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Preliminary study on the necessity of decalcification in DNA extraction from ancient bones

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Abstract When extracting DNA from bone powder obtained by grinding ancient human bones, a process called decalcification was previously necessary. However, decalcification could potentially cause the loss of DNA contained in ancient human bones, which is present only in small amounts. In this study, we investigated whether decalcification is truly necessary when extracting DNA from ancient human bones and how much DNA is lost through decalcification. Here we show that decalcification caused the loss of 90% or more of the DNA from ancient human bones, and also reduced the recovery rate of short DNA fragments. Additionally, it was found that when the amount of bone powder used in the experiment was reduced to one-tenth and DNA was extracted without decalcification, DNA could be recovered without loss. Furthermore, no significant difference was observed in the human DNA content between cases with and without decalcification.
Key Words: Ancient DNA, decalcification, DNA extraction

Introduction

Human bones excavated from archaeological sites contain small amounts of DNA. Since the advent of next-generation sequencers, ancient genome research has been actively conducted to decode the entire genome and elucidate human evolution (e.g., Green *et al.*, 2010). Ancient human bones contain only small amounts of DNA, and most of the DNA is fragmented into pieces smaller than 100 bp. Therefore, the development of technologies to efficiently recover and analyze these DNAs is crucial. Traditionally, to increase the amount of DNA recovered, up to 500 mg of bone powder was scraped from parts such as a molar tooth or femur, from which DNA was then extracted. However, when using up to 500 mg of bone powder, the impurities in the bone (large amounts of inorganic substances such as calcium phosphate and calcium carbonate) act as PCR inhibitors, preventing successful DNA amplification. Therefore, a process called decalcification was necessary to remove these impurities. Decalcification involves soaking the bone powder in an EDTA solution, a chelating agent, to dissolve and remove the impurities. On the other hand, overseas research groups conducting ancient genome research have been minimizing destruction by extracting DNA from as little as 50 mg of samples (e.g., Dabney *et al.*, 2013). While using a small amount of bone powder allows DNA extraction without

Table 1. Sample information and summary of mitochondrial genome analysis

Individual ID	Bone	Extracted DNA	APLP	MtDNA reads	Average Duplicates (x)	Peak of length	Average Depth (x)	Coverage	Haplogrep	Qual	Haplogroup	Mismatch to mutation
906	Right petrous bone	Method A	B	45,762	0.82	53	191.32	1.000	B4f	0.8137	B4f	5.02%
		Dec1	—	52,053	0.24	45	185.48	1.000	B4f	0.8291	B4f	4.85%
		Dec2	—	8,971	0.68	40	29.23	0.999	B4f	0.7746	B4f	7.49%
		PEL	—	5,050	0.49	46	18.34	1.000	B4f	0.7698	B4f	4.63%
		Method D	—	67,027	0.54	42	233.13	1.000	B4f	0.8198	B4f	6.00%
914	Left petrous bone	Method A	B	26,775	0.86	59	121.31	1.000	B4e	0.7955	B4e	5.23%
		Dec1	—	22,486	0.16	45	78.69	1.000	B4e	0.7541	B4e	2.81%
		Dec2	—	3,909	0.66	38	12.30	1.000	B4e	0.7300	B4e	2.24%
		PEL	—	4,859	0.72	47	20.74	0.999	B4e	0.7421	B4e	2.32%
		Method D	—	48,211	0.6	40	153.51	1.000	B4e	0.7541	B4e	3.56%
915	Right petrous bone	Method A	not N	2,536	0.63	63	11.73	1.000	N	0.8211	B4e	29.37%
		Dec1	—	1913	0.14	45	6.54	0.974	B4e	0.6946	B4e	2.02%
		Dec2	—	542	0.44	42	1.74	0.781	R0	0.7278	B4e	28.57%
		PEL	—	426	0.44	42	1.45	0.702	B4e	0.6255	B4e	14.29%
		Method D	—	3,998	0.42	43	12.96	0.996	B4e	0.7220	B4e	6.67%

decalcification, the concern is that using only one-tenth of the bone powder may result in a smaller amount of recovered DNA.

However, it has been reported that decalcification of bone is a time-consuming step and possible cause for the loss of some ancient DNA (Fisher *et al.*, 1993). In addition, our recent reports said that Dabney's updated method tended to identify many SNPs or retrieve more extracted DNA in undecalcified samples than in decalcified samples (Fukumori *et al.*, 2024). This indicates that some of the ancient DNA contained in the bone powder is being dissolved during the decalcification process. Therefore, it is necessary to verify the need to decalcify a large amount of bone powder (up to 500 mg). In this study, we re-extracted DNA from the decalcification solution and remaining pellets obtained using the method we have previously employed, which involves decalcification twice followed by DNA recovery (hereinafter referred to as Method A), to evaluate the extent of DNA leaching caused by decalcification, i.e., the impact of decalcification. Additionally, we extracted DNA using Dabney's updated method (Method D), which does not involve decalcification, and compared the results with those of Method A. The verification was conducted using three human bones (Nos. 906, 914, and 915) excavated from the Naganaka 4 Site, a Gusuku-period to Ryukyu Kingdom Period (Middle Ages to Early Modern Period) archaeological site on Miyakojima Island (Table 1).

