NEXUS: The Canadian Student Journal of Anthropology, Vol. 11: 1-14 (1993)

Ancient DNA: a review of its past, present and future potential in archaeology

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ABSTRACT: This paper provides an extensive review of past and current research involving the biomolecular analysis of nucleic acids. In particular, it focuses on deoxyribonucleic acid (DNA) or ancient DNA that can be recovered from historic and prehistoric human skeletal remains and other archaeological material remains. It also critically assesses the problems that any investigation of ancient DNA poses and points to specific areas for future investigation.

Steven Spielberg's latest film epic *Jurassic Park*, like all good science fiction, has taken established science and pushed it a little further than reality currently allows. After seeing the film, anthropologists may very well ask why study ancient DNA? The reasons are much more compelling than providing Hollywood with a vehicle for conjuring up public misconceptions about the current state of recombinant DNA technology in Molecular Biology. Rather, the main goals of archaeological Molecular Biology are to enhance anthropological understanding of past artefact functions and of human relationships and behaviour in general. Ancient blood proteins and fragments of deoxyribonucleic acid (DNA) molecules have been successfully recovered from a variety of archaeological remains (Cattaneo et al. 1991, 1992; Hanni et al. 1990; Hagelberg and Clegg 1991; Hagelberg 1992; Hagelberg et al. 1991b; Golenberg 1991; Cano et al. 1993; Poinar et al. 1993). Amplification and sequencing of DNA from a 120-135-million-year-old weevil by Cano and colleagues (1993) represents the oldest fossil DNA, to date, ever extracted and sequenced, extending by 80 million years the age of any previously reported ancient DNA (Golenberg et al. 1990; Golenberg 1991).

This paper provides an extensive review of past and current research involving analyses of the DNA content of archaeological remains. In particular, it focuses on the techniques employed for the analysis of ancient DNA content recovered from historic and prehistoric human skeletal remains. It also includes a critical evaluation of the various problems that any investigation of ancient DNA poses and finally, it points to specific areas for future investigation.

Molecular evolution is the historic process through which genes accumulate changes due to stochastic events as well as selective processes. Until recently,

studies of these evolutionary processes were restricted to knowledge of modern structures of proteins and corresponding genes. Means of comparing one individual with another or indeed of identifying an individual as belonging to a particular group, species etc. traditionally relied on blood group antigens and HLA antigens. (Cattaneo et al. 1991; 1992). Although it had been known for a long time that proteins were manifestations of genetic variability engendered by the material contained within the cell nucleus (hence, the term 'nucleic acids'), just how this information was stored and how it was retrieved remained unclear. That is, the genes contained within DNA molecules were inaccessible to scientific investigation due to the enormous size and complexity of the molecules themselves. DNA now occupies a central position in Biology by virtue of its role as the permanent and heritable store of biological information.

This information is preserved in discrete units called 'genes' of which approximately 50,000 are needed to make up a human being (Brown and Brown 1992). Genes encode for specific 'proteins' and genetic information flows in the direction: DNA ➨ messengerRNA ➨ proteins. It is important to note at this stage that not all DNA is transcribed into proteins; at least 80% of the total DNA is not used for this purpose although it nonetheless contains a lot of useful information in what is termed as its 'junk' sequences (Coghlan 1993).

DNA molecules, themselves, are extremely long polymers; that is, they are composed of linear sequences of many smaller subunits called 'nucleotides' arranged in two strands in the form of a double helix. The nucleotides each consist of a phosphate residue in ester linkage to the 5' hydroxyl residue of a deoxyribose molecule, which in turn group to a purine or pyrimidine base. There are four bases in DNA, adenine (A), guanine (G), thymine (T) and cytosine (C). Each of the two DNA strands has a polarity, i.e. a 5' and a 3' end, and the two strands run in opposite directions, i.e. are anti-parallel. A gene, therefore, is simply a segment of a DNA molecule whose information is relayed to a protein as determined by the structure and order of the nucleotide sequence that it contains. The term 'ancient DNA' refers to the DNA molecules that may be preserved in ancient biological materials.

