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Protein and nucleic acid sequences of woolly mammoth cytochrome band the phylogenetic position of *Mammuthus* within the Elephantidae

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The phylogenetic position of *Mammuthus* has remained under debate. Previously published DNA sequences did not provide clear evidence, neither for an association to the African, nor to the Asian elephantid clade. A 335 bp sequence of mitochondrial cytochrome b, which was found identical in three individuals of *Mammuthus primigenius*, had a large number of derived bases as well as four synapomorphic amino acids in common with *Elephas maximus*. It is concluded that *Elephas* and *Mammuthus* may comprise a monophyletic group.

Eiwit en nucleinezuur sequenties van het cytochroom b van de wolharige mammoet en de fyloge - netische positie van het genus Mammuthus *binnen de familie Elephantidae* -
De fylogenetische positie van het genus *Mammuthus* is lang een punt van discussie geweest. De tot nu toe gepubliceerde DNA-sequences brachten geen duidelijkheid, noch voor een verband met de groep van de Afrikaanse olifant, noch met die van de Aziatische olifant. In een DNA-sequence van 335 baseparen van mitochondriaal cytochroom b, die identiek bleek te zijn in drie mammoet-individuen, is een groot aantal afgeleide basen alsmede vier synapomorfe aminozuren hetzelfe als in *Elephas maximus*. De conclusie is dat *Elephas* en *Mammuthus* een monofyletische groep vormen.

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INTRODUCTION

Recently several groups of researchers have published DNA sequences from frozen mammoth carcasses. All of them chose mitochondrial genes that have a higher probability to be accessed over time than single copy nuclear genes. Höss *et al.* (1994) sequenced 93 base pairs of the 12S rRNA gene from four different mammoths of different geological ages. Differences of up to five bases within *Mammuthus* made them suspect that the samples might represent different geographical or temporal subspecies. No phylogenetic conclu-

sions were drawn. Hagelberg *et al.* (1994) sequenced 283 base pairs (bp) of cytochrome b from two mammoths of comparatively high age (beyond the range of radiocarbon dating). They found five variable bases within *Mammuthus* and a slightly higher similarity of mammoth cytochrome b with the orthologous sequence of *Loxodonta* than with that of *Elephas*. The same preliminary result was found by Hauf *et al.* (1995) who sequenced a different fragment of cytochrome b of 118 bp from a late Pleistocene mammoth carcass from Shandrin River (Yakutia). Yang *et al.* (1996) sequenced a fragment of 228 bp of cytochrome b of another mammoth. Though their gene segment largely overlaps with the one of Hagelberg *et al.* (1994), there are significant differences between the sequences of both groups. Using *Mammut americanum* as an outgroup, Yang *et al.* (1996) conclude that their sequence supports a monophyletic Asian *Elephas-Mammuthus* clade. These differing results indicate that additional sequence data of more individual mammoths are needed before conclusions about the phylogenetic position of *Mammuthus* could be drawn. In order to pursue that goal, we continued sequencing a longer part of the cytochrome b gene from three different mammoth carcasses. One of it was obtained from Wrangel Island, where a population of dwarf mammoths had survived the into the Holocene until about 3700 yBP (Vartanyan *et al.* 1995). This population had been described as a subspecies of *Mammuthus primigenius* by Garutt *et al.* (1993).

MATERIALS AND METHODS

DNA was extracted from bone samples of the following mammoth remains:

Mammuthus primigenius primigenius BLUMENBACH, 1799 from Shandrin River, Yakutia, late Pleistocene (radiocarbon dating 28,230 yBP);

M. p. primigenius from Machsounocha River, Yakutia, late Pleistocene (radiocarbon dating 27,330 yBP);

M. p. vrangeliensis GARRUT, AVERIANOV & VARTANYAN, 1993 from Wrangel Island, Russian far east, Holocene (radio carbon dating 7,710 yBP);

a blood sample of *Elephas maximus* from Cologne Zoo.

