PECULIARITIES OF WORKING WITH ANCIENT DNA

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Abstract. This year Nobel Prize in Physiology or Medicine awarded to Swedish paleogeneticist Svante Pääbo demonstrated the significance of ancient genomes investigation, shedding bright light to the aDNA science. As an ancient DNA study has evolved from the 1980s to the present days it has experienced peak of inflated expectations, dramatic fall to trough of disillusionment and slope of enlightenment with exponential data accumulation after the successful introduction of NGS approach. Today ancient DNA study is a rapidly developing and facilitated by advanced technologies science that challenges our assumptions about the past by analyzing over a million-year-old ancient specimens. Here we provide an overview of peculiar properties of ancient DNA analysis of human and microbes genomes.

Keywords: human populations, ancient DNA, paleogenetics, postmortem damage, contamination, ancient bacterial DNA, ancient pathogens.

List of Abbreviations

14C – radiocarbon
A / T / G / C – adenine / thymine / guanine / cytosine
aDNA – ancient DNA
bp – base pairs
C to T – cytosine to thymine replacement
DNA – deoxyribonucleic acid
IntCal20 – International Calibration 2020
kyr – thousand years
Ma – million years
MRCA – most recent common ancestor
mtDNA – mitochondrial DNA
NGS – next-generation sequencing
UDG – uracil-DNA glycosylase
PCR – polymerase chain reaction
rRNA – ribosomal ribonucleic acid

Introduction

Research on aDNA or paleogenetics is defined as the study of degraded DNA fragments that preserved in subfossil material, ranged from hundreds or hundreds of thousands of years old (Pääbo *et al.*, 2004). The number of published papers on paleogenetics has increased exponentially in recent years, and the application of sophisticated new methodologies provided remarkable results in short time.

There are several fields for aDNA research applying. Firstly, in origin study it has revolutionized our understanding of evolutionary history especially of humans. Discovery of previously unknown hominins, analyze of homo group representative's interbreeding and their contribution to the modern human gene pool, including the Denisovans and the Neanderthals (Krause *et al.*, 2010; Pääbo, 2015). Clarifying the migration routes of human from Africa and peopling around the world in conjunction with transition processes from a hunter-gatherer lifestyle to pastoral herding, agricultural farming and domestication, that in turn shed light on the spread of animal pathogens transmission to humans (Frantz *et al.*, 2020; Nielsen *et al.*, 2017; Spyrou *et al.*, 2019). The aDNA data allowed to observe genomic signatures of selection processes in human populations and adaptive introgression: search positively selected genetic loci that accelerated adaptation and improved survival in new environments, thus possibly allowed modern humans to widely expand (Jensen *et al.*, 2022; Racimo *et al.*, 2015; Rees *et al.*, 2020).

Secondly, as already noted, study of paleopathology previously based on examination of fossils, mummified tissue, skeletal remains.

Pathogens and the diseases are one of the most important selective forces experienced by humans during their evolutionary history. Beyond host genomes and their pathogens, metagenomic characterization (analysis of genomes from an environmental sample) of our microbial selves and the identification of epigenetic marks have paved the way for ancient holobiomes that lead to greater understanding of past social, dietary, and environmental shifts and their impact on health. individuals and populations. Paleopathology has emphasized the importance of DNA science not only in understanding our past, but also how our past can affect our lives, our biology and health today.

Thirdly, aDNA has been used to investigate not only human populations but wide-ranging topics, including biogeography and ecology: reconstruction of the phylogeny, extension ways and environmental shifts of controversial species, ecosystem responses to climate change such as impact of Holocene climate change on biodiversity and anthropogenic effect on extinction processes that providing an analogue for understanding potential impacts of future change (de Bruyn *et al.*, 2011; Drummond *et al.*, 2005; Flink *et al.*, 2014; Miller *et al.*, 2012; Mitchell *et al.*, 2014; Rawlence *et al.*, 2014; Shapiro *et al.*, 2004; Willerslev *et al.*, 2014).

DNA Survival

DNA has limited chemical stability and decays without the enzymatic repair mechanisms of living cells. It took about three decades from the beginning of aDNA research to numerous attempts to demonstrate a general relationship between age and DNA preservation. It for a long time could not be proved due to the lack of empirical data on the temporal trends in the rate of DNA decay over time. As a result, studies of radiocarbon-dated DNA from highly closely localized bone specimens showed that DNA damage occurring after death (post-mortem) can be seen as a rate process with a theoretically possible calculation of the upper limit of the DNA preservation (Allentoft *et al.*, 2012).

Theoretical and empirical data presents that age of the upper limit of DNA survival differs between the mitochondrial and nuclear genomes. The limit for PCR amplifiable DNA between 400 kyr and 1,5 Ma, beyond which DNA is either severely crosslinked or non-detectable (Willerslev & Cooper, 2005). mtDNA is the first ancient genome that was sequenced through laborious bacterial cloning due to its short size and the approaches, it was extracted from the dried muscle of a museum specimen of the quagga in 1984 (Higuchi *et al.*, 1984). Until 2013 the oldest ancient genomes were aDNA from a 560,000-780,000 year old horse leg bone (Orlando *et al.*, 2013), the complete mitochondrial genome of a ~400 kyr-old cave bear (Dabney *et al.*, 2013) and archaic hominin (Meyer *et al.*, 2014) from Spain. Last year paper pushed Middle Pleistocene limit to the new ancient record – 1.65 million years old mammoth teeth from Siberian permafrost have produced aDNA obtained 49 million base pairs of nuclear DNA (Meyer *et al.*, 2016; Orlando *et al.*, 2013; van der Valk *et al.*, 2021).

