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Fishing for ancient DNA

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Abstract

The major problems concerning ancient DNA studies are related to the amount of extractable DNA and the precautions needed to avoid contamination. From the very first step of the analyses, the DNA extraction, these problems must be confronted. There are several extraction methods available for DNA in ancient tissue; several of them are complicated and time consuming, and none of the methods have reached an acceptance level such that they are routinely used on a widespread basis. Here we investigate the efficiency of two methods, one based on magnetic separation of the targeted molecules, and one based on silica binding. The efficiency rate of these two on the material studied seems to be identical. The silica binding method has the benefit of relative simplicity, but the magnetic separation technique also has advantages. For example, it is possible to reuse the extract several times for different loci, and it is possible to concentrate all extracted DNA from one locus into one PCR.
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1. Introduction

The possibility to retrieve DNA from ancient tissue has been viewed as holding the future promise of solving a variety of archaeological, anthropological, palaeontological and medical questions. However, working with ancient DNA is a delicate and challenging task, confronting the scientist with several problems specific for this type of material. The obstacles confronted by those who claimed the first successes on DNA from ancient tissue [1,2] are still present now, more than 20 years later, without having been fully resolved.

The main problem when working with ancient DNA stems to some degree from the low amount of starting molecules, and the presence of PCR inhibitors [3,4], but also includes endogenous DNA damage and fragmentation. Early strategies to overcome these problems were to increase the amount of the Taq polymerase, sometimes up to 10 U per PCR reaction [5], or to dilute the DNA extract, thereby diluting the co-extracted PCR inhibitors [6]. Much effort has been focused on improving the extraction and purification procedures. Although some methods have been more influential than others, none has been generally accepted.

Several ancient DNA extraction protocols have been suggested over the years. A few of them have approached the problem in an unconventional manner, such as the use of Dextran Blue as inhibitor carriers [7], the use of pure water [8] and the use of the somewhat unusual reagent Coca Cola [9]. The extraction techniques that have had most impact on general ancient DNA work have focused on purifying extracted DNA with silica binding [10–12], and decalcifying bone with EDTA [3,5,12].

Bone apatite as a DNA adsorber has been recognised in several studies [3,4,13]. Following this, most of the suggested extraction protocols fall within one of three categories: those

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attempting to release DNA by degrading the hydroxyapatite [3,5,12,14–16], those attempting to release the DNA from the bone apatite by adding competing ions [17,18], and those that do not consider the bone apatite [7,11,19,20].

Another way of categorising the methods would be via the purification strategy employed. Most popular are methods based on phenol/chloroform extraction and alcohol precipitation [3,5], and silica binding [11,12]. However other methods have also been suggested, like using chelex [20], centricon filters [21], and Dextran Blue [7]. At present one of the most commonly used methods are based on a combination of EDTA decalcification and silica purification [12,16,22].

The fact that so many different extraction techniques are in use indicates that no single procedure has emerged yet as having clear advantages that would lead to it becoming standardised. Thus, there are as many ancient DNA extraction techniques as there are ancient DNA laboratories. Here we investigate an extraction method, based on hybridisation and magnetic separation that provides pure DNA without any detectable inhibitors while also allowing for multiple analyses based on the same extract. Furthermore, we test the efficiency of the method against a fast and simple method used in several laboratories today.

2. Material and methods

2.1. Material

Twelve samples of cow bones and teeth from the Neolithic to the mediaeval period were used in this study. Samples were extracted in batches of four, with two extraction blanks in each batch (Table 1).

2.2. Initial extraction for fishing and silica purification

Approximately 70 mg of drilled bone powder, and 1 ml of extraction buffer (0.5 M EDTA pH 8.0, 0.5% SDS, 100 mg/ml Proteinase K) were mixed and incubated with constant agitation at 55 °C for 24 h.