The advantage of diagnostic methods based on DNA analysis is that all DNA molecules behave very similarly. In simple terms, they can be considered to be long cylinders with a negative charge of phosphate residues on the outside and with their differences concealed in the middle. All DNA reactions rely on a balance of the attractive forces of the hydrogen bonds in the middle of the helix, and the repulsive forces of the negative charges on the outside. The techniques used to analyse DNA are not mystical in concept nor complicated in practice. In fact, they merely represent a particular approach to the study of disease (Bains 1989). In addition, they are also extremely precise and sensitive. The net result of this is that nucleic acid-based methods are very simple in concept.

Modern DNA can be extracted from any tissue containing nucleated cells by using a variety of techniques. The analytical procedures for investigating DNA

molecules can be divided into two main categories: indirect methods which provide information on the overall organization of a gene and/or intergenic region but not the nucleotide sequence itself, and direct methods which do produce the nucleotide sequence (Brown 1990). Indirect methods are variations of a procedure called 'hybridization' analysis whose principle involves binding a short molecule of a defined nucleotide sequence (ie. the probe) to other DNA molecules (ie. the target) that contain a complementary sequence.

DNA fragments are indirectly produced by cutting the target DNA with restriction enzymes that can then be ordered by electrophoresis in an agarose gel (Kingston 1989). The smaller fragments of DNA migrate faster down the gel than the larger ones, giving a track of DNA fragments of progressively diminishing size. The length of a particular fragment of DNA can then be determined from the distance of its migration in the gel with reference to marker fragments of known size. The DNA fragments in the gel are then denatured into single strands and transferred on to a nitrocellulose filter or nylon membrane by a technique called Southern blotting (Southern 1975). The DNA binds to the membrane, providing a stable array of DNA fragments that can be analyzed by mixing with a DNA probe in what is termed an 'hybridization reaction'. The basis of this reaction is the ability of the complementary DNA strands to bind together.

Direct nucleotide sequencing involves converting the bulk DNA extracted from a tissue into a form suitable for sequencing (Brown 1990). The method is called 'cloning', in which the starting sample is cleaved by a specific restriction enzyme and the resulting fragments introduced into bacterial cells. Each individual bacterium takes up one DNA fragment, replicates and passes it on to daughter cells when it divides, thus giving rise to what are termed 'clones'. In the case of human DNA, there can be several hundred thousand different fragments. Hybridization analysis is used in order to identify and produce the more specific and hence, better defined DNA fragment of interest.

Whereas all of the above recombinant DNA techniques have been routinely used in Molecular Biology to analyze modern genomic and mitochondrial DNA sequences, their application to the study of ancient DNA is a relatively recent occurrence. The earliest indications that molecular genetic information might still persist in ancient materials were demonstrations that peptide bonds can last up to 10^8 years in fossil shells and bones, and that subcellular detail implying the survival of ribosomes and chromatin is still evident in insects from 40 million-year-old amber (Pääbo et al. 1989). These reports spurred further attempts to extract other potentially anthropologically informative biomolecules that may be present in ancient remains, in general.

The presence of ancient DNA was first demonstrated in 1984, when DNA molecules were extracted from nucleated cells derived from the dried muscle tissue of a quagga, an extinct zebra, which had died 140 years ago and was stored in a German museum. After extracting and cloning the DNA in bacteria to increase the amount available for analysis, Higuchi and colleagues (1984) then proceeded to

compare it with DNA sequences obtained from living horses and zebras. They were able to show that the quagga was closely related to the zebra, with a relatively recent time of evolutionary divergence. Shortly, thereafter, Pääbo et al. (1985a, 1985b) reported on the successful extraction of ancient DNA from much older material: the mummy of an Egyptian child, radiocarbon dated to 2430 ± 120 BP. Doran and colleagues (1986) also obtained remnant human DNA from the preserved brain tissue of an individual exhumed from the Windover archaeological site in Florida.

Although these initial reports caused great excitement and publicity including the rather dubious headline 'US scientists clone dinosaurs to fight on after nuclear war' (Hagelberg 1990), negative ancient DNA results were also reported in similarly preserved materials and using the same molecular techniques (Hughes and Connelly 1986). Scientific scepticism arose concerning the plausible use of ancient DNA within archaeology because most of the DNA recovered at the time was apparently derived from bacteria and moulds growing within the ancient tissues (Doran et al. 1986). Also, the tiny quantities of original DNA were heavily degraded and probably modified by the effects of time and environmental conditions, all of which were circumstances that normally interfered with the specificity of the DNA techniques used (Brown 1990). In short, the major criticisms cited by most scientists against the value of ancient DNA research focused on what now have become general conclusions drawn about the unique chemical and physical nature of ancient DNA versus those typically characteristic of modern DNA.

