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Molecular paleontology: Novel insights from ancient DNA

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Abstract

For the past two centuries, paleontologists and archeologists have collected remains of human, animal, and plant life from a more or less distant past. Molecular paleontology entailing recovery and analysis of bio-molecules such as nucleic acids, proteins, lipids, carbohydrates and their derivatives from extinct plants or animals, and has become is integral to question within the sphere of paleontology. The studies have provided important insights into evolutionary events, and their extinction of microorganisms, animals or plant species remains. By applying high throughput molecular analytical techniques to DNA fossils, it is possible to quantify the level of relatedness between the organisms serving as source of nucleic acids. This article provides an overview of the recent molecular biological approaches used in paleontology.

Keywords: *Paleontology, molecular biology, extinction, nucleic acid*

Introduction

Advancements in the field of molecular paleontology have allowed scientists to pursue evolutionary questions on a genetic level rather than relying on phenotypic variation alone. Using molecular biological tools such and recovery and amplification of DNA, it is possible to gain novel insights into the divergence and evolutionary history of various organisms. The study of these ancient remains was performed initially on the basis of the phenotypic attributes. Subsequently, with the help of sequencing and molecular biological techniques, it could be possible to elaborate the extinct organisms at molecular and biochemical levels. The new frontier of paleontology and archeology is represented by the study of DNA. This type of research is of particular interest because DNA serves as repository of the genetic history of an organism; it thus represents a direct clue to evolution. Higuchi *et al*., (1984), for the first time reported a DNA sequence from an old zoological specimen. The researchers utilized an approximately 150-year-old fragment of desiccated muscle of an extinct equid, the quagga, to extract, amplify and analyze its DNA. The DNA was cloned in a phage DNA to determine the nucleotide sequence of a cloned fragment of mitochondrial (mt) DNA. The phylogenetic analysis showed that quagga mtDNA sequence is very close to that for the Burchell zebra (Higuchi *et al*., 1987).

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Few researchers have given credence to claims that samples of dinosaur DNA have survived to the present day, but no one knew just how long it would take for genetic material to fall apart. Now, a study of fossils found in New Zealand is laying the matter to rest - and putting an end to hopes of cloning a *Tyrannosaurus rex*. Following cell death, enzymes start to break down the bonds between the nucleotides that form the backbone of DNA, and microorganisms speed the decay. In the long run, however, reactions with water are thought to be responsible for most bond degradation. As moisture is almost ubiquitous in underground soil, the DNA in buried bone samples should, in theory, degrade at a set rate. Determining that rate has been difficult because it is rare to find large sets of DNA-containing fossils with which to make meaningful comparisons. To make matters worse, variable environmental conditions such as temperature, degree of microbial attack and oxygenation alter the speed of the decay process. But palaeogeneticists Murdoch University in Perth, Australia, have examined DNA-containing leg bones belonging to three species of extinct New Zealand giant birds, called moa. The bones, expected to be 600-8,000 years old, were recovered from three sites within 5 kilometers of each other, with nearly identical preservation conditions including. By comparing the age of specimens and the degrees of DNA degradation, it was calculated that DNA had a half-life of 521 years, meaning that after 521 years, half of the bonds between nucleotides in the backbone of a sample would have leaved. After another 521 years, half of the remaining bonds would have gone; and so on. It was predicted that even in a bone at an ideal preservation

temperature of -5ºC, effectively every bond would be destroyed after a maximum of 6.8 million years. The DNA would cease to be readable much earlier - perhaps after roughly 1.5 million years, when the remaining strands would be too short to give meaningful information.