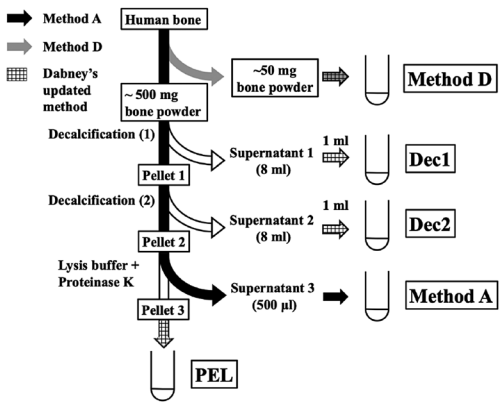

Fig. 1. Workflow of DNA extraction from bone powder.

Materials and Methods

The analysis used the petrous pyramid of the temporal bone, which houses the cochlea and semicircular canals. To extract DNA, we drilled a small hole in the anterior surface of the petrous pyramid to access the inner ear without damaging the bone's shape, and collected up to 500 mg of powder. DNA was extracted following the method of Takahashi *et al.* (2019) (Method A) (Figure 1, Supplementary Figure 1). Eight milliliters of EDTA (pH 8.0) was added to the sample powder, and decalcification was performed

overnight at 56℃ with slow rotation. The solution was then centrifuged at 8000 rpm for 1 minute to remove the supernatant, and another 8 mL of EDTA (pH 8.0) was added to the remaining pellet. Decalcification was performed again overnight at 56℃ with slow rotation. After decalcification, the solution was centrifuged at 8000 rpm for 1 minute to remove the supernatant, and DNA was extracted from the remaining pellet using NucreoSpin DNA Forensic (MACHEREY-NAGEL) according to the following procedure. First, 1000 µL of Lysis Buffer FOL (MACHEREY-NAGEL) and 50 µL of 20 mg/mL proteinase K were added to suspend the sample pellet, and the pellet was rotated at low speed at 56℃ overnight to dissolve the proteins. The dissolved solution was treated with 1500 µL of phenol-chloroform-isoamyl alcohol (25 : 24 : 1) and 1500 µL of chloroform to remove proteins, then collected only the supernatant using Maxtract High Density (QIAGEN). The DNA solution was obtained by following the protocol of the NucreoSpin DNA Forensic. For DNA elution, 130 μL of Buffer EB (QIAGEN) heated to 65℃ was used. Prior to the next-generation sequencing, mtDNA haplogroups of these samples were preliminarily determined by the Amplified Product-Length Polymorphisms (APLP) method described by Kakuda *et al.* (2016).

It is thought that DNA remains in the supernatant (supernatant 1 and 2) and pellet (pellet 3) left after decalcification of Method A. We extracted DNA from these using Dabney's updated method. One milliliter of each supernatant was used for DNA extraction. DNA extraction was manually done according to the instructions by Dabney *et al.* (2013) and Rohland *et al.* (2018), without any modification. The DNA template is normally eluted in a final volume of 100 µL elution buffer. In addition, DNA extraction using Dabney's updated method was also performed from 50 mg of bone powder from the same individual. The extraction solutions were Dec1, Dec2, PEL, and Method D, respectively. The distribution of the lengths of the extracted DNA fragments was confirmed using TapeStation High Sensitivity D5000.

To analyze mitochondrial genomes using a next-generation sequencer (NGS), a DNA library was prepared by modifying the "no uracil-DNA-glycosylase treatment" method of Rohland *et al.* (2015). DNA extraction and library preparation were performed in a clean room, and further work was performed in a laboratory outside the clean room. The DNA library was then amplified twice by PCR to ensure the amount of DNA required for target enrichment of mitochondrial DNA (mtDNA). The distribution of fragment lengths in the DNA library after the first PCR was confirmed using TapeStation High Sensitivity D1000. Target enrichment of human mtDNA was performed using MYbaits Expert Mito (H. sapiens Representative Global Diversity Panel) (Daicel Arbor Biosciences), hybridization was performed according to protocol except for 55℃. The enriched DNA library was sequenced using MiSeq/NexSeq1000 (Illumina) with 150 base paired-end conditions. Sequencing was also performed using NextSeq1000 to determine the percentage of human DNA.

