Multiplex amplification of ancient DNA

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This method is designed to assemble long, continuous DNA sequences using minimal amounts of fragmented ancient DNA as template. This is achieved by a two-step approach. In the first step, multiple fragments are simultaneously amplified in a single multiplex reaction. Subsequently, each of the generated fragments is amplified individually using a single primer pair, in a standard simplex (monoplex) PCR. The ability to amplify multiple fragments simultaneously in the first step allows the generation of large amounts of sequence from rare template DNA, whereas the second nested step increases specificity and decreases amplification of contaminating DNA. In contrast to current protocols using many template-consuming simplex PCRs, the method described allows amplification of several kilobases of sequence in just one reaction. It thus combines optimal template usage with a high specificity and can be performed within a day.

INTRODUCTION

Rationale

Amplification of ancient mitochondrial, chloroplast and nuclear DNA is typically limited by small quantities of endogenous DNA, high levels of DNA degradation, contamination with exogenous DNA and the often restricted quantity of fossil material. Because of these problems, only limited information is usually obtained using standard simplex PCR. This protocol is specifically designed to bypass these limitations and to assemble long, continuous DNA sequences from degraded DNA templates, even if present in low copy numbers. The method is suitable for sequence data retrieval from disparate sources of DNA such as fossils, forensic specimens, noninvasively obtained samples and museum specimens. Thus, its applicability ranges from phylogenetic questions to population genetics, conservation genetics, forensic applications and functional genetics. The technique has been used to amplify both the first complete mitochondrial genome[1] and the first complete nuclear gene (H.R., N.R., T.S. and M.H., unpublished data) from Pleistocene specimens.

The two-step multiplex protocol was initially developed to analyze the sequences of single DNA molecules for genome mapping. When it is applied to ancient DNA, several modifications are required and additional precautions have to be taken.

Working with ancient DNA

Generally, and despite substantial progress in recent years, the retrieval of ancient DNA sequences from archeological and paleontological specimens is not routine. The problems in ancient DNA research arise from the fact that very little, usually extensively degraded and often no DNA survives in ancient tissues, whereas for certain species such as humans, bacteria or domestic animals, contemporary DNA is pervasive in the environment, both inside and outside the laboratory. To address these problems, a number of guidelines have been put forward in recent years. Although different studies require different levels of precautions[2], there are certain standards for ancient DNA work[3–5] to which all studies should adhere in order to obtain authentic sequences. However, it should be noted[2] that even strict adherence to all guidelines may be insufficient to prevent incorrect results.

Physically isolated work area. To avoid amplification of modern DNA contaminants, all work with ancient DNA preceding PCR cycling has to be carried out in a dedicated, isolated environment (in this protocol, Steps 3–7). The risk of cross-contamination with previously amplified PCR products is high, as copy numbers up to 10^{12} are generated for products amplified by PCR. Contamination problems can originate from any previous amplification product containing target sequences for the primers used. This problem becomes even more serious when products are cloned. Therefore, ancient DNA facilities should be located as far as possible from modern DNA laboratories.

General considerations. To sustain the low-DNA status of such facilities, this laboratory should be irradiated with UV, the equipment should be treated with bleach (as far as possible) and personnel should use protective clothing and face shields. Personnel should not enter the low-DNA-status laboratory, after working in a normal molecular biology laboratory, without first changing clothes and showering. Whenever possible, consumables should be irradiated with UV light overnight before use. In general, disposable materials should be used. When chemicals are taken from stocks for preparing buffers, nothing should be returned to the stocks. Chemicals should be poured or transferred using disposable equipment. Aliquots should be prepared for water and all solutions. All tubes should be kept open for as short a time as possible.

Control amplifications. To detect sporadic or low-copy-number contamination, extraction and PCR controls must be performed in each experiment. Although carrier effects may limit their efficacy, a recent study questioned the existence of a strong carrier effect[6]. All results of contamination should be reported, and inclusion of positive controls from modern source DNA should generally be avoided, as they provide a contamination risk.

Appropriate molecular behavior. For most ancient DNA amplifications, both quantity of PCR products and success rate should be inversely correlated with product size[5]. Large products (>500) are unusual, although under exceptional conditions amplification of products of length 1.6 kb[7] to 2 kb[8] have been reported. If single-copy nuclear or pathogen DNA is detected, mitochondrial DNA (mtDNA) should also be obtainable, as multicopy DNA sequences are more likely to survive in ancient specimens owing to their larger initial copy number. Generally, the maximum amplification length for nuclear DNA should be shorter than that for mtDNA.

