Iteratively mapping ancient DNA to reconstruct highly divergent mitochondrial genomes: An evaluation of software, parameters and bait reference

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Abstract

1. The ability to obtain ancient DNA (aDNA) from extinct species has greatly improved our ability to study their evolutionary histories. Most aDNA studies focus on mitochondrial DNA for both population-level and species-level insights, due to its relatively high abundance and increased obtainability. Although several approaches are available to assemble mitogenomes, high levels of DNA damage and non-target contaminating DNA make many approaches unsuitable for aDNA. Further complicating matters for most extinct species is the lack of a sufficiently closely related extant relative to act as a reference.

2. Iterative mapping can assemble mitogenomes without using a close reference sequence, and has been applied to aDNA from various extinct species. Despite its widespread use, the accuracy of the reconstructed assemblies is yet to be comprehensively assessed. Here, we investigated the influence of (i) mapping software (Burrows-Wheeler Alignment [BWA], MITObim and NOVOplasty), (ii) parameters and (iii) bait reference phylogenetic distance on assembly accuracy using two simulated datasets: spotted hyena and various mammalian bait references; southern cassowary and various avian bait references, and two empirical datasets: extinct cave hyena; two extinct sloth species. Specifically, we assessed the accuracy of results through three parameters: pairwise distance to a reference conspecific mitogenome, number of incorrectly inserted base pairs and total length of the assembly.

3. We found large discrepancies in the accuracy of reconstructed assemblies using different mapping software, parameters and bait references. Results also varied depending on the dataset, thereby making broad, generalised conclusions difficult. However, the overall most accurate results were obtained with either BWA using a minimum mapping quality of 20 and relaxed mismatch parameters (-n 0.001 -o 2), or MITObim using a mismatch value of 3 or 5. We also show accuracy could be further improved by combining results from multiple bait references.
1 | INTRODUCTION

The ability to obtain ancient DNA (aDNA) from palaeontological and historic specimens has dramatically expanded our understanding of the evolutionary histories of many extinct species (Paijmans et al., 2013). In aDNA studies, mitochondrial DNA is often the marker of choice, due to the higher proportion of mitochondrial DNA in each cell relative to nuclear DNA (Ho & Gilbert, 2010). Mitochondrial genomes (mitogenomes) have many uses within evolutionary biology, and are routinely utilised at both population level and species level to provide insights into phylogenetic relationships (Paijmans et al., 2017; Westbury et al., 2017) and/or demographic histories (Lorenzen et al., 2011; Stiller et al., 2010). Moreover, due to the unique maternal mode of mitogenome inheritance in vertebrates, it is possible to trace evolutionary lineages to a single common ancestor, and to make specific inferences regarding maternal lineages, without the confounding effects of recombination (Fortes et al., 2016).

Ancient DNA is commonly characterised by degraded and highly fragmented DNA, and an often large proportion of contaminant external/non-target DNA (Ho & Gilbert, 2010). Despite this, complete mitogenomes were first successfully reconstructed for extinct species in the early 2000s (Haddrath & Baker, 2001; Krause et al., 2005). However, technological limitations meant data generation required PCR and the independent sequencing of many short regions across the mitogenome, which is a laborious process. High-throughput sequencing now allows the simultaneous sequencing of millions of DNA fragments, making it easier to generate data from across the entire mitogenome (Knapp & Hofreiter, 2010).

Even with ample data, mitogenome assembly for highly divergent extinct species can be challenging. Short read lengths and high levels of contaminating non-target DNA make de novo assembly difficult. Therefore, the most typical assembly approach is to map sequencing reads to a reference genome from a closely related species. However, many extinct species have no closely related extant relative, and a suitable reference genome is unavailable (Shapiro & Hofreiter, 2014). In the absence of a close relative with which to directly align reads against, iterative mapping has become a popular alternative (Hahn et al., 2013), and has been used to generate mitogenomes from an array of extinct vertebrate species (Mitchell et al., 2014; Paijmans et al., 2017; Westbury et al., 2017; Xenikoudakis et al., 2020). In short, iterative mapping works by (i) mapping short-read data to a bait reference genome, (ii) generating a new consensus sequence from the mapped reads and (iii) using the latter as a new mapping reference. This process is repeated iteratively until either no new reads map or the mitogenome is complete.

