

Short communication

A nuclear DNA phylogeny of the woolly mammoth (*Mammuthus primigenius*)

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1. Introduction

Although the woolly mammoth (*Mammuthus primigenius*) is one of the most intensively studied extinct species at the DNA level, mitochondrial DNA (mtDNA) markers have failed to unambiguously resolve its phylogenetic affiliation within Elephantidae. Most mtDNA-based elephantid phylogenies associate mammoths with African elephants (*Loxodonta africana* and *Loxodonta cyclotis*) to the exclusion of the Asian elephant (*Elephas maximus*) (e.g., Debruyne et al., 2003; Noro et al., 1998). However, other mtDNA studies (Ozawa et al., 1997), including recent sequencing efforts that yielded the complete mitochondrial genomes of two woolly mammoths (Krause et al., 2006; Rogaev et al., 2006), suggested that the Asian elephant is the closest living affine of mammoths. However, relationships inferred from mtDNA may be misleading due to the absence of a closely related outgroup species, or to the radiation of the three elephantid genera in rapid succession, which can produce discordance between a species tree and a gene (mtDNA) tree due to lineage sorting processes. Another difficulty is that in certain species—including elephants—the presence of nuclear insertions of mitochondrial sequences (Numts) can make identifying organellar mtDNA problematic (Greenwood and Pääbo, 1999; Thalmann et al., 2004). Moreover, Numt sequences are a

routine, if unwanted, result of the procedures used in ancient DNA studies (Greenwood et al., 1999). Recently, cytonuclear genomic dissociation has been observed in African elephants, likely due to past hybridization between species (Roca et al., 2005). The existence of such dissociation phenomena could also confound mtDNA analysis within or among other elephantid species.

To date, the only extinct elephantid that has been amenable to confirmable molecular analysis by multiple research groups working with different specimens is the woolly mammoth (for a recent summary, see Greenwood, 2001). Yet, given the lack of consistent results across mtDNA phylogenetic studies, and given the possibility of discrepancies between the mtDNA tree and the species tree due to lineage sorting processes or to cytonuclear dissociation, nuclear DNA offers an alternative approach to studying woolly mammoth phylogeny. Nuclear DNA sequences from mammoths and other well-preserved extinct megafauna have been reported (Greenwood et al., 1999; Greenwood et al., 2001; Poinar et al., 2003; Poinar et al., 2006), and in principle it should be possible to characterize mammoth nuclear DNA sequences for the purpose of phylogenetic analysis. Of additional relevance, several nuclear genes have been investigated in a large number of individuals from different populations of *E. maximus*, *L. africana*, and *L. cyclotis* for the purpose of identifying fixed differences among groups and to establish their phylogenetic relationships (Roca et al., 2001). We have exploited and expanded this dataset to characterize the regions encompassing fixed differences among modern elephants in an

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effort to better ascertain the relationship of *M. primigenius* to extant elephantids.

2. Materials and methods

2.1. Samples

Two mammoth samples were included in this study. The first, from Engineer Creek, Alaska, has a radiocarbon date of $13,775 \pm 145$ years before present; nuclear and mitochondrial sequences for this specimen have been verified independently in different laboratories (radiocarbon dating described in Greenwood et al., 1999). Additional sequences have been reported for this mammoth (Binladen et al., 2006; Greenwood et al., 2001). The second sample, from Naskhok River in northeastern Wrangel Island (East Siberian Sea), has been dated to 4050 ± 40 years before present (Beta-195059; d13 corrected). For extant elephantids, our methods of sample collection, DNA extraction, PCR and sequencing have been previously described (Georgiadis et al., 1994; Roca et al., 2001, 2005).

