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Review

Ancient mitogenomics

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ABSTRACT

The mitochondrial genome has been the traditional focus of most research into ancient DNA, owing to its high copy number and population-level variability. Despite this long-standing interest in mitochondrial DNA, it was only in 2001 that the first complete ancient mitogenomic sequences were obtained. As a result of various methodological developments, including the introduction of high-throughput sequencing techniques, the total number of ancient mitogenome sequences has increased rapidly over the past few years. In this review, we present a brief history of ancient mitogenomics and describe the technical challenges that face researchers in the field. We catalogue the diverse sequencing methods and source materials used to obtain ancient mitogenomic sequences, summarise the associated genetic and phylogenetic studies that have been conducted, and evaluate the future prospects of the field.

1. Introduction

Over the past 25 years, ancient DNA research has maintained a consistently high profile in scientific journals and in the public domain. This is partly owing to appreciation of the attendant technical and methodological challenges, as well as the spectacular, and often controversial, claims that have characterised the field. In addition, ancient DNA work is often associated with charismatic animals such as the woolly mammoth, dodo, and Tasmanian tiger.

Ancient DNA, broadly defined here as any degraded DNA obtained from specimens not deliberately preserved for genetic analysis, differs from modern DNA in several important respects. As a result of post-mortem damage, such as that caused by hydrolytic and oxidative reactions, ancient DNA molecules are often highly fragmented and usually found in low concentrations (Pääbo, 1989). This presents practical obstacles for sequence analysis and heightens the risk of contamination. Consequently, specialised facilities and the implementation of strict laboratory protocols are often regarded as essential for ancient DNA research, especially when human, common domestic, or microbial samples are involved.

The earliest report of ancient DNA appeared in the mid-1980s, when Higuchi et al. (1984) obtained mitochondrial sequence fragments from a 150-year-old museum specimen of the extinct quagga. Shortly afterwards, Pääbo (1985) published a partial mitochondrial DNA sequence from a 2430-year-old Egyptian mummy. Neither of these analyses was reproducible and the sequences are now viewed cautiously (Pääbo et al., 2004). At the time, however, these studies had considerable impact because they suggested that DNA molecules were able to survive for extended periods of time and could still be sequenced. Such ancient genetic data would allow unprecedented access to information that could not be gleaned from palaeontological, archaeological, or documentary evidence alone.

Progress in ancient DNA research gained substantial impetus with the development of the polymerase chain reaction (PCR), which enabled the amplification of very small amounts of starting DNA (Mullis and Faloona, 1987). Recognising its potential for helping to recover genetic information from even trace amounts of nucleic acids, the nascent ancient DNA community rapidly took up this new methodology. However, the application of PCR to ancient specimens soon revealed the ubiquity of DNA sequence damage and the pervasiveness of contamination (Pääbo, 1989; Pääbo and Wilson, 1988). Nevertheless, the magnitude and gravity of these risks did little to dampen enthusiasm within the field, with reports of DNA from Miocene plant fossils (Golenberg et al., 1990; Soltis et al., 1992), a 120-million-year-old insect trapped in amber (Cano et al., 1993), and dinosaurs (Li et al., 1995; Woodward et al., 1994). These optimistic studies, among several others, helped to instigate sensational media coverage of ancient DNA research.

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Subsequent empirical and theoretical evidence has placed the maximum survival time of DNA at under a million years (Lindahl, 1993, 1997; Smith et al., 2001; Willerslev et al., 2003), casting grave doubt on studies claiming the successful retrieval of 'geologically ancient' or 'antediluvian' DNA. Indeed, most or all of these sequences are now strongly suspected to be artefactual (Hebsgaard et al., 2005), and claims of geologically ancient DNA have become far less frequent in the 21st century (but see Fish et al., 2002; Kim et al., 2004; Veiga-Crespo et al., 2007, 2008, 2004; Vreeland et al., 2006). Serious concern over the reliability and credibility of ancient DNA studies has led numerous authors to publish detailed and rigorous guidelines for evaluating the authenticity of ancient DNA sequences (e.g., Cooper and Poinar, 2000; Gilbert et al., 2005; Hofreiter et al., 2001; Pääbo et al., 2004; Ward and Stringer, 1997).

2. Ancient mitochondrial DNA

For a number of reasons, mitochondrial DNA (mtDNA) has been the focus of the majority of ancient DNA studies of animals. First, the mitochondrial genome is usually present in higher copy numbers than the nuclear genome, thus conferring upon mtDNA a greater per-locus chance of being recoverable using common laboratory techniques. Second, the mitogenome has several characteristics that make it particularly amenable to genetic analysis, including maternal inheritance, absence of recombination, and high mutation rate (Ramakrishnan and Hadly, 2009). Third, the availability of modern mitogenomic sequences has made it easier to design and employ targeted sequencing approaches, such as those based on the PCR. We note that some researchers deprecate the use of the term 'mitochondrial genome' to describe the mtDNA molecule, but we will use this concise term throughout our review for the sake of clarity.

In spite of the long-standing interest in mtDNA, ancient mitogenomics is a relatively young field and its course has been strongly influenced by methodological parameters. Owing to the degraded, fragmented nature of ancient DNA, numerous PCRs are required to achieve complete coverage of a mitogenome. This can result in extensive, destructive sampling of ancient, often invaluable, specimens, because each PCR uses extract from the source material. Consequently, it was not until 2001 that the first ancient mitochondrial genomes were sequenced in their entirety, when two independent groups of researchers presented the mitogenomes of four extinct moa (Cooper et al., 2001; Haddrath and Baker, 2001). These represented the products of many years of painstaking efforts in the laboratory.

There was a hiatus of 5 years before the next ancient mitogenomic sequences were published. In 2006, Krause et al. (2006) and Rogaev et al. (2006) independently sequenced the mitogenome of the extinct woolly mammoth. In the past few years, the introduction of high-throughput sequencing methods, such as emulsion PCR and pyrosequencing (Margulies et al., 2005), has vastly improved the yield from source materials and has made the study of ancient mitogenomics much more cost- and time-efficient. In this review, we examine these methodological advances, outline the current state of ancient mitogenomics, discuss the current challenges facing researchers, and describe the studies that have been conducted so far.

