Ancient DNA studies: Common limitations and Genotyping

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Abstract

Ancient DNA (aDNA) research has significantly transformed our understanding of human evolution and genetic diversity. Initiated by the Human Genome Project (HGP), which mapped human genes and established foundational genomic techniques, the field has evolved rapidly. The HGP catalyzed advancements in sequencing technologies, bioinformatics, and analytical methods, enabling the extraction and analysis of aDNA from degraded samples, such as skeletal remains. This review highlights the critical progression from the initial aDNA studies to contemporary techniques, including hybridization capture and high-throughput sequencing, which enhance data quality and processing efficiency. We explore the contributions of aDNA research to evolutionary biology, revealing interbreeding events between archaic and modern humans and identifying ancient genetic variants that influence contemporary traits and disease susceptibility. Despite the tremendous potential of aDNA studies, challenges remain, including post-mortem DNA damage and limitations in genotyping accuracy. Techniques such as pseudohaploid and probabilistic genotyping address these issues, facilitating the analysis of degraded samples. Moreover, ethical considerations in aDNA research are paramount, emphasizing the need for respectful engagement with descendant communities and the preservation of cultural heritage. This review aims to provide a comprehensive overview of the advancements in aDNA research, the methodologies employed, and the ethical frameworks that guide these studies, ultimately underscoring the significant impact of aDNA on our understanding of human history and health.

Keywords: ancient DNA, human evolution, genetic diversity, evolutionary biology, modern health, archaeological insights.

Significance | Mapping human genes transformed genomic research, revealing human evolution, disease susceptibility, and ethical challenges in ancient DNA studies.

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Introduction

The Human Genome Project (HGP) was a groundbreaking initiative aimed at mapping and understanding all the genes of the human species and studying the gene pool. Officially launched in 1990 and completed in 2003, it was led by the National Institutes of Health (NIH) and the U.S. Department of Energy (DOE), with participation from numerous institutions worldwide (Hood and Rowen, 2013). The project's primary goals included identifying the approximately 20,000 to 25,000 genes in human DNA, determining the sequences of the 3 billion chemical base pairs that constitute human DNA, storing this information in databases, improving tools for data analysis, transferring relevant technologies to the private sector, and addressing emerging ethical, legal, and social issues (ELSI). The first significant publication regarding the Human

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Genome Project was the initial working draft of the human genome, announced in June 2000 and published in Nature in February 2001 (Lander et al., 2001).

Over the next two decades, the project grew rapidly, enabling the study of the genome from both evolutionary-historical and species-diversity perspectives. Research has focused on the genomes of prehistoric humans and Neanderthals, as well as those of currently living humans (Dalal et al., 2023). Today, extensive studies have sequenced the genomes of several million living individuals and thousands of prehistoric humans. Another major research area examines the genetic analysis of bacteria and viruses, investigating the origins and mutations of pathogens, as well as human immune cells. This research aims to enhance our understanding of how to combat modern diseases and pathogens, particularly regarding prevention and prophylactic measures (Metz et al., 2023).

The rapid development of the Human Genome Project can be attributed to several interrelated factors. Technological advances, particularly in automated DNA sequencing, have significantly increased both the speed and accuracy of genomic analysis. Techniques such as shotgun sequencing and clone-by-clone sequencing, as well as high-throughput sequencing methods, have enabled researchers to process and analyze enormous amounts of data efficiently (Sakaki, 2019).

Additionally, the development of bioinformatics, which leveraged the growing computing power of the time, was critical. The availability of powerful computers and sophisticated algorithms facilitated the processing, analysis, and storage of the vast genomic data generated by the project. These computational capabilities were essential for assembling the human genome sequence from billions of DNA fragments (Oulas et al., 2019).

International collaboration was another key factor in the project's success. The HGP involved multiple countries and many research institutions, fostering resource sharing, knowledge transfer, and division of labor that together enhanced the project's efficiency and comprehensiveness. This extensive collaboration promoted collective problem-solving, accelerating overall progress (Birney, 2021).