2.2. Ancient DNA (aDNA)

Amplifications and re-amplifications were performed as described by Greenwood et al. (1999). To avoid contamination, processing was carried out in different research institutes: woolly mammoth samples were processed at the Istituto di Medicina Legale (Rome, Italy) while modern elephant samples were analyzed at the Laboratory of Genomic Diversity (Maryland, USA). Each aDNA amplification was performed in duplicate, cloned into a pGEM-T vector (Promega), transformed into electroporation competent bacteria, and five insert-positive clones per amplification sequenced to determine the consensus sequence of the clones (see Supplemental Figures 1–5 for all clone sequences used to derive the consensus sequences used in this study). PCR products ranged from 100 to 180 bp in length. Primer combinations used were, *CHRNA1*: L1 5' GTTTAGTAGGTTGACTTCCA, R1 5' GGAATCCATTATGATCTTTA, L2 5' GTGATGCACAGCATGAACAT, R2 5' AGCAGTTCGAATCCACCAGG, *GBA*: L 5' GTAACCACTATGCTCCTCA, R 5' CAGCCCTGAGGACATCCAC, *BGN*: L1 5' CTGAGCGCTAGGGCCATCCA, R1 5' ATGATGTTGCTGTGCAACA, L2 5' TCACATCCACCAGTACAAAG, R2 5' GTCTGTTTTAAAGCCTTTCC, *LEPR*: L 5' TTATGGACTCTATATTGGAG, R 5' TTGTTGACCATCTGCAAGT. *VWF* sequences were taken from Greenwood et al. (1999).

2.3. Modern DNA

Genomic DNA (~50 ng) underwent amplification by PCR using 200 nM final concentration of each oligonucleotide primer in 1.5 mM MgCl₂, with AmpliTaq-GOLD DNA Polymerase (Applied Biosystems Inc. [ABI]). Primers were as previously reported for *BGN*, *CHRNA1*, and *GBA*

(Lyons et al., 1997; Roca et al., 2001, 2005), but rock hyrax (*Procapra capensis*) *BGN* was amplified using new primers *BGN-F1f* (5'-AAGATCTCCAAGATCCAYGAGAARG) with *BGN-R1f* (5'-CCCARCCTGTACARCTTGGAGTA). *LEPR* used *LEPR-F* (5'-CCAAACCTCGAGGAAA GTTTACC) with *LEPR-R* (5'-AGGCTGCTCCTATGATACCTCAA) for elephants and *LEPR-F2* (5'-GCAGTG TACTGCTGCAATGA) with *LEPR-R2* (5'-TGCAAAGT GCTTCCCACA) for hyrax. *VWF* was amplified using either *vWF-F1a* (5'-GATGGTGTCAACCTCACCTGT) or *vWF-L1* (above) with *vWF-R1a* (5'-CAATGCCACC GGGATCA); hyrax used *vWF-F1a* with *vWF-R1a*. For all primer pairs, PCR consisted of an initial 95 °C for 9:45 min; with cycles of 20 s at 94 °C, followed by 30 s at 60 °C (3 cycles); 58, 56, 54, or 52 °C (5 cycles each temperature); or 50 °C (last 22 cycles), followed by 75 s extension at 72 °C; with a final extension of 3 min at 72 °C. Sequences of several genes had been previously generated for multiple individuals of *E. maximus*, *L. africana*, and *L. cyclotis* (Roca et al., 2001, 2005), while novel elephant, mammoth and hyrax sequences generated for this study have been deposited in GenBank (*BGN*: DQ265804–DQ265820; *CHRNA1*: DQ265821–DQ265838; *GBA*: DQ265839–DQ265855; *LEPR*: DQ265856–DQ265888; *VWF*: DQ265889–DQ265919; Wrangel Island mammoth *BGN*: DQ267154, *CHRNA1*: DQ267155, DQ267156).