Although iterative mapping has been used to assemble mitogenomes from aDNA of divergent extinct species, the accuracy of the reconstructed assemblies has yet to be comprehensively assessed. Several factors may influence the quality of the final reconstruction including aDNA damage, short fragment lengths, endogenous DNA content and the bait reference genome. Here, we investigated the influence of mapping software, parameters and bait reference phylogenetic distance on the accuracy of reconstructing mitochondrial genomes from aDNA. We used two independent simulated datasets, one based on mammals (spotted hyena Crocuta crocuta, and various carnivore bait references) and one based on birds (southern cassowary Casuarius casuarius, and various palaeognath bait references). Furthermore, utilising our findings based on simulated data, we reconstructed mitochondrial genomes from empirical datasets based on extinct cave hyena and extinct sloth mitochondrial genomes and compared those to published sequences.

2 | MATERIALS AND METHODS

A schematic overview of the methodology is presented in Figure 1.

2.1 | Data simulation

To reliably evaluate the accuracy of iteratively reconstructed assemblies while controlling for patterns of aDNA damage and contamination, we generated two simulated mitochondrial datasets, one based on spotted hyena (Genbank accession: MN320452.1) and the other based on southern cassowary (Genbank accession: NC_002778.2) using gargammel (Renaud et al., 2017). In gargammel, we simulated paired-end reads with read lengths of 150 base pairs (bp); 40x coverage of the target mitogenome; 98% of the data to consist of microbial contamination from the database available with gargammel; 1.9% of the data to be made up of a mixture of human (Homo sapiens) chromosome 1 (Genbank accession: NC_000001.11), human mitogenome (Genbank accession: NC_012920.1) and species-specific nuclear genome (spotted hyena—Genbank accession: GCA_008692635.1, and southern cassowary—Genbank accession: PFTA00000000.1); and fragment lengths typical of aDNA (Figure S1). We repeated the simulations twice per species, with damage patterns based on either double-stranded (DS) or single-stranded (SS) aDNA sequencing.
libraries. We trimmed Illumina adapter sequences and merged overlapping read pairs using Fastp v0.20.1 (Chen et al., 2018). Only trimmed and merged reads were considered for downstream analyses unless stated otherwise.

2.2 | Iterative mapping

We selected five bait references of varying phylogenetic distance to each target species (Supplementary Table S1). We calculated pairwise distance (PWD) between each target species and the corresponding bait references by aligning all relevant mitogenomes from each taxonomic group (carnivore or palaeognath). For this, we used Mafft v7.392 (Katoh & Standley, 2013) specifying --globalpair and --maxiterate 16, and the maximum composite likelihood method in MEGA X (Kumar et al., 2018) specifying missing data as a pairwise deletion in the calculation. Due to difficulties aligning the control region among species, we removed this region from the calculation of PWD.

We performed iterative mapping with three different mapping software, Burrows–Wheeler Alignment (BWA) tool v0.7.15 (Li & Durbin, 2009), MITObim v1.8 (Hahn et al., 2013), a wrapper script for the MIRA v4.0.2 alignment tool (Chevreux et al., 1999) and NOVOplasty (Dierckxsens et al., 2017). We investigated BWA and MITObim, as they are most commonly used to generate mitogenomes from extinct species (Anmarkrud & Lifjeld, 2017; Delsuc et al., 2016; Kehlmaier et al., 2017; Westbury et al., 2017; Xenikoudakis et al., 2020), and NOVOplasty as it has proved reliable for a wide range of extant species (Margaryan et al., 2021).

As BWA is not specifically designed for iterative mapping, we created a pipeline using bash tools for this study, which we called ‘ancient ITErative mapper’ (aITE mapper). In short, this method aligns reads to a bait reference using BWA aln, filters the output, removes duplicates using SAMtools v1.9 (Li et al., 2009), creates an indel-aware consensus fasta sequence using kindel v0.4.4 (Constantinides & Robertson, 2017) and uses the output consensus fasta sequence as a new reference sequence in subsequent mappings. This process is repeated until either no new reads map or for a maximum of 100 iterations.

