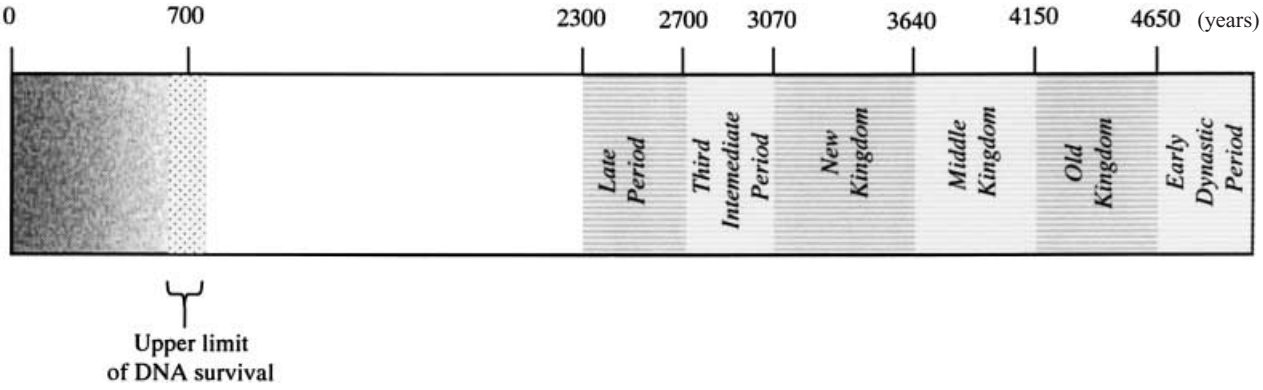

Figure 2. Survival limit of DNA in the Egyptian environment, as inferred from the analysis of ancient and modern papyri [I. Marota, C. Basile, M. Ubaldi, F. Rollo, unpublished data], compared to the chronology of Pharaonic Egypt.

mummies and bone remains [I. Marota, C. Basile, M. Ubaldi, F. Rollo, unpublished data].

In the course of the 1990s, several reports announced the isolation of DNA from paleontological samples many million years old. Although apparently authentic ancient DNA was recovered, these results were later challenged on the basis of empirical and theoretical evidence. For example, the mitochondrial cytochrome b sequence of an 80-million-year-old dinosaur from the Upper Cretaceous Blackhawk Formation in Utah [42], which was recovered from multiple extractions and PCR amplifications, was later recognized by phylogenetic arguments as being most probably of human origin [43].

Among earlier reports of DNA in extremely ancient materials, one of the most celebrated was that from the leaves in the Miocene Clarkia deposits of Idaho [44, 45]. Here too, attempts to replicate the original results in other laboratories failed [46] and only bacterial DNA was detected. Investigations of the other macromolecules in the leaf tissue, on the other hand, showed that the chemical preservation of the Clarkia fossils reflects the thermal immaturity of the sediment. Cellulose, hemicellulose, cutin, and protein have been lost, but lignin and an aliphatic biopolymer were detected using pyrolysis. The lipids from the leaf waxes of Clarkia plants reflect the gross characteristics of the original leaf waxes. However, a comparison of these epicuticular wax lipids with those of modern leaf tissues showed evidence of decay. Such extensive degradation of marker molecules seems incompatible with the preservation of DNA in the Clarkia fossils [47].

The most interesting case is perhaps that of amber-trapped organisms. The fossil resin traps and encapsulates insects, leaves, and other materials in a medium with bacteriocidal properties. Amber inclusions are an important source of information on the taphonomic processes that affect organic remains. For these reasons, amber appeared the most promising source of million-year-old DNA [48–50]. Amber also provides an opportunity to explore the fate of more decay resistant parts of organisms, such as insect cuticle, a complex of chitin and protein that can be extremely tough and durable. Despite these premises, however, no markers characteristic of either chitin or proteins were detected in stingless bees and beetles preserved in 25- to 30-million-year-old Dominican amber [51]. Where a resistant macromolecule like chitin in insects is not preserved, there seems little likelihood that traces of the much more fragile DNA molecule will remain [52].

With regard to this last issue, a particularly accurate investigation was performed by Jeremy Austin and colleagues [25]. They tested 15 specimens of fossil insect, representing three species and body sizes and two different localities and ages (Dominican amber and East African copal) for the presence of insect DNA, using a number of primer pairs designed to bind to insect, fungal, and vertebrate mitochondrial DNA. In no case were they able to amplify authentic insect DNA, not even from the relatively recent (a few million years) copal.

DNA from the frozen fauna of the mammoth steppe

Vast numbers of large mammals including horses, mammoths, moose, wolves, bears, lions, and saber-toothed cats are preserved in Arctic permafrost deposits. The collections at the American Museum of Natural History, New York, and the Canadian Museum of Nature, Ottawa, contain some 100,000 skeletal elements. Permafrost deposits are of great value for understanding the history of populations, for example, how they were affected by climatic and other environmental changes during the last glaciation. The preservation conditions found in permafrost, characterized by low temperature and dehydration of the specimens, are among the best conceivable. It is therefore not surprising that a relatively high rate of success has been reported for permafrost-preserved Siberian mammoths and other mammals, despite the relevant age of the specimens.

Among the extinct genus *Mammuthus*, *M. primigenius* (woolly mammoth) is known as a well-preserved fossil from Siberian permafrost. *Mammuthus* is classified in the subfamily Elephantinae with two extant genera, *Loxodonta* represented by *L. africana* (African elephant) and *Elephas* represented by *E. maximus* (Asian elephant). These three genera are believed to have originated in Africa and to have diverged from *Primelophas gomphoteroides* before the Early Pliocene (approximately 5 million years ago). Phylogenetic relationships among these three genera remain unclear due to lack of fossil evidence clearly showing the divergence process. Paleomolecular analyses can contribute to resolving the *Mammuthus*/*Loxodonta*/*Elephas* trichotomy.

Erika Hagelberg and colleagues extracted DNA from bones of two frozen Siberian mammoths. The first individual, the Khatanga mammoth, consisted of the partial carcass of an adult male excavated in 1977 from alluvial sand in the eastern Taimyr peninsula. The age of the specimen was established to be at least 47,000 years by accelerator mass spectrometry (AMS) radiocarbon dating. The second sample was from a mandible excavated in 1975 from the Allaikha River, northeastern Siberia. The mandible, dated to more than 46,000–47,000 years BP on autochthonous plant vegetation remains and AMS dating, lay in permafrost 16 m below the surface. DNA extraction from the mammoth bone samples carried out in parallel with three forensic and three prehistoric human bone samples and an extraction blank (no bone), followed by PCR amplification with the highly conserved mitochondrial primers L14841 and H15149, consistently gave phylogenetically meaningful sequences which appeared to be slightly closer to African elephant than to Asian elephant [53].