Biochemical preservation. Several studies have shown that there is no simple correlation between sample age and preservation of the DNA[9,10]. These studies revealed that not only the age of a fossil, but also burial temperature and environmental factors, are important for DNA preservation[11,12]. Indirect evidence for possible DNA survival, to support claims of retrieval of authentic ancient DNA from a specimen, can be provided by assessing the total amount, composition and relative extent of diagenetic change in amino acids and other organic molecules[10,13].

Copy number of the template. Often it is useful to assess the copy number of the DNA target using quantitative PCR. When the number of starting molecules is low (<1,000), it may be impossible to exclude the possibility of sporadic contamination, especially in studies on ancient human DNA. Moreover, if amplifications start from very few or even single DNA strands[14], PCR errors, due to template damage, will become incorporated into all molecules of the final PCR products[15]. Furthermore, if nuclear DNA is analyzed, one of the two possibly different alleles will fail to be revealed if the amplification starts from a single molecule.

Reproducibility. As a standard, results should be reproducible from an ancient DNA extract of a specimen, and each nucleotide position should be deduced from at least two independent primary amplifications. This is necessary to avoid incorrect sequence determination due to amplification starting from a small number of damaged templates (see above). In certain cases[16] it may also be necessary to determine sequences from independent extracts from a specimen. If possible, different overlapping primer pairs should be used to confirm that different PCR products are derived from the same DNA source and to increase the chance of detecting nuclear insertions of mitochondrial DNA (NUMTS) or contamination by PCR products.

The strongest proof to exclude intra-laboratory contamination is the extraction and sequencing of separate samples of a specimen in independent laboratories. However, it should be emphasized that this measure will not detect contamination already present on a specimen. Independent replication is particularly important in cases of novel and/or unexpected results.

Cloning of PCR products. In many cases it is recommended that researchers verify sequences obtained by direct sequencing of PCR products by cloning amplified products, both to determine the ratio of endogenous to exogenous sequences and damage-induced errors and to detect the presence of NUMTS. At least three clones per PCR product should be sequenced.

Associated remains. In studies, for example, of human remains, where contamination is a serious issue, evidence that DNA of similar quantity and quality survived in associated faunal material is supporting evidence for the authenticity of the results obtained

for the human remains. Human DNA is prevalent as a source of contamination because of its ubiquity in the environment. In fact, human DNA sequences can readily be retrieved from most ancient animal remains. Ancient animals pose less of a problem, as they are often distinct from, but still similar to, extant species from the same taxonomic group.

Advantage of multiplex PCR

Compared to standard simplex PCR, the two-step multiplex PCR has a number of advantages. In many of the applications mentioned above, the amount of available extract is limited and allows only a few PCRs. The multiplex protocol allows several orders of magnitude more information to be deduced from limited template amounts. This is achieved by amplification of an almost unlimited number of fragments from the same amount of template DNA as would be used for a single standard PCR product. In fact, up to 1,200 loci have already been amplified from single-molecule samples of modern DNA using multiplex protocols[17] (P.H.D., unpublished results). Given an informative length of 50 bp shown to be deducible for nuclear DNA from Pleistocene permafrost samples[18] (H.R., N.R., T.S. and M.H., unpublished data) up to 60,000 bp of nuclear DNA could theoretically be amplified in a single reaction. Although low-number multiplex PCR has been used on ancient DNA before, for example to amplify a number of microsatellites[19,20], the two-step procedure has two important advantages. First, owing to its high specificity when nested primers are used, a very high number of loci can simultaneously and rapidly be amplified without the need of prior optimization of primer combinations. The high specificity is provided by nested primers, which enforce an additional level of sequence selection on the products of the first PCR. This design substantially reduces the occurrence of primer artifacts and false positives due to nonspecific amplification or amplification of contaminating DNA from species different to the target species. Second, only the shorter (nested) fragments from the second step are amplified to high copy number. Thus, the risk of cross-contaminating later samples with PCR products is reduced, as these shorter products cannot serve as templates for the first step.