Mammoth DNA was prepared by a silica-based purification method following the protocol of Höss & Pääbo (1993).
The following precautions were taken in order to avoid contamination:

- Sterile, disposable plastic tubes were used for buffer preparation and aliquotation.
- Glassware, if unavoidable, was sterilized by heat at 180°C for 4h.
- The silica fraction was distributed in sterile, DNA-free reaction tubes (Biopur, Eppendorf).
- Tools for drilling bone were sterilized by open flame and disposed after use.
- DNA was prepared in disposable sterile, DNA-free reaction tubes.

Elephant DNA was prepared with the Dynabeads® DNA DIRECT™- Kit (Dynal®, Oslo, Norway). The kit was exclusively used for preparation of *Elephas maximus* DNA. DNA was amplified by PCR (Saiki *et al.* 1985) using the selective elephant-specific forward primer described previously (Hauf *et al.* 1995) and a newly designed reverse primer covering positions 878 through 853 (5' GCTAGGACACCTCCTAGTTTGTTAGG 3'). The conditions for this initial amplification were the same as in previous experiments (Hauf *et al.* 1995). The resulting 374 bp fragments were amplified successfully 23/26/28 times from each mammoth sample, and 20 times from the sample of *Elephas maximus*. These subsequent amplifications were performed without the addition of BSA. The amplification products were electrophoresed, the desired band excised, purified with the Geneclean II®-Kit (Dianova) and dissolved in 16 µl of double distilled water. The reaction conditions were the same for both, elephant and mammoth DNA. Five µl of the dissolved DNA served as the template for further amplification. Ten µl of the dissolved DNA were used for sequencing according to Sanger *et al.* (1977) as modified by Bachmann *et al.* (1990) and Casanova *et al.* (1990). Primers used for sequencing were the PCR primers as well as two additional primers covering positions 612 to 631

