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Ancient DNA analysis from epoxy resin Biodur®-embedded bones

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ABSTRACT

For microscopic investigation, archaeological bone samples are often embedded in Biodur® epoxy resin. This study wants to test whether it is possible to extract DNA suitable for PCR amplification from this sample type. For eight individuals a set of samples – each consisting of a Biodur-embedded femur sample, a native femur sample and a control sample of different anatomical origin – were submitted to organic DNA extraction. The extraction success was tested by autosomal short tandem repeat amplification. Seven out of eight Biodur-embedded femur samples revealed successful amplification results. If Biodur-embedded bone material exists from earlier microscopic investigations, our results encourage the use of this sample type as a source for genetic research.

METHOD SUMMARY

An organic DNA extraction was performed on archaeological bone samples (femur) embedded in epoxy resin (Biodur®). The DNA extracts were suitable for STR typing of chromosomal DNA.

KEYWORDS:

ancient DNA ● Biodur®-embedded bones ● DNA quantification ● PCR ● STR typing

The success of DNA analysis from bones and any other kind of tissue is highly dependent on the quality of the sample material. Furthermore, other conditions to which the sample material was exposed to may also influence the success of analysis. These may be natural conditions like the burial environment (i.e., the activity of soil-inhabiting microorganisms, pH value, temperature or the presence of humic acids); factors like a constant low temperature and an alkaline pH favor DNA preservation [1–3]. This is demonstrated by numerous studies proving long-term DNA preservation in hard and soft tissues of different ages recovered from permafrost or caves [4–11]. In contrast, high microbial activity, acidic pH values and large proportions of humic acids obviously promote DNA degradation or prevent a successful analysis due to inhibition.

Additionally, chemicals for sample preparation, the embedding process for microscopic investigations or the long-term conservation of soft tissue in fluid media may also influence the DNA preservation. Since the 17th century, zoological, anatomical and pathological samples were usually preserved in ethanol [12] for long-term conservation and the preparation of thin sections for microscopic investigations. In the 19th century, formalin was introduced as a fixative for biological samples [13] and is now one of the prevailing chemicals for microscopic sample preparation [14]. Both chemicals are still utilized for sample preparation, particularly for the embedding process of soft tissue samples in paraffin. DNA extraction from formalin-fixed samples is challenging, because the chemical is known for the formation of methylene bridges between proteins and crosslinks between proteins and nucleic acids [15,16]. Additionally, hydrolytic DNA fragmentation [17] is promoted due to very low pH values in unbuffered formalin solutions. However, back in the 1990s, the first successful attempts to amplify DNA from paraffin-embedded specimens were performed [18], and today the analysis of DNA from either wet preparations in formalin or formalin-fixed and paraffin-embedded soft tissue samples is reported in numerous studies [19–22]. Also, during the same time period, attempts were made to amplify DNA from resin-embedded samples. This comprises approaches to isolate DNA from biogenic materials naturally embedded in amber [23]. Further, other protocols for DNA extraction from sample materials embedded in artificial resins – for example, undecalcified fresh equine bone samples embedded in methyl methacrylate [24] and bone marrow samples embedded in the epoxy resin Durcupan™ [25] – were shown to be successful.

For the embedding process of archaeological bone samples, the epoxy resin Biodur® is commonly used [26]. The embedding is crucial for microscopic investigations of the bones' microstructure to determine the biological age of an individual or enable histopathological investigations [27–30]. There are two prevailing protocols for the embedding process. For the first protocol, the bone sample is dehydrated in an ethanol series with ascending concentrations, transferred to methyl chloride and then embedded in Biodur [30,31]. Although rather time-consuming, this method allows the Biodur to proceed to the very fine canaliculi of the bone, which reduces optical

Table 1. Overview of the investigated samples.

Find No.	Skeletal series	Excavation year	Bone type	Sample name	Embedding material	Duration of embedding	Ref.
GS 53	Goslar (GS), Lower Saxony, Germany, 18th century	1993	Femur compact diaphysis	GS 53 Biodur	Biodur	18 years	[32]
				GS 53 native	NA	NA	
			Tooth	GS 53 control	NA	NA	
GS 97	Goslar (GS), Lower Saxony, Germany, 18th century	1993	Femur compact diaphysis	GS 97 Biodur	Biodur	18 years	[32]
				GS 97 native	NA	NA	
			Petrous bone	GS 97 control	NA	NA	
Roe 11.2	Rödelheim (Roe 1), Hesse, Germany, 19th century	2015	Femur compact diaphysis	Roe 11.2 Biodur	Biodur	2 years	[33]
				Roe 11.2 native	NA	NA	
			Petrous bone	Roe 11.2 control	NA	NA	
Roe 12.2	Rödelheim (Roe 1), Hesse, Germany, 19th century	2015	Femur compact diaphysis	Roe 12.2 Biodur	Biodur	2 years	[33]
				Roe 12.2 native	NA	NA	
			Tooth	Roe 12.2 control	NA	NA	
Goe 50/10 12	Göttingen (Goe 50/10), Lower Saxony, Germany, 19th century	2011	Femur compact diaphysis	Goe 50/10 12 Biodur	EtOH, CH_2Cl_2 and Biodur	1 year	[34]
				Goe 50/10 12 native	NA	NA	
			Vertebra	Goe 50/10 12 control	NA	NA	
Goe 50/10 138	Göttingen (Goe 50/10), Lower Saxony, Germany, 19th century	2011	Femur compact diaphysis	Goe 50/10 138 Biodur	EtOH, CH_2Cl_2 and Biodur	1 year	[34]
				Goe 50/10 138 native	NA	NA	
			Tooth	Goe 50/10 138 control	NA	NA	
Do 67.1	Lichtenstein cave Dorste (Do), Lower Saxony, Germany, Bronze Age	1996–2004	Femur compact diaphysis	Do 67.1 Biodur	Biodur	20 years	[11]
				Do 67.1 native	NA	NA	
			Vertebra	Do 1491 control†	NA	NA	
Do 5518	Lichtenstein cave Dorste (Do), Lower Saxony, Germany, Bronze Age	1996–2004	Femur compact diaphysis	Do 5518 Biodur	Biodur	20 years	[11]
				Do 5518 native	NA	NA	
			Vertebra	Do 2078.02 control†	NA	NA	