Other potential applications of multiplex PCR

In addition to its use for amplification of long continuous stretches from fragmented DNA templates, there are a number of possible applications of this protocol, such as the parallel amplification of multiple non-overlapping short loci, for which a single set of multiplex primers can be used. The technique can also be adapted to single-nucleotide polymorphism (SNP) typing to infer, for example, positive selection, geographical distribution of selected alleles or changes in allele distribution over time and space. Recently, it has been proposed that sequencing of a complete genome from an extinct species to low coverage using the 454 technique[21] will provide a large number of SNPs for extinct species[22]. However, owing to template damage in ancient DNA, many of these SNPs will represent false positives. The two-step multiplex protocol provides a method for rapid evaluation of such SNP candidates and subsequent parallel typing of hundreds to thousands of SNPs from a sample. Using complex sources of DNA, such as feces or sediment, it is also possible to amplify DNA from disparate species such as plants, animals and bacteria in parallel. Finally, this method has many applications in forensics, as it allows simultaneous analysis of multiple

informative markers such as mitochondrial DNA, sex-specific markers, microsatellites and SNPs from samples of limited quantity.

Primer design

Successful amplification depends critically on optimized primer sensitivity, specificity and homogeneity of annealing temperatures. For primer selection and optimization, the program Primer 3.0 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) is recommended. Intensive efforts towards optimal primer design will pay off with primers that can be used directly in multiplex PCR without prior optimization in standard simplex PCR. Primer dimers, which are often observed even in standard simplex PCRs, can be avoided through appropriate primer design. Moreover, the setup of the PCR in two steps, with just one primer pair used in the second step, results in a generally low prevalence of primer dimers. In cases when one primer is nested in the second step, we observed primer dimers for very few of more than 40 primer pairs used in the multiplex step.

The maximum fragment length amplified by any primer pair is limited by the degree of fragmentation of the ancient DNA template and has to be defined prior to multiplex primer design for each individual extract. This is done by designing a number of primer pairs that amplify non-overlapping fragments of increasing length. These primers are used to perform initial multiplex PCRs from the extract in question, prior to design of the complete multiplex sets A and B. When assessing the DNA preservation of a specimen, we typically apply three primer pairs to amplify fragments of 100 bp, 200 bp and 300 bp, including primers. In cases where excellent ancient DNA preservation may be expected, such as in the amplification of mitochondrial DNA from permafrost specimens, amplification of fragments up to 500 bp should be tested. It is possible to refine the maximum amplification length of a specimen using a second pre-run with primer pairs that are designed to amplify fragment lengths covering the range around the longest product from the first prescreening (e.g., if only the 100-bp fragment could be amplified in the first prescreening, then primer pairs generating fragments of about 75 bp, 100 bp, 125 bp and 150 bp can be tested).

Polymerases

Ideally, DNA polymerases should contain no DNA to prevent nonspecific amplification. We recommend using only hot-start enzymes to reduce mispriming and primer-primer amplification, which is particularly important for the multiplex step. Furthermore, polymerases should produce high yields of PCR product even under unfavorable conditions and from small amounts of template. We extensively tested different DNA polymerases and the following were among the best-performing polymerases: AmpliTaq Gold (from Applied Biosystems), Platinum Taq and Accuprime Taq (both from Invitrogen).

Limitations of multiplex PCR

An advantage of multiplex PCR is the simultaneous use of multiple primer pairs in one reaction. However, the number of primer pairs is limited by an increasing possibility of mispriming, although multiplex PCRs using up to 1,200 primer pairs and one copy of template have been found to work successfully with modern DNA[17] (P.H.D., unpublished results).

There are other general limitations that are not specific to the described method but rather are relevant for all PCR-based approaches used for ancient DNA amplification. For example, determination of sequences from species, for which contamination with modern DNA is likely, limits the use of this and any other methods described so far. Also, it is difficult to verify the authenticity of ancient sequences from taxa where the number and sequences of extant species are poorly characterized or where DNA from closely related species is abundant in the environment. For this reason, studies of pathogenic bacteria are hampered by the fact that many bacterial pathogens are closely related to environmental bacteria, and this problem is not ameliorated using the multiplex method.