3. Technical challenges

When an organism dies, its cellular DNA repair mechanisms cease immediately and the cells rapidly undergo autolysis, except in tissues that are already stabilised by processes such as keratinisation or when there are highly favourable preservation situations (Vass, 2001). This decomposition is supplemented by the degrading action of external microorganisms, along with the rapidly-expanding community of commensal bacteria, such as the gut flora. In addition to this microbial attack, biochemical processes such as hydrolysis and oxidation result in DNA strand breakages, cross-linkage between DNA molecules, and baseless sites (Höss et al., 1996b; Lindahl, 1993). Furthermore, some forms of post-mortem damage, such as the so-called miscoding lesions characterised by Pääbo (1989), can cause incorrect nucleotides to be incorporated during enzymatic amplification, resulting in error-laden DNA sequences.

While the overall rate of DNA degradation in any organism is affected by a complex interplay of factors, including temperature, proximity to free water, environmental salt content, and exposure to radiation (Lindahl, 1993), the combined outcome is an irreversible reduction in both the concentration and the length of intact DNA molecules. This degradation has several implications in the context of ancient DNA studies: it limits the amount of DNA available for study, increases the error within the recovered sequence, and significantly raises the impact of contamination from other molecules. These can cause considerable problems for subsequent analyses of the DNA sequence data.

3.1. Post-mortem degradation

The underlying kinetics of DNA degradation are sufficiently complex that they are difficult to model accurately, but it has been repeatedly observed that the result is a reciprocal relationship between the size and number of remaining template molecules (Deagle et al., 2006; Ottoni et al., 2009; Poinar et al., 2003, 2006; Schwarz et al., 2009). Consequently, conventional-PCR assays must settle on a compromise between the length of the target sequences and the number of PCR cycles that are required for adequate DNA amplification. With all other factors being equal, adopting a more-amplicon/shorter-fragment approach may involve lower levels of error because the miscoding lesions are likely to affect fewer molecules at any particular nucleotide position. An additional feature of PCR-based assays in this context is that, owing to the requirement of having template upon which the primers can bind, there is a template limit below which non-primer sequence information cannot be recovered (approximately 40 bases). According to the abovementioned reciprocal relationship between template copy number and size, this greatly limits the materials that can be studied with PCR. One solution to this problem is the ligation of ancient DNA to additional DNA 'adaptor' molecules prior to PCR. In such situations, PCR primers can be ligated outside the ancient target sequence, so that information can be recovered from even the smaller templates. This method forms the backbone of the other approaches used to generate ancient mitochondrial genomes, such as sequencing on the Roche/454 or Illumina GA platforms (see Section 4.4).

In the laboratory, single-base errors due to miscoding lesions can be detected, and thus accounted for, through cloning of PCR products and/or the sequencing of multiple replicate PCRs. If a high-throughput sequencing approach is taken, the manifold coverage of the mitogenome that is often achieved will allow miscoding lesions to be detected in a similar manner. To some extent, single-base errors can also be detected by statistical analysis of the DNA sequences themselves. For example, this can be done by examining singleton mutations in a phylogenetic framework. Existing methods allow the process of post-mortem damage to be modelled either as time-independent (Ho et al., 2007; Mateiu and Rannala, 2008) or as a function of sample age (Rambaut et al., 2009).

3.2. Sample contamination

In a DNA extract from modern, fresh tissue, one might expect that practically all of the DNA molecules are endogenous. In con-

trast, many ancient samples can contain high levels of exogenous DNA, owing to microbiological degradation or absorption from the environment. This problem was recognised in the early studies of ancient DNA, in which the authors conducted DNA-quantitation analyses (Higuchi et al., 1984; Höss et al., 1996a), and has been repeatedly confirmed in subsequent empirical studies (e.g., Malmström et al., 2005; Noonan et al., 2005).

Although the problem of contaminant sequences has long been recognised in the field of ancient DNA, its full magnitude was revealed during the initial high-throughput sequencing (HTS) analyses of Neanderthal and woolly mammoth samples (Green et al., 2006; Noonan et al., 2006; Poinar et al., 2006). The observed levels of contamination were extremely high and, although unsurprising in retrospect, unsettled many researchers who had become accustomed to the reduced contamination problem in PCR-based approaches. For example, only a few percent, at most, of the DNA in temperate-preserved Neanderthal bone is endogenous (Green et al., 2006). An even lower level of 0.27% has been found in a *Myotragus* specimen, which was younger than the Neanderthal bone but had been preserved under warmer conditions in the Balearic Islands (Ramírez et al., 2009). Even in a seemingly well-preserved, deep-frozen mammoth bone, only 45.4% of the DNA could be mapped to the elephant draft genome (Poinar et al., 2006). Although this percentage increases to approximately 54% upon extrapolation to account for missing data in the elephant draft genome, the level of non-endogenous DNA is still high enough to pose a significant challenge to large-scale sequencing projects.

Understanding the causes of the disparity in contamination levels between PCR-based and HTS approaches is instrumental in appreciating the differences between the two techniques. In the conventional PCR approach, contaminants refer to molecules that are competitors for the targeted sequence – that is, sequences that share sufficient similarity to targeted endogenous template molecules that they will be bound by the primers during the PCR, and thus coamplified with the intended target. Despite possibly being at a low overall concentration in the extract, such contaminants can represent a considerable problem because of their potential abundance in relation to the intended target sequences, and they can even be amplified preferentially if they contain lower levels of DNA damage than the endogenous DNA.

The problem is quite different for HTS, which makes no discrimination among the DNA molecules present in the sample. The scale of contamination is then the combined effect of the factors described above, and can lead to a substantially reduced mtDNA proportion in ancient specimens. The ratio of mitochondrial to nuclear DNA template molecules in bone might be, for example, approximately 1:658 (e.g., Poinar et al., 2006; but see Schwarz et al., 2009), but if the endogenous DNA is only 1% of the total DNA in the extract, then only 1 in 65,800 of the templates would be mtDNA.

There are a number of ways to deal with the problem of sample contamination. The first involves the choice of tissue, which could be based on a preservational criterion (e.g., frozen versus temperate bone) or could entail the deliberate selection of tissues that are less permeable to contaminants, or easier to decontaminate, such as hair and nail (Gilbert et al., 2007c; Willerslev et al., 2009). Second, sample decontamination has been suggested as a potential means for optimising HTS analysis of ancient bone (Gilbert, 2008). This is based on the theory of Salamon et al. (2005) and Malmström et al. (2005) that bone can be powdered, then bleached, to reduce contaminant levels. So far, it has only been attempted once, in an analysis of ancient *Myotragus* bones, without noticeable success (Ramírez et al., 2009). Third, the proportion of contamination can be lowered via template enrichment prior to HTS, which can be performed either by multiplex PCR or by 'fishing' with oligonucleotide probes (see Section 4 below) (Briggs et al., 2009).