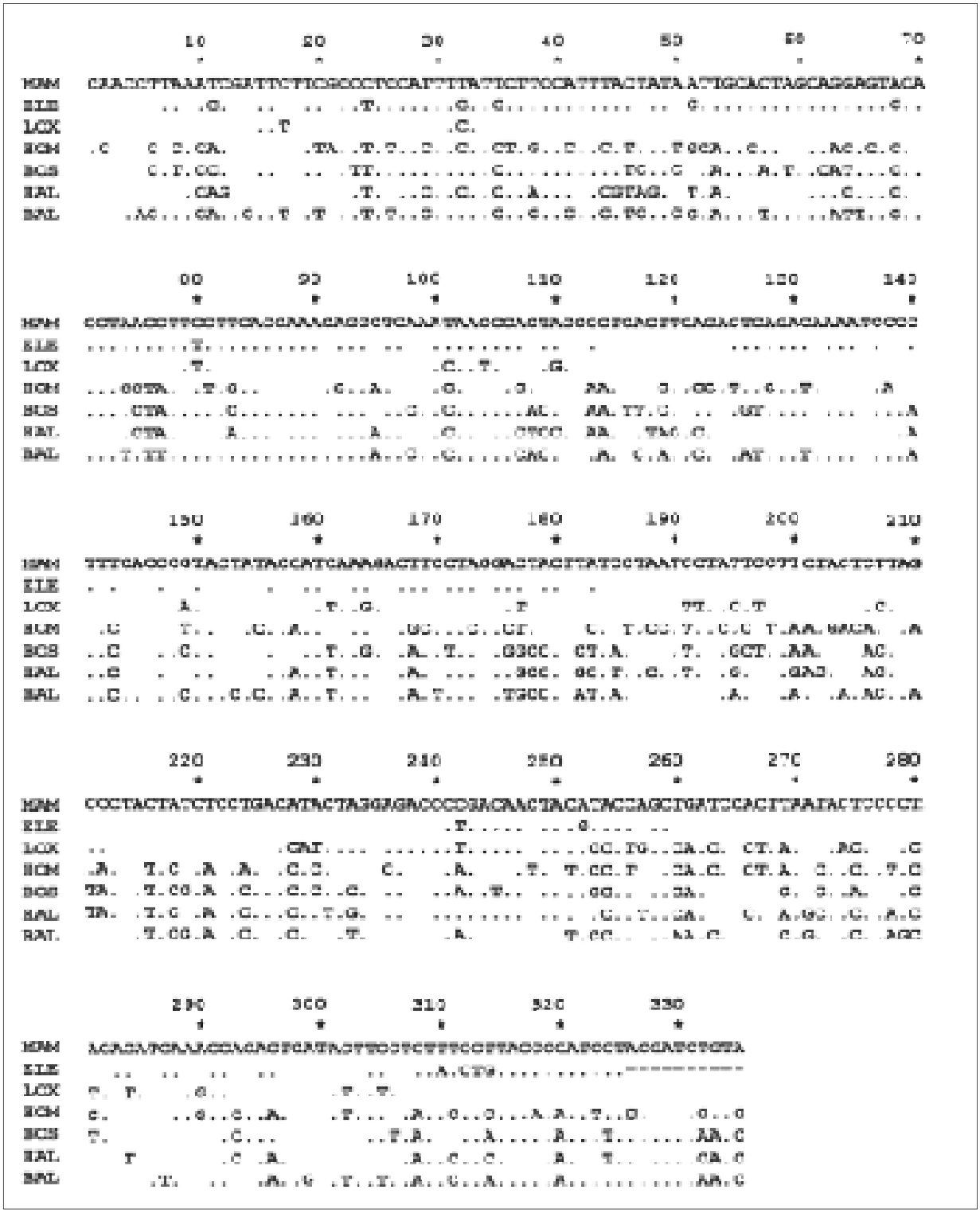

Figure 1 Aligned cytochrome b sequences of *Mammuthus primigenius* (**MAM**, 3 individuals), *Elephas maximus* (**ELE**), *Loxodonta africana* (**LOX**), *Homo sapiens* (**HOM**), *Bos taurus* (**BOS**), *Halichoerus grypus* (**HAL**), and *Balaenoptera physalus* (**BAL**). The sequence shown covers positions 518 to 852 according to the published sequence of *Loxodonta africana*. The human sequence is shown for reference only, the last three sequences were taken as outgroups for the phylogenetic analysis.

(5' CTCCAAATAACCCACTAGGCC 3') and positions 771 to 748 (5' GTG(AT)AGTTGTC(AT)GGGTCTCCTAG 3') respectively. Alignments were made with the 'Align Plus'-programme (version 2.0, Scientific & Educational Software, 1992). Amino acid predictions were made using the mammalian mitochondrial code (Anderson *et al.* 1981). The following published sequence data were used for comparison:
Loxodonta africana (EMBL acc. no. X56285)
Bos taurus (acc. no. V00654)
Halichoerus grypus (acc. no. X72004)
Balaenoptera physalus (acc. no. X61145)
Homo sapiens (acc. no. D38112).
Aligned sequences were analyzed phylogenetically according to the cladistic principle that only synapomorphic (shared derived) character states contain phylogenetic information (Hennig 1950). Base triplets were taken as individual characters with their variants as alternative states. If one of the elephantids shared a state with the outgroup *Bos taurus*, it was regarded as plesiomorphic, and the orthologous states of the other elephantids were regarded as apomorphic. In cases *Bos taurus* was itself apomorphic (e.g., different from elephantids, carnivores and whales) but one of the other outgroups (*Halichoerus grypus*, *Balaenoptera physalis*) shared a state of the respective triplet with elephantids, the latter state was regarded as plesiomorphic. The use of a computer program for tree construction was found neither necessary nor desirable, as only few sequences had to be compared.