MATERIALS

REAGENTS

- AmpliTaq Gold kit (Applied Biosystems, cat. no. N808-0241), including 5 U μl⁻¹ enzyme, GeneAmp 10× PCR Buffer II (100 mM Tris-HCl, pH 8.3, 500 mM KCl) and 25 mM MgCl₂ stock solution
- 25 mM dNTP solution (Amersham Pharmacia, cat. no. 27-2035-02; 25 mM each)
- Primers (Invitrogen), 100 μM stock solution
- BSA (Sigma, cat. no. B4287), 10 mg ml⁻¹,
- PCR reagent-grade water (Sigma, cat. no. W1754), to be used for all solutions and dilutions

EQUIPMENT

- Three independent laboratories, with equipment and disposables: lab for work with ancient DNA, simplex setup lab or dedicated laminar-flow hood, and standard molecular biology lab (see EQUIPMENT SETUP)
- Thermal cycler (MJ Research, cat. no. PTC200)
- 96-well PCR plates (ABgene, cat. no. AB-0900)
- 8-strip PCR tubes (Eppendorf, cat. no. 0030124.359)
- Filter tips (various volume sizes; Greiner BIO-ONE), to be used for all pipetting (Steps 1–14)
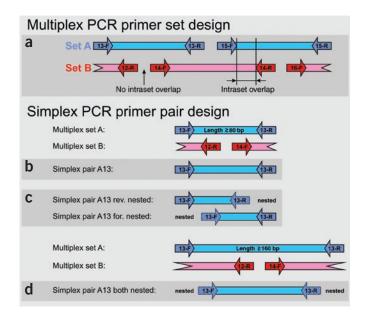
REAGENT SETUP

General properties of primers For designing primers, we recommend the use of the program Primer 3.0. We have successfully used primers with melting temperatures (T_m) ranging from 52 °C to 72 °C and with a maximum T_m difference within a multiplex set of 5 °C (for calculation of these temperatures, please refer to the Primer 3.0 manual). We use the default setting of 20%–80% for primer GC content but we extend the primer length setting to a range of 18–35 bp. For all other parameters, such as 3′ stability or maximum self-complementarity, we use the default conditions.

Primer design for multiplex PCR Start with designing primer set A (see **Fig. 1a**) by selecting primer pairs that amplify non-overlapping fragments (see **Fig. 1**, 'no intraset overlap'). Select a second set of primer pairs (primer set B) to amplify the intervening DNA sequences not covered by primer set A. Make sure that after the primers are subtracted, the deduced sequences from both sets give a continuous sequence (see **Fig. 1a**, 'interset overlap'). Primers for templates with high similarity to extant species with high contamination risk such as human should carry a mismatch to the extant-species sequence at the 3′ end[23] to avoid amplification of contaminating modern DNA. For nuclear sequences, paralogous sequences should be taken into account to decrease the risk of obtaining chimeras or multiple products.

Primer design for simplex PCR The first strategy (**Fig. 1b**) uses the same primer pairs in the second step, for the individual amplification of single fragments, as in the initial multiplex PCR. This approach is time-saving and less expensive but often leads to amplification of nonspecific sequences.

The second strategy (**Fig. 1c**) uses each individual multiplex primer, from each fragment, in combination with a corresponding nested primer. Thus, two

Figure 1 | Primer design strategy for multiplex amplification of ancient DNA. (**a**) The first-step multiplex PCR requires two sets of primers, A and B, that are used in independent multiplex PCRs. Together, primer sets A and B cover the sequence of interest. Within each set PCR fragments do not overlap. However, PCR fragments between set A and B show overlap of non-primer-determined sequences. (**b–d**) Second-step simplex PCRs are used for individual reamplification of each of the fragments obtained in the multiplex PCR. The strategy in **b** uses the same primer pairs used in the multiplex sets. In the strategy shown in **c**, two PCRs with nested primers on one end are used. Both strategies are suitable for amplification of fragmented DNA (>80 bp). In the strategy shown in **d**, completely nested primer pairs are used in the simplex PCRs. This two-sided nested variation can only be used for less fragmented DNA (>160 bp).

simplex PCRs are required for each multiplex PCR product to gain the complete sequence information of the initial multiplex amplification. It should be noted that the two simplex amplifications for each multiplex fragment, in this second strategy (**Fig. 1c**), do not represent independent amplifications according to ancient DNA guidelines, as they go back to the same initial multiplex product, which may have been derived from a single template molecule. Thus, a second multiplex PCR is necessary from which, in order to determine all sequence positions, two simplex amplifications again have to be done, resulting in a total of four simplex PCRs for every targeted multiplex fragment.