Dating methods

Radiocarbon dating

In order to correctly interpret the finds of an ancient specimen, it is important to have an accurate estimate of its age for confirming obtained by the molecular clock method age and verifying the results, for which radiocarbon dating is currently the standard. (Kulkova, 2016) Classic paleomaterials for studying aDNA are rich-collagen bones and teeth perfect fits for radiocarbon age analysis as they contain carbon isotops (Geigl & Grange, 2018).

The method of radiometric dating is based on the measurement of radioactive decay or the formation of specific elements. Prior to the advent of this class of methods, archaeologists determined the age of finds using the method of relative chronology – through iconic artifacts or objects of material culture, as well as the method of stratigraphy. Radiocarbon dating is a method of dating organic remains based on the beta decay of the naturally occurring radioactive isotope carbon (^{14}C). Radioactive decay proceeds at a constant rate and regardless of environmental factors that makes it a convenient dating facility (Bronk Ramsey, 2008).

The ^{14}C radionuclide is formed in the upper atmosphere under the influence of cosmic rays, then the ^{14}C isotopes spreads through all layers of the atmosphere, participating in the biochemical processes of living organisms. Through food chains and chemical transformations, the 14C isotopes enters living organisms, in which carbon isotopes maintain the concentration ratio as in the atmosphere. When an object falls out of carbohydrate metabolism, for example, at the death of an organism, its replenishment with all carbon isotopes stops, while the concentration of stable isotopes does not change, and the radioactive decay of ^{14}C continues at a constant rate - this is the starting point for dating. The radioactive ^{14}C atom is unstable and gradually transforms into nitrogen atoms, the half-life of ^{14}C is 5730 ± 40 years. With the help of radiocarbon analysis, it is possible to estimate the age of samples no older than 50 thousand years, after which the radionuclides will exhaust themselves (Kulkova, 2016).

By comparing the content of the radioactive isotope ^{14}C in the sample and in the atmosphere radiocarbon dating provides age of the object. However, in different time periods and at different latitudes the accumulation of ^{14}C fluctuates due to changes in the intensity of cosmic rays, solar activity and a number of anthropogenic factors. To calculate the absolute geologic age the ^{14}C derived age must be converted to an equivalent calendar through the calibration curve that compensates atmospheric carbon concentration fluctuations. Calibration curves are based on independently dated archives renewing by dendrochronology data or uranium series dating of corals (Bronk Ramsey, 2008). The latest update was released in 2020, IntCal20 (International Calibration 2020), which consider the features of the northern and southern hemispheres, as well as the surface layer of the ocean (Reimer *et al.*, 2020; van der Plicht *et al.*, 2020).

The new version of the IntCal20 calibration curve improves the precision for the study of human evolution in terms of chronological overlap between the presence of H. sapiens and Neanderthals, e.g., directly dated fossil of the oldest H. sapiens so far found in Eurasia from Ust'-Ishim carries a similar amount of Neanderthal DNA ancestry as present-day Eurasians. In this case, the Neanderthals gene flow occurred 7000 to 13,000 years before Ust'-Ishim lived (Fu *et al.*, 2014). In terms of uncalibrated radiocarbon dating, the chronological overlap between the earliest H. sapiens (Bacho Quiro Cave) and the latest H. neanderthalensis (Saint Césaire Cave) in Europe is 6250 ± 910 years. When calibrating against the IntCal13 and IntCal20 curves, this difference decreases to 5000 ± 860 and 3960 ± 710 calendar years, respectively. This conclusion is important for studies of the mixing of Neanderthals and anatomically modern humans in Europe. It also provides better resolution in relation to climatic events (Bronk Ramsey, 2008; van der Plicht *et al.*, 2020).

Genetic dating

Differences in DNA sequences correspond to nucleotide substitutions that have accumulated since their separation from the most recent common ancestor. When the average number of substitutions occurring per unit of time can be determined, the "molecular clock" rate can be estimated. Assuming a constant rate of change among lineages, molecular clocks used to estimate the time of divergence between closely related species or between populations, that allows a direct conversion of the estimate of the degree of replacement due to genetic data into absolute time scales. Fossil evidence has often been used to estimate the MRCA date of two related groups, thus providing a calibration point for the molecular clock (Rohde *et al.*, 2004).

Calibration points are necessary because they anchor the samples and/or population breakdown to some date, which allows extrapolation of the time scale for the remaining samples and nodes (Shapiro *et al.*, 2004). Traditionally, calibration points are found within the fossil record, and/or biogeographic dates (Ho *et al.*, 2015). Clock calibration is usually performed at Bayesian analysis provided by various software.

