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It remains a mammoth DNA fragment. A reply to Binladen *et al*. (2006) and Orlando *et al*. (2006)

Nikos Poulakakis, Aris Parmakelis, Petros Lymberakis, Moysis Mylonas, Eleftherios Zouros, David S Reese, Scott Glaberman and Adalgisa Caccone

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Reply

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Binladen *et al.* (2006) and Orlando *et al.* (2006) raised doubts about the origin of a 43 bp sequence we reported in this journal (Poulakakis *et al.* 2006). Here we address their concerns.

1. BINDING SITES OF PRIMERS AND THE LENGTH OF THE SEQUENCE

Figure 1*a* and the electronic supplementary material of this note provide a detailed description of the amplification strategy and the binding sites of primers used in Poulakakis *et al.* (2006). This information answers the concerns of Binladen *et al.* (2006). We regret that some details were omitted from the original paper. We apologize for this and thank Binladen *et al.* (2006) for allowing us to rectify this mistake. The same authors question why we used only a 43 bp and not the whole amplified 56 bp fragment. The direct sequencing of PCR products produced errors at both ends of the fragment forcing us to discard 13 bp (2 bp from the 5′ and 11 bp from the 3′ end of the fragment).

2. ABILITY TO RETRIEVE DNA FROM AN 800 000 YEAR OLD BONE SPECIMEN

As Binladen *et al.* (2006) and Orlando *et al.* (2006) note, the most well-authenticated aDNA sequences are from material less than 100 000 years old from cold regions (but see Loreille *et al.* (2001) and Willerslev *et al.* (2003) for exceptions), but this age is being constantly pushed back (Valdiosera *et al.* 2006). Based on stratigraphy (Caloi *et al.* 1996; Mol *et al.* 1996), our sample was derived from an approximately 800 000- year- old bone fragment originating from a warm region. But the bone was retrieved from a cave, where environmental conditions remain relatively constant. This, combined with the advantages of the WGA method, may explain the successful retrieval of a small piece of DNA. We note that we were able to retrieve longer pieces (252–258 bp, not 282 bp as Binladen *et al.* (2006) note) from much younger bones (17 000–4200 years ago) from the equally warm areas of Tilos, Cyprus and Iraq (Poulakakis *et al.* 2006), which shows that the success of WGA falls rapidly with the age of the material.

Electronic supplementary material is available at http://dx.doi.org/10.1098/rsbl.2006.0555 or via http://www.journals.royalsoc.ac.uk. The accompanying articles can be viewed at doi:10.1098/rsbl.2006.0536 and doi:10.1098/rsbl.2006.0537.

3. aDNA PROTOCOLS AND THE POSSIBILITY OF SEQUENCE ARTEFACTS

Orlando *et al.* (2006) note that WGA may artificially insert DNA bases into retrieved aDNA fragments and that one way to detect this is cloning (Gilbert *et al.* 2005). Yet recent work (Rogaev *et al.* 2006) suggests that direct sequencing of PCR products is less likely to generate mutations of the type usually found in aDNA, after cloning of PCR fragments (type II mutations in ancient DNA: G/C to A/T changes). No such mutations were found in our sequences (figure 1). Additional support for this comes from the fact that all the three substitutions in the 43 bp sequence were in the same direction in the two independent replicates, and that the sequence from Tilos retrieved by the WGA method was identical to the one produced in a previous study (Poulakakis *et al.* 2002).

We followed all standard aDNA guidelines, corresponding to (i) in Binladen *et al.* (2006), thus minimizing the danger of contamination. The same result was obtained in two different laboratories. Binladen *et al.* (2006) dismiss the significance of this arguing by noting that contamination may have occurred prior to the extraction of DNA (Gilbert *et al.* 2005). But, if so, the contaminant would likely be *Elephas* DNA, since no *Mammuthus* tissue has ever been present in any of the two laboratories.

4. THE PHYLOGENETIC VALUE OF THE 43 BP DNA FRAGMENT

We never claimed that a randomly chosen 43 bp DNA fragment would help to resolve the taxonomic issue of the Cretan sample, as Orlando *et al.* (2006) imply. Based on all published *Mammuthus* and *Elephas* sequences, we designed primers targeted on the fragment that contained the three diagnostic sites for the two genera (G315, G330 and C345). Binladen *et al.* (2006) and Orlando *et al.* (2006) claim that the diagnostic power of these sites is small, when all variable sites of elephantid haplotypes are considered. Yet our aim was to assess the taxonomic position of our aDNA samples with regard to *Mammuthus* and *Elephas*, two genera whose monophyly is undisputed (Noro *et al.* 1998; Krause *et al.* 2006; Rogaev *et al.* 2006). *Loxodonta* has never been considered as a potential source for the Mediterranean elephantids (see electronic supplementary material). Figure 1*b* gives the alignment of the 56 bp fragment of our four samples with the consensus sequences for *Elephas* and *Mammuthus*. Even after the addition of all known elephantid haplotypes, the sites G315/G330/C345 are exclusively found in mammoths, as recognized by Orlando *et al.* (2006). In fig. 1*c* (Poulakakis *et al.* 2006), the fragments from Iraq, Tilos, Cyprus and Crete were compared to one representative sequence from each of the three genera, highlighting only the diagnostic sites 315, 330 and 345. The only variability that affects the *Elephas–Mammuthus* distinction is the presence of A in site no. 315 in the *M. primigenius* sequence U23738 (but see electronic supplementary materials). Orlando *et al.* (2006) question the validity of our phylogenetic analysis on grounds that some elephantid haplotypes were missing. We repeated the phylogenetic analysis using all known