When running aITE mapper, we tested various filtering and mapping options. These included the following: different minimum mapping quality score filtering options (10/20/30), different mismatch values (-n 0.04/-n 0.01/-n 0.001 - o 2). We decided on these parameters as they have been shown to produce the most reliable results when mapping aDNA data to a reference genome (Oliva et al., 2021; Schubert et al., 2012). We specified a minimum read depth in kindel (--min-depth) of three, and otherwise default parameters for all uses.

For MITObim, we implemented default parameters, but with several different mismatch values (0/1/3/5/10/15) and utilised the consensus sequence generated by MITObim. As NOVOplasty requires paired-end reads, we reran Fastp to trim Illumina adapter sequences from our simulated data, but did not merge overlapping read pairs. We ran NOVOplasty specifying both the seed input and reference sequence as the complete mitochondrial genome of the bait reference sequence, a kmer size of either 33 or 23, chose the longest final consensus sequence generated by MITObim. As NOVOplasty requires paired-end reads, we reran Fastp to trim Illumina adapter sequences from our simulated data, but did not merge overlapping read pairs. We ran NOVOplasty specifying both the seed input and reference sequence as the complete mitochondrial genome of the bait reference sequence, a kmer size of either 33 or 23, chose the longest final assembly if there were multiple, and used otherwise default parameters. We tested the smaller kmer size of 23 as suggested by the information in the example config file when read lengths are shorter than 90bp.

We evaluated whether accuracy of the iteratively reconstructed assembly was improved by combining results from
different bait references, but using the same mapper/parameters. We aligned each reconstructed assembly generated using the various bait references for each target species with Mafft, and created a majority rules consensus sequence for each target species, while taking gaps into account in Geneious prime v2021.0.3 (Kearse et al., 2012); we subsequently refer to this as the multispecies consensus sequence. Due to the very short final consensus sequence lengths (~400bp) of the NOVOplasty assemblies from the hyena data, we did not produce a multispecies consensus sequence from the NOVOplasty results.

2.3 | Evaluation

To benchmark our results, we mapped our simulated reads back to the reference conspecific mitogenome of each target species using BWA aln, with default parameters.

We determined the accuracy of the iteratively reconstructed assemblies of each target species using three proxies: (i) PWD to the reference conspecific mitogenome; (ii) number of inserted bp relative to said reference and (iii) total sequence length. We aligned each reconstructed assembly and reference conspecific mitogenome using Mafft. We estimated PWD using a maximum composite likelihood method in MEGA. We manually removed regions that show problem alignments from the PWD calculations, as we deemed this as the product of the aligner tool rather than the iterative mapping process. We calculated the number of inserted bp by counting the number of sites where the iteratively mapped sequence had a nucleotide (even if specified as missing data—N), and the reference conspecific mitogenome was given a gap during alignment; we excluded insertions exceeding the start or end positions of the reference conspecific mitogenome from this calculation, due to the circular nature of the mitogenome. We assessed total sequence length directly from the reconstructed assembly, removing sites with missing data.

2.4 | Empirical data

To evaluate whether our results from simulated data were applicable to empirical data, we downloaded raw single-end reads from extinct cave hyena (Crocuta spelaea) and trimmed paired-end reads from two extinct sloth species (Mylodon darwinii and Nothrotheriops shastensis). The cave hyena dataset contained shotgun sequencing data from an individual with relatively low endogenous DNA content (mitochondrial and nuclear DNA ~1%), termed Ccsp015 (reads accession: SRR5224140), and one individual with relatively high endogenous DNA content (mitochondrial and nuclear DNA ~47%), termed Ccsp041 (reads accession: SRR5224137; Westbury et al., 2020). The sloths contained three datasets; one Mylodon darwinii individual that had been shotgun sequenced, which we termed ‘Myloshot’ (reads accession: ERR1958375; Delsuc et al., 2018); the same individual enriched for mitochondrial DNA, which we termed ‘Lib_67’ (reads accession ERR3308856; Delsuc et al., 2019); and one Nothrotheriops shastensis individual, enriched for mitochondrial DNA, which we termed ‘Lib_X32’ (reads accession: ERR3308859; Delsuc et al., 2019). We merged paired-end reads (sloth datasets) and trimmed adapter sequences (cave hyena datasets) using Fastp. For the sloth dataset, we downloaded mitochondrial genomes from two species (Bradypus tridactylus—Genbank accession: KT815852.1 and Choloepus hoffmanni—Genbank accession: KT213697.1) to use as bait references. For the cave hyena dataset, we used the same bait references as the simulated dataset, with the inclusion of the spotted hyena (Genbank accession: MN320452.1). We ran the iterative mapping three times for each dataset using the best performing parameters based on the simulated data: aITE mapper v3 (minimum mapping quality score 20 and mismatch value -n 0.001 -o 2) and MITObim mismatch 3 and mismatch 5. The final assemblies were evaluated in the same manner as the simulated datasets against published mitochondrial genomes generated from the same sequencing data: Ccsp015 (Accession: MN320457.1), Ccsp041 (Accession:MN320460.1), ‘Myloshot’ (Accession: MF061314.1), ‘Lib_67’ (Accession: MK903501.1) and ‘Lib_ X32’ (Accession: MK903502.1).

