

Phylogenetic resolution within the Elephantidae using fossil DNA sequence from the American mastodon (*Mammut americanum*) as an outgroup

(systematics/molecular evolution/ancient DNA/mitochondrial DNA)

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ABSTRACT DNA was extracted from the extinct American mastodon, the extinct woolly mammoth, and the modern Asian and African elephants to test the traditional morphologically based phylogeny within Elephantidae. Phylogenetic analyses of the aligned sequences of the mitochondrial gene cytochrome *b* support a monophyletic Asian elephant–woolly mammoth clade when the American mastodon is used as an outgroup. Previous molecular studies were unable to resolve the relationships of the woolly mammoth, Asian elephant, and African elephant because the sequences appear to have evolved at heterogeneous rates and inappropriate outgroups were used for analysis. The results demonstrate the usefulness of fossil molecular data from appropriate sister taxa for resolving phylogenies of highly derived or early radiating lineages.

Modern orders of mammals and birds are the result of explosive phylogenetic radiations from a small sampling of surviving taxa following late Mesozoic extinctions. This rapid morphological and ecological evolution is thought to have produced taxonomic orders with long independent evolutionary branches after short periods of shared histories (1, 2). Such a pattern of evolution has two consequences when phylogenetic inferences are estimated from molecular data. The first is that the systematic relationships among such orders are difficult to ascertain or statistically support because terminal representatives of orders retain few unequivocal shared-derived characters and are often equidistantly related to each other. This results in unresolved polytomies or star phylogenies. The second consequence of long independent branches is that resolution of patterns of divergence within clades may also be difficult to estimate if no closely related taxon is available to serve as an outgroup to root the phylogenetic tree. This again is especially problematic when terminal taxa are highly derived. Different choices among seemingly equally suitable outgroups can lead to very different results (3).

Fossil DNA is potentially well suited for phylogenetic studies plagued with the above problems. Within orders, fossils may serve as ancestral sister taxa that can polarize characters and unambiguously root a tree. Among orders, the study of fossil characters may uncover shared traits that are obscured by divergence in modern taxa and may reduce variances of branch lengths by shortening estimated distances between divergence nodes. Unfortunately, with few exceptions (4–6), fossil DNA has not been used for the resolution of systematic problems. Early fossil DNA studies used phylogenetic inference to verify the authenticity of the DNA and thereby to demonstrate the latent potential of fossil DNA (7–12). More recent studies have concentrated on anecdotal reporting of recovery of fossil DNA in response to criticisms of the persistence of DNA over time.

In contrast, this paper demonstrates the usage of fossil DNA for resolving the systematic relationships among genera within a family whose common ancestors became extinct within the recent geological past. We have used the Elephantidae as a paradigm, particularly because of its lack of closely related extant relatives that can be used as an outgroup.

The two endangered species of living elephants, *Elephas maximus* in Asia and *Loxodonta africana* in Africa, are the only remaining representatives of the order Proboscidea. Proboscideans were, however, far more diverse until the Pliocene epoch during which representatives of Deinotheriidae, Mammutidae, Gomphotheriidae, and Stegodontidae, in addition to Elephantidae, were present worldwide (13–15). Of these, the woolly mammoth, *Mammuthus primigenius*, and the American mastodon, *Mammut americanum*, persisted through the Pleistocene and became extinct around 10,000 years ago. Based on paleontological evidence, proboscidean families diverged sequentially into independent lineages, resulting in a hierarchical outgroup, or comblike relationship (13, 14). The Mammutidae, which includes *Mammut americanum*, diverged from the lineage leading to the Elephantidae during the early Miocene or before (24 million years ago). *Elephas* and *Loxodonta* within the Elephantidae diverged from a common ancestor around the Miocene–Pliocene boundary (5 million years ago) and are highly derived morphologically (13, 14).

Based on morphological studies, *Elephas* and *Mammuthus* are considered to form a monophyletic clade with *Loxodonta* as a sister group within the subfamily Elephantinae (16). However, until this present study, no molecular studies have corroborated this hypothesis. Radioimmunoassays were able to identify *Elephas*, *Mammuthus*, and *Loxodonta* as being closely related but could not resolve the relationships within the subfamily (17, 18). Similarly, recent DNA studies were unable to resolve the trichotomy. Hageberg *et al.* (19), based on cytochrome *b* sequences, noted a weakly supported closer affinity of *Mammuthus* to *Loxodonta* than to *Elephas*. Höss *et al.* (20) reported partial *Mammuthus* rDNA sequences, but these were largely uninformative due to the reported genetic distance among mammoth alleles, which was greater than that found between published sequences for the two genera *Loxodonta* and *Elephas*.

