Chapter

Ancient environmental genomics: An Introduction

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Content:

I. Introduction

Environmental DNA (*e*DNA) obtained from ancient samples such as sediments, ice or water are valuable data sources for a wide range of disciplines in past and present biodiversity and biogeography [1-4]. Within the field of ancient metagenomics, the number of published genetic datasets has risen dramatically in recent years and have become an increasingly powerful tool to investigate wide-ranging topics [5]. However, the ancient environmental metagenomics remains many issues that should be to be addressed relating to ancient DNA (*a*DNA) such as degraded nature, incomplete reference databases, sensitivity to contamination by modern DNA [6-8]. This review aims to provide an overview of the use of ancient metagenomics in large-scale ecological and evolutionary studies of individual taxa and communities of both microbes and eukaryotes and illustrate the limitations, risks, and potentiality of this ancient *e*DNA research via high-throughput sequencing (HTS) technologies. Further, paleogenetic and paleogenomics will provide diverse insights into studying evolution and how the present world came to be.

II. Ancient eDNA and ancient environmental metagenomics

In general, *e*DNA was extracted from ancient samples extremely fragmented and chemically modified depending on the sample types [6]. Typically, the size of ancient *e*DNA fragments is from 70 base pairs (bp) to less than 100 bp long [9] and with ends impacted by cytosine deamination [10]. Only in a few cases, where extraordinary preservation such as Antarctic conditions, for example, 500 bp of *a*DNA were recovered from lake sediment [11], respectively. These conditions generally feature anoxic, cold and dry conditions [6]. In the context of isolating *a*DNA from environmental samples, environmental *a*DNA including sedimentary ancient DNA (or *seda*DNA) is used widely and applies to DNA isolated from

sedimentary deposits in lake cores [12-14], marine [15, 16], cave [17-19], ancient forest [20], permafrost [13, 21-23], peat [24], tropical swamp [25]. However, there is potential for many other materials to provide information about the past via *a*DNA analysis as basal ice [20], glacial soil [26], silt-soaked [27]. Analysis of *a*DNA datasets, when combined with traditional proxy results, appears to complement each other, revealing a greater diversity of species than utilizing the methodologies independently [15, 28, 29]. Therefore, *a*DNA should be considered as a complementary, rather than alternative, approach to assays of more traditional established methods [3, 30].

The metagenomics of ancient environmental DNA can be broadly defined as the study of the total genetic content of samples that have degraded over time from several hundred to hundred-thousand years [5, 31]. Despite an extensive application including studies of genome reconstruction of specific microbial taxa [12, 32], host-associated microbial communities [33, 34], and environmental reconstructions using *seda*DNA [5, 25, 35], the major source of ancient eDNA has been almost entirely limited to inventorying taxa through time by using DNA metabarcoding approach [15, 16, 36, 37]. Recent advances of next-generation sequencing (NGS), massively parallel or deep sequencing technology, have the potential to radically change this situation, from sequencing of millions of short DNA fragments to generating datasets of genome-scale from extant and extinct species by bioinformatics analyses [12, 13, 32, 37].

III. The problem of environmental ancient DNA

Despite recent methodological strategies for *a*DNA extraction, Polymerase Chain Reaction (PCR) and/or sequencing, the study of *a*DNA could be negatively affected by the

applicability and the outcome by several inherent technical issues. Part of the challenge is the fact that ancient samples are often rare and precious materials, such as low DNA quantities, DNA damage, high fragmentation, and contamination with modern sources [6]. In general, the ancient *e*DNA sample processing and analysis should be processed with practical recommendations for ancient DNA research to prevent contamination, reviewed in Capo et al., 2021 [35] for lake sediment cores and Armbrecht et al., 2019 [8] for marine sediment cores.

The current *a*DNA extraction protocols were not very different from the protocols used to obtain DNA from environmental settings including silica-based, alcoholic, and phenol-chloroform protocols [22, 38, 39]. For the molecular analyses, the yield and integrity of the recovered *a*DNA obtained will influence the reliability of subsequent results. Therefore, extraction protocols of *a*DNA should be carefully considered and adapted depending on the physical and chemical properties of sediments, DNA-subtracts interaction, or target organisms [8, 15, 40, 41]. Further, quick, simple and direct DNA extraction procedures are needed for use in regular analysis of *a*DNA.

DNA damage alters the base-pairing properties of individual bases and is vastly over-represented in *a*DNA sequences. This increased rate of polymerase misincorporation errors and therefore sequencing errors by incorporating wrong nucleotides opposite modified bases [42, 43]. During PCR, DNA damages cause blocking primer binding/DNA polymerase progression, preventing the amplification of the templates, or hydrolysis of the phosphodiester bond, resulting in a single-strand break [44-46]. For instance, the majority of errors give by deamination of cytosine to uracil, which pairs up with adenine instead of guanine, leading to thymine to cytosine transitions [45-47]. However, well-characterized degradation features of

*a*DNA i.e., damage patterns and high fragmentation, allow us to authenticate 'true' *a*DNA sequences.