Mapping and data filtering of the DNA sequences were performed using the method of Shinoda *et al.* (2017) with the following modifications: DeDup (version 0.11.3) was used to remove PCR duplicates. Deamination and fragment length of the mapped mtDNA sequences were checked using the MapDamage 2.0 software (Jónsson *et al.*, 2013). MtDNA haplogroups were estimated using the HaploGrep (version 2.1.14) software and visually by IGV software, and finally compared with the results of the APLP method. Following Shinoda *et al.* (2017), mitochondrial DNA variants were detected from the mpileup file. Each variant was classified as a SNP if it had a depth of 3 or higher (read at least three times) and if at least 70% of the reads contained a base that differed from the reference genome (rCRS). DNA contamination rates were estimated by checking the percentage of fragments matching the haplogroup (Kanzawa-Kiriyama *et al.* 2017) to confirm the reliability of the obtained sequence information. To determine

the percentage of human DNA in the library, we also sequenced DNA libraries that did not undergo mtDNA target enrichment. The NextSeq 1000 (Illumina) was used for sequencing. The DNA sequence reads were mapped to the human genome reference sequence (hg37d5), and the data were filtered using the method of Shinoda *et al.* (2019).

Results and Discussion

First, we checked whether DNA was present in the extract. In the APLP analysis using DNA extracted by Method A, bands are visible, but they are not clearly defined, indicating that the DNA length and yield are low (Figure 2). As a result, DNA was obtained in all cases, i.e. in Method A, Dec1, Dec2, PEL, and Method D (Figure 3). These results indicate that DNA derived from ancient bones is eluted even during the decalcification steps. However, it is unclear whether the eluted DNA is human DNA derived from human bones or whether it is mostly bacterial DNA that invaded after burial, with human DNA elution being limited. To determine the extent to which human DNA from ancient bones is present in the extract, we created a library of

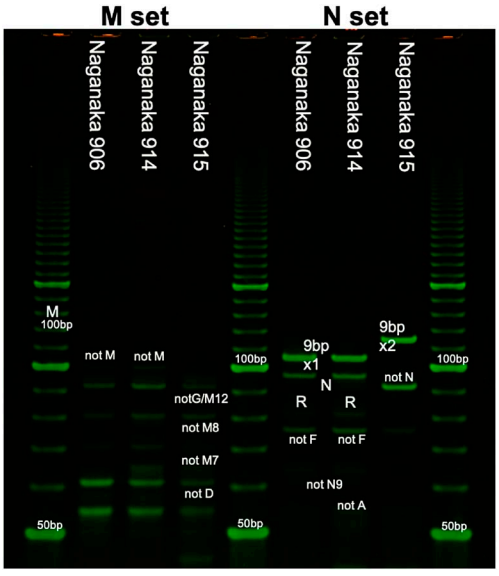

Fig. 2. APLP.

extracted DNA (Supplementary Figure 2) and performed target enrichment targeting human mtDNA to confirm the amount of ancient human mtDNA. As a result, DNA fragments mapped to the mitochondrial genome were present in all extracted DNA (Table 1). These DNA fragments exhibited deamination and fragmentation characteristic of ancient DNA (Figures 4 and 5). This indicates that DNA derived from ancient human bones is present in all extracts. Since DNA contamination from modern humans was also estimated to be low (Table 1), the obtained sequences can be considered to originate from ancient individuals. The estimated mtDNA haplogroups are also consistent for each individual. We also determined individual-specific variants (Table 2). The complete mitochondrial genome sequences determined from DNA obtained by either extraction method were fundamentally consistent. However, No. 915 yielded fewer reads, resulting in many uncalled variants. This No. 915 also showed inconsistencies with the APLP analysis results.

Next, we compared the results of mitochondrial genome analysis of the extracted DNA. First, we compared Method A and Method D. Although the amount of bone powder used was up to 500 mg in Method A and only one-tenth of that amount (–50 mg) in Method D, the number of mtDNA fragments obtained and the depth of the mitochondrial genome were similar (Table 1). This suggests that DNA was lost at some point in the process for Method A. The possible steps are 1st decalcification (Dec1), 2nd decalcification (Dec2), pellet 3 (PEL), and extraction using the NucreoSpin DNA Forensic kit. As shown in Figure 5, mtDNA sequences were obtained from Dec1, Dec2, and PEL, indicating that decalcification causes ancient human DNA to dissolve from bone powder, and even after extraction with lysis buffer + proteinase K, ancient human DNA remains undissolved in the pellet 3. The amount of DNA may also be quite large. By counting the PCR duplicates and unique reads in the sequence data obtained in this study, the total amount of unique mtDNA reads contained in the extraction