First, unlike its modern counterpart, it appears that ancient DNA rarely makes up more than 5 percent of the total DNA extracted (Hagelberg et al. 1989; Brown and Brown 1992). Hence, when recovered, it can never be expected to be pure. Microbial contamination can occur from a variety of sources including fungi, algae and bacteria whilst in the ground and sometimes out of the ground (Pääbo et al. 1989; Hagelberg et al. 1991b). The presence of such contamination not only complicated previous attempts to quantify the amount of ancient DNA recovered, it also rendered its visualization after size-fractionation unuseful since most of the DNA recovered was of modern and foreign origin. Second, it is now well known that ancient DNA undergoes fragmentation during preservation (Pääbo 1989; Lindahl 1993). This fragmentation occurs relatively soon after death and also involves chemical damage in the form of structural base-pair (bp) changes to the individual nucleotides recovered. For example, DNA recovered from dessicated soft tissues is damaged by oxidation, especially the thymine residues. This damage is comparable to that caused by radiation. DNA from wet remains, on the other hand, suffers from depurination due to the acid pH of the environment. Both result in degradation or breakdown of the DNA into small pieces. As a consequence, ancient DNA molecules tend to be relatively short compared to their modern counterparts, rarely more than 100-200 base-pairs in length, with a few molecules recovered from dried skins as long as 500 base-pairs (Pääbo 1989). These figures also may not be

typical as molecules of 375 base-pairs are frequently found in bone (Hagelberg and Clegg 1991) and substantially higher 1000 base-pair in length molecules have been reported for carbonized plant remains (Brown and Brown 1992). Even these are present in very low yields and hence, 'artefactual' DNA as opposed to the 'real' ancient DNA sequences can possibly be visualized making unequivocal results difficult or impossible to obtain. Undoubtedly, the cellular disruption that occurs soon after death which causes breakages and chemical degradation of ancient DNA is an important area that still requires further study.

Recently, many of the analytical difficulties presented by the unique physical and chemical characteristics of ancient DNA have been somewhat obviated. A new molecular procedure for amplifying DNA sequences, the Polymerase Chain Reaction (PCR), was introduced in 1986 and first applied to archaeological material in 1988. PCR is an *in vitro* method for the enzymatic synthesis of specific DNA sequences, using two oligonucleotide primers that hybridise to opposite strands and flank the region of interest in the target DNA (Mullis et al. 1986; Saiki et al. 1986). A repetitive series of cycles involving template denaturation, primer annealing and the extension of the annealed primers by DNA polymerase results in an exponential accumulation of a specific DNA fragment. Because the primer extension products synthesized in one cycle can serve as the template in the next, the number of target DNA copies approximately doubles at every cycle (i.e. 20 cycles of PCR yields a million-fold amplification).

PCR depends on knowing the DNA sequence on either side of the target. It is also able to work with small amounts of starting DNA, theoretically, just a single molecule (Mullis et al. 1986). In short, it should be capable of amplifying the small fragmented stretches of ancient DNA typically hidden within archaeological remains. Exploiting the ability of PCR to 'jump' between fragments, Pääbo and colleagues (1988) recovered longer sequences of DNA from ancient material. Their tests of PCR's precision and efficiency suggested that PCR also appeared to function efficiently even if the ancient DNA had undergone chemical modification. Since PCR does not seem to be as inhibited by chemical damage and requires very small amounts of DNA that need not be very pure, it has now become the preferential choice for obtaining DNA sequences from ancient materials, in general.