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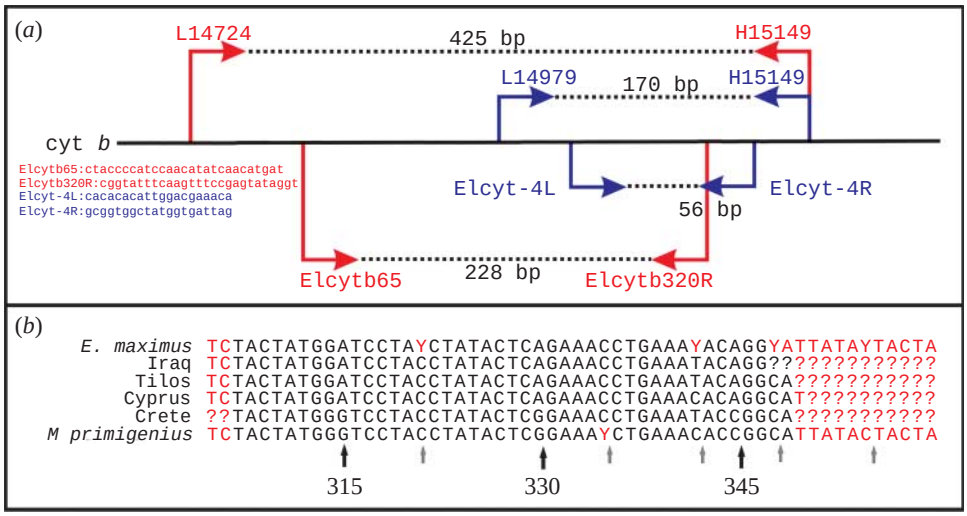

Figure 1. (*a*) Schematic representation of the cytochrome *b* gene and positions of the four primer combinations used in the study of Poulakakis *et al.* (2006). The sequences of the elephantid-specific primers and the size of PCR fragments are also indicated. The primer combination L14724/H15149 produced a fragment of 425 bp, which served as the template in a nested PCR using the primer pair Elcytb65/Elcytb320R for the production of a 228 bp fragment. The L14979/H15149 primer combination produces a 170 bp fragment. This served as the template in the nested PCR using the Elcyt-4L/Elcyt-4R primer pair, leading to the production of a 56 bp fragment. For the Tilos, Cyprus and Iraq bones, the nested PCR was successful for both the 228 bp and the 56 bp fragments. For the bone from Crete, only the PCR for the 56 bp fragment was successful. (*b*) Alignment of the 56 bp cyt *b* sequences for the Crete, Tilos, Cyprus and Iraq specimens together with the *Elephas* and *Mammuthus* consensus sequences. Questions marks represent missing data. The three *Elephas–Mammuthus* diagnostic sites (G315/G330/C345) are identified by black arrows; grey arrows identify all remaining polymorphic sites.

elephantid cyt *b* haplotypes, including newly released *Mammuthus* sequences (figure 2, see electronic supplementary material). The DNA sequence from Crete remains in a monophyletic clade that includes only *Mammuthus* sequences.

5. CONCLUSION

We acknowledge the omission of some laboratory and analytical details in our original paper (Poulakakis *et al.* 2006). However, the substance of our conclusion remains valid. The 43 bp fragment from the bone specimen from Crete clusters with *Mammuthus* sequences. This is supported from identical results in two independent laboratories.

We hope that an on-going collaboration with Michael Hofreiter (Leipzig, Germany) will lead to the retrieval of more DNA fragments from the elephantid material used in our work and that this would lead to the final settlement of the issue.