†Bones were excavated at the secondary burial place (Lichtenstein cave). Bones from one individual were numbered in ascending order according to the finding time and, therefore, the finding numbers of the bones within one individual are different.
NA: Not applicable (native samples served as a reference, control samples served for the comparison of the short tandem repeat typing results).

artifacts to a minimum during microscopic investigations and, therefore, facilitates anatomical and histopathological investigations. For the second protocol, the bone sample is embedded directly in Biodur [27], which may result in the formation of optical artifacts due to remaining air in the ultrastructure of the bone. After the preparation of thin sections, surplus embedded bone material may serve for later genetic analysis.

Although embedded samples are morphologically well preserved over a long time, the DNA extraction of this kind of sample material may be challenging, because the chemicals used for sample preparation or the embedding medium itself may contribute to DNA fragmentation. To the best of our knowledge, there are no studies investigating DNA extraction and analysis from Biodur-embedded bones; therefore we tested whether Biodur-embedded bone samples may be a suitable source for analyzable amounts of DNA.

Materials & methods

Study design & sample material

For this investigation, DNA extractions were performed for eight individuals from different burial sites and time periods. The set of investigated samples for each individual consisted of a femur sample previously embedded in Biodur, a sample obtained from the neighboring part of the respective native femur as a reference, and additionally a control sample derived from the same individual but a different anatomical region (Table 1).

The bone samples of the eight individuals originated from archaeological burial sites ranging from Early Modern Age to Bronze Age. The Biodur embedding of the femur samples had been carried out 1–20 years ago to enable light microscopic examination of the bone microstructures with respect to age determination and pathological processes. The embedding procedure had been carried out following established protocols [27,29–31].

Besides the study group consisting of the Biodur-embedded samples, a reference group was established to enable the comparison of DNA preservation between embedded and native bone samples. Thus, the reference (native) group consisted of eight nonembedded compact femur samples, each derived from diaphysis directly neighboring the original sampling sites for the Biodur embedding.

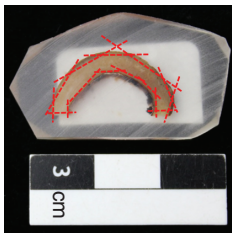

Figure 1. Sampling for the Biodur-embedded bones. The excess epoxy resin and every outer surface of the embedded bones was removed (red dotted lines).

Additionally, a control group comprising eight samples was established. As in the reference group, the samples of the control group were derived from the same skeletal individuals as those represented in the study group. However, the samples were obtained from different anatomical regions (Table 1). The additional control group enables the identification of possible deep contaminations of the femoral samples. The basis for the comparison of the genetic results is autosomal short tandem repeat (STR) typing (also known as genetic fingerprinting) [35].

Methods

Sample preparation & decontamination

All analysis steps (sampling, DNA extraction, STR amplification, DNA quantification, agarose gel electrophoresis and allele determination through capillary electrophoresis) basically followed established protocols [36–38].

The sampling was performed using a hand drill (Dremel® Multi™, WI, USA) with a diamond saw blade (Horico, Berlin, Germany). From the embedded femoral samples, the excess epoxy resin was removed. Given that no particular precautions for the prevention of contamination were taken during the embedding procedure, it was especially important to remove the outer surface of the embedded bone (Figure 1). The native bone samples were sawn out from neighboring parts of the femur diaphysis, while the control samples mainly consisted of teeth or parts of other skeletal elements (Table 1).

All samples were incubated for 10–15 min in 6% sodium hypochlorite solution (Aug. Hedinger GmbH & Co. KG, Stuttgart, Germany) to remove possible adhering contaminations from the bone surfaces [5,39,40] and afterward rinsed with double-distilled water. All further contamination prevention, measurements and monitoring strategy are presented in the Supplementary Material.

Following the rinsing in double-distilled water, the samples were dried overnight at 30°C, then crushed roughly with a steel mortar and milled with a ball triturator type MM 200 (Retsch, Haan, Germany) to a fine powder. In the case of the embedded samples, the powder consisted of bone material and epoxy resin that penetrated the bone during the embedding process. For the native and control samples, the powder consisted of pure bone or tooth root material only.

DNA extraction

The following lysis steps were performed under constant rotation of the samples. Initially, 250 mg of the powdered sample material was incubated with 3900 µl EDTA (0.5 M, pH 8.0, Invitrogen, Waltham, Massachusetts, United States) and 100 µl proteinase K solution in Tris/HCl (pH 7.5, 0.01 mol/l, 600 mAnson-U/ml, Merck, Darmstadt, Germany) for 18 h at 37°C. Afterward, two additional lysis steps were performed by adding 50 µl proteinase K solution (incubation for 2 h at 56°C) and 50 µl sodium dodecyl sulphate (10 mg/ml, Sigma-Aldrich, MO, USA incubation for 5 min at 65°C) to the suspensions. The remaining solid substances, especially the undissolved Biodur residues, were pelleted by centrifugation in a benchtop centrifuge (5430R, Eppendorf, Hamburg, Germany) at 3300 × *g* for 3 min. It has to be noted that the Biodur residues did not always settle completely and therefore may partially remain in the supernatant.

