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Review Article

From the field to the laboratory: Controlling DNA contamination in human ancient DNA research in the high-throughput sequencing era

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Abstract High-Throughput DNA Sequencing (HTS) technologies have changed the way in which we detect and assess DNA contamination in ancient DNA studies. Researchers use computational methods to mine the large quantity of sequencing data to detect characteristic patterns of DNA damage, and to evaluate the authenticity of the results. We argue that unless computational methods can confidently separate authentic ancient DNA sequences from contaminating DNA that displays damage patterns under independent decay processes, prevention and control of DNA contamination should remain a central and critical aspect of ancient human DNA studies. Ideally, DNA contamination can be prevented early on by following minimal guidelines during excavation, sample collection and/or subsequent handling. Contaminating DNA should also be monitored or minimised in the ancient DNA laboratory using specialised facilities and strict experimental procedures. In this paper, we update recommendations to control for DNA contamination from the field to the laboratory, in an attempt to facilitate communication between field archaeologists, anthropologists and ancient DNA researchers. We also provide updated criteria of ancient DNA authenticity for HTS-based studies. We are confident that the procedures outlined here will increase the retrieval of higher proportions of authentic genetic information from valuable archaeological human remains in the future.

Keywords ancient DNA; contaminating DNA; archaeological sampling

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Introduction

After three decades of ancient DNA (aDNA) research (Hagelberg, Hofreiter, and Keyser 2014) and the increasing use of High-Throughput Sequencing (HTS), suspicion of aDNA contamination still sparks debates in the literature. For example, the recent report of mitochondrial sequences from a ~12.5-ky-old skeleton found in a submerged cave on Mexico's Yucatan Peninsula (Chatters et al. 2014) led to a scholarly discussion about aDNA authentication criteria such as the characterisation of the molecular behaviour of aDNA in different environments, experimental replication and data analysis by independent researchers, decontamination procedures, and relevance of bioinformatic analyses of the sequencing data (Prüfer and Meyer 2015; Kemp et al. 2015). The arguments used in this debate might have led to some confusion amongst readers with no expertise in modern aDNA research, but most importantly it illustrates the need for revised standards of aDNA authenticity in the HTS era.

We aim to revisit and update the criteria of authentication of aDNA results in light of the latest HTS-based aDNA studies. We primarily focus on the analysis of human remains, but most recommendations can also be applied to other bioarchaeological materials. Although the issue of DNA contamination in aDNA research has been altered by HTS, strictly controlling for human and environmental contaminating DNA from the time of excavation will ultimately help reduce the sequencing costs and expand the scope of aDNA research.

In this review, we briefly describe aDNA and the latest methods used in aDNA research, and we discuss the importance of DNA contamination. We

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then suggest preventive measures that should be implemented in the field before samples are sent to aDNA laboratories, and explain standard and novel procedures implemented by aDNA researchers to prevent and monitor DNA contamination. Lastly, we provide guidelines to collect samples of interest, including material that emerges as promising for aDNA research and archaeology alike.

A brief technical overview of aDNA research

Ancient DNA refers to DNA molecules that are preserved in historical or pre-historical biological material. Many taphonomic processes influence the preservation of organic material at macroscopic (sample) or molecular (proteins, DNA) level. Therefore, good biomolecular preservation is the exception rather than the rule, and depends on two linked factors: environment and time (Herrmann and Hummel 1994; Brothwell and Pollard 2001; Smith et al. 2001; Smith et al. 2003).

DNA decay starts immediately post-mortem, when enzymes from the organism, bacteria, and fungi start breaking down DNA molecules (Lindahl 1993). This biochemical degradation is limited in certain circumstances (*e.g.* rapid desiccation or low temperature), but slower chemical processes, such as oxidation and hydrolysis, act on the DNA regardless of the conditions (Lindahl 1993). Oxidation and hydrolysis strongly affect the DNA structure and stability, and can result in further DNA degradation and primary sequencing modification in the absence of DNA repair mechanisms (Pääbo et al. 2004; Hebsgaard, Phillips, and Willerslev 2005). As a result, the few remaining DNA fragments that can be extracted from ancient biological material are notoriously short in length, and their integrity is lost, leading to altered sequence information (Pääbo 1989).

More importantly, DNA molecules from the environment (*e.g.* soil matrix, storage facility) or from people who handle the samples can easily outcompete the small amounts of endogenous aDNA (Noonan et al. 2005; Green et al. 2006; Der Sarkissian et al. 2014). A classic example of DNA contamination from the early days of aDNA research is the report of sequences retrieved from Cretaceous dinosaur bone fragments (Woodward, Weyand, and Bunnell 1994), which were later revealed to be human contaminating DNA (Allard, Young, and Huyen 1995; Hedges and Schweitzer 1995; Henikoff 1995; Zischler et al. 1995). This claim and other studies reporting similar false positives forced members of the aDNA field to acknowledge the inherent methodological limitations, revise protocols, and establish guidelines to help editors, reviewers, and readers assess the authenticity of aDNA results (Austin, Smith, and Thomas 1997; Cooper and Poinar 2000; Hofreiter et al. 2001; Poinar 2003; Gilbert, Bandelt, et al. 2005; Willerslev and Cooper 2005).

Techniques used to amplify and sequence DNA molecules have changed dramatically over the past 10 years, and many of the issues with endogenous aDNA retrieval have been at least partially overcome by the recent advent of HTS (Margulies et al. 2005). The Polymerase Chain Reaction (PCR) method that was traditionally used in aDNA research could only amplify a limited number of specific DNA targets at a time when using multiplex assays. On the other hand, HTS combines amplification and sequencing of up to several billions of individual DNA library templates at a time. Most importantly, HTS can sequence short DNA molecules, which is well suited to the extensive fragmentation of ancient DNA templates. As a result, HTS allows aDNA researchers to generate unprecedented amounts of data that were inconceivable using previous techniques.