Nikos Poulakakis[1,2,*], Aris Parmakelis[1,2], Petros Lymberakis[1], Moysis Mylonas[1,2], Eleftherios Zouros[2], David S. Reese[3], Scott Glaberman[4], Adalgisa Caccone[4]

[1]*Natural History Museum of Crete, and*
[2]*Department of Biology, University of Crete, PO Box 2208, 71409 Heraklion Crete, Greece*
[3]*Peabody Museum of Natural History, and*
[4]*Department of Ecology and Evolutionary Biology and Yale Institute of Biospheric Studies, Yale University, PO Box 208118, New Haven, CT 06520-8118, USA*
**poulakakis@nhmc.uoc.gr*

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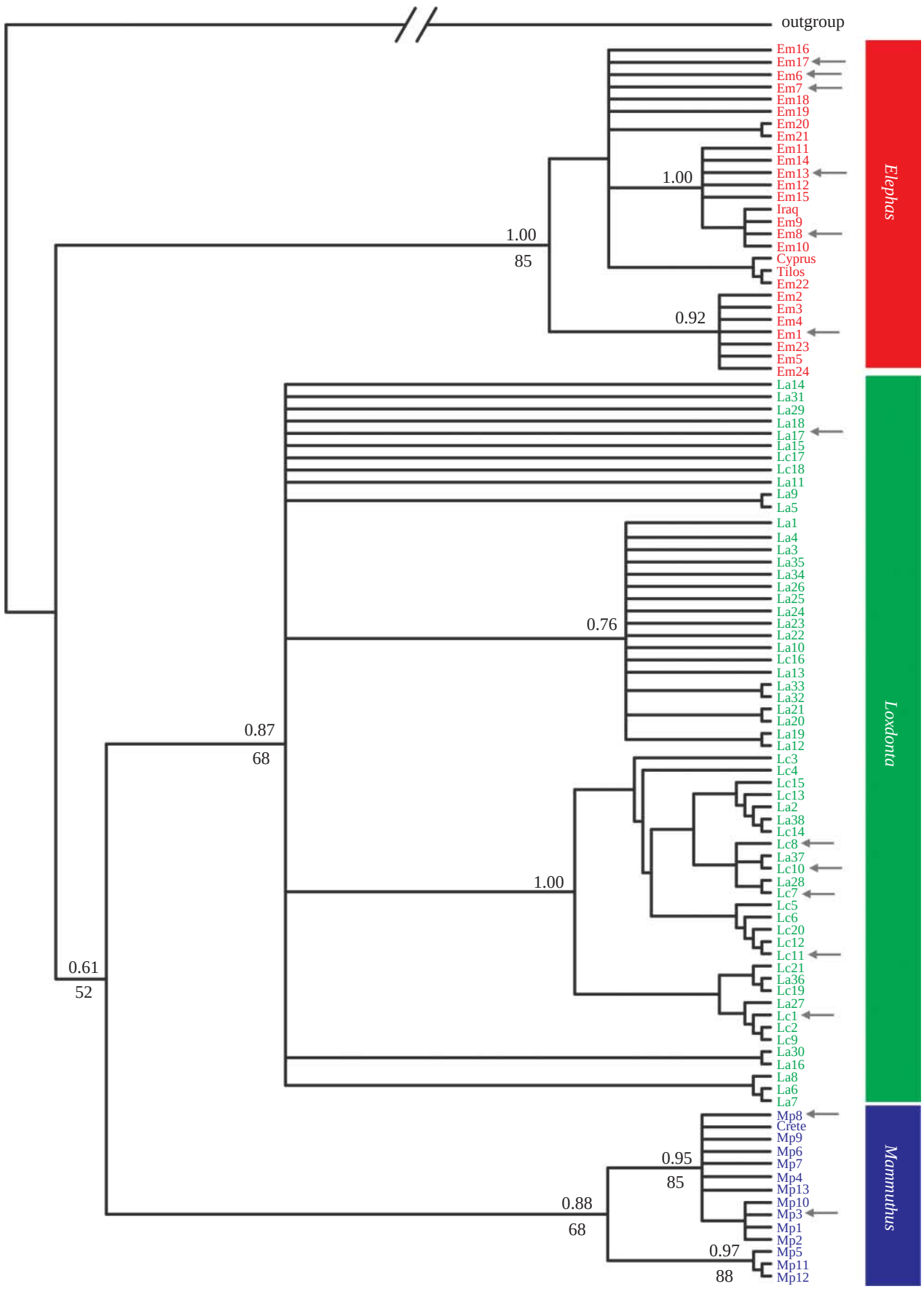

Figure 2. Bayesian Inference (BI) tree (mean ln = −32530.387) of Elephantidae using all different haplotypes that are available in GenBank (50% majority-rule consensus tree). Grey arrows indicate the sequences that Orlando *et al.* (2006) suggested for inclusion in the analysis. Em1–Em15, Lc1–Lc8, La1-2 and Mp1–Mp7 are the same sequences as in Poulakakis *et al.* (2006). Sequences Em16–Em24, Lc9–Lc21, La3–La38 and Mp8–Mp13 are new additions. The topology of the tree (ln = −32840.1539), concerning the major clades of the Elephantidae, obtained by maximum likelihood (ML) analysis was identical to the one obtained by the BI analysis. Numbers above and below branches correspond to the posterior probabilities of the BI and to bootstrap values of ML analyses, respectively.

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