Approximately 4000 µl of the supernatant was rotated in approximately 3000 µl of phenol (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) for 6 min at room temperature and afterward incubated at 56°C for 10 min for phase separation. The organic phases were removed and approximately 4500 µl chloroform (Carl Roth) were added to the aqueous phase. A rotation was performed for 6 min at room temperature. For phase separation, the samples were incubated at 56°C for 10 min. The supernatants were transferred to a mixture of 16 ml Buffer PB (Qiagen, Hilden, Germany) and 100 µl sodium acetate buffer (pH 5.2, 3M, Sigma-Aldrich). The subsequent DNA extraction and purification steps were performed with MinElute™ spin columns (Qiagen) via the QIAvac system (Qiagen) following the manufacturer's protocol. Three washing steps using 700 µl Buffer PE (Qiagen) with a 5-min incubation time were performed. The MinElute spin columns were dry-spun for 1 min at 15,700 × g in a benchtop centrifuge (5415R, Eppendorf) and transferred to a 2-ml collection tube. Three elution steps with 56°C ultrapure water (RNAse-free water, Qiagen), 5 min incubation, and centrifugation for 1 min at 15,700 × g were performed to obtain a total elution volume of 60 µl. The DNA extracts were stored frozen. Extraction blanks were prepared alongside the samples and underwent the same procedure, starting with the lysis step.

For monitoring the DNA extraction success, besides the STR typing a quantification of the DNA by Qubit™ and a qPCR were performed on the Biodur-embedded and the adjacent native femur samples. Details on the quantification approaches are given in the Supplementary Material.

Amplification & gel electrophoresis

For autosomal STR typing, two different kits (miniSTR kit [41] and AmpFlSTR® NGM™, Life Technologies, CA, USA) were used.

The miniSTR kit consists of six autosomal STR markers and the amelogenin marker for sex determination, which are amplified in a heptaplex PCR in total volumes of 25 µl. Amplification duplicates comprising 3 and 5 µl DNA extract were used. A two-step cycling (cycling conditions: initial phase at 95°C for 5 min, 40 cycling steps at 94°C for 1 min and 59°C for 2.5 min, then 60°C for 45 min and hold at 10°C for 10 min) was performed in a Mastercycler™ (Eppendorf). Deviating from the published protocol, Taq PCR Master Mix (Qiagen) was used, the primer pair of D18S51 was applied with a concentration of 0.28 µM each, and the primer pair targeting the amelogenin locus was labeled with 6-carboxyfluorescein (6-FAM) dye.

The NGM kit consists of primers targeting 15 STRs and the amelogenin locus. Amplifications were carried out in total volumes of 25 µl. For each sample, duplicates comprising 3 and 5 µl DNA extract were used. A two-step cycling (cycling conditions: initial phase at 95°C for 11 min, 40 cycling steps at 94°C for 20 s and 59°C for 3 min, then 60°C for 45 min and hold at 10°C for 10 min) was performed in the Mastercycler.

Both the heptaplex and the NGM amplifications were accompanied by extraction blanks, positive and no-template controls; 5 µl of the extraction blanks were used for amplification. As positive controls, DNA extracts from buccal swabs of the processors were used. For the STR typing results, see Table 2 & Supplementary Table 1. For the no-template control samples, ultrapure water (RNAse-free water, Qiagen) was added instead of DNA.

All PCR products were initially separated by standard agarose gel electrophoresis (2.5% agarose gel in 1× Tris-borate-EDTA buffer) in order to verify the amplification success and to estimate the amount of PCR product submitted to capillary electrophoresis [42]. Allele determination was then performed by capillary electrophoresis via 3500 Series genetic analyzer (Applied Biosystems™, MA, USA) using POP-7™ polymer (Applied Biosystems). The resulting data were processed with GeneMapper5™ software (Applied Biosystems).

Results & discussion

This study investigated whether the extraction of analyzable amounts of DNA from epoxy resin Biodur-embedded bones is possible. The consensus STR profiles for the Biodur-embedded samples are presented in Table 2. For each set of samples from eight individuals (consisting of Biodur, native and control), STR typing using two different kits (miniSTR and NGM) was carried out; DNA quantification by Qubit and qPCR was performed for the Biodur and native samples.

The STR typing revealed that for seven out of eight individuals the STR profiles of the Biodur, native and control samples either fully matched (Roe 11.2, Goe 50/10 12 and Do 67.1) or matched in 12–15 of the 17 analyzed STRs (GS 53, GS 97, Goe 50/10 138, Do 5518). However, the deviations are not contradictory: they can be explained by either allelic dropout, overamplified stutter products or – in a single case – a drop-in event (Supplementary Tables 1 & 2). The Supplementary Information for each individual provides all single amplification results and the electropherograms for all sample types.

The STR typing results and the respective calculated matching probabilities (STRidER [43]) of the study samples and the positive controls of the processors show that contamination can virtually be excluded (Supplementary Table 1), because STRs are highly discriminating between individuals [35,44].

For one individual (Roe 12.2), the amplification of the femur samples (Biodur and native) failed almost completely. The control sample (tooth root) of this individual, however, revealed a complete STR profile. Generally, DNA extracted from tooth roots reveals superior amplification results to DNA derived from long bones. The microscopic inspection of the respective thin section of the femur of Roe 12.2 shows that the microstructure is – in contrast to all other samples – severely damaged by microorganisms, which destroyed most of the organic compounds (Figure 2).

The comparison of the agarose gel documentation of the Biodur and native samples indicates that the STR amplification results of embedded and native bones reveal no considerable differences. This applies both to DNA preservation and to questions about possible inhibition of the samples (Figure 3). In particular, the presence of possible inhibiting substances would be noticeable in different primer dimer formation between the samples with 3 and 5 µl DNA added.