2.4. Phylogenetic analyses

Sequences were aligned using ClustalX (Thompson et al., 1997) and visually inspected. Two datasets were analyzed, each with concatenated DNA sequences from the genes *BGN*, *CHRNA1*, *GBA*, *LEPR* and *VWF*. The first dataset included sequences from elephantids and hyrax; 22 bp of the alignment in the 3' fragment of *BGN* was excluded due to saturation of the region between hyrax and elephantids. The 3' fragment of *CHRNA1* was an AfroSINE (Nikaido et al., 2003) present only in each of the elephantids; in hyrax it was coded as gaps and, to maximize resolution within elephantids, the maximum parsimony (MP) analysis treated gaps as a fifth state. The second dataset excluded the hyrax and used only elephantid sequences, including the complete 3' sequence of *BGN*. In both datasets, a deletion (AAACC) was present in *CHRNA1* in one of the chromosomes (i.e., heterozygous) of elephant DS1534 and both chromosomes (homozygous) of LO3508; the deletion was part of the AfroSINE and removed from the alignment to avoid spurious affinity with hyrax. In a poly-T region of *LEPR* there was deletion of a thymine (in LO3505) or addition of a thymine (in LO3517); in each case the indel was present in only one of the chromosomes (heterozygous), and was not coded for analysis. These indels were present only in forest elephants and would not affect relationships inferred among elephantid genera. Modeltest 3.7 (Posada and Crandall, 1998) was used to determine the Akaike Information Criterion model of DNA sequence evolution that best fit the data; the model was implemented

for Neighbor Joining (NJ) and maximum likelihood (ML) methods in PAUP*4.0b10 (Swofford, 2002); MP was also run. Heuristic searches used 50 replicates of random taxon-addition and tree bisection-reconnection (TBR) branch swapping. Bootstrap resampling support was based on at least 100 replicates, with TBR branch swapping of starting trees obtained by stepwise addition. The model of evolution selected by Modeltest for each dataset was as follows. “Base” indicates the base frequencies for A, C, and G, with T inferred. “Nst” lists the number of substitution types listed in a rate matrix; the number of unique types may be

inferred. “Rmat” is the rate matrix. “Rates” indicates the distribution of rates at variable sites. “Pinvar” indicates the proportion of invariant sites. For the elephantids + hyrax dataset: Lset Base=(0.2901 0.2364 0.2236) Nst=6 Rmat=(1.0000 1.9849 0.3926 0.3926 3.7872) Rates=equal Pinvar=0. For the elephantids-only dataset: Lset Base=equal Nst=6 Rmat=(1.0000 1.3818 0.2787 0.2787 3.2657) Rates=equal Pinvar=0. Tree scores are indicated on the Fig. 1 legend.

A Kishino Hasegawa (KH) test was run in PAUP* (Kishino and Hasegawa, 1989) using the following