Methodological improvements have also reduced the cost of HTS analysis of aDNA. Some DNA extraction protocols target ultra-short DNA fragments (Gamba et al. 2015; Dabney et al. 2013) or reduce the fraction of contaminating DNA through sample incubation in buffers or bleach (Der Sarkissian et al. 2014; Korlević et al. 2015; Damgaard et al. 2015; Malmstrom et al. 2007; Salamon et al. 2005). During the DNA library preparation stage, damaged aDNA molecules can be separated from the intact molecules (Gansauge and Meyer 2014), or DNA damage can be enzymatically treated (Rohland et al. 2014; Briggs et al. 2010). Finally, many methods have been developed to enrich the endogenous DNA fraction from highly contaminated aDNA extracts. One such method makes use of cytosine methylation—an epigenetic mark on the DNA that occurs in a different sequence context in humans (or other multicellular organisms) and bacteria—to enrich aDNA libraries with non-bacterial DNA (Seguin-Orlando et al. 2015). However, this method is limited to the analysis of well-preserved aDNA (Seguin-Orlando et al. 2015). The most popular enrichment approach is the selective capture of regions of interest by hybridisation of aDNA with pre-designed oligonucleotide probes (Avila-Arcos et al. 2011; Vilstrup et al. 2013; Carpenter et al. 2013; Enk et al. 2014; Fu, Meyer, et al. 2013; Haak et al. 2015; Burbano et al. 2010; Paijmans et al. 2015; Fehren-Schmitz et al. 2015). The targets of such selective capture assays can be complete mitochondrial genomes (Brotherton et al. 2013; Llamas et al. 2016; Posth et al. 2016), genome-wide SNPs (Haak et al. 2015; Mathieson et al. 2015; Fu et al. 2016), exomes (Castellano et al. 2014), chromosomes (Fu, Meyer, et al. 2013), or complete genomes (Carpenter et al. 2013; Enk et al. 2014).

HTS allowed remarkable achievements during recent aDNA research, such as sequencing the complete mitochondrial genome, hundreds of thousands of nuclear variants, and the nuclear genome from several hundreds of archaeological human remains [see (Ermini et al. 2015; Pääbo 2014; Slatkin and Racimo 2016) for review], including Neanderthals

(Green et al. 2010; Prüfer et al. 2014), 400 ky-old archaic hominins from Sima de los Huesos in Spain (Meyer et al. 2014; Meyer et al. 2016), and archaic hominins from the Denisova Cave in Russia (Meyer et al. 2012). Although some of the latest aDNA research is embedded in large-scale population studies (Allentoft et al. 2015; Mathieson et al. 2015; Haak et al. 2015; Fu et al. 2016; Lazaridis et al. 2016), ancient genomic studies also provide powerful insights into physical anthropology and archaeology, such as genetic relatedness of samples (Vohr et al. 2015) or genetic sex determination (Skoglund et al. 2013; Green et al. 2010). The integration of molecular genetic, anthropological, and archaeological methodologies can help examine the diversity, relationships, and origin of individuals and populations through time. Within an archaeological context, small- and large-scale aDNA research sheds light on kinship, demography, health, subsistence practices, and the social organization of past populations (Haak et al. 2008; Raff et al. 2011; Kirsanow and Burger 2012; Brandt et al. 2015).

Contaminating DNA

Sources of contaminating DNA are diverse and ubiquitous, and can affect ancient remains at any time between the individual's death and when the DNA is sequenced (Brown and Brown 1992; Gilbert, Bandelt, et al. 2005; Yang and Watt 2005; Haile et al. 2007; Pilli et al. 2013). The likely presence of contaminating DNA raises two experimental issues that need to be addressed when interpreting HTS data:

- HTS is based on unbiased amplification and sequencing of any DNA library template present in the reaction mix. HTS does not discriminate between endogenous and contaminating DNA during the sequencing process.
- DNA fragments that are identical or highly similar to the endogenous aDNA are considered a genuine contamination risk. For example, modern human contaminating DNA will likely result in false positives in studies of ancient human samples (Wall and Kim 2007; Skoglund et al. 2014).

Contaminating environmental DNA

A typical aDNA extract will contain less than 1–5% of endogenous DNA fragments that are diluted in a vast majority of exogenous environmental DNA, including microbial (*i.e.* bacteria, fungi, viruses, algae and other protozoans) and metazoan DNA (Noonan et al. 2005; Green et al. 2008; Garcia-Garcera et al. 2011; Der Sarkissian et al. 2014). While contaminating environmental DNA can be useful in studies examining the environment (*e.g.* metagenomics) or taphonomic processes, it can also confound the source DNA from the organism of interest. Ultimately, the relative amounts of environmental and endogenous DNA will determine the shotgun sequencing effort needed to obtain endogenous data, and impact significantly on the research project's budget.

Contaminating human DNA

Contaminating modern human DNA can be found in people's dead skin cells, hair, saliva, dandruff, sweat, and blood. Breathing can also leave trace amounts of DNA behind. PCR-based studies had already shown the extent of human contamination introduced during handling of bone and tooth samples when strict aDNA precautions are not in place (Gilbert, Rudbeck, et al. 2005; Pilli et al. 2013; Sampietro et al. 2006). More recently, HTS data from a Neanderthal sample excavated in the 1980's and handled without precautions revealed that 10.2% of sequences overlapping diagnostic positions originated from modern human contaminating DNA(Skoglund et al. 2014).

