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Ancient DNA analysis

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Abstract

While the first ancient DNA molecules were extracted more than three decades ago, the first ancient nuclear genomes could only be characterized after high-throughput sequencing was invented. Genome-scale data have now been gathered from thousands of ancient archaeological specimens, and the number of ancient biological tissues amenable to genome sequencing is growing steadily. Ancient DNA fragments are typically ultrashort molecules and carry extensive amounts of chemical damage accumulated after death. Their extraction, manipulation and authentication require specific experimental wet-lab and dry-lab procedures before patterns of genetic variation present in past individuals, populations and species can be confidently interpreted. Ancient DNA data help to address an entire array of questions in anthropology, evolutionary biology, and the environmental and archaeological sciences. The data have revealed a considerably more dynamic past than previously appreciated and have revolutionized our understanding of many major prehistoric and historic events. This article provides an overview of the concepts and state-of-the-art methods underlying ancient DNA analysis and illustrates the diversity of resulting applications. It also addresses some of the ethical challenges associated with the destructive analysis of irreplaceable material, emphasizes the need to fully involve archeologists and stakeholders as part of the research design and analytical process, and discusses future perspectives.

[H1] Introduction

In 1984, short DNA fragments were extracted and sequenced from the dried muscle of a museum specimen of the quagga, a species of zebra that became extinct at the beginning of the 20th century, marking the birth of **ancient DNA [G]** (aDNA) research¹. Although the data gathered were limited to 229 base pairs (bp), it was the first time that direct genetic information had traveled through time, adding molecular evidence to the toolkit used by researchers to observe evolution and understand how the modern world came to be.

More than three decades later, the time range amenable to DNA analysis extends to over a half million years (560,000–780,000 years ago²), and many extinct species have now had their genomes completely sequenced, including woolly mammoths³ and cave bears⁴ as have human populations, from Vikings⁵ to Paleo-Eskimos⁶ to Neanderthals⁷⁻¹⁰. With the addition of ancient DNA data, our current atlas of genetic variation is not limited to a snapshot of the diversity found in present-day populations across the world. Instead, it is continuously enriched with temporal information tracking changes in the genetic ancestries of human, animal, plant and even microbial populations as they expanded, collapsed and adapted to new local environmental conditions¹¹⁻¹³.

aDNA has led to the discovery of new branches within the human family tree, including that of the Denisovans, who are close relatives of Neanderthals¹⁴⁻¹⁶. As a result, the genomic consequences of population decline¹⁷⁻¹⁹ and the underlying environmental²⁰⁻²² and/or anthropogenic drivers²³⁻²⁴ of extinctions have been revealed and clarified. Applying aDNA techniques to archaeozoological and archaeobotanical remains has also considerably enhanced our understanding of the transition from hunting and gathering to herding and farming, including how past human groups domesticated wild species showing preferred characteristics¹². Domestication provided new opportunities for zoonotic transfer of animal pathogens to humans, which have also been analyzed using ancient genomic investigation¹³. More generally, aDNA data has transformed our understanding of the historical emergence, virulence and spread of major infectious diseases²⁵. Beyond the genomes of the hosts and their pathogens, the metagenomic characterization of our microbial self²⁶⁻²⁷ and the identification of epigenetic marks²⁸⁻²⁹ have paved the way toward a study of ancient **holobiomes [G]**, which promise to reveal a deeper understanding of past social, dietary and environmental shifts and their impact on the health of individuals and populations.

None of these developments would have been possible without next-generation sequencing (NGS)³⁰, which remains thus far the most transformative technology in the history of aDNA research, profoundly affecting wet-lab and dry-lab activities alike (FIG. 1). At its most basic level, the success of NGS lies in its ability to accommodate the sequencing and analysis of millions of loci in parallel from minute amounts of ultrashort DNA fragments³¹⁻³³. As a result, cumbersome PCR-based analyses of individual loci, which were instrumental in initially establishing the field of ancient DNA analysis, are now almost entirely superseded by genome-scale studies, with the exception of mini-barcodes that remain useful for characterizing past environmental communities³⁴. NGS also importantly allows the study of DNA in its entirety, a cornerstone in modern authentication approaches of aDNA data. In-solution target-enrichment techniques probing specific genomic regions have become instrumental for the cost-effective recovery of sequence data at the gene³⁵, mitogenome³⁶, chromosomal³⁷ and even the whole genome³⁸ scale.

As a complement to more sensitive techniques for extracting³⁹⁻⁴² and integrating DNA into NGS libraries^{16,43}, new sources of archaeological material have broadened the range of aDNA applications. These include dental calculus^{26,44}, wood^{45,46}, mollusk shells⁴⁷, and sediments^{48,49}, as well as biocultural archives such as parchments⁵⁰ and textiles⁵¹. The discovery that particular bone types exhibit better molecular preservation^{52,53} has also facilitated the collection of aDNA time-series data at both population and genome-wide scales. NGS has furthermore revolutionized the analysis of ancient sequence data, providing statistical solutions to overcome vexing problems, such as rampant contamination⁵⁴⁻⁵⁶ and post-mortem DNA damage⁵⁷, while still enabling the detection of subtle changes in population structure by means of genetic drift, admixture and/or selection⁵⁸.

With the addition of new laboratory and computational methods developed in the last decade, aDNA analysis has now come of age. This Primer provides an up-to-date overview of the most commonly used ancient DNA methods and tools, as well as their limitations, and anticipates future innovations that will help propel us beyond the current state-of-the-art as we continue to advance the scope of molecular archaeogenetics.

[H1] Experimentation

Analyzing aDNA requires the destruction of irreplaceable, finite subfossil material that is part of humanity's bio-cultural heritage. As such, ancient DNA studies require careful scientific and

ethical planning and a commitment to responsible research. Once a research plan is established, sample analysis proceeds similarly for most sample types. Samples are taken to a dedicated clean lab facility, and DNA is typically freed from its parent material by a combination of demineralization and digestion. Often, samples have high levels of degradation and are prone to contamination by other DNA sources. Following purification and concentration steps, the ancient DNA is then constructed into an NGS library and sequenced. In addition to these core steps, there are also optional steps that can be performed at various stages to achieve desired effects, such as the complete or partial removal of post-mortem damage prior to library construction or the enrichment of specific genetic targets prior to sequencing.

[H2] Material types

Early aDNA studies considered macroscopic preservation a predictor of molecular preservation and consequently focused on soft tissues from naturally or artificially mummified remains and from stuffed or fluid-preserved museum specimens^{1,59}. Hair shafts provided the DNA source for the first successfully sequenced ancient mammoth⁶⁰ and human genomes⁶, in part owing to keratin's low permeability to contaminants and because it is easier to decontaminate⁶¹. Soft tissues, however, rarely preserve and — with the exception of hair — are usually heavily contaminated by environmental microbes⁶². Mineralized tissues are more abundant and typically better preserved than soft tissues, and consequently the focus of more recent aDNA studies has shifted to vertebrate bones and teeth⁶³. The petrous bone has been singled out for retaining a high degree of endogenous DNA preservation⁵², with ear ossicles⁶⁴ and teeth providing other suitable alternatives. Although tooth cementum can contain high amounts of host DNA⁵³, dentine is generally preferred for genetic analysis since, in addition to host DNA, it also allows for the recovery of ancient blood-borne pathogens^{65,66}.

In studies of ancient plants, suitable materials for genomic analysis include desiccated, charred, waterlogged or mineralized⁶⁷ pollen⁶⁸, cobs⁶⁹⁻⁷¹, pips⁷², herbarium specimens⁷³ or seeds^{74,75}. Seeds have also been found to preserve RNA^{76,77}. New aDNA reservoirs are also still being discovered in the form of purely biological substrates, such as insects⁷⁸, feathers⁷⁹, eggshells⁸⁰, mollusc shells⁴⁷ or wood^{45,46}, but also from “cultural” artefacts including livestock skin parchments⁵⁰ and drinking horns⁸¹, pottery⁸², or birch pitch mastics^{83,84}. Beyond DNA from single focal species, whole communities can also be retrieved from single samples, such as preserved feces (paleofeces or coprolites) and calcified dental plaque (calculus), allowing metagenomic analyses of the gut^{85,86} and

oral microbiota^{26,44}, respectively, as well as the detection of pathogens^{26,44}, parasites⁸⁷ and foods^{88,89}. At larger scales, entire paleo-ecosystems can be reconstructed from environmental archives, such as sediments^{22,49,90}, ice⁹¹ and lake cores^{34,48,92}.

[H2] Ethical aDNA research

There are several ethical considerations that researchers must evaluate before embarking on destructive analysis of irreplaceable archaeological material (FIG. 2). The sampling and destruction of human remains for aDNA research needs to consider cultural, historical and even political implications. Consultation with appropriate local stakeholders, including descendent communities, both prior to the start of a project and during its development, enriches aDNA research and is highly encouraged. This is not limited to human remains, but applies to ancient DNA studies generally, including studies of animals, plants and artifacts.

Ethical issues with regard to aDNA research encompass: conceptualization, sampling, and communication. The conceptualization of the project should proceed in a way that is mindful of historical, cultural and political realities and that involves communication with local stakeholders (excavators, curators/museums, local communities, religious institutions) on equal footing. This should include detailed communication of the relevance and potential outcomes of the analyses as well as possible risks^{93,94}. Second, the strategy and process of sampling should include sample documentation (for example, by photography and/or surface or computed tomography⁹⁵), the use of minimally destructive analytical techniques⁹⁶⁻⁹⁹, and a plan for proper export and storage of samples in accordance with official permissions. The remaining sample material should be returned to the museum or appropriate group, with restoration (or production of a copy) of destroyed parts^{94,100,101} when requested. When working with rare samples, the methods should first be extensively tested and demonstrated to be successful on similar, but more commonly available material (e.g., faunal remains at hominin sites), and sample material should not be fully exhausted but at least partially conserved for future research. Third, communication and consultation should be ongoing with stakeholders, including descendant communities. Ideally, this communication and consultation will include joint decision-making and local capacity building, collaboration on publications and agreement about data management¹⁰². Wagner and colleagues¹⁰³ make five recommendations for establishing successful collaborations between aDNA researchers and descendant communities: consult formally with communities; address cultural and ethical considerations; engage communities

and support capacity building; develop plans to report results and manage data; and develop plans for long-term responsibility and stewardship. Finally, researchers should be mindful of the language that they use in publications, and specifically the potential for misinterpretation or offense given by technical terms — such as specimen, admixture, inbreeding coefficient — that may imply different meanings in scientific and general contexts. Researchers should use a reflected vocabulary that avoids biological essentialism¹⁰⁴, and ensure that their work avoids falling into the pitfalls of racial, nationalist or simplistic narratives¹⁰⁵⁻¹⁰⁷ (BOX 1).

[H2] Ancient DNA facilities

Preserved aDNA is often limited in quantity and has a high degree of degradation, which can be compounded by contamination with modern DNA. Under ideal conditions, samples would be collected immediately from excavated individuals, with minimal handling to limit potential contamination from modern sources, including staff and storage facilities. However, this is not possible for the large archives of already excavated remains in museums and institutes around the world. As such, the field of aDNA has developed a variety of techniques to identify, remove or reduce contamination introduced during post-excavation storage and handling^{40-43,54-57}.

Regardless of sample origins, the extraction and manipulation of aDNA must be carried out in dedicated clean lab facilities to minimize further contamination risks. Such facilities are typically access-regulated and located in separate buildings than those where post-amplification DNA is manipulated. They are maintained as sterile environments through HEPA-filtered positive air pressure systems, UV exposure and daily (bleach) decontamination treatment of bench surfaces¹⁰⁸. Anterooms allow researchers to dress in suitable personal protective equipment (PPE), including disposable full body suits, gloves, sleeves, face masks and overshoes. The workspace is generally divided into multiple, separate rooms in which specific experimental tasks can be performed so as to parallelize work while limiting cross-contamination risks. Lab equipment is routinely decontaminated before and after use by cleaning with bleach and alcohol, while laminar flow hoods, with monitored air extraction and filtering systems, help prevent pollen, powder and aerosol contamination. These strict procedures are necessary to minimize modern DNA entering the facilities through reagents, ventilation and staff personnel.