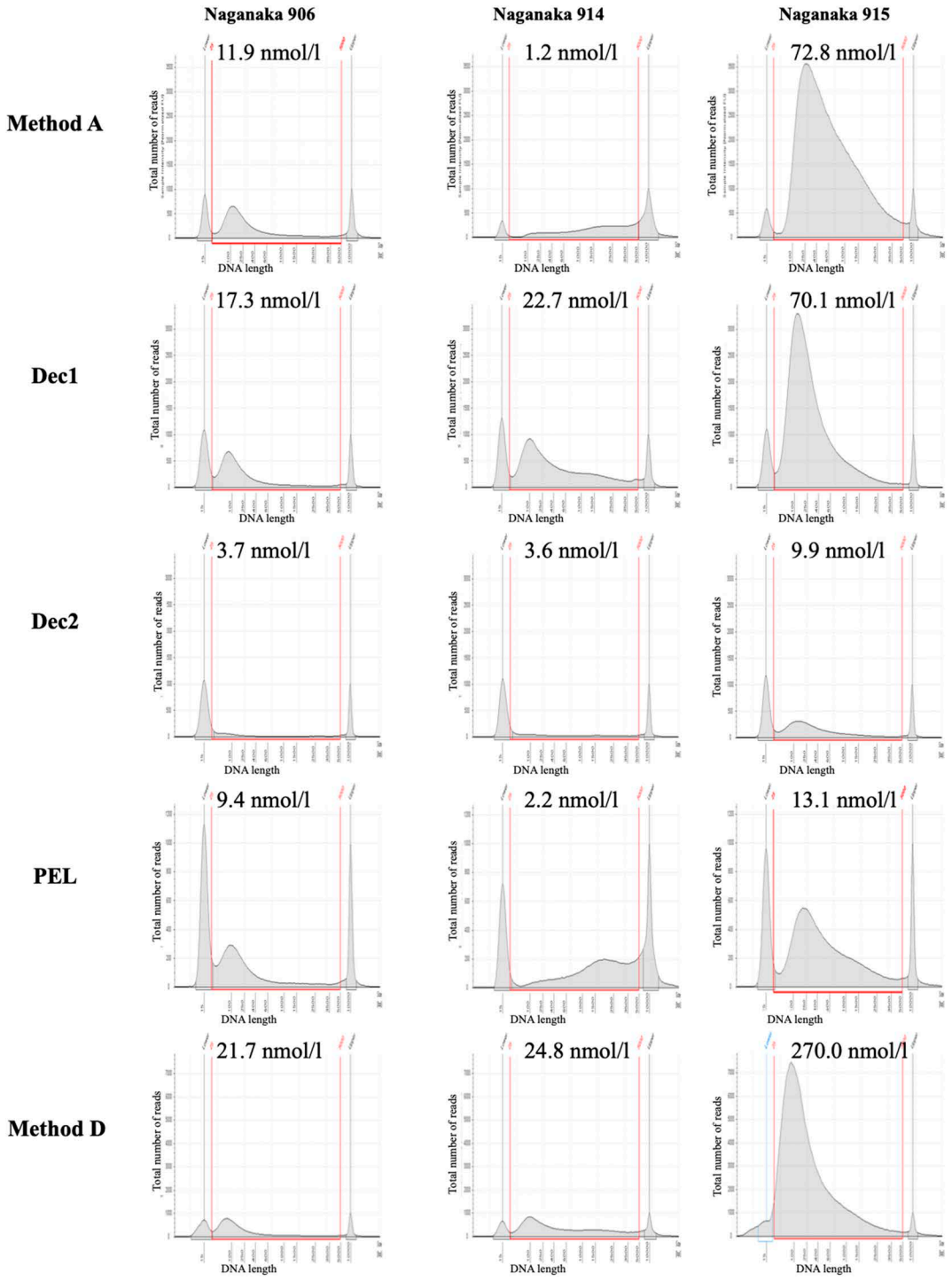

Fig. 3. Length distribution of extracted DNA.

solution can be roughly estimated (Figure 6). Looking at the curve in Figure 6, it appears that the number of unique reads will continue to increase for Dec1. Therefore, the number of