Importantly, the study of ancient DNA (aDNA) has profoundly changed our understanding of human origins and the interactions between archaic humans, such as Neanderthals and Denisovans, and modern Homo sapiens. Research has revealed instances of interbreeding and gene transfer among these groups, providing insight into the genetic diversity and adaptability of early humans. Furthermore, aDNA studies have identified ancient genetic variants that contribute to modern human traits and susceptibility to certain diseases (Pimenoff et al., 2018). This knowledge complements the results of the HGP by linking past genetic adaptations to contemporary health traits and diseases. Advances in technology, spurred by the HGP, such as high-throughput sequencing and bioinformatics tools, have also enhanced the analysis of aDNA, which is often fragmented and degraded. These innovations have enabled more accurate reconstructions of ancient genomes, thereby expanding the scope and resolution of genetic studies of the past (Parks et al., 2015).

Sampling and laboratory work

The process of working with degraded ancient DNA (aDNA) in the laboratory is complex and involves several key steps, from sample preparation to DNA sequencing (Childebayeva and Zavala, 2023). The first step is sample preparation, which is typically performed on skeletal material such as bones or teeth. These samples are thoroughly cleaned to remove potential contaminants before being ground into a powder to isolate the DNA. The extracted DNA, which may be contaminated with microorganisms and non-endogenous DNA from other organisms, is then purified to isolate the genetic material of interest (Stan et al., 2024).

Next, the purified DNA is converted into libraries for sequencing. To achieve this, adaptor sequences required for the sequencing process are added to the DNA fragments (Head et al., 2014). During library preparation, short oligonucleotide indices are also attached to the DNA molecules. These indices serve as molecular barcodes, enabling researchers to distinguish between different DNA libraries during the sequencing process. Depending on the quality of the DNA and specific research objectives, targeted enrichment of specific loci may be performed prior to sequencing (Oguchi et al., 2020). This step, known as hybridization capture, enriches particular regions or types of DNA, making the sequencing process more efficient and cost-effective by focusing on areas of interest (Gasc et al., 2016).

After library preparation and enrichment, the libraries are sequenced using next-generation sequencing (NGS) technologies. The resulting sequencing data undergo bioinformatics analysis to assemble DNA fragments and interpret the genetic information (Cheng et al., 2023). In cases where aDNA samples are relatively well-preserved, such as those less than 30,000 years old, double-stranded library preparation is often combined with partial uracil-DNA glycosylase (UDG) treatment. This process repairs some DNA damage while preserving terminal deamination patterns that are critical for authenticating ancient DNA (Rohland et al., 2015).

Given the limited availability and significance of ancient remains, considerable effort is made to minimize the amount of material used while maximizing the DNA isolated. Although the cranial portion of the temporal bone is a rich source of DNA, its invasive extraction can be problematic. Therefore, teeth and long bones are often used as alternatives, depending on the quality and availability of the samples. To extract highly degraded DNA, techniques such as the preparation of single-stranded libraries are employed, which

are effective for obtaining very short DNA fragments (Harney et al., 2021).

In forensic and ancient DNA studies, the choice of library preparation method—whether full-genomic or targeted enrichment—is crucial as it affects subsequent data analysis. Forensic studies typically focus on common single nucleotide polymorphisms (SNPs) useful for personal identification and genetic ancestry determination, while ancient DNA studies often target SNPs that provide insight into population-level genetic variability (Mandape et al., 2024). A widely used tool in ancient DNA research is the 1240k SNP capture array, which covers about 1.2 million SNPs relevant to global genetic diversity and evolutionary studies. An updated version, the Twist Ancient DNA array, enriches about 1.4 million SNPs and includes additional informative regions not covered by the 1240k array (Mathieson et al., 2015). These arrays facilitate detailed analyses of ancient populations and evolutionary patterns. There is a strong emphasis on open data sharing in the field of ancient DNA research; however, this practice is approached with caution when working with indigenous communities to respect their privacy and cultural sensitivities (Dalal et al., 2023).

After library preparation and sequencing, the next step involves bioinformatics processing of the sequencing data. This includes aligning the short DNA reads to a reference genome, if available, or assembling them de novo if a reference genome is unavailable. The alignment process must consider the unique challenges associated with aDNA, such as postmortem lesions, including characteristic C-to-T and G-to-A substitutions caused by cytosine deamination (Magi et al., 2010).