Laboratory consumables and reagents can be contaminated by human DNA during production in the manufacturing facility, before arriving into an aDNA laboratory (Champlot et al. 2010; Deguilloux et al. 2011; Leonard et al. 2007). Additional DNA contamination could occur during experimental processing if precautions are not in place, and include:

- Cross-contamination between samples when multiple specimens are processed at the same time. For example, cutting and milling the samples into a fine powder are often preliminary steps in the DNA extraction protocols, and the dust generated from a single sample can contaminate other samples.
- Cross-contamination between DNA extracts when multiple samples are processed at the same time. For example, DNA extractions involve pipetting and transfer of liquids that can be carried-over across samples through aerosols.
- Contamination of reaction mixes by DNA carry-over from other experiments. PCR amplification in particular, used to amplify HTS DNA libraries, generates billions of DNA molecules in a volume of 10-50 µl. PCR is thus is a very potent source of contaminating DNA (Willerslev and Cooper 2005).

Prevention of DNA contamination before the arrival in the laboratory

All of the precautions in the laboratory cannot overcome DNA contamination during sample collection and storage. As aDNA studies become increasingly more common and prove to be a powerful addition to archaeological research, it is crucial that aDNA researchers share their experiences, precautions, and protocols with field archaeologists and collection curators. This will facilitate the formal training of field archaeologists and curators for the sampling of

material for aDNA research. We argue that precautions to control or minimise both environmental and human contaminating DNA should always be taken to preserve sample integrity for future generations of researchers, even if aDNA analyses are not anticipated during a specific excavation season. For example, post-excavation microbial growth or unnecessary degradation of the endogenous DNA can affect the ratio of endogenous to exogenous DNA, which will impact significantly on the cost of sequencing — usually the largest expense in HTS-based aDNA research.

Below, we propose guidelines that are equally relevant to archaeologists working in the field, physical anthropologists, and museum curators who handle the remains once unearthed (Brown and Brown 1992; Yang and Watt 2005; Pruvost et al. 2007; Fortea et al. 2008; Allentoft 2013; Pilli et al. 2013). This comprehensive list of procedures provides all the information necessary to balance feasibility and importance of the sampling procedures. The aim is to increase the quality of the data produced and to decrease the costs of analysis, as much as it is reasonable and applicable given a specific situations (*e.g.* constraints during field work) and the anticipated value of the samples. Given adequate training, most of the recommendations can be routinely applied in a majority of archaeological settings. However, the reality of field archaeology is often far from "ideal" (*e.g.* commingled remains, wet caves, limited financial and/or human resources), thus it is advised to keep in mind two key points: protect the samples from DNA contamination, and prevent further endogenous DNA degradation.

Precautions that can potentially make a big difference:

- *Disposable gloves:* Wear disposable medical gloves during excavation and when handling specimens to protect samples from human DNA contaminants. Change gloves between specimens, after touching hair or face, or after touching communal items (*e.g.* trowels, pens). Wearing two pairs of gloves and changing the outer one regularly is particularly efficient, because putting gloves on sweaty hands may be challenging.
- *Do not wash specimens with water:* Water contains contaminating bacterial DNA, and can deeply penetrate into the sample and cause unwanted hydrolytic damage to the endogenous DNA. Light brushing of specimens with a dry brush is preferred to washing with water. Sampling of the surrounding matrix can also be useful for microbial studies, to identify and filter out environmental DNA sequences from the archaeological sample sequencing data.
- *Storage:* Samples should either be completely dry to avoid further contamination with microbial DNA (microbial growth) and damage (hydrolysis), or stored in a cold, dry place as soon as possible (*e.g.* cooler, fridge/freezer).

Optional precautions for maximal effect:

- *Protective gear:* In addition to gloves, other (optional) disposable protective gear may include surgical mask, hair cover/net, and sleeves to cover the arms (Figure 1). If budget and conditions allow, a clean disposable surgical gown or body suit and goggles are desirable.
- *Protect the site:* Protect the site from dust, rain and direct sunlight to limit hydrolysis, irradiation, and further contamination with environmental DNA once the specimen is exposed.
- *Dedicated trained staff:* If possible, assign one or two members of the excavation team to be formally trained and solely responsible for collecting the 'contaminating-modern-DNA-free' samples for aDNA analysis. This should limit the introduction of contaminating DNA from multiple individuals, and can therefore be detected as a systematic ubiquitous signal during computational analyses of the sequencing data.
- *Clean tools:* Clean tools (*e.g.* trowels, dental picks, and brushes) with ≥3% bleach between samples, and dry them prior use on the next sample. The oxidative power of bleach will degrade contaminant DNA.
- *Keep records:* Document soil types, wet/dry conditions, associated biological materials, details about visible treatments to the remains (*e.g.* artificial mummification), and people who handle the specimens. This metadata can be extremely helpful when designing the aDNA study, or when interpreting the results,
- *In situ sampling: In situ* sampling is preferable if conditions allow. In this situation, the following procedures should be implemented:
  - *i.* If the skeletal material is articulated and the orientation of a body can be identified, the skeletal remains to be sampled for aDNA analysis should be freed from the soil and collected first, before excavating the rest of the skeleton.
  - *ii.* If several specimens are mingled (*e.g.* mass grave or collective burial), unearthed skulls should be protected while excavating until all of the individuals have been identified. Samples that are unequivocally assigned to each individual can then be collected (most likely teeth or cranial elements).
  - *iii.* Mummy bundles efficiently protect human remains against contaminating DNA from modern humans. In that specific case, *in situ* sampling should be avoided. Instead, it is recommended to open the bundles and to proceed with sampling in dedicated facilities, such as the archaeology laboratory or the museum.
- *Storage conditions:*
  - *i.* If the sample is too wet or frozen in permafrost, immediate storage in a freezer is