In the course of a similar investigation [54], DNA was extracted from soft tissues of five different mammoths varying in age from 9700 to more than 50,000 years old. Enzymatic amplification of a 93-bp fragment of the mitochondrial 16s ribosomal RNA gene yielded an amplification product from four of the five individuals, specifically from 7 of the 15 extractions performed.

Three proboscidean fossils from different environments were considered in a study in 1996 [55]. One *M. primigenius* sample was a piece of air-dried skin from a frozen woolly mammoth found in 1907 on Lyakhovskiy Island in the Siberian Arctic and now stored in the Musée National d'Histoire Naturelle (Paris). Radiocarbon dating of the sample gave an age of more than 46,000 years. The second *Mammuthus* sample was a cranial fragment collected in 1947 from glacial stream deposits of the Alaskan steppe near Fairbanks. Vertebrate fossils associated with this bone were previously radiocarbon dated to approximately 20,000 years BP. The third fossil was a well-preserved American mastodon (*Mammut americanum*) skeleton excavated in 1968 from Late Pleistocene bog deposits in Oakland County, southern Michigan. The fossil was previously radiocarbon dated as 10,200 ± 170 years old. PCR amplification of DNA extracted from the three specimens using oligonucleotide primers specific for the mitochondrial cytochrome b gene allowed the researchers to obtain stretches of more than 200 bp in length of the gene sequence of both *Mammuthus* and *Mammut*. The comparison of the fossil sequences with the corresponding sequences of contemporary museum specimens of *E. maximus* and *L. africana* supported a monophyletic Asian elephant-woolly mammoth clade, when the American mastodon was used as an outgroup.

Further results were obtained in 1997 for the Enmynveyem mammoth and the baby mammoth known as Dima [56, 57]. The former was discovered in 1986 in the Enmynveyem River Valley of the Chukotka Peninsula of northeastern Siberia. The remains, radiocarbon dated to 32,850 ± 900 years BP, consist of intact portions of the right hind femur, tibia, and fibula with articulated muscle and skin. The latter comes from Magadan and is about 40,000 years old.

In the same year, Noro and coworkers determined the complete sequences of cytochrome b (1137 bp) and 12s ribosomal RNA (961 bp) genes in mitochondrial DNA from the woolly mammoth. The *M. primigenius* specimen was dried muscle tissue of a mammoth excavated from the Pyasna River Valley in the Taimyr Peninsula, Russia, and dated to at least 25,000 years BP by the radiocarbon method. Following its discovery, it was stored in a dessicator at room temperature for over 30 years without any chemical treatment. The phylogenetic analysis of ancient and modern sequence data supported the '*Mammuthus-Loxodonta*' clade, which is contrary to previous morphological reports that *Mammuthus* is more closely related to *Elephas* than to *Loxodonta* [58].

A particularly interesting result was obtained in 1999 by Alex Greenwood and colleagues. They sampled, among other specimens, the molars of two Alaskan mammoths which had previously been excavated from a permafrost deposit at Engineer Creek, Alaska, and a mammoth mandible and soft tissue from the Novosibirskie Islands and the Indigirka Basin in Siberia. A dentine sample from one of the two Alaskan mammoths was dated by AMS at 13,775 ± 145 years, while the Siberian mammoth and the other Alaskan mammoth were assumed to be of Late Pleistocene age.

To determine whether mammoth DNA was present in the Alaskan mammoth bone extract, the researches employed PCR systems targeted to short (< 240 bp) fragments of the mitochondrial 16s ribosomal DNA and cythochrome b gene. These fragments were readily amplified, while attempts to amplify a larger (376-bp) fragment failed, thus demonstrating that the mammoth DNA had undergone substantial degradation. Subsequently, the conservation of nuclear DNA was tested by the use of PCR primers de-

signed to bind to a short portion of the gene for 28s ribosomal RNA (28s rDNA). This gene exists in a few hundred copies per haploid genome in vertebrates. The results showed that the mammoth nuclear DNA could be amplified to give a product of a size similar to that of the African and Asian elephant. On the other hand, the corresponding 28s rDNA amplification products from human and cow DNA were readily distinguishable from those of the mammoth because of their smaller size. In a further series of tests, short (< 100-bp) portions of several single-copy nuclear genes (von Willebrand factor gene, alpha-2B adrenergic receptor gene, interphoto-receptor retinoid-binding protein gene) were also successfully amplified [59].

The brown bear (*Ursus arctos*) is a large, Holoarctic carnivore, whose distribution was dramatically altered by Late Pleistocene events. Bear mtDNA types fall mainly into four clades of related sequences, designated I–IV. Although clade I exists only in western Europe, clades II and IV exist in Europe, Asia, and North America. In North America, the brown bear has had a limited history, appearing in eastern Beringia only 50–70,000 years ago, spreading into the contiguous United States about 13,000 years ago. Within the Americas, clades II, III, and IV are present but in a phylogeographic pattern such that clade II is present only on islands off the coast of northwestern Canada, clade IV occurs in southern Canada and the United States, and subclade IIIb exists in northern Canada and eastern Alaska, whereas subclade IIIa is found in Eurasia and western Alaska. Leonard and colleagues determined mtDNA sequences in seven brown bear bone specimens from permafrost deposits in central Alaska and northwestern Canada, dated from 14,000 to 42,000 years ago. They amplified a 502-bp fragment of the 5′ end of cytochrome b by using three overlapping primer sets, and one control region segment of 173 bp. Subsequent phylogenetic analysis of the cytochrome b and control region sequences indicated that the permafrost sequences could be assigned to the clades I, II, and III, which now occur only in geographically widely separated populations. One interpretation of the existing distribution of the mitochondrial clades is that they represent different waves of immigration by brown bears into North America. The results from the permafrost specimens suggest that clade II represents a first wave and clade IV and clade III, a second and third wave, respectively [60, 61].

