

# Does the 43 bp sequence from an 800 000 year old Cretan dwarf elephantid really rewrite the textbook on mammoths?

Ludovic Orlando<sup>1,\*</sup>, Marie Pagés<sup>1,2</sup>, Sébastien Calvignac<sup>1</sup>, Sandrine Hughes<sup>1</sup> and Catherine Hänni<sup>1</sup>

<sup>1</sup>*Paleogenetics and Molecular Evolution; IFR128, Lyon, F-69007, France; Université Lyon 1, Lyon, F-69007, France; CNRS UMR 5161, INRA LA 1237, Laboratoire de Biologie Moléculaire de la Cellule, Ecole Normale Supérieure de Lyon, 46 Allée d'Italie, Lyon, F-69364 Cédex 07, France*

<sup>2</sup>*Laboratoire de Paléontologie, Paléobiologie et Phylogénie, Institut des Sciences de l'Évolution, Université Montpellier II, Place Eugène Bataillon, 34095 Montpellier Cedex 05, France*

\*Author for correspondence ([ludovic.orlando@ens-lyon.fr](mailto:ludovic.orlando@ens-lyon.fr)).

**Pigmy elephants inhabited the islands from the Mediterranean region during the Pleistocene period but became extinct in the course of the Holocene. Despite striking distinctive anatomical characteristics related to insularity, some similarities with the lineage of extant Asian elephants have suggested that pigmy elephants could be most probably seen as members of the genus *Elephas*. Poulakakis *et al.* (2006) have recently challenged this view by recovering a short mtDNA sequence from an 800 000 year old fossil of the Cretan pigmy elephant (*Elephas creticus*). According to the authors of this study, a deep taxonomic revision of Cretan dwarf elephants would be needed, as the sequence exhibits clear affinities with woolly mammoth haplotypes. However, we point here many aspects that seriously weaken the strength of the ancient DNA evidence reported.**

**Keywords:** pigmy elephants; ancient DNA; molecular phylogeny; whole genome amplification

## 1. COMMENT

Extinct dwarf elephantids from the Mediterranean islands have mostly been considered as *Elephas* rather than *Mammuthus* derivatives, suggesting continental *Elephas antiquus* as single ancestor for the colonization of the region (Poulakakis *et al.* 2002). Alternatively, dwarf elephantids might be seen as members of the *Mammuthus* progeny (Mol *et al.* 1996). The data reported by Poulakakis *et al.* 2006 aims at providing molecular support for this second scenario and a rationale for taxonomic revision of Cretan dwarf elephantids from *Elephas creticus* to *Mammuthus creticus*. However, this conclusion should not be taken for granted, both for experimental and analytical reasons.

The authors recovered a 43 bp sequence of the cytochrome *b* gene from an 800 000-year-old bone of a Cretan dwarf elephant. If true, this would extensively redefine the limit of DNA conservation in the

Mediterranean islands since, so far, such ancient authentic sequences are only known from cold environments (Willerslev *et al.* 2003, 2004) and temperate caves (Valdiosera *et al.* 2006) and until now, authentic ancient Mediterranean sequences are at best *ca* 20 000 years (Poulakakis *et al.* 2002, Lalueza-Fox *et al.* 2005).

The authors allocate their impressive finding to the GenomePhi whole genome amplification (WGA) strategy. GenomePhi WGA restores minute traces of DNA in a two-step procedure (random hexamer annealing followed by strand displacement amplification with Phi29 polymerase). Extremely low yields of DNA are the characteristic of ancient remains (Poinar *et al.* 2003). Consequently, WGA might be highly valuable for fossil genotyping. But extensive chemical degradation of nucleotidic bases (e.g. depurination, deamination) also occurs during diagenesis (Lindahl *et al.* 1993; Hoss *et al.* 1996) and leads to artefactual substitutions during the amplification process (Hofreiter *et al.* 2001; Gilbert *et al.* 2003). As virtually no template would be lesion-free in an 800 000-year-old extract, the WGA-restored DNA fragments might contain large proportions of artefactual substitutions. In this context, cloning the PCR product from at least two independent reactions and sequencing a great number of clones would have allowed the authors to distinguish between true and artefactual substitutions and/or contamination. This strategy—widely documented in the literature—is actually a prerequisite of any ancient DNA analysis, even for much more recent specimens (Gilbert *et al.* 2005). Without further experimental improvement, we should therefore look at the 43 bp sequence reported with caution before interpreting the observed substitutions as diagnostic for the taxonomy of the Cretan sample.

