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Mitochondrial cytochrome *b* of the Lyakhov mammoth (Proboscidea, Mammalia): new data and phylogenetic analyses of Elephantidae

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Abstract

The phylogenetic relationships between recent Elephantidae (Proboscidea, Mammalia), that is to say extant elephants (Asian and African) and extinct woolly mammoth, have remained unclear to date. The prevailing morphological scheme (mammoth grouped with Asian elephant) is either supported or questioned by the molecular results. Recently, the monophyly of woolly mammoths on mitochondrial grounds has been demonstrated (Thomas et al., 2000), but it conflicts with previous studies (Barriel et al., 1999; Derenko et al., 1997). Here, we report the partial sequencing of two mitochondrial genes: 128 bp of 12S rDNA and 561 bp of cytochrome *b* for the Lyakhov mammoth, a 49,000-year-old Siberian individual. We use the most comprehensive sample of mammoth (11 sequences) to determine whether the sequences achieved by former studies were congruent or not. The monophyly of a major subset of mammoths sequences (including ours) is recovered. Such a result is assumed to be a good criterion for ascertaining the origin of ancient DNA. Our sequence is incongruent with that of Yang et al. (1996), though obtained for the same individual. As far as the latter sequence is concerned, a contamination by non-identified exogenous DNA is suspected. The robustness and reliability of the sister group relation between *Mammuthus primigenius* and *Loxodonta africana* are examined: down-weighting saturated substitutions has no impact on the topology; analyzing data partitions proves that the support of this clade can be assigned to the most conservative phylogenetic signal; insufficient taxonomic and/or characters sampling contributed to former discordant conclusions. We therefore assume the monophyly of "real mammoth sequences" and the (*Mammuthus*, *Loxodonta*) clade.

Keywords: *Mammuthus primigenius*; Elephantidae; Ancient DNA; Contamination; Molecular phylogeny; Cytochrome *b*

1. Introduction

In the early 1990s, as the methods for sequencing of ancient DNA became effective, the woolly mammoth *Mammuthus primigenius* (Blumenbach, 1799) rapidly turned to a selected model for molecular systematicts (Hagelberg et al., 1994; Hauf et al., 1995; Höss et al., 1994). The reasons that may explain this overwhelming success are simple. First, the species became extinct but 4000 years ago (Vartanyan et al., 1995). Second, the environmental conditions for specimens having stayed in permafrost for decades were considered as favorable to preserve the DNA. From Adams mammoth excavation in 1799 (Lister and Bahn, 1994) to the rediscovery of "Fishhook mammoth" in 2000 (Mol et al., 2001), more than a dozen of mammoth carcasses have been excavated for the two last centuries, particularly in North-East Siberia and Siberian islands. Yet, only short amplifications could be obtained due to the high level of DNA fragmentation through time (Derenko et al., 1997; Greenwood et al., 1999; Hagelberg, 1994; Hagelberg et al., 1994; Noro et al., 1998). Moreover, chemical alteration of DNA has been put forward to explain the high polymorphism observed in mammoth sequences, relative to polymorphism in elephants (Thomas et al., 2000).

Little agreement on the position of *M. primigenius* on molecular grounds is mainly due to the conjunction of

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physical and chemical degradations, resulting in the production of short and variable sequences. Sister group relationships were proposed between Asian elephant and mammoth (Ozawa et al., 1997; Yang et al., 1996) according to the morphological pattern (Shoshani and Tassy, 1996), or between African elephant and mammoth (Noro et al., 1998; Thomas et al., 2000). Other investigations (Barriel et al., 1999; Derenko et al., 1997) could not resolve this crucial issue due to apparent polyphyly of the mammoths.

We address the question of mammoths monophyly and reappraise the inter-relationships of *M. primigenius*, Asian *Elephas maximus*, and African *Loxodonta africana*. We analyze all mammoth sequences available for the 5′ extremity of the protein coding gene cytochrome *b*: 11 sequences from seven independent studies with a new one (this study). Eleven sequences of *E. maximus* and nine sequences of *L. africana* (two of which are forest African elephants *Loxodonta africana cyclotis*) are added. In this paper, we adopt the conservative classification of African elephants into two subspecies (Laursen and Bekoff, 1978), although a recent paper used genetic divergence as representative of the specific distinction (Roca et al., 2001).

There is conflicting evidence for grouping Proboscidea with either Sirenia or Hyracoidea based on molecular (Kleinschmidt et al., 1986; Madsen et al., 2001) and morphological grounds (Fischer and Tassy, 1993; Rasmussen et al., 1990). Three sirenians (*Dugong dugon*, *Trichechus manatus* and *Hydrodamalis gigas*) and one hyracoid (*Procavia capensis*) were used as outgroups according to Barriel and Tassy's procedure (1998). Yang et al. (1996) questioned the role of a distant outgroup on the basal topology of Elephantidae and added 228 bp cytochrome *b* of the American mastodon (*Mammut americanum*). The accuracy of this alternative outgroup is evaluated.

