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SYSTEMATIC REVISION

Why look back? Methods and relevance of ancient DNA studies

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ABSTRACT

There is an enormous potential in the analysis of ancient DNA, from revealing the genomes of human ancestors to disentangling the origins and evolution of domesticated animals associated with patterns of human settlements and migrations.

In this review we describe the technical aspects for a successful, authentic and reliable recovery of ancient DNA. The aim is to provide an overview of the interesting yet complex process of analyzing DNA obtained from archaeological remains

Keywords: ancient DNA; phylochronology; archaeogenetics; paleogenomics

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RESUMO

Existe um enorme potencial na análise de DNA antigo, desde a descoberta de genomas dos nossos ancestrais até à compreensão sobre as origens e a evolução dos animais domésticos associados a populações humanas e às suas migrações. Nesta revisão descrevemos os aspectos técnicos relacionados com a recuperação de DNA antigo autêntico. O objectivo é proporcionar uma revisão do processo interessante, mas complexo, de análise do DNA recuperado de restos arqueológicos.

Palavras-chave: DNA antigo; filocronologia; arqueogenética; paleogenómica

Introduction

The reconstruction of past events and the understanding of evolutionary processes may be impaired if one relies solely on inferences drawn from modern genetic data (Ramakrishnan and Hadly, 2009). Even though past evolutionary events left specific signatures in the genome of modern species, which to some extent can be assessed by analyzing modern DNA (Barbujani and Chikhi, 2006), some information will remain obscured by the dominance of more recent processes. Understanding the history of populations directly from data obtained from archaeological samples can provide extremely useful information, but it is no trivial task. Adequate samples are difficult to obtain, ancient DNA (aDNA) analysis requires stringent and refined methodological procedures, as well as considerable laboratory expertise.

In studies of the genetic composition of past populations it is important to ensure adequate sample sizes and representative geographic coverage to estimate levels of genetic diversity, to analyze population structure and to infer phylogenies more accurately (Hedrick and Waits, 2005). A phylochronological approach in which populations are studied in space and time using serially sampled genetic data has been proposed (Hadly *et al.*, 2004; Ramakrishnan *et al.*, 2005). Ideally, a phylochronological research makes use of population genetics and phylogenetic methods to analyze genetic data recovered from both archaeological remains and modern populations.

The first successful extraction of old DNA was made in 1984 from a 140 year old museum specimen of a quagga (*Equus quagga quagga*) (Higuchi *et al.*, 1984), an event that saw the beginning of archaeogenetics (Hofreiter, 2008). After the

development of the polymerase chain reaction (PCR) (Mullis and Faloona, 1987), it became possible to amplify small amounts of fragmented DNA from specimens stored in museums for many years. Methods have been developed and criteria established to reproduce results, to identify contamination by modern DNA and to account for sequencing inaccuracies (Paabo *et al.*, 1984; Green *et al.*, 2009). Despite the increasing number of publications in this field over the years (Ramakrishnan and Hadly, 2009), there are several limitations and technical constraints associated with the analysis of aDNA. Several authors consider that it is not possible to amplify DNA molecules older than one million years (Paabo and Wilson, 1991; Lindahl, 1993; Hofreiter *et al.*, 2001). In fact, publications reporting amplification of aDNA obtained from Miocene (23 to 5 million years before present, BP) plant fossils (Golenberg *et al.*, 1990; Soltis *et al.*, 1992) and insects preserved in amber (DeSalle *et al.*, 1992; Cano *et al.*, 1993) have been questioned (Sidow *et al.*, 1991; Austin *et al.*, 1997). Although recent technical advances such as high-throughput DNA sequencing (see the last section of this paper) opened novel opportunities for aDNA-based studies, even at the level of paleogenomics (recovery and analysis of genomes from ancient samples), there are difficulties that arise mainly in assessing the authenticity of whole-genome DNA shot-gun sequences obtained from ancient genomes (Green *et al.*, 2009).

In this review we will describe technical aspects associated with successful, authentic and reliable aDNA recovery. We hope to provide an interesting and comprehensive overview of the process of analyzing DNA obtained from archaeological remains.