Substitution rate estimates can also be derived from the branch length differences ob-

served along the tree. The dating of ancient genomes using genetic calculations based on the assumption that ancient samples have experienced fewer generational changes than living descendant relatives. Recombination occurs at approximately the same rate in all generations, the accumulated number of recombination events provides a molecular clock for the time elapsed or, in the case of an ancient sample, the number of missing generations since it ceased to evolve. This approach gain greater accuracy in substitution rate measurements is to analyze genetic data from ancient specimens for which reliable radiocarbon dates are available. Ancient humans are well suited to provide calibration points for the human mitochondrial molecular clock: reliable radiocarbon dates are available for many specimens, so the number of substitutions accumulated across lineages can be directly converted to the number of substitutions per site per year. Branch shortening - the effect of fewer nucleotide substitutions on ancient branches of a phylogenetic tree compared to modern ones, is commonly seen in phylogenetic studies of ancient humans (Fu *et al.*, 2013). The observed shortening of the branches reflects the relatively shorter time from a common ancestor for ancient human compared to modern: the present-day lineage had more time to accumulate nucleotide substitution. If we know the age of an ancient specimen from a 14C date, we can thus suppose the mutation rate required to produce the observed degree of branch shortening (Moorjani *et al.*, 2016).

Molecular clocks calibrated with ancient DNA have also been used to estimate the age of samples for which direct dates cannot be obtained, such as biological material beyond the range of radiocarbon dating. Phylogenetic trees are reconstructed within a Bayesian framework, and the ages of internal nodes are treated as unknown parameters. The ages of all sampled external nodes are known (e.g., from radiocarbon dating and/or cultural context) except the one that needs to be estimated, which represents an additional model parameter. This method firstly was applied for dating ancient samples on a genome-wide scale research of the Denisova finger bone, also was used to date the Sima de los Huesos hominin to about 400 kyr ago (Leonardi *et al.*, 2016; Meyer *et al.*, 2012, 2014).

An alternative approach of the branch shortening method based on the recombination clock (Hinch *et al.*, 2011). It was developed for dating ancient non-African individuals, leveraging on the size of Neanderthal blocks present in their genome. The basic idea is to estimate the date of Neanderthal introgression separately for ancient and modern non-African genomes, by together modeling recombination rates across the genome and the decay of Neanderthal ancestry through time. Tested on five ancient Eurasian genomes older than 10,000 years ago, this method provided age estimates largely consistent with radiocarbon dates (Chen *et al.*, 2020; Moorjani *et al.*, 2016).

Sources of aDNA

Certain materials have already been proven as classical for aDNA extracting, nevertheless new DNA reservoirs are still being discovered in a huge variety of forms: desiccated, charred, waterlogged or mineralized (Orlando *et al.*, 2021). Depending on the object of study, these can be plants parts (seeds, pollen, cobs, herbarium specimens), animal remains (leathers, eggshells, mollusk shells), «cultural» artefacts including skin parchments and drinking horns, pottery or birch pitch mastics (Orlando *et al.*, 2021). In addition to DNA from a single species, whole communities can also be recovered from a single sample, such as preserved coprolites or calcified dental plaque (calculus). This enables metagenomic analyses of the gut and oral microbiota, as well as the detection of pathogens, parasites, and foods. Environmental archives including sediments, ice, and lake cores can be used to rebuild complete paleoecosystems at greater scales (Warinner *et al.*, 2014).

The first studies of aDNA were focused on extracting DNA material from soft tissues, for example, from mummified tissues, tissue remains from zoological museum stuffeds, and preserved specimens (Higuchi *et al.*, 1984; Pāābo, 1988). But soft tissue, however, is rarely preserved and even if not, it does not mean preserving DNA, e.g., bog bodies have remarkable conservation across thousands of years, how-

ever, the acidic environment of bogs makes recovering genetic material almost impossible even from bones. Djinis, E. (2021) Joshua Levine, P. by C. A. (2017) Thus, the first successful sequenced high-coverage (∼20x) sample of aDNA were isolated in 2010 from a ∼4 kyr-old hair shaft of a palaeo-Eskimo Greenland, which caused the physicochemical properties of hair keratin - resistance to contamination, that are atypical for soft tissues. The use of mineralized tissues for DNA extraction soon became popular due to more abundant e and much better preservation, so the bones and teeth of vertebrates gained popularity (Miller *et al.*, 2008; M. Rasmussen *et al.*, 2010).

The focus of more recent aDNA researches has switched to mineralized tissue – vertebrate bones. Skeletal tissue, such as bones or teeth, can resist post-mortem degradation better than other types of tissues like skin and hair, thereby it is a suitable biological material for studying human ancient DNA. The choice of skeletal element type and its intra-bone part is important because of differences in DNA preservation. The content of endogenous DNA is a key factor. Since DNA degrades over time and skeletal tissues become colonized by microbes, the percentage of endogenous DNA in ancient samples is often less than 1%, that makes genome-scale analysis impossible or unreasonably expensive.

However, not all bone elements are equally effective in preserving DNA. Pore structure of bones is the resulting mineral dissolution, which increases with higher porosity, leading to more mineral loss and lower bone mineral density. The petrous bone, part of the temporal bone, is the hardest and most dense bone in the mammal body (Robling *et al.*, 2006). The otic capsule surrounds and protects the sensory organs of the inner ear, known as the vestibulocochlear organ. With ear ossicles and teeth serving as additional acceptable options, the petrous bone has been selected for retaining a high degree of endogenous DNA preservation (Damgaard *et al.*, 2015; Gamba *et al.*, 2014).

