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Ancient DNA: methodological challenges

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Abstract. The study of ancient DNA offers the possibility of following genetic change over time. However, the field is plagued by a problem which is unique in molecular biology – the difficulty of verifying results by reproduction. Some of the reasons for this are technical and derive from the low copy number and damaged state of ancient DNA molecules. Other reasons are the unique nature of many of the objects from which DNA is extracted. We describe methodological approaches with which these problems can be alleviated in order to ensure that results are scientific in the sense that they can be reproduced by others.

Key words. Ancient DNA; molecular archaeology; PCR; contamination; DNA damage; DNA quantitation; inhibition; jumping PCR.

Introduction

The past few years have seen an increasing interest in the retrieval of DNA sequences from museum collections and archaeological finds. For example, by the study of skins in museum collections that go back some decades, frequencies of mitochondrial types in wild populations could be followed over time[20]. Using dried skins as a source of DNA, it could be shown that the red wolf may be the result of hybridization of grey wolves and coyotes[22]. Remains that are a few thousand years old have allowed conclusions about how extinct species such as moas[3], marsupial wolves[19,10] and saber-toothed tigers[9] are related to extant ones. Finally, DNA sequences have been determined from palaeontological deposits that go back millions of years[5]; and most recently animal and plant inclusions in amber have yielded sequences that are more than a hundred million years old[17]. This young field of molecular archaeology, however, has to overcome some problems before it can be fully regarded as a respectable part of modern science. These problems can be summarized in one word: reproducibility.

There are many reasons why reproducibility represents a problem when ancient DNA is studied. The specimens from which nucleic acids are isolated are in themselves unique and thus an experiment can often not easily be repeated, either by the investigator who originally performed it or by other groups. Yet, it is obvious that if this field is to make a serious contribution to science, investigators need to design projects that ensure reproducible results. An experiment that cannot be replicated is of little or no value, even if the result is correct in the first instance.

Below, we outline some issues that in our opinion are of crucial importance for the further progress of ancient DNA studies. They include simple experiments that characterize the state of preservation of DNA, avoid contamination, and ensure the reproducibility of results.

Characterization

The DNA in a living organism is continuously affected by destructive processes, mainly spontaneous hydrolysis of bonds between base and sugar residues, breakage of the sugar backbone of the DNA, and oxygen radical-induced attacks on bases and sugar residues. It has been calculated that by depurination alone a human cell experiences 2,000–10,000 lesions to its genome per day[12]. Thanks to continuous DNA repair, organisms are able to survive this onslaught on their genetic material. After death, DNA repair ceases whereas many of the destructive processes continue. For example, due to its hydrophilicity, DNA remains hydrated even in dry climates and hydrolytic damage will occur. Although some conditions may increase the chance of survival, all studies examining damage in ancient DNA have shown that the molecules are reduced to a size of some hundred base pairs and that they are heavily affected by oxidative changes[13]. One problem with these studies is that they analyse the bulk of the extracted DNA: whereas only a minute fraction may originate from the organism in question, the rest could be of bacterial or fungal origin. However, it is reasonable to assume that if the majority of the extracted DNA is damaged then the oldest DNA in an extract will be so too.

The behaviour of amplifications by the polymerase chain reaction (PCR) performed from ancient DNA also testifies to its damaged nature. There are basically two phenomena that characterize attempts to do PCR from ancient DNA. First, it is almost always impossible to obtain long amplification products. This restricts work with ancient DNA to short sequences and necessitates the use of overlapping primer pairs if longer sequences are to be obtained. Invariably, we have observed an inverse relationship between amplification efficiency and length of the amplification products. When such a relationship has not been seen, the amplification has always turned out to be due to contamina-

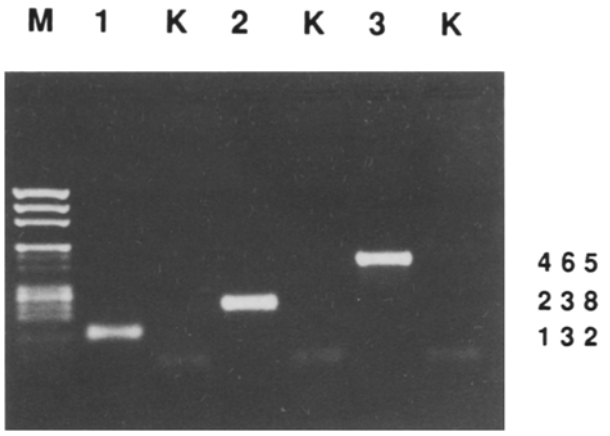

Figure 1A. Agarose gel with PCR products (lanes 1, 2 and 3) from bone extracts of a giant ground sloth using primers for nuclear 18S rRNA genes. There is no inverse relationship between fragment length and amplification efficiency (cf. fig. 4). The lanes referred to as K are PCR controls, lane M refers to marker DNA.