Retrieval of ancient DNA – a demanding task

The emerging field of archaeogenetics undertaken by evolutionary biologists and archaeologists allows one to travel back in time. It relies on the analysis of DNA directly obtained from ancient tissues, avoiding the limitations of genetic inferences based solely on extant samples. The basic procedures for aDNA retrieval can be described as follows: excavation, biometrics, external cleaning, fragmentation or powdering, DNA extraction, PCR amplification and targeted sequencing of a specific genomic region or enrichment and library construction for whole-genome sequencing, bioinformatic analysis and authentication (Schlumbaum *et al.*, 2008). However, poor DNA preservation in archaeological remains can impair genetic studies of past populations. Preservation and DNA integrity are a function of intrinsic factors related to the organic material *post-mortem* degradation and fossilization, *e.g.* fragmentation, organic and chemical modification (Paabo *et al.*, 1989; Lindahl, 1993; Paabo *et al.*, 2004; Geigl, 2008) and, environmental factors. Certain environments (cold, dry and/or low oxygen) are more favorable for molecular preservation of DNA during burials, *e.g.* permafrost regions (Geigl, 2008). In contrast, DNA preservation in temperate regions is much more difficult to obtain. Endogenous DNA degradation can occur even faster after excavation, due to temperature increase, desalting and

decrease of pH during cleaning (Pruvost *et al.*, 2007). Thus, several authors suggest storage of freshly excavated material in dry and cold conditions or even frozen (Burger *et al.*, 1999; Smith *et al.*, 2001; Pruvost *et al.*, 2007).

The use of specifically dedicated facilities for aDNA analysis is mandatory. There are several examples of state-of-the-art aDNA laboratories around the world and these must follow strict rules for the maintenance of an almost sterile environment and control for contamination. There should be a clean area physically separated from general analyses areas where modern samples and PCR products might be present. No modern DNA experiments should ever be carried out in a dedicated aDNA laboratory. Inside the clean area, where ancient samples are handled for DNA extraction and PCR or sequencing set up, the environment must be as clean as possible and have its own independent air system. Frequent decontamination of the laboratory areas (*e.g.* weekly), with for example, 10% bleach solution (or Actril), is essential and should target all working surfaces (benches and equipment), and also walls and ceilings. Environmental controls, as well as the swabbing of work surfaces and other areas, are recommended procedures to check regularly for the presence of DNA from any species by PCR amplification using universal primers (*e.g.* within the cytochrome *b* gene, cyt *b*) (Telechea *et al.*, 2007).

Additionally, molecular techniques for DNA extraction need to be appropriate for each sample type (*e.g.* museum specimens or freshly excavated material) and according to the biological source: faunal (hair, bones, teeth), plants (seeds, pollen and wood), or sediments. The extraction of aDNA itself is challenging. Although a vast number of extraction methods exist (Rohland and Hofreiter, 2007b), there are two major approaches: organic extraction with phenol/chloroform and the silica-binding method. The silica-based method appears to be more efficient in extracting and purifying DNA from bone either in forensic (Davoren *et al.*, 2007) or in aDNA studies. Alternatively, Rohland and Hofreiter (2007a) propose an improved silica-based protocol for maximum recovery of PCR-amplifiable DNA from ancient bones and simultaneously minimum co-extraction of PCR inhibitory substances. This consists of a simplified approach which uses an EDTA buffer and proteinase K for bone digestion, followed by DNA-binding to silica with guanidinium thiocyanate for DNA purification. These authors determined that the addition of bovine serum albumin to the PCR helps to overcome amplification inhibitors present in aDNA extracts and also concluded that the inactive *Taq* DNA polymerase derived from a recombinant *Taq* DNA polymerase (*e.g.* AmpliTaq Gold; Applied Biosystems) is one of the most suitables for aDNA amplification. However, serious contamination problems can arise from the use of reagents containing significant amounts of modern DNA. For example, in studies of domestic animals (*e.g.* cattle or pigs), contamination can occur from BSA, as well as from enzymes containing gelatin derived from animal sources. Some authors have published useful guidelines to avoid contamination from reagents in aDNA studies (Champlot *et al.*, 2010).

Since the success rate for undamaged aDNA isolation tends to be very low, *i.e.* at the level of 10% as reported by Leonard and collaborators (Leonard *et al.*, 2007), numerous samples need to be collected and tested before a representative sample can be attained. The scarcity of ancient remains for a significant analysis both across time and space makes sampling a demanding and time consuming task. It is important to stress at this point that the collection of source material is inevitably done at the expense of precious ancient remains (see Figure 1), although sampling techniques are becoming minimally destructive and cosmetically sensitive (Seco-Morais *et al.*, 2007). Fortunately, advances in molecular biology make it possible to maximize the information obtained from minute amounts of aDNA (see the last section of this paper). In aDNA studies, a serious concern is always present, that can be a source of false positive results: contamination. It remains the responsibility of the researcher to guarantee and demonstrate that proper experimental design and authentication procedures are carried out in each study to avoid and account for contamination (Cooper and Poinar, 2000; Gilbert *et al.*, 2005). In the next section we will provide an overview of the recommended procedures to avoid contamination and to validate aDNA results.