It has been demonstrated that levels of nucleated cells in the apical cementum layer are unaffected by age whereas concentrations of nuclear DNA in the inner dentine layer substantially decrease throughout the individual's life. Furthermore, a quantitative PCR method revealed that the cementum of old teeth typically contains higher concentrations of human mtDNA than the dentine. The cementum layer, which is exposed at the root surface and may be more susceptible to microbial colonization than dentine, would nonetheless show a lower amount of endogenous DNA. Dentine is typically favored for genetic research because, in addition to host DNA, it also enables the recovery of ancient blood-borne pathogens, despite the fact that tooth cementum might contain significant amounts of host DNA (Adler *et al.*, 2011; Higgins *et al.*, 2013).

Tooth and petrous bones are two the most excellent substrates for ancient genomic research but overall, the petrous bones perform better than the teeth. At once petrous bones display a higher C to T damage rate and have smaller ratios of mtDNA to nuclear DNA compared to tooth cementum (Hansen *et al.*, 2017). When choosing a material, it is necessary to assess the harm: there are two temporal bones in the skull and at least a few teeth under various conditions of preservation. The use of the entire otic capsule by drilling can bring great value losses, but the tooth sample can also be poorly represented, at the same time average obtained coverage of genome can differ several times for the same price as a one genome.

The bone portions that had the highest concentrations of dead osteocytes is ones with the highest chances of having endogenous DNA, this part can avoid phagocytosis by osteoclasts during bone remodeling – removal of mineralized bone by osteoclasts followed by the formation of bone matrix through the osteoblasts that subsequently become mineralized (Robling *et al.*, 2006). Petrous bone undergoes minimal remodeling after childhood which may be one of the explanations for its high endogenous DNA concentration (Srensen & Bretlau, 1997). Excellent DNA preservation also results from other in vivo calcification processes, such as those that turn dental plaques (biofilm of microorganisms on teeth surface) into calculus. It is nearly ubiquitous, allowing simultaneous investigation of pathogen activity, host immunity,

and diet, thereby expanding direct research into common diseases in the human evolutionary past (Warinner *et al.*, 2014, 2015).

There is link between visual and molecular preservation, and well-preserved teeth perform on average just as well as the petrous bones (Hansen *et al.*, 2017). Physical and chemical conditions (temperature, humidity, pH, etc.) that affect the bone from the time an organism dies until the time a skeletal component is excavated are the key determinants of long-term degradation. Even ancient petrous bones can be devoid of any endogenous DNA due to the rates of the decay events, which are influenced by these factors. This enormous variation between burial sites is likely caused by these factors. With the exception of empirical data and the thermal age (Smith *et al.*, 2001), there is a lack information that would help to identify the archaeological or palaeontological sites that are best for DNA preservation. Finally, the density of bones may also help to exclude ambient DNA (Geigl & Grange, 2018).

Challenges in aDNA work

Preserved aDNA is often limited in quantity: highly degraded, fragmented and chemically modified, which can be also complicated by contamination with modern DNA. The successful retrieval of aDNA sequences from remains depends on the postmortem instability of nucleic acids. Post-mortem DNA degradation consists of two separate phases that determine the outcome. The first phase of decay accords to the enzymatic decomposition of most of the organic material, including autolysis and putrefaction. This phase of early diagenesis most influences the number and size of surviving DNA molecules and is probably the reason for the lack of correlation between sample age and the average size of surviving DNA molecules (Kistler *et al.*, 2017; Sawyer *et al.*, 2012).

The first phase is likely to be subject to a lot of variation because it cannot be reconstructed. During the second phase of decay, non-enzymatic chemical degradation seems to correlate with the thermal age of the samples and, in particular, with the deamination of cytosines at the ends of DNA molecules. The third factor determining the success of paleogenomic approaches is the varying degree of penetration of DNA from the environment into the bone during burial. This environmental DNA dilutes endogenous DNA and can make shotgun sequencing very costly (Sawyer *et al.*, 2012).

Post-mortem damage

DNA is damaged mainly by two phenomena: hydrolysis and oxidation. In alive organism DNA damage such as from UV radiation is fixed by repair mechanisms of cell system. But DNA damage continues after organism death, while repair pathways no longer function, thereby aDNA strands accumulate various postmortem damage. A diversity of chemical reactions is known to affect aDNA strand breaks and include the fragmentation of aDNA molecules into ultra-short pieces. The most dramatic of these changes is aDNA fragmentation, which occurs through hydrolytic depurination followed by elimination reactions, during this process the aDNA double helix breaks apart into pieces that can be millions of times shorter than their original length during life (Gaeta, 2021).

Hydrolysis can induce misincorporations, most commonly the deamination of cytosine to uracil causing (C to T), also deamination adenine to hypoxanthine (A to G), 5-methylcytosine to thymine (C to T), guanine to xanthine (G to A). The rate of such misincorporations increases towards the ends of reads when mapped against a reference sequence because of cytosine deamination preferentially occurring in the single-stranded overhang termini of aDNA fragments. While the misincorporation of C to T provides convenient genetic signatures for identifying aDNA, it can potentially lead to incorrect results of further sequence analysis (Briggs *et al.*, 2007).