For the amplification and assembly of continuous sequences, the first two strategies (**Fig. 1b,c**) are suitable if template DNA fragments are longer than approximately 80 bp. If the DNA is less fragmented, so that the template molecules are longer than approximately 160 bp, the third strategy (**Fig. 1d**) can be used. In this scenario, both simplex primers are

nested (contrary to the situation with the second strategy, in which only one primer is nested) and only one simplex amplification is done per multiplex fragment. This requires substantial overlap between the initial multiplex fragments, and thus this strategy is restricted to better-preserved samples. This approach can be modified to use only a single nested primer in the simplex amplification; this requires less overall overlap, but still substantially more than in the second strategy. Independent of the strategy chosen, ensure that, after subtraction of the simplex primer sequences, fragments still have an interset overlap, especially if nested primers are used (see **Fig. 1a**, 'intraset overlap').

EQUIPMENT SETUP
Work with ancient DNA requires that three labs be maintained, with absolutely no equipment and consumables exchanged between them. (i) Ancient DNA lab plus equipment and disposables: PCR setup and addition of template DNA to the first-step multiplex PCR should be carried out in this facility. (ii) Simplex setup lab or laminar-flow hood plus equipment and disposables: this lab should be used for opening of the finished multiplex PCRs, dilution and addition of the template to the simplex PCR reaction tubes. No PCR products from the simplex PCRs, modern genomic DNA, plasmids, colony PCRs or other sources of high-copy-number DNA must be handled in this laboratory. (iii) Standard molecular biology lab plus equipment and disposables: the lab is used for gel electrophoresis, product cloning and sequencing.

PROCEDURE
Primer design for multiplex PCR
1| Design two sets of primer pairs for the initial multiplex step covering the sequence of interest, as outlined in the INTRODUCTION ('Primer Setup'). Primer sets A and B are used in independent multiplex amplifications. For a flow diagram outlining the procedure, see **Figure 2**.
? TROUBLESHOOTING

Primer design for simplex PCR
2| Choose the most suitable simplex amplification strategy for amplification of individual fragments, from three different approaches (**Fig. 1b–d**) as described in the INTRODUCTION. Design simplex PCR primers accordingly.
? TROUBLESHOOTING

Multiplex PCR
3| Separately set up the multiplex primer sets A and B:

Multiplex primer set	Volume (µl)	Concentration in multiplex primer set
All primers (100 µM)	2 each	1 µM each primer
Water	To 200	

▲ **CRITICAL STEP** Steps 3–7 must be, and Steps 9–12 may be, carried out in the ancient DNA laboratory.
▲ **CRITICAL STEP** This dilution scheme is applicable for up to 50 primer pairs. If more than 50 pairs are used, either the primer concentration within the mix must be decreased or more concentrated stocks are needed.
? TROUBLESHOOTING

4| Prepare the multiplex master mix for each multiplex PCR:

Multiplex master mix	Volume (μl)	Concentration in PCR
GeneAmp 10× PCR Buffer II	2	1×
BSA (10 mg ml^{-1})	2	1 mg ml^{-1}
MgCl$_2$ (25 mM)	3.2	4 mM
dNTP (25 mM each)	0.2	0.25 mM each
AmpliTaq Gold (5 U μl^{-1})	0.4	2 U
Water	4.2	

▲ **CRITICAL STEP** During the amplification process, the following controls must be performed (see **Fig. 2**): (i) an extraction control, treated identically to the extracts except that no sample is added, and (ii) two or more PCR water controls using water instead of sample (to detect sporadic or low-copy-number contamination). Setting up two or three identical multiplex PCRs is advisable when amplification of the target sequence can be expected, since, as in any ancient DNA study, each nucleotide position has to be determined from at least two independent primary PCRs. Working at this larger scale saves time in obtaining the necessary number of primary amplifications for an authentic ancient DNA reconstruction and prevents loss of ancient template DNA due to damage from freezing-thawing cycles and/or long-term storage.
? TROUBLESHOOTING

5| Combine the mixes prepared in Steps 3 and 4 separately for primer sets A and B for each multiplex PCR:

Multiplex master mix, complete	Volume (μl)	Concentration in PCR
Multiplex primer set from Step 3	3	0.15 μM each primer
Multiplex master mix from Step 4	12	

6| Distribute 15 μl of complete multiplex master mix for each primer set into PCR tubes (at least one extraction control, two PCR controls and one sample).

7| Add 5 μl of one of the following templates: DNA extract, blank extract or water.
▲ **CRITICAL STEP** Several methods have been published for extracting ancient DNA[24–26], but these are not the subject of this protocol. However, ancient DNA standards have to be applied at this very first step, too.