Apart from sample contamination, foreign DNA can also enter the sequencing process from the laboratory. Decontamination of reagents and the laboratory environment can reduce the number of ambient DNA molecules, while the pre-PCR facility itself should be physically isolated from post-PCR areas. Lastly, the influence of laboratory-based contamination can in some situations be minimised by independent replication of the sequencing project by a second research group.

At the post-sequencing stage, there are several methods for detecting contaminants and non-target sequences, including nuclear copies of mitochondrial genes ('numts'). The significance of the problems caused by numts is dependent on the method of data generation. Direct shotgun-sequencing approaches are unlikely to be affected, because the extremely low ratio of numts to mtDNA renders numt-related sequencing errors statistically unlikely (Gilbert et al., 2007a). However, approaches that rely on PCR or targeted sequence capture face a greater challenge because primers or capture probes might bind with equal likelihood to the numts and mtDNA. Should numts or other spurious sequences be sequenced, they can detected in several ways. First, high depth of sequencing coverage (either through cloning or through HTS) can alert an analyst to the presence of alternative sequences over any particular mtDNA region, thus indicating that caution and close investigation are required. Second, such sequences can potentially be identified by their unusual placement in estimated phylogenetic trees, or by the presence of stop codons or frame-shift mutations in protein-coding genes. Recent contaminants in geologically ancient samples can be identified by relative-rates tests, because their sequences exhibit fewer nucleotide substitutions than would be expected on the basis of the sample's age (Hebsgaard et al., 2005). However, this method would not have sufficient statistical power for identifying contaminants in relatively young samples.

4. Methods

Ancient mitogenomic sequences have been obtained using a variety of methods, of which the basic characteristics are summarised in Table 1. A workflow diagram showing the relationships of the different extraction, amplification, and sequencing steps is provided in Fig. 1. Sample collection, storage, and extraction steps are common to all ancient DNA studies and have been reviewed in detail elsewhere (e.g., Campos et al., 2009; Rohland et al., 2004). We do not discuss these steps here, but point interested readers to the reviews cited.

It is worth noting that the generation of sequence data, no matter which method is used, rarely represents the completion of the sequencing task. Sequence data often require subsequent computational parsing to sort out true mtDNA from other co-sequenced DNA, and ultimately require assembly into the final mitogenome sequences. The methods used to achieve this vary from one study to another, but the complexity of this step is generally dependent on two main factors. First, the length of recovered sequences is an important determinant of how confidently they can be identified as authentic, and thus assigned to a particular part of the mitochondrial genome: the shorter the sequence, the greater the difficulty. In this regard, one can speculate about the limits of usability for several of the available HTS platforms – although they yield millions of bases per run, they are limited under current sequencing chemistry to relatively short read lengths (in particular the Illumina GA and Applied Biosystems SOLiD). The second major factor is the availability of mtDNA sequences from closely related species, which are used to guide the screening and assembly of new sequences. In this context, perhaps the most commonly used resource is the Organelle Genome Resource at NCBI Genbank (current URL: http://www.ncbi.nlm.nih.gov/genomes/Genomes-

Table 1
Characteristics of mitogenomic sequencing methods.

Method	Use of template	Speed	Cost	Technical requirements
1. Simplex PCR	High	Very slow	Medium to high, depending on how much cloning used	Basic laboratory equipment and skills
2. Multiplex PCR then simplex PCR	Low	Slow	Medium to high, depending on how much cloning used	Ability to design robust multiplex PCR assay
3. Multiplex PCR then FLX	Low	Fast, once a balanced assay can be developed	Low	Access to HTS, ability to design robust multiplex PCR assay
4. Shotgun	High	Medium to fast, depending on mtDNA content of the library	Medium to high, extract dependent	Access to HTS, bioinformatics skills to process the large amounts of sequence data
5. Fish with probes then shotgun	Low	Fast	High initially, low subsequently	Access to HTS, bioinformatics skills to process the large amounts of sequence data

