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Evaluation of DNA extraction protocols from ancient bones and teeth: comparison of three extraction methods and the necessity of decalcification

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Abstract In general, ancient bones contain a small amount of DNA, and the remaining DNA is often highly fragmented. Because human bones consist large amount of inorganic substances which act as PCR inhibitors, it was often decalcified prior to extracting DNA. Although decalcification is beneficial for removing those inhibitors and bacterial DNA, it is time-consuming step and possible cause for the loss of some ancient DNA. Despite this, there is little known about the necessity of decalcification prior to DNA extraction. In addition, few studies have compared the elution efficiency of ancient human DNA between the extraction methods. In this study, we examined the effective DNA extraction method from degraded ancient bones and teeth with short DNA fragments. First, we tested the necessity of decalcification of the samples prior to DNA extraction. Second, we compared the size distribution of the extracted DNA and the human DNA among three extraction methods. We found that the decalcified samples tended to yield higher human DNA and many SNPs than the undecalcified samples when using Qiagen's QIAamp® DNA mini kit (hereafter called "QIAamp method"). On the other hand, the undecalcified samples identified many SNPs or large amount of the extracted DNA compared with the decalcified samples when using the Dabney's method using Roche column technology (hereafter called "Dabney's updated method") and the Promega's Maxwell® FSC DNA IQ™ Casework Kit (hereafter called "Maxwell method"). These results suggest that decalcification is not an essential step in the extraction of DNA, and instead, it may depend on the extraction methods and sample conditions. We also found that DNA fragments shorter than 100 bp were efficiently retrieved by the Dabney's updated method compared with the other two methods. Our study suggest that all three methods can be used to extract ancient DNA, but in cases where the sample is highly degraded and most of DNA is diminished to short fragments, the Dabney's updated method is the best extraction method.

Key Words: ancient DNA, decalcification, DNA extraction, size distribution

Introduction

In general, ancient bones contain a small amount of DNA, and the remaining DNA is often highly fragmented to less than 100 bp (Sawyer *et al.*, 2012). In earlier studies, mitochondrial DNA which exists in several hundred copies in cells, have been used for the ancient studies (Adachi *et al.*, 2013). The recent advance of next-generation sequencing has made it possible to obtain a great deal of genetic information not only from the mitochondrial genome but also from the nuclear genome of ancient humans (Rasmussen *et al.*, 2010). In addition, optimized DNA extraction and library preparation methods have been developed one after another, providing many important insights for anthropological study (Rohland and Hofreiter, 2007a; 2007b; Miura *et al.*, 2023). In ancient bones, it is difficult to get DNA because cell structures were rarely preserved, and chemical modification of nucleic acids was detected during many years of deposition. Besides, most genetic information of ancient humans resides in short DNA molecules, thus it is important to recover short DNA fragments during extraction.

Another difficulty of recovering DNA from human

bone is that it contains large amount of inorganic substances such as calcium phosphate and calcium carbonate, which have been identified as PCR inhibitors that interfere with DNA amplification. In addition, ancient bones and teeth excavated from soils and sediments often contain inhibitory substances such as environmental bacteria and virus. Moreover, in general, hard tissue samples such as bones and teeth are difficult to soluble in buffer, especially when large amount of samples are used. Thus, it was common practice to decalcify it with EDTA prior to DNA extraction (Krings *et al.*, 1997, Rohland and Hofreiter, 2007a; 2007b; Adachi *et al.*, 2018; Finaughty *et al.*, 2023). Indeed, several studies have reported that more DNA can be recovered from samples that have been decalcified than those that have not (Loreille *et al.*, 2007; Finaughty *et al.*, 2023). On the other hand, however, it has also been reported that some DNA is simultaneously removed when decalcification is performed (Fisher *et al.*, 1993). Despite the fact that decalcification is an important process in DNA extraction from ancient bones, the necessity has not been studied in detail.

After the decalcification procedure, ancient bone samples are processed extracting DNA. In earlier studies, the phenol/chloroform method has been used to extract DNA from ancient bones (Hänni *et al.*, 1995; Kalmár *et al.*, 2000). The most widespread extraction method in recent years is binding DNA to silica. The extraction method using silica is superior to the phenol/chloroform method in terms of high DNA yields and the removal of PCR inhibitors. Promega's extraction kit (Maxwell® FSC DNA IQ™ Casework Kit, hereafter called "Maxwell method") is based on silica-coated magnetic beads to bind the DNA followed by a number of washing steps, which was designed for highly calcified samples (Hakim *et al.*, 2019; Lisman *et al.*, 2023). The Qiagen's extraction kit (QIAamp® DNA mini kit, hereafter called "QIAamp method") is also an extraction method based on silica spin columns. Automated nucleic acid extraction systems are available for these two methods, allowing extraction with minimal risk of contamination and labor. However, these extraction methods may have low recovery rates of short DNA fragments, which are important for ancient human genome analysis. Subsequently, a silica-based DNA extraction technique have been introduced by Dabney *et al.* (2013), which enables the retrieval of DNA fragments shorter than 50 bp. Furthermore, a recently updated extraction method using a Roche's preassembled large-volume silica spin column (hereafter called "Dabney's updated method") can also efficiently recover very short DNA fragments (Rohland *et al.*, 2018). Thus, several methods for ancient DNA extraction exist, however, few studies have compared the elution efficiency of ancient human DNA between the extraction methods.

In this study, we evaluated several published extraction methods from ancient human bones and teeth. First, we tested the necessity of decalcification prior to extraction in each three methods: QIAamp method, Maxwell method, and Dabney's updated method. In QIAamp method, we compared the size distribution of the extracted DNA and the human DNA among the same samples with different decalcification treatments: 1) decalcification treatment, 2) undecalcification treatment, and 3) undecalcification but adding EDTA as a lysis buffer (hereafter called "undecalcification + EDTA treatment"). In the Dabney's updated method, we compared the size distribution of the extracted DNA and the human DNA among the same samples with presence and absence of decalcification. We also compared the size distribution of the extracted DNA among the decalcified and undecalcified samples using the Maxwell method. Second, we compared the size distribution of the extracted DNA and the human DNA in the three extraction methods to determine which method was more efficient in extracting fragmented ancient human DNA.

Materials and Methods

Decalcification and DNA extraction

The bone and teeth samples were collected from the remains of "highly likely to be Japanese" during World War II in southern areas. The outer surface of the bone samples was removed to a depth of 1 mm by using a dental drill, and were cut out a 15 mm piece. The teeth samples were used without surface treatments. The bone and teeth samples were cleaned by brushing and ultrasonic cleaning for 20 min. Samples were then immersed in 50% sodium hypochlorite solution (197-