3 | RESULTS

When mapping our simulated reads of spotted hyena and southern cassowary back to the reference conspecific mitogenome using BWA, we found no errors (PWD = 0) in the mapped assembly, regardless of whether the sequencing reads contained DS or SS aDNA damage patterns. However, a small number of sites had missing data: spotted hyena DS = 2, SS = 2; southern cassowary DS = 8, SS = 4. A detailed summary of all iterative mapping results are presented in Supplementary Tables S2–S7.

3.1 | aITE mapper

For both the carnivore and palaeognath datasets, PWD and number of inserted bp in general had no obvious correlation with phylogenetic distance to the bait reference, but total sequence length generally declined (Figures 2 and 3, Supplementary Tables S2–S5). The relationship between PWD and minimum mapping quality was not as clear. Either we saw little difference, or lower PWD with increasing minimum mapping quality (Figures 2 and 3). Changing minimum mapping quality also had little influence on the number of inserted bp. However, increased minimum mapping quality led to shorter total sequence length (Supplementary Tables S2–S5). Again the influence of relaxing the mismatch parameters showed no obvious correlation with PWD. However, we do see a general trend of less inserted bp and longer overall assembly lengths with -n 0.001 -o 2 relative to other mismatch values (Figures 2 and 3).

Overall, the multispecies consensus sequence from multiple bait references was more accurate than a single bait reference, and in general resulted in a decrease in PWD (Figures 2 and 3). However,
we retrieved highly elevated inserted bp levels when using the default mismatch parameter (~n 0.04) and a minimum mapping quality of 30, but generally less inserted bp when using more relaxed mismatch values. In every comparison, we recovered equal if not longer total sequence lengths than when using only a single bait reference (Supplementary Tables S2–S5).

### 3.2 | MITObim

Overall, there was no obvious relationship between PWD and phylogenetic distance of the bait reference when using MITObim, regardless of mismatch parameter (Figures 2 and 3). This was also mostly true for the number of inserted bp. In the carnivore dataset, the *Parahyaena* bait reference led to the highest PWD in the DS dataset, whereas *Proteles* overall led to the highest PWD in the SS dataset (Figure 2). We saw the most elevated levels of inserted bp when mapping to *Ursus* as bait reference followed by *Parahyaena*, again suggesting the number of inserted bp does not correlate with phylogenetic distance of the bait reference.

For the carnivore dataset, most tests recovered near-complete mitogenomes, to the exclusion of the *Suricata* and *Ursus* bait references and a mismatch value of 0, and in some cases the total sequence length exceeded the total linear length of the mitogenome (Supplementary Tables S2 and S3). With the palaeognath dataset, only the bait reference *Dromaius* resulted in near-complete mitochondrial genomes (16,732bp as opposed to the expected linear length of 16,740bp). All other bait references resulted in a total sequence length of ~14,886bp (Supplementary Tables S4 and S5).

With the carnivore dataset, we generally found the highest values of PWD to be at the lower mismatch values (0 and 1) and higher values (10 and 15; Figure 2). All mismatch values resulted in comparable numbers of inserted bp and total sequence lengths to the exclusion of 0, which always resulted in shorter total lengths. In the palaeognath dataset, the largest PWD always arose with a mismatch of 0 (Figure 3). However, we did not see any obvious relationship
between mismatch value and assembly accuracy; most PWD and inserted bp for the remaining mismatch values (1–15) were 0, and the recovered sequence lengths were all near-identical.