Here we present a molecular phylogeny based on previously unavailable cytochrome *b* sequence data from the extinct *Mammut americanum*, along with novel sequences from *Mammuthus primigenius*, *Elephas maximus*, and *Loxodonta africana*, which resolves the elephantid relationships and is consistent with previous morphological analyses of these species (13, 14, 17, 21). In addition, our results indicate that the rate of nucleotide substitution in cytochrome *b* in the *Elephas*–

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Mammuthus lineage may have been faster than that in *Loxodonta*.

MATERIALS AND METHODS

Samples. Three fossil and two contemporary museum specimens were used for DNA extraction and analysis. The contemporary samples were salt-preserved skin samples from *Elephas maximus* (died 1980) and *Loxodonta africana* (died 1992) females. The fossils were preserved in different paleoenvironments. One *Mammuthus primigenius* sample (referred as *Mammuthus*-1) was a piece of air-dried skin from a frozen woolly mammoth found in 1907 on Lyakhovskiy Island in the Siberian Arctic and now stored in the Museum National d’Histoire Naturelle (Paris). Radiocarbon dating of the sample gave an age of >46,000 years (Beta Analytic, Miami; ref. no. Beta 79731). The second *Mammuthus* sample (*Mammuthus*-2) was a cranial fragment collected in 1947 from glacial stream deposits of the Alaskan steppe near Fairbanks. This disarticulated bone was washed away from Pleistocene rock mucks. The sand and silt deposits near Fairbanks are frozen during most of the year and contain vertebrate fossils from bone fragments to nearly complete mummies. Vertebrate fossils associated with this bone were previously radiocarbon dated to ≈20,000 years (J. P. Alexander, personal communication). The bone was stored in the American Museum of Natural History (New York). The third fossil was a well-preserved *Mammuthus americanum* skeleton excavated in 1968 from late Pleistocene bog deposits in Oakland County of southern Michigan. One-third of the skeletal remains of the individual mastodon was found in a semiarticulated fashion under a near-surface burial in a glacial outwash plain. According to a nearby water well drilling (Michigan Department of Conservation no. 2A-DDCA), the late Pleistocene sediments that contained the mastodon fossil consist of well-sorted gray-colored fine sands, silts, and clays, representing secondary bog deposition of reworked material from nearby glacial moraines. Such paleoenvironmental interpretations are consistent with the regional geological history and information from contemporaneous fossil sites of the area. The *Mammuthus* skeleton is now displayed at the Highland Lake Campus, Oakland Community College, Union Lake, MI. Two samples from different ribs of the same individual were analyzed. The fossil was previously radiocarbon dated as 10,200 ± 170 years old (Westwood Laboratories, Westwood, NJ; ref. no. I-3774). The particular

mastodon specimen was chosen as a likely source for DNA because previous studies demonstrated that proteins were preserved in them (17).

DNA Extraction, Amplification, and Sequencing. DNA was extracted using previously published extraction methods (22–24). Equipment and reagents were dedicated solely for ancient DNA work and extractions, and amplifications were carried out in a laboratory where no mammalian DNA except human had been previously used. Disposable equipment was used whenever possible, and reusable equipment was soaked in 0.5% sodium hypochlorite and then exposed to UV light for 1 hr prior to use. Independent extracts from the same sample or samples from different parts of the same animal were used as template for PCR amplification using primers L14724 and H15149 (25). *Mammuthus*-2 and *Mammuthus* DNA were amplified using two-stage nested PCR with newly designed Elcytb65 (CTACCCCA-TCCAACATATCAACATGAT) and Elcytb320R (CGGTAT-TTCAAGTTTCCGAGTATAGGT) as internal primers. PCR assembly was carried out under a laminar flow hood, and the PCR reaction solutions were exposed to UV light for 45 min before adding template DNA and enzyme. All sample reactions were accompanied by appropriate extraction and negative PCR controls. Primary PCR amplifications were performed on a Coy Tempcycler II thermocycler with temperature settings of 94°C (40 sec), 50°C (40 sec), and 72°C (1 min) for 40 cycles. In the second stage of the nested amplification, the primary PCR product was used as a template without further purification. The secondary amplification was performed in an Idaho Technologies air temperature cyler with denaturation and annealing times of 12 sec each and elongation times of 30 sec. Extraction and negative PCR controls were carried through the secondary amplification to monitor contamination. The sequences were derived by direct dideoxy sequencing of PCR products (26). Each sequence was read from both strands.