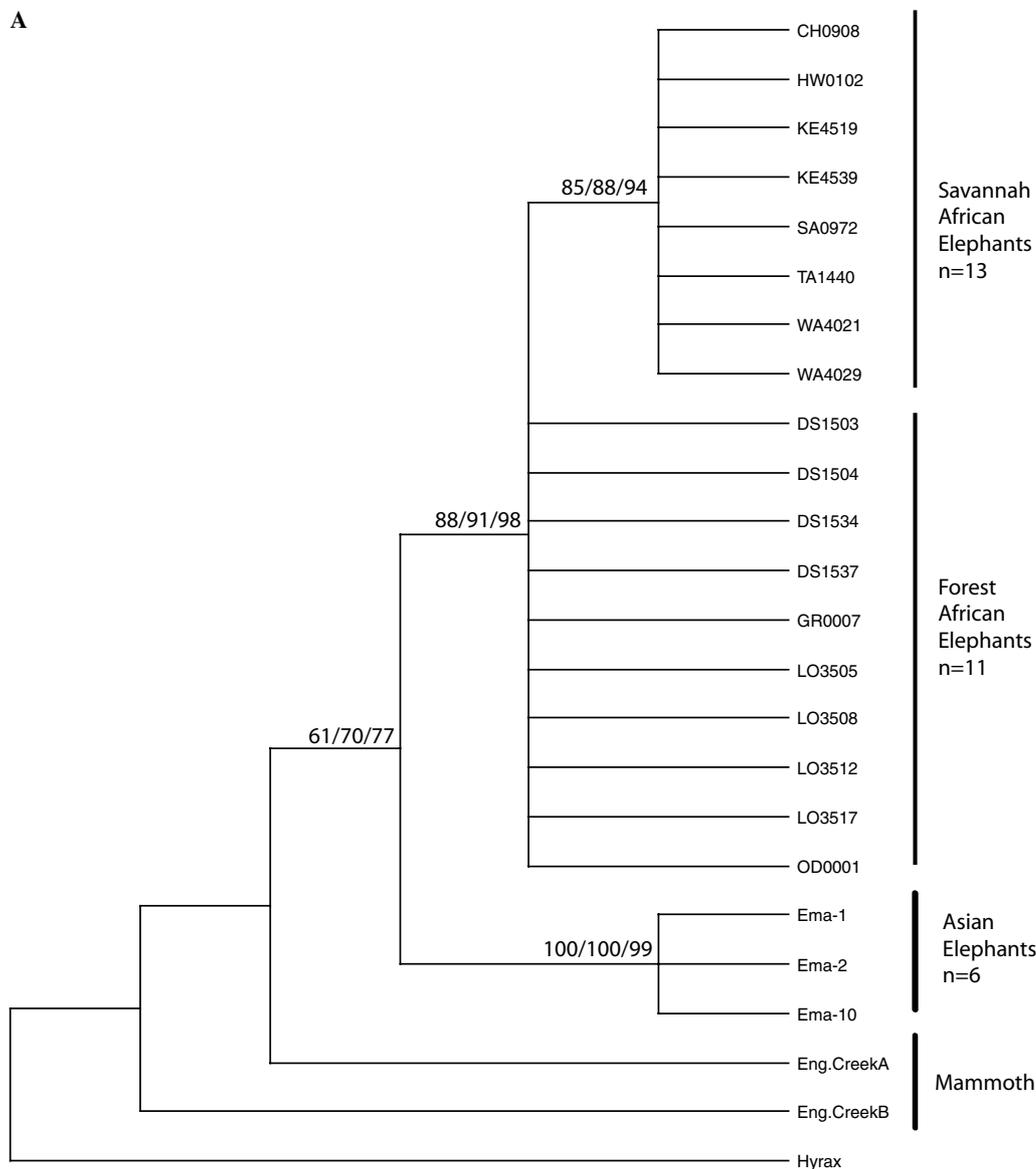


Fig. 1. Phylogenetic trees showing relationships among mammoths and living elephantids using DNA sequences from five nuclear genes (*BGN*, *CHRNA1*, *GBA*, *LEPR*, *VWF*). For both trees, bootstrap scores above 50% are shown for (left to right) maximum parsimony, Neighbor Joining, and maximum likelihood methods; “ns” indicates less than 50% bootstrap support for a given method. Modern elephant designations are taken from Table 1. Numbers indicated by species labels reflect the presence of additional individuals with duplicate sequences, not listed on the tree but shown in Table 1. (A) Strict consensus of 211,697 equally parsimonious trees produced by maximum parsimony analysis of 701 bp using hyrax as an outgroup, excluding a saturated portion of the 3' *BGN* sequence and treating gaps as a fifth state. The same interspecies relationships were suggested by MP (length 295; CI 0.990; RC 0.950), NJ (ME-score = 0.22282) and ML (-Ln likelihood = 1448.6734) methods. (B) The NJ tree, midpoint rooted for a 677 bp alignment excluding the hyrax sequence. The same interspecies relationships were suggested by MP (number of trees = 1000 [maxtrees], Length 62; CI 1.000; RC 1.000), NJ (ME-score = 0.03703) and ML (-Ln likelihood = 0.03758) methods.