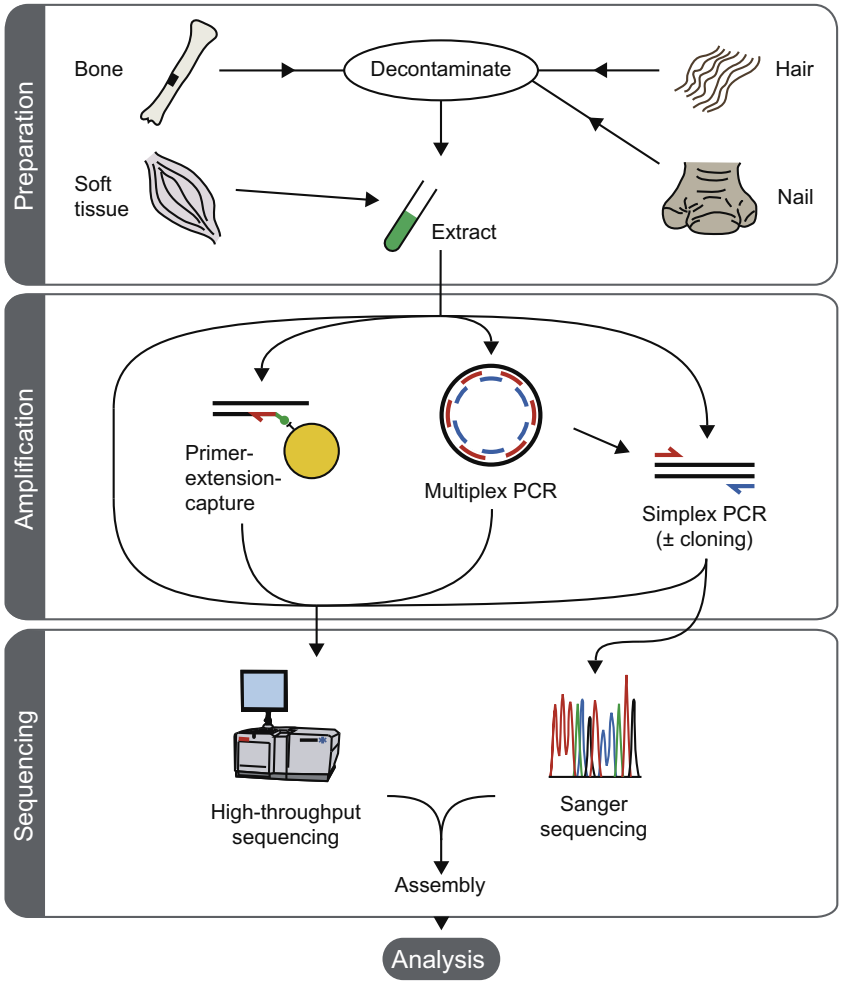

Fig. 1. Workflow diagram showing the relationships among the various amplification and sequencing techniques used in studies of ancient mitogenomes. The process begins with an ancient sample and produces an assembled DNA sequence for analysis.

Home.cgi?taxid=2759&hopt=html), a large database that contains all publicly available mitogenome sequences.

4.1. Conventional (simplex) PCR

The first published ancient mitogenomes were generated using conventional PCR, followed by either direct Sanger sequencing (Haddrath and Baker, 2001) or molecular cloning followed by Sanger sequencing (Cooper et al., 2001; Rogaev et al., 2006). In some recent studies, the amplicons of conventional PCR have been pooled and then sequenced on a Roche FLX or other HTS platform (e.g., Ermini et al., 2008). Such methods are both time- and resource-consuming, as they require many hundreds of PCRs, each targeting a small DNA fragment. Ultimately, these short, overlapping sequences are assembled into the final consensus sequence.

A considerable disadvantage associated with conventional PCR is that substantial amounts of DNA extract are required, given the large total number of PCRs that are performed. Thus, this approach makes considerable demands on ancient specimens, making it prohibitive for most mitogenomic studies. Additionally, as with all primer-based methods, conventional PCR is somewhat limited by the need to have some *a priori* sequence information upon which to design the primers. For extinct species, known sequences from related taxa need to be used for primer design.

4.2. Multiplex then simplex PCR

Krause et al. (2006) and Römpler et al. (2006) improved significantly on the conventional method by demonstrating that the initial simplex PCR amplifications could be replaced with a small number of multiplex PCRs. The concept behind these multiplex PCRs is simple: one includes multiple pairs of primers in a single PCR, each targeting a unique, non-overlapping part of the mitochondrial genome. Each primer set yields an amplicon, thus resulting in a significant enrichment of each by the end of the reaction. To increase the yield of any particular fragment, the product from the first reaction is diluted and simplex PCRs are performed using individual primer pairs. The primers can be identical to those used in the multiplex reaction, or can be designed to target a sequence nested within each of the multiplex products. The amplified fragments are then sequenced using one of the available techniques.

Although conceptually simple, designing primers that work together efficiently in such a situation is by no means trivial. Despite this, however, the method has been used successfully in several cases, yielding, for example, the first mammoth and mastodon mitogenomes (Krause et al., 2006; Rohland et al., 2007), the cave bear mitogenome (Krause et al., 2008), and part of those of the Romanov family (Rogaev et al., 2009). The principal benefit of approaches based on multiplex PCR is that they require far less initial PCR template, thus conserving precious extracts. As with conventional PCR, however, primer design relies upon some degree of *a priori* information about the genome of the target species or its close relatives.

4.3. PCR amplification followed by FLX sequencing

High-throughput DNA sequencers, such as the Roche FLX or Illumina GA, make it possible to sequence multiple PCR products rapidly and efficiently (e.g., Thomas et al., 2007). The extremely large number of sequences that are generated means that, for the most part, the content of any PCR product will be sequenced in depth, mimicking conventional molecular cloning and DNA sequencing. With an array of both commercial and custom sequence-labelling tools now available (e.g., Binladen et al., 2007; Meyer et al., 2007), many PCR products can be pooled in such a way that subsequently allows segregation; thus, these methods offer a rapid and economical option. In the context of mitogenomics, the FLX sequencer was first used in this way to sequence the simplex products of the mitogenome of the Tyrolean Iceman (Ermini et al., 2008). Recently, the approach was taken further with the publication of the first ancient mitogenomes that were directly FLX-sequenced from the products of multiplex reactions (Stiller et al., in press). This represents is a considerable technical achievement, given the theoretical problems of balancing multiplex reactions – in poorly balanced reactions, unless a large amount of redundancy is incorporated into the sequencing, dominant amplicons will be sequenced at the cost of those that are rare. The authors overcame this challenge by limiting the number of PCR cycles employed to amplify the DNA, and in doing so successfully obtained near-complete mitogenome sequences from 31 cave bears.

4.4. Shotgun

For researchers with sufficient funds and access to the required platforms, HTS methods represent a tempting alternative to the conventional approach. The methods are fundamentally based on the principle of shotgun sequencing, which involves the random selection of large numbers of DNA molecules from those available in an extract. The frequency of any resulting sequence is a direct function of its original frequency. In any extract, depending on the tissue type (and some other considerations discussed below), mtDNA will represent a certain percentage of the total DNA. In humans, for example, the size of the diploid genome is ~6 Gb. Although the mitogenome is tiny in comparison (~16.5 kb), there can be up to 10,000 copies in a single cell. Thus, the ratio of mitochondrial to nuclear DNA can be as high as 165 Mb:6 Gb, whereby we would expect that one in every 36.4 sequence fragments is mitochondrial. When combined with the very large number of sequences generated by HTS (hundreds of thousands to millions of sequences, depending on the platform and chemistry used), this makes the method an attractive option even for ancient mitogenomics. An additional benefit of HTS is that, as no primers are required, much smaller template molecules can be targeted than in a conventional PCR approach.

Adopting HTS methods for ancient mitogenomics has not been straightforward, however, and several surprises have been encountered. One positive finding has been that the relatively large amounts of starting material recommended by the manufacturers for building libraries (in the order of micrograms) has turned out to be an overestimate for ancient samples. There are several reasons for this. First, ancient DNA rarely requires fragmentation prior to library construction, which is normally a key requisite for modern DNA and represents a stage in which very large amounts of DNA are lost. Second, more sensitive ways of building and measuring the libraries have been proposed that significantly reduce the required amount of starting material (Maricic and Pääbo, 2009; Meyer et al., 2008). Third, many ancient DNA extracts actually contain plenty of DNA molecules, but often the majority is derived from environmental microorganisms and other contaminants.