8| Put the multiplex tubes in a thermocycler outside the ancient DNA lab and amplify using the following durations and temperatures:

Step	Temperature	Duration
Initial denaturation	94 °C	9 min
30 steps of:		
Denaturation	94 °C	20 s
Annealing	Primer dependent	30 s
Elongation	72 °C	30 s
Final elongation	72 °C	4 min
Storage	4 °C	Until use

▲ **CRITICAL STEP** Times and temperatures have to be adapted to the specific primers and to the targeted lengths.
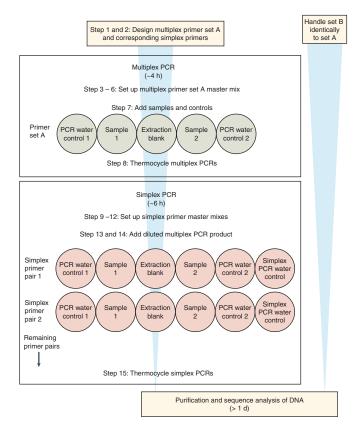**? TROUBLESHOOTING**

Figure 2 | Flow diagram for the steps of the detailed protocol, including chronology, estimated duration and references to the corresponding protocol steps.

Simplex PCR

9| Set up simplex primer pairs:

Simplex primer pair	Volume (µl)	Concentration in mix
Forward primer (100 µM)	20	10 µM
Reverse primer (100 µM)	20	10 µM
Water	160	

10| Prepare the simplex master mix for each simplex PCR:

Simplex master mix	Volume (µl)	Concentration in PCR
GeneAmp 10× PCR Buffer II	2	1×
BSA (10 mg ml^{-1})	2	1 mg ml^{-1}
MgCl$_2$ (25 mM)	3.2	4 mM
dNTP (25 mM each)	0.2	0.25 mM each
AmpliTaq Gold (5 U µl^{-1})	0.05	0.25 U
Water	4.55	

▲ **CRITICAL STEP** Simplex PCRs must be performed from all multiplex PCR reactions, including all controls. All controls must be performed with all primer pairs used in simplex PCRs. These controls include (i) a diluted extraction control from multiplex PCR to detect contamination during extraction process, (ii) diluted PCR water controls from multiplex PCR (testing each control separately) to uncover PCR contamination during multiplex or simplex setup, and (iii) a simplex control using water instead of template from multiplex PCR to identify PCR contamination during simplex setup.

11| Combine the mixes prepared in Steps 9 and 10 for each simplex PCR:

Simplex master mix, complete	Volume (µl)	Concentration in PCR
Simplex primer pair from Step 9	3	1.5 µM each primer
Simplex master mix from Step 10	12	

12| Pipette 15 µl of complete simplex master mix into PCR plates and store at 4 °C until continuing with Step 14.
▲ **CRITICAL STEP** Store no longer than a few hours.

13| Dilute a portion of each completed multiplex PCR with water in a ratio of 1:20 to 1:100 (we routinely use a dilution of 1:25). From each multiplex PCR, a single dilution that provides enough template for the subsequent simplex PCRs is sufficient. If the complete multiplex reaction is diluted, a 1:20 dilution results in sufficient template for 80 simplex amplifications and a 1:100 dilution suffices for 400 simplex reactions. If template for more than 400 simplex amplifications is required, a higher dilution factor than 1:100 is necessary.
? TROUBLESHOOTING
▲ **CRITICAL STEP** To avoid contamination and cross-contamination, Steps 13 and 14 are carried out in the simplex PCR laboratory or a dedicated laminar-flow hood with equipment reserved only for these two steps.

14| Set up the simplex PCRs by adding 5 µl diluted multiplex PCR from Step 13 to the corresponding wells of the plate prepared in Step 12.

15| Place the tubes in a thermocycler and amplify using the cycling protocol described in Step 8.