Accounting for contamination and results authentication

Contamination - a trap for ancient DNA research

Contamination can be a very tricky issue when analyzing aDNA, as it can lead to false positive results. It can occur in the field before the samples are analyzed in the laboratory, from 'environmental' DNA (*e.g.* soil, handling and animal products) or from inter-sample contamination before PCR and sequencing. Other sources of contamination can also be animal DNA present in PCR reagents, and/or previous PCR products (Pruvost and Geigl, 2004; Willerslev and Cooper, 2005; Leonard *et al.*, 2007). The sources of contamination vary according to the object being studied: for example, for sediments there is the risk of vertical DNA migration across strata (Hofreiter, 2003; Willerslev *et al.*, 2003; Allendorf *et al.*, 2008), whereas for bones contamination can occur during handling at the excavation sites (Yang and Watt, 2005). Physical methods, such as the use of sandpaper for polishing or electric drills, can be used to clean the outermost surface of some types of specimens to prevent contamination before aDNA extraction. Chemical decontamination of bones is possible; for example, by using an aggressive treatment with 0.1M HCl, 0.5% bleach on powdered samples (Malmstrom *et al.*, 2007) or ultraviolet irradiation, but with some risks (O'Rourke *et al.*, 2000). In genetic studies of ancient fauna, the focus of this review, contamination with exogenous DNA can originate from cross-sample contamination in the same deposit and/or from modern reference specimens used during identification of the remains (Yang and Watt, 2005). Human DNA contamination, from manipulation, is prone to occur as well (Malmstrom *et al.*, 2005).

Methods to prevent and to detect contamination, and also authenticity criteria,

have been presented and improved by many authors (*e.g.* Cooper and Poinar, 2000). Prevention of contamination is much easier to guarantee and more efficient than decontamination procedures, which can be invasive or, worse still, destructive. Specific recommendations for the sampling of excavated and stored remains, including hominids, have been thoroughly described by Yang and Watt (2005) and Hublin *et al.* (2008). These guidelines involve prevention of endogenous DNA degradation and its contamination, as well as minimizing invasive sampling. Briefly, avoidance of pre-laboratory contamination should start at the excavation site and be carried out efficiently by field archaeologists, who are the first to handle the remains. Excavations should be carried out as aseptically as possible by using gloves and bleached instruments when manipulating specimens for subsequent genetic analysis. Remains should be stored individually in sterile plastic bags and preferably frozen at –20ºC as soon as possible. Screening for DNA contamination from field and laboratory workers, especially when human remains are being investigated, is important. Within the framework of the Neanderthal genome project, biotinylated oligonucleotide primers have been used to capture target sequences from adaptor-ligated aDNA libraries (primer extension capture, PEC) allowing for high specificity and contamination control (Briggs *et al.*, 2009b). The development of modern high-throughput sequencing techniques raises new questions regarding aDNA contamination and analysis, and most probably will require the definition of new guidelines to guarantee the authenticity and accuracy of results. For example, due to the high levels of environmental contaminants present in bone specimens, it is necessary to generate an enormous number of shotgun sequences to be able to recover entire aDNA genomes (Briggs *et al.*, 2009a). Additionally, many parallel amplification reactions are needed to sequence hundreds of very short overlapping PCR fragments obtained from heavily degraded aDNA, such as that retrieved from Neanderthal specimens older than 40,000 years, which increases the probability of cross-contamination and makes use of large amounts of precious biological material (Briggs *et al.*, 2009a).

Figure 1 - Sampling techniques are becoming minimally destructive and cosmetically sensitive. Here a little hole made on the surface of the bone with a Dremel tool to collect bone tissue (powder) from the inside.