4.5. Fish with probes then shotgun

Recently, an appealing solution to the target-enrichment problem of HTS was published by Briggs et al. (2009) in their analysis of five Neanderthal mitogenomes. Termed 'primer-extension-capture', the method involves large numbers (>600) of 5′-biotinylated oligonucleotide probes, designed to bind specifically to small mitogenomic fragments present in HTS libraries prepared on ancient DNA extracts. Following a series of enzymatic elongation, magnetic streptavidin bead capture, and amplification steps, the authors demonstrated significant enrichment of the target in the final library to be sequenced, increasing from a fraction of a percent to 40% in Neanderthal samples. With such enrichment, libraries from multiple individuals can be sequenced simultaneously by HTS (using a Roche FLX platform in this case), yielding extremely well-covered, and thus reliable, mitogenomic sequences. The method is able to recover very small targets, because the probes only require a small capture region (approximately 20 bases) and primer-binding sites are not needed. This extends the range of material from which ancient DNA can be retrieved. Another benefit is the economy that this method brings, although this only comes once the probes have been purchased. With hundreds of biotinylated oligonucleotides required, the initial cost required to set up the system can sit in the tens of thousands of Euros.

5. Source materials

The nature of ancient DNA research, which is often based on samples that have been preserved in suboptimal conditions, creates a challenge with regard to potential source materials. There is differential DNA survival across tissues, but the choice of source material is usually determined by opportunity rather than by technical considerations. To date, a range of source materials have been utilised in ancient DNA research, and several of these have been explored in mitogenomic analyses.

5.1. Soft tissue

In animals, soft tissues are generally the most metabolically active and have the highest *in vivo* concentration of mitochondria and, consequently, mtDNA molecules. In most situations, however, soft tissues undergo rapid post-mortem decay and are usually unavailable for palaeogenetic analysis. To date, ancient mitogenomic sequencing from soft tissue has only been performed for two specimens. The first was the permafrost-preserved leg of a woolly mammoth, which was analysed by Rogaev et al. (2006). The leg, radiocarbon dated at 32,850 ± 900 years old, had been kept frozen since its discovery in north-eastern Siberia in 1986. The second specimen was the 5000-year-old corpse of the Tyrolean Iceman, which had been discovered in a mummified state high in the Alps. Ermini et al. (2008) successfully sequenced the Iceman's mitogenome after extracting DNA from a 70 mg rectal sample.

5.2. Bone

Bones represent the most commonly used type of source material for conventional ancient DNA studies of animals. One obvious reason for this is their widespread occurrence and relative abundance in the archaeological and fossil record, a consequence of the fact that their structure allows them to survive comparatively well while other biomaterials degrade The observed quality of the endogenous DNA is also relatively high, with bones tending to retain the highest DNA concentration and longest fragments compared to other commonly used tissues. Thus, it is not surprising that bone represented the first source of DNA to be shotgun-sequenced with HTS approaches (e.g., Green et al., 2006; Noonan et al., 2006; Poinar et al., 2006).

It soon became apparent, however, that bone suffers from a number of problems that do not necessarily affect some of the other tissues. Above all, their relatively high porosity results in the presence of high levels of contaminants in extracts (e.g., Green et al., 2006, 2008; Noonan et al., 2006). Consequently, bones yield comparatively low amounts of endogenous DNA unless they are suitably decontaminated or enriched (Briggs et al., 2009), or are exceptionally well preserved (e.g., Poinar et al., 2006).

5.3. Hair shafts

Viewed for a long time as a viable, though poor, source of DNA (e.g., Wilson et al., 1995), hair shafts have received renewed interest, in particular as a source for HTS of ancient mitogenomes (Gilbert et al., 2008a,b, 2007c; Miller et al., 2009, 2008; Willerslev et al., 2009). In this context, hair shafts have several useful properties. Where available, they tend to come in large quantities, allowing sampling to be performed with less disfiguration than for bone. Second, hair shafts appear to be relatively resistant to contamination, even when degraded, and seem to be easier to decontaminate than bone (Gilbert et al., 2006, 2007c; Jehaes et al., 1998). Third, although the process of keratinisation means that the DNA in hair shafts initially exists in lower amounts and in shorter fragments, the rate of DNA damage (and thus the rate of sequence error) appears to be lower (Gilbert et al., 2007b, 2004).

In comparison to bone, hair shafts have enriched mtDNA relative to nuclear DNA. For example, Poinar et al. (2006) found that the ratio of mitochondrial to nuclear DNA in a mammoth bone was 1:658, whereas Gilbert et al. (2007c) detected a much higher ratio in the hair shafts of various mammoth specimens, ranging from 1:217 to 1:48. Moreover, it was found that the levels of DNA damage were lower in the hair shafts than in the bone, despite the latter having a lower thermal age (Gilbert et al., 2007c).

5.4. Nail

Nail has been validated as a source of DNA for conventional PCR (Anderson et al., 1999; Gilbert et al., 2007a; Tahir and Watson, 1995). The structural characteristics shared by nail and hair, particularly the fact that both materials are composed of keratinised cells, suggested that they might be of equivalent value in HTS. Using century-old toenail samples from black and Javan rhinoceroses, Willerslev et al. (2009) investigated the utility of nail as a source of mtDNA. These samples yielded 1.21% and 0.41% endogenous mtDNA, respectively, compared with 6.6% from the hair shaft of an ancient woolly rhinoceros. Given the aforementioned variation observed in hair mtDNA levels themselves, however, it is unclear whether these particular results are representative of nail samples in general.

6. Study organisms

Ancient mitogenomes have been sequenced for a limited number of vertebrate taxa, including three genera of birds, one marsupial, and species from four orders of placental mammals (Table 2; Fig. 2). The mitogenomic sequences have been analysed in studies of phylogenetic relationships, divergence times, population genetics, DNA degradation, and forensics.

