biology letters

Molecular evolution

Biol. Lett. (2007) 3, 55–56
doi:10.1098/rsbl.2006.0537
Published online 25 October 2006

800 000 year old mammoth DNA, modern elephant DNA or PCR artefact?

Jonas Binladen, M. Thomas P. Gilbert and Eske Willerslev*

Centre for Ancient Genetics, Niels Bohr Institute and Biological Institute, University of Copenhagen, Juliane Maries vej 30, 2100 Copenhagen, Denmark
**Author for correspondence (ewillerslev@bi.ku.dk).*

Poulakakis and colleagues (Poulakakis *et al.* 2006: *Biol. Lett.* 2, 451–454), report the recovery of 'authentic' mammoth DNA from an 800 000-year-old fragment of bone excavated on the island of Crete. In light of results from other ancient DNA studies that indicate how DNA survival is unlikely in samples, which are recovered from warm environments and are relatively old (e.g. more than 100 000 years), these findings come as a great surprise. Here, we show that problems exist with the methodological approaches used in the study. First, the nested PCR technique as reported is nonsensical—one of the second round 'nested' primers falls outside the amplicon of the first round PCR. More worryingly, the binding region of one of the first round primers (Elcytb320R) falls within the short 43 base pair reported mammoth sequence, specifically covering two of the three reportedly diagnostic *Elephas* polymorphisms. Finally, we demonstrate using a simple BLAST search in GenBank that the claimed 'uniquely derived character state' for mammoths is in fact also found within modern elephants.

Keywords: ancient DNA; mammoths; taxonomy; nested PCR; Crete

1. INTRODUCTION

Poulakakis *et al.* (2006) have recently published the remarkable claim of a mammoth (*Mammuthus primigenius*) 43 base pair (bp) mitochondrial DNA (mtDNA) sequence recovered from an 800 000-year-old specimen excavated on the Greek island of Crete. This is an important finding as if correct a revision of the origin of the small elephants of Crete is warranted.

Poulakakis and colleagues obtain their sequence using a novel three-round PCR approach. In the first round, non-specific amplification of all DNA in the extraction is performed using multiple displacement amplification (MDA). In the subsequent steps, a mammoth-specific nested PCR is performed, initially amplifying a 282 bp fragment of mammoth mtDNA, followed by a second round of PCR resulting in the final 43 bp of 'novel' mammoth sequence.

On examination of the paper, we have found several remarkable aspects about the findings that lead us to question the authenticity of the recovered 43 bp DNA fragment.

2. DISCUSSION

Firstly, the warm Cretan environment (present average yearly temperature of 18–19°C. Matzarakis *et al.* 2005) contrasts strongly to the cold environments where well-preserved DNA is mainly found (Smith *et al.* 2003). Most models of DNA degradation assume that the rate of DNA degradation is exponentially linked to temperature (Lindahl 1993; Smith *et al.* 2001; Marota *et al.* 2002; Smith *et al.* 2003; Willerslev *et al.* 2004; Gilbert *et al.* 2005*a*,*b*), and experimental data seem to confirm this fact (e.g. Kumar *et al.* 2000; Hansen *et al.* 2006). Therefore, the fact that an initial 282 bp fragment could be recovered, when others struggle to get fragments half that size from cold preserved samples (e.g. Handt *et al.* 1994), is interesting.

Even if the survival of authentic DNA is accepted, we note a second problem that is apparent in the reported methodology. Ancient DNA (aDNA) studies are highly susceptible to contamination (e.g. Richards *et al.* 1995), and as such aDNA methods are designed to separate the low-level DNA containing samples and DNA extractions from the higher concentrations of DNA represented by modern DNA or PCR amplicons (e.g. Willerslev & Cooper 2005). The method section of the manuscript is written ambiguously—The authors claim that 'All procedures, except DNA sequencing' were done in aDNA conditions. This can be interpreted in two ways.

(i) Making a clear distinction between the handling of the vulnerable sample and DNA extractions in a dedicated, isolated pre-PCR facility, while running PCRs and manipulating amplified PCR products in a dedicated PCR area that is spatially isolated from the first one (following regular aDNA guidelines, e.g. Cooper & Poinar 2000)

(ii) Alternatively, the sentence could be read as sample and extraction handling being performed in the same area as the subsequent manipulation of PCR products, followed by a spatial separation of DNA sequencing (here referring to the cyclic PCR and use of a sequencing machine). The latter adds an enormous risk of contamination to any aDNA study. Clearly, this may simply be a misinterpretation, but in a field with many dubious reports (Gilbert *et al.* 2005*a*,*b*; Hebsgaard *et al.* 2005), precise reporting of methods and working techniques is vital, especially when reporting an aDNA sequence 10-fold older than majority of reported ancient sequences (c.f. Willerslev & Cooper 2005).