Advanced era of molecular paleontology

With the development of polymerase chain reaction (PCR), it became possible to routinely amplify and study surviving ancient DNA molecules even if only in a single copy (Pääbo *et al*., 1989a,b; Thomas *et al*., 1989). However, due to the enormous power of PCR to amplify even a few copies of DNA sequences, modern DNA contamination has become a crucial problem. For this reason, many of the most extravagant reports on ancient DNA, including claims of DNA sequences surviving for millions of years in plants (Golenberg *et al*., 1990; Soltis *et al*., 1992; Kim *et al*., 2004) and dinosaur bones (Woodward *et al*., 1994), have been disputed and actually disregarded. Studies on ancient DNA need to deal with technical problems that are specific to this field. The first difficulty is the production of sufficient quantities of authentic DNA sequences to make the investigations conclusive. This difficulty is a consequence of post-mortem DNA degradation processes, which can cause miscoding lesions, potentially leading to sequence errors, or physical destruction of the DNA molecule, thus increasing the risk for preferential amplification of exogenous contaminant sequences (Table 1). To deal with this issue, researchers have guidelines to ensure the quality of DNA data and the reliability of consequent conclusions. Over the years, these guidelines have gradually evolved into a more detailed and extensive list of requirements, resulting in the nine "gold criteria" in "Ancient DNA: do it right or not at all" (Cooper and Poinar 2000) (Table2).

Table 1 The summary of factors causing damage to ancient DNA

Sl. No.	Damaging factors	Type of process	Effects on DNA molecule	Possible solutions in aDNA classical sequencing methodologies
1.	Oxidative damage	Formation of single-stranded nicks	Cleavage of the phosphodiester Backbone	PCR of overlapping fragments of short length
			Depurination resulting in a baseless site	Multiple independent PCR cloning and sequencing of several clones
			Breakage of the sugar backbone through β-elimination	Uracil-N-glycolase treatment
			Results in lesions blocking the polymerase enzyme, and promoting chimeric sequences through 'jumping' PCR	Blocking primers Single primer extension
2.	Degradation by microbial nucleases in post mortem cell	Strand breaks	Short fragment length	PCR of overlapping fragments of short length
3.	DNA crosslink's	Inter-strand crosslinks by alkylation	May prevent the amplification of endogenous template molecules	PTB (N-phenylacyl thiazolium bromide)
		Intermolecular crosslink's by Maillard reaction	Increases the risk of Contamination	
4.	Hydrolysis damage	Miscoding lesions, e.g., deamination of cytosine and adenine to uracil and hypoxathine, respectively	Results in the incorporation of erroneous bases during amplification and change of coding	Multiple independent PCR Cloning and sequencing of several clones UNG treatment

Table 2 Summarized criteria of authenticity according to Cooper and Poinar (2009)

Measure	Justification
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 devoted and isolated milieu. Labs concerned in amplification of large amounts of target DNA should be avoided.
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 may be avoided as they serve as contamination risk.
Suitable molecular behavior	PCR amplification strength should be inversely related to product size. Reproducible miDNA should be obtainable if single-copy nuclear or pathogen DNA is detected. Deviations from these expectations should be justified.
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 nuclear mitochondrial DNA sequence. 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.
Quantization	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.

In particular, it is suggested that, in absence of full compliance with all nine criteria, the reliability and authenticity of results remains uncertain. In the last few years, with the advent of new sequencing technologies, the field of ancient DNA is experiencing a new era wherein what was once impossible is now possible, as for example drafting genomes of extinct organisms like *Homo neanderthalensis* (Green *et al*., 2010), or distinguishing endogenous from contaminant DNA in archaic *Homo sapiens* specimens (Krause *et al*., 2010). This article reviews the history of studies on ancient DNA from a methodological point of view, ranging from the most significant ones performed with the so-called "classical methodology" comprising of PCR amplification, cloning, and sequencing by Sanger sequencing, to the more recent ones performed by next-generation sequencing technologies (NGS).

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 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 (Lindahl and Nyberg, 1972, 1974). 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 the buried remains. Sugar residues are also subject to attack by oxygen, resulting finally in strand breakage (Lindahl *et al*., 1993, 1997). 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 (Lorenz and Wackernagel, 1994). 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.

