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DNA extraction from Pleistocene bones by a silica-based purification method

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The polymerase chain reaction has made it possible to include extinct species and past populations in molecular studies of phylogeny and evolution (1). This emerging field, however, is marred by problems, mainly because archaeological remains often yield no amplifiable DNA, extracts often contain components which inhibit the *Taq*. polymerase, and contamination of trace amounts of contemporary DNA can yield misleading results (2, 3). We have found that the following method, which is a modification of a protocol published by Boom *et al.* (4), is highly useful in alleviating the former two types of problems and in several cases allows the study of late Pleistocene animal remains that often are not amenable to other extraction procedures.

A layer of approximately 1 mm is removed from the surface of the bone samples by grinding with a drilling machine in order to reduce contamination from previous handling. The sample is ground to a fine powder under liquid nitrogen in a freezer mill (Spex Industries Inc., Edison, NJ). About 0.5 g of bone powder is added to 1 ml of extraction buffer consisting of 10 M guanidinium thiocyanate (GuSCN), 0.1 M Tris–HCl pH 6.4, 0.02 M EDTA pH 8.0 and 1.3% Triton X-100. This is then incubated at 60°C for one to several hours with sporadic agitation. After centrifugation for 5 min at 5,000 rpm about 500 μl of the supernatant is recovered and added to a mixture of 500 μl of extraction buffer and 40 μl silica suspension prepared as in ref. 4. The mixture is incubated for 10 min at room temperature. Subsequently, the silica pellet is washed twice with a buffer consisting of 10 M GuSCN and 0.1 M Tris–HCl, pH 6.4, twice with 70% ethanol and once with acetone. After drying the pellet at 56°C, nucleic acids are eluted at 56°C in two aliquots of 65 μl water or TE and stored at −20°C.

Five μl of the extract are added to a 30 μl PCR, in which a wax-mediated hotstart is performed where the upper phase contains BSA, 0.75 units *Taq*. polymerase (Perkin Elmer, Roche, NJ) as well as the extract. The lower phase contains primers, dNTPs and $MgCl_2$. Both phases are buffered in Tris. The final concentrations after fusion of the phases are: 67 mM Tris–HCl pH 8.8, 2 mM $MgCl_2$, 1 mM dNTPs, and 1.3 mg/ml BSA. Each of the 40 cycles consists of denaturation at 92°C for 40 s, annealing at 55°C for 60s, and extension at 72°C for 60 s. After electrophoresis of the PCR products in low-melting agarose gels, bands are cut out and melted in 100 μl of dH_2O. Five μl are added to a second 50 μl PCR which is carried out without hotstart and BSA and at 60°C annealing. The PCR products are purified using Geneclean (BIO 101 Inc., La Jolla, CA) and sequenced (5). To avoid contamination by contemporaneous DNA, extraction and PCR preparation steps are carried out in a laboratory exclusively dedicated to this purpose. Contamination is monitored by two extraction controls (mock extractions submitted to PCR) per extraction and a PCR control (PCR without extract).

This method allows the extraction of DNA from bone samples of a 25,000-year-old Alaskan member of the horse family, identified as putatively *Equus hemionus*. Unambiguous direct

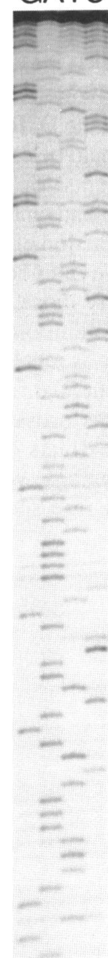

Figure 1. Direct sequencing reaction of amplification product from the mitochondrial 16S rRNA gene of a 25,000-year-old equide.

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                     10          20          30          40          50          60          70          80          90
Equus sp.    AACAAAACAA  CCTCCGAGTG  ATTTAAAT--  CTAGACTAAC  CAGTCAAAAT  ATAGAATCAC  TTATTGATCC  AAACTATTGA  TCAACGGAAC  A
E. hemionus  ..........  ..........  ..........  ..........  ..........  .C.T......  ..........  ....C.....  ..........  .
E. caballus  ..........  ..........  ..........  .C........  ..........  ...T......  ..........  ....C.....  ..........  .
B. taurus    ..T....A.T  ........C.  ....T..AGA  ......CC..  A.......TC  .CTCT...G.  .C........  ...AAC....  ..........  .
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Figure 2. Alignment of a 25,000-year-old putative *Equus hemionus*, contemporary *Equus hemionus* (10), horse (*Equus caballus*) and cow (*Bos taurus*). Dots represent sequence identity to the ancient sample.

sequence determinations were obtained, e.g. for a 91 bp fragment of the mitochondrial 16S rRNA gene (Figure 1). The sequences show this Pleistocene horse form to be closely related, but not identical, to a contemporary domestic horse, but much further from other ungulates, such as cow (Figure 2). Three other methods for extracting DNA from ancient tissues (6–8) failed in extracting amplifiable DNA from this sample. The same drastic improvement over previously used methods has been achieved also for several bone samples of an extinct ground sloth of late Pleistocene age (Höss *et al.*, in preparation) as well as for mummified remains of Native American populations (Handt *et al.*, in preparation).

It should be noted that due to the DNA-binding capacity of the silica, all buffers, but especially the GuSCN-containing solutions, are very liable to contamination by modern DNA. To overcome this, buffers are prepared in sterile 50 ml tubes and preincubated with silica for several hours in order to bind contaminating nucleic acids. After centrifugation for 3 min at 3,000 rpm, the supernatant is recovered, aliquoted and stored in the dark. It should also be noted that even minimal amounts of silica particles will inhibit the PCR. Thus, to ensure the absence of silica in the extract, it should be centrifuged for 2 min at 12,000 rpm before removing aliquots for PCR.

This procedure has several advantages for extraction of ancient DNA. Besides its high extraction efficiency, it is simple and fast and therefore allows large numbers of bone samples to be screened in order to identify those that contain surviving DNA molecules. Furthermore, inhibition of the PCR by components of archaeological extracts is abolished. We encountered no example of inhibition in about 50 different bone samples analyzed. In addition, this method has proved very useful for removing inhibitory activities from extracts of other complex sources of DNA, such as animal droppings, which can be of great use for studies of endangered and rare species (9).

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