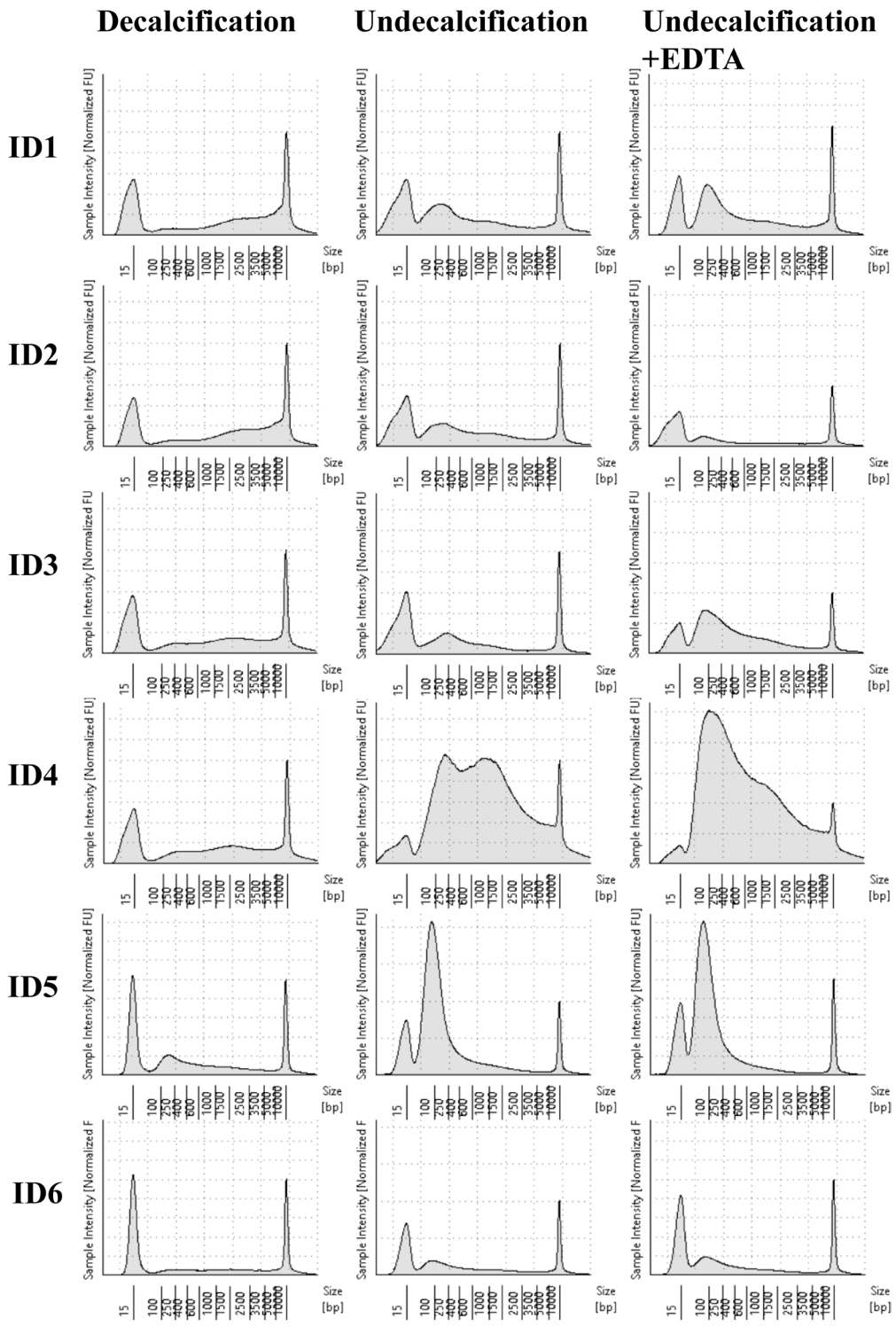

Figure 1. The size distribution of the extracted DNA based on the decalcification (left), the undecalcification (middle), and the undecalcification + EDTA (right) treatments using the QIAamp method. The lower marker (15 bp) and upper marker (10,000 bp) are the first and last peaks, respectively. The right panels show the same samples as in the left and the middle panels.

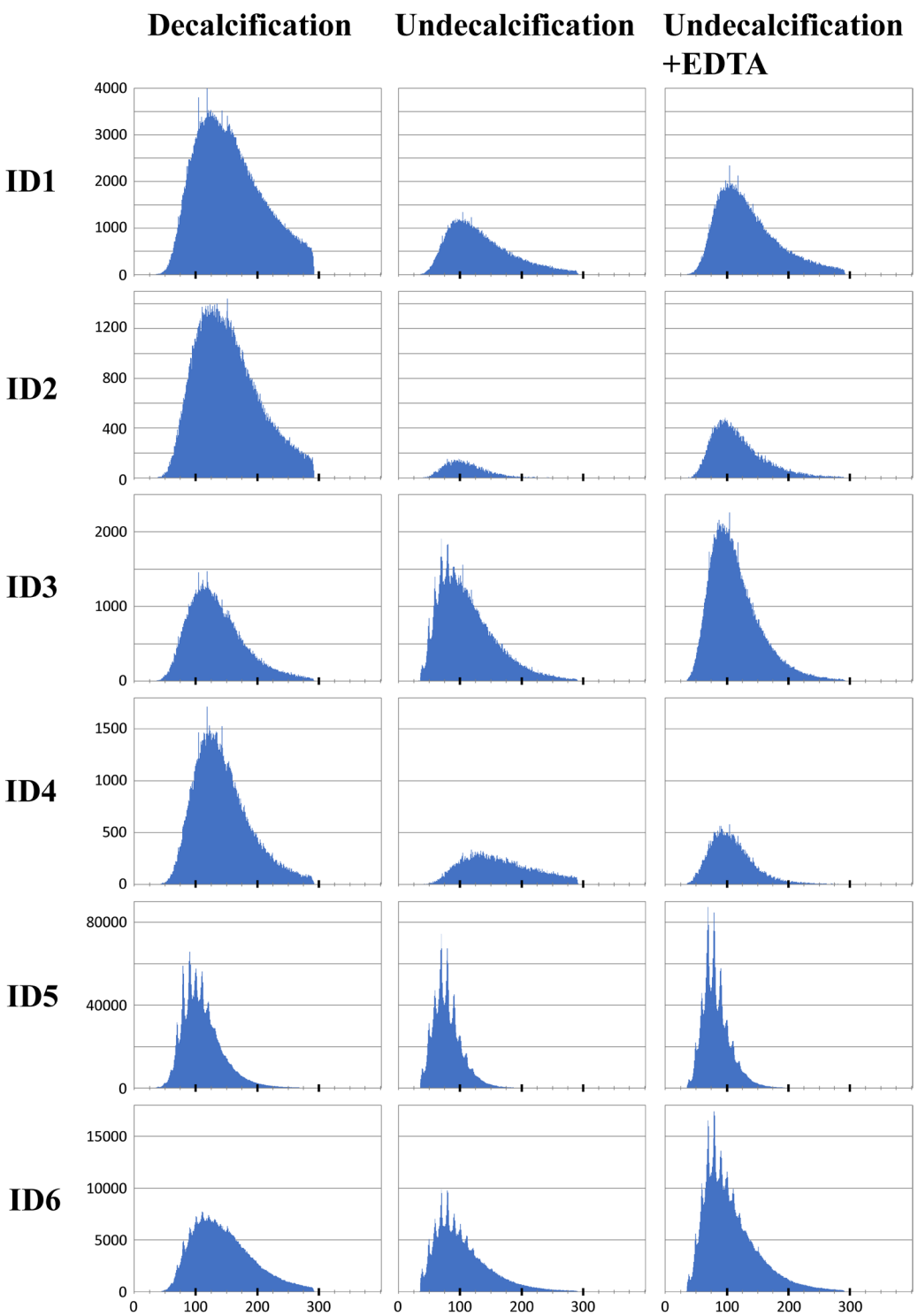

Figure 2. The size distribution of the human DNA based on the decalcification (left), the undecalcification (middle), and the undecalcification + EDTA (right) treatments using the QIAamp method. The right panels show the same samples as in the left and the middle panels. Note that the y-scale is not the same for each sample.

Table 1. The sample ID, the DNA extraction method, the mapped reads of human (%) and the number of identified SNPs under the presence/absence of decalcification. The high mapped reads of human (i.e., > 5%) were shown in bold.

ID	DNA extraction method	Mapped reads of human (%)	Number of identified SNPs		
			Decalcification	Undecalcification	Undecalcification + EDTA
1	QIAamp	1.33	254415	90313	134972
2	QIAamp	0.77	121185	8762	30573
3	QIAamp	0.8	86588	97246	118912
4	QIAamp	—	101217	31940	30200
5	QIAamp	**28.3**	743103	621208	671966
6	QIAamp	**8.14**	408408	315739	443056
7	Dabney's updated	0.65	1128186	1208327	—
8	Dabney's updated	<0.01	45863	51475	—
9	Dabney's updated	0.04	29677	32277	—
10	Dabney's updated	1.39	34343	46955	—
11	Dabney's updated	0.04	29577	26297	—
12	Dabney's updated	0.02	44429	39807	—
13	Dabney's updated	0.03	21662	23016	—
14	Dabney's updated	0.04	51370	59547	—

02206, FUJIFILM Wako) for 15 min and rinsed several times with tap water. Then, samples were irradiated with ultraviolet radiation using a UV crosslinker (CL-3000, 254 nm, Analytik jena) for 20 minutes (i.e., 10 minutes per side), followed by air dry for 48 h. Next, the samples were powdered using a mill (Multi-beads Shocker®, Yasui Kikai, Osaka, Japan). DNA of powdered samples were extracted using the following three methods.

1) QIAamp method

The first method used in the comparison was using the QIAcube connect instrument with the QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany). In the decalcification treatment, 100 mg of powdered samples were decalcified with 1.5 mL of 0.5 M EDTA (pH 8.0) at 56℃ overnight in a shaking incubator. The mixture was centrifuged at 2,000 *g* for 15 minutes, and the pellet was used as a decalcified sample. After the decalcification treatment, we followed the manufacturer's protocol. In the undecalcification treatment, samples were processed without EDTA procedure. In the undecalcification + EDTA treatment, samples were similarly processed as the undecalcification treatment but 0.5 M EDTA was added as a lysis buffer. The final elution step was done twice, with 30 µL and 20 µL of AE buffer added to the membrane and incubated, then centrifuged for a total elution volume of 50 µL.

2) Dabney's updated method

The second method used in the comparison was first described by Dabney *et al.* (2013) and then improved by Rohland *et al.* (2018) using the High pure viral nucleic acid large volume kit (Roche, Mannheim, Germany). In the decalcification treatment, 100 mg of powdered samples were decalcified with 1.5 mL of 0.5 M EDTA (pH 8.0) at 56℃ overnight in a shaking incubator. After the decalcification treatment, DNA extraction was manually done according to the instructions by Dabney *et al.* (2013) and Rohland *et al.* (2018), without any modification. In the undecalcification treatment, 50 mg of powdered samples were similarly processed without EDTA procedure. The final elution step was done twice, with 50 µL and 50 µL of TET added to the membrane and incubated, then centrifuged for a total elution volume of 100 µL.