IV. How to study ancient metagenomic

The application of several technologies, from PCR and the earlier methods, including Sanger sequencing, to HTS, also known as Next-Generation Sequencing (NGS) [48] for short-read (shotgun) sequencing [49] or long-read sequencing, dramatically started a new revolution in ancient DNA research **(Figure 1).** While traditional PCR methods could only amplify a small number of specific target sequences, HTS combines amplification and sequencing of up to several billions of individual DNA library templates at a time. DNA/RNA metabarcoding approach is an extension of DNA barcoding, which relies on HTS technologies [36, 50-53]. Furthermore, HTS can sequence shorter DNA fragments - shotgun [37] and event recover whole genome sequences for the study of paleogenomics [12, 54, 55]. These technologies generate large quantities of highly accurate DNA sequences at lower costs than it was possible by using first-generation sequencing technologies.

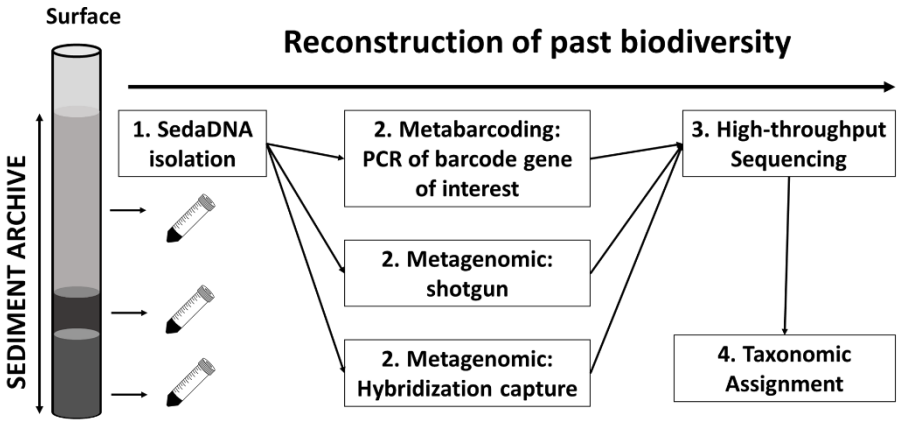

Figure 1. Conceptual workflow of ancient metagenomic approach applied to DNA preserved in environmental archives (e.g., marine and freshwater sediment cores) to reconstruct the past diversity.

In brief in **Figure 1**, two main approaches to the study of *a*DNA are metabarcoding, the taxonomic identification of the community via analysis of short DNA sequences of one or a few genes, and metagenomics, the analysis of total DNA of the community via whole-genome sequencing. For workflow of the wet laboratory, total DNA is initially isolated from the sample, for example, sediment cores. Next, the DNA metabarcoding standard steps include PCR amplification, library preparation, and sequencing followed by bioinformatic analyses. Depending on the targeted organisms, the specific primers are used to amplify DNA fragments, e.g., the mitochondrial COI region [56], foraminiferal 37f hypervariable region [57-59], and the internal transcribed spacer (ITS) region [60]. For distinguishing samples during bioinformatic processing, specific tags or indexes are added using ligation or other PCR-round. After quantification and normalization steps, the final library is then sequenced on one of the various available sequencing platforms, e.g., Illumina, Ion Torrent, PacBio, or Oxford Nanopore. In contrast, after collecting suitable samples under the guideline of *a*DNA research, the wet lab workflow for (shotgun) metagenomics can be roughly divided into three steps: DNA extraction, library preparation, and sequencing, without PCR.

4.1. Metabarcoding and its limitations

To date, most paleoecological *a*DNA investigations have employed the widely used DNA metabarcoding method, usually, with a focus on a particular organismal group [61]. DNA metabarcoding represents a molecular approach to contemporary taxonomy and identification, e.g., plant [50, 62-65], fungi [60], foraminifera [57, 58], metazoan [56, 66, 67]. The PCR-

metabarcoding approach uses primer pairs to target and maximize portions of the hypervariable regions of the phylogenetic marker genes. Amplicons from separate samples are then given molecular barcodes, pooled together, and sequenced by amplicon-based HTS approaches. Fragments of *a*DNA are analyzed with a bioinformatics pipeline and identified from environmental archives, by comparison, them against sequences of reference database taken from modern reference organisms [29, 36, 68, 69].