2.3. Fishing extraction method

The fishing technique is partly based on ideas published by Tofanelli and Nencioni [23]. The samples were centrifuged at 12,000 rpm for 5 min and the supernatant was added to a 30,000 MWCO Amicon® Ultra-4 Centrifugal filter device (Millipore) and spun for 20 min in a swinging bucket centrifuge at 4000 × *g*. One millilitre of binding and washing buffer (1 M NaCl, 5 mM Tris–HCl pH 7.5, 0.5 mM EDTA) was subsequently added to the centrifugate and the filtering process repeated twice with centrifugation for 15 min, each time adding 1 ml of binding and washing buffer to the washed sample. The final retention, usually about 50 μl, was mixed with 1 ml of binding and washing buffer.

Biotinylated primers, 0.05 pmol of each (Table 2), were added to the sample and incubated at 100 °C for 15 min, on ice for 15 min and at 50 °C for 30 min. After letting the sample reach room temperature, DNA was immobilised to 0.04 mg Dynabeads® M-280 Streptavidin-coated beads, using a Dynal Magnetic Particle Concentrator following manufacturer's

Table 1
Archaeological samples exposed to two different DNA extraction methods and thereafter amplified for two different D-loop fragments

Sample	Element	Y frag III	F frag III	Y D-loop	F D-loop	Outcome	Age
DD1	Tooth	1	1	–	–	2	Early neolithic
DD2	Mandible	1	1	1	–	3	Early neolithic
DD3	Mandible	2	2	–	–	4	Early neolithic
DD12	Tooth	1	–	–	–	1	Early neolithic
DD13	Tooth	–	–	–	–	–	Early neolithic
DD46	Bone	2	2	2	2	8	Medieval
DD47	Bone	2	2	1	2	7	Medieval
DD48	Bone	–	2	–	2	4	Medieval
DD66	Tooth	2	2	2	1	7	Younger bronze age
DD67	Bone	2	–	2	–	4	Late bronze age
HM8	Bone	1	–	–	–	1	Neolithic
Lzz2419	Bone	–	2	–	–	2	Late pleistocene
E. blank		–	–	–			
E. blank		–	–	–	–		
E. blank		–	–	–	–		
E. blank		–	–	–	–		
E. blank		–	–	–	–		
E. blank		–	–	–	–		
PCR blank		–	–	–	–		
PCR blank		–	–	–	–		
PCR blank		–	–	–	–		
Outcome		14	14	8	7		

Sample extracted with the Yang method is listed under Y, those extracted with the fishing method is listed under F. The numbers in the columns refers to the amount of successful amplifications out of two for each fragment, extraction method, and sample. The short fragment (Frag III) was amplified using primers An1F and An3R, the long fragment (D-loop) was amplified using primers An2F and An3R. Time periods for the samples are provided in the table. Genebank accession no are: EF506876-EF506884

Table 2
Primers used in the study

Name	Sequence 5′ → 3′	Application
An2F	GCCCCATGCATATAAGCAAG	PCR
An1F	CTTAATTACCATGCCGCGTG	PCR
An3R	CGAGATGTCTTATTTAAGAGG	PCR
B-Ko1	Biotin-ACCATTAGATCACGAGCTTAA	Extraction
B-Ko2	Biotin-GAAGAAAGAACCAGATGCC	Extraction
B-An1R	Biotin-CACGCGGCATGGTAATTAAG	Extraction
B-An1F	Biotin-CTTAATTACCATGCCGCGTG	Extraction

recommendation. The immobilised template was washed twice with binding and washing buffer and twice with PCR buffer (10 mM Tris–HCl pH 8.0, 50 mM KCl). Finally, the immobilised template was re-suspended in 40 μl of ddH_2O (Sigma).