[H2] DNA extraction

In order to maximize preservation of the remains' integrity and allow potential further molecular or morphological analyses, minimally-destructive methods have been proposed to sample bones⁹⁶, teeth⁹⁷, insects⁹⁸ or plants⁹⁹, for example. Optimization at every experimental and computational step of the aDNA pipeline has significantly increased the sensitivity of aDNA methods, thereby decreasing the amounts of sampled material necessary for successful analyses. Small amounts of preserved DNA and extensive DNA fragmentation to lengths <100 bp¹⁰⁹ pose serious challenges for extracting DNA molecules from ancient samples. 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 downstream enzymatic reactions^{110,111}. Optimized protocols have been adapted for each experimental step to accommodate a variety of sample types and preservation states, and some allow the simultaneous extraction of proteins for radiocarbon dating¹¹² and proteomic analyses¹¹³. In most cases, samples are first mechanically reduced to a powder and DNA is then released through incubation in buffers that decalcify mineral matrices, break down proteins and lipids and disrupt DNA interactions with organic and inorganic compounds. Removal of loosely bound contaminants can be achieved through pre-digestion cleaning steps consisting of either a bleach treatment⁴⁰ to oxidize and destroy contaminant DNA and chemical inhibitors, or a staged digestion^{41,53}, whereby the first extracted fraction (which is likely to contain the majority of contaminants and inhibitors) is set aside or discarded. The methods may also be combined⁴², but both approaches also result in varying degrees of endogenous aDNA loss, and therefore aggressive cleaning should be viewed as a trade-off that is subject to diminishing returns.

Because it is highly fragmented, aDNA behaves differently than high molecular weight genomic DNA from fresh specimens, and it requires custom protocols for efficient recovery³⁹. Methods most often used for aDNA isolation rely on the adsorption of DNA molecules to silica particles¹¹⁰ in a chaotropic binding buffer carried out in solution¹¹⁴, on spin columns^{41,42,53,115} or on suspended silica-coated magnetic beads^{115,116}. DNA is then eluted in a low-salt buffer after ethanol washes. In some instances, a phenol-chloroform purification step can advantageously be used as an alternative¹¹⁷ or complementary approach¹¹⁸. Optimizing DNA extraction and library construction to recover fragments as short as 35 bp has been decisive in characterizing DNA from samples showing extensive molecular degradation^{39,119}.

[H2] Post-mortem DNA damage removal

A wealth of chemical reactions are known to affect DNA after death, and include: the fragmentation of DNA molecules into ultra-short DNA fragments; the conversion of the four nucleotides into various derivatives; and the cross-linking of DNA to other molecules. These post-mortem alterations potentially hinder the manipulation of aDNA and reduce the amount of retrievable genetic information^{109,120}. The most dramatic of these changes is DNA fragmentation, which occurs through hydrolytic **depurination** followed by **beta-elimination reactions [G]** that ‘nick’ (break) the DNA backbone (FIG. 3); during this process, the DNA double helix breaks apart into pieces that can be millions of times shorter than their original life during life. The next most common hydrolytic degradation reaction is the deamination of cytosines, which are converted into uracils and thereafter sequenced as thymine analogues¹²¹(FIG. 3a). This process is responsible for the sequencing artefacts observed as C-to-T misincorporations (and also G-to-A misincorporations for double-stranded DNA libraries) (FIG. 3b). The rate of such misincorporations increases towards the end of reads when mapped against a reference sequence due to cytosine deamination preferentially occurring in the single-stranded overhanging termini of aDNA fragments¹²¹. Post-mortem damage can accumulate with the age of the sample¹²², but degradation kinetics are ultimately driven by local environmental conditions^{2,45}. As a result, some recent samples can appear more damaged than older ones. In addition, degradation processes are best studied in mineralized tissues, and are less well understood in other kinds of remains.

Although C-to-T misincorporations provide handy genetic signatures that can be used to authenticate aDNA sequences^{54,57}, they can also bias sequence analyses, potentially leading to incorrect conclusions^{123,124}. To lower the impact of damage-induced sequence errors, DNA extracts can be optionally treated before library construction with a commercialized enzymatic mix of uracil–DNA–glycosylase (UDG) and endonuclease VIII (Endo VIII) known as the USER reagent (New England Biolabs). This reagent removes uracils and cleaves the resulting abasic sites, thereby cutting out damage but also shortening the length of the DNA molecule¹²⁵. While having the beneficial effect of reducing sequencing errors, USER treatment also has the negative effect of eliminating the damage patterns that are needed to authenticate aDNA sequences and differentiate them from contaminating; however, in the case of mammalian nuclear DNA, this damage signal can still be observed by examining CpG dinucleotides (FIG. 3c). For non-mammalian DNA, some labs first

perform screening of a non-treated (non-UDG) DNA library [G] to determine sequence authenticity, and then build a second USER-treated (full-UDG) library for analysis. However, the need to build multiple libraries per sample can dramatically increase costs. As a solution, a modified USER protocol has been developed (UDG-half) that removes most damage but retains a single uracil at each end of the molecule¹²⁶, thereby allowing aDNA authentication but limiting damage to the terminal base where it can be easily masked or clipped computationally during downstream analysis. Decisions on the use of USER treatment are generally study dependent, and involve calculations regarding the relative importance of sequence fidelity, sequence length and cost for a given study design and research question.

[H2] Mini-barcode PCR amplification

One of the few areas of aDNA research that still directly amplifies raw DNA extracts by PCR is in the field of palaeoenvironmental reconstruction, which attempts to recover information on past species communities through DNA barcoding [G]¹²⁷. This is achieved through the use of PCR capable of amplifying target loci from a wide range of organisms, typically targeting the *trnL* loop of the chloroplast genome in plants¹²⁸, and the mitochondrial targets *cytb*⁹⁰, *CO1*⁹¹, *12S* or *16S*¹²⁹ in animals. Such targets are selected on the basis of their relative high variability such that each species is expected to be defined by distinct haplotypes — like barcodes — thereby distinguishing them from other species.

High taxonomic resolution is often achieved through meta-barcoding, an approach that combines barcode PCR with NGS^{22,130-132}, which uses tagged (indexed) primers, eliminating the need for conventional molecular cloning to separate out individual sequences from the amplicon pool. Multiple samples can be simultaneously sequenced due to the incorporation of short sample-specific index sequences into the PCR primers, usually of 4–6 bases, thereby allowing later bioinformatic recovery of specific sample sets. This approach is popular because of its cost effectiveness and perceived sensitivity. However, it also has limitations. First, the termini of the aDNA fragments are generally not amplified using this method, so damage parameters such as cytosine deamination cannot be used as an authentication criterion. Second, allelic drop out, the failure of certain templates to amplify, can occur as a result of the clonal biases inherent in PCR or from length polymorphisms in the target region¹³³, both of which are especially problematic when DNA is degraded and template copy number is low, giving rise to low or skewed sequence complexity. As

with PCR-based aDNA approaches used in the 1990s, the authentication of **metabarcoding [G]** results relies instead on reproducibility across multiple PCR replicates¹²⁸ and demonstrating that negative controls are either blank or contain different sequence signatures than that seen within the samples. Alternative **shotgun sequencing [G]** and/or target-enrichment approaches are increasingly being applied in palaeoenvironmental reconstructions^{48,49,134,135}, along with authentication approaches suitable for low representation metagenomic data¹³⁶ and the use of phylogenetic assignment algorithms that are robust to poor database representation of taxa¹³⁷.

[H2] DNA library construction

Molecular cloning of PCR amplicons into bacterial plasmids was the first methodology used to build aDNA fragments into DNA libraries, which were then cultured to retrieve sufficient material for sequencing using capillary electrophoresis¹³⁸. The limitations of capillary instruments, however, never provided sufficient sequencing throughput to obtain sequence data beyond the megabase scale^{138,139}. Culture-free NGS DNA libraries provide a substantially less labor-intensive solution as they can be virtually immortalized by PCR for unlimited reuse prior to sequencing. There are two main library preparation methods currently available for **sequencing by synthesis [G]** (for example, Illumina sequencing¹⁴⁰), the main type of NGS used 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^{121,141,142}, while in the single-stranded preparation method, heat-denatured aDNA templates and adapters are ligated as single-stranded molecules^{16,143,144} (FIG. 2). The latter approach shows increased sensitivity for low biomass samples since overhanging 3'-OH termini and even strands with nicks are amenable to ligation. These features are enzymatically removed or improperly built using double-stranded library preparation. Adding biotinylated adapters one-at-a-time during a single-stranded preparation also prevents the formation of constructs refractory to sequencing and reduces material loss during library purification. Another 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 costs⁴³. While initially cost prohibitive, the single-stranded methodology has now been modified to allow ligation using inexpensive double-stranded **DNA ligases [G]**^{145,146}.

Experimental procedures in which libraries are prepared within single tubes are also available¹⁴⁷, which further reduces manipulation and hands-on time while facilitating parallelization¹⁴⁴.

All NGS aDNA libraries include short unique identifying sequences (or indexes) integrated within their adapters to ensure traceability from preparation to sequence production. These indexes allow pooling of multiple samples within sequencing runs and also greatly reduce contamination risks within the laboratory as only sequences deriving from samples should carry the appropriate adapter-index combination. Their sequence can be read as part of the main sequencing reads¹²⁶ or through external sequencing primers¹⁴² or both¹⁴⁸. Using indexes on both adapters is highly recommended to allow the detection of chimeric DNA templates that can form through jumping-PCR¹⁴² and index hopping (or index switching) during cluster generation¹⁴⁹.

[H2] DNA library amplification

Although single molecule sequencing (for example, Helicos, Pacific Biosciences, and Oxford Nanopore) has provided a PCR-free alternative for sequencing aDNA libraries², the generation of almost all aDNA data to date has required library PCR amplification to boost signal prior to sequencing. PCR, however, does not simply restore sufficient amounts of material for downstream manipulation; it can also significantly alter the library composition as DNA polymerases show various preferences for templates of particular sizes and/or base composition¹⁵⁰, as well as differing in ‘proofreading’ capabilities for bypassing post-mortem DNA damage^{48,151}. Pfu Turbo Cx, Herculase II and Accuprime Pfx represent some of the most commonly used DNA polymerases for their capacity to perform even amplification of fragments with varying base composition and length, and to preserve the original library complexity. Pfu Turbo Cx is a non-proofreading enzyme capable of amplifying damaged, uracil-containing templates without enzymatic stalling, and it is typically used during library construction. Herculase II and Accuprime Pfx are proofreading, high fidelity enzymes that are typically used for subsequent amplification steps during target enrichment and sequencing preparation, but may also be used to produce libraries prepared from USER-treated templates. Increasing PCR cycle number drives the formation of PCR duplicates [G], resulting in clonality, and must be mitigated to avoid saturation during sequencing unless target-enrichment is performed. Real-time PCR quantification of library concentration can help identify the optimal number of cycles required for final library amplification, as well as estimate the library complexity in order to decide on sequencing depth¹⁵². Performing independent amplifications of library aliquots in parallel also

helps to maintain complexity and reduce sequencing redundancy, and further PCR reconditioning can be helpful in reducing heteroduplex formation [G]¹⁵³. Technical guidance for determining optimal amplification conditions, including numbers of PCR cycles, has been released for a number of protocols (for example, see¹⁵⁴ and¹⁴³).