Purification and sequencing
16| Final steps to obtain DNA sequences include PCR product purification (using either classical phenol/chloroform extraction or commercially available silica-based kits such as the QIAquick gel extraction or QIAquick PCR purification kits), Topo TA cloning and sequencing of the products. These standard procedures are not described, but all these steps should be carried out in a standard molecular biology lab with strict temporal and spatial separation from previous steps.
? TROUBLESHOOTING

● **TIMING**
Steps 3–8: about 4 h (1 multiplex set of ~50 primer pairs and 8 vials)
Steps 9–15: about 6 h (~50 simplex PCRs)
We find it more convenient to set up both the multiplex PCRs (Steps 3–6) and the simplex PCRs (Steps 9–12) in the clean room before adding the extracts and controls to the multiplex PCRs (Step 7). While the simplex PCRs are being set up, the multiplex PCRs can be stored in the refrigerator, as can the simplex PCRs during Steps 7, 8 and 13. Performing the steps in this order allows the setup of all PCRs using the low-DNA status of the ancient DNA facilities before handling any DNA. After adding extracts and controls to the multiplex PCRs, take all multiplex and simplex tubes and plates for each primer set (from Steps 7 and 11) out of the ancient DNA laboratory. Put the simplex reactions in the refrigerator and place the multiplex tubes in a thermocycler (Step 8).

? TROUBLESHOOTING
Troubleshooting is discussed in **Table 1**.

TABLE 1 | Troubleshooting table.

PROBLEM	MOST LIKELY CAUSE	SOLUTION
Step 16: amplification product too short	Combining multiplex primer set A and B (Step 1 or 3)	Separate primer pairs into two sets to prevent the amplification of short overlapping products. For example, combining set A and B (as in **Fig. 1a**) creates a primer pair 13F and 12R whose PCR product is much smaller than the intended product of 13F and 13R. This shorter product is preferentially amplified not only for kinetic reasons but also owing to the fact that shorter target DNA is present in higher template numbers due to ancient DNA fragmentation.
Step 16: no amplification product	Targeted PCR fragment too long (Step 1)	Redesign the multiplex primers, taking into account that the maximum fragment length amplified by any primer pair is limited by the degree of fragmentation of the ancient DNA template and has to be defined before multiplex primer design. Design a number of primer pairs that amplify non-overlapping fragments of increasing length. Use these primers for initial multiplex PCRs from the extract to determine the likely target fragment length. Consider these results for final multiplex PCR primer design. Generally, better results are obtained when the amplification length is below the maximum amplification length observed for a sample.
	Primers do not anneal (Step 1 or 2)	Primer design is based on the sequence of interest either from the same or closely related species. Sequence data with reasonably high sequence similarity are necessary to avoid failure of primer binding. Thus, it may be necessary to sequence suitable extant species prior to primer design. In the case of highly variable target sequences or the lack of sequence data from closely related species, it may be necessary to design degenerate primers.
	Unsuitable PCR conditions (Step 4 or 8)	For some targets, recommended buffers or *Taq* polymerases might not work (e.g., because of a high GC content). Therefore, further optimization is needed. Try PCR additives such as glycerol or DMSO; optimize annealing temperature and elongation time.

TABLE 1 | Troubleshooting table (continued).

Step 16: multiple products or smears	Degenerate primers are too nonspecific (Step 1 or 2)	Use less degenerate primers. The most degenerate primer pair we used so far within a multiplex primer set was composed of 320 different primers. However, to increase success rates, number and degeneracy of degenerate primers in a multiplex set should be kept as low as possible.
	Primers are too nonspecific (Step 1 or 2)	Redesign primers and avoid known repeat regions, sequences that are similar between the target species, and potentially contaminating DNA such as human or bacterial DNA or sequences similar between paralogs of large gene families. Primers should be chosen by use of both a mispriming library (as provided with Primer 3.0) and comparison of potential primers to public sequence data using Blast Search[27]. If working with nuclear targets, consider paralogs to avoid chimeras.
	Insufficient nesting (Step 2)	If strategy 1b was used, switch to another strategy and use at least one nested primer per simplex PCR. Try to nest simplex primers as much as possible by designing simplex primers that they have no overlap with the multiplex primers and by nesting both simplex primers of a fragment.
Step 16: product in PCR water control	Contamination in PCR reagents and/or during PCR setup (Steps 3–14)	Design primers in a way that amplification of human DNA is excluded. Sequence water control bands to ensure no cross contamination with previous PCR products has occurred. If this is the case, make sure that no member of the lab works in the clean room facilities the same day after having done work in the modern lab. Replace PCR reagents and run a small number of controls only to test for contamination before using extract again.
Step 16: product in extraction control	Contamination during extraction process	Design primers in a way that amplification of human DNA is excluded. Sequence contamination product to clarify its origin. If identical to target species, cross contamination either during extraction or with previous PCR products has occurred. In such case discard extracts and repeat extraction.
Step 16: product in simplex water control	Contamination in PCR reagents or during dilution (Step 13)	Repeat simplex PCRs. If contamination persists, perform a new multiplex PCR.
Step 16: Topo TA cloning failed	Unsuitable 5′ ends of one primer (Step 1 or 2)	Redesign primers so that they do not end with a 5′ thymine[28].