2.4. Silica extraction method (Yang et al. [12])

The alternative method is based on the Rapid QIAquick method identified by Yang et al. [12], as outlined by Bouwman and Brown [24]. Each sample was centrifuged at 2000 rpm for 5 min and 0.5 ml of the supernatant was transferred into a 15 ml Falcon tube containing 2.5 ml PB buffer (Qiagen). After mixing, 0.5 ml was added to a QIAquick column (Qiagen), which was then centrifuged at 13,000 rpm for 1 min. The flow-through was discarded and the step was repeated until all PB/DNA mix had passed through the filter column. PE buffer (0.75 ml, Qiagen) was added to the column, which was centrifuged as above, and the run through discarded. The column was then transferred to a new 1.5 ml microtube and 50 ml EB buffer (10 mMTris–HCl pH 8.5, Qiagen) was added. After 1 min at room temperature the column was centrifuged at 13,000 rpm for 1 min and the elute was collected. This was repeated once and the two elutes were pooled giving a total volume of approximately 96 μl.

2.5. Amplification of prehistoric samples

PCR was carried out using 9 μl of the hybridised extract when the fishing method was used, alternatively 2 μl when the silica column method was used. PCR parameters were: 3 U HotStarTaq™ DNA polymerase (Qiagen), 1× Qiagen PCR buffer, 2.5 mM $MgCl_2$, 200 μM of each dNTP, and 0.2 μM of each primer [25,26] (Table 2) in a total volume of 25 μl. The PCR profile was 10 min at 95 °C, followed by 45 cycles of 15 s at 94 °C, 30 s at 55 °C, and 15 s at 72 °C, with a final extension step of 7 min at 72 °C. At least one PCR blank was amplified alongside each batch. One master mix was made for the two extraction batches and samples from the two techniques tested were amplified together to minimise experimental differences. Seven microlitres sterile water was added to the samples extracted using the silica column method to equalise the total volume.

PCR products were visualised on 2% agarose gels and all positive products were purified using Exosap-IT™ and sequenced using the Mega BACE 1000™ system.

3. Results

No contamination was evident in any of the PCR or extraction blanks. Each PCR was repeated once to ensure that the results were consistent. Out of the 64 PCRs 43 yielded amplicons. The fished hybridised extracts provided 21 positive results and the Yang method provided 22 positive results. Expected molecular behaviour was evident, since the shorter fragment amplified nearly twice as often as the longer fragment (28:15), and in no sample was only the long fragment amplified.

4. Discussion

In this study two different ancient DNA extraction methods are compared and evaluated. The method relying on silica binding columns is commonly used on museum material of moderate age, as well as on DNA from prehistoric tissue. The other is based on a purification concept that has yet not been widely exploited in ancient DNA extractions, purifying ancient DNA with magnetic separation [23]. However, a limited number of studies on ancient DNA have employed this technique [27–29]. Here, when the two methods were applied to the same 12 ancient cattle samples, we could not detect any difference in efficiency.

The silica-binding protocol is conventional and relies on strategies that have been well tested over the last decade. The major advantages of this method compared to other ancient DNA extraction is that it is fast, low cost, and requires little hands-on time. In less then 2 days, it is possible to process a significant number of DNA extractions and subsequent PCR reactions.

The fishing method provides other advantages, at least in theory. Firstly, the extract used in the PCR is extremely pure, and only contains the specific fragment targeted, minimising co-extracted inhibitors. Secondly, all DNA from the extract may be concentrated into one PCR, since all molecules from one locus is fished out and added to the reaction. Yet another advantage is the possibility to re-use the extract for other fragments of interest by simply hybridising in the extract again with a new set of biotinylated probes. This must be considered a major advantage from an antiquarian perspective, as it will minimise the amount of bone sample needed if several markers are to be studied on the same individual. The disadvantages with the fishing-based method are practical aspects of cost and complexity. It could also be argued that the higher level of sample manipulation does add a higher risk for contamination. However, the main source for contamination is not in the laboratory, but is introduced during the excavation of the specimen, or during storage in museums or in other collection facilities [30].

Another matter that needs to be addressed is how traditional control rules should be applied. For instance, it has been suggested that a minimum of seven PCRs should be set up from one extraction of highly degraded material in order to control for allelic dropout [31]. How does this apply to fished extracts? At least in principle, it should be possible to concentrate the target DNA fragment into one PCR reaction. Is it therefore

better to concentrate all DNA into one or two amplification reactions to provide enough starting molecules to avoid allelic drop out, or should the DNA be diluted enough to provide for seven PCRs? This is a matter that needs to be investigated further, for example by quantitative PCR experiments.