To determine the ancient origins of DNA, researchers analyze the nature of the damage. For instance, terminal deamination is a hallmark of aDNA, and observing these patterns helps confirm the authenticity of ancient sequences and distinguish them from possible modern contamination. Additionally, contamination testing is conducted by comparing aDNA sequences to known modern human sequences to ensure that no modern DNA has been inadvertently introduced (Peyrégne and Peter, 2020).

After data validation, various analyses can be performed depending on the study's goals. Population genetics studies may focus on haplotype and phylogenetic analyses to understand the evolutionary relationships and migration patterns of ancient populations. Functional genomics approaches can identify genetic variants that provided adaptive advantages or promoted specific phenotypic traits in ancient populations (Feng et al., 2023).

In forensic science, analyses often focus on identifying SNPs specific to individuals, which can help establish identity, parentage, or determine physical traits. Researchers studying ancient DNA can utilize arrays such as the 1240k or Twist Ancient DNA array to obtain SNPs informative for population genetics. Data from these arrays can assist in reconstructing ancient population structures, migration events, and interactions with archaic humans such as Neanderthals and Denisovans (Yousefi et al., 2018).

In some cases, targeted enrichment strategies like hybridization capture are employed to selectively sequence regions of interest. This method reduces sequencing costs and increases the resolution of specific genomic regions, allowing for a detailed examination of genes or loci relevant to the research questions. For example, targeted capture can focus on SNPs that inform the genetic basis of certain traits or study the evolutionary pressures that have shaped human genomes (Gasc et al., 2016).

Post-mortem DNA damage removal

The process of laboratory work with degraded ancient DNA (aDNA) involves numerous chemical reactions that occur post-mortem and affect the integrity of DNA molecules. These reactions include the fragmentation of DNA into ultrashort fragments, the conversion of nucleotides into various derivatives, and the cross-linking of DNA with other molecules. Such postmortem changes can create significant challenges in handling aDNA and reduce the amount of genetic information that can be recovered (Dabney et al., 2013).

One of the most notable postmortem changes is DNA fragmentation, which results from hydrolytic depurination followed by beta-elimination reactions. This process breaks down double-stranded DNA into fragments that can be millions of times shorter than their original lengths during a person's lifetime (Danielewski et al., 2023). Another common reaction is hydrolytic degradation, specifically the deamination of cytosines, which are converted to uracils and subsequently sequenced as thymine analogs. This process leads to sequencing artifacts, such as C-to-T and G-to-A misplacements, which increase toward the end of sequencing because cytosine deamination predominantly occurs in the single-stranded overhangs of aDNA fragments (Carpenter et al., 2006).

It is important to note that postmortem damage can accumulate over time; however, the kinetics of degradation ultimately depend on local environmental conditions. Notably, the level of damage does not always correlate with the age of the remains or the DNA within them: some more recent specimens may appear more damaged than older ones. Additionally, degradation processes are better understood in mineralized tissues than in other types of remains (Krassner et al., 2023; Zhu et al., 2017).

While C-to-T mislocalizations provide convenient genetic signatures for authenticating aDNA sequences, they can also skew sequence analysis, potentially leading to erroneous conclusions. To mitigate the impact of sequencing errors caused by damage, DNA extracts can be treated with an enzymatic mixture of uracil-DNA glycosylase (UDG) and endonuclease VIII (Endo VIII), known as the USER reagent (Flores Bueso et al., 2020). This treatment

removes uracil and cleaves the resulting abasic sites, thereby repairing damage and shortening DNA molecules. However, this process has both positive and negative effects: while it reduces sequencing errors, it also repairs the damage that is necessary for authenticating aDNA sequences. Nevertheless, in mammalian nuclear DNA, damage signals can still be observed when analyzing CpG dinucleotides (Krokan et al., 2002; Krokan and Bjørås, 2013).

To analyze mammalian DNA, some laboratories first examine an unprocessed (non-UDG) DNA library to determine sequence authenticity, and then create a second USER-processed (full-UDG) library for further analysis. This approach allows researchers to assess both the authenticity of aDNA sequences and the impact of lesion repair on the resulting data (Wibowo et al., 2021).