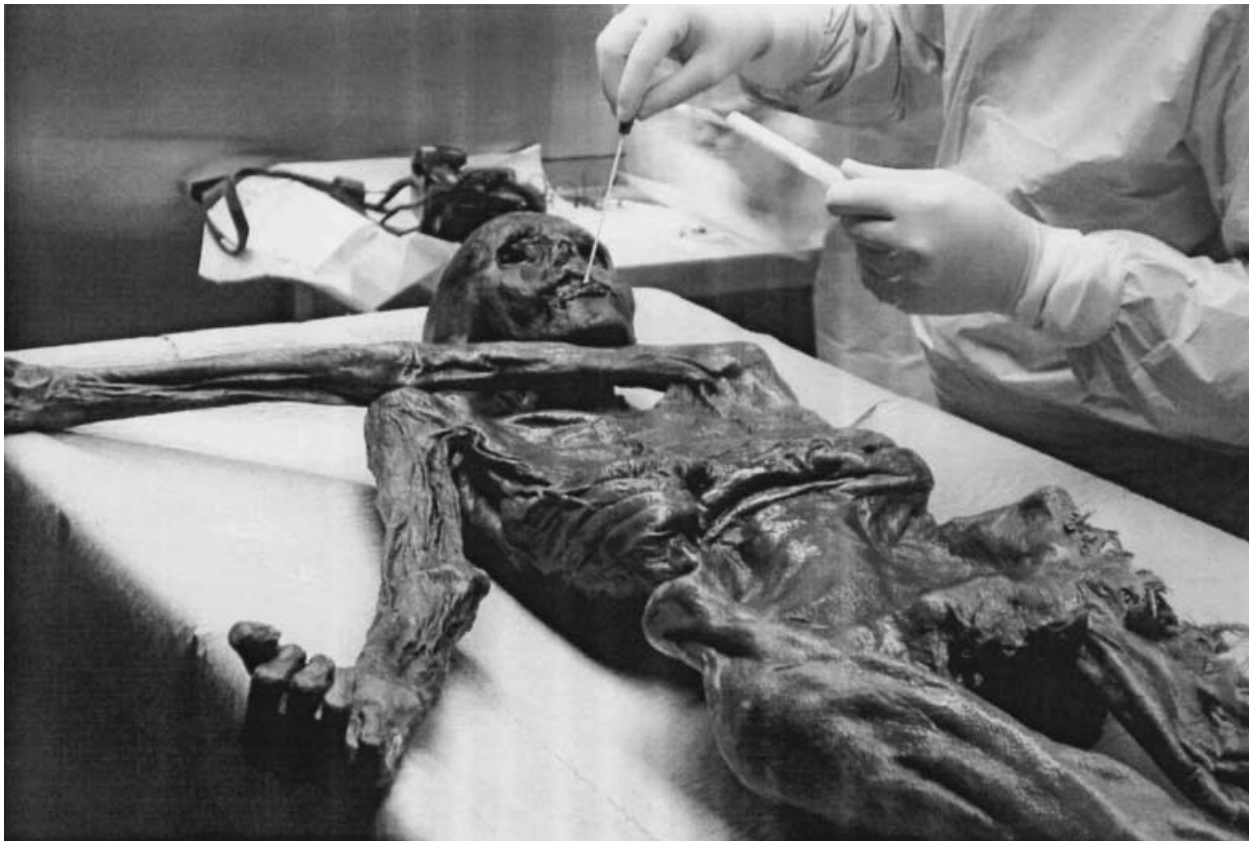

Figure 3. The Tyrolean iceman during the 25 September 2000 survey [36].

A witness from the neolithic: the Tyrolean iceman

The so-called Tyrolean iceman, or Ötzi, is a mummified human body found in an Alpine glacier on 19 September 1991 at 3270 m above sea level. The most relevant feature of the find, later radiocarbon dated to 3350–3100 BC, corresponding to the Late Neolithic, is the amazing state of preservation of the mummy and of the clothing and equipment found on the body and near it (fig. 3). They include, among many other items, extremely perishable ones such as a large fragment of a cloak made of knotted tufts of grass, a fur hat, and a wooden bow with arrows and quiver [62].

The iceman lay in a chamber-like depression, below a rocky ledge, sheltered from the shearing flow of glacial ice. So trapped, the corpse was not expelled with the regular glacial turnover. In the years following the discovery, several hypotheses have been made regarding the process of mummification. Until 1995, the prevailing idea was that the corpse had undergone rapid dehydration by a warm wind (an autumn föhn) and been subsequently covered by snow. An alternative speculation was that body and equipment froze rapidly, and were then covered by a porous layer of snow which allowed the body to air desiccate. Paul Bahn, on the other hand, suggested that the corpse was preserved in the same way as the many frozen carcasses of mammoths and other Ice Age animals in Siberia and Alaska. These were preserved by the build-up of ice in the sediments that enveloped the bodies: the ice layers desiccated the soil and dehydrated the carcasses. Unlike freeze-drying, where the original form remains intact, this process shrivels the body [63].

None of these hypotheses, however, can entirely account for a number of features which are being progressively revealed by the investigations carried out independently in several laboratories since the first exploration of the iceman's site. For example, histological and biochemical analyses have shown an almost complete loss of the iceman's epidermis, acompanied by profound postmortem alterations of skin triacylglycerols, which implies a prolonged (up to several months) immersion in water before dry weather and, possibly, cold winds desiccated the corpse [64].

The Tyrolean iceman has been the object of a detailed molecular analysis by research teams in Munich and Oxford. To assess whether some of his original DNA remained, the copy number of fragments of mtDNA (D-loop region) was estimated by competitive PCR, starting from extracts obtained from a total of eight samples of muscle, connective tissue, and bone removed under sterile conditions from the left hip region of the mummy, which had been damaged during the first salvage attempt. The PCR-amplified mtDNA was subsequently cloned into a plasmid vector and sequenced. Alignment of a number of amplicon sequences showed that the body had been heavily contaminated by modern human DNA, as witnessed by the contemporaneous presence of several different mitochondrial sequences in the same specimen. To remove contaminating DNA, two of the samples were selected that were of sufficient size to allow the superficial parts to be removed. Finally, by applying the principle that the ancient mtDNA should be of low molecular weight (< 150 bp in length), the Munich team succeeded in indicating one sequence as the most likely candidate for the iceman's original mtDNA. This result was independently confirmed in the Oxford laboratory. Once compared with the mtDNA of contemporary populations, the iceman mtDNA was found to occur most frequently among Europeans north of the Alps, having been found in 7 out of 155 individuals from northern Germany, Denmark, and Iceland, and twice among British Caucasoids. It was also found once in Swiss populations and three times in the Mediterranean region, but not among 120 sub-Saharan Africans, 143 Siberians, 419 Native Americans, and 16 individuals from the valley where the body was found. When the mean pair-wise sequence difference between the iceman sequence and sequences from various regions of the world was determined, the iceman sequence was found to be most distant from sub-Saharan Africans and closest to the individuals from the Alpine area [65].