3) Maxwell method

The third method used in the comparison was using the automate Maxwell® RSC instrument (Promega Corporation, Madison, USA) with the Maxwell® FSC DNA IQ™ Casework Kit (Promega Corporation, Madison, USA). In the decalcification treatment, 100 mg of powdered samples were decalcified with

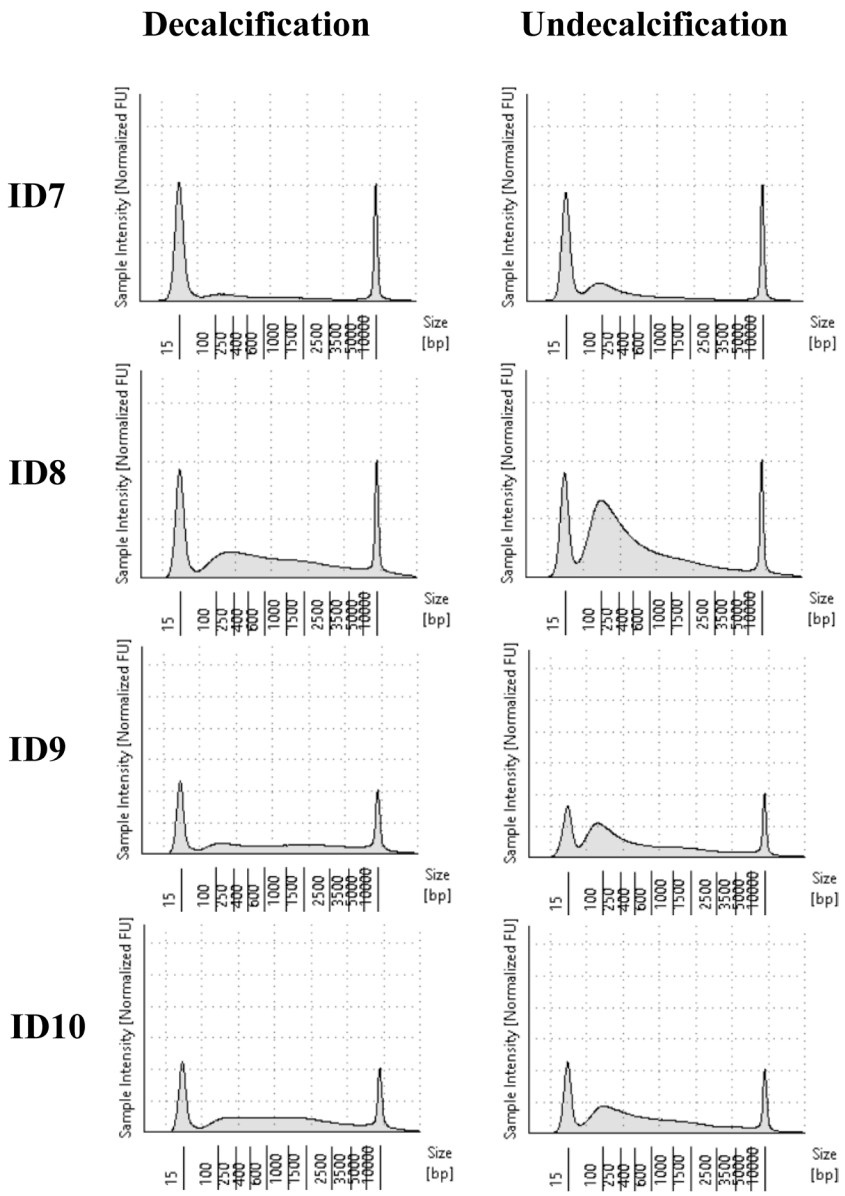

Figure 3A, B. The size distribution of the extracted DNA based on the decalcification (left) and the undecalcification (right) treatments using the Dabney's updated method. The lower marker (15 bp) and upper marker (10,000 bp) are the first and last peaks, respectively. The right panels show the same samples as in the left panels.

0.5 M EDTA (pH 8.0) at 56°C overnight in a shaking incubator. After the decalcification treatment, we followed the manufacturer's protocol. In the undecalcification treatment, samples were similarly processed without EDTA procedure. The DNA template was eluted in a final volume of 50 µL elution buffer.

The size distribution of the extracted DNA was measured using the 4150 TapeStation Instrument with a High Sensitivity D 5000 Screen Tape Kit (Agilent Technologies). After the DNA extraction, all samples were kept at 4°C until the genetic analysis.

Library preparation and Sequencing

15 µL of DNA was used for preparing DNA libraries

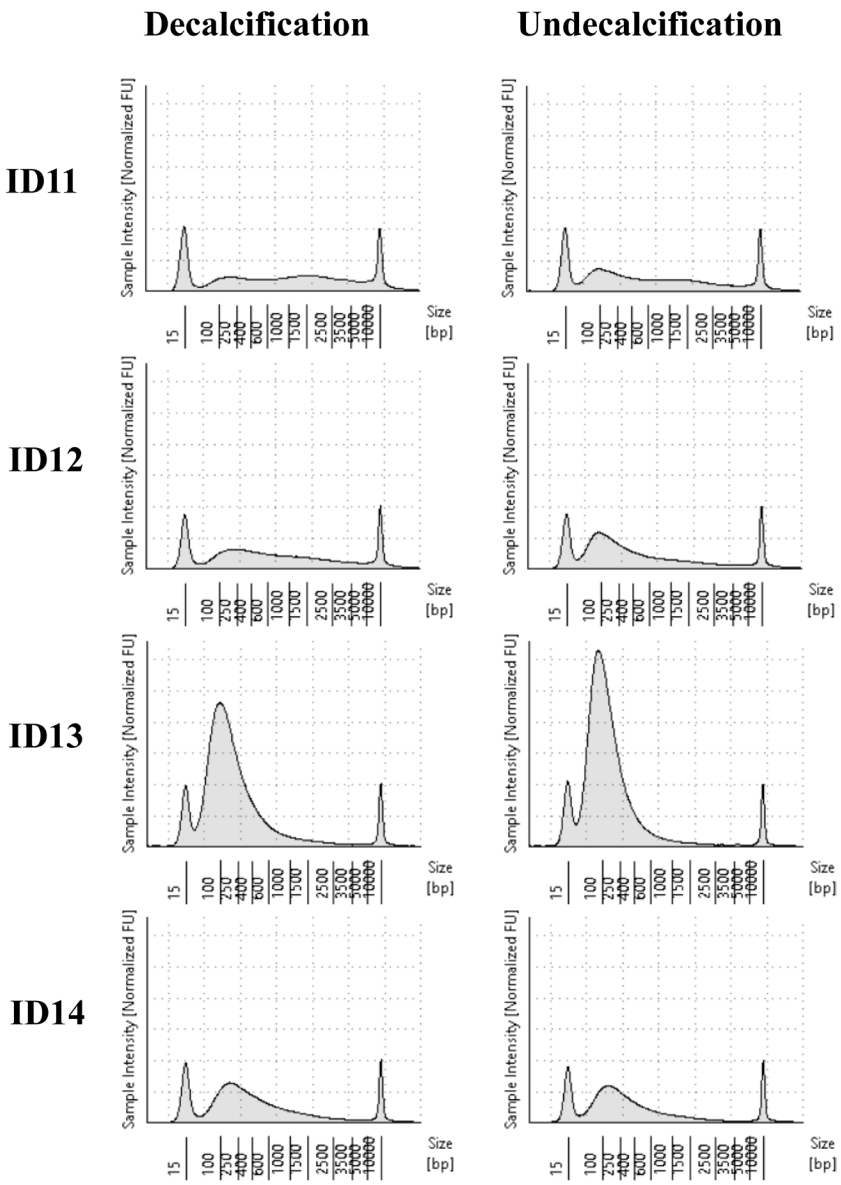

Figure 3A, B. Continued

using the NEBNext Ultra II DNA Library Prep Kit for Illumina. (New England Biolabs, Massachusetts, USA). For adapters, KAPA Universal Adapter (Roche) was diluted to 3 µM and added to the solution. NEBNext® Multiplex Oligos for Illumina® (96 Unique Dual Index Primer Pairs) was used for the index. DNA libraries were amplified using the NEBNext Q5U enzyme (New England Biolabs) under the conditions of the manufacturer's protocol. Eight libraries with different indices were pooled and target capture of mitochondrial and nuclear DNA was performed. Target capture of mitochondrial DNA was performed using Mybaits Mito Human (Arbor Bioscience, Michigan, USA) and target capture of nuclear DNA was performed using Mybaits Human Affinities Kit Prime Plus (Arbor Bioscience) or the Twist Diversity SNP and Ancient SNP panels (Twist bioscience, California, USA). Experiments were performed according to the manufacture's protocols. The prepared DNA libraries and target capture products were quantified using a