Initially samples were analyzed under a blind testing design, in which the taxonomic identities of the samples were known only to one of the authors (J.S.), who was not performing the laboratory analysis. Duplicate samples from the same animal and samples from different individuals of the same species were provided with only sample numbers. Correct identifications of contemporary species and duplicates were achieved when sequences determined in the laboratory were compared with previously published data. Fossil sequences were similar but not identical to the two modern sequences, demonstrating the robustness and cleanliness of the laboratory procedure.



FIG. 1. Aligned DNA sequences of a 228-bp fragment of cytochrome *b* from extant (*Loxodonta africana* and *Elephas maximus*) and extinct (*Mammuthus primigenius* and *Mammuthus americanum*) proboscideans. Specimen numbers follow the description in the text. Dots represent identical bases to *Loxodonta*-1, and ? represents one unresolved base. The codon numbering follows the *Homo sapiens* system (28).



FIG. 2. Autoradiograph of a segment of the cytochrome *b* gene from *Mammuthus americanum*, the extinct American mastodon. Arrows indicate variable sites relative to the *Loxodonta*-1 sequence, and the asterisk identifies the unique first position nonsynonymous substitution. The numbers refer to the homologous human position (28).

Phylogenetic Analyses. Phylogenetic analyses were performed using maximum parsimony with exhaustive search and equal character weighting (27) and by neighbor-joining analysis using two-parameter sequence distance estimates with a 10:1 transition to transversion ratio (28, 29).

RESULTS AND DISCUSSION

Fragments (228 bp) of the mitochondrial gene cytochrome *b* from positions 14,841 to 15,068 (human sequence numbering) (30) were sequenced (Fig. 1). The two *Mammuthus* sequences were confirmed by identical sequences from four independent extractions and PCR amplifications each. The *Mammuthus* sequence was confirmed by four identical sequences derived from independent DNA extractions from two rib bones, each amplified and sequenced twice. The *Mammuthus* sequence differs from the most similar sequence (*Loxodonta*-1) by 10 substitutions, including a first position, nonsynonymous substitution

(tyrosine → asparagine) in codon 75 (human codon numbering) (Fig. 2). The two *Mammuthus* sequences differ from each other by 4 synonymous, third-position transitions. In comparison, the *Loxodonta*-1 sequence from this study differs from a published *Loxodonta* sequence (25) (*Loxodonta*-2) by 1 transition and 1 transversion, both of which are synonymous third-position substitutions, and the two *Loxodonta* sequences differ from the *Elephas* sequence by 11 third-position transitions and either 1 or 2 third-position transversions, respectively. Kimura's two-parameter estimates of genetic distances (Table 1) indicate that the *Mammuthus*-2 sequence is marginally more similar to the *Loxodonta* sequences than to the *Elephas* sequence, consistent with previously published data (19).

To assess the effects of outgroups on the analysis within Elephantidae, separate phylogenetic analyses were conducted without and with *Mammuthus americanum*. The five Elephantidae sequences were initially analyzed by maximum parsimony (27) using homologous sequences from *Homo sapiens* (human) (30), *Diceros bicornis* (black rhinoceros) (25), and *Sus scrofa* (domestic pig) (25) as outgroups. An exhaustive search excluding *Mammuthus* resulted in four equally parsimonious trees (Fig. 3 A–D). Two trees had *Elephas* alone as the first diverging lineage in the order and differed only in the relative positions of *Mammuthus*-1 and *Mammuthus*-2 sequences (Fig. 3 A and B) within a *Mammuthus*–*Loxodonta* branch. The two other trees (Fig. 3 C and D) differed only in the placement of *Sus* relative to *Homo* and had *Elephas* with *Mammuthus*-1 diverging as one monophyletic group and *Mammuthus*-2 with *Loxodonta* as another within Proboscidea. A neighbor-joining tree using the two-parameter model to estimate distances supported a *Mammuthus*–*Loxodonta* lineage as shown in Fig. 3B (28, 29). The bootstrap resampling analysis using maximum parsimony supported grouping the two *Loxodonta* sequences in 82% of the 1000 tests but could not resolve the relationships among *Elephas*, *Mammuthus*, and *Loxodonta* above the 50% consensus level.