On the other hand, the vast majority of the endogenous DNA of the iceman appeared to be degraded. This conclusion was confirmed by the failure of attempts to amplify single copies of the human amelogenin gene, which is located on the X and Y chromosomes, and is currently used for molecular sexing of skeletal remains [66, 67]. The possible presence of remnants of the original nuclear DNA is still under scrutiny [68].

A particularly interesting sector of molecular paleontology is the study of ancient microorganisms, bacteria, filamentous fungi, yeasts, algae, protozoans, and viruses. The analysis of ancient microorganism DNA in ancient human and animal remains can contribute to an understanding of issues as various as the spread of a new disease, a mummification process, and the effect of diet on historical human populations.

To identify the composition of the cadaveric flora in the iceman and to shed new light on the mummification process [69], we analyzed about 80 sequences of bacterial DNA (16s ribosomal RNA gene) in samples of skin and muscle from the mummy. The investigation was complicated by the fact that the surface of the mummy had been swabbed with phenol soon after its discovery. Our results showed that no trace of microbial DNA was left on the actual surface of the body, while the untreated skin bore the remains of large numbers of bacteria belonging to the genera *Sphingomonas*, *Afipia*, *Curtobacterium*, *Microbacterium*, *Agromyces*, and others. Compared to the untreated skin, the iceman's muscle was also very rich in bacterial DNA. However, this DNA comes, with few

exceptions, from the species *Clostridium algidicarnis*. The sharp difference in the bacterial DNA composition of skin and muscle suggests that the remains of the original cadaveric microflora of the latter have not disappeared during the iceman's taphonomic history. On the other hand, the massive presence of *C. algidicarnis*, a cold-adapted sporigenous, the DNA of which was previously found in the tissue of a naturally desiccated Andean mummy [70], indicates that the hypothesis that the iceman's corpse underwent rapid dehydration by the effect of a warm wind (föhn) is no longer plausible. The results best fit with the hypothesis that the body was first covered by snow and ice, and then underwent thawing and, finally, desiccation.

All external body surfaces, including the digestive tract, have a normal resident bacterial flora. Because of cell turnover, gut surfaces are coated with dead and desquamating cells which provide an excellent basal nutrient source, to which can be added nutrients passing through the lumen and the gut. The bacterial flora represents a not insignificant component of the complex intestinal ecosystem. Its distribution along the intestinal tract is, however, highly variable in quantity and composition and can be influenced by diet, interaction among the different groups of microorganisms and ingestion of drugs toxins, and carcinogenic substances. Under normal conditions, the upper part of the small bowel (duodenum, jejunum) is considered to have a low microbial content, while its lower portion (ileum) is characterized by higher cell numbers. The highest microbial content is found in the large bowel (colon) [71].

A pilot study on the bacterial flora of the iceman has been performed [72]. To check whether 5000 years lying under the glacier had totally abolished traces of the bacteria that dwelt in the gastrointestinal tract of the Neolithic herdsman/hunter, or whether some remains of prehistoric bacteria are left, three types of sample were selected. The first comprised grass fragments from the boots and the cloak, and the second and third, biopsies from the iceman's stomach and colon, respectively. DNA was extracted from the three groups of samples and PCR amplified using universal primer pairs targeted to portions of the 16s rDNA. Amplification products were cloned and amplicons from the three groups of libraries sequenced and used for database scanning. The results showed that the bacterial flora associated with the grass clothing included, among others, species belonging to the genera *Zoogloea*, *Curtobacterium*, *Arthrobacter Desulfitobacterium,* and *Sphingobacterium*. On the other hand, bacterial sequences obtained from stomach biopsies were few in diversity and consisted mainly of *Burkholderia* spp. and *Pseudomonas* spp. Finally, the colon showed a wide array of *Clostridium* and *Eubacterium*, most of which are recognized common inhabitants of human intestines, and *Vibrio*. The marked differences found in the composition of the bacterial flora of the grass clothing (possibly representative of the microbiological situation of the glacier), stomach, and colon clearly show that the body was colonized by microorganisms from the external environment only to a limited extent during its long taphonomical history [72].

To investigate the origin of the fungal hyphae that cover the grass clothing (cloak, boots) of the iceman, Franco Rollo and colleagues extracted DNA from two radiocarbon-dated samples of grass. The DNA was then PCR amplified using primer pairs specific for the region containing the internal transcribed spacers and the 5.8s rDNA (ITS), and primer pairs specific for an approximately 600-bp-long fragment of the nuclear small-subunit ribosomal DNA (SSU rDNA) repeat units of eukaryotes. The amplification products were cloned and sequenced. Sequence analysis of 20 ITS and 10 SSU rDNA amplicons indicated that three types of fungal DNA had been extracted from the Neolithic grass. Phylogenetic analyses, using 5.8s and SSU rDNA fungal reference sequences from databases, showed that the DNAs came from a basidiomycete, phylogenetically close to *Leucosporidium scottii*, a psycrophilic yeast-like microorganism isolated, among other substrates, from soil, plant material, and water in Antartica and Canada, and from two ascomycetes, one of which is possibly related to the Eurotiales [73–76].

It may be of interest here to mention the results of a molecular study of deep ice cores. Although fossil plant pollen and spores from several deep ice cores have been characterized and have provided information about ancient flora composition and dispersal, most organic material found in fossil glacial ice is not amenable to morphological identification. DNA analysis of deep ice cores from North Greenland dated approximately 2000 and 4000 years BP [77], showed an unprecedented diversity of plants, algae, and protists of both local and distant origin, thus providing valuable information about ancient communities and their change over time.

