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Brief Communication

Forensic strategy to ensure the quality of sequencing data of mitochondrial DNA in highly degraded samples

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ABSTRACT

Mitochondrial DNA (mtDNA) is widely used for DNA analysis of highly degraded samples because of its polymorphic nature and high number of copies in a cell. However, as endogenous mtDNA in deteriorated samples is scarce and highly fragmented, it is not easy to obtain reliable data. In the current study, we report the risks of direct sequencing mtDNA in highly degraded material, and suggest a strategy to ensure the quality of sequencing data. It was observed that direct sequencing data of the hypervariable segment (HVS) 1 by using primer sets that generate an amplicon of 407 bp (long-primer sets) was different from results obtained by using newly designed primer sets that produce an amplicon of 120–139 bp (mini-primer sets). The data aligned with the results of mini-primer sets analysis in an amplicon length-dependent manner; the shorter the amplicon, the more evident the endogenous sequence became. Coding region analysis using multiplex amplified product-length polymorphisms revealed the incongruence of single nucleotide polymorphisms between the coding region and HVS 1 caused by contamination with exogenous mtDNA. Although the sequencing data obtained using long-primer sets turned out to be erroneous, it was unambiguous and reproducible. These findings suggest that PCR primers that produce amplicons shorter than those currently recognized should be used for mtDNA analysis in highly degraded samples. Haplogroup motif analysis of the coding region and HVS should also be performed to improve the reliability of forensic mtDNA data.

1. Introduction

In forensic DNA casework, short tandem repeat (STR) analysis is the first choice due to its high power of discrimination. However, STR analysis frequently does not work when DNA samples are highly degraded because there are only two copies of nuclear DNA in a cell. In such cases, mitochondrial DNA (mtDNA) analysis is mainly applied for forensic DNA testing because of its high number of copies in a cell. Because endogenous DNA in such samples would be fragmented, the primer sets used for PCR and direct sequencing are generally designed to obtain relatively short amplicons (approximately 200–400 bp) to increase the robustness of PCR [1–4]. Additionally, as reported by Deguilloux et al. [5], mtDNA analysis of badly deteriorated samples is exceptionally susceptible to contamination with exogenous mtDNA, because endogenous DNA in such specimens is frequently very scarce. Consequently, endogenous DNA molecules may be outnumbered by exogenous mtDNA in the analysis of highly degraded samples.

Generally, forensic mtDNA testing is performed by sequencing hypervariable segments (HVS) 1 and 2 [1–4]. Recently, coding region analysis is considered to be indispensable for reliable sequencing of highly degraded mtDNA because postmortem damage hotspots exist in mtDNA, and these correlate with nucleotide positions (nps) which are known to have high *in vivo* mutation rates in HVS 1 [6]. Further, the amount of postmortem damage in the coding region is significantly lower than in HVS 1 [6]. These findings suggest that when analyzing badly deteriorated forensic samples it should be confirmed if single nucleotide polymorphisms (SNPs) observed in the coding region and the HVS make phylogenetic sense by using haplogroup motif analysis [7].

In the previous studies, we successfully identified the genealogy of mtDNA in ancient specimens [8,9] by detecting the SNPs in the coding region using multiplex amplified product-length polymorphisms (APLP) [10]. Besides the merit of detecting multiple phylogenetically-important mutations simultaneously, this system is especially useful for analyzing highly degraded samples because of its small amplicon size (45–151 bp). In the current study, we report the risks of conventional forensic mtDNA analysis with highly degraded material, and suggest a strategy to ensure the quality of the sequencing data.

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2. Material and methods

2.1. Authentication methods

Intensive precautionary measures to avoid exogenous DNA contamination were carried out as described previously [8]. The DNA sample was collected only by the first author while wearing disposable laboratory equipment.

2.2. DNA samples

The right lower second molar of a Jomon skeleton (Miyatojima No. 16) excavated from Satohama shell midden, Miyagi, Japan was used as the DNA sample. This skeleton is considered to belong to the Final Jomon era, about 3000–2300 years ago.

2.3. DNA extraction

DNA was extracted from the tooth of the skeleton according to previously published protocols [9] with slight modifications. In brief, the tooth was dipped in 13% bleach solution for 15 min, rinsed with DNase-/RNase-free distilled water, and allowed to air-dry. The outer surface of the tooth was removed using a dental drill, and the tooth was again rinsed with DNase-/RNase-free distilled water and allowed to air-dry under UV irradiation. The sample was then encased in silicone rubber, and the dentin around the cavitas dentis and the dental pulp was powdered and removed through the cut plane of the root tip, as described by Gilbert et al. [6]. The powdered tooth was decalcified with 8 ml of 0.5 M EDTA (pH 8.0) at 56 °C for 2 days before DNA extraction.

To obtain DNA solution, a commercial DNA extraction kit (Kit A) was used, which is not intended to be used for human DNA extraction. As described in the next section 2.4 (SNPs typing and sequencing), the kit we used was contaminated by exogenous human DNA. The decalcified sample was lysed in 1000 μl of the buffer supplied with Kit A with 50 μl of 20 mg/ml Proteinase K at 56 °C overnight. DNA was extracted from the lysate using Kit A in accordance with the technical manual. One hundred microliters of DNA extract was obtained from the sample.

