DNA analysis of the Metro Chinese Cemetery samples

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Report on the Extraction of DNA from the "Metro Chinese cemetery" samples

Summary

The eight samples tested for DNA analysis failed to exhibit any usable DNA that could be characterized for diagnostic substitutions. Minor adjustments to the protocols were made to increase the chances of success, however, these modifications also failed to produced usable results.

Background: Mitochondrial DNA (mtDNA) Properties and mtDNA Variation

MtDNA is an extra-nuclear genome found in the mitochondria of cells. Each mitochondrion contains one or two copies of the genome and each cell contains approximately 700 mitochondria (resulting in estimates of 1000-1500 copies of the genome per cell versus nuclear genes that are only found in only two copies per cell). The high copy number of the mtDNA genome partial compensates for the fact that DNA degrades with time, and has become the choice genetic marker for the investigation of DNA from skeletal remains.

Other unique characteristics of the genome further illustrate its utility in studies of biological relationships. Human mtDNA is strictly maternally inherited (Giles et al., 1980), reflecting only female movement/history, and is particularly useful in discerning ancestor-descendant relationships because it does not recombine during meiosis. Rapid evolution of the mitochondrial genome (Brown et al., 1979) allows one to use this molecule in studies of populations that share recent common ancestry. Lastly, the genome has been fully sequenced (Anderson et al., 1981) and its variation investigated in world-wide populations, having resulted in a large comparative database.

Contamination Control

As DNA extracted from ancient remains tends to be in low copy number and is highly degraded (Lindahl, 1993; Pääbo, 1990), ancient DNA (aDNA) extractions are highly susceptible to contamination originating from modern sources. Modern contaminating DNA can be in higher copy number and more fully intact than the endogenous aDNA and, thus, can compete with aDNA during polymerase chain reaction (PCR) amplification. Ancient DNA extractions can become contaminated via two sources: surface contamination of the bone or tooth from handling the material or later in DNA laboratory, during DNA extraction and analysis.

The former source of contamination can originate at any step of an aDNA study from the time of excavation of the remains to the time of DNA extraction. Modern contamination of the bone or tooth surface can arise from anyone who has had direct contact with the material, including the archaeologist that excavated the remains, any archaeological researchers that analyzed (e.g. cataloging, measuring) the remains, as well DNA

laboratory personnel. That a skeletal or tooth surface can become contaminated, it is particularly important to successfully remove the contamination before DNA extraction begins. To accomplish this goal, the remains were treated with a bleach solution to remove surface contamination (Kemp and Smith, 2006)

The latter source can originate from reagents, labware, PCR carryover, or DNA lab personnel. As such, procedures that reduce contamination were implemented, including: the use of DNA free lab-ware and reagents, all processing of ancient materials was performed in a laboratory physically separated from the one in which modern DNA is examined, and the use of negative controls in both DNA extraction and amplification to monitor contamination, if present (following the advice of Kelman and Kelman, 1999).

DNA Extraction

The samples were submerged in 6% sodium hypochlorite (bleach) for 15 min (Kemp and Smith, 2006). The bleach was removed by rinsing the sample with DNA free water (Gibco). The sample was then powdered mechanically and approximately 0.25 grams from each sample was submerged in 2 mL of molecular grade (DNA free) 0.5 M EDTA, pH 8.0 (Gibco) for 2 days. An extraction control, to which no sample was added, accompanied the extraction and was subject to all of the steps that follow. To the samples 3 mg of Proteinase K was added, and then was incubated at 65° C for 11 hours.

DNA was extracted from the digested samples using a three-step phenol/chloroform method: two extractions adding an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) to the EDTA, followed by one extraction with an equal volume of chloroform:isoamyl alcohol (24:1).

To aid in the removal of co-extracted polymerase chain reaction (PCR) inhibitors, DNA was precipitated out of solution with isopropanol (Hanni et al., 1995). This was performed by adding one half volume of room temperature 5 M ammonium acetate and, to this combined volume, one volume of room temperature absolute isopropanol, then storing the solution overnight at room temperature.