RESULTS AND DISCUSSION

The aligned DNA sequences of Woolly Mammoth, African and Asian Elephant and of the outgroups are shown in Figure 1. Figure 2 shows the respective amino acid sequence. The three individual sequences of *Mammuthus* were identical. This is astonishing, in particular as with *M. primigenius vrangeliensis*, a distinct subspecies is involved. Previous authors (see below) reported instead a high intraspecific variability in mammoth mitochondrial genes. There are four possible alternative explanations for this discrepancy:
(1) Our sequence portion might be less variable than the ones investigated by others;
(2) Other authors used mammoth remains from a greater time range than ours;
(3) Previous authors might have amplified partially degraded DNA or used a polymerase with a higher error rate;
(4) We might have amplified the same individual sequence several times, instead of three different ones (contamination of tools or buffer solution). We cannot completely exclude possibility 4, although we used our possibilities to avoid contamination. A solution will be sought with an independent repetition and with additional samples.

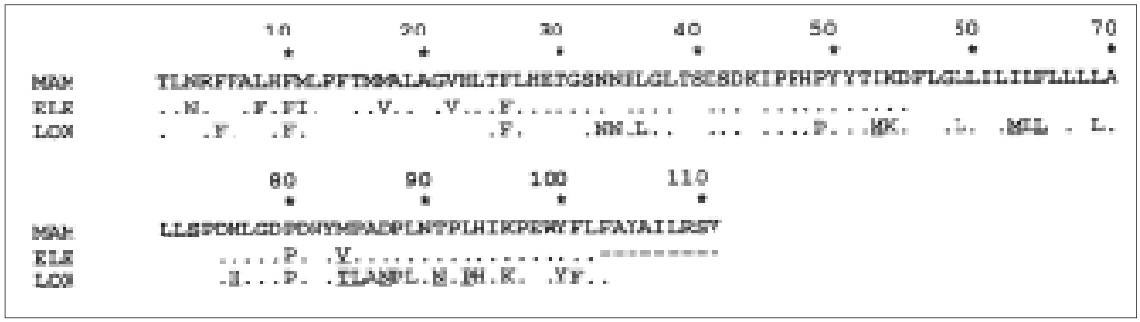

Figure 2 Amino acid sequence of cytochrome b of the investigated elephantids. The sequence covers the amino acid positions 174 to 284 of the published sequence of *Loxodonta africana*. Differences from *Mammuthus* are underlined. All amino acids of ELE and LOX denote sites with silent mutations with different nucleotide sequences, whereas the amino acid sequence remains unchanged.

Table 1 Possible synapomorphies in cytochrome b sequences.

	Mam + Lox	Ele + Lox	Mam + Ele
Hagelberg *et al.*, 1994 (283 bp)[1]	2 or 3 bases	1 base	2 bases
Yang *et al.*, 1996 (228 bp)[1]	2 bases	none	3 bases
both Hagelberg and Yang *et al.*[1]	(1 base)[2]	none	1 base[3]
this study (335 bp)	3 to 5 bases[4] 1 transversion 1 amino acid	1 base[5] no transversion no amino acid	16 or 17 bases[6] 3 transversions 4 amino acids

1) reanalyzed using the sequence of *Mammut americanum* (Yang *et al.*, 1996) as outgroup, not counting sites which vary within *Mammuthus primigenius*.

2) In Hagelberg's data set, one mammoth individual is plesiomorphic at this site (no. 112).

3) Site no. 14,920 of Yang *et al.* (1996).

4) Sites no. 11, 24, 35, (68), (314)

5) Site no. 80

6) Sites no. 101, (149), 161, 191, 195, 197, 207, 228, 230, 253, 254, 260, 261, 269, 275, 280, 281