The fact that the embedded and native bone samples revealed no considerable differences was unexpected, because 250 mg of powdered sample material from the Biodur-embedded samples contained portions of epoxy resin and thus consisted of less bone material compared with the native samples; hence we expected less DNA in the Biodur-embedded samples, which was only partially supported by the data of the quantification approaches. The data for the overall DNA content measured by the Qubit 4 approach show that the total DNA amount was lower in half of the Biodur-embedded samples compared with the native ones. Furthermore, the results of the relative quantification acquired by qPCR revealed lower DNA content in six out of eight individuals in the Biodur-embedded compared with the native samples. In these six cases, the similar PCR product amounts of the Biodur-embedded and native samples seem to indicate a more effective amplification for the Biodur samples. This is supported by the calculated PCR efficiencies to an average of F = 96.8% for the Biodur-embedded bone samples and F = 88.4% for the native samples during the qPCR (Supplementary Table 3). The

Table 2. Short tandem repeat typing results for Biodur-embedded samples and positive controls.

		individual (finding number), Biodur-embedded								Positive controls (investigator initials)						
		GS53	GS97	Roe 11.2	Roe 12.2	Goe 50/10 12	Goe 50/10 138	Do 67.1	Do 5518	ALF	BG	MBT	MFE	MKG	MSZ	SH
Amelogenin‡	Alleles	X	X	X	–	X	X	X	X	X	X	X	X	X	X	X
		Y	Y	Y	–	Y	X	Y	X	X	X	Y	X	X	Y	X
D13S317†	Alleles	8	11	8	9	9	10	11	12	NI	NI	NI	NI	NI	NI	NI
		11	12	11	–††	12	11	13	13	NI	NI	NI	NI	NI	NI	NI
D21S11‡	Alleles	28	30.2	30	–	29	28	30.2	28	30.2	29	29	30	27	29	30
		30	31.2‡‡	30	–	30	30	33.2	29‡‡	31	32.2	30	30.2	28	30.2	31.2
			32.2						30							
D18S51‡	Alleles	15	14	13	15¶	14	14	14	12	13	12	13	16	14	10	14
		16	16	15	–	21	17	20	15	19	15	17	17	15	17	18
TH01‡	Alleles	8	6	6	8¶	8	7	9.3	6	9	6	7	7	7	6	9.3
		9	9.3	8	–	9	9.3	9.3	9.3	9.3	9.3	9	9.3	9.3	9	9.3
D5S818†	Alleles	11	12	11	–	12	11	10¶	11	NI	NI	NI	NI	NI	NI	NI
		13¶	–††	13	–	–††	–††	12	12	NI	NI	NI	NI	NI	NI	NI
FGA‡	Alleles	20	18	21	–	22	20	21	23	22	23	21	20	23	19	19
		22	20	24	–	22	24	25	24	23	25	22	23	26	23	23
D10S1248§	Alleles	14	13	13	–	14	14	14	13	13	14	14	14	14	13	13
		15¶	–††	15	–	15	15	–††	–††	15	16	17	15	15	16	14
VWA§	Alleles	18	18	17¶	–	17	17	15	14	14	16	17	17	17	16	17
		19	19	20	–	18	18	16	15	17	17	19	19	17	16	17

† Heptaplex amplification only.
‡ Heptaplex and NGM amplification.
§ NGM amplification only.
¶ Occurred once in two amplifications.
†† No amplification product as a consequence of homozygosity or allelic dropout/null allele.
‡‡ Most likely overamplified stutter artifacts. Stutter artifacts are well known in STR amplifications.
–: No amplification product; NI: Not investigated; STR: Short tandem repeat; X: *AMELX* allele; Y: *AMELY* allele.

Table 2. Short tandem repeat typing results for Biodur-embedded samples and positive controls (cont.).

		individual (finding number), Biodur-embedded								Positive controls (investigator initials)						
		GS53	GS97	Roe 11.2	Roe 12.2	Goe 50/10 12	Goe 50/10 138	Do 67.1	Do 5518	ALF	BG	MBT	MFE	MKG	MSZ	SH
D16S539§	Alleles	9	12	11	–	9	13¶	11	10	12	12	11	8	11	10	11
		12	–††	12¶	–	12	14¶	12	13	13	12	13	9	11	11	11
D2S1338§	Alleles	17	17	16	–	23	17	17	18	21	17	19	20	17	16	20
		18¶	19¶	19	–	25	21¶	18	25	27	17	19	24	17	24	24
D8S1179§	Alleles	14	10	12	–	14	15	12	11	12	12	10	12	10	13	13
		15	13	14	–	15	16¶	13	14	13	14	14	15	13	13	15
D22S1045§	Alleles	16	15	15	15¶	15	15	15	15	15	11	15	11	16	16	15
		–††	16	16	–	16	–††	16	17	16	15	16	15	16	17	16
D19S433§	Alleles	13	14	14	–	13	14	13	13	15	14	14	13	15	14	13
		14	15.2	–††	–	–††	–††	15	15	17.2	14	16	17	15.2	14.2	16
D2S441§	Alleles	10	14	10	10¶	11	14	10	11	11	11	12	14	11	12	10
		11.3	–††	11	–	14	–††	14	–††	14	14	14	14	13	14	14
D3S1358§	Alleles	18	15	16	17¶	15	15	15	14	15	14	14	16	16	15	16
		–††	17	–††	–	19	–††	17	16	16	16	16	18	18	15	16
D1S1656§	Alleles	13	15.3	12	–	11	15	18.3	14	17.3	11	14	13	16	11	11
		–††	17.1	–††	–	16	17.3¶	–††	16	17.3	11	17.3	14	16	15.3	16
D12S391§	Alleles	20¶	18	19	–	19	17	20	20	18	18	18	20	17	18	21
		21	23	21	–	21	24¶	21	23	24	19	20	22	22	20	23
		24¶														

† Heptaplex amplification only.
‡ Heptaplex and NGM amplification.
§ NGM amplification only.
¶ Occurred once in two amplifications.
†† No amplification product as a consequence of homozygosity or allelic dropout/null allele.
‡‡ Most likely overamplified stutter artifacts. Stutter artifacts are well known in STR amplifications.
–: No amplification product; NI: Not investigated; STR: Short tandem repeat; X: *AMELX* allele; Y: *AMELY* allele.