tion with non-ancient templates such as PCR products from former amplifications. For example, when different primers specific for the 18S ribosomal gene were used to amplify what was expected to be nuclear rDNA sequences from a 13,000-year-old ground sloth, no inverse relationship was observed between amplification efficiency and lengths of the amplification products (fig. 1A). These were sequenced and found to be of fungal rather than mammalian origin (fig. 1B). Thus, the products are derived from fungi that may have colonized the sloth remains recently. In our experience it is therefore highly valuable to characterize the DNA with respect to the lengths of the amplification products it allows. Any indication that it fails to show signs of degradation should be regarded as a serious warning signal.

A second feature of PCR products originating from ancient DNA molecules is that the sequences often indicate that some sort of recombination has taken place during the PCR. The reason for this is a phenomenon called 'jumping PCR'. Because of the large amount of damage present in ancient DNA, the polymerase often reaches template positions which carry either a lesion or a strand break that stops the polymerase. The partially extended primer can anneal to another template fragment in the next cycle and be extended up to another damaged site. Thus, in vitro recombination can take place until the whole stretch encompassed by the two primers is synthesized[4,11,14] and amplification enters the exponential part of the PCR. When working with a homogenous system (haploid organisms, mtDNA) jumping PCR can be an advantage rather than a drawback, since it helps to amplify longer fragments than are actually present in the extract. However, when working with di- or polyploidic systems, or if contamination occurs, jumping will complicate the interpretation of the results[6,6a]. In such cases, molecular cloning and sequencing of multiple clones will not only help to sort out the different species of molecules present in the amplification products but the fact that jumping occurs furnishes another indication that the DNA may be of ancient origin.

Finally, it is highly desirable that additional molecular and chemical characterization of nucleic acids and other biomolecules be explored in the future to increase knowledge of post mortem processes affecting DNA as well as to establish additional criteria for the evaluation of results from ancient extracts.

Quantitation

A quantitation of the number of endogenous molecules in an ancient DNA extract is valuable since it makes possible a direct comparison of the state of preservation among specimens and extracts. However, as the amounts of bacterial and fungal DNA in extracts from

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                    10         20         30         40         50         60         70         80         90
Neomylodon?  TTTATTTGAT AG-----TAC CTTACTACAT GGATACCTGT GGTAATTCTA GAGCTAATAC ATGCTAA-AA ACCTCGACTT C---------
Eurotium     .......... .......... .......... .......... .......... .......... .......... .......... .........
C. elegans   CC.TA.CCGG GATCCGGAT. ...---C.T. .....A...C ..A...A..G .......... ....A.CT.T ...C.A.--- -...CGCAAG
Drosophila   ..CC..A... C.TTAACAGT T------..T. .....A.... .......... .......... ....A.TT.. .A.AT..ACC TTAT......
Human        .AC-C.G.T. GATCCTGCCA G.AG.-.T.. .CT.GT..CA AAG.T.AAGC C.TGC.TGT. TAAG..CGC. CGGC..GTAC AGTGAAACTG

                   100        110        120        130        140        150        160        170        180
Neomylodon?  GGAAGGGGGT GTATTTATTA GATAAAAA-- ------ACCA ACGCCCTTCG GGGCTC---- ---------- ---------- CTTGGTGAAT
Eurotium     .......... .......... .......... .......... .......... .......... .......... .......... .........
C. elegans   GC.GG---.. .C.A...... ..AC.---.. .....G.... .ACGTT.... .ACG.TG... .......... .......... T...T...C.
Drosophila   ...G.C.T.. .CT..... .GCT....CC AAGC..---- ---------- ------GATC GCAAGATCGT TAT.ATTGGT TG----A.C.
Human        C.A.T..CTC A.TAAATCAG TTATGGTTCC TTTGGTCG.T CGCT...CTC CCA..TGGAT AACTGTGGTA ATTCTAGAGC TAATACATGC

