

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

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**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

		3		3		3
		1		3		4
		5		0		5
<b>CRETE</b>		<b>TACTATGGGTCTTACCTATACTCGGAAACCTGAAATACCGGCA</b>				
<i>Elephas maximus</i>	ht1	.....A.....	.....A.....	.....A.....	.....A.....	.....A.....
	ht2	.....A.....	.....A.....	.....NNNNNNNNN	.....NNNNNNNNN	.....NNNNNNNNN
	ht3	.....A.....	.....T.....	.....A.....	.....NNNNNNNNN	.....NNNNNNNNN
	ht4	.....A.....	.....T.....	.....A.....	.....C..A.....	.....C..A.....
	ht5	.....A.....	.....T.....	.....A.....	.....C..A..T.	.....C..A..T.
	* ht6	.....A.....	.....A.....	.....A.....	.....C..A..T.	.....C..A..T.
<i>Loxodonta sp.</i>	ht1	.....A.....	.....A.....	.....A.....	.....A.....	.....A.....
	* ht2	.....A.....	.....A.....	.....A.....	.....A.....	.....A.....
	ht3	.....A.....	.....A.....	.....T.....	.....T.....	.....T.....
	ht4	.....A.....	.....A.....	.....T.....	.....T.....	.....T.....
	ht5	.....A.....	.....A.....	.....T.....	.....NNNNNNNNN	.....NNNNNNNNN
	* ht6	.....A.....	.....A.....	.....A.....	.....NNNNNNNNN	.....NNNNNNNNN
<i>Mammuthus primigenius</i>	ht1	.....A.....	.....A.....	.....A.....	.....A.....	.....A.....
	ht2	.....A.....	.....A.....	.....T.....	.....T.....	.....T.....
	* ht3	.....A.....	.....NNNNNNNNN	.....NNNNNNNNN	.....NNNNNNNNN	.....NNNNNNNNN
<i>Mammuthus americanus</i>	ht	.....C..A.....	.....NNNNNNNNN	.....NNNNNNNNN	.....NNNNNNNNN	.....NNNNNNNNN

Figure 1. Alignment of cytochrome *b* haplotypes of extant and extinct elephantids. The asterisk refers to haplotypes absent from the Poulakakis *et al.* (2006) dataset. Missing data are reported with N. Accession numbers: *Elephas maximus*, ht1 = AF132522, ht2 = Y13886, ht3 = Y13883, ht4 = AF132520, ht5 = D50846, ht6 = AF298855; *Loxodonta sp.*, ht1 = AY359266, ht2 = AY359271, ht3 = D84150, ht4 = AY741069, ht5 = AJ132955, ht6 = LAF132954; *Mammuthus primigenius*, ht1 = DQ316067, ht2 = AF517567, ht3 = U23738; *Mammuthus americanus*, ht = U23737.

		1		2		3		4	4
		1		0		0		0	3
<b>Takouatz</b>		<b>ACTACTATTT TACTCCATGT CCTATTCATT TCATATATAC CAT</b>							
<i>Ursus maritimus</i> (AF303111)		.....	.....	.....	.....	.....	.....	.....	.....
Clade I (Lineage.8, W)		.....	.....C..	.....	.....	.....	.....	.....	.....
Clade II (X75872)		.....	.....	.....T.	.....	.....	.....	.....	.....
Clade II (X75869)		.....	.....C..	.....T.	.....	.....	.....	.....	.....
Clade II (Lineage.26, W)		.....	.....C..T.	.....T.	.....	.....	.....	.....	.....
Clade II (Lineage.27, W)		.....	.....C..T.	.....T.	.....	.....	.....	.....T	.....
Clade II (Lineage.31, W)		.....	.....C..	.....T.	.....	.....	.....	.....	.....
Clade III (Lineage.21, W)		.....	.....C..	.....	.....	.....	.....	.....	.....
Clade IV (Lineage.37, W)		.....T.	.....	.....T.	.....	.....	.....	.....	.....
Clade V (X75864)		.....	.....G..	.....T.	.....	.....	.....	.....	.....
Clade V (X75871)		.....	.....C..G..	.....T.	.....	.....	.....	.....	.....
Clade V (X75868)		.....C.	.....C..G..	.....T.	.....	.....	.....	.....	.....
Clade V (X75878)		.....	.....C..GC.	.....T.	.....	.....	.....	.....	.....

Figure 2. Alignment of mtDNA CR haplotypes of extant and extinct bears. Takouatz refers to an authentic sequence of a brown bear (*Ursus arctos*) that lived in the Algerian Atlas approximately 7500 years ago. Roman numbers refer to the common names of the different brown bear clades. Accession numbers are given between parentheses when available. Some sequences (identified by a W in our alignment) are not available from GenBank but only upon request from Waits *et al.* (1998).

bear (*Ursus arctos*) that lived in North Africa 7500 years ago (Takouatz, figure 2, Calvignac *et al.* submitted). Considering only the first 43 bp misidentifies the specimen as a polar bear (*Ursus maritimus*). Interestingly, the phylogenetic analysis reported by Poulakakis *et al.* 2006 presents mammoths as the sister species of African and not Asian elephants, contrarily to what complete mitochondrial genomes (Krause *et al.* 2006; Rogaev *et al.* 2006), nuclear genes (Capelli *et al.* 2006) and genomic screenings (Poinar *et al.* 2006)

have recently suggested. Thus, we highly recommend gathering further information (at the mtDNA and nuclear levels) before definitely revising the taxonomy of Cretan dwarf elephants. Such information would concomitantly demonstrate the real efficiency of the WGA approach. We hope to see such data soon since this strategy would considerably facilitate access to ancient nuclear loci, which are still only rarely studied (Greenwood *et al.* 1999; Noonan *et al.* 2005; Capelli *et al.* 2006; Poinar *et al.* 2006; Römpler *et al.* 2006).

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