[H2] Target-enrichment

The often-limited endogenous fraction (i.e. DNA of the species of interest) present in aDNA libraries can make shotgun sequencing of entire ancient genomes uneconomical, especially at high depths-of-coverage. Target-enrichment approaches have thus been developed to focus sequencing efforts on library content of particular interest. Early approaches relied on library annealing to microarray-bound probes¹⁵⁵ but now various in-solution hybridization approaches involving short DNA³⁵⁻³⁷ or RNA^{156,157} oligonucleotides are favored. These generally consist of 52-¹⁵⁸, 60-¹⁵⁹ and 80-mers⁷² and can target from thousands to millions of loci¹⁶⁰, including entire chromosomes^{37,161} and whole genomes^{38,157}. However, probe design must attend to base compositional and structural considerations¹⁵⁹ to avoid biasing the recovery of sequence variants. In addition, **ascertainment bias** [G] introduced by the selection of particular probe sets can also affect estimates of population affinities. For example, targeting SNP variants that are common in a given population can result in overlooking non-targeted SNPs that may be more frequent in other populations. Probe ascertainment should therefore be controlled or corrected for whenever intended for population inference¹⁶². This can be achieved in silico for species in which extensive genome sequence data have been generated, by comparing population genetics statistics based on whole genome data to those estimated when conditioning on specific genome locations, as well as through various modelling approaches¹⁶³.

Target-enrichment [Au:OK?] probes can be purchased directly from provider companies, or probes can also be generated in-house using oligonucleotide synthesizers or by following lab procedures converting fresh DNA extracts or RNA transcripts of closely-related taxa into DNA libraries that can be amplified and/or transcribed to produce probes on demand^{36,38,164,165}. A wide range of predesigned and custom in-solution target-enrichment kits are available through companies such as Arbor Biosciences and Agilent Technologies, but large probe sets, such as those used in comparative studies with world-wide human genotype panels¹⁶⁰, are commercially restricted to some laboratories. Although large probe sets are available on commercial microarrays, such as the Axiom

Genome-Wide Human Origins 1 Array, microarrays require high quality genomic DNA for input and are generally incompatible with aDNA. Although some labs have negotiated access to large custom in-solution probe sets on an ad hoc basis, the field as a whole would benefit from collective bargaining to make the manufacturing of large high-demand probe sets more accessible.

Performing two successive rounds of enrichment generally increases on-target coverage rates¹⁶⁶, while the use of short adapters can favor on-target library-to-probe annealing and enhance enrichment efficacy¹²⁶. Further rounds of enrichment are generally not beneficial, as they tend to increase library clonality and therefore lead to diminishing returns. Finally, probe-free enrichment approaches leveraging the affinity of methyl-binding domains for methylated CpGs have been proposed to separate the microbial fraction from the vertebrate and plant DNA templates present in aDNA extracts, but their efficacy is generally limited by the short size of aDNA fragments^{62,167}.

[H2] DNA sequencing

Different NGS instruments have been used to retrieve aDNA data from subfossil and museum specimens. The Roche 454 system was first used in 2006³¹ and remained dominant until 2010⁷ when the first ancient human genome was sequenced using Illumina technology's sequencing-by-synthesis technology⁶. Although other technologies have been sporadically used (for example, SOLiD¹⁶⁸, Helicos², BGI-SEQ500¹⁶⁹, Ion Torrent¹⁷⁰), the vast majority of aDNA sequence data have been produced using Illumina instruments, due to their ubiquity, high data output, cost-effectiveness and relatively low error rates. They also work optimally on relatively short DNA sequences (<300 bp) and are well suited to sequence DNA in the 50–150 bp range, which characterizes most aDNA. Illumina platforms are not devoid of problems, however, and the assessment of possible batch effects may be required. This is usually achieved through a recalibration of individual base quality scores using spiked-in PhiX control libraries^{171,172} to account for variability specific to each individual sequencing run. During sequencing preparation, it is also important to take steps to mitigate index hopping when using Illumina platforms, especially those using patterned flow cells and isothermal cluster generation by exclusion amplification, such as the HiSeq4000, HiSeqX and NovaSeq platforms¹⁷³. These steps include removing heteroduplexes and free adapters from sequencing pools prior to sequencing and computationally removing chimeric sequences after sequencing based on dual index combination analysis^{142,149}.

[H2] Information management systems

Truly large-scale paleogenomics projects are now a reality for aDNA laboratories. Such projects may involve an extensive network of collaborators with large numbers of samples that are analyzed using a wide range of methods. Documentation of metadata and experimental steps is thus becoming increasingly important in order to optimize work coordination and collaboration, as well as to ensure quality control and ethical handling of the samples. This information is generally digitized in most, if not all, aDNA laboratories, and Laboratory Information Management Systems (LIMS) specifically developed for aDNA such as CASCADE¹⁷⁴ are becoming freely available.

[H1] Results

Numerous tools and pipelines are available for aDNA sequence analysis, and the selection of appropriate procedures depends on the nature of the research project objectives — for example, whether aimed at paleo-environmental reconstruction, microbial profiling or modelling population history. There are, however, a number of steps in the analytical workflow that are common to most projects. These involve the processing of raw sequencing data, alignment against reference genomes and/or sequence databases, and the assessment of authenticity and error rates, including miscoding lesions resulting from post-mortem damage. Analytical tools used in aDNA analysis are listed in Table 1.

[H2] Read processing and alignment

DNA molecules can be sequenced in one or both directions, resulting in single reads or paired-end reads. The first processing steps include index demultiplexing [G] to identify those reads belonging to each individual library based on their index combination, followed by read trimming to remove adapters and/or low-quality terminal sequences and finally the collapsing of overlapping read pairs into consensus sequences^{175,176}. Although with exceptions¹⁷⁷, the limited length of aDNA and the presence of often dominant environmental microbial DNA contamination have precluded widespread usage of de novo sequence assembly. Processed reads are instead typically aligned using *BWA*¹⁷⁸ or *Bowtie2*¹⁷⁹ to reference genomes of the focal species, such as the human genome but also to potential microbial pathogens that may have infected the individuals during life. Parameters maximizing mapping specificity and sensitivity can be identified using aDNA read simulators such as

*gargammel*¹⁸⁰, and depend on the evolutionary distance to the reference genome used, DNA fragment size and the amount of post-mortem damage^{111,181-184}. Mappers can show different performance depending on the experimental procedure followed during sample preparation, such as the type of DNA library constructed¹⁸⁵ and whether or not extracts were USER-treated¹⁸⁴.

Single haploid genomes provide only one version of the structural and sequence diversity present in a species. Mapping to single reference genomes can thus introduce substantial reference bias, especially as short reads carrying the alternate allele can prove particularly difficult to map¹⁶². Mitigation solutions include the use of variation-aware read aligners such as *vg*¹⁸⁶ or read filtering procedures post-mapping¹⁴⁸. Bioinformatic pipelines such as *Paleomix*¹⁸⁷ and *EAGER*¹⁸⁸ provide automated solutions to convert raw sequencing reads into alignments available for downstream analyses, and more.

[H2] Authentication

Authentication of genomic DNA from single individuals (see below for authentication of microbial DNA) requires both the observation of post-mortem nucleotide misincorporation arising from cytosine deamination^{57,120} (FIG. 3A-H) and the estimation of contaminating DNA based on heterozygosity levels found in haploid chromosomes⁵⁴ (FIG. 3I). Post-mortem DNA damage patterns can be quantified in non- or partial USER-treated libraries using the tool *mapDamage*²⁵⁷. Cytosine deamination can also be estimated using the tool *PMDtools*⁵⁵ on data generated following full USER-treatment by conditioning on CpG dinucleotides¹²⁵, since these are heavily methylated in the nuclear genomes of vertebrates and protected from USER degradation. Estimating the degree of contamination using ploidy can be challenging as it generally requires access to a polymorphism database to account for the probability that contaminating molecules are undetected because the contaminant and the endogenous genome share the same variants. However, multiple tools exist for assessing contamination levels in haploid chromosomes, both from mitochondrial DNA data (for example *schmutzi*¹⁸⁹) and using the X-chromosome in males (for example *ANGSD*^{190,191}). Emerging methods such as *AuthentiCT*¹⁹², which uses a model of cytosine deamination to estimate contamination, promise to generalize contamination inference across organisms, sex and chromosome types, thereby mitigating the need for the ploidy-based approaches. Additional approaches for estimating contamination work by isolating sequences with evidence of cytosine

deamination^{55, 193, 194} and then detecting differences in ancestry¹⁹⁴ or linkage disequilibrium¹⁹⁵ between the damaged (alleged ancient) fraction and the total data using reference panels.

[H2] Error rates

The total number of nucleotide misincorporations present in a reconstructed genome depends on the amount of sequencing data produced, the amount of post-mortem DNA damage that has accumulated and whether or not USER treatment was used in the experimental workflow. Reconstructed ancient genomes generally contain sequences with both genuine and artefactual variants, which may impact population-level analyses of allelic sharedness and genetic distances. Relative error rates can be estimated by comparing the genetic distance of an ancient specimen to an **outgroup [G]**: the inflation of genetic distance between the ancient genome and the outgroup genome relative to the genetic distance separating the same outgroup and a high-quality modern genome provides a conservative measure of the error rate^{2, 190}. Breaking down individual error rates per nucleotide substitution type (for example, C-to-T, G-to-A, C-to-A and G-to-T) generally reveals a pervasive excess of transitions (C-to-T, G-to-A and their reciprocal changes) due to post-mortem cytosine deamination⁷. This can be done within the framework of the ANGSD software¹⁹¹ that covers a wide analytical range specific for NGS and ancient DNA data, including the calculation of **genotype likelihood scores [G]** that are aware of individual base qualities (i.e. the confidence in each base called during sequencing) and many of the analyses presented below. Read simulation with *gargammel*¹⁸⁰, conditioning calculations on transversions⁷ or other substitution classes showing non-inflated error rates¹⁴⁸, filtering CpGs¹⁹⁶ (FIG. 3E-H) or subtracting total counts of mutational patterns proportionally to error rates¹⁹⁷ can help ensure the validity of downstream conclusions.

[H2] Molecular sexing and kinship

Biological sex, as determined by the karyotype of sex chromosomes in species such as humans (XY in males, XX in females and exceptional karyotypes such as XXY, XYY and others), is straightforward to estimate even with only a few thousand random sequences from a human individual¹⁹⁸. To account for misalignments between the sex chromosomes, different approaches compare the number of sequences aligning to the X, Y and autosomal chromosomes in different

ways to establish calibrated expected values for each biological sex^{16,198,199}. For species in which reference assemblies lack the Y-chromosome, X-to-autosomal coverage ratios are often used²⁰⁰, and require not more than 1,000–10,000 high-quality read alignments.

Methods detecting genetic kinship generally make use of population panels in which expectations of **identity-by-descent [G]** (or allelic mismatch rates) can be derived in unrelated individuals²⁰¹⁻²⁰³. They are implemented in user-friendly tools, such as *lcMLkin*²⁰¹ and *READ*²⁰³, providing inferences up to second degree relationships and potentially more. The latter, however, requires that at least three individuals from a given group are available to assess the sequence similarity thresholds corresponding to similar kin. Together with observed shared matrilineal and patrilineal markers on the mitochondrial DNA and Y-chromosomes (which require a minimal 3–5× sequence coverage²⁰⁴), extensive pedigrees can then be reconstructed²⁰⁵. However, such approaches are sensitive to the allelic frequencies within the population panel used, which ideally should be close to the allelic frequencies of the population investigated. The need for a population baseline can be avoided by computing individual heterozygosity at loci with coverage $>1 \times 206$.