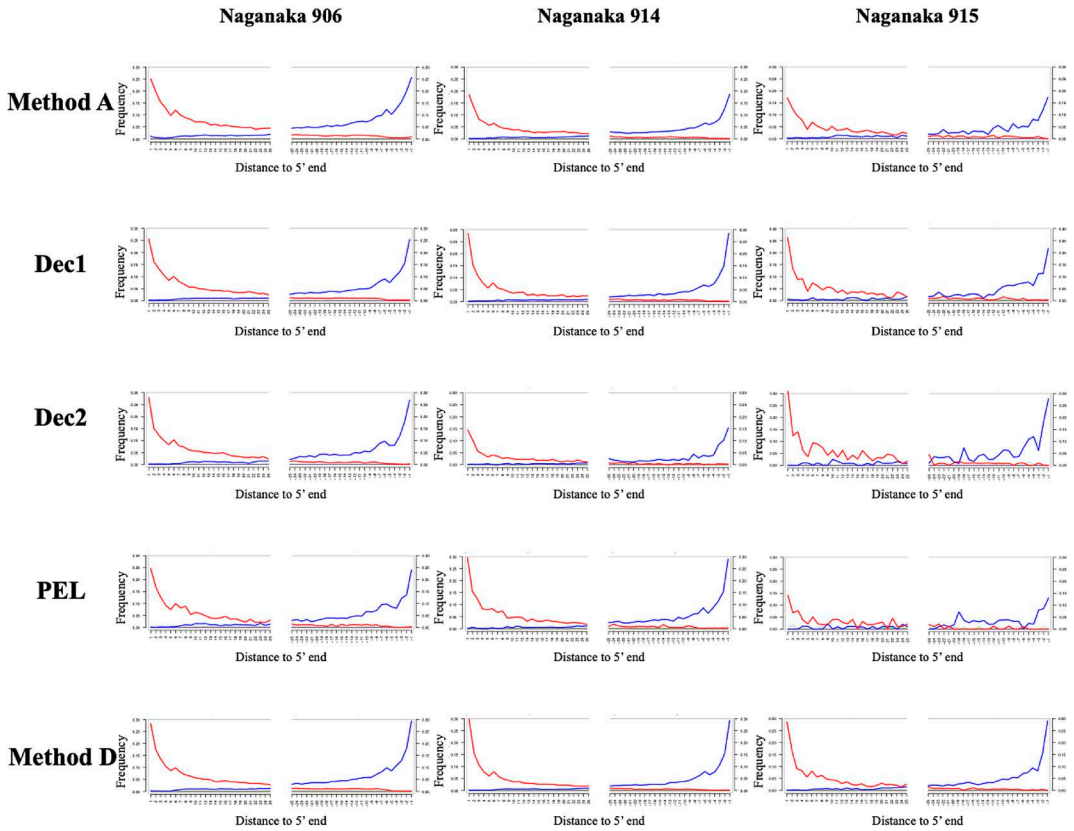

Fig. 4. Deamination.

unique reads obtained was large in Dec1 compared to Method A, while Dec2 was only a fraction of that obtained by Method A. Although caution is required when comparing the results due to differences in extraction methods, it is clear that Dec1 is significantly higher. In addition, we note that only 1 ml of the 8 ml decalcification solution was used in Dec1 and Dec2. In other words, the actual decalcification supernatant contains eight times the amount of mtDNA unique reads shown in Figure 6 in Dec1 and Dec2. These results show that 90% or more of the total DNA is lost through two rounds of decalcification.

The distribution of mtDNA fragment lengths in Method A shows that the peak length is approximately 10–20 bp longer than in Dec1, Dec2, PEL, and Method D extracted using Dabney's updated Method. This difference may be due to short DNA being lost during decalcification, or to a low recovery rate of short DNA using the NucreoSpin DNA Forensic kit in Method A. However, since short DNA is also recovered in PEL, short DNA is likely to be included in the lysis buffer + Proteinase K elution stage. This suggests that the longer peak may be due to the latter possibility. Additionally, the DNA extracted using Method A shows an overall tailing distribution toward longer lengths. This may be because decalcification causes short DNA fragments to dissolve more easily, leaving longer DNA fragments preferentially in the pellets (Pellets 1, 2, and 3) after the removal of the supernatant. In the case of human bone No. 914, longer DNA fragments were recovered from DNA extracted using PEL compared to Method D (Figure 5, Supplementary Figure 3). However, the same trend was not observed in No. 906. Fukumori *et al.* (2024) also noted that, when