The third, and most worrying problem, is the three-step PCR method used. Post-MDA, the authors report the use of the primers published by Yang *et al.* 1996 (Elcytb65, Elcytb320R) to amplify an initial 282 bp fragment, on which a second nested PCR (Elcytb4_L and Elcytb4_R) is performed in order to generate a 57 bp amplicon that contains the reported 43 bp fragment. However, a simple alignment of the two primer sets with published mammoth and modern elephant sequences indicates several perplexing findings. Firstly, one of the primers used in the secondary amplification (Elcytb4_R) lies *outside* the region amplified in the first amplification (figure 1).

Received 7 June 2006
Accepted 14 August 2006

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                            1          11         21         31         41         51         61         71         81         91         101        111        121
                            |          |          |          |          |          |          |          |          |          |          |          |          |
E. maximus AB002412         CCACCCCATC TAACATCTCA ACATGAT//T ATACACACAC ATTGGACGAA ACATCTACTA TGGATCCTAC CTATACTCAG AAACCTGAAA TACAGGCATT ATATTACTAC TAATTACCAT AGCCACCGC
E. maximus D50846           .......... .......... .......//. .......... .......... .......... .........T .......... .......... C.....T... ...C...... ....C..... .........
M. primigenius D50842       .T........ .......... .......//. .......... .......... .......... ...G...... ........G. .......... ...C...... ...C...... ....C..... .........
M. primigenius AF517567                                  //. .......... .......... .......... ...G...... ........G. ...T...... ...C...... ...C...... ....C..... .........
M. primigenius AF309074                                  //. .........T .......... .......... ...G...... ........G. .......... ...C...... ...C...... ....C..... .........
CRETE                                                                                  ..... ...G...... ........G. .......... ...C....
Loxodonta spp. AY359268     .T........ C......... G......//. .......... ........G. .......... .......... ........G. .......... ...C...... .......... ....C..... .........
Loxodonta spp. AF132527     .T........ C......... .......//. .......... .......... .......... ...G...... ........G. ....T..... ...C...... .......... ....C..... .........
Loxodonta spp. AJ132954     .T........ C......... G......//. .......... ........G. .......... ...G...... ........G. .........
Elcytb65 (PCR1)             .T........ C.....A... .......//
Elcytb32OR (PCR1)                                                                                      .. ........G. ....T..... ...C.
Elcytb4_L (PCR2)                                             ...... .......... ...
Elcytb4_R (PCR2)                                                                                                                         . ....C..... .........
Poulakakis et al. sites                                                                    ↑315           ↑330           ↑345
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// - indicate the removal of 188 bp. Dots indicate identity to top sequence. 'CRETE' is the sequence reported by Poulakakis *et al.* (2006)

Figure 1. Alignment of the reported sequence together with genbank sequences of *Mammuthus*, *Elephas*, *Loxodonta* and the two primer sets from the 'nested PCR'.

Thus, the second PCR is performed on template that lacks one of the primer-binding sites. This in turn indicates the worrying notion that the final amplified and reported sequence *includes the original primer-binding site* (figure 1). Furthermore, and somewhat incredulously, two of the three nucleotide positions (no. 330 and no. 345 in figure 1) that Poulakakis *et al.* refer to as 'diagnostic' positions, which discriminate *Elephas* from *Mammuthus* and *Loxodonta* actually lie within the original primer-binding site, thus they are likely to be generated from one of the original primers. In addition, a simple BLAST search in GenBank reveals that these two nucleotide positions are also found among some modern African elephants (*Loxodonta spp*; e.g. GenBank sequence AY359268), thus they are hardly 'diagnostic' to mammoths. This is also the case for the third supposedly diagnostic nucleotide position (no. 315). Although the authors claim that this is a 'uniquely derived character state for the *Mammuthus*–Crete lineage', it is also found among some modern African elephants (*Loxodonta spp.*; e.g. GenBank sequence AJ132954).

In passing, we also note that if the primers were used as the authors report, and if the specimen is similar to the other seven published mammoth sequences that span the same region, the final amplified PCR product (excluding primers) should be 57 bp. Thus, the reported sequence apparently lacks 14 nucleotides at the terminal part of the sequence. Poulakakis *et al.* do not describe the reason for this.

To conclude, serious theoretical and methodological flaws exist in this paper, questioning the credibility of the reported sequence. Additionally, the *Mammuthus*–Crete diagnostic site is found in modern elephants. The authors rely on the use of independent replication to justify their results, the dangers of which have recently been discussed (Gilbert *et al.* 2005*a*,*b*). Therefore, we do not believe the results offer solid support for transferring *Elephas creticus* to the genus *Mammuthus*.

This work was supported by the Marie Curie Actions FP6 'Formaplex' grant (M.T.P.G.), the Wellcome Trust, UK, the Carlsberg Foundation, DK and the National Science Foundation, DK (J.B. and E.W.).

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