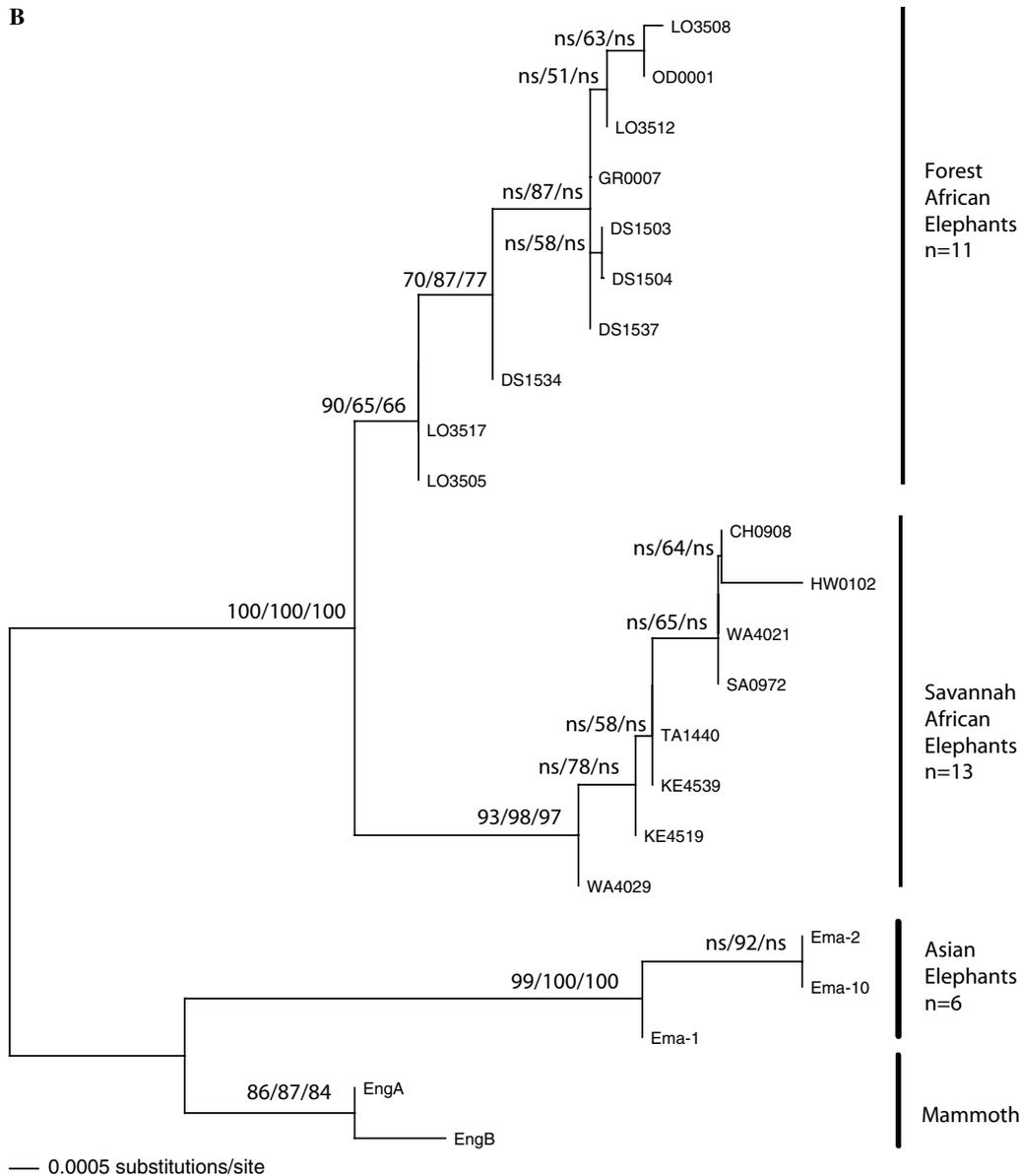


Fig. 1. (continued)

tree based on MP analysis of the dataset: (((((((((CH0809, HW0102, SA0972, WA4021), KE4519, KE4539, TA1440, WA4029), (DS1503, DS1504, DS1537, GR0007, (LO3508, OD0001), LO3512), DS1534), LO3517), LO3505), (Ema-1, (Ema-2, Ema-10))), (Eng.CreekA, Eng.CreekB)), hyrax). This tree was compared to two other trees, both with the same intra-generic but different inter-generic relationships among the individuals. In the first tree used in both KH tests, *Loxodonta* and *Elephas* formed a clade excluding *Mammuthus*; in one comparison tree *Loxodonta* and *Mammuthus* were grouped to form a clade excluding *Elephas*, while in the other *Elephas* and *Mammuthus* formed a clade excluding *Loxodonta*.