Thus, even if the two methods compared here seem to be equal in efficiency, we claim that all characteristics of the fishing method have yet not been fully studied. Therefore there may be other advantages than PCR success rate that argue for fishing. Until these aspects have been investigated, the choice between these two methods must be based on factors other than efficiency.

References

[1] R. Higuchi, B. Bowman, M. Freiberger, O.A. Ryder, A.C. Wilson, DNA sequences from the quagga, an extinct member of the horse family, Nature 312 (1984) 282–284.

[2] S. Pääbo, Molecular cloning of Ancient Egyptian mummy DNA, Nature 314 (1985) 644–645.

[3] E. Hagelberg, J.B. Clegg, Isolation and characterization of DNA from archaeological bone, Proc. R. Soc. Lond. B: Biol. Sci. 244 (1991) 45–50.

[4] N. Tuross, The biochemistry of ancient DNA in bone, Experientia 50 (1994) 530–535.

[5] C. Hänni, T. Brousseau, V. Laudet, D. Stehelin, Isopropanol precipitation removes PCR inhibitors from ancient bone extracts, Nucleic Acids Res. 2 (1995) 881–882.

[6] F. Kaestle, Report on DNA analysis of the remains of ‘‘Kennewick Man’’ from Columbia Park, Washington, in: F.P. McManamon, J.C. Roberts, B.S. Blades (Eds.), Report on the DNA Testing Results of the Kennewick Human Remains from Columbia Park, Kennewick, Washington, 2000.

[7] T. Kalmár, C.Z. Bachrati, A. Marcsik, I. Rasko, A simple and efficient method for PCR amplifiable DNA extraction from ancient bones, Nucleic Acids Res. 28 (2000) E67.

[8] V.N. Petrishchev, A.B. Kutueva, G. Rychkov Iu, A simple and effective method for isolation of DNA from fossil bones for subsequent amplification using the polymerase chain reaction, Genetika 29 (1993) 690–693.

[9] M. Scholz, C. Pusch, Isolation from ancient bone samples using a mixture of Coca-Cola (TM) and gall-soap, Bulletin der Schweizerischen Gesellschaft für Anthropologie (1998).

[10] R. Boom, C.J.A. Sol, M.M.M. Salimans, C.L. Jansen, P.M.E. Wertheim-van Dillen, J. van der Noordaa, Rapid and simple method for purification of nucleic acids, J. Clin. Microbiol. 28 (1990) 495–503.

[11] M. Höss, S. Pääbo, DNA extraction from Pleistocene bones by a silica-based purification method, Nucleic Acids Res. 21 (1993) 3913–3914.

[12] D.Y. Yang, B. Eng, J.S. Waye, J.C. Dudar, S.R. Saunders, Improved DNA extraction from ancient bones using silica-based spin columns, Am. J. Phys. Anthropol. 105 (1998) 539–543.

[13] A. Götherström, A. Angerbjörn, M. Collins, K. Lidén, Bone preservation and DNA amplification, Archaeometry 44 (2002) 395–404.

[14] S. Horai, K. Hayasaka, K. Murayama, N. Wate, H. Koike, N. Nakai, DNA amplification from ancient human skeletal remains and their sequence analysis, Proc. Jpn. Acad. B 65 (1989) 229–233.

[15] D.L. Fisher, M.M. Holland, L. Mitchell, P.S. Sledzik, A.W. Wilcox, M. Wadhams, V.W. Weedn, Extraction, evaluation, and amplification of DNA from decalcified and undecalcified United States Civil War bone, J. Forensic Sci. 38 (1993) 60–68.

[16] M. Krings, A. Stone, R.W. Schmitz, H. Krainitzki, M. Stoneking, S. Pääbo, Neandertal DNA sequences and the origin of modern humans, Cell 90 (1997) 19–30.