However, metabarcoding which is applied to environmental *a*DNA is complicated by its natural degradation. The PCR-based approach for sequencing can generate incorrect sequence data from *a*DNA for several reasons. The total amplified sequence count is likely to reflect the original abundance of different DNA sequences in the sample. Damages of *a*DNA could inhibit DNA polymerase progression or prevent primers from binding to templates during PCR. The *a*DNA fragments are extremely short and low-yields, while preferential random amplification is longer or requires abundant DNA molecules. As a result, a lot of PCR cycles are needed, and false-positive findings are more frequent, and heavily biased towards well-preserved or more abundant sequences, possibly from present-day DNA contamination during the first few cycles [37, 70]. It can be induced predictably biased in multi-template PCR and significantly distort the final output. To solve this problem, PCRs can be repeated independently and increase the total number of replicates for each sample as well as using negative controls should be applied [71]. This approach makes short and rare sequences more likely to be identified than if only one replicate were used since they are likely to be missed in a single PCR but should be expected in one or more of the repeat PCRs. Further, based on using genetic markers in molecular studies of previous paleo-microbiome research, the length of taxonomic marker genes is a major cause of differential amplification resulting in a taxonomic bias in ancient reconstructions [72].

4.2. Shotgun sequencing and Whole Genome Sequencing

Shotgun sequencing is the untargeted (shotgun) sequencing of all genetic material (metagenomics) present in a sample, which has the potential to look for population genomic variation from multi-taxon mixtures and independent of DNA fragment size [36, 72]. Compared to metabarcoding, the shotgun approach is less subject to bias introduced by laboratory processing, ever-reducing sequencing costs. Generally, shotgun sequencing randomly breaks DNA sequences of the entire chromosome or entire genome into many small fragments and reassembles the sequences by computers via observing the overlapping sequences or regions. The shotgun approach can detect this genomic variation of the population by utilizing extensive intraspecific genomic reference datasets [73, 74] or assembling *de novo* genomes [75, 76]. Furthermore, the whole-genome shotgun (WGS) method entails sequencing many overlapping DNA fragments in parallel and then using a computer to assemble the small fragments into larger contigs and, eventually, chromosomes within a short period. NGS has also been used to obtain RNA and pathogen genome sequences from ancient plant remains [77]. The adoption of NGS technologies significantly expanded the range of *a*DNA studies possible, enabling the analysis of full chloroplast [54, 78], and mitochondrial and nuclear genomes [79, 80] from ancient samples. For instance, chloroplast and mitochondrial genomes of single-celled microalgae (*Nannochloropsis limnetica*) were successfully reconstructed from 20 000-year-old lake sediments [12].

Shotgun sequencing is a faster method and cheaper to carry out compared with traditional sequencing. Usefully, the advent of the shotgun approach permits statistical data analyses to detect specific substitutions that are normally present at the ends of ancient DNA fragments, therefore confirming whether a sequence or set of sequences is relatively ancient

and not modern contamination, as well as improving the specificity and sensitivity of taxonomic identification [81, 82]. In some cases, as for eukaryotes in *seda*DNA, if the targeted DNA is rare compared to the total genomic DNA, producing large numbers of short sequencing reads [83] is required to recover sufficient genetic information and perform meaningful statistical analyses, particularly useful for *a*DNA analysis for its fragmentation and degradation [84]. Usefully, the ends of older sequences retrieved using a shotgun approach will show deamination damage, which can confirm whether a sequence or set of sequences is relatively ancient and not modern contamination. Although whole ancient genomes are becoming more readily accessible, mitochondrial [13, 85, 86] or chloroplast [12, 54, 78] genomes are an alternative choice in *a*DNA studies dealing with samples with high DNA degradation, and low DNA yields. Before sequencing, another alternative option applies the hybridization capture technique [78, 87]. The constraint of shotgun sequencing might be solved by using the hybridization capture approach before sequencing to enrich the DNA of the targeted species in the samples. To do this, small segments of DNA from the species and target sites of interest can be used as baits, with the matching sites of interest in ancient DNA libraries being hybridized. This technique, originally developed for modern DNA, is commonly applied in ancient DNA studies, particularly for use on single specimens [88] and with a focus on mammals, mostly using mitochondrial DNA [89, 90], chloroplast and nuclear DNA [78, 91-93], cave sediments [19], permafrost samples [22].