Table 1 gives the numbers of possible synapomorphies for pairs of elephantids taken from our sequence of cytochrome b and from those of previous publications. Yang *et al.* (1996) state that their choice of the Mastodontid *Mammut americanum* as outgroup prevented them from being misled in the phylogenetic interpretation of mammoth sequences. As Hagelberg*et al.* (1994) sequenced the same part of the cytochrome b gene as Yang *et al.*, we reanalyzed their data using the Mammut sequence of Yang *et al.* (1996) as the plesiomorphic state. Even with this new outgroup, the mammoth sequences of Hagelberg *et al.* still contain more possible synapomorphies for *Mammuthus* and *Loxodonta* than for a *Mammuthus-Elephas* clade. The two researchers groups' sequence data are so different that when their four orthologous mammoth sequences are compared, only one of their possible synapomorphies with *Elephas* remains unambiguous (i.e., shared by all four *Mammuthus* and two *Elephas* individuals, and not by *Mammut* and *Loxodonta*.). In addition, one of the two possible synapomorphies of *Loxodonta* and *Mammuthus* of Yang *et al.* (1996) is shared by one of the mammoth sequences of Hagelberg *et al.* (1994) Both of these base substitutions are silent transitions and can therefore be regarded as weak characters which can easily evolve several times independently. Therefore, contrary to the opinion of Yang *et al.* (1996), we conclude that their database is not sufficient to solve the question of the phylogenetic position of *Mammuthus*. The same must be said about our preliminary data (Hauf *et al.* 1995).

In contrast, our new data set points to another direction (although we lack an orthologous mastodontid sequence). The overwhelming number of possible synapomorphies in our sequence provide evidence in favour of a monophyletic clade [*Elephas-Mammuthus*] (Table 1). The number of four synapomorphic amino acids as well as three transver-

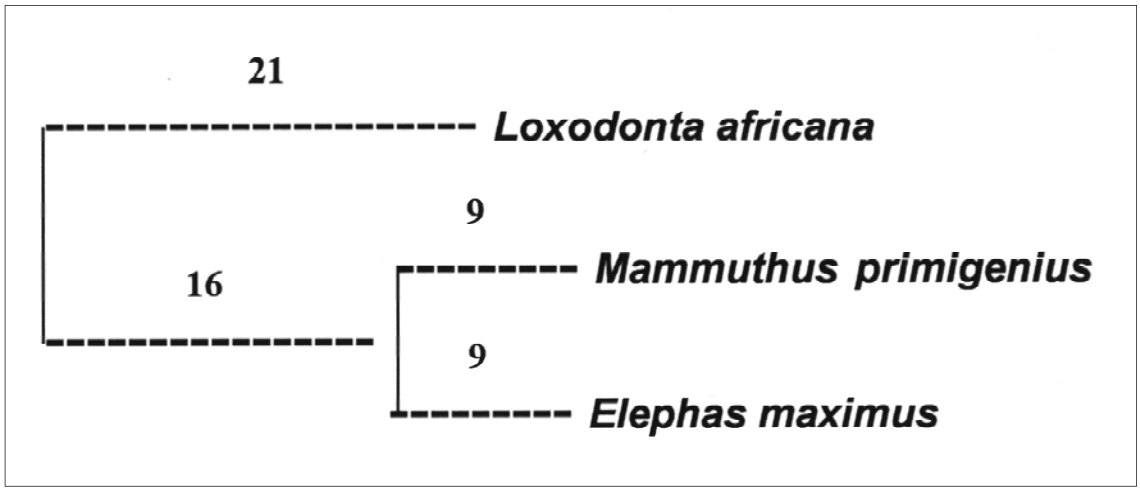

Figure 3 Phylogenetic relationships of elephantid cytochrome b sequences. Additive cladogram, composed of apomorphic bases (numbers). The few apomorphic bases shared by *Loxodonta* with *Mammuthus* or *Elephas* are regarded as convergent autapomorphies. At two sites, all three species apparently had autapomorphic bases.

sions give a high support for this clade. Mutations supporting a *Mammuthus-Loxodonta* assemblage are concentrated to the first 100 bases of our sequence. On total evidence, the phylogram depicted in Figure 3 appears the most probable. The *Mammuthus-Elephas* lineage shows an increased evolutionary rate, which had already been noticed by Yang *et al.* (1996).

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Note added in proof

The cytochrome b sequence from *Loxodonta africana* (Irwin *et al.* 1991) proved to be wrong in several positions after sequencing the whole mitochondrial genome of *Loxodonta africana* in our lab. Therefore, some of the conclusions drawn in the paper may be newly evaluated.

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