Oxidation as a result of interaction with ionizing radiation or the action of free radicals can induce base misincorporations or block polymerases and either stop amplification or lead to "jumping PCR" and the production of chimeric sequences. Cross-links either within or between DNA strands via alkylation (Paabo, 1989) will also block the polymerase and stop amplification. Strand breaks caused by nuclease activity,

microorganism degradation, preservation conditions, direct break (hydrolysis), depurination causes abasic site (hydrolysis). These post-mortem changes potentially interfere the manipulation of aDNA and reduce the amount of recoverable genetic information. Most saved DNA strands in ancient remains are short in length (fewer than100 bp).

To reduce the impact of sequence errors caused by damage, DNA extracts can be further processed prior to library generation with a commercially available uracil-DNA glycosylase (UDG) and endonuclease VIII enzyme mixture known as the USER reagent (New England Biolabs). This reagent removes uracils and cleaves the resulting abasic sites, thus repairing the damage but also shortening the DNA molecule (Briggs *et al.*, 2010). Despite the positive effect of reducing sequencing errors, USER processing also has the negative effect of removing damage patterns that are necessary to authenticate aDNA sequences and differentiate them from contaminating DNA. However, despite the elimination of uracils in the template molecules, the sequencing of aDNA USER-treated samples can still shows a slight signal of C to T at the aDNA fragments termini. This signal is mostly driven by the presence of methylated CpG epialleles in the sequence data (Pedersen *et al.*, 2014). For non-mammalian DNA analysis, a raw (non-UDG) DNA library can be initially screened to determine the authenticity of sequence origin, and then second library processed by USER (full-UDG) can be generated for analysis. To decrease the cost of library preparation, there is the USER (partial UDG-treatment) protocol, which eliminates most of the damage, but retains one uracil at each terminus, thereby providing DNA authentication and preserved damage signal at the terminal bases can be cut off computationally during subsequent analysis (Rohland *et al.*, 2015).

Simple modification to high-throughput sequencing library preparation removes uracil residues from ancient DNA and subsequently repairs the DNA fragments, greatly increasing the accuracy of the DNA sequences determined while maintaining DNA sequence yield from precious DNA sources.

Post-mortem damage can accumulate with the age of the sample, but the amount of damage is sample-dependent and linked to preservation conditions, as a result, some recent samples can be more damaged than older ones (Dabney *et al.*, 2013). Under favorable conditions, DNA can survive for thousands of years in the remains. Cold, dry, temperature-stable environments such as permafrost regions and caves are among the best sources of well-preserved specimens. Preservation in cold environments may reduce nuclease activity, reducing some of the damage that occurs immediately after death. The rate of depurination is influenced by temperature, among other factors (Shapiro *et. al.*, 2004), which explains why the most extreme survival of DNA was recorded in ice cores (Willerslev & Cooper, 2005). With rare exceptions, it is possible to effectively conduct aDNA study of samples extracted from the remains found in the equatorial climate, therefore sampling for paleogenetic studies poorly represented at the territory of Africa, such as genome-wide DNA data from 4 children from Shum Laka (Cameroon) buried 3-8 kyr – one of the earliest known archaeological sites within the probable homeland of the Bantu language group (Lipson *et al.*, 2020). Also, some of the populations stay understudied due to various burial practices in cultures.

Contamination

Not all ancient samples are equally possible to be affected by contamination. Studies of ancient humans or microorganisms are at highest risk for contamination due to the pervasive nature of both potential contaminants and challenges distinguishing a potential contaminant from an authentic ancient sequence. Human contamination can be introduced to ancient human samples in several ways: during excavation, for example, bones and teeth are typically handled in non-sterile environments by bare hands. Bones and teeth are also sometimes cleaned by washing in water, which can contain human cells like skin flakes. This is a problem because hydroxyapatite, which is the main mineral component of bone, tooth enamel, and den-

tin, absorbs DNA in a liquid environment (Korlević *et al.*, 2015). Ancient samples can also be contaminated during museum storage, both through touching and contact with other samples.

At any point during the processing of an aDNA sample, contamination can happen. The sample itself can be contaminated. Additionally, contamination may be introduced during subsequent stages of an experiment, such as DNA extraction, the creation of a sequencing library, or PCR setup. Reagents may be contaminated, laboratory staff may introduce their DNA, or any DNA brought into the lab on their person, their shoes, or their clothing, and airborne particles may enter through the building's air supply. Another potential source of contaminated DNA is previously amplified DNA found in the lab setting. Over a million copies of the template may be present in even the minuscule amount of DNA that is released when a tube is opened. The laboratory where prehistoric samples are created and any laboratory where samples are processed after amplification should be kept strictly separate (Fulton & Shapiro, 2019).

The reduced size of the DNA molecules makes it difficult to separate endogenous DNA of interest from contaminating DNA and from co-extracted small molecules that could act as inhibitors in toward enzymatic reactions. After death, tissues are colonized by microbial decomposers, which can introduce exogenous contamination by microbial DNA that in some cases represents >99% of recovered DNA (Green *et al.*, 2010a). The most challenging complication of aDNA research stems from the small proportion of surviving copies of endogenous DNA in an extract, compared to the ubiquitous nature of DNA in the environment. In an ideal experiment setup collected from excavated remains samples should be immediately with minimal handing processed to limit potential contamination from modern sources, including staffs and storage facilities (Fulton & Shapiro, 2019). There are numerous examples of studies where contamination has led to erroneous results, the field of aDNA has developed various techniques to identify, remove or reduce contamination introduced during post-excavation storage and handling (Gamba *et al.*, 2016; Korlević *et al.*, 2015).