When the *Mammuthus* sequence is added to the parsimony analysis, two equally parsimonious trees are found, both of which support *Mammuthus* as the earliest diverging proboscidean among the studied taxa and a monophyletic *Elephas*–*Mammuthus* lineage (Fig. 3 E and F). The two trees differ in the identity of the most recent common *Loxodonta* ancestor being unique or shared with the *Elephas*–*Mammuthus* clade. A neighbor-joining analysis supported *Mammuthus* as the earliest diverging proboscidean and *Loxodonta* and *Elephas*–*Mammuthus* as two subsequently diverging monophyletic sister groups (Fig. 3F). In a bootstrap resampling analysis using parsimony, there is 100% support for the monophyly of all proboscidean sequences in 1000 bootstrap samples. Using *Mammuthus* as the outgroup, the *Elephas*–*Mammuthus* sequences are monophyletic in 74% of 1000 bootstrap samples. This level of support is relatively strong when it is considered that the two intraspecific *Loxodonta* sequences are

Table 1. Sequence distances among cytochrome *b* fragments

	<i>Mammuthus</i>	<i>Loxodonta</i> -1	<i>Loxodonta</i> -2	<i>Elephas</i>	<i>Mammuthus</i> -1	<i>Mammuthus</i> -2
<i>Mammuthus</i>		8/2	8/3	13/1	11/2	11/2
<i>Loxodonta</i> -1	0.0455 (0.0146)		1/1	11/1	6/0	5/0
<i>Loxodonta</i> -2	0.0502 (0.0154)	0.0088 (0.0063)		11/2	7/1	6/1
<i>Elephas</i>	0.0652 (0.0180)	0.0554 (0.0164)	0.0600 (0.0170)		4/1	6/1
<i>Mammuthus</i> -1	0.0600 (0.0170)	0.0317 (0.0122)	0.0409 (0.0139)	0.0223 (0.0101)		4/0
<i>Mammuthus</i> -2	0.0600 (0.0170)	0.0224 (0.0101)	0.0316 (0.0121)	0.0316 (0.0121)	0.0179 (0.0090)	

The upper right matrix includes number of transitions/number of transversions. The lower left matrix includes Kimura two-parameter distances with standard errors in parentheses (26).