[H2] Multivariate analyses

Principal component analysis (PCA) is a classic, exploratory statistical method that is used to represent genetic affinities among individuals within simple graphs. It summarizes genetic variation measured from individual genotypes in hundreds to thousands of individuals for thousands to millions of SNP loci, into a reduced number of dimensions that are shaped by ancestry^{207,208}. Those dimensions represent the principal components and provide the main axes of the graphical representation of genetic affinities. Ancient DNA data from an individual are often projected onto present-day genetic variation¹⁹⁴ (FIG. 4A), but when sufficient data are available, ancient specimens can be included in the principal component computation itself. In cases where ancient genetic coverage is low, it is generally necessary to pseudo-haploidize modern data by randomly selecting one read at each polymorphic position, unless approaches are used that explicitly account for varying coverage across sites and individuals²⁰⁹. Differences in coverage across ancient individuals can also be mitigated using **Procrustes analysis [G]** of independent PCAs¹⁹⁴ followed by least square projection²¹⁰. It is notable that PCA clustering is sensitive to the sample size of different ancestries and also to their amount of genetic drift, which exaggerates principal component distances^{207,208,211,212}.

Therefore, individuals from the same population but distant in time may be misleadingly separated in PCA space. Multi-dimensional scaling (MDS) has been proposed as an alternative to PCA in cases where only minimal sequence coverage is available, typically $0.001\text{--}0.1\times^{213}$ for human data. For both methods, accurate clustering can be achieved even in the absence of sequence overlap between different ancient individuals, as long as sufficient sequence data enable the estimation of genetic distance to a predefined panel of relevant ancestries.

[H2] Population ancestry modelling

While PCA results are most often visualized as biplots and in practice analyzed in two-dimensional comparisons of two principal components at a time, alternative clustering methods such as those implemented in ADMIXTURE²¹⁴ make higher-dimensional assessments possible. There is a broad suite of software that implements an explicit genetic model aimed at partitioning individuals into ancestries, fitted to genotype frequencies²¹⁴⁻²¹⁶, that have now been extended to model the temporal structure of the data²¹⁷. However, these approaches are sensitive to (and thus can be biased by) genetic drift between time periods and differences in relative sample size between ancestries. This contrasts with methods based on f -statistics²¹⁷⁻²¹⁹, which leverage covariation of allele frequencies between populations, and can retrieve unbiased admixture proportions. Although more cumbersome than clustering approaches for initial data exploration, f -statistic-based methods can in many cases also provide a statistical test for proposed ancestry models.

Three individual statistics are central to the f -statistic framework. f_2 statistics measure distances between pairs of populations and/or individuals. f_3 statistics can reveal unambiguous evidence for situations in which one individual (or population) is formed by the admixture of two others²¹⁹, but they can also provide a measure of shared genetic drift when an outgroup is included²²⁰. Finally, f_4 statistics test for asymmetries between populations that are indicative of gene flow²¹⁹ (FIG. 4B). If a five-population history of a specific topology can be assumed, a ratio of two f_4 statistics can be used to estimate ancestry proportions unbiased by genetic drift²¹⁹. In more generalized developments of the f -statistics approach in the tool *qpAdm* (and *qpWave*), the predicted f -statistics of a 1-source²²¹ or 2- to 4-source ancestry model^{158,210} can be obtained if a set of reference populations or genomes can be posited that are more distantly related to the ancestry sources than

populations defined by users to represent sources. This approach then provides a p -value for the ancestry model tested and, for valid models, also unbiased ancestry proportion estimation.

[H2] Genotype imputation

With some exceptions, ancient genomes and genome-wide enrichment of SNPs are sequenced at limited depth-of-coverage, which precludes the determination of genotypes. Statistical inference of missing genotypes is possible using a process called genotype imputation that assumes that the distribution of haplotypes present in the population is known and that sufficient coverage is available, typically around $1\times^{222}$. In practice, the haplotype distribution is often approximated from large-scale reference panels such as the Haplotype Reference Consortium²²³ for human populations. This may be adequate for relatively recent historical time periods²²⁴ and/or areas such as Europe where many genomes from modern human populations have been collected²²², but may be underpowered in cases of limited reference panels, as is true for many populations from elsewhere in the world²²⁵. Imputation can help genotype single loci — such as lactase persistence²²⁶ — to full genomes^{52,222,227}, and estimate runs-of-homozygosity and inbreeding coefficients from low-coverage data²²⁷. Promisingly, new approaches relying on linkage information from reference panels of modern haplotypes that are compatible with a minimum of $0.3\times$ coverage data are now emerging²²⁸. Patterns of non-random allele association within haplotypes can also improve the inference resolution of population structure and outperform methods using unlinked variation^{222,229}. Nevertheless, imputation can also infer false genotype calls²³⁰, and so care must be exercised when using imputation to investigate the evolutionary history of specific traits.

[H2] Microbiota profiling

In addition to containing genomic fragments from the focal species, aDNA extracts also generally play host to an entire metagenomic diversity of environmental microbes that mainly colonized the subfossil material after death^{7,231}. The presence of often dominant non-host DNA templates within aDNA libraries can considerably increase genome sequencing costs but can be filtered out using target-enrichment techniques or enzymatic digestion of over-represented bacterial sequence motifs⁷. In studies of pathogens (see¹³ for a review) and oral, gastric and fecal microbiota,

however, the metagenomic content itself can be the intended research target if pathological lesions, dentine, dental calculus²⁶, stomach contents^{231,232} or coprolites²³³⁻²³⁵ are analyzed.

Taxonomic profiling of library metagenomic content is typically carried out using sequence identity threshold against comparative databases (such as *MALT*²³⁶; FIG. 4C) or k-mer representativity patterns (for example *Kraken*²³⁷), which breakdown the frequency distribution of each sequence motif of a predefined length of k nucleotides. The sensitivity and specificity of taxonomic assignment largely depends on the representation characteristics of the comparative database but are robust to post-mortem DNA damage^{238,239}. Curated databases of microbial markers such as *MetaPhlAn2*²⁴⁰ ensure specificity but may lack sensitivity for environmental and/or non-human associated microbial taxa, while large uncurated sequence repositories such as the NCBI nucleotide database may lack specificity (see²⁷ for a review). Several bioinformatic pipelines automating analyses from mapping to statistical profiling are available, such as *HOPS*²⁴¹ and *metaBit*²⁴². In addition, *SourceTracker*²⁴³ and *CoproID*²³³ can estimate mixture proportions from known candidate sources, such as soil, gut and oral microbiota. Authentication of microbial taxonomic profiles is difficult and requires cross-validation through different software²³⁸ and analyses of specific sequence characteristics, including through mapping against the genomes of candidate species and recovery of post-mortem signatures of molecular degradation²⁷.

Although **16S metabarcodes [G]** are extensively used for profiling modern microbiota, the approach is impracticable on ancient material because of amplification biases introduced during PCR due to extensive DNA fragmentation¹³³. 16S ribosomal RNA (rRNA) gene amplification is designed to target hypervariable regions in order to distinguish microbial taxa, and these sequences are clustered based on similarity ($\geq 97\%$) with bacterial taxa. However, most aDNA sequences are smaller than 200 bp in length, the minimum for 16S rRNA variable region amplification. For example, a 2015 study¹³³ showed that amplified libraries of the 16S V3 region did not accurately reflect microbial taxa within ancient DNA samples when compared to shotgun metagenomic data. This was in part due to biased amplification of length polymorphisms whereby taxa with deletions were overrepresented and taxa with insertions were underrepresented¹³³. Shotgun metagenomic approaches are, thus, recommended.

[H2] DNA methylation

Two main experimental methods have been applied to map DNA methylation [G] signatures on ancient genomes. The first builds on methods commonly used on fresh DNA such as bisulfite conversion [G]^{244,245} and immunoprecipitation [G]^{62,167}. Bisulfite conversion uses sodium bisulfite to convert unmethylated cytosines into uracils, similar to the C-to-U conversion that occurs in post-mortem DNA damage. However, bisulfite conversion is also extremely harmful to DNA and, thus, generally not recommended for aDNA, despite a few successes on limited numbers of samples^{244,245}. Immunoprecipitation is likewise extremely limited in practice due to the fast postmortem decay of CpG dinucleotides¹⁶⁷ and DNA fragmentation⁶², which reduce the number of potential DNA methylation targets available per fragment.

The second category of methods for ancient DNA methylation analysis relies on indirect statistical inference based on patterns of post-mortem DNA decay that are ubiquitous in plants²⁴⁶ and animals^{28,29}. The idea is implemented in open-source statistical packages such as *epiPALEOMIX*²⁴⁷ and *DamMet*²⁴⁸. It leverages NGS data generated following USER-treatment, which maintains C-to-T misincorporations only at methylated sites²⁹. While obtaining reliable estimates at single nucleotides resolution requires impractical sequence coverage ($\geq 80 \times^{248}$), the methodology has been applied successfully to assess DNA methylation levels within genomic regions.

[H1] Applications

The application potential of aDNA analysis has a broad scientific scope and relevance to key archaeological, ecological and evolutionary questions. The following section highlights some of the areas that have received heightened scholarly attention and include human population history, plant and animal domestication, and the origins and evolution of pathogens and microbiomes, as well as the impact of global climatic changes on past faunal and floral communities.

[H2] Human genomics

No area of aDNA analysis, except that of microbial archaeology¹³, has benefited as much from NGS as that of past human population genetics (FIG. 5). The wealth of newly available ancient human genomic data has been instrumental in transforming our understanding of the human

past, from a previous narrative of long-term population continuity and isolation to one in which mobility and population mixture have played much more prominent roles¹¹.

The first ancient human genome was sequenced in 2010 from the hair of an approximately 4,000-year-old Saqqaq-PaleoInuit man from Greenland⁶. It made the possibility of sequencing of authentic ancient human genomes a reality, something that was until then considered unlikely due to pervasive sequence contamination in the pre-NGS era²⁴⁹. The draft genomes of two archaic hominins, the Neanderthals⁷ (1.3× coverage, composite of 3 individuals) and Denisovans¹⁵ (1.9× coverage), were released shortly after the high-coverage Saqqaq-PaleoInuit genome⁶ (20× coverage). Both archaic genomes revealed evidence of admixture amongst various hominin lineages⁸, in which Neanderthals and Denisovans contributed significant ancestry to modern non-African populations and modern populations of Australasia and Oceania, respectively²²¹, contributing to potential phenotypic and health consequences today²⁵⁰.

Since 2010, ancient human genomics has moved forward with incredible speed, and current publications typically include genome-scale data and/or whole genome sequences from dozens to hundreds of individuals^{5,251-253}. Current understanding of genomic variation within pre-Holocene populations is lagging behind that of more recent populations due to the scarcity of the fossil record and relatively limited DNA preservation, although partial nuclear genomes from pre-Neanderthal groups¹⁹³ and genomes from multiple Upper Palaeolithic modern humans^{37,254-257} have been successfully recovered. Europe, and more generally west Eurasia, has been the focus of most research²⁵⁸, although significant progress has also been made in understanding the genomic history of other regions, such as East Asia^{257,259}, the Americas^{6,220}, Oceania^{190,260}, Southeast Asia^{261,262}, the circum-Mediterranean²⁶³ and Africa²⁶⁴. These other regions include climatic zones that were previously believed not to be suited for long-term DNA preservation.

Studies of west Eurasia have revealed that the genetic diversity of present-day Europeans is primarily formed by three major ancestry components²¹⁰, consisting first of early hunter-gatherers^{194,265} who later admixed with farmers leaving Anatolia approximately 9,000 years ago, and who brought with them agricultural innovations such as domesticated crops and livestock and introduced the Neolithic lifestyle into Europe²⁶⁶. A third genomic component was later overlaid following the migration of pastoralists culturally associated with the Yamnaya horizon from the Pontic steppe around 5,000 years ago^{158,226} (FIG. 4A,B). It is now believed that it was this pastoralist migration, and not earlier Neolithic population movements, that likely brought the proto-Indo-European language to Europe^{158,226}. This was then followed by the westward expansion of the Bell

Beaker complex that is genetically associated with an almost total **population replacement [G]** of groups in Britain²⁰⁶.