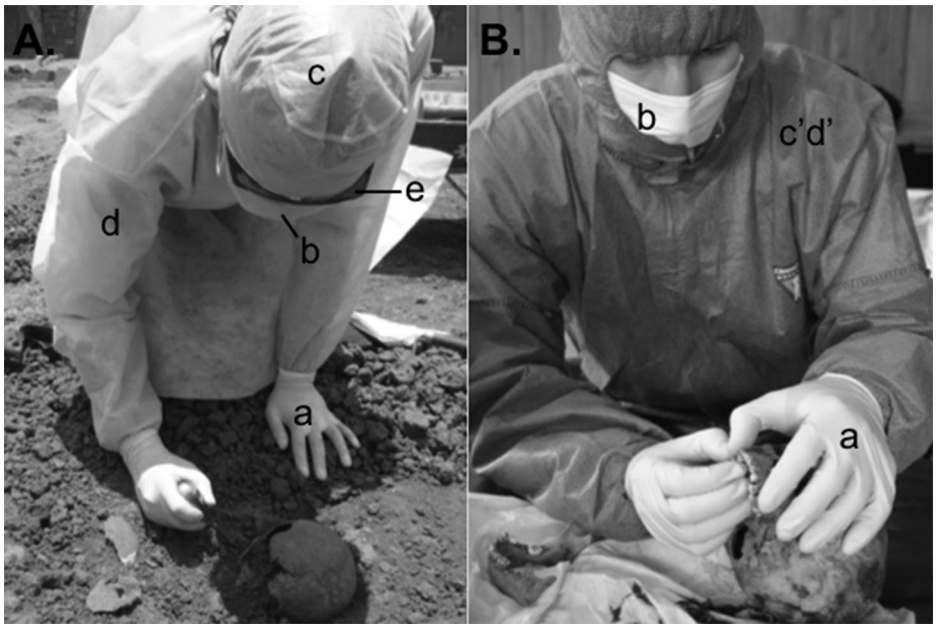

Figure 1 Examples of protective gear sported during excavation (A) and museum (B) sampling. Medical gloves (a; 2 pairs for convenience, the outer one being changed regularly) are critical. Optional items include surgical mask (b), hair cover (c), and long sleeves to cover the arms. If budget and conditions allow, a clean disposable surgical gown (d) or body suite (c'd'), and goggles (e) are desirable.

highly recommended. Repeated humidity changes and freeze and thaw cycles should be minimised/avoided, as condensing water and its crystallisation will promote diagenesis. If freezing the sample and preserving the cold chain is not possible, wrap wet remains in clean paper towel and let dry on a clean surface (out of direct sunlight) to prevent mould formation.

ii. Store samples individually in a clean, dry, airtight container (*e.g.* plastic Ziploc bag or tube) to avoid cross-contamination by contact with other samples.

iii. Clearly label the sample container with date, site location, sample type, name of excavators, etc.

- *Chemical treatment:* The use of preservative agents, hardeners, varnish glue, adhesive tape, or any chemicals such as sodium fluoride should be avoided, as they can inhibit experimental enzymatic reactions or introduce contaminating DNA.
- *DNA analysis of samples:* In cases where samples are sent or carried to an aDNA facility shortly after the specimen is excavated, it is advised to perform aDNA analysis as soon as possible for optimal aDNA preservation. If immediate aDNA analysis is not planned, storage of samples in a dry and cold environment (-20 to +4°C) is recommended to limit further DNA degradation and microbial growth.
- *Museum specimens:* Specimens stored in museums or in other facilities are extremely valuable for aDNA research, despite the potential lack of contextual information, storage at room temperature, and extensive manipulation with bare hands. When sampling museum specimens, we strongly advise the routine implementation and use of protective gear and appropriate sampling protocols as outlined above.

Controlling contaminating DNA in the laboratory

Three essential steps should be taken to limit DNA contamination while performing aDNA research:

- Only ancient samples should be processed in a dedicated aDNA laboratory.
- The potential risks of DNA contamination should be identified and eliminated if possible.
- Exposure to contaminating DNA should be controlled and limited in the aDNA laboratory.

Laboratory setup

A dedicated aDNA laboratory should be isolated both physically and logistically from post-PCR facilities (Cooper and Poinar 2000; Poinar 2003; Knapp et al. 2012; Pääbo et al. 2004) and fitted with HEPA-filtered ventilation (Knapp et al. 2012). Ideally, the aDNA laboratory should be equipped with positive air-pressure to prevent the uptake from air outside the lab (Knapp et al. 2012). In addition, chemical cleaning procedures with ≥3% sodium hypochlorite (bleach) and/or DNA degrading detergents (*e.g.* Decon®90, DNA-Exitus-Plus™, DNA AWAY®) should be in place for all laboratory surfaces and equipment (Champlot et al. 2010; Knapp et al. 2012). The surface of small laboratory

equipment and consumables should also be irradiated using ultra-violet (UV) light bulbs (254 nm) at 10 cm distance for one hour, corresponding to a measured energy of 1,45 J/cm^2 (Champlot et al. 2010). DNA amplification and sequencing must under all circumstances be performed in a physically separated post-PCR molecular biology laboratory (Knapp et al. 2012). Modern reference sample preparation must also be performed in a separate clean room of the molecular biology laboratory, or a separate facility.

Access to the aDNA laboratory should be restricted to personnel trained for aDNA research (Knapp et al. 2012). It is advised to enforce a one-way rule for movements from the ancient to the modern DNA laboratories for both personnel and laboratory supplies. Workers should wear disposable clean room overalls, surgical facemask, a visor, a minimum of two layers of medical gloves (the outer layer being changed regularly), and disposable shoe covers or dedicated footwear cleaned with bleach (Knapp et al. 2012). Upon arrival of the laboratory items or samples, packaging should be discarded before entering the aDNA laboratory. Containers (*e.g.* plastic bags or tubes) holding the samples or laboratory supplies should be thoroughly decontaminated with bleach and UV irradiation (as described above), or replaced with sterile bags. It is recommended to store samples, DNA extracts, and PCR/HTS reagents in separate fridges/freezers or rooms.