4.3. Bioinformatics considerations

Now the shotgun approach provides an alternative approach to metabarcoding for determining for taxonomic and functional profiling of metagenome-assembled genomes. The amount of genetic data has risen exponentially and vast amounts of that are mostly uploaded

to and stored on public archives, for example, European Bioinformatic Institute's (EBI) European Nucleotide Archive (ENA, https://www.ebi.ac.uk/ena/) or the US National Center for Biotechnology Information (NCBI)'s Sequence Read Archive (SRA, https://www.ncbi.nlm.nih.gov/sra). However, it brings huge challenges at the stage of bioinformatics for its analysis. A vast of bioinformatics tools, protocols and studies have been introduced to improve efficiency in analyzing ancient metagenomic data. Bioinformatics tools designed for *a*DNA metagenomics as mapDamage [94-96], PyDamage [97] or open-sourced/mapping guidelines pipeline [98, 99] for estimating DNA damage, SourceTracker [100] for identifying the proportions of endogenous and contaminant signals in each sample; resolving the sequencing errors [96, 101]; MEGAN [102, 103], PIA [104] for taxonomic identification; KEGG [105], EGGnog [106], SEED [107] protein databases for functional profiles can be analysed in MEGAN, reference-free alternative approaches based on k-mer counts [108] to annotate metagenomes. However, differences between metagenomic analysis pipelines produce systematic biases [25], which will require the development of more accurate analysis pipelines for ancient DNA.

Nevertheless, several issues currently limit the shotgun sequencing approach. Cytosine deamination patterns of *seda*DNA molecules impede *de novo* assembly of contigs [10, 109]. The limitation of sufficiently curated genome-scale reference data substantially reduces the potential for success of the bioinformatic analyses with metagenomic data, for example, plants [77, 110], and eukaryotic [111, 112]. The large fraction of taxa present in the environment, but not represented in databases is still problematic. In these cases, metagenomic data can vary in content across samples from the same or similar environments. In contrast, there are more than 130,000 genome or near-complete sequences available from different phyla that have been sequenced along with a variety of microorganisms, including archaea, fungi, and viruses [113-

115]. Based on the annotated reference genomes or clade-specific [116] or universal markers [117], appropriate normalization by genome size [55], and taxon relative abundances can be estimated. This led to the development of the field of paleomicrobiology [1, 32], to the analysis of deposited microbial DNA to study microbial diversity, ecology, and evolution in environmental archives.

4.4. Applications of ancient environmental metagenomics

The shotgun of *seda*DNA in paleoecology from lake sediment cores combined a multi-proxy approach [14], and marine environments [37, 40], which has provided greater taxonomic resolution and extended the historical record of aquatic ecosystems to centennial or even millennial time scales. These *seda*DNA archives can be used to characterize biodiversity trends, illuminate past food web dynamics, and reconstruct long-term environmental changes in aquatic ecosystems. As ecology and paleoecology merge, both short-term and long-term trends as a consequence of human actions on aquatic ecosystems have been traced using paleogenomic research in freshwater ecosystems [118-120] and marine sediments [121, 122].

Paleogenomics is a branch of research concerned with reconstructing and analyzing genetic data from extinct organisms. Ancient genomes may be used to explore the evolution of present species in great detail by sequencing ancient DNA preserved in subfossil remains [54, 123] or environmental archives [1, 12]. By analyzing large-scale environmental DNA metagenomic study of ancient plant and mammal communities, tracking the ancient population origins, movements and interrelationships, the evolutionary genomic changes at both macro- and micro-evolutionary temporal scales of the microbiome, vegetation, animals and *Homo species* [12, 13], as well as identification of phenotypic features over large temporal and

geographical scales [89, 90, 124]. For example, a study on DNA retrieved from Arctic permafrost and lake sediment samples by Wang et al. [13] demonstrated that steppe–tundra flora dominated the Arctic during the Last Glacial Maximum, followed by the regional divergence of vegetation during the Holocene epoch. The extinction of several now-extinct megafauna species enabled the survival of some ancient plants and animals. Moreover, analysis of mammoth environmental DNA reveals a previously unsampled mitochondrial lineage. Additionally, the genetic material preserved in sedimentary archives offers a unique way to uncover the role of microorganisms in past ecosystems and their responses to environmental perturbations. Genomic reconstruction of historical and present microbial communities from ancient permafrost samples in Siberian broadened our understanding of biogeochemical changes [32]. Furthermore, this study provides insights into microorganisms' long-term survival strategies from the past paleoenvironment to present-day freezing-temperature conditions.

V. Summary

In conclusion, the fields of *a*DNA are increasingly turning to the environmental archives and provide great potential for entire paleoecosystems and paleoclimate reconstructions. As technology advances and procedures are optimized, metagenomic-based approaches, from metabarcoding (amplicon-based) to shotgun and true ancient metagenomics, are part of the next breakthrough in paleogenetic, offering the potential for better species identification and quantitative estimations of their abundances in large-scale biodiversity comparisons over both time and place. Importantly, further basic studies are needed to use a full understanding of its potential and limitations for applications of the use of metagenomics for ancient eDNA.

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