Ancient animal and plant genetics

(a) Population genetics

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

populations have repeatedly shown that modern patterns of distribution and diversity are often owing to relatively recent events, and can give misleading views of past evolutionary processes (Cooper *et al*. 1996; Barnes *et al*., 2002; Goldstein and DeSalle 2003; Shapiro *et al*., 2004). This can have major conservation implications, such as the legal ability to reintroduce extirpated taxa (e.g. Laysan duck, Anas laysanensis), or the apparent creation of new conservation units or species when formerly clinal distributions are disrupted (e.g. northeastern beach tiger beetle, Cicindela dorsalis dorsalis). Conversely, studies of several ancient populations of large vertebrates have shown extensive gene flow between taxa recognized as different species or subspecies, suggesting that the morphological plasticity in some fossil groups may have been considerably underestimated (Bunce *et al*. 2003; Huynen *et al*. 2003; Shapiro *et al*. 2004). The combination of ancient sequences and coalescent methods has considerable power to reconstruct demographic histories, test models of population genetics and reveal much novel information about microevolutionary processes. These methods can even recover demographic data for taxa that have been through recent population bottlenecks, which would normally remove genetic signals (Shapiro *et al*. 2004). Such methods also provide an opportunity to directly estimate evolutionary rates of nucleotide substitution and directly date phylogenetic events without using an external palaeontological calibration for a molecular clock (Lambert *et al*. 2001; Ritchie *et al*. 2004). The results show that palaeontologically calibrated rate estimates are often significantly slower than those calibrated from aDNA population studies and may reflect differences in sequence substitution processes operating at short and long timeframes.

(b) Nuclear DNA

Sequences from multi-copy loci are easier to recover from fossil remains than single-copy sequences, probably owing to the presence of larger numbers per cell. Consequently, there has been an almost complete reliance on mtDNA and cpDNA sequences in ancient DNA research. However, these markers may not reflect the overall history of the genome when closely related species or population genetic questions are addressed (Hofreiter *et al*. 2001b). Studies show that multi- and single-copy nuclear DNA (nuDNA) sequences can also be obtained from fossil remains, such as Pleistocene sloth coprolites from Nevada (Poinar *et al*. 2003). This study used single-copy nuDNA sequences to resolve the phylogenetic position of the extinct Shasta ground sloth (Nothrotheriops shastensis), and demonstrated that Pleistocene DNA survival was also possible in specimens preserved in warm arid climates and not just permafrost-preserved fossils (Greenwood *et al*. 1999, 2001). Plant aDNA sequences have been retrieved from samples such as seeds (Rollo and Amici 1991; O'Donoghue *et al*. 1996; Brown 1999) and ancient maize cobs (Jaenicke-Despre´s *et al*. 2003). Interestingly, the maize study recorded how variants were selected at three nuDNA genes that effect architecture, storage protein synthesis and starch production. The results show that these genes were largely homogenized as early as 4.4 kyr ago by Mexican farmers, although one of the loci was not fixed until ca. 2 kyr ago. Encouragingly, this study indicates that aDNA studies of selection will become increasingly possible as phenotypically important loci are identified by genome projects.

(c) Organellar genomics

The majority of aDNA research has relied on relatively short sequences (ca. 1 kb) obtained through the amplification of numerous small overlapping fragments (ca. 100– 500 bp). However, the field was recently brought into the genome era when the sequences of two complete mtDNA genomes (ca. 16.5 kb) were determined for extinct species of New Zealand moa (Cooper *et al*. 2001b). Partial mitochondrial genome sequences of two other moa were also generated (Haddrath and Baker 2001), demonstrating that it is possible to reconstruct long genetic sequences from fossil remains. Such large amounts of sequence data are desirable as they permit detailed phylogenetic analyses and statistically precise estimates of molecular divergence dates. It is important to note that even if it becomes possible to reconstruct ancient nuDNA sequences, it would still be impossible to bring extinct organisms back to life. Among many other requirements, the cloning of complex organisms needs a complete and undamaged nuDNA genome, packaged correctly, and a compatible maternal host.