The tube was centrifuged for 30 min at 3100 rpm to pellet the DNA. The liquid was discarded and the tube was air-dried for 15 minutes. The pelleted DNA was washed with 1 mL of 80% ethanol by vortexing for about 30 seconds (making sure to dislodge the pellet from the side of the tube). Centrifuging the tube for 30 min at 3100 again pelleted the DNA. The ethanol was poured off and the tubes air-dried for 15 min. To further remove co-extracted PCR inhibitors, the pelleted DNA was re-suspended in 300 microliters of DNA-free ddH_2O and silica extracted (Höss and Pääbo, 1993) using the Wizard PCR Preps DNA Purification System (Promega), following the manufacturer's instructions (except that the DNA was finally eluted with 100 microliters of ddH_2O). The final solution was stored at $-20^{O}C$.

PCR Amplification and DNA sequencing

PCR amplification reactions contained 8.76 microliters of DNA-free ddH_2O (Gibco), 2.4 microliters of 2 mM dNTPs, 1.5 microliters of 10X PCR Buffer, 0.45 microliters $MgCl_2$ (50millimolar), 1.8 microliters of each primer (20 millimolar), 0.06 microliters of Platinum *Taq* (Invitrogen), and 1.5 microliters DNA template. Negative controls (PCR reactions to which no DNA template was added) accompanied every set of PCR reactions to monitor the presence of contaminating DNA. Coordinates, numbered according to the Cambridge Reference Sequence (Anderson et al., 1981), for primers used are found in Table 1.

PCR conditions were as follows: 94° C for 3 min, 40 cycles of 15 second holds at 94° C, 55° C, and 72° C, followed by a final three minute extension period at 74° C. 5-6 microliters of the amplicon was electrophoresed on a 6% polyacrylamide gel. The gel was stained with ethidium bromide and visualized under UV light, to confirm the successful amplification of the amplicon.

Results

The eight samples failed to produce amplification for the PCR primer pairs used. Three amplicons were visualized but were from a PCR reaction where the negative control also contained an amplicon. These amplicons were sequenced using the protocol below.

Sequencing Hypervariable Region

The sequencing PCR reactions differ from the one used to amplifying the regions containing the haplogroup-defining polymorphisms. PCR amplification reactions contained 17.52 microliters of DNA-free ddH_2O (Gibco), 4.8 microliters of 2 millimolar dNTPs, 3.0 microliters of 10X PCR Buffer, 0.9 microliters $MgCl_2$, 3.6 microliters of each primer (20 millimolar), 0.06 microliters of Platinum *Taq* (Invitrogen), and 3.0 microliters of DNA template. The primers used are listed in Table 1. For the amplification of sequencing products "touchdown" PCR was utilized (Don et al., 1991). The PCR conditions were as follows: 5 min hold 94° C, 60 cycles of 15 second holds 94° C, the annealing temperature (which was decreased 0.1^{O} C after each successive round of amplification), and 72° C, followed by a final three minute extension period at 74° C. The starting annealing temperatures are listed in Table 1. About 3-4 microliters of the amplicons were run on 6% polyacrylamide gels, stained with ethidium bromide and visualized with UV, as described above, to confirm success in amplification. The remaining PCR product was ExoI digested by added to the amplified product 60 microliters of ddH_2O, 2 microliters of the ExoI buffer, and 0.2 microliters of the ExoI enzyme. This reaction was incubated at 37° C for 90 min and then at 80° C for 20 min to denature the ExoI. The ExoI digested DNA was filtered through a Millipore plate, and re-suspended in 25 microliters ddH_2O. This product was directly sequencing on an ABI 3100 DNA sequencer.

Results of Sequencing Hypervariable Region I

The DNA sequence from the amplicons matched the DNA sequence identified in the negative control. This sequence was for HVI-3 PCR primers and contained a T position at nucleotide position 16256, relative to the Cambridge Reference Sequence (Anderson et al., 1981). This is indicative of contamination, as the mtDNA of one of the researchers in the laboratory also contains a T at position 16256.

Conclusions

The lack of amplification of DNA extracted from the samples, indicate that DNA is not well enough preserved to characterize the DNA sequence of the samples.

Works Cited

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Table 1. Primers used in this study.

Target Region	Primer	Coordinates*	Annealing Temperature
HVI-1	15986F	15986-16010	62° C[#]
	16153R	16132-16153	
HVI-2	16106F	16106-16126	62° C[#]
	16251R	16230-16251	
HVI-3	16190F	16190-16209	58° C[#]
	16355R	16331-16355	
HVI-4	16232F	16232-16249	58° C[#]
	16404R	16383-16404	

* Coordinates, numbered according to the Cambridge Reference Sequence (Anderson et al., 1981).
[#] Touch-down PCR used, decreasing the annealing temperature 0.1^{O} C after each cycle.