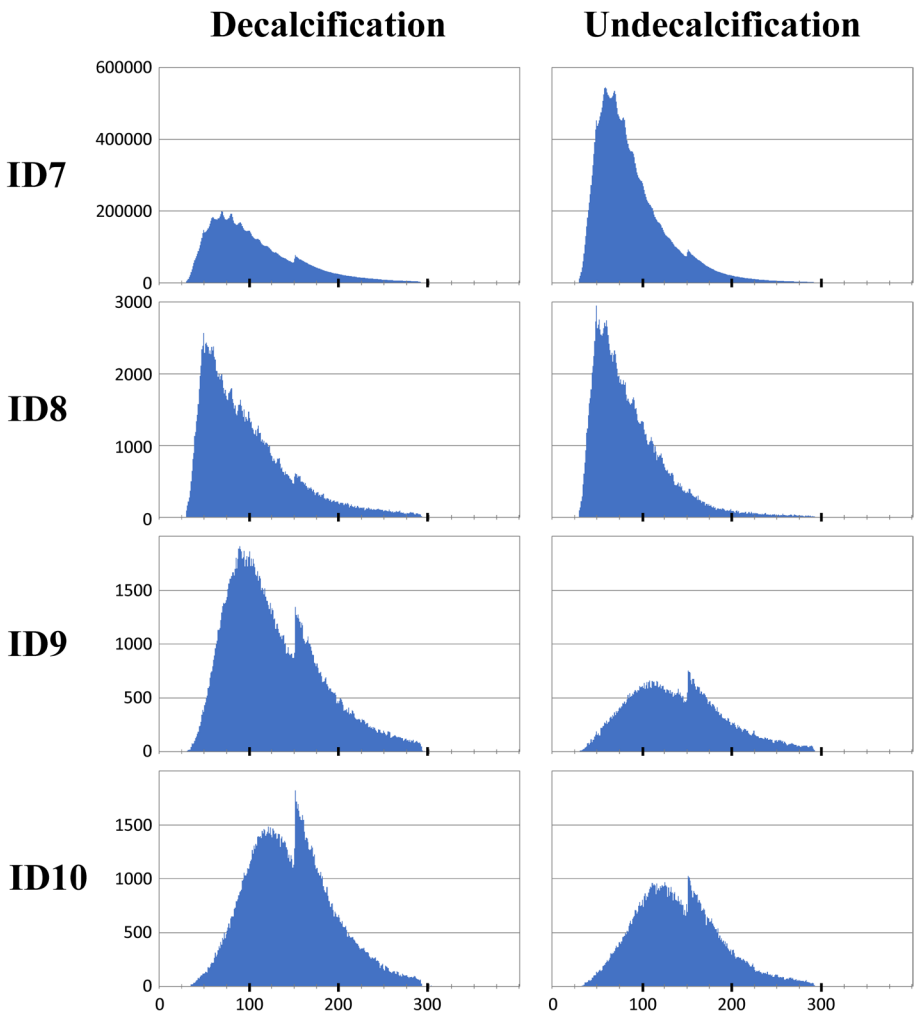

Figure 4A, B. The size distribution of the human DNA based on the decalcification (left) and the undecalcification (right) treatments using the Dabney's updated method. The right panels show the same samples as in the left panels. Note that the y-scale is not the same for each sample.

4150 TapeStation Instrument with a D1000 ScreenTape Kit. (Agilent Technologies, California, USA). Sequencing was performed on Illumina MiSeq and v2 Reagent 300-cycle kit at the National Museum of Nature and Science or on one lane of the Illumina NovaSeqX Plus 10B flow cell at Macrogen, Inc. (Tokyo, Japan).

Sequence analysis

Adapter sequences were trimmed with AdapterRemoval v2 (Schubert *et al.*, 2016). Sequences with less than 30 bp and low-quality bases on 5′/3′ ends were also eliminated with the software (—trims —trimqualities—minlength 30—minquality 20). After the adapter trimming, the remaining reads were mapped to the human reference genome (hs37d5) using BWA with "mem" algorism (Li and Durbin, 2009). After eliminating hard-clipped, soft-clipped and unmapped reads, the remaining reads were merged and sorted with SAMtools (Danecek *et al.*, 2021). After removing PCR duplicates using DeDup v0.12.8 (Peltzer *et al.*, 2016), reads with a mapping quality of 30 or more were extracted.

The authenticity of the obtained data was estimated by following Kanzawa-Kiriyama *et al.* (2019). The

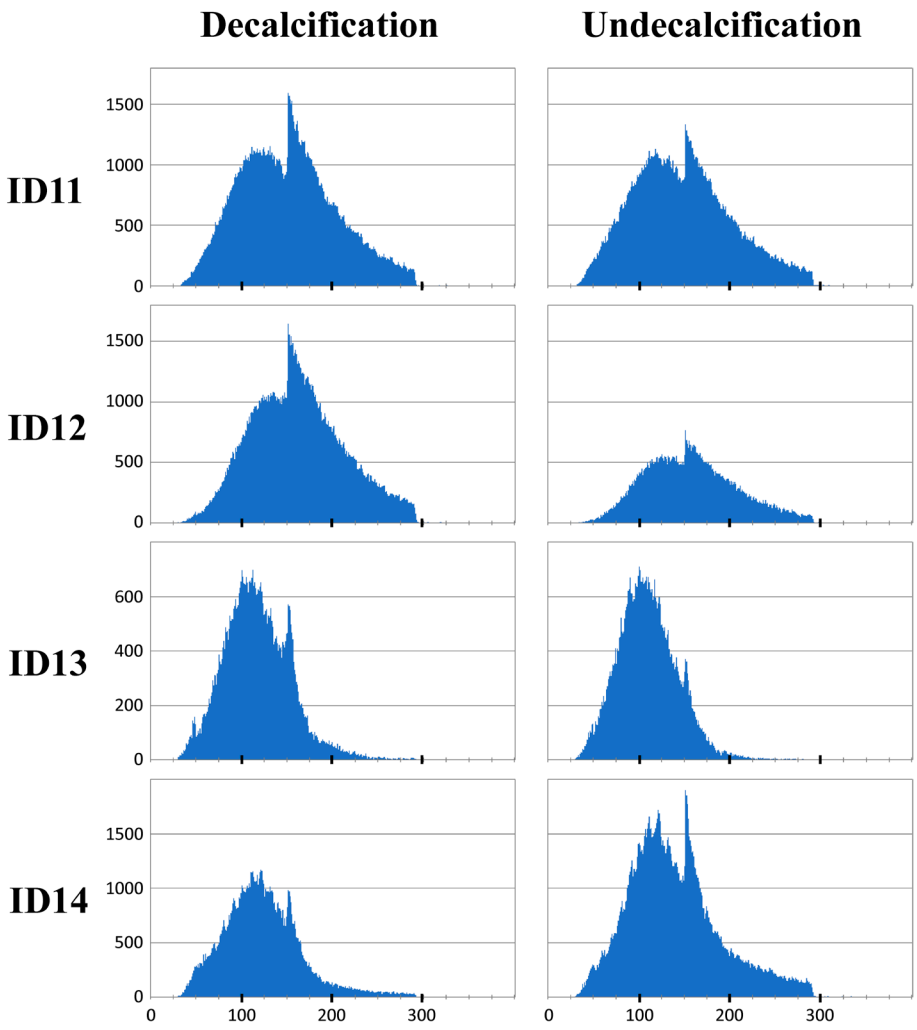

Figure 4A, B. Continued

length distribution file (output of mapDamage2.0; Jónsson *et al.*, 2013) was used to plot the histogram of unique human reads. The software READ (Relationship Estimation from Ancient DNA, Monroy Kuhn *et al.* (2018) was used to estimate genetic kin relationship between the libraries of the decalcification and the undecalcification treatment.

Ethics statement

This study was permitted by the Ethics Committee at the National Museum of Nature and Science.

