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Phylogenetic position of *Elephas, Loxodonta* and *Mammuthus*, based on molecular evidence

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SUMMARY: Previous authors have disagreed about the phylogenetic relationships between *Mammuthus, Elephas* and *Loxodonta*. Phylogenies based on the mitochondrial cytochrome b gene were contradictive. We sequenced a 450 base pair fragment of the mitochondrial control region. A phylogenetic tree based on 76 parsimony informative characters shows a well supported sister group relationship of *Mammuthus* with *Elephas*, whereas *Loxodonta* retains a basal position.

1. Introduction

The three main genera of Elephantidae, *Elephas, Loxodonta* and *Mammuthus*, originated during the Pliocene from the African genus *Primelephas* (Maglio 1973). *Elephas* and *Mammuthus* migrated to Eurasia and became extinct in Africa, where only *Loxodonta* persisted.

The question which of the two living genera of elephantids, *Elephas* or *Loxodonta*, is closer to *Mammuthus* phylogenetically, or whether *Mammuthus* branched off from the common stem first, is a simple three-taxon problem. However, despite a number of efforts, this problem has presented considerable resistance to being solved.

As *Elephas* and *Mammuthus* share a relatively derived molar structure, they were traditionally grouped together (Shoshani *et al.* 1985). In another morphological character, the shape of the tip of the trunk, *Mammuthus* is more similar to *Loxodonta* (Vereshagin & Tikhonov 1990).

Well preserved mammoth remains from the Siberian permafrost soil do not only allow the reconstruction of soft tissue anatomy like trunk shape, but also extraction of intact pieces of macromolecules. Therefore in recent years, several teams have begun sequencing fragments of mammoth DNA, preferably mitochondrial DNA, which is present in multiple copies in each living cell and has a good chance of being preserved many years after death. Table 1 gives an overview of these studies and their differing results. It is noteworthy that, even when the same genes were sequenced (cytochrome b in most cases), the results were strikingly different: Ozawa *et al.* (1997) identified a monophyletic group *Elephas-Mammuthus*, whereas Noro *et al.* (1998), using more or less the same fragment but different individuals, found a clade *Loxodonta-Mammuthus*. Both groups reported a good bootstrap support for their results, but suffered from low number of individuals analysed (one or two mammoth samples only). Recently, we re-analysed the data of Ozawa *et al.*, but used our own new *Loxodonta* sequence for comparison (Hauf *et al.* 2000), which resulted in an association *Elephas-Loxodonta*, whereas *Mammuthus* occupied a more basal position.

Thus cytochrome b appears to support either of the three alternative groupings, depending on the particular sequences used for comparison and possibly also on the particular region of this gene chosen (see Joger *et al.* in press). We therefore decided to sequence a non-coding mitochondrial region, the control region (d-loop) in order to have an independent indicator for phylogenetic relationships.

Tab.1 - Attempts to determine the phylogenetic position of *Mammuthus* within the Elephantidae using mitochondrial DNA sequences.

Year	Authors	No of sequenced base pairs	Gene	Results: sister groups
1994	Höss et al.	92	16s RNA	not resolved
1994	Hagelberg et al.	283	cytochrome b	uncertain (*Mammuthus-Loxodonta*?)
1995	Hauf et al.	115	cytochrome b	uncertain (*Mammuthus-Loxodonta*?)
1996	Yang et al.	228	cytochrome b	*Mammuthus-Elephas*
1997	Derenko et al.	331	cytochrome b	not resolved
1997	Ozawa et al.	1005	cytochrome b	*Mammuthus-Elephas*
1998	Noro et al.	1137	cytochrome b	*Mammuthus-Loxodonta*
		961	12s RNA	*Mammuthus Loxodonta*
1999	Hauf et al.	335	cytochrome b	*Mammuthus-Elephas*
1999	Barriel et al.	varying	cytochrome b	uncertain *(Mammuthus-Loxodonta*?)
2000	Thomas et al.	545	cytochrome b	uncertain (*Mammuthus-Loxodonta*?)
2000	Hauf et al.	1005	cytochrome b	uncertain (*Elephas-Loxodonta*?)

2. Materials and Methods

Bones of eight mammoth individuals from Wrangel island, Chukotka (NE Siberia) were used as sources of DNA. These bones belong to the subspecies *Mammuthus primigenius vrangeliensis* Garrutt, Averianov & Vartanyan 1993, which survived well into the Holocene (Vartanyan *et al.* 1995). Radiocarbon datings were done for two of our individuals from Wrangel (DM 13: 4250 BP, DM 5a: 5280 BP). Blood samples from five specimens of *Elephas maximus* were obtained from German zoos (origins: Vietnam, Thailand and Myanmar). The sequence of *Loxodonta africana* obtained earlier (Hauf *et al.* 2000) was used for comparison. A short nuclear insertion of a d-loop fragment discovered by Greenwood *et al.* (1998) in Asian elephants was also included in the analysis. *Dugong dugong* was used as outgroup.