Further to the east, archaeogenomic studies have shown that Central and Inner Asia underwent a population history very different from that of Europe, even though they were impacted by some of the same events. These areas were initially populated by groups of hunter-gatherers who were among the first to domesticate the horse²⁵¹ and who were related to contemporary Native Americans. However, in contrast to the hunter-gatherers of Europe, these peoples did not experience migration from Anatolia and never underwent the same processes of Neolithization²⁵³. Nevertheless, much of Asia has witnessed major migrations since the Bronze Age^{251-253,267}, including from Yamnaya-related and subsequent western steppe populations who spread pastoralism across the continent. These population dynamics resulted in Central and Inner Asia shifting from initially being occupied by groups of peoples related to Native Americans, to being occupied by peoples of mixed western Eurasian genetic ancestry, and then finally to becoming inhabited by peoples with greater Northeast and East Asian ancestry. In the process, the language topology of the region also changed from being largely Indo-Iranian to becoming predominantly Turkic or Mongolic today^{252,253}.

Beyond Eurasia, studies of ancient human genomes have also substantially contributed to our understanding of the population history of the Americas, from its initial peopling and the subsequent dispersal and diversification of indigenous populations²⁶⁸⁻²⁷⁰ to the impact of European contact and colonialism²⁷¹. Recent studies are also informing the population history of tropical regions previously thought to be beyond the reach of ancient DNA due to extensive DNA degradation conditions, such as the Caribbean, which now appears to have been colonized in at least three waves from migrants originating in both North and South America²⁷². The study of ancient human genomes has also contributed to the reconstruction of events for which the historical record is limited or intentionally concealed. One such example is the application of ancient genomics to the study of the Transatlantic Slave Trade, which has allowed the identification of specific places of origin of African-born enslaved individuals who were forcibly taken from Africa to the Americas, revealing the diversity of their ethno-geographical origins within West Africa and beyond, as well as aspects of their lives in the Americas²⁷³⁻²⁷⁴.

[H2] Domestication

The domestication of plants and animals represents a key stage in human history and has long been an area of unresolved debate about the timing, location and number of domestication

sources²⁷⁵. Pre-NGS era studies identified key loci under early selection in crops such as maize⁶⁹ and pigs²⁷⁶, providing glimpses into the process as well as into their likely routes of spread. Now domestication models are being re-written as genomic data reveal a dynamic and complex history that includes complete population turnover, adaptive introgression between wild and domesticated forms, an unexpected temporal and spatial distribution of origin and selection and a larger role for natural dispersal processes (see^{12,277} for recent reviews on these topics).

Large-scale population replacement occurred in dogs²⁷⁸, wolves^{196,279,280}, horses^{148,281}, pigs²⁸² and crops such as potato²⁸³. For example, indigenous American dogs were completely replaced by later European dogs²⁷⁸ with some introgression from Inuit dogs²⁸⁴, and modern domestic horses did not arise from the Eneolithic Botai culture of central Kazakhstan as previously thought²⁸¹, but from a later Bronze Age expansion¹⁴⁸. In contrast, a staggering level of constancy is evident in grapes with over 900 years of uninterrupted vegetative propagation⁷².

Adaptive introgression has emerged as a recurrent feature in response to past environmental dynamics. For example, maize assimilated wild adaptive variation enabling northwards expansion²⁸⁵, as did flax in Europe²⁸⁶. Cattle were introgressed by wild aurochs²⁸⁷ and with zebus, thereby gaining alleles adaptive for response to drought²⁸⁸. Such findings overturn the concept of domestication as a process of isolation between wild and domesticated populations and suggest instead complex instances of gene flow, as is evident in the mosaic ancestry of ancient goats²⁸⁹ and in the multiple emergence events of semi-domesticated maize varieties^{70,74}, which were later fully domesticated at secondary centers in a stratified domestication process²⁹¹. Paleogenomic data also do not support the presence of a strong demographic bottleneck during early domestication stages (see²⁹² for a review in major crops), but rather studies of wheat²⁹³, sunflowers²⁹⁴, and horses¹⁴⁸ support more recent losses of genetic diversity.

Finally, time-stamped genomes have enabled the temporal stratification of both phenotypes and selective regimes. In sorghum, early selection was related to plant architecture, and only later switched to increased sugar metabolism²⁹⁵. In maize, complex phenotypes like days-to-flowering have been reconstructed from genomes showing latitudinal adaptation to day length²⁹⁶. Early selection of coat color is evident in pigs²⁸² and goats²⁸⁹, but some traits were acquired later such as behavior-related and productivity traits in chickens during the medieval period^{297,298}, and morphotype and speed in horses^{148,299}. Importantly, damaging mutation load was a feature of later selective episodes and breeding but not part of the initial domestication of horses²⁹⁹, maize²⁹⁶ and sorghum²⁹⁵.

Continuing to characterize past genomic variation in ancient crops and animal breeds will be instrumental for developing the future sustainability of agriculture.

[H2] Pathogens and microbiomes

Together with classical approaches in paleopathology and paleodemography, aDNA from microbes, including pathogens and commensals, can provide insights into the health of ancient peoples as well as shifts in diets and disease ecology. Initial studies of ancient microbes used PCR-based methods to identify specific pathogens (such as those causing skeletal lesions characteristic of tuberculosis and leprosy)³⁰⁰, to analyze the first historic pathogen genome from the 1918 influenza virus³⁰¹, and to explore the ancient microbiome²⁷. However, such approaches suffered from an inability to distinguish ancient and modern contaminating microbial DNA^{27,302}. More recently, NGS-based methods have resulted in the successful recovery of authenticated pathogen genomes from not only *Mycobacterium tuberculosis* and *Mycobacterium leprae*, but also many pathogens that leave no visible evidence in the skeleton, such as *Yersinia pestis*, *Helicobacter pylori*, *Vibrio cholera*, *Salmonella enterica Paratyphi C*, Variola virus, human parvovirus, and hepatitis B virus (see¹³ for a review). For example, analyses of the victims of both the Plague of Justinian and the Black Death have shown that *Y. pestis* was indeed the cause of both pandemics^{65,303}, and further genomic analysis has provided insight into the course of these pandemics over time, including parallel changes in the pathogen itself³⁰⁴⁻³⁰⁶. Surprisingly, extinct strains of *Y. pestis* have also been identified in ancient individuals dating as early as the Late Neolithic and Bronze Ages throughout Eurasia, and genomic data show the progression of evolutionary changes that have affected its virulence through time, including the acquisition of a transmission mode via fleas (see¹³ for a review).

While the analyses of ancient pathogen genomes have shown plague to be older than expected, they suggest that the origins of the tuberculosis complex (*M. tuberculosis* and related strains) in humans may have evolved more recently (~3,000–6,000 years ago)³⁰⁷⁻³⁰⁸. PCR data exist that putatively identify tuberculosis in older individuals, however, genomic data from bone samples that would distinguish contaminating microbial DNA are not yet available^{27,302}. Additional genomic data from ancient tuberculosis strains could show that the current estimate of time to the most recent common ancestor reflects a bottleneck or other population dynamics in this clonal pathogen. The ancient tuberculosis data also show the significance of pathogen exchange among animals and humans. In South America, before European colonization, aDNA has shown that people were affected by tuberculosis strains that are most closely related to *M. pinnipedi*, a zoonotic form of the

pathogen that usually infects seals and sea lions. This strain was likely transmitted from South American seals to humans through consumption of undercooked seal meat or during butchering, after which human-to-human transmission may have begun³⁰⁷.

Similarly, NGS has facilitated microbiome analyses from a range of ancient contexts including dental calculus, coprolites, latrine sediments and mummies^{309,310}. Comparisons between modern and ancient oral microbiota have suggested possible shifts in relation with increasingly carbohydrate-rich diets during the Neolithic and Industrial revolution²¹³, although further work revealed the need to account for other possible factors driving oral microbiota profiles, such as the biofilm maturation stage³¹¹, tooth type and surface³¹². Likewise, studies of the ancient gut microbiome have revealed previously unknown microbial diversity in the human gut, as well as the loss of key microbial symbionts in present-day industrialized populations^{85,235}. The microbiome is also the source of several recently evolved human pathogens, including the causative agents of diphtheria (*Corynebacterium diphtheriae*), gonorrhoea (*Neisseria gonorrhoeae*), bacterial meningitis (*Neisseria meningitidis*) and pneumonia (*Streptococcus pneumoniae*, *Haemophilus influenzae*) (see³¹⁰ for a review), and thus understanding its evolution and changing ecology through time is critical to understanding infectious human disease.

[H2] Extinction and climate change

Ancient DNA has fed debates not only on the phylogenetic placement of many extinct species but also on the causes of their disappearance. This can be investigated by correlating known human activities or climatic events with population expansions and declines as revealed from serially-sampled DNA data. Dynamic population size trajectories can be estimated from calibrated gene genealogies within a serial coalescent statistical framework (see³¹³ for a review). Additionally, the regions showing paleoclimatic conditions compatible with the presence of a given species can be inferred from spatio-temporal fossil distribution data and bioclimatic data. Key to all such analyses is the availability of radiocarbon dates for the specimens analyzed using genetic techniques. Radiocarbon dating should thus be highly recommended wherever possible.

Evidence currently available in bison, horses, reindeer, musk oxen, woolly mammoths, and woolly rhinos, suggest that climate change is a common driver of population size changes, but also that species show individualistic responses in the face of climatic and anthropogenic pressure²⁰. Human activities have been found to possibly mediate species demise through various mechanisms,

including direct over-exploitation, as shown in moas³¹⁴ and great auks³¹⁵. Extinctions can also have many indirect causes that can be difficult to disentangle, such as human-mediated habitat disturbance or introduction of pathogens or predators. For example, the occupation of caves by Aurignacian humans competed with the natural homing behavior of cave bears for places in which they could hibernate to survive the winter^{23,24}. As for the extinct huia birds of New Zealand, the drastic reduction of forest cover and the translocation of mammal predators during the European settlement may have been fatal to these endemic passerines³¹⁶.

Complete genome data from single diploid individuals and/or populations have provided new avenues for reconstructing paleodemographic trajectories using the pairwise and multiple sequentially Markovian coalescent (PSMC³¹⁷ and MSMC³¹⁸, respectively) modelling frameworks. This approach has revealed that archaic hominin populations survived extinction over long periods of time despite highly reduced effective population sizes⁸⁻¹⁰. Analysis of the complete genome from a late-surviving woolly mammoth has identified two episodes of severe demographic collapse in the Early-Middle Pleistocene and at the Pleistocene-Holocene transition³. The resulting reduced genetic diversity and the accumulation of numerous potentially deleterious mutations may have precipitated the population's extinction through the alteration of important functions in development, reproduction, and olfaction¹⁷⁻¹⁹. Demographic reconstructions can, however, be biased by strong natural selection acting on genomes, especially in extreme cases of large population sizes. This was observed for the passenger pigeon that went from a census population size of several billion to extinction in only decades³¹⁹. By reconstructing temporal baselines prior to environmental and/or human impact, it is increasingly clear that aDNA from museum and archaeological remains can advantageously complement current genetic diversity assessments to establish extinction risks and conservation priorities³²⁰, as previously hinted for Przewalski's horses³²¹ and arctic foxes³²².