6.1. Moa

In 2001, two research groups published four mitogenome sequences from three extinct moa species (Cooper et al., 2001; Haddrath and Baker, 2001): one each from the South Island giant moa (*Dinornis giganteus*) and bush moa (*Anomalopteryx didiformis*), and two from the Eastern moa (*Emeus crassus*). In both cases, conventional PCR was used to generate sets of overlapping sequences ranging from 350 to 600 bases in length, which were then assembled to form complete mitogenomes.

The two research teams conducted phylogenetic analyses using these sequences, along with those from closely related birds, in order to investigate the evolution of ratites. This avian superorder is of particular interest because of its Gondwanan distribution: the ostrich is found in Africa, the emu in Australia, cassowaries in Australia and New Guinea, rheas in South America, kiwi and extinct moa in New Zealand, and extinct elephant birds in Madagascar. The studies by Cooper et al. (2001) and Haddrath and Baker (2001) found that the estimated timing of evolutionary divergence events among ratite species did not entirely coincide with the break-up of Gondwana, leading to the conclusion that the kiwi and ostrich reached their present locations by dispersal.

6.2. Elephantids

Within the space of a few weeks, Krause et al. (2006) and Rogaev et al. (2006) independently presented complete mitogenomic sequences of the extinct woolly mammoth. Rogaev et al. (2006), who initiated their mitogenomic project in January 2000, obtained their sequence by assembling a series of overlapping PCR products. In contrast, Krause et al. (2006) employed multiplex PCR with 46 primer pairs before reamplifying each product individually. Rohland et al. (2007) used a similar multiplex-PCR approach, with 78 primer pairs designed to amplify fragments ranging from 139 to 334 bases in length, to sequence the mitogenome of the mastodon from the root of a molar tooth.

Elephantid relationships were one of the key questions that these new studies attempted to address. Specifically, previous analyses of partial mitochondrial sequences were unable to determine decisively whether woolly mammoths were more closely

Table 2
Details of ancient mitochondrial genomes that have been completely or near-completely sequenced.

Species	Binomial	Number of genomes	Mean age estimate (yr BP)[a]	Source material	PCR method	Sequencing method	Reference
Birds							
South Island giant moa	*Dinornis giganteus*	1	*613*	Bone	Simplex	Sanger	Cooper et al. (2001)
Eastern moa	*Emeus crassus*	1	*1245*	Bone	Simplex	Sanger	Cooper et al. (2001)
		1	Unknown	Bone	Simplex	Sanger	Haddrath and Baker (2001)
Bush moa	*Anomalopteryx didiformis*	1	Unknown	Bone	Simplex	Sanger	Haddrath and Baker (2001)
Mammals							
Woolly mammoth	*Mammuthus primigenius*	1	*32,850*	Muscle	Simplex	Sanger	Rogaev et al. (2006)
		1	*27,740*	Bone	Emulsion	Pyrosequencing	Poinar et al. (2006)
							Gilbert et al. (2007a)
		1	*12,170*	Bone	Multiplex	Sanger	Krause et al. (2006)
		10	*17,125–50,200*	Hair	Emulsion	Pyrosequencing	Gilbert et al. (2007b)
		5	*13,995–>63,500*	Hair	Emulsion	Pyrosequencing	Gilbert et al. (2008a)
Mastodon	*Mammut americanum*	1	~90,000	Tooth	Multiplex	Sanger	Rohland et al. (2007)
Woolly rhinoceros	*Coelodonta antiquitatis*	1	Unknown	Hair	Emulsion	Pyrosequencing	Willerslev et al. (2009)
Giant short-faced bear	*Arctodus simus*	1	22,417	Bone	Multiplex	Sanger	Krause et al. (2008)
Cave bear	*Ursus spelaeus*	1	44,160	Bone	Multiplex	Sanger	Krause et al. (2008)
		1	31,870	Bone	Simplex	Sanger	Bon et al. (2008)
		31	Unknown	Bone	Multiplex	Pyrosequencing	Stiller et al. (in press)
Neanderthal	*Homo neanderthalensis*	1	38,310	Bone	Emulsion	Pyrosequencing	Green et al. (2008)
		5	38,790–~65,000	Bone	PEC	Pyrosequencing	Briggs et al. (in press)
Human (Paleo-Eskimo)	*Homo sapiens*	1	3950	Hair	Emulsion	Pyrosequencing	Gilbert et al. (2008b)
Human (Iceman)	*Homo sapiens*	1	5225	Intestine	Simplex	Pyrosequencing	Ermini et al. (2008)
Tasmanian tiger	*Thylacinus cynocephalus*	2	~100	Hair	Emulsion	Pyrosequencing	Miller et al. (2009)
Black rhinoceros	*Diceros bicornis*	1	~100	Nail	Emulsion	Pyrosequencing	Willerslev et al. (2009)
Javan rhinoceros	*Rhinoceros sondaicus*	1	~100	Nail	Emulsion	Pyrosequencing	Willerslev et al. (2009)
Human (Romanovs)	*Homo sapiens*	4	~100	Bone	All	Sanger	Rogaev et al. (2009)

[a] Dates in italic type are given in uncalibrated ^{14}C years before 1950, while dates in roman type are given in calendar years before present. Dates of unknown status (calibrated/uncalibrated) are assumed to have been given in calendar years.

related to Asian or African elephants. The new mitogenomic studies found compelling support for a sister relationship between the woolly mammoth and Asian elephant. This conclusion was strengthened by the analysis that included a mastodon mitogenome (Rohland et al., 2007), which overcame the previous problem of using distant outgroup taxa (hyrax and dugong).

Poinar et al. (2006) analysed a mammoth bone using emulsion PCR and pyrosequencing, generating about 13 million bases of endogenous DNA. From 137,527 sequence reads, 209 were identified as being mtDNA, spanning 10,964 unique bases (~65%) of the mitogenome. From this output, along with subsequently generated data, Gilbert et al. (2007b) assembled a complete mitogenomic sequence.

More recently, the emergence of ancient population mitogenomics was marked by the sequencing of 15 mammoth mitogenomes by HTS analysis of DNA extracted from ancient hair shafts (Gilbert et al., 2008a, 2007c). Combined with the three previously published mitogenomes, the new sequences enabled the study of woolly mammoths using population-genetic and phylogenetic methods. These studies confirmed the existence of a deep mitochondrial split between two mammoth populations, one of which went extinct at least tens of thousands of years earlier than the other. Upon modelling the structures of the mitochondrial proteins, the authors did not find evidence of any important structural or putative functional disparities that could have been responsible for the differential survival of the two populations (Gilbert et al., 2008a).