(d) Dirty DNA

The discovery that diverse vertebrate mitochondrial DNA(mt DNA) and plant chloroplast DNA (cpDNA) sequences may be preserved in permafrost and cave sediments, has opened the way for ancient DNA analyses of biological change at the ecosystem level (Hofreiter *et al*. 2003; Willerslev *et al*. 2003a). It has been possible to recover DNA from small samples (ca. 2 g) of sediments up to 300–400 kyr old, including sequences of herbs, shrubs, trees, mosses, and megafauna such as mammoth, bison and horse (Willerslev *et al*. 2003a). Sedimentary DNA offers the potential to link fauna and flora directly in space and time over broad areas, and could provide an important complement to pollen records which are complicated by variations in influx rates, long-distance dispersal and vegetative plant growth (Guthrie 1990; Anderson *et al*. 1994). As angiosperm pollen generally does not contain cpDNA (Shi-yi 1997), the majority of the plant cpDNA sequences in sediment are envisaged originating from seeds or somatic cells and tissues (Willerslev *et al*. 2003a). Although the potential of sedimentary DNA is remarkable, important concerns include the effects of water movement on DNA mobility in non-frozen areas, and possible biases in the deposition and survival rates of different taxa. Encouragingly, free DNA molecules in the soil are shown to bind tightly to

the edges of humic acids, clays, feldspar and quartz, which limits their movement and degradation by endonucleases (Lorenz and Wackernagel 1994).

Ancient human DNA: A contentious issue

While animal and plant aDNA studies have seen rapid progress, contamination issues have undermined promises that such research would also revolutionize bioarchaeology (Herrmann and Hummel 1996). Early studies using material from hot environments such as Egypt (Pääbo, 1985) and Florida (Hauswirth *et al*. 1994) are recognized as probable contaminants. For example, the Egyptian mummy sequence was a very large (3.4 kb) fragment of nuDNA, which is highly unusual, and was recovered from a region where temperatures make DNA survival very unlikely (Marota *et al*. 2002; Gilbert *et al*. 2005a). Several reports show that despite rigorous protocols (Cooper and Poinar 2001; Hofreiter *et al*. 2001b), modern human contamination is widespread in amplification products from ancient extracts (Kolman and Tuross 2000; Hofreiter *et al*. 2001b). It also appears impossible to clean contemporary human DNA from human bones and teeth despite extensive treatment with UV irradiation and bleach (Gilbert *et al*. 2005b). It appears that the porosity of bone and dentine in teeth are the main entry routes for DNA generated from sweat, skin fragments and exhaled cells, reinforcing the urgent need for disposable gloves and face-masks during excavation and handling of archaeological specimens. Interestingly, hair may be a more reliable source for ancient human DNA studies, as it appears less susceptible to contamination than bone and teeth (Gilbert *et al*. 2004a). Many excavated archaeological remains appear to contain DNA from multiple individuals (Gilbert *et al*. 2003a,b), raising the issue of how to authenticate ancient human DNA when 'unique' sequences, such as the Neanderthal (Krings *et al*. 1997) or distinct modern human groups like the Andaman Islanders (Endicott *et al*. 2003), are not reproducibly obtained. A good example is the analysis of Italian Cro-Magnon specimens (Caramelli *et al*. 2003), where comprehensive protocols of authentication (Cooper and Poinar 2000; Hofreiter *et al*. 2001b) were followed. However, because the resulting sequences were indistinguishable from modern Europeans, sample contamination must remain the null hypothesis. By contrast, Serre *et al*. (2004a) assume that it is impossible to authenticate any modern human sequence obtained from archaeological specimens, and instead confirm the absence of Neanderthal-specific mtDNA sequences from five European early modern human (EMH) specimens. Since coalescence theory indicates that the (inferred) modern human mtDNA sequences of the five EMH specimens are unlikely to exactly match the 5–7 ancestral lineages of modern populations, this effectively doubles the number of modern human mtDNA lineages known to exist in the Late Pleistocene. This value was used with population genetic models to calculate that the maximum Neanderthal genetic contribution to EMH is likely to have been less than 25% (Serre *et al*. 2004a). Although not independently replicated, this study demonstrates how aDNA can increase the resolving power of population genetics studies (Cooper *et al*. 2004). The retrieval of putative Neanderthal mtDNA sequences (Krings *et al*. 1999, 2000; Ovchinnikov *et al*. 2000; Schmitz *et al*. 2002) is the major highlight in ancient human DNA studies because it allowed direct testing of hypotheses about the origin of the modern human gene pool. Importantly, some suggestions that ancient sequences such as the Neanderthal results might be due to PCR artifacts (Pusch and Bachmann 2004) appear unjustified, and may result from poor experimental design and methodology (Serre *et al*. 2004b). Until recently there seemed little hope for obtaining DNA sequences from other extinct hominids (e.g. Homo erectus). Otherwise, advances in protein sequencing techniques and the stability of certain proteins (Nielsen-Marsh *et al*. 2002), may also provide a means for such comparisons, although the limited phylogenetic utility of short amino acid sequences constrains the resolving power possible. A final complicating issue in ancient human mtDNA studies is the authentication of haplogroup designations. Mutational hot spots may generate erroneous, but potentially credible results when PCR reactions are initiated from small numbers of DNA molecules (Gilbert *et al*. 2003a,b). This is exacerbated in studies of human control region sequences, where haplogroup identifications are routinely categorized using fewer than five site changes, and in extreme cases (e.g. some European groups) by as few as one. In such situations, the use of real-time PCR and UNG treatment is highly advisable (Table 1). A far more reliable approach is to characterize multiple variable positions around the mitochondrial genome to define a haplotype, as shown by Maca-Meyer *et al*. (2004) in a study of the colonization of the Canary Islands.

