Current trends in 'ancient DNA studies' – A review

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It is just over a decade since the first published report on 'ancient DNA'. Today it is becoming increasingly clear that this area has enormous potential, and is fast developing to be recognized as an important research area with implications to a number of diverse fields of modern science. It is therefore pertinent to review the current status of research and the future vistas in this area.

RECENT advances in molecular biology have facilitated the understanding of man and his environment at the genetic level. Genetic studies at the DNA sequence level has – for quite sometime now – been possible on contemporary life forms. Molecular methods and approaches have helped unravel the evolutionary relationships among the different organisms. The study of 'ancient DNA' has attracted the attention of scientists in the recent past. Initially studies were mostly confined to soft tissue specimens. But, with the advent of the polymerase chain reaction (PCR) technique, there has been a revolution in the bio-molecular study of DNA in ancient specimens[1–14], popularly called 'ancient DNA studies'. Successful studies of mitochondrial DNA (mtDNA)[12–19] and even nuclear DNA[9,20–24] from ancient specimens are beginning to be reported.

A number of inherent advantages make mtDNA a preferred tool in 'ancient DNA studies'. (i) mtDNA accumulates mutations at a much higher rate and provides adequate calibration for reading even recent population history. (ii) The mitochondrial genome comes only from the mother. Because of this maternal mode of inheritance, there is no recombination of maternal and paternal genes, which sometimes blurs the history of the genome as read by geneticists. Potentially therefore, mtDNA offers a powerful way of inferring population history, unhindered by the genetic fog of recombination. (iii) High copy number of mtDNA is perhaps the most important with respect to analysis of ancient specimens.

For more than a decade biologists have been using the data from mtDNA to uncover population histories of many species. mtDNA analysis[25] relating to the origin of modern humans has led to the mitochondrial eve theory, which claims that modern humans can be traced back to an ancestral population that lived in Africa relatively recently, close to 150,000 years ago. Furthermore, the theory suggests that descendants of this ancestral population migrated into the rest of the Old World, completely replacing the then existing population of archaic humans. With the generation of additional data from other laboratories, the Mitochondrial Eve hypothesis seems to be strengthening, though the opinion is not always uniform[26,27]. Ancient mtDNA studies have basically been an extension of studies being done on contemporary populations. Thanks to the advancement in bio-molecular sciences[28,29], vital information regarding human origins and population history are now being unravelled[9,30].

The area of 'ancient DNA studies' holds enormous potential in a number of diverse fields. Today the interest in 'ancient DNA' has grown considerably ever since Higuchi *et al.*[31] reported the recovery of mtDNA from dried muscle of the quagga, an extinct member of the horse family. It is therefore timely to survey the organismal range of preserved DNA currently being analysed, and the spectrum of questions being answered.

DNA and its survival

Bio-molecules have varying degrees of stability over time. Soon after cell death, these molecules begin rapid degradation. Nucleic acids have limited life expectancies under physiological conditions, and DNA particularly is a chemically unstable molecule that decays spontaneously, mainly through hydrolysis and oxidation. Hydrolysis causes deamination of the nucleotide bases and cleavage of the base-sugar bonds, creating baseless sites. Deamination and depurination are the two main types of hydrolytic damage[32]. Baseless sites weaken the DNA strand, causing strand breaks that fragment the DNA gradually into smaller pieces. Oxidative damage is basically by the modification of the nucleotide bases leading eventually to the destruction of the ring structure of the base and sugar residues in the DNA molecule. DNA degradation is also caused by nonenzymatic methylation and by other enzymatic pathways.

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Due to the presence of DNA repair mechanisms in the living organisms, DNA undergoes constant repair to counteract any damage it incurs. On death, however, spontaneous degradation of the DNA molecule occurs, even in fully protected environments[33]. It therefore appears that the chances of DNA survival in 'million-year-old' specimens is very little unless special conditions for preservation exist (see Table 1). Theoretical calculations have suggested that DNA may not survive for more than 10,000–100,000 years[32,34], and even if it does it is expected to be highly fragmented and chemically modified[2,35].