6.3. Bears

In 2008, the complete mitochondrial genome of the extinct cave bear was sequenced independently by two teams of researchers. Bon et al. (2008) obtained a mitogenomic sequence from a bone in the Palaeolithic painted cave of Chauvet-Pont d'Arc, southern France, whereas Krause et al. (2008) analysed a femur discovered in Gamssulzen Cave, Austria. The groups used conventional PCR and multiplex PCR, respectively, to amplify DNA for sequencing. Krause et al. (2008) also presented the mitogenomic sequence of the North American giant short-faced bear, acquired from a calcaneus bone found in the Yukon Territory, Canada.

In both studies, the authors investigated the phylogenetic relationships among bears by including the complete mitogenomes of all eight extant ursid species. Several general conclusions were shared by the two reports. First, compared with previous studies, the increased size of the data set led to an improvement in phylogenetic resolution. Second, the bear family experienced a relatively rapid succession of evolutionary divergence events. However, Krause et al. (2008) and Bon et al. (2008) produced disparate estimates of the overall evolutionary timescale, disagreeing also with a third recent analysis of ursid mitogenomes (Yu et al., 2007). These disparities appear to have been the result of differences in the age calibrations employed by the three studies (Ho, 2009).

More recently, Stiller et al. (in press) employed a novel approach to obtain complete or near-complete mitogenomic sequences of 31 cave bears. The authors amplified DNA using multiplex PCR, then tagged the products with barcodes before sequencing them in two successive HTS runs. This resulted in good coverage of the mitogenomes, ranging from 13,631 to 16,393 nucleotides and representing an overall completeness of about 96%. Based on a phylogenetic analysis, Stiller et al. (in press) found that the 31 bears were placed into three strongly-supported monophyletic groups, consistent with previously proposed subspecies.

6.4. Rhinoceroses

The phylogenetic relationships among the rhinoceroses have been a source of ongoing dispute, with the publication of partial mitochondrial sequences failing to settle the issue convincingly (Orlando et al., 2003; Tougard et al., 2001). In an attempt to address this outstanding problem, Willerslev et al. (2009) used HTS

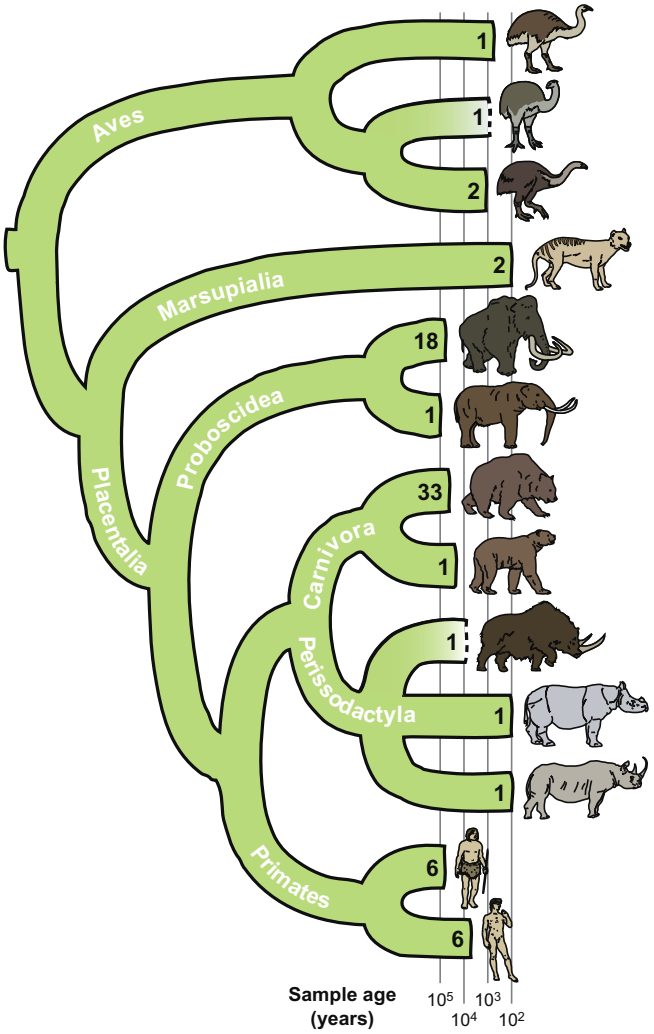

Fig. 2. Cladogram showing the evolutionary relationships among the animal mitogenomes that have been completely sequenced from ancient samples. From the top, the organisms are bush moa (*Anomalopteryx didiformis*), South Island giant moa (*Dinornis giganteus*), eastern moa (*Emeus crassus*), Tasmanian tiger (*Thylacinus cynocephalus*), woolly mammoth (*Mammuthus primigenius*), mastodon (*Mammut americanum*), cave bear (*Ursus spelaeus*), giant short-faced bear (*Arctodus simus*), woolly rhinoceros (*Coelodonta antiquitatis*), Javan rhinoceros (*Rhinoceros sondaicus*), black rhinoceros (*Diceros bicornis*), Neanderthal (*Homo neanderthalensis*), and modern human (*Homo sapiens*). Numbers at the tips denote the number of ancient mitogenomic sequences that have been published for each organism. The tips of the tree are positioned on a logarithmic scale, indicating the age of the oldest sample yielding a complete mitogenome. Note that the ages of the South Island giant moa and woolly rhinoceros samples are unknown.

methods to obtain mitogenomic sequences from a Pleistocene hair sample of the extinct woolly rhinoceros, and from historic samples of the extant black and Javan rhinoceroses (see Section 6.6).

Willerslev et al. (2009) were unable to resolve the higher-level relationships within Rhinocerotidae, even though their analysis included complete mitogenomes from all five extant rhinocerotid species and the woolly rhinoceros. There was substantial conflict in the phylogenetic signal among genes, while the choice of outgroup taxon (tapir and/or horse) had a noticeable effect on the inferred tree.

6.5. Humans and Neanderthals

Ever since the publication of the human mitochondrial genome in 1981 (Anderson et al., 1981), mtDNA has been used extensively in studies of human evolution and demography. For example, our current views on the timing of prehistoric human migrations are largely based on mitochondrial estimates (for a recent review, see Endicott et al., 2009). Obtaining DNA sequences from ancient hominins, however, has been a difficult and contentious exercise because of the heightened risk of contamination (Handt et al., 1994; Richards et al., 1995).