                   190        200        210        220
Neomylodon?  CATAATAACT --AAGCGAAT CGCATGGCCT T-GCGCCGG
Eurotium     .......... .......... .......... .........
C. elegans   .TG.....-- ...-...AGT. TA.TGTCAG. .TCGA.T.A
Drosophila   .TAG....GA T.--..AG.. ..T....T.. ...TA...A
Human        .GACGGGCGC TG.CC.CCT. ...GG..GGG AT...TGCA
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Figure 1B. Sequence alignment of fragments of the 18S rDNA from different species. The sequence obtained from one of the amplification products from figure 1A is compared to the sequences of different organisms. There is little or no similarity between the putative ground sloth sequence (*Neomylodon*) and a mammalian sequence (human). However, it is identical to a fungus (*Eurotium*).

ancient remains often vastly exceed those of the endogenous DNA, a straightforward quantitation of total nucleic acids, by photometrical measurements makes little sense. Furthermore, the recovered amounts of endogenous DNA are often too small to be directly detectable by radioactive probes in dot blots. Thus, PCR-based techniques have to be used. Most useful is the use of primers specific for the organism under study. These are used to perform a series of amplifications to which a constant amount of the ancient extract is added as well as a dilution series of a template that differs in length from the endogenous template by a few bases (fig. 2A). After agarose gel electrophoresis (fig. 2B) the concentration of the longer template that approximately matches that of the extract template can be determined and thus a calculation of the numbers of amplifyable ancient molecules is possible. It should be noted that the use of an internal standard which is amplified by the same primers as the ancient template is preferable to the use of a control template that is amplified by another primer pair since these can differ in their sensitivity to components in the PCR which affect amplification efficiency.

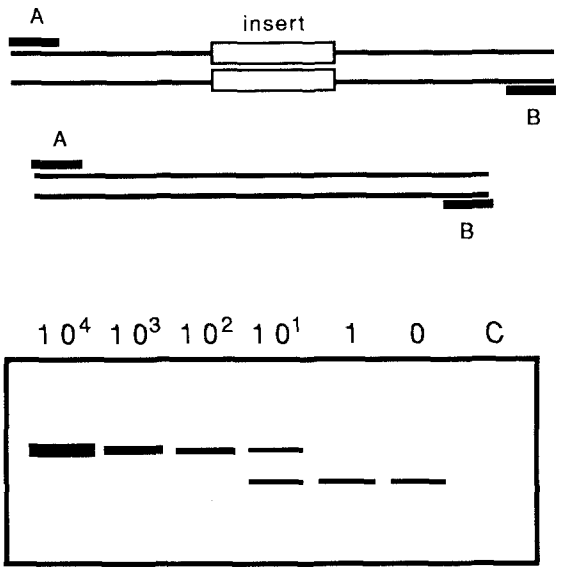

Figure 2A. Schematic illustration of quantitative PCR. Above, the template from a tissue extract which is amplified by primers A and B is shown as well as a template into which an insert has been introduced. Apart from this insert, the template is identical to the template from the tissue extract. To a constant amount of extract, a dilution series of a known amount of the insert template is given. Above the lanes, the number of added molecules is indicated. C refers to a control amplification without templates. It can be seen that approximately ten copies of the insert template allow the extract template to amplify to equivalent amounts, indicating that there are approximately ten copies of the target sequence in the extract added to the PCR.

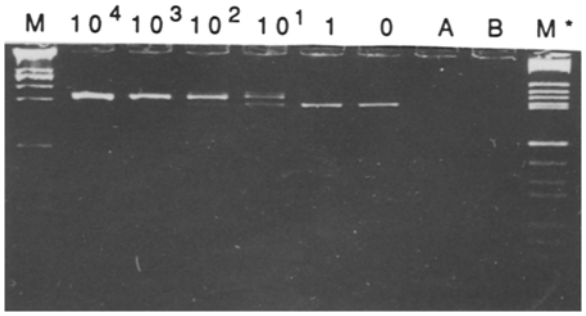

Figure 2B. A quantitation of the amount of amplifiable human mitochondrial in a 5000-year-old mummy performed as in the schematic picture above. A and B refer to PCR and extraction controls, respectively. M are molecular size markers.

Contamination by carry over from PCR products can often be detected by quantitation when the results show that one template exists in vastly greater amounts than other templates from the same genetic compartment. Also, a quantitation determining that only a few mitochondrial DNA molecules are present in an extract will save the investigator fruitless attempts to amplify single copy genes from the same extracts and alternatively alert them to the fact that 'success' with such amplifications might be suspicious.