Ancient DNA studies

Ancient DNA has been discovered in a variety of preserved biological material. This includes bones, mummies, museum skins, insects in amber, and plant fossils. 'Ancient DNA studies' is today an important research tool in disciplines as diverse as archaeology, conservation biology and forensic sciences. Reports of the application of this technology to fields such as archaeology[36–39], palaeontology[8,10,40–42], forensic sciences[22,43,44], conservation and population biology[45,46], botany[38,42,47], zoology[6,13,31,48–51], genetics and human evolution[12,52,53] are being published.

During the pre-PCR era scientists attempting 'ancient DNA studies' almost relied on well-preserved specimens. Much of the status that the field has attained has been after the invention of PCR[28,29], and most of the success has been from phylogenetic studies of extinct animals[6,13,50,54].

The spectrum of questions being addressed

'Ancient DNA studies' are being pursued by various groups to answer questions pertaining to their respective fields of interest.

In archaeology, ancient DNA has been contributing both to the interpretation of individual sites and to the development of hypotheses about past populations. Site interpretation is aided by DNA-based sex typing of fragmentary human bones, and by the use of genetic techniques to assess the degree of kinship between the remains of different individuals[55].

Analysis of ancient human remains has provided information about sex ratios[56], tribal relationships, and the colonization of the New World[57–59]. Ancient human DNA studies can provide a temporal perspective to important issues in human history, such as changes in population structure and movement patterns, language evolution and the origins of infectious disease[53,56,57,60–62]. However, the difficulty associated with extracting and authenticating ancient human DNA presents unique research problems, and limits the scope of such studies. Modern human DNA can contaminate experiments at many stages and phylogenetic criteria are often not appropriate test of authenticity[63–65]. In this context, the path-breaking study of Neanderthal origins by the sequencing of a segment of mtDNA d-loop[12] has been acclaimed as a mighty step forward and has set new standards of experimental rigour, especially for studies on ancient human DNA.

Ancient human DNA studies are being pursued by a number of groups against all odds of maintaining strict contamination controls. Study of the human skeletal remains – in general – has been for a long time now providing us considerable information on the historic, protohistoric and even prehistoric human populations. Ancient DNA from such skeletal materials is just beginning to unravel information of greater precision and detail. The present focus of such studies is to track the origin of populations by tracing the chronology of appearance/disappearance of mutations, and by phylogenetic analyses. The amplifying cloning and sequencing of the Tyrolean Ice Man DNA, from a 5000-year-old mummified specimen found in the Alps in the year 1991, was done by Handt *et al.*[66]. Comparison of the mtDNA sequence from the Ice Man with those of some contemporary populations, including populations from Northern Europe, the alpine region and Oetztal (the valley where the body was found), demonstrated that the Ice Man sequence corresponds to mtDNA types existing in central and northern European populations. The report by Hagelberg and Clegg[17] on the study of a fragment of human fibula from pre-1778 Polynesia, showed that DNA extracted and amplified from this specimen, exhibited the typical deletion of one copy of a 9-bp repeat in the region V of mtDNA, present in modern Polynesians. In another study Hagelberg *et al.*[37] showed that, mtDNA of ancient Easter Islanders shared many polymorphisms with today's Polynesian populations, confirming theories about early settlers of this island. By a phylogenetic analysis, Horai *et al.*[67] found the affiliation of the ancient and contemporary populations at Hokkaido Island, Japan. According to this study, *Jomon* and *Ainu* people are at the origin of most of the modern Japanese. Oota *et al.*[59] have analysed mtDNA from 2000-year-old human skeletal remains from Kyushu and found that genetic diversity in the ancient populations were comparable to contemporary Japanese and Ainu populations. In a similar attempt, Hanni *et al.*[68] found that the commonly encountered reference sequence[69] in France was already present 6,000 years ago, and the cytosine at positions 16249 and at 16362 were already present in Spain 5,000 years ago. Hauswirth *et al.*[70] studied the mtDNA from 14 individuals from a burial (dated 6980–8120 ± 100 years BP) in Florida. This study showed that all individuals from the burial site were

related, but some individuals lacked the Native American characteristics, raising interesting questions regarding their origins.

Ancient DNA is beginning to reveal the origin and history of genetic and infectious diseases. DNA has been extracted from archaeological remains of a child's skull exhibiting cranial porotic hyperostosis, a bone lesion that may be attributed to thalassaemia or malnutrition. Analysis of the β-globin gene mutation showed that the subject was suffering from β-thalassaemia[71]. An analysis of the 1000-year-old mummified remains of a pre-Columbian American woman has provided evidence of the early presence of human tuberculosis in the New World[39].