Results

Comparison of decalcified and undecalcified samples

In QIAamp method, the volume zones of the extracted DNA for the undecalcification treatment and the undecalcification + EDTA treatment ranged from 100 to 400 bp, while those for the decalcification treatment ranged from 1500 to 2500 bp and an overall lower peak (Figure 1). The size distribution of human DNA mapped to the human reference genome after sequencing was 60-280 bp for all three decalcification treatments (Figure 2). DNA yields and the number of identified SNPs of the human DNA tended to increase in the decalcification treatment compared with the

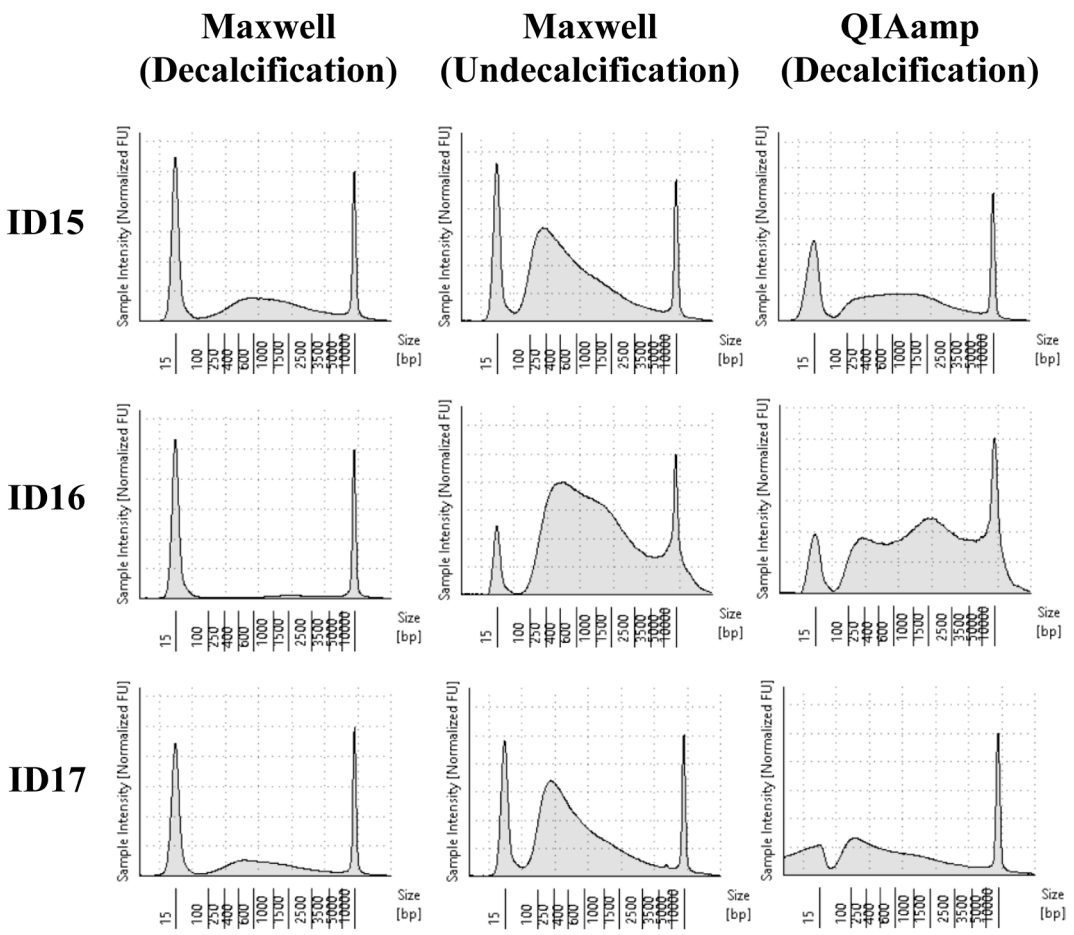

Figure 5. The size distribution of the extracted DNA based on the Maxwell (decalcification: left, undecalcification: middle), and the QIAamp (decalcification, right) methods. The lower marker (15 bp) and upper marker (10,000 bp) are the first and last peaks, respectively. The right panels show the same samples as in the left and the middle panels.

undecalcification and the undecalcification + EDTA treatments (Table 1, Figure 2). In two samples of high mapped reads of human (i.e., ID5 and ID6), DNA yields of the human DNA were higher than those of other four samples (i.e., ID1 to ID4, Table 1, Figure 2).

In the Dabney's updated method, the peak of extracted DNA fragments was short (approximately 100 bp) for both decalcification treatments (Figure 3A, B). Despite half the amount of sample was used, the yield of extracted DNA fragments (i.e., peak height) were slightly higher in the undecalcification treatment than in the decalcification treatment (Figure 3A, B). In addition, the peak position of the extracted DNA fragment was about 10 bp shorter in the undecalcification treatment than in the decalcification treatment (Figure 3A, B). DNA yields and the peak position of the human DNA were similar to both decalcification treatments (Figure 4A, B). The number of identified SNPs tended to be higher in the undecalcification treatment than in the decalcification treatment, despite half the amount of sample was used in the undecalcification treatment (Table 1).

In the Maxwell method, the volume zone of the extracted DNA fragments ranged from 250 to 600 bp, and DNA yields tended to be higher in the undecalcification treatment than in the decalcification treatment (Figure 5).

Based on these results, we used the size distribution

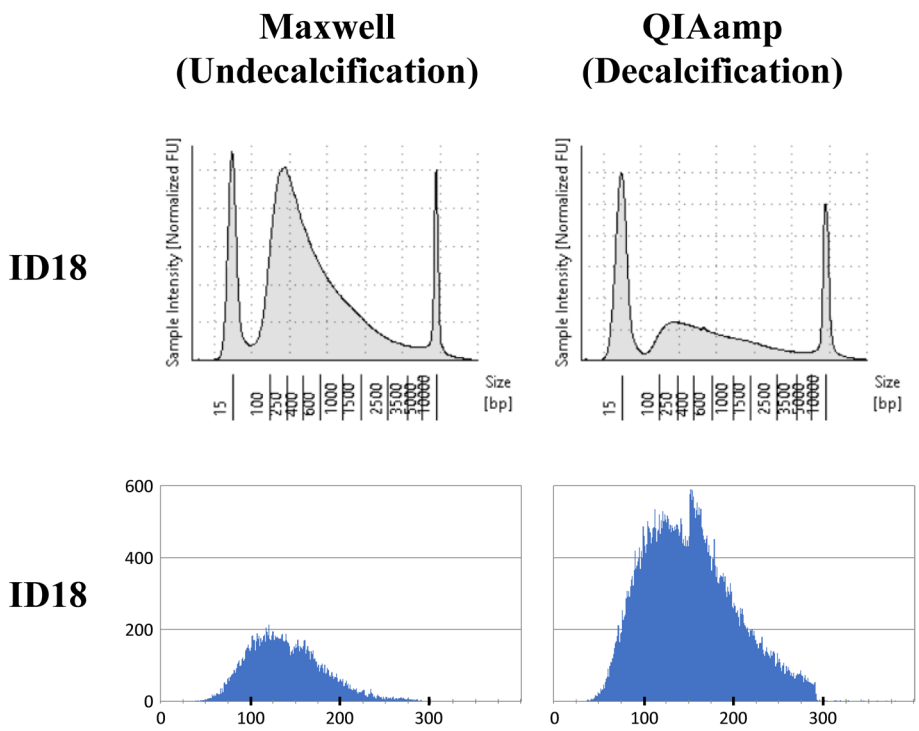

Figure 6. The size distribution of the extracted DNA (upper) and the human DNA (lower) based on the Maxwell (undecalcification, left), and the QIAamp (decalcification, right) methods. In the above two panels, the lower marker (15 bp) and upper marker (10,000 bp) are the first and last peaks, respectively. All four panels show the sample ID18.

data of the decalcification treatment in the QIAamp method, and those of the undecalcification treatments in the Dabney's updated method and the Maxwell method in the following comparison of extraction methods.

Comparison of three DNA extraction methods

Comparison of the Maxwell method (undecalcification treatment) and the QIAamp method (decalcification treatment) showed that the peak of extracted DNA fragment size was around 250 bp for both, with the Maxwell method yielded higher DNA concentrations than the QIAamp method (Figure 5, 6). When size distribution of the human DNA was compared between these two methods, the peak of DNA fragment size was around 120 bp for both, and the DNA yields were higher in the QIAamp method than in the Maxwell method (Figure 6).

Next, we compared the size distribution of the DNA extracts between the Maxwell method (undecalcification treatment) and the Dabney's updated method (undecalcification treatment). Overall DNA yields were higher in the Maxwell method than in the Dabney's updated method, but the peak position of DNA fragment size was shorter in the Dabney's updated method (110 bp) than in the Maxwell method (250 bp, Figure 7). Hence, the yield of extracted DNA fragments shorter than 100 bp increased in the Dabney's updated method compared to the Maxwell method (Figure 7). Because the amount of bone powder used in the Dabney's updated method (i.e., 50 mg) was half that of the Maxwell method (i.e., 100 mg), DNA yields of the Dabney's updated method were not inferior to those of the Maxwell method. The number of mapped reads of human for the sample ID19 were very low (i.e., 0.02), however, there were sufficient amount of the DNA yields shorter than 100 bp in the Dabney's updated method compared with the Maxwell method (Table 2, Figure 8).