It was only in 2008 that an ancient human mitogenome was finally sequenced in its entirety. Gilbert et al. (2008b) employed HTS methods to analyse the mtDNA of an approximately 4000-year-old male Palaeo-Eskimo from Greenland. By determining the sequence of the individual's mitogenome, the authors were able to assign him to mitochondrial haplogroup D2a1, which includes modern Aleuts and Siberian Sireniki Yuit (Eskimos). Most importantly, the Palaeo-Eskimo did not fall within the diversity of modern Native Americans or Eskimos, indicating that the earliest migrants into Greenland were not directly related to the maternal gene pool of the modern native inhabitants of North America (Gilbert et al., 2008b).

More recently, Ermini et al. (2008) used conventional PCR and pyrosequencing to obtain the mitogenome of the Tyrolean Iceman, popularly known as Ötzi, whose body had been preserved by chance high in the Tyrolean Alps following his putative violent death over 5000 years ago. Upon comparing his mitogenomic sequence to those of 115 modern individuals from mitochondrial haplogroup K, the authors confirmed previous reports that the Iceman belonged to a subgroup of K1 (Endicott et al., 2007; Rollo et al., 2006). Ermini et al. (2008) were unable to find any exact modern matches to the Iceman's sequence.

Archaeological specimens can also yield genetic information. By analysing partial mtDNA sequences extracted from remains from two graves near Yekaterinburg, various studies have produced positive identifications of members of the Romanov Imperial family, who were believed to have been murdered in 1918 during the Russian Civil War (Coble et al., 2009; Gill et al., 1994; Ivanov et al., 1996). Rogaev et al. (2009) sequenced the complete mitogenomes of Emperor Nicholas II and Empress Alexandra from the first grave, along with two of their children, Crown Prince Alexei and Grand Duchess Maria, in the second grave. The authors successfully used three different PCR methods: conventional PCR, emulsion PCR, and multiplex PCR. Together with corroborating evidence from nuclear DNA, anthropological examination, and previous studies, the findings of Rogaev et al. (2009) provide a conclusive demonstration that the remains in the two graves represent the entire Romanov family and several servants.

The mitogenome of the Neanderthal was sequenced by Green et al. (2008), adding to the 15 published partial control-region sequences. Ranging in age from 29,000 to 100,000 years, these sequences collectively indicate that Neanderthals did not contribute to the extant mitochondrial diversity in modern humans (Hebsgaard et al., 2007; Hodgson and Disotell, 2008). To obtain their sequence data, Green et al. (2008) had to overcome a number of significant methodological challenges. Their sample was a 38,310-year-old bone which had been excavated from Vindija Cave, Croatia. The influence of post-mortem sequence damage was minimised by the manifold sequencing coverage (averaging 35-fold coverage throughout the mitogenome), and the contamination rate was estimated to be very low. The phylogenetic analysis performed by Green et al. (2008) confirmed that the Neanderthal mtDNA lies well outside the modern human diversity, with the two lineages having diverged about 660,000 years ago. Observing an excess of nonsynonymous changes along the Neanderthal lineage, the authors suggested that Neanderthals had a smaller effective population size than humans.

A further five Neanderthal mitogenomes have subsequently been sequenced by Briggs et al. (2009), who developed a primer-extension-capture method to isolate specific sequences from a

library of DNA fragments. As a result, mtDNA constituted 18–40% of the output of their HTS analyses, compared with a native, un-enriched content of about 0.001% in typical Neanderthal specimens. Bayesian phylogenetic analysis of the six available Neanderthal mitogenomes, along with representative sequences from modern humans, yielded age estimates for various events in hominin evolution: 85,000–139,000 years for the mitochondrial ancestor of Neanderthals, 95,000–179,000 years for the mitochondrial ancestor of modern humans, and 322,000–554,000 for the mitochondrial divergence between the two species (Briggs et al., 2009).

6.6. Archival specimens

Mitogenomic sequences can be obtained from museum specimens that are decades or even centuries old, despite the usually suboptimal conditions of preservation. For example, the common preservative formaldehyde is known to react adversely with DNA (Feldman, 1973). The possibility of sequencing museum specimens allows genetic studies of species that are difficult to find in the wild, or that went extinct in historical times. In a recent study, the mitogenomes of the black and Javan rhinoceroses were obtained from museum specimens (Willerslev et al., 2009). Using an HTS approach, Miller et al. (2009) obtained mitogenomic sequences from two specimens of the Tasmanian tiger, or thylacine. This species went extinct in 1936, when the last known individual died at Hobart Zoo. The specimens used by Miller et al. (2009), which comprised a dried skin as well as an entire alcohol-preserved organism, dated from 1905 and 1893, respectively.

7. Future prospects

The advent of high-throughput sequencing methods has allowed the rapid production of large amounts of DNA sequence data. This technology has been exploited in the field of ancient DNA to generate not only mitogenomic sequences, but also large volumes of nuclear sequence data (Blow et al., 2008; Green et al., 2006; Miller et al., 2008; Noonan et al., 2006; Poinar et al., 2006). The recent publication of a draft nuclear genome of the woolly mammoth (Miller et al., 2008), along with the impending release of the Neanderthal genome, provides a compelling signal that past technical and methodological barriers have been largely overcome.

In spite of the recent progress in ancient nuclear genomics, there is evidently a place for ancient mitogenomics. Mitochondrial genome sequences are useful in population genetic, phylogenetic, and phylogeographic analyses. This is largely due to the high substitution rate observed in mtDNA, particularly in the non-coding control region, which allows sequences to exhibit an appreciable degree of variation even at the intraspecific level. Nuclear markers often used in population genetics, such as microsatellites, are not well characterised in ancient organisms, but will become increasingly important in the future (Allentoft et al., 2009). Compared to nuclear DNA, the higher survival rate and greater degree of sequencing coverage of ancient mtDNA leads to a markedly reduced influence from post-mortem damage and contamination. The function and properties of several mitochondrial proteins have been investigated in some detail, while secondary structural models exist for mitochondrial RNA molecules. For these reasons, in spite of its shortcomings as a genetic marker, it appears that the mitogenome will continue to play an important role in ancient population genetics and phylogenetics in coming years.

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