Plant and animal remains in archaeological sites can also be analysed with species-specific primers[64,72]. DNA from early cattle remains in Britain has shown that modern cattle are derived from a diverse ancestral population with a recent expansion event in Europe[73]. Similarly, a recent Holocene population expansion has been suggested after the analysis of Bronze Age rabbits[74].

Ancient DNA in preserved plant remains can provide information on the development of agriculture[55]. DNA has been analysed and sequences obtained from Iron Age wheat specimens from England[75,76] and 4700-year-old maize specimens from Peru[38].

A number of interesting investigations have been carried out and exciting results obtained in the field of systematics and conservation biology. The analysis of the 18S rRNA gene from the tissue remains of the extinct quagga has revealed its phylogenetic affiliation[31]. A study using the ancient 12S rRNA gene and 16S rRNA gene sequences retrieved from a 13,000-year-old extinct ground sloth, *Mylodon darwinii*, has helped resolve its phylogeny[50]. Analysis of the mtDNA in the subfossil bones preserved in lava tubes has helped in identifying and studying the endangered ducks in the Laysan Island[71]. Microsatellite analysis of wolf-like canines has shown that the red wolf had extensively hybridized with grey wolves and coyotes, well before its extinction[77].

Studies on the loss of genetic variation have been done in recently bottlenecked populations using museum specimens obtained before the bottleneck. Analysis of the DNA obtained from skin and feather samples of the San Clemete Island Shrike[78] has revealed marked reductions in the genetic variability in the modern populations. Analyses done by Shankaranarayanan *et al.*[79] on 50 to 125-year-old skin samples of Indian tigers have revealed vital information about the heterozygosity levels in the species, which existed at that time. This information has led to the conclusion that the extent of genetic variation in Indian tigers was approximately the same 150 years ago as it is today. Reduced genetic variation found today in this species therefore could not be due to recent inbreeding. It may be the inherent characteristic of the species. Ancient DNA has not only helped track endangered species using their dung[80] but also has helped knowing the dietary habits of those species[13]. Tracking violations of wildlife protection treaties, such as the sale of meat from endangered whale species in the markets of Japan and Korea, have also been demonstrated[81].

Published reports of ancient DNA from million-year plus samples are very infrequent now after theoretical experiments have drawn limits to the stability of DNA[32]. The other reason why scepticism has been expressed about ancient DNA, beyond 100,000 years old[33,62,82–85], is because none of the results have been replicated and verified independently[33,86]. Amber seems to be an exception where million-year-old ancient DNA can be expected preserved according to the evidence from a comprehensive study done by Poinar *et al.*[82]. This study observes that the extent of amino acid racemization of aspartic acid (Asp), alanine (Ala) and leucine (Leu) provides a criterion for assessing the presence of endogenous DNA in ancient samples. According to this study, samples in which *d/l* (optical characteristic) ratio of Asp exceeds 0.08, ancient DNA sequences could not be retrieved. Reports on ancient DNA purportedly millions of years old from palaeontological finds were examined in this study, and scepticism expressed about the validity of such claims. The only exceptions – the study observes – to the presence of ancient DNA, is in some representative insects in amber and copal.

Amino acid racemization analysis, as an indicator of the extent of DNA degradation in ancient specimens is gaining wide acceptance ever since the first study by Poinar *et al.*[82]. The spectacular study of Neanderthal DNA by Krings *et al.*[12] has strengthened the argument in favour of the use of amino acid racemization analyses, preceding any 'ancient DNA studies'. In this regard, it is pertinent to take a close look at the physico-chemical aspects of the link between amino acid racemization and ancient DNA degradation. Studies have shown that, DNA depurination, the one major hydrolytic reaction responsible for spontaneous degradation of nucleic acids[87,88] is affected by some of the same factors that affect racemization[82]. The activation energy, and the rate constants of Asp racemization and DNA depurination are similar (at neutral pH) over a wide temperature range[87–89]. There is a tremendous potential that biogeochemical studies of this kind have to elevate the status of ancient DNA to new heights, although they might not attract that much media attention as the spectacular molecular genetic studies. A conscious effort is needed to encourage interdisciplinary studies between diverse fields such as geology, biology, archaeology, and chemistry to tap the potential of ancient DNA in full.