Initially, PCR amplification of ancient DNA molecules involved their extraction from exceptionally well preserved specimens such as mummies and bog bodies (Brown and Brown 1992). The first successful use of PCR amplification of DNA sequences in archaeology — the 7,000 year old brain from the Windover site in Florida (Pääbo et al. 1988) — has since been corroborated with other studies on similar tissues (Pääbo 1989; Thueson and Engberg 1990; Lawlor et al. 1991). These reports have been quickly followed up by other studies of a variety of zoological, archaeological and palaeontological material (Thomas et al. 1989; Ellegren 1991; Hagelberg and Clegg 1991; Hagelberg et al. 1989, 1991b; Hanni et al. 1990; Hummel and Herrmann 1991; Thuesen and Engberg 1990; Rollo et al. 1988; Niklas 1990; Golenberg et al. 1990; Golenberg 1991; Cano et al. 1993; Poinar et al. 1993).

In particular, the initial demonstration of the presence of ancient DNA in archaeological human bones (Hagelberg and Clegg 1991; Hagelberg et al. 1989, 1991b; Hanni et al. 1990; Horai et al. 1989, 1990; Hummel and Herrmann 1991; Hummel et al 1992), the oldest dated to 5450 BP (Hagelberg et al. 1989), and the subsequent demonstration of ancient DNA in human bones dated to 4500-5000 BP (Hanni et al. 1990) can be considered a major breakthrough since the preservation of hard tissues over soft tissues in archaeological contexts is a much more frequent archaeological event. Plant, animal and insect remains form the final type of archaeological material from which ancient DNA has been extracted and successfully amplified by PCR (Rollo et al. 1988; Brown and Brown 1992; Golenberg et al. 1990; Golenberg 1991; Poinar et al. 1993; Cano et al. 1993).

These studies all suggest that much older material will also eventually yield DNA sequences (Brown and Brown 1992). Nevertheless, the very sensitivity and robust technical power that makes PCR so advantageous to the study of ancient DNA in archaeology also threatens to render it ineffective, particularly if used haphazardly, ie. without tight controls over experimental designs. The technique is sensitive to contamination from extraneous DNA sources (Erlich 1989; Cherfas 1990, 1991). Even under the most sterile conditions, some contamination is always possible when using PCR technology in laboratories that routinely handle modern DNA samples (Anderson 1989; Annas 1992). Careful laboratory procedure and prealiquoting reagents, the use of specialized pipets, and the physical separation of the reaction preparations from the analysis of the amplification reaction products are used to minimise the risks of contamination within the laboratory (Kwok and Higuchi 1989; Sarkar and Sommer 1990).

Brown and Brown (1992:19-20), however, have identified four major stages of potential contamination with extraneous human DNA which could possibly occur in archaeological and forensic contexts, the former two of which still plague ancient DNA analyses. These include the handling of individuals between death and inhumation; between inhumation and exhumation; during excavation; and finally, post-excavation, ie. during analysis in the laboratory. The possibility of extraneous prehistoric human DNA contamination is more than simply a technical issue and has broad implications for archaeology and the study of ancient DNA.

As noted above, the potential for modern laboratory human DNA contamination can be consistently monitored using good experimental design, appropriate controls and repetitive analyses of samples. Little is known to date about the potential for the movement of DNA within and between burial matrices during inhumation. The presence of microbial versus human DNA can be monitored relatively successfully using specific human versus non-human primers as controls (Hagelberg and Clegg 1991). Successful attempts to minimize risks of contamination in ancient human bone studies has also involved utilizing various mechanical extraction methods whereby the surface bone is scraped and the sample is taken from the underlying surfaces, presumably less affected by both diagenetic and post-excavation processes (Hagelberg et al. 1991b). Selecting what appears to be more 'visually' sound bone

samples for DNA analysis has also been shown to be a reasonable means for avoiding these problems, although a recent study indicates that there may be no correlation at all between morphologically sound samples and their DNA content, (Jeffreys et al. 1993). Hence, this is another area where future study is definitely needed in order to enhance our knowledge about the affects of diagenetic and/or post-mortem processes on DNA samples derived from archaeological contexts.