Ancient DNA Genotyping

The process of ancient DNA (aDNA) genotyping involves the processing and interpretation of genetic data obtained from highly degraded samples, often requiring specialized methods that consider the quality of the data and the goals of the analysis. aDNA genotyping can be divided into two categories: pseudohaploid genotyping and probabilistic genotyping, each of which is suitable for different levels of data quality and downstream analysis requirements (Bonfigli et al., 2023).

Pseudohaploid genotyping is typically used with low-coverage data, genomic arrays, or in situations where a large number of genomes are analyzed, most of which have low coverage. In this method, instead of determining the true diploid haplotype, one read at each position is randomly selected (Barlow et al., 2020). This approach simplifies the process when the data are insufficient to reliably determine diploid genotypes. Programs such as pileupCaller and bam-caller are commonly used for pseudohaploid genotyping (Das et al., 2019). These tools allow users to specify which single nucleotide polymorphisms (SNPs) should be genotyped and provide filtering based on coverage, mapping quality, and base quality. Users can either randomly select a read from the pile or, if coverage permits, choose the allele supported by the most reads. This step is critical after trimming DNA ends to minimize the impact of deamination (Etter et al., 2011; Homer and Nelson, 2010). For single-stranded DNA (ssDNA) libraries that have not undergone uracil-DNA glycosylase (UDG) treatment or have undergone partial UDG treatment, the effects of deamination or contamination can be further minimized by restricting the calling of C-to-T SNPs to reverse strands and G-to-A SNPs to forward strands. Additionally, after genotyping, the observed number of transitions and transversions (such as C>T, A>G, A>C, etc.) can be quantified to determine whether their ratio differs from the expected value (a Ti/Tv ratio of 2-2.1 for full-genome sequencing) (Psonis et al., 2021; Weiß et al., 2020). However, this method is not suitable for capture lattices, where the Ti/Tv ratio can deviate significantly from the expected norm.

Probabilistic genotyping is better suited for aDNA samples with higher coverage. Several programs, such as snpAD, ATLAS, bcftools, GATK, and ANGSD, can be used to determine the probability of genotypes in ancient samples (Gill et al., 2021). These tools often utilize a reference set of modern genotypes, such as the 1000 Genomes reference set, to support the genotyping process. Trimming DNA ends remains important before determining diploid genotypes to reduce errors (Garrido Marques et al., 2024; Ebler et al., 2019). Tools like ATLAS can account for aDNA damage when estimating genotype probability, eliminating the need for extensive pre-processing prior to genotyping. Furthermore, when working with data that has not undergone UDG processing, genotyping can be restricted to transversions, which are less susceptible to deamination in aDNA, providing more reliable results for subsequent analyses. Damaged reads can also be filtered out using tools like PMDtools. Non-UDG ssDNA libraries allow reads to be processed separately, offering an additional level of control over the data (Sharko et al., 2023).

Limitations and considerations

The limitations of genotyping ancient DNA (aDNA) primarily revolve around the accuracy of genotype calls and their subsequent impact on analyses. Genotyping aDNA often struggles with low coverage, which can lead to allele dropout and increased stochastic sampling, complicating the differentiation between heterozygous and homozygous loci (Ausmees et al., 2022; Sousa da Mota et al., 2023). Forensic genotyping typically employs high coverage to mitigate damage present in DNA from historical and forensic samples. In contrast, aDNA studies can benefit from trimming reads to remove damage or using probabilistic genotyping that accounts for post-mortem damage, thereby enabling the analysis of more degraded samples (Morozova et al., 2016).

Despite the challenges of low coverage and the theoretical absence of limits in pseudohaploid calling, contamination can reduce the likelihood of correctly sampling endogenous reads. Limiting the analysis to presumed deaminated fragments can provide a sanity check to evaluate whether certain signals are driven by contamination (Renaud et al., 2015). Another method involves using f4 statistics, which measure allele frequency correlations across four populations. In the absence of contamination, these statistics should approximate zero. Reference bias is another concern that can be assessed using f4 statistics when high- and low-coverage data are analyzed together, revealing a potential attraction between pseudohaploid data and the reference genome (Peter, 2022; Patterson et al., 2012). In probabilistic genotyping, reference bias remains an issue, particularly when higher genotype

probabilities are assigned to alleles that are homozygous for the alignment reference, which predominantly includes individuals of European and African ancestry. This bias became evident when researchers noted that the reference set may not fully represent genetic variability in forensic or ancient samples, where individuals could display different linkage disequilibrium patterns and potentially shorter haplotypes due to the mixed ancestry of modern individuals (Günther, T., & Nettelblad, 2019).