When comparing the three extraction methods, the

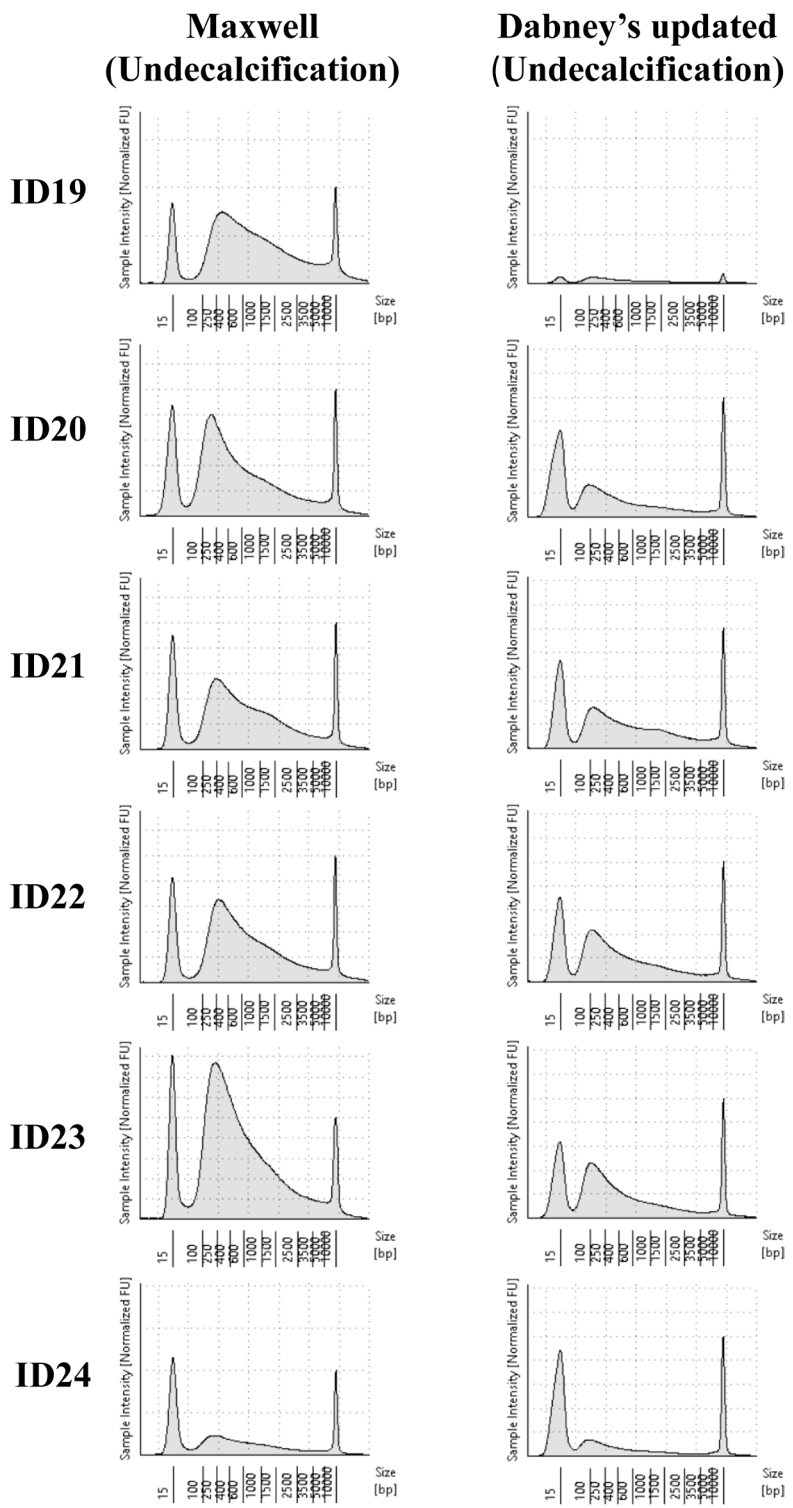

Figure 7. The size distribution of extracted DNA using the Maxwell method (left) and the Dabney's updated method (right). The lower marker (15 bp) and upper marker (10,000 bp) are the first and last peaks, respectively. The right panels show the same samples as in the left panels.

fragment size of the extracted DNA in the Dabney's updated method was short ranging from 100 to 250 bp, and this method efficiently retrieved high amount of very short DNA fragments compared to other two methods (Figure 9A, B). The same was true for human DNA, with the Dabney's updated method yielded higher amount of short human DNA fragments than the other two methods (Figure 10A, B). Since the average contamination rates of all samples were low at 3.12% and no differences were observed between three methods, the increase in yield was not due to contamination but to differences in DNA recovery by the extraction method. Despite half the amount of sample was used in the Dabney's updated method, the number of identified SNPs were not inferior to those of the other two methods (Table 3).

In the sample ID7 to ID14, we conducted the kinship estimation between the libraries of the decalcification and the undecalcification treatment. The analysis showed that all libraries except ID11 were categorized as "identical twins/same individual". In the sample ID11, there was little variation, and the relationship between the libraries of the decalcification and the undecalcification treatment was categorized as "First Degree". We also analyzed the SNP duplication between the libraries of the decalcification and the undecalcification treatment, and confirmed that there is enough duplication among the samples (ID7: 1,097,934, ID8: 3,211, ID9: 945, ID10: 1,809, ID11: 875, ID12: 1,875, ID13: 648, ID14: 4,269). These results suggested that our SNP analysis was consistent between the decalcified and the undecalcified samples.

Table 2. The sample ID and the mapped reads of human (%).

ID	Mapped reads of human (%)
15	0.07
16	<0.01
17	0.03
18	<0.01
19	0.02
20	0.01
21	<0.01
22	<0.01
23	0.02
24	0.08

Discussion

Decalcification and undecalcification treatments

Until now, the common method for extracting DNA from ancient bones has decalcified the sample prior to DNA extraction (Krings *et al.*, 1997; Rohland and Hofreiter, 2007a; 2007b; Adachi *et al.*, 2018). Since ancient bone consists of many inorganic components such as calcium phosphate and calcium carbonate, which may act as PCR inhibitors, decalcification has been considered beneficial for the following DNA analysis. In this study, decalcified samples yielded a greater amount of human DNA data than undecalcified samples using QIAamp method. Based on this result, it was suggested that decalcification is preferred when using QIAamp method. Previous studies have reported that more DNA can be recovered when bone is decalcified than when it is not (Loreille *et al.*, 2007;

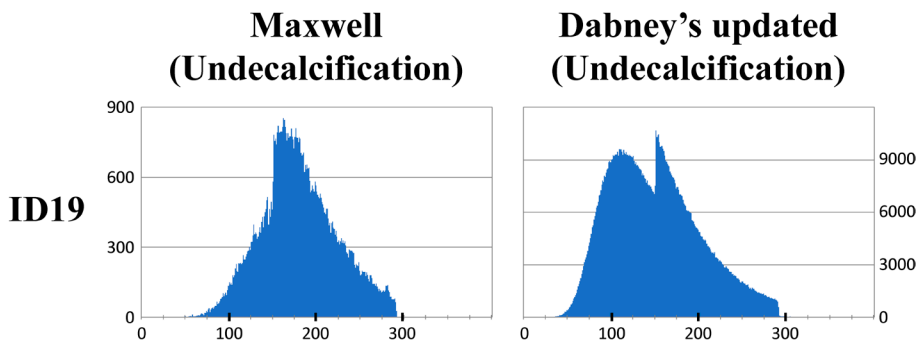

Figure 8. The size distribution of human DNA using the Maxwell method (left) and the Dabney's updated method (right) in the sample ID19. Note that the y-scale is not the same for each panel.