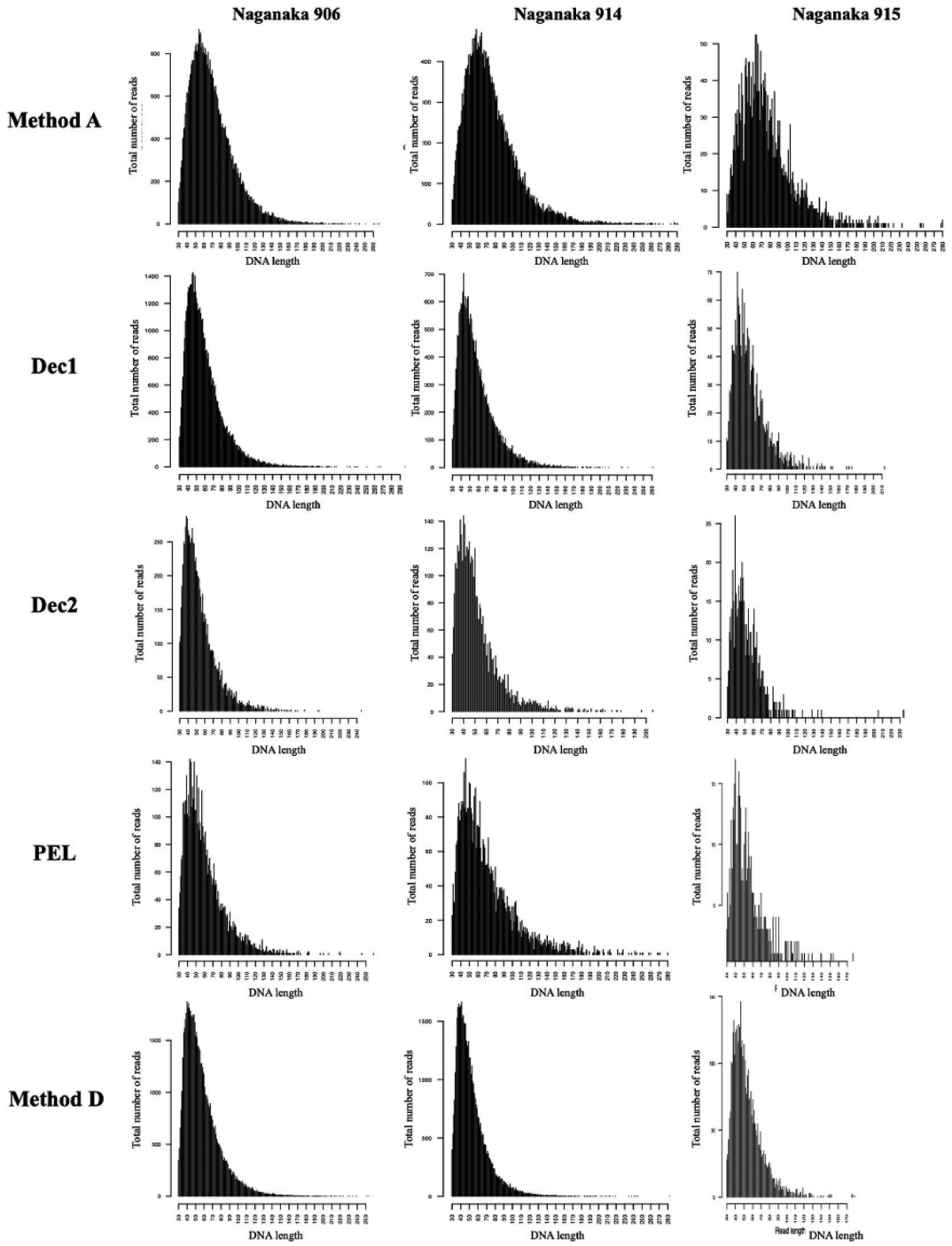

Fig. 5. Length distribution.

comparing the distribution of DNA fragment lengths, the peak of the DNA fragment length distribution was approximately 10 bp shorter in cases where decalcification was not performed than in cases where it was, supporting the results of this study.