The authors took advantage of three sites (G315/G330/C345) to identify the fossil as a *Mammuthus* specimen. We must note, however, that the dataset used misses a large part of the published elephantid haplotypic diversity (figure 1). Contrary to what the authors presented in fig. 1c in Poulakakis *et al.* 2006, the region exhibits not three but at least nine polymorphic sites (figure 1). More specifically, G315 is not autapomorphic of mammoths since some African elephant and mammoth sequences exhibit G315 (Accession numbers D84150-LAF132954-AY741069-AJ132955) and A315 (Accession number U23738), respectively. A330 is not autapomorphic of *Elephas maximus* either since it is present in one *Loxodonta* sequence (Accession number AY359271). It is true that though the G315/G330/C345 haplotype has formally been observed only in mammoths so far, but the nearest *Loxodonta* haplotypes (ht1 and ht3; figure 1) are only one substitution away, and *Loxodonta* ht6 even exhibits perfect identity over 34 nucleotides (figure 1). Moreover, haplotypes display up to four nucleotidic differences in a given genus (figure 1). Consequently, we find the support for taxonomic revision from *Elephas creticus* to *Mammuthus creticus* rather weak, and similarly suggestive of a *Loxodonta* specimen.

Finally, very short sequences might mislead taxonomic identification, as illustrated later. Following scrupulous respect of authentication standards, we recovered a 269 bp control region sequence of a brown



We are indebted to Marilyne Duffraisse and Eric Morency for critical reading of the manuscript.