Sample preparation

Sample treatment prior to DNA extraction is required to remove or minimise pre-laboratory surface contamination. Irradiation with UV light of all sample surfaces is necessary but not sufficient. UV light does not destroy DNA, but creates DNA structures (*e.g.* thymine dimers) that prevent DNA from being amplified by PCR (Ou, Moore, and Schochetman 1991). Other decontamination methods, depending on the nature, the size, and the shape of the sample, include: *i*) physical removal of surface contaminating DNA by abrasion of the sample surface using sand paper, sandblasting, or abrasive disks, with an exhaust system to evacuate dust; *ii*) release of surface contaminating DNA by pre-incubation in a phosphate buffer (Korlević et al. 2015) or EDTA (Damgaard et al. 2015; Der Sarkissian et al. 2014); or *iii*) chemical destruction of contaminating DNA by treating the sample with bleach (Adler et al. 2013; Kemp and Smith 2005; Korlević et al. 2015; Malmstrom et al. 2007; Salamon et al. 2005). The latter method could be applied to small or delicate samples (*e.g.* hair, dental calculus, small bone fragments) despite its harmful effect on endogenous DNA (Malmstrom et al. 2007; Korlević et al. 2015). Bleach will also fragment modern contaminating DNA in a depurination-dependent fashion similar to what is observed in ancient molecules (Garcia-Garcera et al. 2011). Irrespective of which decontamination method is used, aDNA researchers have to balance the removal of contaminating DNA with preserving the remaining endogenous DNA.

Updated criteria of aDNA authenticity for HTS-based studies

HTS data and bioinformatics

The large amount of HTS data allows researchers to assess the level of contamination after sequencing, through *post hoc* bioinformatic comparisons. HTS studies on ancient humans showed that endogenous aDNA and contaminating modern DNA differ markedly in several features, despite their high sequence similarity. Ancient DNA fragments tend to be shorter than modern contaminating fragments (Malmstrom et al. 2007; Green et al. 2009; Krause et al. 2010), despite conflicting reports for a correlation between fragment length and ancient samples age (Allentoft et al. 2012; Sawyer et al. 2012). The observation that post-mortem DNA fragmentation occurs preferentially at depurinated sites is a second criterion to characterise aDNA (Briggs et al. 2007), although it does not seem to correlate with ancient samples age (Sawyer et al. 2012). A third characteristic feature is C-to-T (and complementary G-to-A) misincorporation patterns observed near the ends of HTS DNA sequences due to cytosine deamination that occurs preferentially in single-strand overhangs of aDNA molecules (Briggs et al. 2007; Orlando et al. 2011; Meyer et al. 2012). In contrast to fragment length, the proportion of these misincorporations does correlate with the age of the ancient samples (Sawyer et al. 2012). Consequently, fragment length and characteristic aDNA damage patterns are used to assess the authenticity of aDNA, and have been implemented in several recent bioinformatics programs (Helgason et al. 2007; Jónsson et al. 2013; Korneliussen, Albrechtsen, and Nielsen 2014; Skoglund et al. 2014; Renaud et al. 2015; Peltzer et al. 2016; Ginolhac et al. 2011). It should be noted that the molecular tools used during the aDNA library preparation, or the sequencing technology, might impact on the characteristic aDNA damage profile (Briggs et al. 2010; Meyer et al. 2012; Orlando et al. 2011; Rasmussen et al. 2010; Rohland et al. 2014; Seguin-Orlando et al. 2013; Tackney et al. 2015). As a consequence, criteria of authenticity should be revised depending on the experimental HTS protocols.

Patterns of aDNA damage are also used to computationally quantify and discard contaminating sequences, assuming the putative contaminating DNA comes from modern-day humans and is relatively intact (Skoglund et al. 2014; Fu et al. 2015; Meyer et al. 2016; Meyer et al. 2014; Renaud et al. 2015). Further work is needed to assess the contamination levels in cases where exogenous contaminating DNA is also ancient and display damage patterns similar to authentic endogenous aDNA under independent decay processes. This issue has been shown using PCR and cloning (Sampietro et al. 2006) but has not been formally addressed in HTS datasets yet, and all current

computational methods consider that contaminating DNA is mostly intact.

Updated criteria of aDNA authenticity

Since HTS-based studies might not primarily rely on experimental replication anymore (a cornerstone of previous guidelines for PCR-based protocols), but rather on a strict filtering and interpretation of the data (Green et al. 2009; Kircher 2012; Schubert et al. 2012), we argue that data processing and statistical methods should be scrutinised. The bioinformatic processing and analysis of HTS data should be carefully conducted by researchers, as well as thoroughly evaluated by peer reviewers. As a consequence, measures should be taken to ensure mistakes are not carried throughout the analyses, which might impact the conclusions of the study.

The biochemical properties of aDNA molecules have not changed despite the development of HTS technologies. In addition, PCR is still widely used to amplify DNA libraries used in HTS, and low sequence complexity mixtures (*e.g.* poorly preserved samples, post-capture DNA libraries) may lead to previously reported PCR issues in aDNA studies. Consequently, most criteria of authenticity proposed for PCR-based research (Cooper and Poinar 2000; Poinar 2003; Pääbo et al. 2004; Austin, Smith, and Thomas 1997; Hofreiter et al. 2001) are still valid, although they require updating to conform with new techniques (Knapp, Lalueza-Fox, and Hofreiter 2015). These updates include:

- **Negative controls:** Extraction blanks and no-template controls are recommended to monitor contaminating DNA during the laboratory procedures. While clean-room barcoding of DNA libraries allows the detection of cross-contamination during library amplification and HTS sequencing (Green et al. 2009; Knapp and Hofreiter 2010; Kircher, Sawyer, and Meyer 2012; Knapp, Stiller, and Meyer 2012), all steps prior to the ligation of barcoded adapters to the DNA templates are still susceptible to contamination. No-template controls are therefore crucial and absolutely essential for studies of the diversity of the microbial communities associated with the remains, in order to build a reference database of laboratory-specific contaminating DNA.
- **Experimental replication:** In order to maximise the molecular complexity for HTS, aDNA experiments often involve the preparation of DNA libraries from multiple aDNA extracts from the same sample, or multiple DNA libraries from the same aDNA extract [*e.g.* (Meyer et al. 2014; Orlando et al. 2011)]. More importantly, this experimental replication also informs about the reproducibility of the results. In addition, the replication of DNA extraction and/or library preparation steps in independent laboratories allows monitoring for intra-laboratory contamination [*e.g.* (Llamas et al. 2016)].
- **Fragment length:** The distribution of aDNA fragment lengths should be skewed toward short fragments, with the vast majority of them typically shorter than 100 bp (Sawyer et al. 2012).
- **DNA preservation and variant coverage depth:** The massively parallel sequencing of DNA library templates has superseded cloning of PCR products, but the resulting sequencing data can be verified using the same criteria: determination of the ratio of endogenous versus exogenous sequences, detection and quantification of DNA damage, and coverage of each variant position by multiple individual sequences to confirm the authenticity of genetic variation. Of note, damage patterns have been characterised for a relatively limited range of temperatures, environments and samples (mostly humans). Little is known about the relative rates of damage in other organisms, especially microorganisms. This aspect still requires detailed investigation and researchers should thus be careful drawing final conclusions based on damage patterns alone.
- **Associated remains:** An associated non-human sample can be included as a control to assess the presence of modern human contaminating DNA [*e.g.* (Rasmussen et al. 2010; Skoglund et al. 2012)].
- **Contamination estimates:** Modern human DNA contamination levels in extinct human HTS datasets can be estimated by comparing the sequences at nucleotide positions that are well characterised in present-day human populations. In the case of haploid loci such as the mtDNA and the non-recombining part of sex chromosomes in males, only one allele is expected at a given site in absence of contamination. The presence of additional alleles allows a direct quantification of contamination (Fu, Mittnik, et al. 2013; Green et al. 2010; Rasmussen et al. 2011; Kousathanas et al. 2016). A method relying on a similar principle is also available for the autosomes (Racimo, Renaud, and Slatkin 2016), although it is highly specific and requires full population history modelling for both endogenous and contaminating sequences. It is important to note that contamination levels estimated from mtDNA and nuclear genome data are not necessarily proportional (Green et al. 2009), so one should consider quantifying both mitochondrial and nuclear contaminating DNA if the data is available.
- **Validation of sequence variation:** Ultimately, genetic variation should be verified in phylogenetic or population genetic analyses to confirm its authenticity. In any case, and especially if the results seem to lead to a paradigm shift, it is of paramount importance to involve

colleagues as well as independent expert collaborators to inspect carefully every step of the data analysis. The aDNA sequence data should be carefully re-processed independently, and the complex statistical analyses of very large datasets should also be replicated independently. For example, the conclusions of a recent study about the extent of Eurasian back migration in African human populations (Gallego Llorente et al. 2015) had to be revised after some problems were detected by another team (Gallego Llorente et al. 2016).

What to sample?

The choice of sampling will depend entirely on the general aim of the study. If the research focuses on ancient humans, bones, teeth and hair are the most suitable material for aDNA analysis (Campos et al. 2011; Bengtsson et al. 2012; Higgins and Austin 2013). If the research focuses on infectious disease, sampling targets depend on the infectious agent and/or clinical manifestations. Mycobacterial diseases, such as tuberculosis or leprosy, will leave focal lesions on some skeletal parts that can be sampled for aDNA analysis (Stone et al. 2009). All systemic diseases (including mycobacterial diseases) may be studied using residual infected blood that can be trapped in the teeth pulp chamber (Bos et al. 2011; Wagner et al. 2014). Finally, recent ancient microbiome studies have also opened new research opportunities, and require specific samples and sampling methods (see below).

In general, the specimen selection should be based on good preservation and minimal diagenetic alteration. Damage often varies considerably within a single specimen, so one should focus on the most intact areas. As aDNA analysis is destructive, material with potential for museum display should be sampled wisely. Likewise, regions of the skeleton that are informative for morphological and pathological studies (*e.g.* areas of muscle attachment on long bones, areas affected by diseases) should not be sampled, except if required for aDNA analysis of diseases. Of note, even 'non-destructive' methods (Bolnick et al. 2012; Hofreiter 2012; Mohandesan, Prost, and Hofreiter 2012) will irreparably damage the sample. For example, non-destructive methods often involve incubating the sample in buffers, which can result in decolouration of the sample and increase in surface contaminating DNA in the final aDNA data (Damgaard et al. 2015; Korlević et al. 2015).

Bones

Several visual factors can indicate good macroscopic preservation, and high chances of endogenous DNA survival. For example, fresh-looking compact bones or bone fragments with smooth and intact surfaces are indicators for good macroscopic preservation (Figure 2). The weight of the skeletal element may also indicate good preservation, but requires