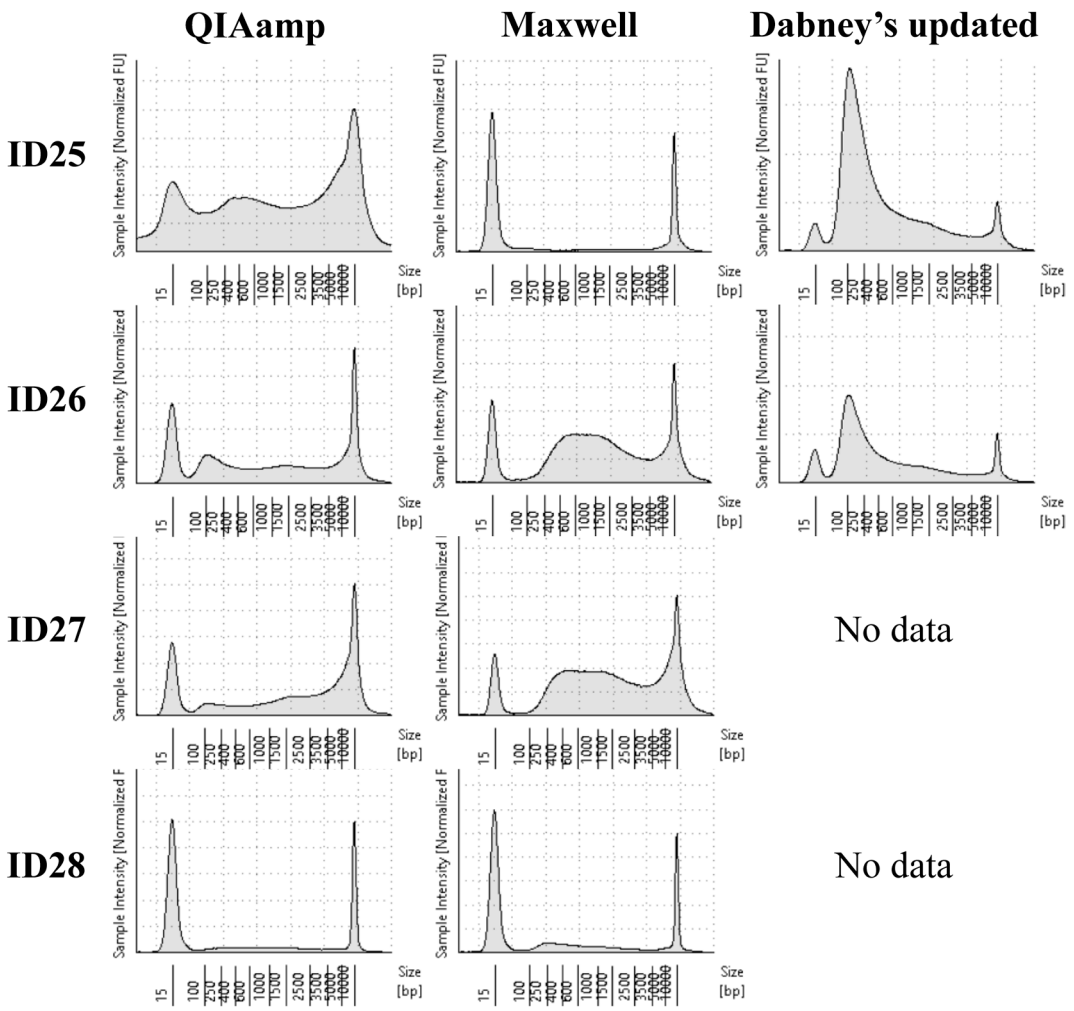

Figure 9A, B. The size distribution of the extracted DNA using the QIAamp (left), the Maxwell (middle), and the Dabney's updated (right) methods. The lower marker (15 bp) and upper marker (10,000 bp) are the first and last peaks, respectively. The right panels show the same samples as in the left and the middle panels. Note that there are no available data on six samples extracted using the Dabney's updated method.

Finaughty *et al.*, 2023). Finaughty *et al.* (2023) showed that cancellous bone yielded higher DNA yields compared to dense cortical samples when it was decalcified. Cancellous bones were likely to expose to environmental bacteria for a long time compared to dense cortical bones, suggesting that it may be better to decalcify sample with high contamination. In addition, the duration and temperature of the decalcification process can greatly affect the yields of extracted DNA (Finaughty *et al.*, 2023), so these factors should also be considered when decalcification is performed.

On the other hand, the Dabney's updated method and the Maxwell method tended to identify many SNPs or retrieve more extracted DNA in undecalcified samples than in decalcified samples. In the Dabney's updated method, despite half the amount of sample was used in the undecalcification treatment, DNA yields of the human DNA were similar to both decalcification treatment. This suggests that the undecalcified samples yielded sufficient human DNA compared to decalcified samples. It has been reported that decalcification of bone is time-consuming step and possible cause for the loss of some ancient DNA (Fisher *et al.*, 1993). If bone samples contain a large amount of

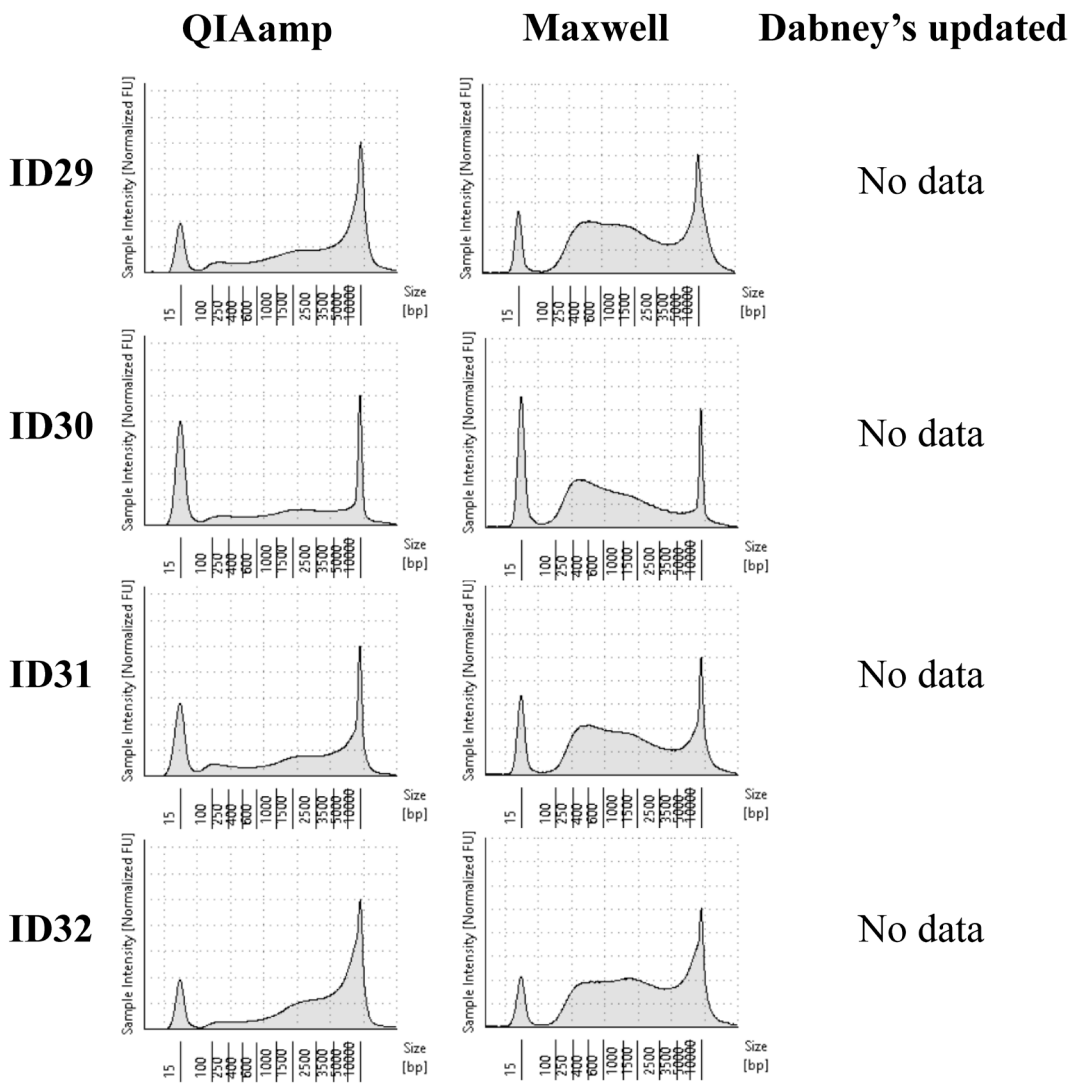

Figure 9A, B. Continued

human DNA and show high mapped reads of human, decalcification will not cause any problem for the subsequent analysis. However, because most ancient bones contain a small amount of DNA, and very short DNA fragments are necessary for analysis, it may be better to extract such samples without decalcification. More importantly, this study suggested that the peak position of extracted DNA was shorter and many SNPs were found when bone was undecalcified. This means that when the samples were not decalcified, the loss of short DNA fragments is minimized, resulting in the short, fragmented human DNA may be efficiently recovered. This would ultimately lead to an increase in the number of identified SNPs in the decalcification treatment. In this study, the fragment size of human DNA after mapping was longer than 100 bp, thus the difference in the yield of human DNA between decalcified and undecalcified samples was not so pronounced. However, in case of degraded samples with shorter fragment sizes, the yield of human DNA may be higher in the undecalcified samples than in the decalcified samples. From these results, we conclude that the decalcification of bone and teeth samples is not a necessary step in the extraction of DNA, and it