[H2] Environmental DNA

The retrieval of animal and plant DNA from sediments, ice and lake cores, commonly known as **environmental DNA [G]** (eDNA), is probably the only research area that traces its origins to the field of aDNA itself rather than being adopted from contemporary genetics. Early pioneering work successfully retrieved DNA fragments from extinct mammals and birds from only a few grams of Siberian permafrost and New Zealand cave sediments⁹⁰. If requiring large drilling equipment, sampling procedures should be experimentally tested for contamination, for example by introducing synthetic plasmids of known sequences at the surface of the equipment, whose penetration into the

drilling core can be tracked^{22,48}. The experimental workflow typically includes DNA extraction and the sequencing of biomarkers providing taxonomic resolution³²³, and special attention must be given to rule out on-site contamination⁴⁸ and **stratigraphic leaching [G]**³²⁴. Early analyses relied on molecular cloning of PCR-amplified meta-barcodes, but massively parallel sequencing facilitated access to the entire molecular diversity of individual animals and plants³²⁵. Current approaches build on shotgun metagenomics⁴⁸ and more economical techniques involving target-enrichment^{49,326}.

One of the great benefits of eDNA is that it allows the detection of all domains of life, from microbes to vertebrate species alike, even in the absence of macrofossils^{326,327}. Taxonomic resolution is, however, limited due to the extensive fragmentation of eDNA, and it varies with the completeness of comparative sequence databases. The extent to which quantitative assessments can be obtained is currently unknown, although new biomass proxies aim at this objective¹³⁶. The exact sources of eDNA fragments and the conditions governing their preservation remain largely unknown. eDNA is assumed to derive from skin cells, feces, urine and microfossils, and various mineral particles have been suggested to favor its preservation within sediments⁹⁰. Microscopic bone and tooth fragments that are too small to be identified and are thus confused with sediments have also been speculated to potentially represent a significant source of eDNA⁴⁹.

Environmental DNA has proved a powerful approach to assess the first and last appearance of taxa in the fossil record, and it has been applied to investigate the timing of the extinction of woolly mammoths in mainland Alaska³²⁷ and Yukon³²⁶, and the survival of spruce in Scandinavian refugia during the last Ice Age¹³⁰. Environmental DNA has also provided insights into the spatial and temporal distribution of animal species, including extinct hominins⁴⁹, and the impact of global climatic change^{22,48,90,91,328}. All these studies have in one way or another challenged the validity of climate niche predictions and commonly accepted models of extinction, biogeography and archaeology.

[H1] Reproducibility and data deposition

[H2] Possible confounding factors

Given that numerous tools and approaches are available for analyzing ancient genomic data, reproducibility of results can be hampered by the lack of sufficiently detailed descriptions of each

analytical step in the methods sections of publications. While some guidelines exist for the analysis of ancient genomic data, for example for mapping^{162,181,183} or data authentication^{56,57,329}, and automated tools embedding critical analytical procedures within open-source pipelines are available^{187,188,241,242}, there is still no consensus on a “gold-standard” way for carrying out (or reporting) most analyses. More often, the selection of programs and parameters is research group-based, with publications reporting different analytical approaches and criteria. The list of factors to consider extends far beyond the minimal data requirement for specific analyses³³⁰ and includes: platform-specific sequence error profiles¹⁶⁹; the magnitude of post-mortem DNA damage and contamination and their impact on downstream inference⁵⁵; base quality rescaling⁵⁷; read alignment parameters^{180,182}; database selection and taxonomic classifier parameters^{27,238}; awareness of the applicability conditions of statistical methods; and many more. In the face of the complexity and diversity of possible confounding factors, it is of the utmost importance that all relevant analytical parameters, including software versions and parameters (even if default), are described in full detail in the methods section of any scientific article reporting ancient DNA data.

[H2] Public repositories

To be accepted as valid forms of evidence, raw sequence data and alignments against reference genomes must be made freely available through public repositories such as the [Sequence Read Archive](#) (SRA) and the [European Nucleotide Archive](#) (ENA) in order to ensure research reproducibility and future analyses. Entirely curated datasets are also made available upon publication through individual laboratory websites (for example the [Reich laboratory website](#)). Full traceability of the underlying experimental procedures, including sequencing chemistry and base calling software, is necessary to account for possible data structure deriving from technical artefacts. We recommend that raw, unfiltered sequencing data be uploaded as compressed fastq files together with the alignment files underlying the analyses presented — in BAM format for individual (mito)genomes and in multifasta format for barcode alignment or phylogenetic reconstruction. Labels — for example read groups and sample names — should match those provided in the supplemental sections of the original publication.

[H2] Long-term legacy

The destructive sampling underpinning ancient genomic work has raised severe concerns pertaining to the long-term viability of the data produced. Current approaches are mainly based on whole-genome shotgun sequencing or targeted SNP capture. While the latter is both time and cost effective¹⁶⁰ and remains the only methodology showing sufficient sensitivity to gather data from extremely degraded specimens¹¹⁹, it considerably restricts the amount of information retrieved from samples showing better molecular preservation. In ancient human genome studies, this approach typically queries 1.2 million positions known to be polymorphic amongst a worldwide panel of contemporary human populations¹⁶⁰, leaving other positions and other DNA material present in the extracts, like those from pathogens or microbiota, unexplored. Whole-genome shotgun sequencing is instead not limited to any pre-selected target regions or genomic variants, but rather aims to uniformly cover the entire metagenome. While more expensive to generate and more demanding to analyze, this un-targeted approach can reveal new informative variants and produce data that are applicable to future research questions. Regardless of approach, however it is also possible to create from the original extracted aDNA an immortal DNA sequencing library that can serve as a long-term genetic archive of the sample, and which could be stored in a museum or laboratory cryo-facility. If prepared correctly, this library can be theoretically reamplified indefinitely without exhaustion or loss of complexity¹⁴³. We advise curators to balance the uniqueness of the material considered, known or estimated DNA preservation rates, experimental costs and long-term plans for both DNA library and data archiving when authorizing destructive sampling.

[H1] Limitations and optimizations

[H2] Wet-lab

Due to the sensitive nature of aDNA, preparation and manipulation of aDNA must be carried out in lab facilities with positive air-pressure, UV surface irradiation and strict cleaning procedures to ensure minimal contamination. Clean lab facilities must be physically separated from other lab areas where DNA is amplified, captured and/or sequenced. Samples must be selected according to the main objectives of the study. For example, while petrosal bones are generally preferred for retrieving genome-scale data from host species, their metagenomic potential for

pathogen studies is limited³³⁰. Rates of aDNA preservation also vary at microscopic scales. Therefore, multiple analyses repeated on small amounts of material can increase the chances of success compared to a single analysis from a large sample, which averages out preservation rates and maximizes destruction. No DNA analyses should be carried out on precious, rare material in the absence of preliminary analyses suggesting feasibility and supporting molecular preservation on site. Specific chemical treatment of bone powder, for example with bleach^{40,42}, can reduce the exogenous DNA fraction and may be attempted in cases of repeated failure due to contamination; however, such treatment can also artificially age modern contaminant DNA, and so authentication of bleach-treated samples should be performed with care. DNA libraries should contain multiple indices to eliminate the inclusion of spurious recombining DNA templates in downstream analyses¹⁴². PCR amplification of DNA libraries must be carried out in conditions maximizing molecular complexity, and no DNA extracts and/or DNA libraries should be exhausted for a single analysis. In case of target-enrichment, two successive rounds of capture generally augment on-target recovery rates¹⁶⁶. Library pooling prior to sequencing helps to reduce sequencing costs but requires that the base composition is balanced, especially for Illumina platforms, as a balanced base composition within the first six nucleotides is instrumental for calibrating the fluorescence measurement of nucleotide bases. This can be achieved by spiking library pools with sufficient amounts of a PhiX DNA library prior to sequencing. Large-scale projects can benefit from nascent automation procedures both for the preparation of DNA libraries and their capture^{49,126,144}. In all circumstances, the experimental procedures implemented from sampling to sequencing must be recorded and fully described (and ideally shared through online repositories, like [protocols.io](https://www.protocols.io)) so as to assess the possible impact of experimental differences in the sequence data.

[H2] Dry-lab

With genome-scale sequence data, the number of possible statistical analyses is virtually limitless. Common caveats include the impact of contamination and post-mortem DNA damage and of read alignment parameters. For highly degraded or contaminated material, analyses may be conditioned on reads showing evidence of post-mortem DNA damage^{55,189,192} and/or limited to transversions⁷ so as to mitigate both rampant contamination and damage-related nucleotide misincorporation. This approach, however, significantly reduces the amount of data available. Quantitative assessment of damage and contamination followed by explicit simulation¹⁸⁰ can help

establish the robustness of the conclusions. Microbial taxonomic assignment should be cross-validated through multiple approaches and the presence of potential pathogens must be established by checking that the read edit distance distribution against the reference genomes of close relatives indicates closer proximity with the candidate²⁷. The amount of sequence data necessary for a given analysis depends on the underlying population history and genetic diversity present in a species. For example, a minimal threshold of 10,000–30,000 SNPs is commonly used for assessing genetic relationships of human individuals at intra-continental scales^{331,332} but molecular sexing of mammal species and identification of first-generation hybrids generally requires no more than 1,000–10,000 mapped reads²⁰⁰. The computational procedures must be fully described with explicit reference to analytical parameters so as to ensure reproducibility (and ideally shared through online repositories like [GitHub](#) or [Bitbucket](#)).

[H1] Outlook

[H2] Working together

Within the last decade, aDNA research has come of age and has complemented the toolkits of archaeologists and evolutionary biologists with techniques providing an unprecedented resolution of detail on past environments, societies and individuals. Perhaps the most important contribution of aDNA is that it offers access to a wealth of biological information that can help shape and test working hypotheses about the past in a quantitative manner. Adopting such a hypothesis-testing framework comes naturally for evolutionary biologists who share similar methodologies, including phylogenetic reconstruction and population genetics. This approach, however, differs from that of archaeologists, whose research is primarily grounded within the epistemological frameworks of the social sciences and humanities. As a fast-evolving and inherently interdisciplinary field, ancient DNA research is where these distinct disciplinary approaches mix and meet — to both great effect and occasional mutual misunderstanding. For example, most aDNA work to date has focused on big picture studies comprising large temporal and spatial scales. While necessary for delineating major trends and new working hypotheses, such studies have sometimes divided the archaeological community, as they necessarily over-simplified the inherent complexity of past societies and the

archaeological record itself. Moreover, the often unreflected use of problematic terms and the simplistic equivalence of genetic and cultural groupings (BOX 1) have found major criticism^{104,107}. Whether seemingly rapid shifts in ancestry detected in aDNA data were the result of sudden massive population replacements and/or long-term individual human mobility due to institutions such as patrilocality has also been a matter of discussion and debate^{333,334}. However, now that studies have created a basic overview of the population genomic developments in many regions of the world, it is possible to zoom into more local contexts. Future projects can, for instance, study to what extent biological relatedness was decisive for the constitution of social belonging expressed, for example, through the joint burial of individuals in collective graves over time. Paleodemographic profiles augmented with aDNA evidence on the virulence of past pathogens, as well as paleopathological data, markers of violence and activity and dietary isotopic signatures, can help to establish the underlying causes of health changes in past societies. The reconstruction of ancient trade routes and networks represents another area that could greatly benefit from joint research efforts combining ancient DNA, archaeological fieldwork and remote sensing, for example by integrating genetic markers of migration and metallurgic signatures of material provenance into Geographic Information System (GIS) data analysis. Encouragingly, in the last few years, scholars with different backgrounds in the fields of (bio-)archaeology have increasingly learned to work together and have developed many initiatives to establish and strengthen the crucial interdisciplinary dialogue and mutual understanding that forms the basis for a future reflective bioarchaeology¹⁰⁴. It has become clear that the full community of stake-holders (including descendant communities, archaeologists, biological anthropologists, and museum curators) needs to be embraced, from sampling design to data interpretation, to avoid mis- or over-interpreting the evidence and to collaboratively build a strong foundation for future work (BOX 2).