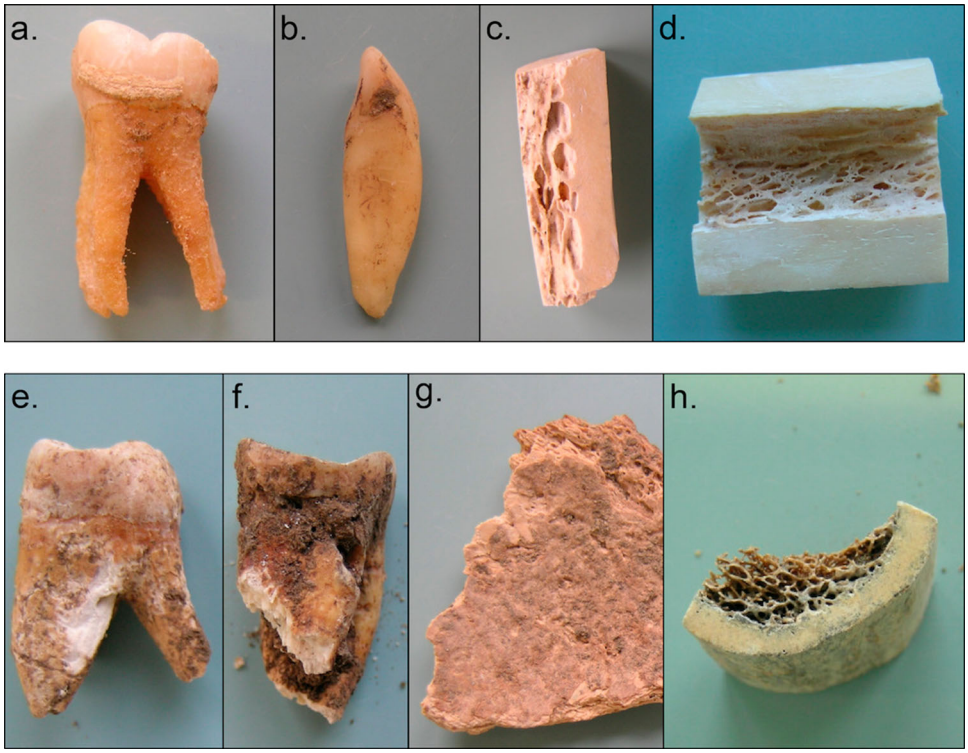

Figure 2 Examples of archaeological human tooth and bone samples and their suitability for aDNA research. a-d: fresh-looking, intact samples with smooth surfaces have a good potential for aDNA research; in the case of bones (c, d), the periosteum should be dense and compact. e-h: evidence of mineral alteration and/or microbial attack (low density, dull surface, crumbly broken ends) usually indicates a poor preservation of aDNA.

experience with handling well and poorly preserved samples for comparison. Fragmented bones indicate good preservation when they harbour strong and jagged edges, as opposed to crumbly, chalky, and broken ends. In difficult cases, a thin, smooth surface layer (especially under articulation faces) can often hide a chalky interior matrix, but low bone density and cracks should reveal underlying diagenetic alteration. Another sign of good organic preservation is a burning smell when cutting bone or teeth. This should smell like scorched hair, rather than the acrid inorganic smell of cutting rock.

Strong cortical bones (tibia, femur) are less vulnerable to contaminating DNA due to their density that provides a protective crystal matrix for endogenous DNA (Campos et al. 2011; Orlando et al. 2011; Gilbert, Rudbeck, et al. 2005). Given its extremely high density, the petrous part of the temporal bone has been shown to yield 4- to 16-fold more endogenous DNA than teeth, and up to 183-fold more than other bones (Gamba et al. 2014). The petrous bone is therefore possibly the best material for aDNA studies. In addition, removal of the petrous bone is inconspicuous, and will not alter the cranium for museum display.

Bench surfaces, cutting tools, the hood, and immediate surrounding area should be cleaned thoroughly with ≥3% bleach between samples. Gloves should be changed in between the cutting and drilling of individual samples. In general, 0.1–0.5 g of material is needed for DNA extraction, but up to 1 g may be required for badly preserved samples. Small samples (tooth, cortical fragment, or hand/foot bone) can be sent whole to the aDNA laboratory. If the sample needs to be cut, a 1–2 cm square section can be removed using a clean hacksaw or a drill with thin cutting discs at low speed to avoid dust/burn. Cutting should be performed in a well-ventilated area, preferably with an exhaust system to avoid cross-contamination by bone dust. The petrous pyramidal apex projects into the brain cavity and can be accessed through the foramen magnum. A clean and efficient sampling method consists in placing a small cold chisel (~5 mm cutting edge) against the base of the petrous pyramid and giving a sharp hard tap with a hammer, applying the force sideways. It is important to sample the dense bone surrounding the inner ear and the inner ear itself, as these parts contain orders of magnitude more DNA than the porous apex of the petrous pyramid (Pinhasi et al. 2015).

Teeth

Sampling teeth for aDNA analysis provides several unique benefits, in contrast to bone sampling. The non-porous enamel coating on teeth protects the sample from contamination (Gilbert, Rudbeck, et al. 2005; Pilli et al. 2013). Teeth roots can also be sampled in an aDNA laboratory without destroying the crown, allowing for subsequent morphological or epigenetic dental trait studies. Teeth can be placed back in the jaw socket after root sampling, therefore preserving the specimen for potential museum display. Finally, sampling teeth is logistically less demanding, and teeth can be unequivocally assigned to an individual skull. An added benefit to sampling teeth is the possibility of analysing systemic pathogens that can be found in the blood trapped in the dental pulp chamber (Bos et al. 2011; Schuenemann et al. 2011).

Similar to bone sampling, smooth and intact surfaces are indicators for good macroscopic preservation (Figure 2). Tooth extractions should be preferably performed with gloved fingers, by repeated gentle side-to-side movements. Gloves should be changed in between individual samples. Tools, such as pliers, often break the enamel or tooth roots. Single-rooted teeth, such as canines and premolars, can be removed easily and are sufficiently large samples (compared to incisors). Molars do require some experience to extract, but often provide the most material to sample.

Hair

Hair samples are rare, but provide a valuable source of aDNA (Rasmussen et al. 2010; Bengtsson et al. 2012; Llamas et al. 2016). Hair is often found on mummified remains from very arid or cold climates, including parts of South America, Central Asia, Australia, and South Africa. The hair structure provides efficient protection against exogenous contaminating DNA, and sampling generally causes minimal damage to the specimen (Bengtsson et al. 2012; Campos and Gilbert 2012). In general, the hair shaft (the keratinized part of the hair) contains short fragments of mitochondrial DNA and some highly fragmented nuclear DNA, but the quality varies unpredictably and is consistently poor (Bengtsson et al. 2012). In comparison, the hair root and the attached follicles, which are the metabolically active parts of the hair, often contain better preserved DNA (Bengtsson et al. 2012). Because PCR analysis of DNA retrieved from hair is challenging, these samples are much better suited for advanced HTS technologies (Rasmussen et al. 2010).