2.4. SNPs typing and sequencing

To confidently assign mtDNAs to the relevant haplogroups, 26 haplogroup-diagnostic SNPs, and a 9-bp repeat variation in the non-coding cytochrome oxidase II/tRNA^{Lys} intergenic region, were analyzed by multiplex amplified product-length polymorphisms (APLP) [10]. Additional SNPs were also analyzed for sub-haplogrouping of M7a, as described previously [9].

Segments of mtDNA that covered parts of the tRNA^{Pro} gene and HVS 1 (np: 15999–16366) were amplified and sequenced. Amplifications were carried out in a total reaction volume of 20 μl containing a 2-μl aliquot of DNA extract, 0.2 μM of each primer (Fig. 1 and Table 1), and reagents of a multiplex PCR kit (Qiagen, Hilden, Germany). The PCR conditions were: at 95 °C for 15 min; followed by 38 cycles at 94 °C for 20 s, 54 °C for 20 s, and 72 °C for 15 s.

When we confirmed the PCR products on 2% agarose gel, unambiguous band was observed in the negative extraction control lane as well as the sample lane. On the other hand, no band was observed in the negative PCR lane. Therefore, the Kit A we used was suspected to be contaminated by exogenous DNA. Mitochondrial DNA sequence of the negative extraction control was T16126C-T16224C-C16294T-C16296T-C16301T, relative to the revised Cambridge reference sequence [11]. By using BLAST search (http://blast.ddbj.nig.ac.jp/), this sequence is very similar to that of the individual whose accession number is JF707633 (only T16224C is not observed in this individual). Interestingly, JF707633 is assigned to haplogroup T2, which is commonly observed in Caucasians but is rare in East Asians including Japanese. We reported the result to the manufacturer, and it was consequently revealed that the Kit A we used was contaminated by exogenous human DNA during the manufacture process. For Kit A distributed after our indication, the problem of human DNA contamination has been solved by the manufacturer.

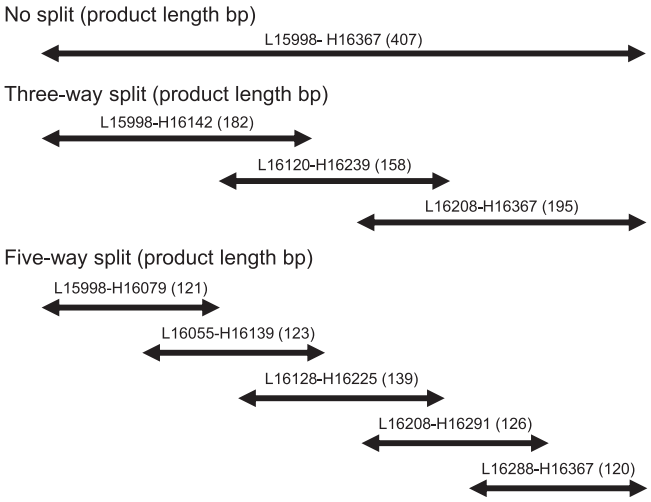

Fig. 1. Scheme of the split sequencing of mitochondrial DNA.

Table 1
Primers used for amplification and sequencing of mtDNA.

Primer	Sequence (5′–3′)[a]
L15998	CCA TTA gCA CCC AAA gCT A
L16055	gAA gCA gAT TTg ggT ACC AC
L16120	TTA CTg CCA gCC ACC ATg AA
L16128	CCA gCC ACC ATg AAT ATT gTA C
L16208	CCC CAT gCT TAC AAg CAA g
L16288	CCA CTA ggA TAC CAA CAA ACC T
H16079	TgT ACg AAA TAC ATA gCg gTT g
H16139	TAC TAC Agg Tgg TCA AgT AT
H16142	ATg TAC TAC Agg Tgg TCA Ag
H16225	gCA gTT gAT gTg TgA TAg TTg
H16239	Tgg CTT Tgg AgT TgC AgT Tg
H16291	ATg TAC TAT gTA CTg TTA Agg gTg g
H16367	ATC TgA ggg ggg TCA TCC AT

[a] Guanine is indicated with a lower case letter (g) to avoid confusion with cytosine (C).

Following PCR, the products were subjected to direct sequencing using the same primers and a BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on a 310 DNA Sequencer (Applied Biosystems) with Ridom TraceEdit software (Ridom, Würzburg, Germany). Both L and H strands were sequenced for all segments.

Each APLP and sequence analysis was performed at least twice to confirm reproducibility.

3. Results

By APLP analyses, the mtDNA being examined was assigned to haplogroup M7a2 (Fig. 2). From the phylogenetic tree [12], mtDNA assigned to this haplogroup is expected to have distinctive HVS1 sequence motif, T16140C-T16209C-C16223T (M7a2a) or T16093C-T16140C-T16209C-C16223T (M7a2a1). However, by using the primer set L15998 and H16367, mutations observed in segment