Total DNA was extracted from the mammoth bone samples (or elephant blood samples). To avoid contamination by extraneous DNA, the bone surface was removed using a hand grinder. A 8 mm Ø perforation was made into the bone to obtain about half a gram of clean powdered sample. DNA was prepared by a silica-based purification method using the GENECLEAN® Kit (BIO 101, Inc., La Jolla, USA). The primers to amplify a fragment of 450 base pairs of the mitochondrial control region were chosen from the known mitochondrial sequence of *Loxodonta africana* (Hauf *et al.* 2000). The DNA amplification was performed in a reaction volume of 50 µl containing 1x PCR buffer (Qiagen), 2.5 mM $MgCl_2$, 150 µM dNTPs, 1.6 mg/ml BSA (MBI Fermentas), 0.5 mM of each primer and 1.25 units of *Taq* polymerase (Qiagen) in a Perkin Elmer Thermocycler (GeneAmp® PCR System 9700). The amplification conditions were: 95°C for 2 minutes for initial denaturation, followed by 40 cycles consisting of 94°C for 10 seconds, 54°C for 10 seconds 71°C for 40 seconds, followed by 72°C for 5 minutes for a final extension. No amplification was detected by electrophoresis in extraction and PCR blanks.

PCR fragments were sequenced in both directions according to the chain-termination method of Sanger *et al.* (1977), using the cycle sequencing technique. The sequencing reactions contained approximately 300 ng of amplified DNA as sequencing template and 5 pmol of the respective primer. To this mixture the appropriate amount of Big Dye® Terminator Cycle Sequencing Ready Reaction Sequencing Mix (PE Applied Biosystems, Weiterstadt,

Germany) was added, following the manufacturer's instructions. The cycling conditions were: the denaturation step at 96°C for 10 seconds, followed by the annealing step at 50°C for 5 seconds and the extension/termination step at 60°C for 4 minutes, total of 25 cycles. The sequencing samples were electrophoresed on a ABI PRISM® 377 DNA sequencer and analyzed using the ABI PRISM™ Sequencing Analysis software, version 3.2 (PE Applied Biosystems, Weiterstadt, Germany).

Sequences were compared with the GeneDoc program, version 2.5.000. Trees (maximum parsimony and neighbor joining algorithms without weighting) were reconstructed with PAUP version 4.0.

3. Results and Discussion

Of the 450 base pairs of the mitochondrial d-loop sequenced, 76 were parsimony informative.

Neighbor Joining (Fig. 1) and Maximum Parsimony reconstructions revealed identical branching patterns which were statistically confirmed by 1000 bootstrap replicates. *Mammuthus* turned out as sister genus of *Elephas*, thus confirming the classical, morphologically based concept, whereas *Loxodonta* remained well outside the *Mammuthus-Elephas* clade. The short nuclear insertion sequence from Elephas took a position at the base of the *Mammuthus* lineage, thus providing additional evidence of a common ancestor of these two genera. We therefore conclude that current evidence is in favour of a sister group relationship of *Elephas* and *Mammuthus*. However, additional genes (especially nuclear genes) should be sequenced before definite conclusions can be drawn.

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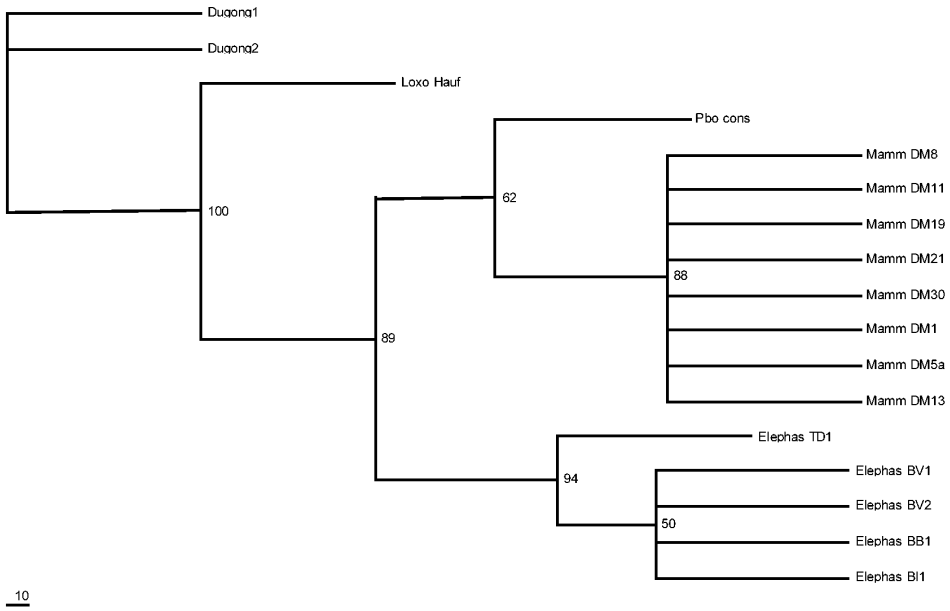

Fig.1 - Maximum paresimony tree of a 450 base pair sequence of the mitochondrial d-loop region of eight individual *Mammuthus primigenius vrangeliensis* (Mamm), five individual *Elephas maximus* and one *Loxodonta africana* (Loxo Hauf), including a short nuclear insertion from *Elephas* (Pbo cons, from Greenwood *et al.* 1998). Numbers indicate percent bootstrap values of 1000 replicates. The tree was rooted using the outgroup *Dugong dugong*.

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