[H2] Future directions

In studies of humans, aDNA research has been thus far most successful when reconstructing ancestry profiles of past individuals and their population affinities. Future work may increasingly leverage patterns of linkage disequilibrium across sites to gain finer scale genetic resolution. This will prove especially useful for identifying subtle genetic affinities not captured by unlinked SNP information alone²²⁹ and when DNA preservation limits the amount of genetic information recovered. However, gaining further insights about the past will require molecular

investigations beyond humans, their diseases and their domesticates, to include the full range of species and biomaterials preserved in the archaeological and paleontological record, from textiles and ceramics to sediments. Integrating DNA data with other proxies, including stable and radiogenic isotopes¹¹², microremains and small molecule metabolites³³⁵ and paleoproteomic data¹¹³, will greatly improve our knowledge about the lives of past peoples including their diets^{26,336}, environments, mobility³³⁷, craft activities³³⁸, drug and medicinal use³³⁹ and their health status and stress^{26,340}. The successful application of such multi-proxy approaches to the study of the 5,300 year old stomach contents of the Iceman has, for example, provided unprecedented resolution into the nutritional habits and food-processing methods during the European Copper Age³⁴¹.

An increasing body of work has shown that genetic data in tandem with archaeological and paleopathological data can illuminate social rules governing past societies, including marital patrilocality and social inequality²⁰⁵, consanguinity²²², inbreeding avoidance²⁵⁵, and care for individuals with genetic disorders. Recent case studies (for example, for the Early Bronze Age in Southern Germany²⁰⁵ and the Neolithic in Ireland²²²) have shed new light on past social institutions and societal transformations by deciphering the complexity of households and individual mobility as well as the dependency of wealth and status on biological relatedness. Here, archaeogenetic studies have the potential to dramatically advance our understanding of past social structures and the interplay of biological relatedness, health and mobility with social practices and worldviews. Armed with ancient epigenetic markers, future aDNA work will likely inform us about age-at-death of past individuals^{29,248} and the biological and medical consequences of their social status.

Given that all of these approaches are destructive and archaeological remains are finite, however, it is critical that resources are used with the aim of maximizing the amount of data collected and that efforts to develop multi-proxy methods compatible with minute sampling are explored^{112,113}. Efforts to better understand post-mortem DNA degradation and to repair aDNA lesions¹⁰⁹ should be pursued to help further minimize destructive sampling. Such efforts would facilitate DNA analyses into the Upper Paleolithic and Middle Pleistocene, and possibly beyond the current record for the oldest genome around 560,000–780,000 years ago². Increasing focus on environmental aDNA fragments preserved in sediments rather than in macro-fossils will also provide additional opportunities for minimizing destruction while perhaps enriching and providing a fuller accounting of the taxonomic diversity of plant and animal communities in past ecosystems^{22,34,48,49}. In addition, applying these approaches to marine and deep-sea sediments may increasingly inform us about the resilience of the ocean system to past global environmental

change³⁴², and reveal ecosystems and past events in landscapes that became submerged during the Holocene^{134,136}. Such studies promise to have a transformative impact on our understanding of the peopling of the Americas and island southeast Asia, as well as early human expansions out of Africa.

Over the past decade, short read high throughput DNA sequencing technologies have fundamentally changed the field of ancient DNA, but even now new sequencing technologies are on the horizon³⁴³. At first glance, long read sequencers such as PacBio and Oxford Nanopore seem to have little relevance for highly degraded ancient DNA; however, concatenating ancient DNA fragments separated by spacers within single library templates may offer new opportunities for aDNA sequencing, including the direct detection of base modifications. In the longer term, this may even represent the only economical solution for future paleogenomic studies as short-read sequencing technologies are slowly phased out. New advances in de novo genomic and metagenomic assembly likewise hold promise for moving beyond reference-based mapping and towards a more accurate and complete reconstruction of ancient microbial genomes and metagenomes²³⁸, including improved strain separation, genomic architecture reconstruction, and the identification and recovery of novel genes. The last decade has shown that aDNA researchers can be extremely creative in developing innovative solutions to harness the full power of available high throughput sequencing technologies. In the future, it will be essential to continue to adapt our research toolkits to incorporate new and emerging technologies as we strive to fully access the complete amount of information preserved in the fossil and sedimentary record.

New developments in inferring function from ancient genetic sequences are also afoot. Genome editing technologies such as CRISPR-Cas9³⁴⁴, pluripotent iPS stem cells³⁴⁵ and miniaturized organoid systems mimicking simplified organs³⁴⁶ now offer the possibility to investigate the functional consequences of virtually any change in the sequence of a given genome. The comparison of our genome and that of archaic hominins has, for example, provided a preliminary list of the genetic changes that made us humans, and attempts to understand their consequences on human brain development have already started³⁴⁷. Future research will likely increasingly rely on these technologies and genome time-series data of model organisms to assess the adaptive consequences of past genetic changes.

Over almost forty years, aDNA research has taught us surprising things about the human story and life on this planet. It has moved forward in parallel with technological and computational advances in the life sciences, and it has grown and matured in response to new developments in the

social sciences and humanities. The discovery that a vast and invisible molecular past survives in the archaeological record has had a transformative effect on the field of archaeology, and promises many new and unexpected findings to come. In the future, there is no doubt that our ability to increasingly detect and reconstruct the molecular archaeological record will enrich the human story and contribute to the transdisciplinary research endeavor of understanding our shared human past.

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Competing interests

The authors declare no competing interests.

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Related links

Sequence Read Archive (SRA): <https://www.ncbi.nlm.nih.gov/sra>

European Nucleotide Archive (ERA): <https://www.ebi.ac.uk/ena>

Reich laboratory website: <https://reich.hms.harvard.edu/datasets>

Protocols.io: <https://www.protocols.io>

GitHub: <https://github.com>

Bitbucket: <https://bitbucket.org/product>

Max Planck Harvard Research Center for the Archaeoscience of the Ancient Mediterranean:
<https://www.archaeoscience.org/>

International Symposium for Biomolecular Archaeology (ISBA):
<https://isba9.sciencesconf.org>

Standards and Precautions and Advances in Ancient Metagenomics (SPAAM):
<https://spaam-workshop-github.io>

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Glossary

16S metabarcodes. Selected variable regions of the 16S rRNA gene whose sequence provides taxonomic resolution amongst bacteria and archaea.

Ancient DNA. Ultrashort and degraded DNA fragments that are preserved in subfossil material, including hard tissues such as bones, teeth and shells, and soft-tissues, such as mummified skin and hair, as well as sediments.

Ascertainment bias. Statistical bias resulting from the collection of genetic data at a subset of loci that do not reflect the overall genetic diversity present at the whole genome scale.

DNA Barcoding. Taxonomic assignment of metagenomic DNA content on the basis of DNA fragments that show limited intra-specific sequence diversity but large inter-specific sequence diversity.

Bisulfite conversion. A chemical reaction using sodium bisulfite that converts un-methylated CpG dinucleotides into UpGs but leaves methylated CpGs intact, thereby allowing the detection of DNA methylation by sequencing.

Demultiplexing. Process by which pools of sequences originating from different DNA libraries are assigned back to their original samples on the basis of short synthetic sequence(s) added during library indexing.

DNA library. Molecular construction in which DNA fragments are ligated to DNA adapters of known sequences in order to be amplified and optionally captured prior to sequencing; different sequencing platforms require different library constructs.

DNA ligases. Class of enzymes that are capable of stitching together different DNA fragments.

DNA methylation. Biological process by which the activity of a DNA segment is modified without changing the underlying sequence but by adding methyl groups to the DNA molecule.

Environmental DNA. Fragments of DNA that are preserved within sediments and water that can be used for a fast, cost-effective monitoring of the ecology of a given region.

Holobiome. Total sum of the DNA fragments making up the genome of a host organism and all of its microbiota.

Identity-by-descent. DNA segments between two or more individuals are identical-by-descent when they are inherited from a common ancestor in the absence of recombination.

Immunoprecipitation. Molecular lab technique by which specific molecules are purified on the basis of their chemical affinities for particular protein groups, such as antibodies.

Outgroup. Represents an individual, a population, or a group of populations and/or species that are genetically-close but different than those under study.

Population replacement. Population process by which the gene pool of one local population is at least partially replaced by that coming from another, genetically-distinct, population.

Procrustes analysis. Or Procrustes superimposition, is a statistical method allowing the translation, rotation and scaling of multidimensional objects within a single analytical space where they can be compared.

Shotgun sequencing. Non-targeted sequencing of DNA library content.

Stratigraphic leaching. Migration of DNA across strata in sediments caused by water movement, micro-organism growth or bioturbation and compromising the reliability of the stratigraphy, i.e., the order, position and age of the geological layers formed by the different piles of sediments.

Highlighted References

Briggs et al. 2007¹²¹. This article presents the first statistical model of post-mortem DNA degradation and its impact on nucleotide misincorporation patterns, which provides the basis of important criteria for data authentication.

Rasmussen et al. 2010⁶. This article reports the first ancient human genome sequenced from the hair-shaft of a 4,000 year-old Paleo-Inuit. The authors find evidence for genetic discontinuity with modern Greenlanders, supporting multiple migration waves into Arctic Greenland.

Green et al. 2010⁷. This article reports the first Neanderthal genome obtained from the DNA extracts of three paleontological bones. It establishes the Neanderthal genetic legacy within modern human genomes and describes important wet-lab and dry-lab methodologies that have shaped the following decade of aDNA research.

Pedersen et al. 2014²⁹. This is the first report of ancient epigenomes, leveraging post-mortem DNA degradation signatures to statistically infer DNA methylation and nucleosome positioning.

Orlando et al. 2013². This article presents the oldest sequenced genome to date, from one horse metapodial preserved in permafrost for 560,000–780,000 years.

Warinner et al. 2014²⁶. The authors report the first metagenomic and paleo-proteomic analysis of ancient dental plaque and demonstrate the preservation of oral microbial signatures, diet content and inflammation markers.

Meyer et al. 2012¹⁶. This article presents the first high-quality genome from an archaic hominin and describes the first experimental procedure for DNA library preparation from single-stranded DNA templates. The approach outperforms competing technologies in sensitivity and complexity, and minimizes loss of authentic DNA molecules.

Willerslev et al. 2003⁹⁰. This article reports the first analysis of environmental DNA prior to the advent of next-generation DNA sequencing, and establishes the long-term DNA persistence of paleo-communities within sediments.

Fu et al. 2013³⁷. This study presents the first application of in solution target-enrichment at the genome scale and describes sequence for a full chromosome of an approximately 40,000 year old anatomically modern human from China.

Poinar et al. 2006⁸⁸. This article reports the first application of next-generation DNA sequencing to ancient specimens. It describes the metagenomic nature of paleontological remains together with megabase-scale data from the woolly mammoth genome.

Krause et al. 2010⁵⁴. This article establishes important criteria for aDNA data authentication, including the presence of post-mortem cytosine deamination signatures and DNA fragmentation through depurination. It reports a complete mitochondrial genome from an approximately 32,000 year old anatomically modern human from Kostenki, Russia.

Patterson et al. 2012²¹⁹. The authors describe the statistical methodology forming the core of most following aDNA studies and aimed at investigating population structure and admixture patterns.

Gamba et al. 2014⁵². This study reports the first matched empirical evidence establishing better DNA preservation rates in petrosal bones, which paved the way for future studies at the population scale. It also presents complete genomes of Mesolithic hunter gatherers and Neolithic farmers in the Caucasus, supporting direct early contact between both groups.

Bos et al. 2011⁶⁵. The authors report the first complete genome of an ancient bacterial pathogen, *Yersinia pestis*, from osseous human remains of individuals who died from the Black Death in 1347-1348.

Dabney et al. 2013³⁹. The study reports a new DNA extraction method from ancient osseous remains that is tailored to the ultrashort and extensively damaged nature of aDNA molecules. This

methodology allowed the retrieval of full mitochondrial genome sequences from 300,000 year old cave bear specimens preserved in Atapuerca, Spain.