ANTICIPATED RESULTS

Step 1, primer design for multiplex PCR: to obtain the complete mitochondrial genome of a mammoth from a fossil bone, we designed multiplex primer pairs based on database sequences of African (GenBank ID: AJ224821) and Asian (ID: NC005129) elephants, mammoth (ID: DQ188829) and dugong (ID: AJ421723). This was done to obtain amplification products even if the sequence of an individual differed from the publicly available data. The resulting multiplex PCR products from set A and B range in size between 139 bp and 334 bp (including primer). **Table 2** shows examples of three randomly chosen primer pairs. Complete primer sets A and B include 39 primer pairs each.

The multiplex PCR setup (Steps 3–8) was carried out, with DNA extract from mammoth bone used as template, as described above including one extraction control, two PCR water controls and four samples (two with 1:5 and two with 1:10 diluted extract). Thermocycling was performed using the parameters stated above with an annealing temperature of 52 °C.

TABLE 2 | Examples of three randomly chosen primer pairs.

Primer name	Forward primer sequence 5′→3′	Primer name	Reverse primer sequence 5′→3′	Length of PCR product
B6-F	ATTTATTCCAGTACGAAAGGA	B6-R	TTACGAAGTTGTATRTAGCCTA	300 bp
A22-F	CTTAGGAGTCTACTTCACACTTCT	A22-R	GATCCTCATCAGTAAATDGATA	279 bp
A30-F	CCACTTTATTCACAGCYATCTG	A30-R	CAGTGAGTGCTARGCTGCC	294 bp

TABLE 3 | Primer combinations chosen for simplex PCRs.

Primer name	Forward primer sequence 5′→3′	Primer name	Reverse primer sequence 5′→3′	Length of PCR product
B6n-F*	ACAGAAAAAATGAGGCCA	B6-R	TTACGAAGTTGTATRTAGCCTA	280 bp
A22-F	CTTAGGAGTCTACTTCACACTTCT	A22n-R*	TGGATAAATAGAGGAATAGTCATAC	263 bp
A30-F	CCACTTTATTCACAGCYATCTG	A30n-R*	GGTATTGTTTTATAGAGTCCTCCTA	250 bp

Step 2, primer design for simplex PCR: the third strategy described (see **Fig. 1d**) was employed for simplex PCR design, but using only one nested primer per primer pair. Nesting was done either completely (no overlap between the original and nested primer) or partially (some overlap between the original and nested primer). **Table 3** shows the primer combinations chosen for simplex PCRs; nested primers are marked with asterisks (*).

The setup of simplex PCRs (Steps 9–15) was done as stated above. Each multiplex reaction was diluted 1:25 and separately amplified with all simplex primer pairs. Additionally, a simplex PCR water control was included for all simplex primer pairs. Thermocycling was performed as described above at an annealing temperature of 53 °C.

Step 16, Purification and sequencing: PCR products were visualized by electrophoresis on ethidium bromide–stained agarose gels as shown in **Figure 3**. The gel picture shows single bands of the expected size in the sample lanes and no amplification products in the control lanes. Moreover, no primer dimers or other secondary products are visible.
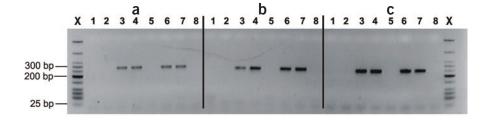

Figure 3 | Anticipated results. Agarose gel electrophoresis of three different simplex PCR setups (**a**–**c**) and the NEB Low Molecular Weight DNA ladder (X). For each setup, lanes are loaded as follows: 1 and 5, PCR water controls (carried along from multiplex PCR); 2, extraction control; 3 and 4, a mammoth extract dilution of 1:10; 6 and 7, a dilution of 1:5; and 8, a 1:5 dilution of the PCR water control for the simplex PCR. Different primer pairs (see ANTICIPATED RESULTS) are presented as **a** for B6, **b** for A22 and **c** for A30.

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