In India there are no published reports yet of 'ancient DNA studies' except for the study done by Shankaranarayanan *et al.*[79]. mtDNA analysis of contemporary human populations to understand population history and relationships is in its infancy in India[90-93]. In general, a comprehensive database of contemporary sequences is an essential prerequisite to any study based on ancient DNA sequences. Ancient DNA as a tool to understand aspects of human populations has never been pursued. In this regard, a collaborative effort is on, involving the Centre for Cellular and Molecular Biology (CCMB), the new Centre for DNA Fingerprinting and Diagnostics (CDFD) at Hyderabad, Deccan College, Pune, and Jawaharlal Nehru University, New Delhi, to use 'ancient DNA' in the study of human populations. The study aims to understand the genetic relationships between the ancient Mesolithic and Chalcolithic human populations, and their contemporary relatives.

Some of the key publications that give a rough overview of the advances in this field of research are tabulated (Table 1, Appendix).

Technical advances, pitfalls in the area of 'ancient DNA'

It can be stated without much doubt now that the area of 'ancient DNA' would not have grown to the state that it has now, but for the PCR technique. Ancient DNA studies heavily rely on PCR-based techniques. PCR amplification enables us to amplify a specific DNA fragment from a few intact DNA molecules in the presence of an excess of damaged molecules and other nontarget DNA. However, the retrieval of authentic and unambiguous ancient DNA sequences, using PCR, can be problematic[63,65].

The obstacles to any ancient DNA analysis lie in the sensitivity of PCR as much as the key to any such analysis. Highly sensitive experiments could be designed as in the case of the amplification of Neanderthal DNA using Neanderthal-specific primers[12]. On the contrary, minute amounts of contaminating DNA may be preferentially amplified, especially when the ancient extract contains few or no endogenous DNA molecules, and contaminating DNA could out-compete endogenous DNA during PCR because it is usually of more recent origin and therefore, less damaged[33,63]. When amplifications are targeted at DNA fragments that are longer than any template molecules present in the ancient extract, chimeric DNA sequences may be produced via 'jumping' PCR[94,95]. These chimeric DNA sequences could be derived from endogenous ancient template, contaminating DNA, or a combination of both, and can confuse and mislead any investigator[55,63].

The problem of contamination is one of the biggest in any work with ancient DNA, and this can occur at many stages[33]. Stringent laboratory conditions and quality controls during experiments are therefore advocated[63] during any ancient DNA analysis.

Reproducibility of the results is one of the basic requirements of any field to be considered as a 'scientific discipline'. Doubts have been cast on the validity of reports, which have published results that could not be repeated. For example, studies reported on 17–20 million-year-old Miocene plant fossils, such as *Magnolia* leaves[8] and *Taxodium* specimens[40], could not be replicated[86,96]. Also reports on the recovery of ancient DNA from 120 to 135 million-year-old weevil[41], and 30 million-year-old termite trapped in amber[10] are now considered with caution[32,33,97]. There seem to be some genuine constraints and reasons why reproducibility represents a problem in 'ancient DNA studies'. For example, the ancient specimens used for the DNA analysis could themselves be unique, and thus an experiment can often not be easily repeated. Yet it is now becoming quite obvious that the results should be verifiable if any study in this area is to pass the basic test to be recognized as a 'scientific report'.

This young area of 'ancient DNA' has to overcome at least some of the above-mentioned problems before it can be fully regarded as a respectable part of modern science.

Authenticating 'ancient DNA'

Establishing the authenticity of an ancient sequence obtained is done by rigorous genetic observations[64,98]. In case of across-the-species studies, species-specificity is followed as a good genetic test of authenticity[6,38,48,50,71,99]. In case of within species studies, a large number of modern sequences all over the world of the same species are needed to verify their authenticity. This, for example, is the case in humans. In humans, sequences should match the human phylogeny in the particular geographic region from where the samples come from, or they should be explained by any valid theory of human migrations[17,66,68,100]. Today the database of human sequences is fast aiding the development of a powerful molecular genetic tool to trace human population migrations[30,101-104]. Such tools are already proving useful in knowing the authenticity of reports. For example, the report on the sequence of dinosaur DNA[105] was later understood by phylogenetic tests as most likely to be of human origin[98,106].