Contamination

The study of ancient DNA became possible with the invention of the PCR. Its crucial advantage for molecular archaeology is that it both allows the amplification of minute amounts of DNA in the presence of large amounts of damaged and degraded DNA, and specific amplifications in the presence of vast amounts of irrelevant DNA from e.g. micro-organisms which are often present in the extracts. However, in this invaluable property of the PCR lies a curse that plagues the field – even a few molecules of modern DNA which make their way into extracts prepared from ancient tissues will be amplified and cause incorrect results. These contaminating DNA molecules can be present in the specimens before samples are removed or they may be introduced during DNA extraction and PCR. A typical example of contamination comes from studies of moas, giant flightless birds of New Zealand (fig. 3). Using moa-specific primers no specific PCR product is detected, whereas human-specific primers yield a band. Sequence determination confirmed the human origin of the band (not shown), suggesting a contamination with human DNA of either the sample itself or of the reagents used. This clear-cut case of contamination illustrates not only the ever-present spectre of contamination but also the serious problems encountered when working with ancient human material as opposed to animal remains. Since human DNA is the most likely source of contamination, a phylogenetic criterion can in most cases not be used to exclude contamination. In some regions of the world, however, enough knowledge about mitochondrial sequence variation in present populations has been accumulated so that it is possible to predict what sequences can be reasonably expected.

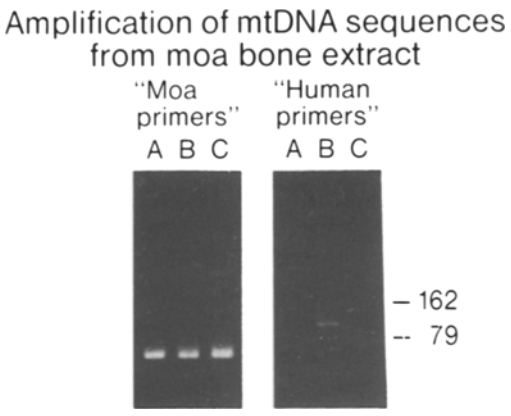

Figure 3. Agarose gels showing amplification products from moa bone extracts using moa-specific primers (left panel) and human-specific primers (right-panel), respectively. No specific products can be seen with the moa specific primers (bands are due to primer artefacts). However, the human-specific primers yield a specific band, revealing its contamination with modern human DNA. Lanes A and B represent extractions from moa bones, lane C an extraction control.

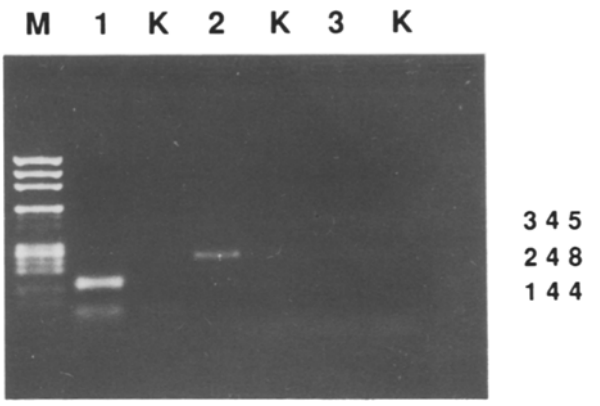

Figure 4. Agarose gel with PCR products from bone extracts of a giant ground sloth using primers for the mitochondrial 16S rRNA gene. With progressively longer fragment sizes (lanes 1, 2 and 3, respectively) a decrease of the amplification efficiency is noticeable (cf. fig. 1A). Lanes K are PCR controls, lane M is a size marker.

This, for example, is the case in the New World and Oceania.

Controls and criteria of authenticity

The extensive frustrations and set-backs which we and others have experienced in working with DNA from museum specimens and archaeological finds have stimulated us to suggest a number of conditions that should be fulfilled before a DNA sequence is regarded as potentially of ancient origin. These are:

1) Strict physical separation of the laboratory where ancient DNA is extracted and amplifications are set up from laboratories where other work is carried out.

2) Precautionary measures in the specially dedicated laboratory to curb contamination from DNA originating from the investigators or from PCR. These include specially dedicated clothing, equipment and reagents which are used exclusively in the extraction laboratory. Furthermore daily irradiation by UV light, washing of the benches and equipment with 5% sodium hypochlorite, microwave treatment of water used for solutions and separate ventilation systems should be used.

3) Appropriate controls to monitor contamination. This routinely includes two extraction controls (i.e. DNA extractions performed in parallel with the ancient extracts to which no tissue is added) and a PCR control (i.e. no extract added to the PCR).

4) At least two extractions per sample should be carried out on different occasions and preferentially from different parts of the sample. These should yield identical results and, when this is not the case it should be reported in publications.

5) The sequence should make phylogenetic sense. If one studies an extinct species, the sequences should indicate the general phylogenetic affiliation of the creature in question.

6) An inverse relationship between amplification efficiency and amplification length is expected in ancient DNA (fig. 4).