Even when the level of contamination is extremely low, PCR will preferentially amplify modern DNA over damaged ancient molecules. Copies of the targeted fragment may contain blocking lesions, for example, which affect polymerase processing, or may simply be in low abundance so that PCR enters the exponential phase many cycles after the reaction has begun. If only a few contaminant molecules are present and amplified during the initial cycles of the PCR, these will rapidly outnumber (and outcompete) amplification of the authentic ancient DNA (Fulton & Shapiro, 2019).

Computational pipelines have been developed to detect the presence of contaminating DNA after DNA sequencing has been performed. It is more difficult to identify authentic and contaminated DNA when the aDNA is more closely related to the potentially contaminating DNA. In archaic hominins such as Neanderthals, all mitochondrial genome sequences published so far fall outside the variation of modern humans, making present-day human contamination estimates achievable if mitochondrial coverage is sufficiently high (Green *et al.*, 2010b; Reich *et al.*, 2010; Renaud *et al.*, 2019).

aDNA research design

Work with ancient DNA is time-consuming and expensive. However, when care and appropriate precautions are taken from the outset, it can be a powerful tool for investigating evolutionary processes that cannot be addressed using modern data alone.

The peculiarities of working with aDNA require that the laboratory should be maximally optimized in order to exclude contamination of ancient samples. The researcher is responsible for creating a critical study design, with thoughtful experimental objectives. Not all laboratories can afford full equipment, methods and materials, some of the requirements may be optional, but do not neglect the conditions for working with ancient samples. With the move from PCR-based analysis to NGS, it has be-

come easier to identify and prevent contamination, but be attentive, stringency and scientific soundness of action is a key for work (Cooper & Poinar, 2000; Gilbert *et al.*, 2006).

Appropriate setup of the aDNA workspace is critical. The aDNA facility should be isolated from locations where PCR is performed routinely, preferably in a separate building that does not house any PCR labs. Ideally, the room will be positively pressurized, so that air does not flow in from the adjoining room/hallway when the door is opened.

One should assume that all reagents and tools are contaminated with human DNA, even those marked as sterile. All equipment should be decontaminated before use, for example, by UV irradiation, baking, acid-treatment or bleach. All of the methods are aimed at destroying DNA and must be strictly followed according to the protocol in order to exclude the remains of fragmented DNA. In an effort to clean ancient bones and teeth of surface pollutants, numerous techniques have been proposed. These include physically removing the outer surface, washing it using chemicals like water, EDTA, bleach, ethanol, acid, or hydrogen peroxide for an extended period of time, UV-lighting the sample, and/or extracting the interior material (Kemp & Smith, 2005). Bleaching the bone powder appears to degrade contaminant DNA more quickly than endogenous DNA if the material is well-preserved (Salamon *et al.*, 2005).

It is important rule in the laboratory to isolate samples as much as possible before the amplification step and to follow the concentration gradient of potential contaminants. According to this rule, equipment and reagents can only be moved in one direction – from a clean area to a dirty one, or in the direction of the previous stages. Division into zones: sample preparation zone; decontamination and preparation for extraction; zone for purification and preparation of libraries for sequencing. If it is necessary to move against the gradient, the worker must change suit and take a shower.

Negative extraction and PCR controls obligatory. Extraction and PCR controls containing no DNA (negative controls) should be carried out next to the samples set at every step. Appropriate molecular behavior for PCR-based studies, an inverse relationship should be observed between the length of the targeted PCR fragment and the strength of the amplification. Reproducibility within-lab replication of PCR amplifications, overlapping PCR products, and amplifications from multiple DNA extractions must be consistent. Cloning – at minimum 10% of subset of PCR amplifications should be cloned to assess damage and detect nuclear mitochondrial insertions, chimeric sequences from jumping PCR, and contaminants (Handt *et al.*, 1996).

Biochemical preservation of extracted DNA and quantitation of starting material. DNA from associated remains using for confidence. Use of a «carrier DNA» negative in PCR-based assays for cleaning from low concentrated pollutants. Time-dependent or preservation-dependent pattern of DNA damage and sequence diversity. Critical assessment of the sensibility of the results obtained from an ancient DNA experiment is an important aspect of aDNA research.

Obtaining almost all aDNA data to date requires PCR amplification of libraries to amplify signals prior to sequencing. There are 2 types of NGS applying for aDNA: double-stranded library preparation and single-stranded library preparation. Each has different characteristics in terms of costs, hands-on time and sensitivity. In the double-stranded preparation method, aDNA molecules are end-repaired and ligated to double-stranded adapters whereas in the single-stranded preparation method, heat-denatured aDNA templates and adapters are ligated as single-stranded molecules (Briggs *et al.*, 2007; Gansauge *et al.*, 2020; Gansauge & Meyer, 2013; Kircher *et al.*, 2012). Advantage of the single-stranded preparation method is that it enables the molecular selection of DNA templates carrying evidence of post-mortem DNA damage, which can increase the fraction of endogenous DNA incorporated into sequencing libraries and thus reduce downstream sequencing costs 145 146.