Whenever possible, the inner layers of hair should be selected, as they are more protected from environmental contaminating DNA than the exposed outer layers. If possible, hair with scalp attached may improve the aDNA analysis, because hair roots are richer in nuclear DNA than the shaft.

Other valuable sources of DNA for microorganism studies

The study of ancient microbiomes is an emerging new field of anthropological and archaeological research, as the microorganisms co-evolving with the human body can provide contextual information on health, diet, and migration. Calcified dental plaque (calculus) is a complex biofilm of oral microorganisms, which preserves human oral bacteria for ancient DNA and protein studies (Adler et al. 2013; Warinner, Rodrigues,

et al. 2014; Warinner, Speller, and Collins 2014; Warinner, Hendy, et al. 2014; Weyrich, Dobney, and Cooper 2015), while preserved ancient faeces (coprolites) record some information about microorganisms that once lived in the human gut (Tito et al. 2012; Tito et al. 2008). Broad research questions are now the focus of aDNA analysis of the microorganisms exceptionally well preserved in archaeological dental calculus, and include investigating oral microbial diversity through time, bacterial evolution, ancient human diets, and human migratory and admixture patterns (Warinner et al. 2015; Weyrich, Dobney, and Cooper 2015).

When sampling dental calculus, larger robust samples are better, although samples <10 mg and only a few millimetres in size can be successfully examined. Typically, the best locations for calculus are on the lingual side of the molar teeth in the mandible, or the buccal side of the teeth of the maxilla (upper jaw). When removing dental calculus, facemasks and clean gloves should be worn to avoid contamination with modern oral microorganisms, which can be easily transferred from sneezing, coughing, and even breathing. Once the calculus specimen to be removed is identified, the skull or mandible should be placed over clean aluminium foil or wax paper. The tooth should be held in place with fingers, and a plastic dentist scapula should be used to avoid damaging the tooth surface. Slight pressure should be placed on the scapula such that the largest exposed ridge of the calculus is receiving the applied pressure. The calculus should disassociate quickly and will fall onto the aluminium foil. The researcher should also be careful when applying pressure, so that the calculus sample will fall downward, and not be propelled into the air (Weyrich, Dobney, and Cooper 2015).

Coprolites are prone to rapid degradation in most environmental conditions, and post-depositional alterations caused by the metabolism of undigested nutrients can both result in large biases in microbial community composition (Tito et al. 2012). In addition, coprolites do not offer an ideal dense matrix for DNA preservation and present a high risk of contamination by environmental DNA (Tito et al. 2012). Nevertheless, in ideal cases coprolites are a valuable source of ancient human DNA and can provide a unique opportunity to study ancient gut microbiomes (Gilbert et al. 2008; Jenkins et al. 2012; Tito et al. 2012).

Human pathogens are also popular in aDNA studies, although criteria for authentication of results had often been questioned in the PCR era (Stone et al. 2009). HTS technologies and enrichment methods have now allowed the sequencing of complete pathogen genomes, providing a higher level of confidence in identifying authentic pathogen DNA in several recent publications: *Mycobacterium tuberculosis* responsible for tuberculosis (Bos et al. 2014), *Mycobacterium leprae* responsible for the leprosy (Schuenemann et al. 2013), *Yersinia pestis* responsible for the plague (Bos et al. 2011; Schuenemann et al. 2011; Devault, McLoughlin, et al. 2014; Gilbert 2014; Wagner et al. 2014), and *Vibrio cholerae* responsible for the cholera (Devault, McLoughlin, et al. 2014; Devault, Golding, et al. 2014). Most pathogens are present in large numbers in specific soft tissues or in blood, but only a few pathogens will leave sufficient amounts of genetic material in ancient bones and teeth. For example, *Yersinia pestis* DNA seems to be more concentrated in residual dried blood cells inside the pulp chamber of teeth than in bones (Schuenemann et al. 2011). Studies of tuberculosis should directly target the elements showing relevant characteristic lesions (e.g. vertebra, ribs) where the pathogen of interest is most likely highly concentrated (Stone et al. 2009; Muller, Roberts, and Brown 2013; Bos et al. 2014). However, periosteal rib lesions do not necessarily arise from infection by the tuberculosis complex, and attempts to identify a wide range of respiratory pathogens should be employed if examining these lesions (Raff, Cook, and Kaestle 2006; Stone et al. 2009).

Museum and private collections

Most of the above recommendations are also applicable to previously excavated specimens stored in anatomical or osteological collections at museums or universities. However, the most prominent problem with such collections is that they can be tens if not hundreds of years old. This obviously complicates the reconstruction of a 'post-excavation' history for a particular sample set or can even render it impossible (Gilbert, Bandelt, et al. 2005). Further, storage conditions can also adversely impact on the overall DNA preservation (Pruvost et al. 2007). Gapless collection records that provide information about the treatment and handling of the samples will assist the researcher in selecting samples.

Concluding remarks

After three decades of aDNA research, limitations and pitfalls encountered in aDNA studies are well characterised. The aDNA community has reacted to initial reports of false positives by establishing strict experimental protocols. Given the latest technical advances, we encourage archaeologists and aDNA researchers to engage in a direct and open dialogue regarding these recommendations to design efficient protocols to reduce and prevent DNA contamination. Only a close interaction between archaeologists and geneticists will put aDNA research in a strong position to generate more exciting and authentic results in the future, eventually at a reduced cost. In our view, this will be done via *i)* targeting samples with the best endogenous DNA preservation (*e.g.* petrous bones) readily available in fossil collections worldwide or yet to be excavated, *ii)* excavating and storing new specimens using *ad hoc* precautions to prevent contamination by modern human DNA or growth of microbial contaminants, and *iii)* continuous development and

optimisation of molecular and computational techniques for the analysis of aDNA.

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