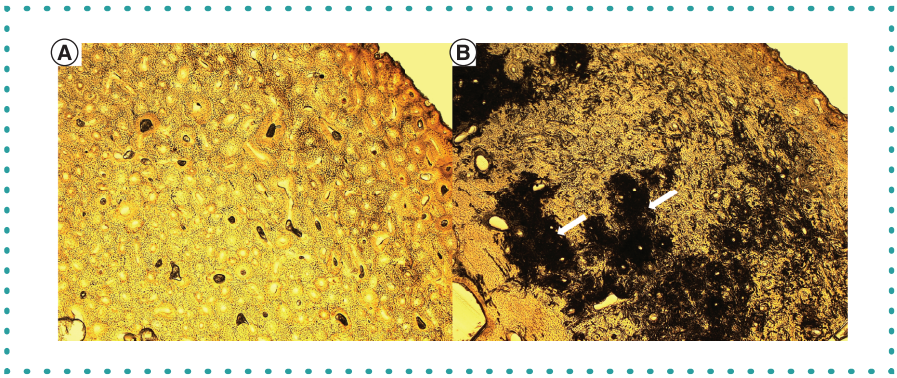

Figure 2. Microscopic images of thin sections (plain light, 60 μm, 25× magnification) of the microstructures of the femurs of the individuals (A) Roe 11.2 and (B) Roe 12.2. In contrast to Roe 11.2, the bone substance of individual Roe 12.2 is severely damaged by microorganisms (arrows), which have destroyed most of the organic compounds.

Table 3. Results of the DNA quantification.

Sample ID	Total DNA amount (exogenous + endogenous) measured by Qubit 4 (ng/μl)	Calculated endogenous DNA amounts based on qPCR results[#]		Proportion of endogenous DNA from total DNA amount (%)
		Average (ng/μl)	SD	
GS 53 Biodur[‡]	1.330	0.031	0.011	2.331
GS 53 native[‡]	1.760	0.085[§]	0.031	4.830
GS 97 Biodur[†]	4.060	0.086[§]	0.002	2.118
GS 97 native[‡]	4.480	0.058	0.006	1.295
Roe 11.2 Biodur[†]	2.420	0.017[§]	0.001	0.702
Roe 11.2 native[‡]	1.960	0.043	0.002	2.194
Roe 12.2 Biodur[†]	2.780	0.001[§]	0	0.036
Roe 12.2 native[‡]	2.840	0.005	0.006	0.176
Goe 12 Biodur[†]	7.820	0.219	0.017	2.801
Goe 12 native[‡]	6.940	0.302	0.033	4.352
Goe 138 Biodur[†]	3.140	0.065	0.009	2.070
Goe 138 native[‡]	5.600	0.064	0.002	1.143
Do 67.1 Biodur[†]	0.596	0.116	0.001	19.463
Do 67.1 native[‡]	0.440	0.239	0.003	54.318
Do 5518 Biodur[†]	1.570	0.085	0.02	5.414
Do 5518 native[‡]	0.836	0.273[§]	0.017	32.656
Ex-blank 1[†]	too low	0.001	0	NA
Ex-blank 2[‡]	too low	0[¶]	0	NA

[†]qPCR1.
[‡]qPCR2.
[§]1 Ct value excluded (e.g., due to a PCR failure).
[¶]2 Ct values excluded (e.g., due to a PCR failure).
[#]Calculated endogenous DNA amounts base on qPCR results of the undiluted DNA extracts. The qPCR results of the 1:20 dilutions revealed stochastic values indicating too low amounts of DNA.
NA: Not applicable.

most plausible explanation for the outcome of the experiment is that the powdered epoxy resin in the Biodur samples acts in a similar way to Chelex®100, a chelating resin that can be used for DNA extraction from different types of sample material due to its positive purification characteristics [45]. Comparable to Chelex, it can be supposed that the pulverized epoxy resin is also capable of binding protein fragments and humic acids, which would lead to more purified DNA extracts for the Biodur samples and thereby positively influence the efficiency of the PCR. In this case, the purification effect of the pulverized epoxy resin, which is a portion of the weighed sample amount, may compensate for the respective lesser amount of bone material and thus DNA in the Biodur samples.

The total DNA amounts assessed by Qubit and the qPCR results determining the relative amount of human specific endogenous DNA for an 81-bp fragment are presented in Table 3 (for more detailed information, see Supplementary Table 3).