Although single-molecule sequencing has provided a PCR-free alternative for sequencing aDNA libraries, the generation of almost all

aDNA data to date has required library PCR amplification to boost signals prior to sequencing. (Dabney & Meyer, 2012). Conventional DNA polymerases exhibit different preferences for templates of a particular size and/or base composition and differ in their ability to «correct» to bypass post-mortem damage, so the use of other polymerases such as Pfu Turbo Cx, Herculase II and Accuprime Pfx is necessary. They can perform uniform amplification of fragments with different base compositions and lengths, as well as maintain the original complexity of the library (Seguin-Orlando *et al.*, 2015).

Endogenous DNA due to low concentration in libraries can make gun sequencing of entire ancient genomes uneconomical, target-enrichment approaches have thus been developed to focus sequencing efforts on library content of particular interest Target enrichment is a DNA preparation step prior to sequencing whereby DNA is either directly amplified (based on PCR) or captured (hybrid capture-based). These improved DNA fragments quality that can then be sequenced using DNA sequencers. Targeted enrichment is a targeted sequencing technique, also known as resequencing. Targeted enrichment improves and sequences only a portion of the entire genome, or regions of interest, without sequencing the entire genome of the sample. To focus only on the detection of a genome or DNA sample, targeted enrichment is required (Lang *et al.*, 2020; Suchan *et al.*, 2016).

Despite occasional use of alternative methods, Illumina instruments have produced the vast majority of aDNA sequence data due to its accessibility, large data output, cost-effectiveness, and generally low error rates. Additionally, it functions best with relatively short DNA sequences (300 bp) and is ideally suited for sequencing DNA with a length of 50–150 bp, which is the average for aDNA.

The choice of the most suitable methods for aDNA sequence analysis depends on the the research project's objectives, such as whether they are focused on population history modeling, microbiological profiling, or paleoenvironmental reconstruction. However, there are several processes in the analytical workflow that apply to the majority of projects. Raw sequencing data processing, alignment against reference genomes and/or sequence databases, and the evaluation of authenticity and error rates, including miscoding lesions brought on by post-mortem damage, are all part of these processes.

Sequencing DNA molecules in one or both directions can provide single readings or paired-end reads. Index demultiplexing, read trimming, and consensus sequence creation are the first processing steps. Read trimming removes adapters and/or low-quality terminal sequences, and consensus sequence creation collapses overlapping read pairs into a single read pair. Processed reads are usually aligned to reference genomes of the focal species, such as the human genome, as well as potential microbial pathogens that may have infected the person during life. This is often done using BWA or Bowtie2 (Li & Durbin, 2010; Schubert *et al.*, 2016).

The next steps in the analysis are error deletion and endogenous DNA authentication, partly discussed in the Challenges in aDNA work chapter.

Ancient microbiome

Together with classical approaches in paleopathology and paleodemography, aDNA from microorganisms, including pathogens and commensals, can provide insights into the health of ancient peoples as well as shifts in diets and disease ecology.

Just as in the study of ancient genomes, a significant breakthrough in the ancient microbiomes has occurred since the introduction of NGS technology. The accumulated array of sequence data allowed authentication based on template sequences. The analysis of poorly preserved ancient material is facilitated by the use of library enrichment for NGS. Targeted enrichment contributed to the study of the first ancient bacterial genome of Yersinia pestis, which was extracted from the teeth of a 14th-century plague victim with a 30-fold coverage (Bos *et al.*, 2011). Up to this point, there had been controversy about the possibility of isolating

plague bacteria from teeth (Drancourt & Raoult, 2014).

The material for the study of microbiome DNA contains endogenous and exogenous DNA reflecting the pre-mortem and post-mortem diversity of the microbiome respectively. Endogenous DNA consists of host-associated commensal species and epidemic pathogens while exogenous DNA contains bacteria from the environment, contamination from human interaction with samples and other contaminants (Warinner *et al.*, 2017). False-positive results often occur due to the relationship of pathogenic bacteria and bacteria due to the environment. Authentication is also complicated by the inconvenience of applying signature analysis of postmortem damage since postmortem colonization leads to a number of similar damage patterns from ancient to modern, making it difficult to distinguish (Weiß *et al.*, 2020).

In this case of the problem of contamination and exogenous and endogenous DNA mixing is solved by using mapDamage software to calculate damage and authenticate ancient samples in such genetically mixed samples (Ginolhac *et al.*, 2011). Another method effectively amplifies age associated degradation patterns in microbial ancient and modern mixtures is sequencing DNA libraries enriched in molecules carrying uracils. This facilitates the discovery of authentic ancient microbial taxa in cases where degradation patterns are difficult to detect due to large sequence divergence in microbial mixtures (Weiß *et al.*, 2020).

Another difficulty of the analysis is that the balance of the microbiome shifts after death, while anaerobic bacteria survive more successfully, such obtained results may be misinterpreted. The composition of a microbe's cellular membrane plays a key role in the postmortem preservation Gram-positive bacteria, whose nuclear acids are rich in GC content have advantage, also like mycobacteria, whose membranes includes mycolic acid that perform a protective function (Rivera-Perez *et al.*, 2016). Glycosidic ether lipids and hopanoids are components of bacterial membranes (not found in eukaryotes) protect DNA from enzymatic degradation (Schouten *et al.*, 2010).