Choosing genotyping methods requires balancing the need for accuracy with the degree of uncertainty that is permissible. In aDNA studies, less stringent quality control metrics and reliance on population-level estimates allow for the use of lower-quality and lower-quantity data compared to forensic contexts, where individual identification is paramount and the consequences of errors are more severe (Pavan et al., 2020). Interestingly, aDNA findings can directly impact modern communities, such as providing evidence for Native American tribes seeking federal recognition in the United States. This underscores the profound implications that genotyping accuracy and data interpretation in aDNA research can have beyond academic and scientific realms, influencing cultural and legal outcomes (Wagner et al., 2020).

Ethics in a DNA research

Ethical considerations are of paramount importance in ancient DNA (aDNA) research due to the sensitive nature of the samples and their potential impact on descendant communities and cultural heritage. Several key ethical considerations should be kept in mind throughout the research process (Ávila-Arcos et al.,2022).

When working with ancient human remains, researchers must adhere to the principles of respect for individuals, beneficence, and justice. This involves obtaining informed consent from relevant stakeholders, such as indigenous communities or descendants, whenever possible. Respecting cultural protocols and traditions is crucial, and researchers should consult with these communities constructively to ensure that their views and interests are incorporated into the research process (Lovo et al., 2021).

Preservation of cultural heritage is another critical ethical consideration in aDNA research. Researchers must approach the processing and analysis of ancient remains with sensitivity and respect for their cultural significance. This includes minimizing destructive sampling, preserving the integrity of burial sites, and adhering to ethical principles and legal requirements governing the excavation and study of archaeological remains (Alpaslan-Roodenberg et al., 2021).

Additionally, researchers must consider the potential social and cultural implications of their findings. Genetic research has the power to reshape our understanding of human history and identity, so researchers need to be aware of the possible consequences of their work. This includes issues related to genetic ancestry, population mixing, and the legacy of colonialism and discrimination, which can profoundly affect contemporary social dynamics and relationships (Roth et al., 2020).

Finally, transparency and accountability are essential ethical principles in aDNA research. Researchers must openly communicate their methods, results, and interpretations to both the scientific community and the general public. This involves acknowledging uncertainties and limitations in the data, engaging in peer review and scientific debate, and promoting responsible data sharing to facilitate independent verification and replication of results.

Conclusion

Ancient DNA studies have opened new avenues for understanding human history, genetic diversity, and disease susceptibility. By leveraging technological advancements and international collaboration, researchers have made significant progress in reconstructing ancient genomes and unraveling the genetic complexities of our past. The integration of bioinformatics tools, high-throughput sequencing methods, and targeted enrichment strategies has enhanced the accuracy and resolution of aDNA analyses.

Despite the challenges posed by post-mortem DNA damage and limited sample availability, researchers have developed innovative approaches to optimize data quality and interpret genetic information from degraded samples. The distinction between pseudohaploid and probabilistic genotyping methods reflects the evolving landscape of aDNA research, catering to varying levels of data quality and analytical needs.

Ethical considerations play a critical role in shaping the future of aDNA research, emphasizing the importance of respecting individual and cultural rights, preserving heritage, and addressing the societal implications of genetic findings. Transparent communication, collaboration with descendant communities, and adherence to ethical guidelines are essential for ensuring responsible and culturally sensitive research practices.

As the field of ancient DNA continues to evolve, researchers must navigate the complexities of genotyping ancient samples while upholding ethical standards and engaging with stakeholders. By embracing interdisciplinary approaches, fostering collaboration, and promoting transparency, the scientific community can harness the power of ancient DNA to unlock the mysteries of our genetic past and pave the way for a more inclusive and informed understanding of human evolution.

Author contributions

A.V.P. was responsible for the original draft preparation, while V.N.S., A.N.O., I.V.K., T.I.K., I.A.S., and D.F.B. contributed to the writing, review, and editing of the manuscript.

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Competing financial interests

The authors have no conflict of interest.

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