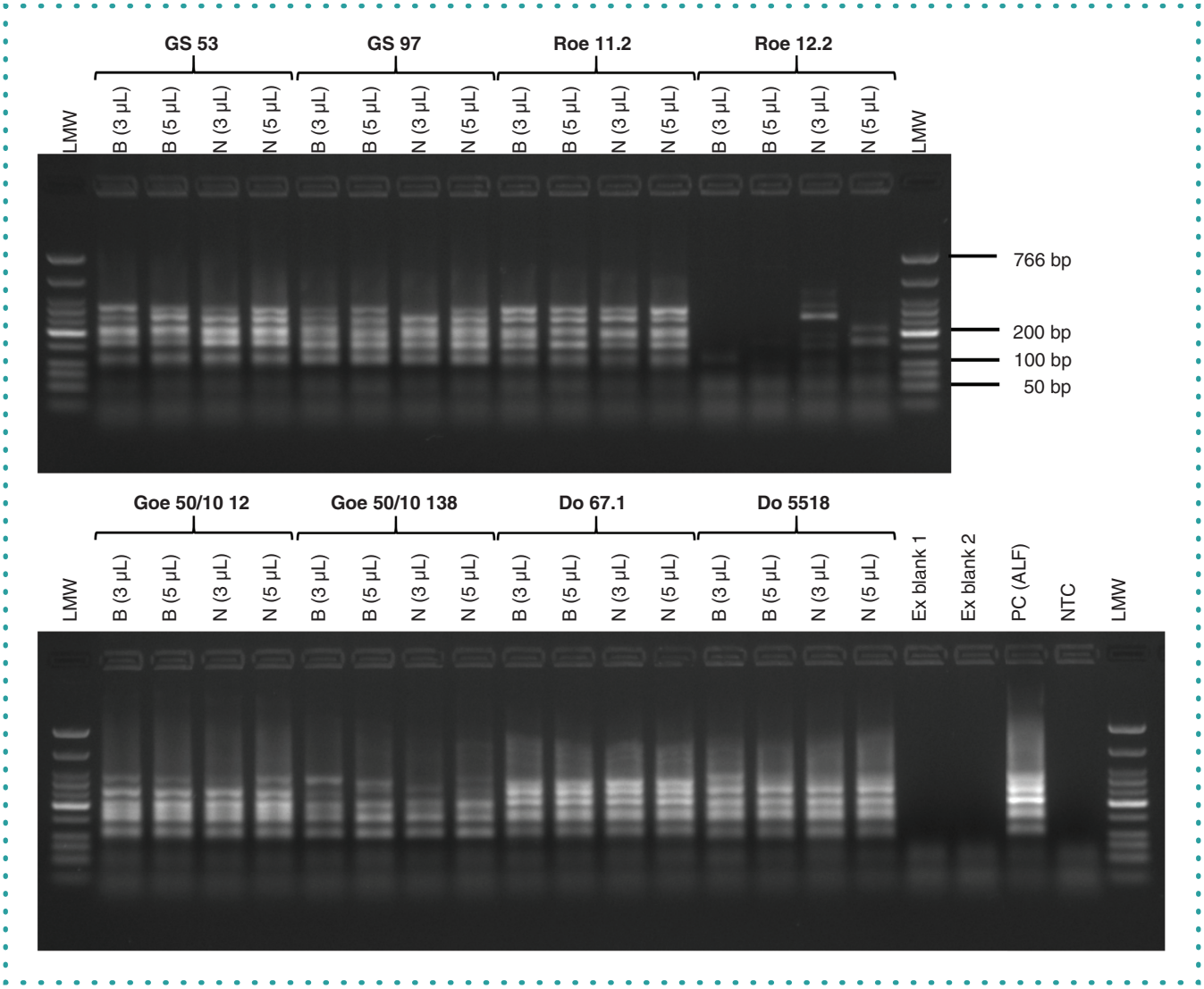

Figure 3. Results of amplification with the NGM kit. The kit consists of primers targeting 15 short tandem repeats and the amelogenin locus for both sample types, Biodur-embedded (B) and *native* (N), of the investigated individuals (DNA inset is bracketed). Gel bands are located between approx. 100 and 360 bp of the DNA ladder. For every individual, with the exception of Roe 12.2, the amplification resulted in several visible bands for both DNA extracts (Biodur-embedded and native bone samples), indicating a successful amplification of all systems. In contrast, the amplification failed almost completely for both samples of the individual Roe 12.2. However, neither sample shows apparent signs of inhibition which would result in a decrease or failure of primer dimer formation (at ~50 bp). The extraction blanks (Ex Blank) show no bands. As a positive control, contemporary DNA of the processor was used (for the short tandem repeat typing results, see Table 2 & Supplementary Table 1). For the no-template control, water was used instead of DNA. Electrophoresis parameters: 8 µl PCR product with 2 µl loading dye, 3 µl size standard: low-molecular-weight ladder (LMW, New England Biolabs, MA, USA), 2.5% agarose gel, 110 V, exposure time: 0.4 s.

The question remains why the relative amounts of DNA in the Biodur-embedded and the native samples are not consistent in some individuals. In the case of the determination of the total amount of DNA, a possible explanation for the observed inconsistencies can be found in deviating microbial invasion even in directly adjacent regions of compact bone. This phenomenon may be caused by chemical impregnation of parts of the bone through, for example, grave goods or clothing, which may promote or – more often – prevent the invasion of bacteria and fungi. This may also influence the degree of DNA fragmentation and therefore may cause seemingly inconsistent values for endogenous DNA, as determined by the 81-bp fragment in two individuals.

Conclusion

Although detailed interpretation about the influence of Biodur on DNA preservation and PCR efficiency is complex and may require larger sample size, we can conclude that the extraction and amplification of DNA from Biodur-embedded sample material was successful. This

was demonstrated by STR typing results in seven out of eight individuals. Our results therefore indicate that this sample type is a suitable source for genetic research.

Future perspective

The study demonstrated that it is possible to extract DNA from Biodur-embedded archaeological bone material and that this DNA is suitable for PCR-based amplification. Therefore, given an existing permission for subsequent processing, our protocol allows genetic analyses, even when the skeletons are already reburied or invaluable skeletal material ought to be saved. More research would be necessary to fully understand the effects of Biodur on DNA preservation and PCR efficiency, as it could be shown via qPCR that some of the Biodur-embedded samples contained lesser amounts of endogenous DNA fragments of suitable length for STR typing on average, although this fact was obviously compensated by an increased amplification efficiency.