Overall, the multispecies consensus sequences were more accurate than when using a single bait reference. In the carnivore dataset, we found the lowest overall levels of PWD and inserted bp, as well as the longest sequence lengths relative to using a single bait reference (Figure 2). This pattern was also true for the palaeognath dataset, the multispecies consensus sequences were also more accurate than when using a single bait reference; to the exclusion of the SS dataset with a 0 mismatch value which had elevated numbers of inserted bp (270bp).

3.3 | NOVOplasty

With the carnivore dataset, both the DS and SS datasets resulted in either relatively short (400–500bp) or relatively long (16,000–17,000bp) assemblies (Supplementary Tables S2 and S3). A kmer size of 33 resulted in more of the longer assemblies. All assemblies had no inserted bp, but the longer assemblies did have slightly elevated PWD (up to 0.0065). With the palaeognath dataset, all runs resulted in 0 PWD, 0 inserted bp, and relatively long assemblies of ~15,000 bp (Supplementary Tables S4 and S5). NOVOplasty requires paired-end data, which may explain why it has been shown to be better at resolving repetitive regions (Dierckxsens et al., 2017). However, the short fragment lengths of aDNA mean paired-end information is not always available, which may limit the applicability of this software with aDNA samples.

3.4 | Empirical data

Unlike with the simulated data, we could not be sure of the correct sequence of empirical data. However, we assume the published sequences are correct when discussing our results. For Ccsp015, we
observe that aITE mapper was better overall relative to MITObim, generating smaller PWD and less inserted bp for all bait references apart from Proteles and Suricata, and comparable overall sequence lengths (Figure 4 and Supplementary Table S6). For Ccsp041, MITObim appeared to perform better in all cases apart from with Suricata and Ursus as bait references (Figure 4 and Supplementary Table S6). For the sloth dataset, both bait references, Bradypus and Choloepus, produced similar results in all comparisons (Figure 4 and Supplementary Table S7). For Myloshot, aITE gave elevated PWD and more inserted bp compared to MITObim. However, this could be explained by the much shorter total sequence length of ~4000bp when using MITObim, compared to ~17,000bp when using aITE (Figure 4 and Supplementary Table S7). The mitochondrially enriched data, Lib_67 and Lib_X32, produced more accurate results than Myloshot, with lower PWD and fewer inserted bp. MITObim produced slightly less inserted bp but again with shorter overall sequence lengths (Figure 4 and Supplementary Table S7).

### 4 | DISCUSSION

We investigated the influence of mapping software, parameters and bait reference sequence on reconstructing mitogenomes from aDNA through iterative mapping, providing a reference for informed decision making on how to best reconstruct mitogenomes with aDNA data. Overall, our results show that there is no clear, decisive best approach. Depending on the dataset, the most optimal approach slightly changed. However, there were combinations of software and parameters clearly better than others. We found aITE mapper v3 (the pipeline developed here using BWA, minimum mapping quality 20 and mismatch value -n 0.001 -o 2) or MITObim using a mismatch value of 3 or of 5 gave the most reliable results overall. Therefore, for future studies, we recommend these approaches as the best starting point. One option would be to use a combination of mapping approaches, comparing results, and only considering sites where there is consensus across multiple approaches.

An accurately reconstructed assembly is crucial for the reliability of downstream analyses; the incorrect incorporation of nucleotides may bias evolutionary inferences. A single mitogenome from an extinct species is commonly used to estimate when the species diverged from its closest living relative (Mitchell et al., 2014; Westbury et al., 2017; Xenikoudakis et al., 2020). However, the inclusion of errors would artificially inflate (in the case of random insertions/substitutions) or deflate (in the case of mapping biases towards the bait reference allele) divergence estimates, leading to erroneous inferences of the driving forces of divergence events, for example, climatic shifts, natural disasters, continental drift. Furthermore, as the mitogenome includes protein coding genes, which undergo selective processes (Atlas & Fu, 2021; Pavlova et al., 2017), incorrect reconstruction may also influence selection analyses. Population-level analyses may also be impacted, if sequencing errors and damage patterns are incorporated when using an incorrectly assembled mitogenome as mapping reference for DNA read data from conspecific specimens.