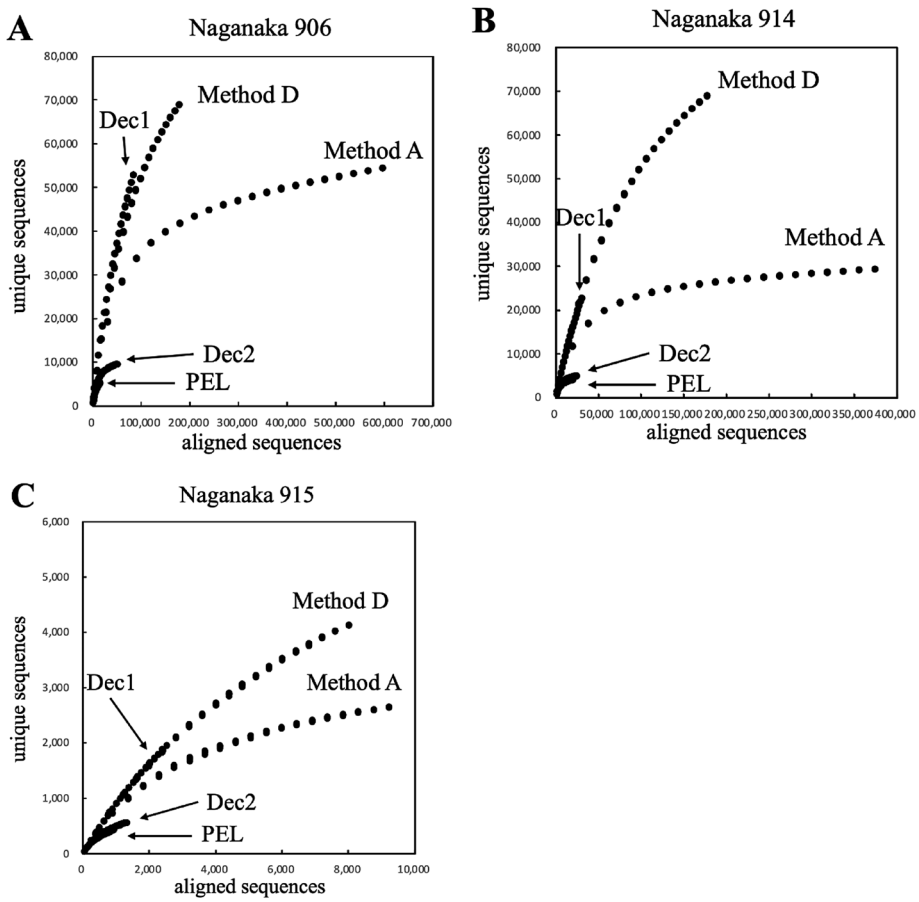

Fig. 6. Unique read.
A) Naganaka 4 No. 906, B) Naganaka 4 No. 914, C) Naganaka 4 No. 915

Based on the results above, Method A appears to result in the loss of a significant amount of ancient human DNA. However, the decalcification process in Method A may have advantages, such as eluting impurities and bacterial DNA. Does this mean that Method A yields a lower proportion of bacterial DNA and a higher proportion of human DNA than Method D? Unfortunately, the results did not meet expectations. As shown in Table 3, no differences in the three samples analyzed were observed between Methods A and D. Additionally, no differences were observed between Method A and Dec1 or Dec2. Since PEL has a low human DNA content, it is evident that the lysis buffer + proteinase K eluted most of the human DNA.

In summary, a significant amount of ancient human DNA was found to be lost due to decalcification. It was also suggested that shorter DNA fragments were lost during this process. If a sufficient amount of DNA can be obtained from a small piece of sample, Method D may be preferable to Method A for preserving samples. However, this conclusion is based on only three samples, so it is difficult to say that the DNA extraction method has been sufficiently evaluated. In the future, it will be necessary to verify the method using ancient samples under various conditions.

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Table 2. Individual-specific variants from each mitochondrial DNA haplogroup

Individual ID	Extracted DNA	Haplogroup	Individual-specific variants	Not called mitochondrial DNA hapogroup specific variants
906	Method A	B4f	C186T, A8158G, C13677T, G15884A, A16183C	—
	Dec1	B4f	C186T, A567ACCCCC, A8158G, C13677T, G15884A, A16183C	—
	Dec2	B4f	C186T, A8158G, C13677T, G15884A, A16183C	—
	PEL	B4f	C186T, A8158G, C13677T, G15884A, A16183C	16325
	Method D	B4f	C186T, A567ACCCCC, A8158G, C13677T, G15884A, A16183C	—
914	Method A	B4e	T196C, G4048A, C4926T, G5460A, A12358G, A14133G, C16223T, T16243C, C16291T	513
	Dec1	B4e	T196C, G4048A, C4926T, G5460A, A12358G, A14133G, A16182C, C16223T, T16243C, C16291T	513
	Dec2	B4e	G4048A, C4926T, C5499T, A12358G, A14133G, CAA16179C, A16183ACCC, C16223T, T16243C	513
	PEL	B4e	T196C, G4048A, C4926T, G5460A, A12358G, A14133G, A16183C, C16223T, T16243C, C16291T	513
	Method D	B4e	T196C, G4048A, C4926T, G5460A, A12358G, A14133G, C16223T, T16243C, C16291T	513
915	Method A	B4e	A750G G4048A C7028T G9921A G12771A A14133G C14766T A15326G C16223T	146, 185, 189, 195, 513, 10750, 15535, 16189, 16217
	Dec1	B4e	G4048A, C4926T, A14133G	513, 1438, 2706, 6614, 16189, 16217
	Dec2	B4e	A263G C14766T A15326G	—
	PEL	B4e	—	73, 146, 185, 189, 195, 263, 513, 827, 1438, 2706, 4769, 6614, 8860, 10750, 11719, 15535, 16189, 16217
	Method D	B4e	T196C, G4048A, C4926T, G5460A, A14133G, C16291T	513, 16189

* A302ACC, T310TC, AGC512A and T16519C were removed from the list.

Table 3. Human DNA frequency

Sample ID	Extracted DNA	Human DNA (%)
906	Method A	1.351%
	Dec1	1.664%
	Dec2	0.825%
	PEL	0.179%
	Method D	1.474%
914	Method A	0.644%
	Dec1	0.626%
	Dec2	0.478%
	PEL	0.129%
	Method D	0.416%
915	Method A	0.011%
	Dec1	0.029%
	Dec2	0.020%
	PEL	0.005%
	Method D	0.025%

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References

Dabney, J., Knapp, M., Glocke, I., Gansauge, M.-T., Weihmann, A., Nickel, B., Valdiosera, C., García, N., Pääbo, S., Arsuaga, J.-L., and Meyer, M. (2013) Complete mitochondrial genome sequence of a Middle Pleistocene cave bear reconstructed from ultrashort DNA fragments. *Proceedings of the National Academy of Sciences of the United States of America*, **110**: 15758–15763.