The carrier effect

Occasionally we have noted that even though multiple extraction and PCR controls were negative, a sequence turned out to be due to contamination, sometimes from other organisms studied in the laboratory. This can be explained by a carrier effect of the ancient extract. A very low number of contaminating molecules in the extraction or amplification reagents may not yield any amplification product as they may be adsorbed to the plastic ware and therefore cannot serve as template molecules in the PCR. However, when a tissue extract is added which may contain DNA from micro-organisms or other molecules such as sugars, these act as carriers by displacing the contaminating molecules from plastic surfaces and thus allow the contaminating molecules to become available for amplification. This very insidious type of contamination is difficult to detect and is a continuous source of worry for any work with extremely low copy-number sequences.

Inhibition

When nucleic acids are extracted from ancient tissues by methods using proteinase K and phenol extraction, the extracts often contain components that inhibit the *Taq.* polymerase and force the investigator to dilute the extract until it becomes amenable to PCR. This is one of the major problems in the ancient DNA field[18]. A new extraction method seems to overcome this problem in several cases. This method, which is a modification of a protocol by Boom et al.[2] to purify DNA after extrac-

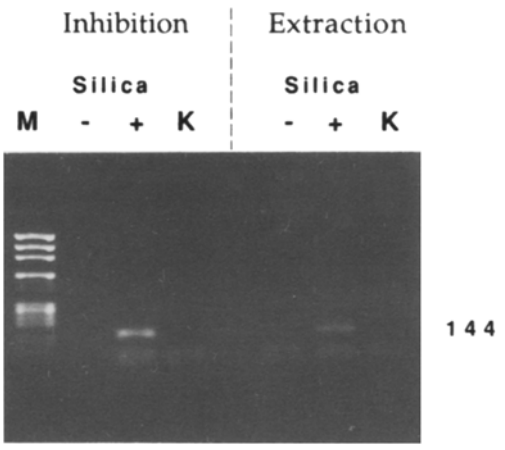

Figure 5. Comparison of a silica-based DNA extraction method to conventional methods. The agarose gel shows a comparison of amplifications from DNA extracted by conventional methods (here: Proteinase K digestion followed by Phenol:chloroform extraction and centrifugation driven dialysis using Centricon 30. Two other methods using phosphate buffer[16] and Chelex®[21] gave similar results) and a silica based extraction method[8]. The left part of the figure shows amplifications from DNA extracts from brown bear excrements[7] which are notorious for inhibition. Using the conventional methods (lane −) the PCR is inhibited as can be seen by the lack of primer dimers. Using the silica method (lane +), the amplification is successful.
The right part of the gel depicts the efficiency of the two methods in extracting amplifiable DNA from Pleistocene bone material. Though the PCR is not inhibited, the conventional method yields no specific amplification product (lane −) whereas with the silica method amplifiable DNA can be extracted from the same bone sample (lane +). Lanes labelled K are PCR controls.

tion, uses high concentrations of guanidinium thiocyanate to solubilize tissues and to cause the DNA to bind to silica particles from which the DNA can then be retrieved[8]. Apart from efficiently removing inhibitory agents from DNA extracts, this method has in several cases yielded a higher extraction efficiency than other methods, as illustrated in figure 5.

Reproducibility and credibility

Above, we have focused on the methodological horrors that plague everyone who works in this field. However, provided that we are all aware of the pitfalls and problems that may cause even the most cautious worker to regard a sequence as genuinely antique when it is in fact a novel or especially insidious form of contamination, we believe that it is possible to use ancient DNA to add to our knowledge of the history of our own species as well as of other parts of the biota. This is feasible when we not only adhere meticulously to criteria and controls to detect contamination as outlined above but also attempt to design projects in a way that maximises the possibility of verifying a result by its reproduction in the same laboratory as well as in other laboratories. This can be done, for example, when several specimens exist of an extinct species. Thus, the sequences initially published from the marsupial wolf[19] could later be confirmed and extended by others[10]. Another way to achieve reproducibility is to analyse several related species of a group[3] or several individuals of the species. The fact that such specimens do not show the same, but different and closely related sequences, will strengthen the argument that the sequences are of ancient origin. Furthermore, when a specimen is truly unique, the confidence in a result can be increased if two laboratories independently remove samples from a specimen and arrive at compatible results[6a]. Finally, it would contribute significantly to the credibility of the field if investigators would publish also the frequency of negative results and contamination encountered in the course of a study.

By these means our results may not end up being irreproducible and anecdotal in character (e.g. ref. 1) but may contribute to our understanding of the history of life.

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