While the simulated data provided an easily controllable environment, various factors of aDNA make it unpredictable. Fragment lengths, damage patterns and endogenous DNA content can all
vary greatly, even within samples from the same individual (Alberti et al., 2018). The results from our empirical data show this; aITE mapper on the cave hyena with low endogenous DNA content (Ccsp015) generally outperformed MITObim, but vice versa for the cave hyena with high endogenous DNA content (Ccsp041). We also observed that data enriched for the mitochondrial genome led to more accurate results than shotgun data. While it is difficult to know the overall endogenous DNA content of Myloshot due to the lack of a conspecific nuclear genome to map against, it can be assumed that the mitochondrial enrichment resulted in much higher mitochondrial endogenous DNA content in Lib_67, aiding in the reconstruction by removing non-target DNA fragments.

The circular nature of the mitogenome is both a pro and a con in mitogenome reconstruction. While the presence of circularity in the final consensus sequence can be used to evaluate the completeness of the reconstruction (Hahn et al., 2013), we observed both sides of the linear sequence are extended in MITObim, and reads no longer uniquely map to a single location, decreasing the accuracy at the terminal ends of the sequence. However, it may be possible to circumvent this problem by linearising the mitogenome from a different starting point, and repeating the mapping process.

Linearising the mitogenome from a different starting point may also offer a means to overcome repetitive elements, but further investigations are required to confirm this. In the southern cassowary mitogenome, there is a repetitive element of a single A followed by many G at -14,800 bp. Presumably due to the short read lengths of our simulated aDNA data (Supplementary Figure S1), MITObim was unable to reconstruct any sequences after this repeat from any bait reference with a PWD >0.19. This was likely due to the high divergence from the bait reference in the region, as much of this post-repeat region comprised the highly divergent control region. In fact, the control region appeared to be the most difficult region to reconstruct in most tests, potentially due to the repetitive and rapidly evolving nature of the region.

We found that caution should be applied when considering results from a single bait reference, as bait reference-specific biases can occur. Both BWA and MITObim resulted in incorrectly inserted bp; manual inspection revealed these mostly arose from few insertions but with long stretches of bp. Long stretches of inserted bp could be caused by insertions–deletions (indels) between the bait reference and the target species. The bait reference may have had an insertion not found in the target species’ reference mitogenome, leading to the false mapping of reads to said region. A single insertion could explain why we consistently observed a ~15 bp insertion when using Dromaius as bait reference in the palaeognath dataset, but none using any other bait reference. Therefore, multiple bait references may be necessary to ensure the highest accuracy possible and to avoid species-specific insertions. However, it should be noted that we found elevated numbers of inserted bases in the combined consensus. This may be the result of effectively summing all the inserted bases in the consensus sequences from each individual reference, which may superficially make this approach appear to be worse compared to a single reference. Therefore, this should be considered when combining results from multiple bait reference sequences.

Finally, although we have performed the method evaluations using aDNA datasets, the principles are also applicable to modern data from extant species. Modern datasets are much less volatile, containing relatively high-quality DNA and little-to-no damage. However, many extant species also lack reference mitochondrial genomes, with the closest reference genome being highly divergent, which our results show is not a large influencer in the accuracy of iterative mapping. Depending on the quality of the DNA, de novo assembly is a viable option. However, performing de novo assembly requires a higher level of computational expertise relative to iterative mapping. As we show iterative mapping to be a useful approach for much more problematic aDNA, it should function equally as well, if not better than shown here, when assembling mitochondrial genomes from modern DNA when no close reference genome is available.

**AUTHOR CONTRIBUTIONS**

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**CONFLICT OF INTEREST**

The authors declare that they have no conflicts of interest.

**PEER REVIEW**

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**DATA AVAILABILITY STATEMENT**

Scripts for aITE mapper, MITObim and consensus sequence building used can be found on github.com/Mvwestbury/Iterative_mapping or under the fixed DOI: https://doi.org/10.5281/zenodo.7077708.

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