If prehistoric human remains have been handled by other individuals prior to their burial, contamination with their extraneous human DNA is a very real possibility. Although there would appear to be 'no' solution to this specific problem within archaeological contexts (Brown and Brown 1992), the situation could possibly be obviated by the actual choice of tissue selected for ancient human DNA studies. On the one hand, the majority of the prehistoric human DNA studies have concentrated on the use of bone and/or soft tissue as the medium of analysis. On the other hand, it is well known that dental material is the hardest and most resistant tissue in the human skeleton, even when found in burial contexts. The exterior enamel matrix of a tooth is particularly resistant to environmental degradation (Cole and Eastoe 1988). It surrounds a soft pulp interior of potentially rich genomic and mitochondrial DNA cells that are stabilized chemically and structurally by hydroxyapatite which forms a significant component of the tooth. The relative isolation of the circum-dental pulpal cavity should, in theory, protect the DNA from environmental assault. In practice, the initial demonstration of ancient DNA extracted from a single prehistoric dog canine (Hanni et al. 1990) and its presence in several modern forensic human teeth (Ginther et al. 1992) suggests that utilizing this region for the analysis of human DNA samples derived from archaeological contexts, as opposed to samples taken from bones or soft tissues, should minimize the risks of extraneous human DNA contamination from both the handling of individuals shortly after their death and prior to their inhumation, excavation procedures and post-excavation activities such as museum curation and/or osteological investigations.

Given the pitfalls and limitations posed by any study of ancient DNA as described above, what can archaeologists learn from the analysis of ancient DNA molecules? To begin with, the research to date demonstrates that the investigation of variable sequences of ancient DNA has the potential to provide insights into the origins of past human population migrations and/or diffusions (Brown and Brown 1992). Several recent studies have been published addressing archaeological questions concerning the past peopling of several parts of the world using data derived from DNA analyses (Hagelberg and Clegg 1993; Gibbons 1993). At present, this work is held up by one major problem. As noted above, PCR depends on knowing the sequences that flank the target DNA of interest. To be used within archaeological contexts, it requires comprehensive comparative DNA sequencing studies of modern populations. Preliminary research in this area has been carried out for various regions of the world (Di Rienzo and Wilson 1991; Horai et al. 1991; Ward et al. 1991; Vigilant et al 1989; Allen et al. 1993). Nevertheless, Foo and

colleagues' (1992) preliminary efforts to specifically devise PCR libraries of ancient DNA without prior knowledge of the sequences using single oligonucleotide primers may prove fruitful in resolving this concern. Population movements within continents or islands are of equal interest to archaeologists and social geographers alike (McKie 1993). Ancient DNA analysis, in conjunction with traditional blood group analysis, could be used to enhance our understanding of the past mobility behaviour of specific regional groups.

Assessing biological affinity has been a long term aim in osteology. In modern forensic contexts, PCR has been used to amplify highly variable 'microsatellite' regions that exist in human mitochondrial DNA in order to identify specific individuals (Brown 1991). This area of focus is commonly termed genetic 'fingerprinting'. The potential of genetic 'fingerprinting' DNA analysis has been demonstrated in recent historical archaeological contexts by Hagelberg et al. (1991a) and Ginther et al. (1992), whose analyses of human bone and teeth DNA both confirmed the personal identities of murder victims by comparing these microsatellite mtDNA sequences with living relatives of the individuals in question. Corroborative DNA 'fingerprinting' studies for the purpose of identification of specific individuals have recently appeared, including identification of Vietnam war victims (Holland et al. 1993; Fisher et al. 1993) and other major figures of historical import such as Joseph Mengele (Jeffreys et al. 1993). The potential still exists for unravelling family relationships both within and between archaeological populations based on the DNA content of human skeletal remains.

The study of ancient DNA using PCR technology can also be used in order to deduce some biological traits such as sex. Conventional osteological methods of human skeletal sex determination include morphological features of the bony pelvis and skull, dimensions of post-cranial joint surfaces, and various discriminant functions derived from measurements taken on the skull, pelvis and post-cranial skeleton (Krogman and İşcan 1986). Typical accuracies for sex determination of well preserved adult human skeletal remains are 95-100% using the whole skeleton, 90-98% using the pelvis alone, and 80-90% using the cranium or postcranial skeleton. Archaeological human skeletal remains are often fragmentary and incomplete, limiting the effectiveness of traditional osteological sex determination techniques. Furthermore, the conventional methods are difficult to apply to cremated bone and to subadult remains.