Future research on the iceman might focus on the quest for bacterial pathogens. Study of the history and evolution of infectious diseases is a major issue of molecular research applied to archeology. Significant results have already been obtained in the case of tuberculosis which has been demonstrated, for example, in pre-Columbian America by DNA analysis [78]. Starting material was a lung lesion of a spontaneously mummified body of an adult who died 1000 years ago in southern Peru. DNA was extracted from pathological tissue and enzymatically amplified using a primer pair designed to bind to a 123-bp-long sequence which is part of a repetitive insertion sequence-like element (IS6110). This first result was confirmed by PCR analysis of the DNA extracted from a vertebral lesion in a 12-year-old girl with Pott's disease from pre-Columbian (about 1000 AD) Chile [79].

More recently, the presence *of Mycobacterium tuberculosis* was detected in skeletal remains from Lithuania, dated to the 15th–17th centuries. Most interestingly, DNA of the bacillus was identified in both pathological and normal tissues (bones and even teeth) of the same individual, thus proving hematogenous spread of mycobacteria. Moreover, the presence of *M. tuberculosis* DNA was demonstrated in skeletal remains of individuals without specific lesions [80]. Further evidence for the incidence of tuberculosis in historical populations comes from a study of *M. tuberculosis* complex DNA in 1400-year-old calcified pleural tissue [81].

Human genes from the glacial era

During the last (Würmian) glacial era, Europe and western Asia were inhabited by a group of hominids called the Neandertals (*Homo neanderthalensis* or *Homo sapiens neanderthalensis*). As is known, this name comes from the valley of Neander near Düsseldorf, Germany. In a cave (the Feldhof cave), human skeletal remains of previously unknown morphology were found in 1856. Since then, the evolutionary position of the Neandertals has been the subject of debate among paleoanthropologists. While it is now sufficiently clear that the Neandertals stem from *H. erectus*, the relationship between Neandertals and modern Europeans remains enigmatic. In the past, the Neandertals were believed to be the direct ancestors of modern Europeans. While this theory is now abandoned, some specialists are convinced that at least some genes of the Neandertals were passed to modern humans. Others, however, maintain that the Neandertals were completely replaced by modern humans without contributing any gene.

In 1997, a team led by Svante Pääbo succeeded in unravelling the nucleotide sequence of a 379-bp portion of the mitochondrial DNA control region of a Neandertal. The specimen analyzed was the Neandertal-type specimen found in 1856 and the starting material was a 3.5-g section of the right humerus. Samples were taken from different points of the specimen (periostal surface, compact bone, endostal surface, varnish coat) and tested for amino acid racemization. The results showed that the ratio of the D to L enantiomers of aspartic acid in the three bone samples varied between 0.11 and 0.12. These figures were considered to be in a range compatible with DNA survival.

Subsequently, DNA was extracted from a portion of cortical bone and amplified using a set of primers designed to amplify a 105-bp-segment of the human mitochondrial DNA control region.

The amplification product was cloned in a plasmid vector and sequenced. While three clones were similar to the human reference sequence [82], the remaining 27 exhibited substantial differences. The conclusion was drawn that the first group represented contaminating DNA while the second was DNA endogenous to the Neandertal bone.

In a second phase of the experiments, the researchers utilized sets of primers designed on the basis of the putative Neandertal sequence to amplify the DNA extracted from the bone. In this way, the entire sequence of the hypervariable region 1 (HVR-1) of the mtDNA control region was determined. Since the DNA state of preservation allowed only short fragments to be amplified, this was achieved by several overlapping amplifications. When the Neandertal sequence was compared to the human reference sequence, 27 differences were detected. In particular, the comparison of the sequence to a collection of 2051 human and 59 common chimpanzee sequences showed that the DNA of Neandertal and modern human differ by 20–34 substitutions, while the differences within present-day populations range between 1 and 24. On the other hand, the base substitutions between modern humans and chimpanzee range between 46 and 67 (average 55).

It must be added that comparison of modern human sequences shows no effect of lineage or geographical distribution: whatever the geographical origin of the individuals, the mean substitution number is always the same. This observation contradicts the hypothesis of a closer proximity of modern Europeans with the Neandertals.

Furthermore, a distance tree, rooted using 16 chimpanzee lines, shows a first separation between Neandertals and modern humans followed by a series of dichotomies, the first of which separates an African line from the others. This result strengthens the picture according to which modern humans arose in Africa and then migrated to the other continents with the final result of completely replacing the pre-existing populations of *H. erectus* and *H. neanderthalensis*.

On the basis of the estimated divergence date between humans and chimpanzees, one can calculate when the most recent ancestral sequence common to the Neandertal and modern human mtDNA existed. This is between 550,000 and 690,000 years BP, when using an estimated divergence date between humans and chimpanzees of 4–5 million years ago [83, 84]. This first report was followed by another describing the extraction, amplification, and sequencing of DNA from 29,000-year-old archeological bone material of a Neandertal infant recovered from the Mezmaiskaya Cave in the northern Caucasus. In this case, the preservation of collagen-type debris was used as an indicator of macromolecule preservation in the bone. Two sections of one rib were used for DNA extraction in two independent laboratories (Glasgow and Stockholm) and the DNA was amplified using oligonucleotide primers specific for Neandertal mtDNA. When the Mezmaiskaya and the Feldhofer mtDNA sequences were phylogenetically compared with those of modern humans, they grouped together in a distinct clade [85].

Further confirmation came from the analysis of 15 Neandertal bone specimens from the Vindjia Cave, Croatia. Amino acid composition and extent of racemization indicated that seven samples had undergone only moderate diagenesis. One of them was radiocarbon dated by AMS to over 42,000 years BP and used for five DNA extractions. In three extractions, N-phenacylthiazolium bromide, a compound that cleaves sugar-derived condensation products, was added to the extraction medium. Amplification of multiple overlapping mtDNA fragments allowed the reconstruction of 357 bp of HVR-1 and 288 bp of HVR-2. When compared with the type specimen sequence, they differed by nine substitutions and in the length of a stretch of cytosine and thymidine residues in HVR-2. In this case too, phylogenetic analysis showed that the two Neandertal (the Feldhof and the Vindjia) sequences group together apart from all modern humans [86].

Taken together, these results do not exclude the possibility that interbreeding between Neandertals and modern humans may have taken place, but they show that even if it occurred, Neandertals did not end up contributing mtDNA to the contemporary human gene pool.