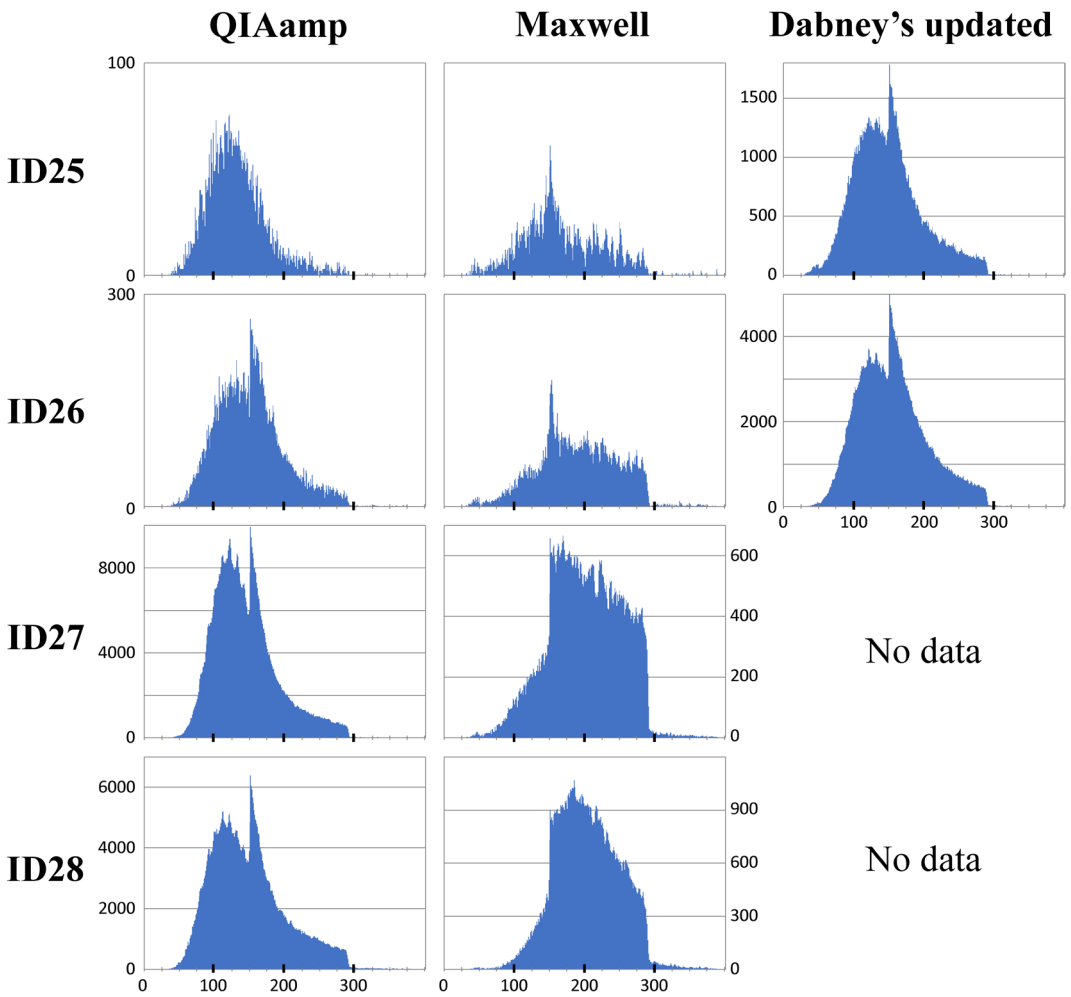

Figure 10A, B. The size distribution of the human DNA using the QIAamp (left), the Maxwell (middle), and the Dabney's updated (right) methods. The right panels show the same samples as in the left and the middle panels. Note that there are no available data on six samples extracted using the Dabney's updated method, and the y-scale is not the same for each sample.

should be considered depending on the extraction methods and sample conditions.

Comparison of three DNA extraction kits

Since the majority of DNA in ancient bones is fragmented into less than 100 bp, the key to successful ancient DNA analysis depends on how efficiently those short DNA fragments can be recovered. In this study, DNA from bone and teeth samples was extracted using three methods, and the results showed that Dabney's updated method was superior to the Maxwell and the QIAamp methods in recovering DNA fragments shorter than 100 bp. In addition, for human DNA data obtained after sequencing, the Dabney's updated method accumulated more data of short fragmented human DNA than other two methods. This suggests that the Dabney's updated method is a superior extraction method for efficiently recovering short human DNA fragments. In some longer DNA fragments extracted with the Maxwell and the QIAamp methods may have contained not only human DNA but also contaminating DNA from bacteria and viruses (Willerslev and Cooper, 2005). In the present analysis, human DNA could be extracted from ancient bones

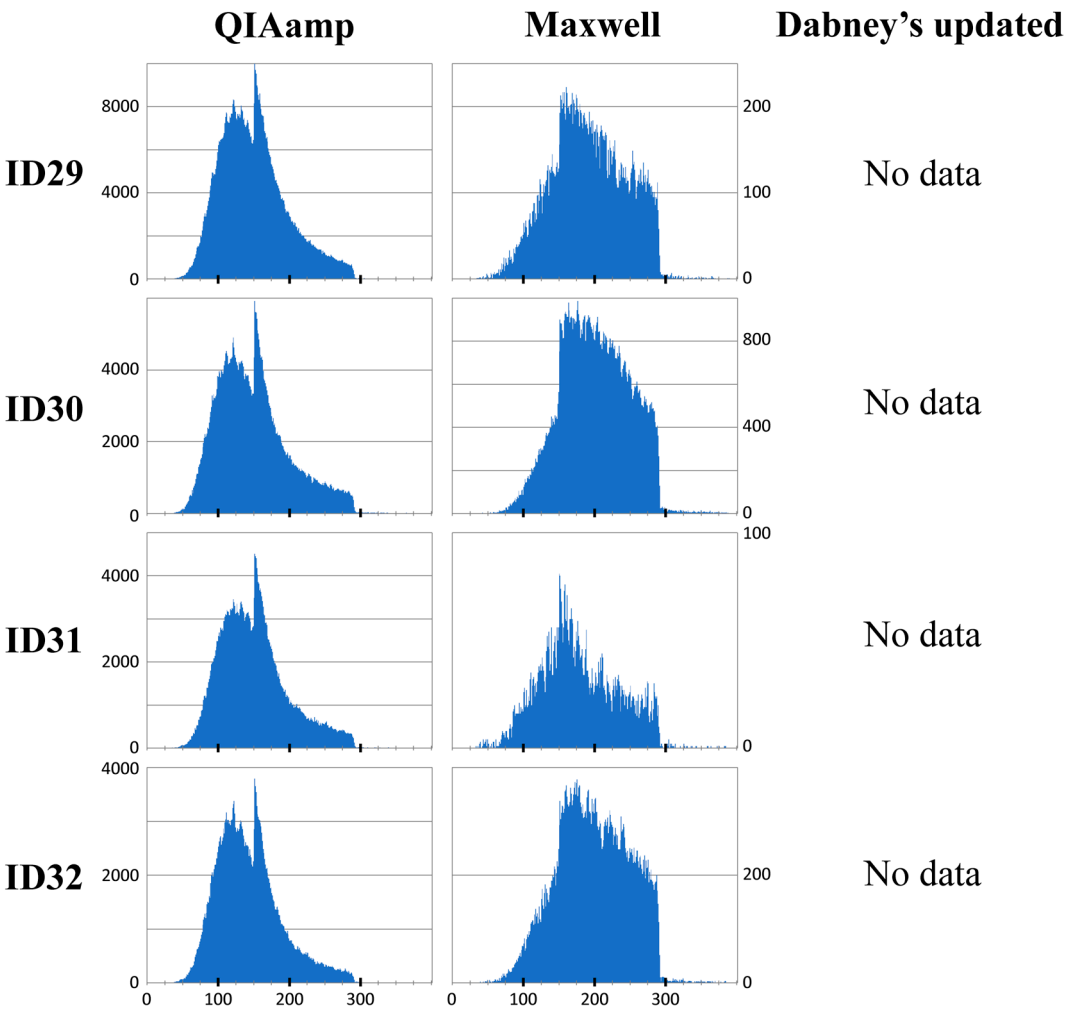

Figure 10A, B. Continued

Table 3. The sample ID, the mapped reads of human (%) and the number of identified SNPs obtained by the three DNA extraction methods.

ID	Mapped reads of human (%)	Number of identified SNPs		
		QIAamp	Maxwell	Dabney's updated
25	0.01	*	144	22214
26	0.02	*	1283	24622
27	0.12	78232	9872	No data
28	0.11	17313	5828	No data
29	0.09	65133	5297	No data
30	0.05	20698	16225	No data
31	0.05	18809	891	No data
32	0.05	23977	9000	No data

*Two samples have no data because the target capture kits were different from the other samples.

and teeth using all three extraction methods. Since DNA fragments that are long enough (i.e., 200 bp or more) often remained in ancient bones in good condition, the Maxwell and the QIAamp methods would enable to extract enough amount of DNA for using the analysis (e.g., STR analysis) that targets long loci ranging from 100 to 400 bp. In addition, these two extraction methods have automated nucleic acid extraction systems that allow analysis with minimal risk of contamination and labor. On the other hand, in most ancient bones, DNA is diminished to fragments shorter than 100 bp, and it is important to recover a sufficient amount of short DNA for the subsequent analysis. Although the Dabney's updated method does not have an automated nucleic acid extraction system, it would be the best method to extract DNA from degraded human bones.

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