Authentication – no absolute criteria

Regarding within-laboratory prevention and detection of contamination, it has been suggested that work in aDNA-dedicated laboratories, with characteristics as described

in the previous section is fundamental. There are several renowned laboratories around the world, which have the conditions and expertise to conduct such studies. Ancient DNA is of sub-standard quality and, therefore, strict controls must be established and demonstrated in order to guarantee the reliability of data (Gilbert *et al.*, 2005). In addition, it is important to assess the quality and utility of the extracted aDNA prior to further molecular analysis. Different methods can be used: indirectly by amino acid racemization analysis (Poinar *et al.*, 1996), or the more recently proposed real-time qPCR (Hofreiter *et al.*, 2001; Morin *et al.*, 2001; Pruvost and Geigl, 2004; Schwarz *et al.*, 2009). Imprecision of the first method has recently been pointed out (Schwarz *et al.*, 2009). The latter has the advantage of being informative regarding possible inhibition, and it allows determination of the suitable volume, dilution factor and purification of the extracted aDNA for downstream analysis (*e.g.* Pruvost and Geigl, 2004). When the DNA amount is limiting in the beginning of a PCR reaction and also degraded, several repetitions, as well as high-coverage sequencing (either through cloning of several PCR products or by employing high-throughput methods) are necessary to obtain accurate results (Handt *et al.*, 1996; Cooper *et al.*, 2001).

Cloning can be used to detect the presence (number and percentage) of different types of sequences and potential NuMts (nuclear copies of mitochondrial sequences). For example, Ermini *et al.* (2008) were able to identify a mutated position in the mtDNA genome of an Iceman simply by sequencing a large number of clones, which had been missed in a previous study where fewer clones were screened. The development of high-throughput sequencing methods (*e.g.* 454 GS Junior sequencing, Roche) make it possible to obtain large numbers of aDNA sequences in a short time, *i.e.* high-coverage, which allows the identification of contaminant sequences and to authenticate results.

Additionally, along with the process of aDNA analysis, the inclusion of multiple blank extractions as well as negative PCR and sequencing controls (at high ratios) is critical to monitor for contamination. The use of modern positive PCR controls should be avoided due to obvious contamination risks (Willerslev and Cooper, 2005). Reproduction of results (at least at the 10% level) from independent extractions from the same biological source within the same laboratory, and when needed in an independent laboratory, is common practice in high-standard aDNA research.

Some useful indicators can inform whether reliable aDNA data were obtained: inverse relationship between amplification efficiency and fragment length (Handt *et al.*, 1994), as well as standard tests of the data phylogenetic sense (Handt *et al.*, 1996). Altogether the above methods will help to demonstrate whether aDNA is authentic and not a contamination by modern DNA, although no absolute criteria prove the antiquity and quality of the aDNA. Authentication criteria have been described and updated thoroughly in several publications over time (Handt *et al.*, 1994; Willerslev *et al.*, 1999; Cooper and Poinar,

2000; Hansen *et al.*, 2001; Hofreiter *et al.*, 2001; Gilbert *et al.*, 2003; Paabo *et al.*, 2004; Gilbert *et al.*, 2005; Willerslev and Cooper, 2005; Schlumbaum *et al.*, 2008; Green *et al.*, 2009; Krause *et al.*, 2010). Gilbert and collaborators (2005) make a pertinent observation about the importance of researchers pursuing these authentication procedures for the validation of data and at the same time following a cognitive approach towards their conduct.

Human DNA contamination in animal samples is common and can be identified, limited or circumvented if specific PCR primers are carefully designed and used in the reactions. Moreover, subsequent cloning will allow the detection of sequences of human origin. For Neanderthal extracts, the case is complicated due to the similarities between human and Neanderthal genomes. Contamination should be assayed using fixed differences in the Neanderthals nuclear genome where differences from modern humans are identified (Green *et al.*, 2009). Malmstrom *et al.* (2007), tested the reliability of one criterion of authentication – ‘‘appropriate molecular behaviour’’ (Cooper and Poinar, 2000) of *bona fide* aDNA. The authors concluded that authentic and contaminant DNA behaves differently and that this asymmetry can be used to identify authentic aDNA. For example, bleach treatment has a more detrimental effect on contaminant DNA and as the PCR target fragment size decreases there is a substantial increase of the authentic DNA proportion. Using different quantification methods and different types of data sets this asymmetry is apparently the only known detectable and quantifiable difference between contaminating modern human and ancient human DNA (Malmstrom *et al.*, 2007). In the framework of the recently released complete mitochondrial genome of a Siberian hominin (Krause *et al.*, 2010), the patterns of aDNA degradation were used to ascertain the authenticity of the sequences obtained in comparison to the potentially contaminant modern human DNA. The level of post-mortem damage of DNA depends on the time elapsed since the death of the organism, and on the effects of exposure of biological remains to taphonomic processes during site deposition.