A different methodological approach has been employed in the work of Scholz and colleagues [87]. These researchers introduced Southern blot hybridization of genomic DNA as a simple fossil DNA-based approach to classify remains of Neandertals. When hybridized with genomic DNA of either human or Neandertal origin, DNA extracted from two Neandertal specimens from Warendorf-Neuwarendorf, Germany, and Krapina, Croatia, was shown to yield hybridization signals that differed by at least a factor of two compared to the signals obtained with fossil DNA of an early *H. sapiens* from the Vogelherd cave (Stetten I), Germany. When labelled chimpanzee DNA was used as a probe, Neandertal and human DNA, however, revealed hybridization signals of similar intensity. These results were interpreted as indicating that the genome of Neandertals differs significantly from the genome of anatomically modern humans, due to the contrasting composition of repetitive DNA. In turn, this supported the hypothesis that the Neandertals were not ancestors of anatomically modern humans.

The work by Scholz and colleagues has generated some debate among specialists [88, 89]. The main issues of contention are the effect of soil microorganisms and the discrimination power of the hybridization techniques applied to the analysis of ancient-DNA samples.

Conclusions

Sixteen years after the first results, the research in molecular paleontology now encompasses a wide range of themes and materials. Frozen-desiccated remains, either of animal or human origin, can conserve important traces of the original DNA even after many thousands of years. On the other hand, considerably less ancient materials, such as human mummies from Egypt, probably offer very little, if any, chance of success. A still unsolved puzzle is how authentic 'Mousterian' DNA may have been preserved in hominid bones dating to > 40,000 years, as these remains come from sites in central Europe and western Asia which are not characterized by particularly favorable environmental conditions [90]. This is all the more enigmatic since the analysis of animal teeth and bones from Neandertal and Cro-Magnon sites has failed to produce amplifiable DNA [91].

Acknowledgements. Work in the authors' laboratory is supported by Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST), research project 'Fonti biologiche e storico-letterarie per la conoscenza delle condizioni di vita e delle malattie nella Roma antica' (MM10264872).

1 Higuchi R., Bowman B., Freiberger M., Ryder O. and Wilson A.C. (1984) DNA sequences from a quagga, an extinct member of the horse family. Nature **312:** 282–284
2 Higuchi R. G., Wrischnik L. A., Oakes E., George M., Tong B. and Wilson A. C. (1987) Mitochondrial DNA of the extinct quagga: relatedness and extent of postmortem change. J. Mol. Evol. 25**:** 283–287
3 Pääbo S. (1985) Preservation of DNA in ancient Egyptian mummies. J. Archaeol. Sci. **12:** 411–417
4 Pääbo S. (1985) Molecular cloning of ancient Egyptian mummy DNA. Nature **314:** 644–645
5 Rollo F. (1985) Characterization by molecular hybridisation of RNA fragments isolated from ancient (1400 B.C.) seeds. Theor. Appl. Genet. **71:** 330–333
6 Saiki R. K., Scharf S., Faloona F., Mullis K. B., Horn T., Erlich H. A. et al. (1985) Enzymatic amplification of beta globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science **230:** 1350–1354
7 Mullis K. B. and Faloona F. A. (1987) Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. Methods Enzymol. **155:** 335–350
8 Pääbo S., Higuchi R. G. and Wilson A. C. (1989) Ancient DNA and the polymerase chain reaction. J. Biol. Chem. **264:** 9709–9712
9 Herrmann B. and Hummel S. (1994) Ancient DNA. Springer, Berlin
10 Audic S. and Béraud-Colomb E. (1997) Ancient DNA is thirteen years old. Nat. Biotechnol. **15:** 855–858
11 Kelman L. M. and Kelman Z. (1999) The use of ancient DNA in paleontological studies. J. Vertebr. Paleontol. **19:** 8–20
12 Pääbo S., Irwin D. M. and Wilson A. C. (1990) DNA damage promotes jumping between templates during enzymatic amplification. J. Biol. Chem. **265:** 4718–4721
13 Lindahl T. and Nyberg B. (1972) Rate of depurination of native deoxyribonucleic acid. Biochemistry **11:** 3610–3618
14 Lindahl T. and Nyberg B. (1974) Heat induced deamination of cytosine residues in deoxyribonucleic acid. Biochemistry **13:** 3405–3410
15 Lindahl T. (1993) Instability and decay of the primary structure of DNA. Nature **362:** 709–715
16 Lindahl T. (1997) Facts and artifacts of ancient DNA. Cell **90:** 1–3
17 Lorenz M. G. and Wackernagel W. (1994) Bacterial transfer by natural genetic transformation in the environment. Microbiol. Rev. **58:** 563–602