Executive summary

- For microscopic age determination or the investigation of pathological alterations, archaeological bone samples are often embedded in Biodur® epoxy resin, and remnants of embedded bone material usually exist.
- Biodur-embedded femur bone samples from eight individuals were pulverized and a phenol–chloroform technique was used to extract DNA. Additionally, a native femur sample and a control sample from a different anatomical region were included in the experiment for each individual.
- DNA extraction from Biodur-embedded bone samples and PCR amplification was possible for seven out of eight individuals.
- Quantification of endogenous DNA by qPCR often revealed lower amounts of DNA in the Biodur-embedded material compared with the native femur samples. This corresponds to the fact that part of the initial standardized weighed samples consisted of pulverized resin in the Biodur samples.
- In these six cases, similar PCR product amounts of the Biodur-embedded and native samples were observed during agarose gel electrophoresis, indicating a higher PCR efficiency for the embedded samples.
- Given an existing permission for subsequent processing, our protocol allows genetic analysis, even when the skeletons are already reburied or the skeletal material ought to be saved.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.2144/btn-2022-0056

Author contributions

M Schultz conceived the experiment; AL Flux and S Hummel designed and performed the experiments and analyzed the data; AL Flux, M Schultz and S Hummel wrote the paper

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References

Papers of special note have been highlighted as: • of interest; •• of considerable interest

1. Lindahl T. Instability and decay of the primary structure of DNA. *Nature* 362(6422), 709–715 (1993).
2. Hummel S, Schultes T, Bramanti B, Herrmann B. Ancient DNA profiling by megaplex amplications. *Electrophoresis* 20(8), 1717–1721 (1999).
3. Gilbert MTP, Hansen AJ, Willerslev E *et al.* Characterization of genetic miscoding lesions caused by postmortem damage. *Am. J. Hum. Genet.* 72(1), 48–61 (2003).
4. Hofreiter M, Capelli C, Krings M *et al.* Ancient DNA analyses reveal high mitochondrial DNA sequence diversity and parallel morphological evolution of late pleistocene cave bears. *Mol. Biol. Evol.* 19(8), 1244–1250 (2002).