Reliable ancient DNA research: how far back can we look?

Kinetic studies of DNA degradation indicate that DNA fragments might not survive more than 10,000 years in temperate regions and around 100,000 years in colder regions (Poinar *et al.*, 1996; Smith *et al.*, 2001; Binladen *et al.*, 2007b). However, this limit can be exceeded in especially well-preserved specimens, such as those recovered from permafrost (Orlando *et al.* 2013). Diachronic studies (longitudinal studies over time) using aDNA sequences are probably limited to approximately the past 100,000 years (Hofreiter *et al.*, 2001). However, aDNA has been obtained from cave bear samples dating from ~110,000 years to as old as 400,000 years old, where the authors were able to screen single nucleotide polymorphisms (SNPs) by targeting short cyt *b* fragments through pyrosequencing

(Valdiosera *et al.*, 2006). The samples analysed in this study were collected in caves, where temperature and humidity are reasonably constant. Therefore, this should be regarded as an example of exceptional aDNA recovery from >100,000 year old specimens preserved under optimal environmental conditions. Case studies of DNA retrieval from specimens as old as millions of years are suspicious, simply because they violate the theoretical limit of DNA survival (Cooper and Wayne, 1998). So far, aDNA has been extracted from several biological sources such as teeth, bones, hair, skins, mummified tissue, fish scales, fingernails, feathers, eggshell, coprolites and seeds obtained from museum collections or archaeological sites (Brown and Brown, 1994; Hofreiter *et al.*, 2001; Gilbert *et al.*, 2004; Leonard, 2008; Oskam *et al.*, 2010).

The length of the average aDNA molecule is generally around 100-200 bp (Green *et al.*, 2006; Poinar *et al.*, 2006), but most probably no longer than a few hundred base pairs, depending on the environmental conditions under which the specimens were preserved. With endogenous DNA repair mechanisms no longer operating *post-mortem*, DNA starts to decay and suffers chemical modifications. These DNA lesions have been thoroughly reviewed by Pääbo and collaborators (2004) and can consist of strand breaks, oxidative lesions, hydrolytic lesions and crosslinks. Lesions that may occur and accumulate in the course of millennia can result in highly fragmented aDNA, and substantially altered sequences can be obtained after PCR amplification and sequencing. Template DNA with such modifications produces typical PCR errors and evident sequencing artifacts, *e.g.* a CG (cytosine guanine) to TA (thymine adenine) bias due to hydrolytic deamination of cytosine to uracil and thymine, and/or formation of chimeric sequences through "jumping PCR". Depending on time and environmental conditions, DNA degradation may actually leave no intact molecules. All these base lesions may cause nucleotide misincorporations when aDNA sequences are replicated during enzymatic amplification and large-scale sequencing (Stiller *et al.*, 2006). Ancient DNA is invariably damaged but, fortunately, there are methods that can improve the quality of the template to be amplified and/or sequenced, or the PCR performance such as Uracil-N-glycosylase (UNG) and N-phenacylthiazolium bromide (PTB). High-fidelity polymerase enzymes, such as Pfu, Taq HiFi (Willerslev and Cooper, 2005), Phusion polymerase (Finnzymes as suggested in the Illumina library preparation protocol) (Rasmussen *et al.*, 2010), as well as a novel hybrid *Taq* polymerase variant are capable of extending DNA despite base mismatches and limiting misincorporations (Gloeckner *et al.*, 2007). The challenge when studying aDNA is directly related to the quality of the preserved molecule, but also to the genomic region targeted, as we explain in the following section.

Molecular markers: Nuclear versus organellular ancient DNA

Various aDNA evolutionary studies rely on the analysis of organellular rather than nuclear DNA and focus on neutrally evolving

and non-coding loci. The mitochondrial genome has some interesting peculiarities: it is only 1/200,000 the size of the nuclear genome, has a rate of evolution that is five-to-ten times greater than the nuclear genome, occurs in many copies per cell and is maternally inherited almost without recombination. However, in particular for population genetic studies in closely related species, there is the need to use nuclear DNA as well for a multi-locus approach to make more precise inferences (Bruford *et al.*, 2003). Y-chromosome markers, for example, are very informative regarding patrilineal inheritance, and may tell a different story than that of the maternal lineages. Nuclear microsatellites are also useful in detecting signatures of more recent processes and in inferring relationships among modern populations, but their use is limited in aDNA studies due to difficulties in targeting repetitive regions. In addition, the analysis of functional nuclear loci is extremely informative, for example, to investigate economic and domestication-related traits, genetic variation of the immune system, to identify selection for specific functions and to detect environmental adaptation (Jaenicke-Despres *et al.*, 2003; Svensson *et al.*, 2007).