The microbial concerns

Many studies have reported the survival of ancient microbial DNA from archaeological remains (Cano *et al*. 2000; Rollo *et al*. 2002; Raoult *et al*. 2000; Fletcher *et al*. 2003), but unfortunately very few have used independent replication to authenticate results. As a consequence, there is some doubt over the validity of many studies, especially when research is carried out in microbiology departments where the microbial DNA sequences might be expected as a local contaminant.

It is a matter of concern that many of the ancient microbial sequences are similar or identical to modern strains, or come from environments unlikely to preserve DNA. This is unfortunate, as the rapid evolutionary rates of many pathogens provide a unique opportunity to use phylogenetic analyses to verify the sequences as ancient, i.e. ancestral to modern diversity. Where independent replication has been attempted, such as studies of *Yersinia pestis* sequences in European plague victims (Raoult *et al*. 2000), the results have been negative (Gilbert *et al*. 2004b). Reports of viable microbial cells and DNA from

materials hundreds of millions of years old, such as fossil plant and animal remains, deep sediments, amber and halite (Kennedy *et al*. 1994; Vreeland *et al*. 2000; Fish *et al*. 2002; Vreeland and Rosenzweig 2002) also seem surprising. By contrast, a recent study of bacterial DNA under ideal deep-frozen conditions suggests a maximum survival time for amplifiable bacterial DNA of between 400 kyr and 1.5 Myr (Willerslev *et al*. 2004a), which is close to theoretical predictions. Claims regarding ancient micro-organisms are extremely difficult to authenticate because a novel sequence cannot be used as a criterion for authentication since only ca. 1 % of the potential modern contaminants (i.e. extant microbial diversity) are thought to be known (Ward *et al*. 1992). Furthermore, like human DNA, microbial contamination is ubiquitous (Willerslev *et al*. 2004b), and it is shown that contemporary microbes have a global distribution and that endemics are rare, i.e. everything is everywhere (Fenchel *et al*. 1997; Finlay 2002). As a consequence, the independent replication of results will be a less rigorous criterion of authenticity in ancient microbial studies, and clear time-dependent patterns of diversity and DNA degradation may be needed to demonstrate authenticity (Willerslev *et al*. 2004b). Despite these problems, nearly all of the geologically ancient microbial claims have not been checked by either the analysis of DNA preservation states, or more disconcertingly, by independent replication. For example, reports of bacterial DNA and viable cells in halite were published without verification of the result by real-time PCR, DNA damage data or independent replication (Vreeland *et al*. 2000; Fish *et al*. 2002). Several papers have commented on the exceptional similarity of these microbial DNA sequences to contemporary ones (Graur and Pupko 2001; Nickle *et al*. 2002) including Gram -ve proteobacteria with fragile cell walls, and no known adaptations to long-term DNA preservation. It is also notable that members of nearly all major microbial groups have been recovered from multimillion- year-old materials, despite possessing huge differences in hardiness and resistance to DNA degradation (Kennedy *et al*. 1994), strongly suggesting that contamination may be involved.