5. Hummel S. *Ancient DNA Typing. Methods, Strategies and Applications*. Springer-Verlag, NY, USA (2003).
- **Concise review on the first two decades of ancient DNA research focusing on methods, strategies and applications.**
6. Gilbert MTP, Kivisild T, Grønnow B *et al.* Paleo-Eskimo mtDNA genome reveals matrilineal discontinuity in Greenland. *Science* 320(5884), 1787–1789 (2008).
7. Krause J, Briggs AW, Kircher M *et al.* A complete mtDNA genome of an early modern human from Kostenki, Russia. *Curr. Biol.* 20(3), 231–236 (2010).
8. Ishida Y, Roca AL, Fratpietro S, Greenwood AD. Successful genotyping of microsatellites in the woolly mammoth. *J. Hered.* 103(3), 459–464 (2012).
9. Keller A, Graefen A, Ball M *et al.* New insights into the Tyrolean Iceman's origin and phenotype as inferred by whole-genome sequencing. *Nat. Commun.* 3, 698 (2012).
10. Orlando L, Ginolhac A, Zhang G *et al.* Recalibrating Equus evolution using the genome sequence of an early Middle Pleistocene horse. *Nature* 499(7456), 74–78 (2013).
11. Flindt S, Hummel S. Rätsel Lichtensteinhöhle – Eine Großfamilie aus der Bronzezeit [Internet]. Wbg Theiss Verlag, Darmstadt, Germany. www.lehmanns.de/shop/geisteswissenschaften/56541578-9783806243642-raetsel-lichtensteinhoehle
12. Artelt W. Die anatomisch-pathologischen Sammlungen Berlins im 18. Jahrhundert – Universitätssammlungen in Deutschland. *Klinische Wochenschrift* 15, 96–99 (1936).
13. Blum F. Der Formaldehyde als Haertungsmittel. *Zeitschrift Wissenschaftliche Mikroskopie* 10, 314–315
14. Musiał A, Gryglewski RW, Kielczewski S, Loukas M, Wajda J. Formalin use in anatomical and histological science in the 19th and 20th centuries. *Folia Med. Cracov* 56(3), 31–40 (2016).
15. Brutlag D, Schlehuber C, Bonner J. Properties of formaldehyde-treated nucleohistone. *Biochemistry* 8(8), 3214–3218 (1969).
16. Kim TH, Dekker J. Formaldehyde cross-linking. *Cold Spring Harb. Protoc.* 2018(4), pdb.prot082594 (2018).
17. Gilbert MTP, Binladen J, Miller W *et al.* Recharacterization of ancient DNA miscoding lesions: insights in the era of sequencing-by-synthesis. *Nucleic Acids Res.* 35(1), 1–10 (2007).
18. Scholte GH, van Doorn LJ, Quint WG, Lindeman J. Polymerase chain reaction for the detection of *Helicobacter pylori* in formaldehyde-sublimate fixed, paraffin-embedded gastric biopsies. *Diagn. Mol. Pathol.* 6(4), 238–243 (1997).
19. Sato Y, Sugie R, Tsuchiya B, Kameya T, Natori M, Mukai K. Comparison of the DNA extraction methods for polymerase chain reaction amplification from formalin-fixed and paraffin-embedded tissues. *Diagn. Mol. Pathol.* 10(4), 265–271 (2001).
20. Paireder S, Werner B, Bailer J *et al.* Comparison of protocols for DNA extraction from long-term preserved formalin fixed tissues. *Anal. Biochem.* 439(2), 152–160 (2013).
- **Comparison and evaluation of ten DNA extraction methods from long-term formalin-fixed and paraffin-embedded soft tissues with respect to DNA yield, purity and fragment length.**
21. Hühns M, Röpenack P, Erbersdobler A. Molecular and immunohistochemical characterization of historical long-term preserved fixed tissues from different human organs. *PLOS ONE* 10(8), e0135297 (2015).
22. Bonnet E, Moutet M-L, Baulard C *et al.* Performance comparison of three DNA extraction kits on human whole-exome data from formalin-fixed paraffin-embedded normal and tumor samples. *PLOS ONE* 13(4), e0195471 (2018).
23. Peris D, Janssen K, Barthel HJ *et al.* DNA from resin-embedded organisms: past, present and future. *PLOS ONE* 15(9), e0239521 (2020).
24. Leutenegger CM, von Rechenberg B, Huder JB *et al.* Quantitative real-time PCR for equine cytokine mRNA in nondecalcified bone tissue embedded in methyl methacrylate. *Calcif. Tissue Int.* 65(5), 378–383 (1999).
25. Krenacs T, Bagdi E, Stelkovics E, Bereczki L, Krenacs L. How we process trephine biopsy specimens: epoxy resin embedded bone marrow biopsies. *J. Clin. Pathol.* 58(9), 897–903 (2005).
26. von Hagens G. Impregnation of soft biological specimens with thermosetting resins and elastomers. *Anat. Rec.* 194(2), 247–255 (1979).
- **Application of resins and elastomers to impregnate biological specimens.**
27. Herrmann B, Grupe G, Hummel S, Piepenbrink H, Schutkowski H. Prähistorische Anthropologie: Leitfaden der Feld- und Labormethoden. Springer-Verlag, Heidelberg, Germany (1990).
28. Schultz M, Drommer R. Möglichkeiten der Präparateherstellung aus dem Gesichtsschädelbereich für die makroskopische und mikroskopische Untersuchung unter Verwendung neuer Kunststofftechniken. In: *Fortschritte der Kiefer- und Gesichts-Chirurgie 28. Experimentelle Mund-Kiefer-Gesichtschirurgie. Mikrochirurgische Eingriffe*. Hoppe W (Ed.). Georg Thieme Verlag, NY, USA, 95–97 (1983).
29. Schultz M. Methoden der Licht- und Elektronenmikroskopie. In: *Anthropologie: Handbuch der vergleichenden Biologie des Menschen. Band I, 1 Wesen und Methoden der Anthropologie*. Knussmann R (Ed.). Gustav Fischer Verlag, NY, USA, 698–730 (1988).
30. Schultz M. Light microscopic analysis of macerated pathologically pathologically changed bones. In: *Bone Histology*. Crowder C, Stout S (Eds). CRC Press, FL, USA, 253–296 (2012).
31. Schultz M. Paleohistopathology of bone: a new approach to the study of ancient diseases. *Am. J. Phys. Anthropol.* Suppl. 33, 106–147 (2001).
- **Brief summary on the history of paleohistopathological investigation and different methods for microscopic investigation of macerated bones.**
32. Bramanti B. Genetic characterization of the Goslar skeleton collective by aDNA analysis. In: *Anlage 3 zum Abschlußbericht "Der Harz alsfrühmittelalterliche Industrielandschaft" Teilprojekt Anthropologie AZII 70959* (2000).
33. Hampel A. Gräber der napoleonischen Befreiungskriege in Frankfurt a. M.-Rödelheim. Kriegsrelikte des frühen 19. Jahrhunderts im Frankfurter Stadtgebiet. In: *Hessen Archäologie 2015 Jahrbuch für Archäologie und Paläontologie in Hessen. hessenARCHÄOLOGIE des Landesamtes für Denkmalpflege Hessen*. Theiss–Verlag, Stuttgart, Germany (2015).
34. Grosskopf B. Die Bestatteten vom Michaelis Friedhof - Anthropologische Auswertung. In: *Der vergessene Friedhof. Kulturgeschichtliche und naturwissenschaftliche Ergebnisse zur Ausgrabung des neuzeitlichen katholischen Friedhofes in Göttingen*. Universitätsverlag, Göttingen, Germany, 37–68 (2015).
35. Butler JM. *Forensic DNA Typing*, 2nd Edition. Elsevier Academic Press, London, UK (2004).
36. Morin PA, Chambers KE, Boesch C, Vigilant L. Quantitative polymerase chain reaction analysis of DNA from noninvasive samples for accurate microsatellite genotyping of wild chimpanzees (*Pan troglodytes verus*). *Mol. Ecol.* 10(7), 1835–1844 (2001).
37. Frischalowski M, Seidenberg V, Grosskopf B, Wulf F-W, Hummel S. Molekulargenetische Untersuchung des Verwandtschaftsverhältnisses von möglichen Mutter-Kind-Bestattungen aus dem frühneuzeitlichen Eldagsen. In: *Nachrichten aus Niedersachsens Urgeschichte* Theiss–Verlag, Stuttgart, Germany, 193–206 (2015).
38. Flux AL, Mazanec J, Strommenger B, Hummel S. *Staphylococcus aureus* sequences from osteomyelitic specimens of a pathological bone collection from pre-antibiotic times. *Diversity* 9(4), 43 (2017).
39. Kemp BM, Smith DG. Use of bleach to eliminate contaminating DNA from the surface of bones and teeth. *Forensic Sci. Int.* 154(1), 53–61 (2005).
40. Llamas B, Willerslev E, Orlando L. Human evolution: a tale from ancient genomes. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 372(1713), 20150484 (2017).
41. Seidenberg V, Schilz F, Pfister D, Georges L, Fehren-Schmitz L, Hummel S. A new miniSTR heptaplex system for genetic fingerprinting of ancient DNA from archaeological human bone. *J. Archaeol. Sci.* 39(10), 3224–3229 (2012).
- **Development and application of the miniSTR system for genetic fingerprinting especially suitable for highly degraded DNA.**
42. Wittmeier P, Hummel S. Agarose gel electrophoresis to assess PCR product yield: comparison with spectrophotometry, fluorometry and qPCR. *BioTechniques* 72(4), 155–158 (2022).
43. Bodner M, Bastisch I, Butler JM *et al.* Recommendations of the DNA Commission of the International Society for Forensic Genetics (ISFG) on quality control of autosomal short tandem repeat allele frequency databasing (STRidER). *Forensic Sci. Int. Genet.* 24, 97–102 (2016).
44. Hummel S, Bramanti B, Schultes T, Kahle M, Haffner S, Herrmann B. Megaplex DNA typing can provide a strong indication of the authenticity of ancient DNA amplifications by clearly recognizing any possible type of modern contamination. *Anthropol. Anz.* 58(1), 15–21 (2000).
45. Walsh PS, Metzger DA, Higuchi R. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *BioTechniques* 10(4), 506–513 (1991).
- **The use of Chelex 100 for DNA extraction and its influence on PCR-based amplification from various forensic sample materials.**