Briggs et al. 2010¹²⁵. The authors present an experimental procedure based on enzymatic digestion that eliminates nucleotide misincorporations from ancient sequence data. This provided the basis for robust statistical inference, minimally impacted by spurious allelic variation, while has paved the way to aDNA methylation maps.

Reich et al. 2010¹⁵. The authors report the discovery of Denisovans, a previously-unknown lineage of archaic hominins that lived in southern Siberia at least 50,000 years ago. This was the first time that a representative of the *Homo* evolutionary tree was described from molecular data and in the absence of key macrofossil remains showing clear morphological characteristics.

Rohland et al. 2015¹²⁶. This study reports the first method for DNA library preparation that is compatible with full automation and also describes various approaches aimed at authenticating data while minimizing the impact of post-mortem DNA misincorporation on downstream analyses.

Table 1. List of analytical tools used in ancient DNA analysis.

Software	Link	Description
CASCADE ¹⁷⁴	Available by contacting authors	LIMS for aDNA experimental workflow
PALEOMIX ¹⁸⁷	https://paleomix.readthedocs.io/en/latest/	Read alignment and processing, phylogenomics
nf-core / EAGER ¹⁸⁸	https://eager.readthedocs.io/en/latest/index.html https://github.com/nf-core/eager	Read alignment and processing
mapDamage ²⁵⁷	https://ginolhac.github.io/mapDamage/	Post-mortem DNA damage assessment
PMDtools ⁵⁵	https://github.com/pontuskk/PMDtools	Selection of reads showing signatures of post-mortem DNA damage
Schmutzi ¹⁸⁹	https://grenaud.github.io/sc_hmutzi/	Contamination estimates based on mitochondrial DNA data
DICE ³⁵⁰	https://github.com/grenaud/dice	Contamination estimates based on nuclear data
VerifyBamID ³⁵¹	https://github.com/statgen/verifyBamID/releases	Identification of contamination and/or sample swaps

Gargammel ¹⁸⁰	https://grenaud.github.io/gargammel/	aDNA read simulator
metaBIT ²⁴²	https://bitbucket.org/Glouvel/metabit/src/master/	Taxonomic profiling of (ancient) metagenomic data
HOPS ²⁴¹	https://github.com/rhuebler/HOPS	Taxonomic profiling of (ancient) metagenomic data
MEx-IPA	https://github.com/jfy133/MEx-IPA	Interactive viewer of MALT taxonomic assignments
coproID ²³³	https://github.com/nf-core/coproid	Identification of the host sources of fecal material
epiPALEOMIX ²⁴⁷	https://bitbucket.org/khanghoj/epipaleomix/wiki/Home	Inference of aDNA methylation and nucleosome mapping
DamMet ²⁴⁸	https://github.com/KHanghoj/DamMet	Inference of aDNA methylation, accounting for DNA damage, sequencing and genotyping errors
ANGSD ¹⁹¹	http://www.popgen.dk/angsd/index.php/ANGSD	Variant identification, Population genetics inference
ATLAS ³⁵²	https://bitbucket.org/wegmannlab/atlas/wiki/Home	Variant identification
ADMIXtools ²¹⁹	https://github.com/DReichLab/AdmixTools	Population genetics inference
smartPCA	https://github.com/chrchan/eigensoft/wiki/smartpca	PCA & Procrustes PCA projection

bammds ²¹³	https://savannah.nongnu.org/projects/bammds/	Multidimensional scaling
PCAngsd ²⁰⁹	http://www.popgen.dk/software/index.php/PCAngsd	PCA, admixture & selection signatures
DATES ²⁵³	https://github.com/priyamoorjani/DATES	Inference of admixture timing
LSD ³⁵³	https://bitbucket.org/plibrado/LSD/src	Selection signatures
GRoSS ³⁵⁴	https://github.com/FerRacimo/GRoSS	Selection signatures
ROHan ³⁵⁵	http://grenaud.github.io/ROHan/	Heterozygosity estimates & runs-of-homozygosity
hapROH ²²⁸	https://pypi.org/project/hapROH/	Inbreeding inference from low-coverage data
lcMLkin ²⁰¹	https://github.com/COMBINE-lab/maximum-likelihood-relatedness-estimation	Kinship inference
READ ²⁰³	https://bitbucket.org/tguenter/read/src/default/	Kinship inference
SourceTracker ²⁴³	https://github.com/danknigh/sourcetracker	Metagenomic authentication

Figures

Fig. 1. Analytical milestones in aDNA research. Key milestones pertaining to wet-lab method improvement (a) or dry-lab advances (b).

Fig. 2. Experimental workflow

A wide range of remains are amenable to aDNA analysis. Prior to sample destruction, a research plan should be agreed amongst the different stakeholders. The different wet-lab procedures must be carried out in specific aDNA facilities minimizing environmental contamination and include all pre-amplification experimental steps, including sample preparation, DNA extraction, optional USER-treatment and DNA library construction. Target-enrichment and PCR amplification are carried out in regular molecular genetics facilities. Following NGS sequencing, the sequence data are processed on computational servers and uploaded in public repositories. Results should be communicated to the stakeholders and any remaining sample should be returned as per the initial agreement.

Fig. 3. Post-mortem DNA damage and data authentication.

A typical ancient DNA molecule is shown at the centre. Fragmentation through purine loss results in the formation of overhanging ends, shown as 5'-overhangs (a), where cytosine deamination is considerably faster than within double stranded parts. The base composition and nucleotide misincorporation profiles around read starts and read ends are shown for data generated on double-stranded DNA libraries both in the absence (b) and in the presence (c) of USER treatment^{125,126} (data from²⁵⁵). Positions 1–10 refer to the first 10 read positions, while positions -5–1 correspond to the five genomic positions located upstream of reads. The base composition indicated corresponds to that of the reference genome. Positions N-9–N refer to the last 10 read positions, while positions N+1–N+5 correspond to the five genomic positions located downstream of reads. In the absence of USER treatment, sequence data are enriched in purines (G and A) at genomic positions flanking read starts and show an increasing excess of C-to-T misincorporations toward read starts), regardless of CpG contexts. The same happens with G-to-A at read ends. Such cytosine deamination profiles are lost for sequences generated using the same sample and methodology but applying USER to DNA extracts, except within CpG contexts. DNA processing during the construction of single-stranded DNA libraries result in different base compositional profiles, in which profiled read ends

are symmetrical to those observed at read starts, instead of being reverse complemented. ReadF and ReadR form the read pair resulting from Paired-End sequencing.

Fig. 4. Examples of standard aDNA statistical analyses applied to human and microbiome data.

(a) Principal Component Analysis (PCA) of prehistoric individuals from Britain (circles), projected on the diversity of present-day Europeans (triangles, data from^{158,206,348}). The gene pool of people who lived in present-day Britain has considerably changed through time, reflecting a complex migration and admixture history. During the Paleolithic and Mesolithic periods (1), individuals in Britain genetically resembled Western Hunter Gatherer (WHG, blue) populations found at that time throughout much of Western Europe. During the Neolithic (2), the arrival of farming populations dramatically shifted the genetic profile of individuals in Britain towards Anatolian Neolithic Farmers (ANF, yellow). Then, beginning in the Chalcolithic period and continuing through the Bronze Age (Early Bronze Age, EBA) (3), the genetic profile of individuals in Britain shifted again, this time towards Western Steppe Herders (WSH, pink) from the Pontic steppes associated with the expansion of the Bell Beaker cultural phenomenon across Europe. (b) Genetic hypotheses generated using PCA can be formally tested using f -statistics. Here we show f_4 -statistics of the form (Mbuti, Yamnaya; X, Y), where X and Y are pairs of ancient individuals from Britain associated with the Neolithic, Bell Beaker and the Chalcolithic/EBA. Scores significantly different from zero ($|Z\text{-score}| > 3$) are shown in pink, while non-significant scores are shown in blue. Individuals from Britain and associated to the Bell Beaker culture and the Chalcolithic/Early Bronze Age show a significant excess of genetic sharedness with Bronze Age Western Steppe Herders associated with the Yamnaya culture of the Pontic steppes. (c) Principal Coordinate Analysis (PCoA) of genus-level taxonomic frequency profiles of ancient (circles) and modern (diamonds) microbiota reconstructed from feces, dental calculus, dental plaque, dentine, bone, soil and sediments (data from³⁴⁹). Ancient dental calculus have metagenomic diversity profiles similar to modern dental calculus, while paleofeces resemble modern feces from nonindustrialized populations. Microbes colonizing skeletal material generally originate from soil, but some dentine samples show evidence of being decomposed by the dental plaque bacteria.

Fig. 5. Geographic and temporal distribution of ancient specimens analyzed at the genome scale. (a) Humans (data from Ancient Human DNA uMap, accessed on July 28th 2020 and available at https://umap.openstreetmap.fr/en/map/ancient-human-dna_41837#6/51.000/2.000). (b) Non-human animals. (c) Plants. (d) Pathogens (data from³⁴⁹). (e) Metagenomes (data from³⁴⁹). The minimum (square) and maximum (diamond) age of the samples analyzed at a given location are provided. Only squares are shown in case the temporal range overlaps only one time interval. Colors correspond to approximately 1,000 year time intervals, except for the oldest, which includes genomes dating to 13,000 years ago or more, including Late and Middle Pleistocene specimens. The size of squares and diamond is proportional to the number of samples analyzed at a given location, but is adequately scaled in each panel for clarity and according to the total number of specimens (N=) for which both geographic and temporal data are available.

Boxes

Box 1. Writing Archaeogenetic Prehistory.

In the last decade, archaeogenetic studies have provided radically new insights into prehistory worldwide. However, several early publications did not fully appreciate the related history of research in archaeology. The inherent complexity of relevant terms such as culture, migration or people in the social sciences was insufficiently acknowledged, in part due to different disciplinary publishing norms and to the strict limitations on word count and number of references imposed by high-profile journals, which resulted in very negative feedback by the archaeological community. Now, sustainable collaborations between archaeogeneticists, archaeologists and historians have been established and even institutionalized (such as with the [Max Planck Harvard Research Center for the Archaeoscience of the Ancient Mediterranean](#)), and awareness has been raised on both sides — for the use of challenging terms on the one hand and the potential of archaeogenetic historiography on the other. Increasingly, a new generation of scholars is being trained within an interdisciplinary framework that allows narratives of the past to be told in more integrative, nuanced and sophisticated ways that address the complexity of archaeological and archaeoscientific datasets.

Box 2: Who conducts ancient DNA research?

The field of ancient DNA research has diverse and heterogeneous disciplinary origins. Initial studies were largely led by biochemists and primarily focused on evolutionary questions relating to extinct mammals, but the field quickly diversified to also encompass anthropological and forensics questions. Today evolutionary biology and anthropology have become the two main disciplinary homes of aDNA research, and there has been tremendous growth in the number of ancient DNA labs over the past decade. Housed in museums, research institutes and university departments, the size of these labs ranges from small labs serving a single principal investigator (PI) to large, multi-PI labs supporting dozens of technicians, students and postdocs. Funding is also highly varied, with some labs receiving core government funding while others depend entirely on third-party research grants. Moreover, funding agencies, grant types and funding levels also differ substantially across countries and disciplines, and such differences contribute to large disparities across different aDNA labs in terms of their resources and research capabilities. International collaboration and community

building, the establishment of enduring teaching and training networks and the formation of academic societies and research consortia are needed to leverage resources across the field, remove barriers to access for smaller labs and support the growth of the field as a whole. Initiatives such as the biannual [International Symposium for Biomolecular Archaeology](#) (ISBA) conference and the [Standards and Precautions and Advances in Ancient Metagenomics](#) (SPAAM) workshop aim to address some of these challenges, but additional collaborative efforts, such as lobbying of funding agencies and collective bargaining to make large in-solution probe sets available through commercial vendors, are needed.