Metagenomics, Next Generation Sequencing of Ancient DNA and paleontology

Culture-independent techniques have contributed significantly to investigate the complex microbial ecologies of skin, gut and genitourinary tract, and developing therapeutic interventions (Prakash *et al*., 1997; Singh *et al*., 2008).

Table 3 Fossils DNA studies using NGS for sequence data collection

Sl.No.	**Research achievements**	**Target region**	**Sequencing/Target enrichment Strategy (References)**
1.	Metagenomics to paleogenomics: large-scale sequencing of mammoth DNA	genome	Shotgun (Poinar *et al.* 2006)
2.	Analysis of one million base pairs of Neanderthal DNA	genome	Shotgun (Green *et al.* 2006)
3.	Whole-genome shotgun sequencing of mitochondria from ancient hair shafts	mitochondrial genome	Shotgun (Gilbert *et al.* 2007)
4.	Intraspecific phylogenetic analysis of Siberian woolly mammoths using complete mitochondrial genomes	mitochondrial genome	Shotgun (Gilbert *et al.* 2008)
5.	Paleo-Eskimo mtDNA genome reveals matrilineal discontinuity in Greenland	mitochondrial genome	Shotgun (Gilbert *et al.* 2008)
6.	A complete Neandertal mitochondrial genome sequence determined by high-throughput sequencing	mitochondrial genome	Shotgun (Green *et al.* 2008)
7.	Sequencing the nuclear genome of the extinct woolly mammoth	genome	Shotgun (Miller *et al.* 2008)
8.	The mitochondrial genome sequence of the Tasmanian tiger (Thylacinus cynocephalus)	mitochondrial genome	Shotgun (Miller *et al.* 2009)
9.	Identification of microsatellites from an extinct moa species using high-throughput (454) sequence data	microsatellites	Shotgun (Allentoft *et al.* 2009)
10.	Paleogenomics in a temperate environment: shotgun sequencing from an extinct Mediterranean caprine	genome	Shotgun (Ramírez *et al.* 2009)
11.	Analysis of complete mitochondrial genomes from extinct and extant rhinoceroses reveals lack of phylogenetic resolution	mitochondrial genome	Shotgun (Willerslev *et al.* 2009)
12.	Targeted retrieval and analysis of five Neandertal mtDNA genomes	mitochondrial genome	Capture (Briggs *et al.* 2009)
13.	Tracking the past: interspersed repeats in an extinct Afrotherian mammal, Mammuthus primigenius	genome	Shotgun (Zhao et al., 2009)
14.	Direct multiplex sequencing (DMPS)--a novel method for targeted high-throughput sequencing of ancient and highly degraded DNA	mitochondrial genome	multiplex PCR (Stiller *et al.* 2009)
15.	A complete mtDNA genome of an early modern human	mitochondrial	Capture (Krause *et al.* 2010)