There are, however, either intrinsic or technical limitations associated with each of the above mentioned molecular markers when doing aDNA research: nuclear DNA is expected to be harder to retrieve, whereas multicopy DNA, such as that in organelles (mitochondria in animals or chloroplasts in plants), is more likely to be present. Microsatellite loci, in particular, can have lengths over 300 base pairs (bp) exceeding the limit of aDNA amplification; but more importantly, polymerase can slip during PCR, and wrong allele sizes can be scored due to degradation of the template DNA (Schlumbaum *et al.*, 2008). Although extensively used, there are also problems regarding mitochondrial DNA. Its validity for population level studies has been questioned due to several particularities. Being a single genetic locus, it may not thoroughly reveal the history of the genome (Hofreiter *et al.*, 2001). As evidenced by Blow and collaborators (2008), it is susceptible to potential biases and quantitative errors, since undamaged modern contaminant DNA can be preferentially amplified. Events such as trafficking of genetic material from the mitochondria to the nucleus, (NuMts, see section “Accounting for contamination and results authentication”) may impair the ascertainment of organellular DNA sequences (Kolokotronis *et al.*, 2007). Assumptions regarding its clonality, or strict clonal transmission, neutrality and clock-like nature have been challenged (see the review of Galtier *et al.*, 2009). There is evidence for heteroplasmy due to recombination or biparental transmission (Eyre-Walker, 2000; Hey, 2000; White *et al.*, 2008); Bazin and colleagues (2006) demonstrated the non-neutrality of mtDNA by a meta-analysis of over 1,600 animal species, and it is clear that the dynamics of the mtDNA genome and its diversity patterns are not clearly understood. Variations in mutation rates were detected across various lineages questioning the clock-like nature of mtDNA (Galtier *et al.*, 2009). Thus, caution is required when using mtDNA as a molecular marker. Additionally, most of the molecular markers applied to aDNA have

been developed in modern samples, which can introduce ascertainment bias (Leonard, 2008). We may or may not find the same variation as detected in modern samples, and we will certainly miss some of the variation exclusive to ancient populations. That is why direct screening of ancient populations for molecular markers is important to correctly assess past genetic diversity (Leonard, 2008). Complete genomes of several species are now being sequenced and many genes will be fully described and eventually their functions characterized (Xia *et al.*, 2009), oppening new possibilities for the development of additional molecular markers. Fortunately, new strategies are being developed that will allow the use of small amounts of archaeological material which contain degraded endogenous DNA, to analyze nuclear aDNA at the level of genomics.

Powerful technical approaches for ancient DNA analysis

As already mentioned, samples for aDNA studies may contain very little endogenous aDNA, and the template has suffered chemical modifications such as presented by Pruvost and collaborators (2004). The traditional strategy adopted to increase the amounts of target DNA is PCR (Mullis and Faloona, 1987). However, there are some limitations associated with conventional PCR amplification when working with aDNA. The original template is likely to have become damaged over time and so constitutes a poor substrate for *Taq* polymerases. PCR can be unreliable for various reasons such as its high sensitivity to contaminant DNA and the presence of PCR inhibitors. Specific methodological procedures are, therefore, needed.

There are non-PCR based methods to increase the quantity of aDNA recovered, such as whole genome amplification (WGA) using the multiple displacement amplification (MDA) technique (Dean *et al.*, 2002). This method has been successfully applied, for example by Druzhkova *et al.* (Druzhkova *et al.*, 2013), to increase the initial amount of aDNA retrieved from the remains of a putative 33,000 year old Pleistocene dog from Altai (Siberia), and as a preliminary procedure for PCR and sequencing experiments.