18 Poinar H. N., Höss M., Bada J. L. and Paabo S. (1996) Amino acid racemization and the preservation of ancient DNA. Science **272:** 864–866
19 Bada J. L., Wang X. S., Poinar H. N., Pääbo S. and Poinar G. O. (1994) Amino acid racemization in amber-entombed insects: implications for DNA preservation. Geochim. Cosmochim. Acta **58:** 3131–3135
20 O'Connell T. C., Hedges R. E. M. and Klinken G. J. van (1997) An improved method for measuring racemization of amino acids from archaeological bone collagen. Ancient Biomol. **1:** 215–220
21 Bada J. L., Wang X. S. and Hamilton H. (1999) Preservation of key biomolecules in the fossil record: current knowledge and future challenges. Phil. Trans. R. Soc. Lond. B **354:** 77–87
22 Handt O., Höss M., Krings M. and Pääbo S. (1994b) Ancient DNA: methodological challenges. Experientia **50:** 524–529
23 Handt O., Krings M., Ward R. H. and Paabo S. (1996) The retrieval of ancient human DNA sequences. Am. J. Hum. Genet. **59:** 368–376
24 Clementi M., Menzo S., Bagnarelli P., Manzin A., Valenza A. and Varaldo P. E. (1993) Quantitative PCR and RT-PCR in virology. PCR Methods Appl. **2:** 191–196
25 Austin J. J., Smith A. B. and Thomas R. (1997) Palaeontology in a molecular world: the search for authentic ancient DNA. Trends Ecol. Evol. **12:** 303–306
26 Cooper A. and Poinar H. K. (2000) Ancient DNA: do it right or not at all. Science **289:** 1139
27 Kolman C. J., and Tuross N. (2000) Ancient DNA analysis of human populations. Am. J. Phys. Anthropol. **111:** 5–23
28 Hackett C. J. (1981) Microscopical focal destruction (tunnels) in exhumed human bones. Med. Sci. Law **21:** 243–265
29 Marchiafava V., Bonucci E. and Ascenzi A. (1974) Fungal osteoclasia: a model of dead bone resorption. Calcif. Tissue Res. **14:** 195–210
30 Grupe G. and Dreses-Werringloer U. (1993) Decomposition phenomena in thin sections of excavated human bones. In: Histology of Ancient Human Bone: Methods and Diagnosis, pp. 27–36, Grupe G., and Garland A. N. (eds), Springer, New York
31 Tuross N. (1994) The biochemistry of ancient DNA in bone. Experientia **50:** 530–535
32 Hedges R. E. M., Millard A. R. and Pike A. W. G. (1995) Measurements and relationships of diagenetic alteration of bones from three archaeological sites. J. Archaeol. Sci. **22:** 201–211
33 Colson I. B., Bailey J. F., Vercauteren M. and Sykes B. C. (1997) The preservation of ancient DNA and bone diagenesis. Ancient Biomol. **1:** 109–118
34 Vernesi C., Caramelli D., Carbonell i Sala S., Ubaldi M., Rollo F. and Chiarelli B. (1999) Application of DNA sex tests to bone specimens from three Etruscan (VII–III century B.C.) archaeological sites. Ancient Biomol. **2:** 295–305
35 Rollo F. and 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
36 Rollo F., Ubaldi M., Marota I., Luciani S. and Ermini L. (2001) DNA diagenesis: effect of environment and time on human bone. Ancient Biomol. in press
37 Cooper A. and Wayne R. (1998) New uses for old DNA. Curr. Opin. Biotechnol. **9:** 49–53
38 Kuyl A. van der, Dekker J., Attia M., Iskander N., Perizonius W. and Goudsmit J. (1994) DNA from ancient Egyptian monkey bones. Ancient DNA Newsl. **2:** 19–21
39 Kuyl A. C. van der, Kuiken C., Dekker J.T., Perizonius W. R. K. and Goudsmit J. (1995) Nuclear counterparts of the cytoplasmic mitochondrial 12s rRNA gene: a problem of ancient DNA and molecular phylogenies. J. Mol. Evol. **40:** 652–657
40 Höss M., Jaruga P., Zastawnj T. H., Dizdaroglu M. and Pääbo S. (1996) DNA damage and DNA sequence retrieval from ancient tissues. Nucleic Acids Res. **24:** 1304–1307
41 Krings M., Salem A. H., Bauer K., Geisert H., Malek A. K., Chaix L. et al. (1999) mtDNA analysis of Nile river valley populations: a genetic corridor or a barrier to migration? Am. J. Hum. Genet. **64:** 1166–1176
42 Woodward S. R., Weyand N. J. and Bunnell M. (1994) DNA sequence from Cretaceous period bone fragments. Science **266:** 1229–1232
43 Hedges S. B. and Schweitzer M. H. (1995) Detecting dinosaur DNA. Science **268:** 1191
44 Golenberg E. M., Giannasi D. E., Clegg M. T., Smiley C. J., Durbin M., Henderson D. et al. (1990) Chloroplast DNA sequence from a Miocene *Magnolia* species. Nature **344:** 656–658
45 Golenberg E. M. (1991) Amplification and analysis of Miocene plant fossil DNA. Phil. Trans. R. Soc. Lond. B **333:** 419–427
46 Sidow A., Wilson A. C. and Pääbo S. (1991) Bacterial DNA in Clarkia fossils. Phil. Trans. R. Soc. Lond. B **333:** 429–433
47 Logan G. A and Eglinton G. (1994) Biogeochemistry of the Miocene lacustrine deposit at Clarkia, northern Idaho, USA. Org. Geochem. **21:** 857–870
48 Cano R. J., Poinar H. N., Pieniazek N. J., Acra A. and Poinar G. O. (1993) Amplification of DNA from a 120–135-million-year-old weevil. Nature **363:** 536–538
49 DeSalle R., Barcia M. and Wray C. (1993) PCR jumping in clones of 30-million-year-old DNA fragments from amber preserved termites (*Mastotermes electrodominicus*). Experientia **49:** 906–909
50 Cano R. J., Borucki M. K., Higby-Schweitzer M., Poinar H. N., Poinar G. O. and Pollard K. J. (1994) *Bacillus* DNA in fossil bees: an ancient symbiosis? Appl. Environ. Microb. **60:** 2164–2167
51 Briggs D. E. G. (1999) Molecular taphonomy of animal and plant cuticles: selective preservation and diagenesis. Phil. Trans. R. Soc. Lond. B **354:** 7–17
52 Priest F. G. (1995) Age of bacteria from amber. Science **270:** 2015
53 Hagelberg E., Thomas M. G., Cook C. E., Sher A. V., Baryshnikov G. F. and Lister A. M. (1994) DNA from ancient mammoth bones. Nature **370:** 333–334
54 Höss M., Pääbo S. and Vereshchagin N. K. (1994) Mammoth DNA sequences. Nature **370:** 333
55 Yang H., Golenberg E. M. and Shoshani J. (1996) Phylogenetic resolution within the Elephantidae using fossil DNA sequences from the American mastodont (*Mammut americanum*). Proc. Natl. Acad. Sci. USA **93:** 1190–1194
56 Derenko M., Malyarchuk B. and Shields G. F. (1997) Mitochondrial cytochrome b sequence from a 33000 year-old woolly mammoth (*Mammuthus primigenius*). Ancient Biomol., **1:** 149–153
57 Ozwa T., Hayashi S. and Mikhelson V. M. (1997) Phylogenetic position of mammoth and steller's sea cow within Tethytheria demonstrated by mitochondrial DNA sequences. J. Mol. Evol. **44:** 406–423
58 Noro M., Masuda R., Dubrovo I. A., Yoshida M. C. and Kato M. (1998) Molecular phylogenetic inference of the woolly mammoth *Mammuthus primigenius*, based on complete sequence of mitochondrial cytochrome b and 12S ribosomal RNA genes. J. Mol. Evol. **46:** 314–326
59 Greenwood A. D., Capelli C., Possnert G and Pääbo S. (1999) Nuclear DNA sequences from late Pleistocene megafauna. Mol. Biol. Evol. **16:** 1466–1473
60 Leonard J. A., Wayne R. K. and Cooper A. (2000) Population genetics of ice age brown bears. Proc. Natl. Acad. Sci. USA **97:** 1651–1654
61 Pääbo S. (2000) Of bears, conservation genetics, and the value of time travel. Proc. Natl. Acad. Sci. USA **97:** l320–1321
62 Spindler K. (1995) The Man in the Ice, Orion London
63 Bahn P. G. (1996) Tombs, Graves and Mummies. Barnes and Noble, New York