	from Kostenki, Russia	genome	
16.	Ancient human genome sequence of an extinct Palaeo-Eskimo	genome	Shotgun (Rasmussen *et al.* 2010)
17.	A complete mitochondrial genome sequence from a mesolithic wild aurochs (Bos primigenius)	mitochondrial genome	Shotgun (Edwards *et al.* 2010)
18.	Complete mitochondrial genome of a Pleistocene jawbone unveils the origin of polar bear	mitochondrial genome	Shotgun (Lindqvist *et al.* 2010)
19.	The complete mitochondrial DNA genome of an unknown hominin from southern Siberia	mitochondrial genome	Capture (Krause *et al.* 2010)
20.	Targeted investigation of the Neandertal genome by array-based sequence capture	nuclear genome	Capture (Burbano *et al.*2010)
21.	A draft sequence of the Neandertal genome	genome	Shotgun (Green *et al.*2010)

In addition, the metagenomics has been used for elucidating the complex microbiota of sea, soil compost and ancient biomaterials (Singh *et al*., 2008). The invention of NGS has revolutionized almost all fields of genetics, but few have profited from it as much as the field of ancient DNA research. From its beginnings as an interesting, but rather marginal discipline, ancient DNA research is now on its way into the centre of evolutionary biology. In less than a year from its invention, the NGS has increased the amount of DNA sequence data available from extinct organisms by several orders of magnitude. Ancient DNA research is now not only adding a temporal aspect to evolutionary studies, and allowing for the observation of evolution in real time; it also provides important data to help understand the origins of species. Ancient DNA studies of human archaeological samples routinely generate complete mitochondrial genomes via DNA hybridization-based enrichment of mtDNA target sequences (Knapp *et al*. 2012), and the creation of barcoded/indexed DNA libraries, followed NGS. Multiple samples can be processed in parallel in a high-throughput fashion (Knapp *et al*., 2012), greatly reducing processing contamination risks, labour and costs compared to traditional DNA sequencing. These kinds of DNA capture strategies generally rely on the hybridization of target DNA sequences to probes that are either immobilized on a surface or in solution (Hodges *et al*., 2007; Gnirke *et al*., 2009). Despite the significant potential of these new approaches, they have not been applied or examined in a forensic context for human identification. More than 20 studies have already used NGS to obtain sequence data from ancient biomaterials (Table 3)

The three key steps for generating reads are: (1) library preparation, (2) library amplification and (3) sequencing. The new generation of NGS sequencers has made it possible to increase the number of bases sequenced per run with a concomitant decrease in sequencing costs. The most important NGS platforms used in the field of ancient DNA analyses are the 454/Roche FLX and the Illumina Genome Analyzer. Both technologies share the same rationale for the production of sequences (reads), but differ in the amplification procedure and sequencing chemistry resulting in different throughputs. In spite of the high sensitivity and productivity of NGS sequencers, their signal detection system is not sensitive enough to measure the sequencing signal originating from a single molecule. Detection systems, such as the CCD camera for both 454/Roche and Illumina, can identify a signal only if it is generated by millions of DNA molecules, thus amplification of the sequencing library is necessary.

Conclusion

Molecular paleontology is an emerging and highly important field, with respect to recovery and analysis of truly ancient molecules. Paleogenomes isolated from pathogenic organisms have indicated that DNA preservation extends further back in time and across a wider range of environments. As the number and range of published palaeogenomes grows, paleogenomics is poised to play an increasingly important role in improving our understanding of evolutionary processes over the short and medium term. Enrichment protocols of DNA samples and specific bioinformatics pipelines have been successfully developed to increase data reliability. However, other steps in the procedures and analyses need to be optimized to generate a robust ancient DNA data set. The NGS technologies have accelerated the change in the focus of research. Major insights have been gained from the multidisciplinary nature of the studies on ancient DNA, which exploit data combined from different technologies capable of generating genome-wide data. Moreover, which have been used to study complex processes, such as human evolution, domestication or demographic events, need to be confirmed even if the availability of ancient well-preserved specimens is always a critical and intrinsic feature of palaeogenomic projects.

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