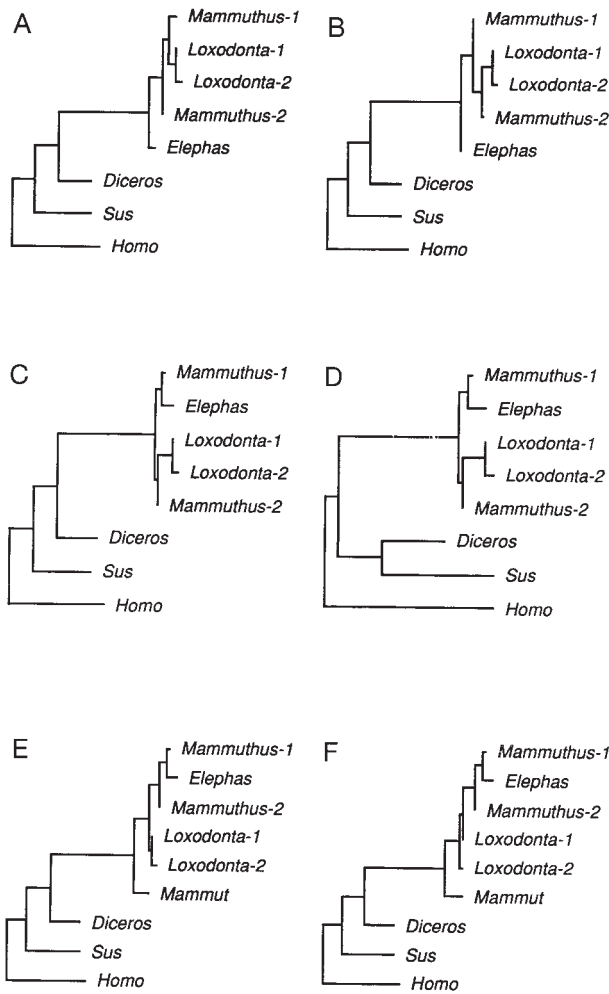


FIG. 3. Phylogenetic trees based on analysis of Proboscidea sequences using *Homo sapiens* (28), *Dicerus bicornis* (23), and *Sus scrofa* (23) as outgroups. *Loxodonta-2* (*Loxodonta africana*) was also previously published (23). Trees were generated using maximum parsimony with exhaustive search and equal character weighting (25). Branch lengths are scaled to the number of substitutions on each branch. Trees A–D are equally parsimonious trees generated without *Mammuthus*. Trees E and F are equally parsimonious trees generated including *Mammuthus*. Neighbor-joining analysis was performed using two-parameter sequence distance estimates with a 10:1 transition to transversion ratio (26, 27). The neighbor-joining tree without and with *Mammuthus* have the same topologies as B and E, respectively. Previously published *Mammuthus* cytochrome *b* sequences (19) contained an apparent typographical error, which deleted six bases spanning codons 95–96 in their sequences. This region is variable in *Mammuthus* in this study; therefore, the previously published sequences were not used in our analysis.

monophyletic with 80% support. The relationships among the two *Mammuthus* and one *Elephas* sequences could not be resolved based on the present data. In comparison, when *Homo* is used as an outgroup instead of *Mammuthus*, a monophyletic *Loxodonta*–*Mammuthus* clade is supported in 69% of bootstrap samples.

Examination of the branch lengths of the neighbor-joining trees and the distance data from which the tree are estimated (Table 1) indicates a 25–36% increase in distances between *Loxodonta* and *Elephas*–*Mammuthus* sequences and the common outgroup sequence, *Mammuthus*. These differences in branch lengths are unexpected considering that all but one of the substitutions (in *Mammuthus*) are synonymous and hence are expected to be neutral and that the estimated time of divergence of *Loxodonta*, *Elephas*, and *Mammuthus* is only about 5 million years ago (13, 14, 21). Using a relative rate test (31–34),

the differences in branch lengths are not statistically significant at the $P < 0.05$ level (*Loxodonta*, *Elephas*–*Mammuthus*: $K_{13} - K_{23} = -0.01175 \pm 0.01039$; *Loxodonta*, *Mammuthus*–*Mammuthus*: $K_{13} - K_{23} = -0.01735 \pm 0.01583$); however, the sample size (sequence length) is too small to reject the null hypothesis of rate homogeneity given the estimated branch lengths. The nominal differences in distances are notable as morphological, and strato-phenetic studies of proboscideans have also indicated a rapid evolution of *Mammuthus* and *Elephas* relative to *Loxodonta* (16, 21). This apparent correlation between morphological and molecular results warrants expansion of the DNA sequence data in cytochrome *b* and other loci within the same linkage group.

The fossil American mastodon serves as an outgroup for the Elephantidae and, therefore, is critical both for resolving the relationships within this family and for uncovering potential differences in evolutionary rates. Even though not ancestral to *Loxodonta*, *Mammuthus*, or *Elephas*, *Mammuthus* aids in the definition of primitive and ancestral states within the Elephantidae. This has the effect of reducing branch lengths to more distant outgroups in phenetic terms and of identifying shared ancestral characters in cladistic terms. For example, when the data set is analyzed in the absence of *Mammuthus*, the monophyletic *Loxodonta* lineage is distinguished by four uniquely derived states (autapomorphies) at positions 14,866, 14,914, 14,920, and 15,016 (Fig. 1). However, three of the four (14,866, 14,914, and 14,920) are shared with *Mammuthus*. Assuming that *Mammuthus* is indeed an informative outgroup for the Elephantidae, these three characters become proboscidean plesiomorphies. Additionally, four other characters (14,938, 14,995, 15,013, and 15,061) become shared characters with the non-proboscidean sequences when *Mammuthus* is added to the analysis. Prior analyses of proboscidean sequences were unable to resolve the relationship within Elephantidae because the outgroups used are too distant, and the rates of evolution within the group may not be homogeneous. The apparent grouping of *Mammuthus* and *Loxodonta* in these prior analyses is the result of a distant outgroup effectively rooting the family at the midpoint. Indeed, distances from *Mammuthus*, *Loxodonta*, and *Elephas* to *Homo* in this study are nearly identical, differing by at most 6%.

The present study demonstrates the utility of fossil material to resolve phylogenetic polytomies and to highlight heterogeneities in evolutionary rates by establishing closely related outgroups within clades that are presently species poor, both in absolute and in phylogenetic diversity. It is further expected that fossil sequences will be powerful in assessing relatedness between clades when available taxa are limited to highly derived groups (6, 35).

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