64 Bereuter T. L., Mikena T. and Reiter C. (1997) Iceman's mummification-implications from infrared spectroscopical and histological studies. Chem. Eur. J. **3:** 1032–1038
65 Handt O., Richards M., Trommsdorff M., Kilger C., Simanainen J., Georgiev O. et al. (1994) Molecular genetic analyses of the Tyrolean Ice Man. Science **264:** 1775–1778
66 Stone A. C., Milner G. R., Pääbo, S. and Stoneking M. (1996) Sex determination of ancient human skeletons using DNA. Am. J. Phys. Anthropol. **99:** 231–238
67 Lassen C., Hummel S. and Herrmann B. (1996) PCR based sex identification of ancient human bones by amplification of X- and Y-chromosomal sequences: a comparison. Ancient Biomol. **1:** 25–34
68 Schiermeier Q. and Stehle K. (2000) Frozen body offers chance to travel back in time. Nature **407:** 550
69 Rollo F., Luciani S., Canapa A. and Marota I. (2000) Analysis of bacterial DNA in skin and muscle of the Tyrolean iceman offers new insight into the mummification process. Am J. Phys. Anthropol. **111:** 211–219
70 Ubaldi M., Luciani S., Marota I., Fornaciari G., Cano R. J. and Rollo F. (1998) Sequence analysis of bacterial DNA in the colon of an Andean mummy. Am. J. Phys. Anthropol. **107:** 285–295
71 Hill M. J. (1995) The normal gut bacterial flora. In: Role of Gut Bacteria in Human Toxicology and Pharmacology, pp. 3–17, Hill M. J. (ed.), Taylor and Francis, London
72 Cano R. J., Tiefenbrunner F., Ubaldi M, Del Cueto C., Luciani S., Cox T. et al. (2000) Sequence analysis of bacterial DNA in the colon and stomach of the Tyrolean iceman. Am. J. Phys. Anthropol. **112:** 297–309
73 Rollo F., Asci W., Antonini S. and Ubaldi M. (1994) Molecular ecology of a Neolithic meadow, the DNA of the grass remains from the archaeological site of the Tyrolean Iceman. Experientia **50:** 576–584
74 Rollo F., Asci W., Marota I. and Sassaroli S. (1995) DNA analysis of grass remains found at the iceman's archaeological site. In: Der Mann im Eis, pp. 91–105, Spindler K., Rastbichler-Zissernig E., Wilfing H., Nedden D. zur and Nothdurfter H. (eds), Springer, Vienna
75 Rollo F., Sassaroli S. and Ubaldi M. (1995) Molecular phylogeny of the fungi of the Iceman's grass clothing. Curr. Genet. **28:** 289–297
76 Antonini S., Luciani S., Marota I., Ubaldi M. and Rollo F. (2000) Compilation of DNA sequences from the iceman's grass clothing. In: The Iceman and his Natural Environment, pp. 157–162, Bortenschlager S. and Oeggl K. (eds), Springer Vienna
77 Willerslev E., Hansen A. J., Christensen B., Steffensen J. P. and Arctander P. (1999) Diversity of Holocene life forms in fossil glacier ice. Proc. Natl. Acad. Sci. USA **96:** 8017–8021
78 Salo W. L., Aufterheide A. C., Buiskra J. and Holcomb T. (1994) Identification of *Mycobacterium tuberculosis* DNA in a pre-Columbian Peruvian mummy. Proc. Natl. Acad. Sci. USA **91:** 2091–2094
79 Arriaza B. T., Salo W. Aufderheide A. C. and Holcomb T. A. (1995) Pre-Columbian tuberculosis in northern Chile: molecular and skeletal evidence. Am. J. Phys. Anthropol. **98:** 37–45
80 Faerman M., Jankauskas R., Gorski A., Bercovier H. and Greenblatt C. L. (1997) Prevalence of human tuberculosis in a medieval population of Lithuania studied by ancient DNA analysis. Ancient Biomol. **1:** 205–214
81 Donoghue H. D., Spigelman M., Zias J., Gernaey-Child A. M. and Minnikin D. E. (1998) *Mycobacterium tuberculosis* complex DNA in calcified pleura from remains 1400 years old. Lett. Appl. Microbiol. **27:** 265–269
82 Anderson S., Bankier A. T., Barrel B. G., Bruijn M. H. L. de, Coulson A. R., Drouin J. et al. (1981) Sequence and organisation of the human mitochondrial genome. Nature **290:** 457–465
83 Krings M., Stone A., Schmitz R. W., Krainitzki H., Stoneking M. and Pääbo S. (1997) Neandertal DNA sequences and the origin of modern humans. Cell **90:** 19–30
84 Krings M., Geisert H., Schmitz R. W., Krainitzki H. and Paabo S. (1999) DNA sequence of the mitochondrial hypervariable region II from the neandertal type specimen. Proc. Natl. Acad. Sci. USA **96:** 5581–5585
85 Ovchinnikov I. V., Götherström A., Romanova G. P., Kharitonov V. M., Lidén K. and Goodwin W. (2000) Molecular analysis of Neanderthal DNA from the northern Caucasus. Nature **404:** 490–493
86 Krings M., Capelli C., Tschentscher F., Geisert H., Meyer S., Haeseler A. von, et al. (2000) A view of Neandertal genetic diversity. Nat. Genet. **26:** 144–146
87 Scholz M., Bachmann L., Nicholson G. J., Bachmann J., Giddings I., Rüschoff-Thale B. et al. (2000) Genomic differentiation of Neanderthals and anatomically modern man allows a fossil-DNA-based classification of morphologically indistinguishable hominid bones. Am. J. Hum. Genet. **66:** 1927–1932
88 Geigl E. M. (2001) Inadequate use of molecular hybridization to analyze DNA in Neanderthal fossils. Am. J. Hum. Genet. **68:** 287–290
89 Bachmann L. (2001) Reply to Geigl. Am. J. Hum. Genet. **68:** 290–291
90 Caldararo N. and Gabow S. (2000) Mitochondrial DNA analysis and the place of Neandertals in *Homo*. Ancient Biomol. **3:** 135–158
91 Cooper L., Poinar H. N., Pääbo S., Radovcic J., Debénath A., Caparro M. et al. (1997) Neandertal genetics. Science **277:** 1021–1025