[17] P. Persson, A method for recovery of DNA from ancient bones, Anc. DNA Newslett. 1 (1992) 31–32.

[18] A. Götherström, K. Lidén, A modified DNA extraction method for bone and teeth, J. Nord. Archaeol. Sci. 9 (1996) 53–56.

[19] H. Meijer, W.R. Perizonius, J.P. Geraedts, Recovery and identification of DNA sequences harboured in preserved ancient human bones, Biochem. Biophys. Res. Commun. 183 (1992) 367–374.

[20] M. Faerman, D. Filon, G. Kahila, C.L. Greenblatt, P. Smith, A. Oppenheim, Sex identification of archaeological human remains based on amplification of the X and Y amelogenin alleles, Gene 167 (1995) 327–332.

[21] T. Anzai, T.K. Naruse, K. Tokunaga, T. Honma, H. Baba, T. Akazawa, H. Inoko, HLA genotyping of 5,000- and 6,000-year-old ancient bones in Japan, Tissue Antigens 54 (1999) 53–58.

[22] H. Malmström, E. Svensson, T. Gilbert, E. Willerslev, A. Götherström, G. Holmlund, More on contamination: the use of asymmetric molecular behaviour to identify authentic ancient human DNA, Mol. Biol. Evol. 24 (2007) 998–1004.

[23] S. Tofanelli, L. Nencioni, Recovering ancient DNA by streptavidin-coated magnetic beads and biotinylated oligonucleotides, Anc. Biomol. 2 (1999) 307–320.

[24] A.B. Bouwman, T.A. Brown, Comparison between silica-based methods for the extraction of DNA from human bones from 18th to mid-19th century London, Anc. Biomol. 4 (2002) 173–178.

[25] J.F. Bailey, M.B. Richards, V.A. Macaulay, I.B. Colson, I.T. James, D.G. Bradley, R.E. Hedges, B.C. Sykes, Ancient DNA suggests a recent expansion of European cattle from a diverse wild progenitor species, Proc. R. Soc. Lond. B 263 (1996) 1467–1473.

[26] C.S. Troy, D.E. MacHugh, J.F. Bailey, D.A. Magee, R.T. Loftus, P. Cunningham, A.T. Chamberlain, B.C. Sykes, D.G. Bradley, Genetic evidence for near-Eastern origins of European cattle, Nature 410 (2001) 1088–1091.

[27] J. Burger, W. Rosendahl, O. Loreille, H. Hemmer, T. Eriksson, A. Götherström, J. Hiller, M.J. Collins, T. Wess, K.W. Alt, Molecular phylogeny of the extinct cave lion Panthera leo spelaea, Mol. Phylogenet. Evol. 30 (2004) 841–849.

[28] C. Anderung, A. Bouwman, P. Persson, J.M. Carretero Díaz, A.I. Ortega Martínez, J.L. Arasuaga, R. Elburg, C. Smith, H. Ellegren, A. Götherström, Prehistoric contacts over the Straits of Gibraltar indicated by genetic analysis of Iberian Bronze Age cattle, Proc. Natl. Acad. Sci. USA 102 (2005) 8431–8435.

[29] A. Götherström, C. Anderung, L. Hellborg, R. Elburg, C. Smith, D.G. Bradley, H. Ellegren, Cattle domestication in the near East was followed by hybridization with aurochs bulls in Europe, Proc. R. Soc. Lond. B 272 (2005) 2345–2350.

[30] H. Malmström, J. Storå, L. Dalén, G. Holmlund, A. Götherströmm, Extensive human DNA contamination in extracts from ancient dog bones and teeth, Mol. Biol. Evol. 22 (2005) 2040–2047.

[31] P. Taberlet, S. Griffin, B. Goossens, S. Questiau, V. Manceau Escaravage, L.P. Waits, J. Bouvet, Reliable genotyping of samples with very low DNA quantities using PCR, Nucleic Acid Res. 24 (1996) 3189–3194.