Optical characteristics (*d/l* ratio) of the amino acids[82], the extent of preservation of collagen[100], and the presence of hydantoin residues[35] have been correlated to the presence of retrievable ancient DNA. These tests could serve as useful indicators and could presage long and laborious procedures to the sequencing and analysis of the region of interest. More importantly, such indicators could avoid indiscriminate crushing up of precious

ancient specimens. If perfected, these indicators could serve as useful tools to verify the authenticity of claims in the area of ancient DNA.

Conclusion

The area of 'ancient DNA' is fast evolving from a research tool to a scientific discipline by itself, thanks to the technical advances specially PCR and path-breaking papers in this area. But there is almost a limit being fixed on the time scales that can be dealt, due to the limitations in the technical know-how as on date. 'It would require another technical breakthrough of the order of PCR to go back any further in time', according to S. Paabo[107]. The focus of most of the recent studies has been the ancient DNA from specimens, which are at-the-best tens of thousand years old.

Permafrost or cave deposits and amber could be some of the only alternative places where scientists could cross the time limits set by theoretical studies, and look for ancient DNA that is millions of years old. Permafrost and amber are compared to genetic museums and zoos[62,108], and ancient DNA from specimens present in them can help realize the potential of ancient DNA in full.

Ancient DNA that meets the authentication criteria could provide valuable data that is hitherto unavailable through any source or approach. A comprehensive understanding of aspects, such as human and faunal population migration, etc. look quite possible today. The other exciting avenues that could be explored are verifying theories and hypotheses pertaining to the effect of climate change on aspects such as genetic diversity, population migrations, etc.

Ability to study ancient DNA is already empowering scientists to explore new avenues and areas. Scientists have always wondered if amino acid and DNA sequences could be used for geochronological purposes[109,110]. The chronometric properties of molecular sequences, if standardized, could be exploited for geochronological purposes. Use of properties of bio-molecules present in ancient samples for geochronological purposes is not new. Amino acids are being used for quite sometime now for geochronological purposes. 'Amino acid geochronology' is a well-established tool today. A vast array of utilities is about to unfurl, and it is premature therefore to enlist even the broad range of subject areas that could possibly impact from 'ancient DNA'.

Appendix

Table 1.

Organism/Specimen	Type of material and preservation conditions	References in an ascending order of the year of publication	Reports of DNA within (✓) and beyond (✗) Theoretical limits (100,000 years) to stability
Quagga (horse family)	~	Higuchi *et al.*[31]	✓
2,400 year old Egyptian mummy	~	Paabo[1]	✓
Brain from bog at Florida	~	Doran *et al.*[3]	✓
Marsupial wolf	~	Thomas *et al.*[6]	✓
Human bone	β	Hagelberg *et al.*[18]	✓
Kangaroo rat	~	Thomas *et al.*[7]	✓
Miocene leaf fossils	Ξ	Golenberg *et al.*[8]	✗
Maize seeds	θ	Rollo *et al.*[47]	✓
Red wolf	~	Wayne and Jenks[46]	✓
Moa	©, β	Cooper *et al.*[54]	✓
Saber-tooth	β	Janczewski *et al.*[48]	✓
Termites	A	DeSalle *et al.*[10]	✗
Weevil	A	Cano *et al.*[41]	✗
Wheat seeds	θ	Allaby *et al.*[75]	✓
Dinosaur	β	Woodward *et al.*[105]	✗
Tyrolean Iceman	©, ~	Handt *et al.*[66]	✓
Cave bear	©, β	Hanni *et al.*[111]	✓
Mammoth	*f*, ©, β	Hagelberg *et al.*[19]	✓
Laysan duck	©, β	Cooper *et al.*[71]	✓
Mastodon	β	Yang *et al.*[51]	✓
Extinct ground sloth *Mylodon darwini*	©, β	Hoss *et al.*[50]	✓
Neanderthal	©, β	Krings *et al.*[12]	✓
Sea cow	©, β	Ozawa[112]	✓
Extinct ground sloth *Nothrotheriops shastensis*	Φ	Poinar *et al.*[13]	✓

Codes used: β, bone; A, amber; ©, cold site; Ξ, compression fossil; θ, mummified or charred seeds; *f*, permafrost site; ~ soft tissue; Φ, coprolite.

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