Fisher, D. L., Holland, M. M., Mitchell, L., Sledzik, P. S., Webb Wilcox, A., Wadhams, M., and Weedn, V. W. (1993) Extraction, evaluation, and amplification of DNA from decalcified and undecalcified United States Civil War. *Journal of Forensic Sciences*, *JFSCA*, **38**: 60–68.

Fukumori, K., Kameda, Y., Nagata, N., Matsumoto, T., Nam, K.-O., Tsujimura, I., and Kanzawa-Kiriyama, H. (2024) Evaluation of DNA extraction protocols from ancient bones and teeth: comparison of three extraction methods and the necessity of decalcification. *Bulletin of the National Museum of Nature and Science*, *Series D*, **50**: 1–19.

Green, R. E, Krause, J., Briggs, A. W., Maricic, T., Stenzel, U., Kircher, M., Patterson, N., Li, H., Zhai, W., Fritz, M. H., Hansen, N. F., Durand, E. Y., Malaspinas, A. S., Jensen, J. D., Marques-Bonet, T., Alkan, C.,

Prüfer, K., Meyer, M., Burbano, H. A., Good, J. M., Schultz, R., Aximu-Petri, A., Butthof, A., Höber, B., Höffner, B., Siegemund, M., Weihmann, A., Nusbaum, C., Lander, E. S., Russ, C., Novod, N., Affourtit, J., Egholm, M., Verna, C., Rudan, P., Brajkovic, D., Kucan, Ž., Gušic, I., Doronichev, V. B., Golovanova, L. V., Lalueza-Fox, C., de la Rasilla, M., Fortea, J., Rosas, A., Schmitz, R. W., Johnson, P. L. F., Eichler, E. E., Falush, D., Birney, E., Mullikin, J. C., Slatkin, M., Nielsen, R., Kelso, J., Lachmann, M., Reich, D., and Pääbo, S. (2010) A draft sequence of the Neandertal genome. *Science*, **328**: 710–722.

Jónsson, H., Ginolhac, A., Schubert, M., Johnson, P., and Orlando, L. (2013) mapDamage2.0: Fast approximate Bayesian estimates of ancient DNA damage parameters. *Bioinformatics*, **29**: 1682–1684.

Kakuda, T., Shojo, H., Tanaka, M., Nambiar, P., Minaguchi, K., Umetsu, K., and Adachi, N. (2016) Multiplex APLP system for high-resolution haplogrouping of extremely degraded East-Asian mitochondrial DNAs. *PLoS ONE*, **11**: e0158463.

Kanzawa-Kiriyama, H., Kryukov, K., Jinam, T. A., Hosomichi, K., Saso, A., Suwa, G., Ueda, S., Yoneda, M., Tajima, A., Shinoda, K. I., Inoue, I. and Saitou, N. (2017) A partial nuclear genome of the Jomons who lived 3000 years ago in Fukushima, Japan. *Journal of Human Genetics*, **62**: 213–221.

Rohland, N., Harney, E., Mallick, S., Nordenfelt, S. and Reich, D. (2015) Partial uracil-DNA-glycosylase treatment for screening of ancient DNA. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **370**: 20130624.

Rohland, N., Glockle, I., Aximu-Petri, A., and Meyer, M. (2018) Extraction of highly degraded DNA from ancient bones, teeth and sediments, high-throughput sequencing. *Nature Protocols*, **13**: 2447–2461.

Shinoda, K., Kanzawa-Kiriyama, H., Kakuda, T. and Adachi, N. (2017) Mitochondrial DNA analysis of human skeletal remains excavated from the Iwashita Cave and Shimo-motoyama rock shelter sites. *Anthropological Science (Japanese Series)*, **125**: 49–63.

Shinoda, K., Kanzawa-Kiriyama, H., Kakuda, T., and Adachi, N. (2019) Genetic characteristics of Yayoi people in Northwestern Kyushu—Ancient genome analysis of human bones excavated from Shimomotoyama Rock Shelter, Sasebo, Nagasaki Prefecture, Japan—. *Anthropological Science (Japanese Series)*, **127**: 25–43.

Takahashi, R., Koibuchi, R., Saeki, F., Hagihara, Y., Yoneda, M., Adachi, N., and Nara, T. (2019) Mitochondrial DNA analysis of the human skeletons excavated from the Shomyoji shell midden site, Kanagawa, Japan. *Anthropological Science*, **127**: 65–72.