Minimum spanning tree analysis was performed for the aligned elephantid sequences (without hyrax) using the TCS program (Clement et al., 2000).

3. Results

Two individual mammoths were genotyped at multiple nuclear DNA loci chosen for the potential presence of fixed nucleotide differences between *Elephas* and *Loxodonta*. A total of 681 bp of mammoth sequence was determined for loci *BGN* (175 bp), *CHRNA1* (193 bp), *GBA* (62 bp), *LEPR* (137 bp), and *VWF* (114 bp), with sequences for *BGN*, *CHRNA1*, and *VWF* amplified in two non-overlapping fragments. The mammoths were from different continents (Wrangel Island in northeastern Asia and Engineer Creek in Alaska) and chronologically separated by thousands of years. Thus, recovered sequences are likely to be minimally representative of geographic variation among mammoths for the loci characterized. In addition, little variation among mammoths has been

excluded yielded a similar network but reduced the number of steps along some branches (for example L03508 would be the same as OD0001; see also Table 1). The distance and diversity exhibited by *L. cyclotis* reflects a long history of reproductive isolation from *L. africana*.

4. Discussion

Although a small number of sites uniquely group woolly mammoths and Asian elephants, the phylogeny of the Elephantidae could not be resolved with the current dataset. However, the trend does not suggest a strong *Mammuthus*–*Loxodonta* association as has been reported in several mtDNA based studies (Greenwood et al., 1999). By contrast, the *VWF* gene suggests a mammoth–*Elephas* association, as does the *BGN* gene. While *GBA* is ambiguous, *CHRNA1* favors a mammoth–*Loxodonta* association and *LEPR* slightly favors *Loxodonta*–*Mammuthus* as there are heterozygous forest elephant individuals with only one difference compared to mammoth while Asian elephants uniformly display two fixed *LEPR* differences versus the mammoth sequence. Nonetheless, none of our analyses combining all the sequences produced a *Mammuthus*–*Loxodonta* grouping.

The three elephantid genera radiated in quick succession in the late Miocene/early Pliocene (Maglio, 1973; Vignaud et al., 2002). Their evolutionary patterns may be comparable to that produced by the contemporaneous rapid radiation of the gorilla, chimpanzee and human lineages, in which the correct (gorilla (human, chimpanzee)) relationship is supported by only 60% of nuclear loci and phylogenetically informative sites, due to random sorting, recombination, genetic drift or homoplasy (O’Higin et al., 2002; Satta et al., 2000). An added difficulty for interpreting elephantid relationships is that one target group is extinct. Lack of an appropriate outgroup sequence is another difficulty. Hyracoids and sirenians are the groups most closely related to proboscideans, but since their divergences occurred at the beginning of the Cenozoic 63 Ma, they are poor candidates for determining among-species branching patterns. Although mtDNA sequences have been reported for the mastodon (*Mammuth americanum*), the results have not been independently replicated and nuclear DNA has never been retrieved from a mastodon (Yang et al., 1996).

Nonetheless, the results of this study suggest that further sequencing of woolly mammoth nuclear genes should resolve their phylogeny conclusively, although it will require a substantial increase in the number of informative sites and independent loci examined. Recent developments in sequencing technology suggest that whole genome analysis of extinct animals, particularly mammoths will be feasible (Poinar et al., 2006). We also conclude that the application of nuclear markers is now practicable and indeed preferable for systematic study of a wide variety of extinct animals represented by well-preserved remains in museum collections.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.ympev.2006.03.015](https://doi.org/10.1016/j.ympev.2006.03.015).

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