Moreover, next-generation DNA sequencing methods (Metzker, 2010) requiring minute amounts of nucleic acids have enabled the unprecedented study of ancient nuclear genomes, even of human ancestors (Green *et al.*, 2006; Noonan *et al.*, 2006; Poinar *et al.*, 2006; Shendure and Ji, 2008; Rasmussen *et al.*, 2010). These new DNA technologies have revolutionized the field of archaeogenetics. Sequences produced by next-generation technologies are detected as they are being synthesized – sequencing by synthesis (SBS) (Fuller *et al.*, 2009). Several methods have been designed: (1) emulsion-based technology and (2) bridge amplification, which both require DNA amplification; and (3) a high-definition optical system that is able to detect single-base additions to single DNA or RNA strands (Millar *et al.*, 2008).

Several platforms for massive detection of short sequencing reactions have been

developed: Roche/454 FLX, Illumina/Solexa Genome Analyzer, Applied Biosystems SOLiDTM System, Helicos HeliscopeTM, and Pacific Biosciences SMRT instruments. The basis of these systems and their performances are comprehensively presented elsewhere (Mardis, 2008; Millar *et al.*, 2008; Schuster, 2008; Shendure and Ji, 2008; Metzker, 2010). Briefly, and quoting Green *et al.* (2009, p. 2495), "they rely on the construction of sequencing libraries by the ligation of DNA adapters to the ends of DNA molecules in a sample. These adapters then serve as priming sites both for amplification and for sequencing, which occur either on beads or on a solid surface in which each bead or cluster on a surface represents an amplified copy of a single original template molecule."

More recently, target enrichment methods have been developed and a few examples for the 454 FLX platform are: (1) the use of multiplex identifiers (MIDs) (molecular barcodes), which enable unique samples to be specifically tagged in a sequencing run allowing for the combination of template DNA from multiple individuals (see Binladen *et al.*, 2007a); (2) the primer extension capture (PEC) method developed by Briggs *et al.* (2009a), which directly isolates specific DNA sequences from complex libraries of highly degraded DNA, and (3) the direct multiplex sequencing (DMPS) developed by Stiller *et al.* (2009) that combines standard multiplex PCR with sample barcoding and high-throughput sequencing. For other target enrichment approaches, such as the uniplex PCR, the molecular inversion probes (MIP) and the hybrid capture, see the recent review by Mamanova *et al.* (2010).

These second-generation sequencing methods require DNA enrichment (or DNA capture) through the generation of sequencing libraries and therefore can introduce biases into the data.

Third Generation Sequencers (TGS) are now available which rely on direct sequencing and allow for single molecule sequencing (Rizzi *et al.*, 2012), and provide sequence information and quantitation for many different applications. True Single Molecule DNA Sequencing (tSMS), using the Helicos HeliScope platform, has been successfully applied to study a Pleistocene horse preserved in permafrost (Orlando *et al.*, 2011), and its performance was improved by the use of two different template preparation methods (Ginolhac *et al.*, 2012). For illustration of some of the methods mentioned above see Figures 3, 4 and 5 in Rizzi et al. (2012).

Although the measures previously described for prevention of contamination and authenticity of aDNA are still valid with these new technologies, novel difficulties have arisen with the recent advances in high-throughput DNA sequencing. Referring to Poinar *et al.* (2006), these new methods allow filtering and correcting for aDNA damages because they generate overlapping reads and multifold coverage of the target regions. However, this versatile large-scale data production requires new and substantial computational resources (Venter, 2010) for downstream data storage, laboratory information management systems and

bioinformatic support (Mardis, 2008). Challenges for bioinformatics, due to the average short read-lengths resulting from these new technologies, are, amongst others, sequence quality scoring, alignment, assembly and data release. For a review on high-throughput sequence alignment and assembly algorithms, see Flicek and Birney (2009). Numerous software packages are now available for analyzing these kinds of sequencing data (for a review see Shendure and Ji, 2008). Accuracy of data is a major issue with these high-throughput sequencing systems, as calling precision (determination of the correct nucleotide) is highly dependent on redundancy (coverage), sequencing raw error rate (Fuller *et al.*, 2009), and on the use of closely related genomic sequences for correctly identifying and classifying *bona fide* endogenous DNA fragments (Prufer *et al.*, 2010). The establishment of consensual guidelines for reporting and archiving short-read sequence data is a subject still under development (Shendure and Ji, 2008; Shendure *et al.*, 2008). Albeit the need for extraordinary computational processing, large research teams and generous budgets, these new methods are ideal for the analysis of aDNA and are likely to result in the recovery of entire ancient genomes.

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