- Calvignac, S., Hughes, S., Tougard, C., Michaux, J., Thévenot, M., Hamdine, W. & Hänni, C. Submitted. Ancient DNA evidences the loss of a highly divergent brown bear clade during historical times. *Mol. Ecol.*
- Capelli, C., Macphee, R., Roca, A., Brisighelli, F., Georgiadis, N., O'Brien, S. J. & Greenwood, A. D. 2006 A nuclear DNA phylogeny of the woolly mammoth (*Mammuthus primigenius*). *Mol. Phylogenet. Evol.* **40**, 620–627.
- Gilbert, M. T., Hansen, A., Willerslev, E., Rudbeck, L., Barnes, I., Lynnerup, N. & Cooper, A. 2003 Characterization of genetic miscoding lesions caused by postmortem damage. *Am. J. Hum. Genet.* **72**, 48–61. (doi:10.1086/345379)
- Gilbert, M. T., Bandelt, H., Hofreiter, M. & Barnes, I. 2005 Assessing ancient DNA studies. *Trends Ecol. Evol.* **20**, 541–544. (doi:10.1016/j.tree.2005.07.005)
- Greenwood, A. D., Capelli, C., Possnert, G. & Pääbo, S. 1999 Nuclear DNA sequences from late Pleistocene megafauna. *Mol. Biol. Evol.* **16**, 1466–1473.
- Hofreiter, M., Jaenicke, V., Serre, D., Haeseler Av, A. & Pääbo, S. 2001 DNA sequences from multiple amplifications reveal artifacts induced by cytosine deamination in ancient DNA. *Nucleic Acids Res.* **29**, 4793–4799. (doi:10.1093/nar/29.23.4793)
- Hoss, M., Jaruga, P., Zastawny, T. H., Dizdaroglu, M. & Pääbo, S. 1996 DNA damage and DNA sequence retrieval from ancient tissues. *Nucleic Acids Res.* **24**, 1304–1307. (doi:10.1093/nar/24.7.1304)
- Krause, J. *et al.* 2006 Multiplex amplification of the mammoth mitochondrial genome and the evolution of Elephantidae. *Nature* **439**, 724–727. (doi:10.1038/nature04432)
- Lalueza-Fox, C., Castresana, J., Sampietro, L., Marques-Bonet, T., Alcover, J. A. & Bertranpetit, J. 2005 Molecular dating of caprines using ancient DNA sequences of *Myotragus balearicus* an extinct endemic Balearic mammal. *BMC Evol. Biol.* **5**, 70.
- Lindahl, T. 1993 Recovery of antediluvian DNA. *Nature* **365**, 700. (doi:10.1038/365700a0)
- Mol, D., DeVos, J., van den Bergh, G. D. & Sondaar, P. Y. 1996 The taxonomy and ancestry of the fossil elephants of Crete: faunal turnover and a comparison with proboscidean faunas of Indonesian islands. In *Pleistocene and Holocene fauna of Crete and its first settlers* (ed. D. S. Reese), pp. 81–98. Madison, WI: Prehistory Press.
- Noonan, J. P. *et al.* 2005 Genomic sequencing of Pleistocene cave bears. *Science* **309**, 597–599. (doi:10.1126/science.1113485)
- Poinar, H., Kuch, M., McDonald, G., Martin, P. & Pääbo, S. 2003 Nuclear gene sequences from a late Pleistocene sloth coprolite. *Curr. Biol.* **13**, 1150–1152. (doi:10.1016/S0960-9822(03)00450-0)
- Poinar, H. *et al.* 2006 Metagenomics to paleogenomics: large-scale sequencing of mammoth DNA. *Science* **311**, 392–394. (doi:10.1126/science.1123360)
- Poulakakis, N., Theodorou, G. E., Zouros, E. & Mylonas, M. 2002 Molecular phylogeny of the extinct Pleistocene dwarf elephant *Palaeoloxodon antiquus falconeri* from Tilos Island, Dodekanisa, Greece. *J. Mol. Evol.* **55**, 364–374. (doi:10.1007/s00239-002-2337-x)
- Poulakakis, N., Parmakelis, A., Lymberakis, P., Mylonas, M., Zouros, E., Reese, D., Glaberman, S. & Caccone, A. 2006 Ancient DNA forces reconsideration of evolutionary history of Mediterranean pygmy elephants. *Biol. Lett.* **2**, 451–454. (doi:10.1098/rsbl.2006.0467)
- Rogaev, E., Moliaka, Y., Malyarchuk, B., Kondrashov, F., Derenko, M., Chumakov, I. & Grigorenko, A. 2006 Complete mitochondrial genome and phylogeny of Pleistocene Mammoth *Mammuthus primigenius*. *PLoS Biol.* **4**, e73. (doi:10.1371/journal.pbio.0040073)
- Römpler, H., Rohland, N., Lalueza-Fox, C., Willerslev, E., Kuznetsova, T., Rabeder, G., Bertranpetit, J., Schöneberg, T. & Hofreiter, M. 2006 Nuclear gene indicates coat-color polymorphism in mammoths. *Science* **313**, 62. (doi:10.1126/science.1128994)
- Valdiosera, C., García, N., Dalén, L., Smith, C., Kahlke, R.-D., Lidén, K., Angerbjörn, A., Arsuaga, J.-L. & Götherström, A. 2006 Typing single polymorphic nucleotides in mitochondrial DNA as a way to access Middle Pleistocene DNA. *Biol. Lett.* **2**, 601–603. (doi:10.1098/rsbl.2006.0515)
- Waits, L., Talbot, S. L., Ward, R. & Shields, G. F. 1998 Mitochondrial DNA Phylogeography of the North American brown bear and implications for conservation. *Conserv. Biol.* **12**, 408–417. (doi:10.1046/j.1523-1739.1998.96351.x)
- Willerslev, E. *et al.* 2003 Diverse plant and animal genetic records from Holocene and Pleistocene sediments. *Science* **300**, 791–795. (doi:10.1126/science.1084114)
- Willerslev, E., Hansen, A. & Poinar, H. 2004 Isolation of nucleic acids and cultures from fossil ice and permafrost. *Trends Ecol. Evol.* **19**, 141–147. (doi:10.1016/j.tree.2003.11.010)