Due to the conservation advantages and despite particular problems, researchers have been able to gain a wealth of information from the study of bacterial aDNA. The history of bacterial DNA study begins with the analysis of 16S rRNA of a single species and ends with the reconstruction and comparison of complete genomes from several genera. This is a widely used method for identifying microorganisms by comparing the nucleotide sequences of the gene encoding 16S rRNA. Low-cost and fast amplification and sequencing of the 16S rRNA gene can be easily carried out in mixed microbial communities without cultivation, since the sequence of this gene is similar in a large pool of microbes, and it can be used universal primers. Further analysis allows diversity to be distinguished as taxonomic units (OTUs), convenient measurable proxies for microbes related by origin (Kowalchuk *et al.*, 2004).

Ancient microbial DNA has been isolated from various sample types, including permafrost, halites, amber, bones, internal organs, dental pulp, and coprolites. Coprolites are mummified or fossilized feces and are perhaps one of the richest sources of ancient microbes. Coprolites have been found in dry, cold and even tropical environments (Poinar *et al.*, 2003; Santiago-Rodriguez *et al.*, 2013). Early studies used morphological observations to determine the parasitological composition of coprolites. Similarly, the possible diet of an individual was determined on the basis of grains and seeds preserved in the feces. Also, it was shown that helminth eggs (Tito *et al.*, 2012). have persisted for thousands of years, although it is not clear if they were still infectious. Paleoparasitological studies of coprolites were among the first to suggest that the dietary habits and even lifestyles of ancient cultures (such as hunter-gatherers and farmers) could be discerned from ancient specimens (Cano *et al.*, 2014).

In caprolites, it is possible to determine both pathogenic and microorganisms characteristic of a healthy organism (52). A more interesting direction is the study of the reflection of diet on the intestinal microbiome of ancient organisms, especially humans by not

only food remains but using metabolites remaining in coprolites (Kowalchuk *et al.*, 2004). This demonstrates that the presence or absence of DNA from spe-cific microorganisms in coprolites may be important to investigate diet but also that the metabolic pathways for the degradation of key compounds may be a great source of information as well. Geographically as well as ethnically distinct populations may have different variations in microbiome diversity (Tito *et al.*, 2012; Yatsunenko *et al.*, 2012).

The transition to farming and agriculture with the changes to a sedentary lifestyle contributed to the growth of infectious diseases and the emergence of new infections (Harper & Armelagos, 2013). The progression to denser, more fertile human populations in close proximity to domestic animals has been termed the Neolithic demographic transition. The use of the molecular clock method showed that at least three paleontological pathogens are associated with this event in human history: Y. pestis, M. tuberculosis, and M. leprae (Pearce-Duvet, 2006).

As the causal agent of plague, Y. pestis is responsible for multiple human pandemics with millions of deaths through human history and is therefore a crucial species for epidemiological research According to paleogenetic data, the first appearance of the plague occurred about 5 thousand years ago, which coincides with the expansion from central Eurasia and eastern and central Europe (de Barros Damgaard *et al.*, 2018; Rasmussen *et al.*, 2015). The first and second plague pandemics take place in 6th -8th and 14th -18th centuries, the third pandemic started in the mid-19th and is still going on (Wagner *et al.*, 2014).

Y. pestis is a slowly evolving and well-studied pathogen with a well-documented historical record and many paleogenomes available. However, it still lacks a reliable chronology of evolution, highlighting the difficulty of mapping the bacterial past (Arning & Wilson, 2020). Studying the spread of come across pathogens between host and microorganism is weighty to discover the mechanisms by which these pathogens interact with humans and animals; it also helps in finding new treatments (Dcosta *et al.*, 2011).

The human body, with its microbiome diversity, is a site for horizontal gene transfer, potentially making it a vessel for the development of antimicrobial resistance. Studies of the oral microbiome of modern humans have found genetic sequences associated with antibiotic resistance on dental calculus (Xie *et al.*, 2010). Later, sequences homologous to antibiotic resistance genes found in oral and pathogenic bacteria were identified in ancient calculus. Despite the poorly researched role of these genes, their presence indicates that even before the use of therapeutic antibiotics, the molecular mechanisms of antibiotic resistance were already at a low level (Sommer *et al.*, 2009).

Although the exact function of these genes in our samples is unclear, their presence nonetheless demonstrates that the low-level biomolecular mechanism for broad-spectrum antibiotic resistance has long been present in the human microbiome, showing how the oral microbiome functions as a source and reservoir of new antibiotic resistance (Warinner *et al.*, 2014, 2017).

Conclusion

To obtain valid data on ancient genomes, that is necessary to responsibly carry out research at all its stages: from archaeological excavations to strict laboratory processes and ethical standards. Successful work requires close collaboration between geneticists and archaeologists, as well as interdisciplinary specialists.

Paleogenetics is becoming more and more widespread and provides an amount of sciences by fast accumulating data with the remarkable rate. If the basic aim of ancient DNA research was more driven by enthusiasm and a desire to discover new human's ancestors and evolutional processes, then today's data show how much paleogenetics has advanced and highly specialized. Bringing practically applied genetic data, especially to medicine, paleogenetics has developed a respectful attitude towards itself through a difficult path with restrictions and failures to incredible annual news.

Acknowledgments

This work has been supported by the grants of the Russian Science Foundation, RSF 22-24-00681.

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