As an alternative to morphological sex determination, the prospect of determining biological sex by chemical analysis has been a long term prospect in human skeletal biology. Although considerable attention has been focused on chemical methods of sex determination of archaeological skeletal remains (Lengyel 1968; Dennison 1979; Beattie 1982; Duffy 1989; Gibbs 1991; Teshima 1992), it has only recently become possible to determine sex based on the molecular content of teeth and bones utilizing Y-chromosome-specific PCR amplification.

PCR amplification of Y-chromosome-specific DNA sequences has been eminently suitable for sex-typing in modern clinical and forensic contexts, as primers directed

at sequences specific to the Y-chromosome will produce amplified DNA only from males (Handyside et al. 1990; Kogan et al. *1987*; Bobrow et al. 1971; Cooke 1976; Nakahori et al. 1986). With ancient DNA this has proven less straightforward (Sykes 1991; Brown and Brown 1992) but not wholly unsuccessful (Hummel and Herrmann 1991; Hummel and Herrmann et al. 1992). The challenge in this area of ancient DNA research is that, although a failure to find a Y-specific sequence would most likely mean that the skeleton tested is a female, it could also mean that the relevant bit of ancient DNA was not amplified for technical reasons (Sykes 1991; Cherfas 1991). This problem can be alleviated by altering Hummel and Herrmann's (1991) initial experimental design. Utilizing XY-specific primers such as those delineated from the amelogenin gene (Nakahori et al. 1992) along with Y-specific primers during amplification procedures (which should result in positive PCRs for both males and females), should also provide a positive control and thus demonstrate that the ancient DNA has been successfully extracted and amplified. Also, inclusion of the X-and Y-chromosome specific primer sets as modified by Gnaesslen and colleagues (1992) should indicate a female specimen. Research in this direction is currently being pursued by the author. If the results prove to be accurate, they will be very useful in the analysis of prehistoric remains from around the world and in addressing historical and archaeological evidence which may relate to gender-specific behaviourial practices. These include testing theories of infanticide, determining the sex of cremated human skeletal remains, and corroborating gender-specific mortuary practices suggested by differential treatment of grave goods etc.

Finally, the study of ancient DNA could also be used to reveal the presence of genetically linked diseases within and between prehistoric populations. The potential feasibility of this type of research has initially been demonstrated by two independent preliminary studies involving amplifications of the cystic fibrosis gene from ancient human DNA (Sykes 1991). Lawlor and colleagues (1991) also recently successfully amplified, cloned and sequenced a segment of the major histocompatibility complex (MHC) from the ancient brain tissue of an individual found at the aforementioned Windover archaeological site, in Florida. Many other erosive arthropathies, including rheumatoid arthritis, are known to be associated with the glycoproteins of the MHC gene complex (Ortner et al. 1992). The ability to recover genes of specific genetically related diseases, such as cystic fibrosis, from the DNA content of archaeological human remains opens up the possibility of assessing susceptibility or resistance of ancient populations to infectious agents. The concrete contribution that ancient DNA can immediately provide is the ability to test independently whether putative morphological manifestations of a genetically linked disease can be confirmed at the molecular level. In addition, affected individuals without skeletal manifestations of a particular genetically transmitted disease could also be determined.

To conclude, it is clear from this review of ancient human DNA studies that research, thus far, has only scratched the surface of what promises to be a major

new area of biomolecular Archaeology within Anthropology. To date, the situation is somewhat analogous to that of a child learning to crawl before he/she learns how to walk. In short, a lot of tedious and seemingly unrewarding work still needs to be done, particularly in the area of understanding the unique nature and physical characteristics of ancient DNA, itself. The major bugbear with ancient DNA analysis is extraneous DNA contamination. The implications are clear: Archaeologists must exercise extreme care when excavating specimens destined for DNA analysis. Despite the pitfalls, the study of ancient DNA promises to be a new independent means for enhancing archaeologists' knowledge and understanding of the evolutionary behaviour of past human populations. 'You may have seen the film or read the book, now it is not quite *Jurassic Park* — but it is fast becoming an anthropological reality' (Redford 1993). Archaeology and Molecular Biology may be two very different disciplines and few people as yet have the confidence to call themselves biomolecular archaeologists. Setting up the collaborations that will link the disciplines of Molecular Biology and Anthropology, once and for all, is a major challenge for the future.

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