2. Materials and methods

2.1. Sample source and DNA extraction

The sequences from Barriel et al. (1999), listed in Table 1, were completed in accordance with the same protocol. In addition, a sample of cranial bone of a *L. a. cyclotis* was provided by the Musée Royal d'Afrique Centrale (Tervuren, Belgium). This animal was killed in the 1950s in the north of the Democratic Republic of Congo (former Zaire).

The specimen of mammoth used in this study is from the main Lyakhov Island (Siberia) and was radiodated of at least 49,000 years BP (^{14}C dating, LSM-10145, CNRS Gif-sur-Yvette, France). It was excavated in 1902 and suffered no particular treatment until being offered to the Muséum National d'Histoire Naturelle (Paris, France) in 1912. The dried skin and the skeleton were preserved. Two different types of samples were collected: a piece of dry skin, like Yang et al. (1996) on the same specimen and a sesamoid bone of the right hindfoot. Only the core of the bone was treated while the external part was discarded. DNA extractions were performed for approximately 0.5 g of these tissues according to a phenol/chloroform protocol (Hassanin et al., 1998). Final volumes for four positive extracts ranged from 50 to 100 μl. The whole procedure was conducted in a chamber, separated from the rest of the laboratory. No other mammoth DNA had ever been treated before in this laboratory. DNA-free tubes were used after autoclaving at 120 °C for 15 min. All equipment was exposed to UV light for at least 40 min before use.

2.2. PCR amplification and assessment of maximum size for recovering ancient DNA

Primers were designed as specific for Elephantidae or mammoth with OLIGO version 4.0-s. Those specific primers (listed in Appendix A) were used to avoid co-amplification of contaminated DNA (Richards et al., 1995). Several primers (suffixed -n) were taken or modified from Noro et al. (1998). The different pairs of primers produced fragments with overlapping domains to check their authenticity.

A complete range of fragment sizes for PCR products spanning 100–250 bp were constituted. The 32 pairs of primers thus determined permitted to establish the maximum size of amplifiable DNA that could be recovered from our samples of mammoth. Symmetric PCRs with different reaction conditions were adapted for elephants and mammoth. For the latter, they were as follows: a hot start at 94 °C for 2 min, followed by 10 cycles of [94 °C for 1 min, 50–55 °C for 90 s, and 72 °C for 1 min], and then 30 cycles of [94 °C for 40 s, 55–60 °C for 1 min, and 72 °C for 1 min], and afterwards a final incubation of 2 min at 72 °C was performed.

Amplifications used 2 μl mammoth genomic DNA each. They were performed in a total volume of 25 μl containing 1 U *Taq* DNA polymerase (Appligene), 10 mM Tris–HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 250 $\mu g \cdot ml^{-1}$ Bovine Serum Albumin (Sigma), 0.7 μl DMSO (Sigma), 200 μM dNTPs (Boehringer), and 20 pmol of each primer.

All sample reactions were accompanied by negative PCR controls. For a check of DNA amplification, 5 μl PCR product was electrophoresed on 2% agarose gel and stained with ethidium bromide. No amplification was detected in extraction and PCR controls.

2.3. Direct sequencing of PCR products

Recent data (Noro et al., 1998; Ozawa et al., 1997) confirmed that a prior cloning of PCR products is not

Table 1
Data set studied in this paper: 36 terminals and their (partial or complete) cytochrome *b* gene sequences

Order	Taxon	Geographic origin	Fragment length (sites)	Accession No. (GenBank)	Reference
Proboscidea	*Elephas maximus*	—	1137 bp (1–1137)	D83048	Ozawa et al. (1997)
Proboscidea	*E. maximus*	—	1137 bp (1–1137)	AB002412	Noro et al. (1998) (a)
Proboscidea	*E. maximus*	—	1137 bp (1–1137)	D50844	Noro et al. (1998) (b)
Proboscidea	*E. maximus*	—	1137 bp (1–1137)	D50846	Noro et al. (1998) (c)
Proboscidea	*E. m. indicus*	India	1130 bp (1–1130)	AF132520	Barriel et al. (1999)
Proboscidea	*E. m. indicus*	Burma	1137 bp (1–1137)	AF132521	Barriel et al. (1999) (a)
Proboscidea	*E. m. indicus*	Burma	1137 bp (1–1137)	AF132522	Barriel et al. (1999) (b)
Proboscidea	*E. m. indicus*	Bhutan	1128 bp (1–1128)	AF132524	Barriel et al. (1999)
Proboscidea	*E. m. indicus*	Thailand	1132 bp (1–1132)	AF132525	Barriel et al. (1999)
Proboscidea	*E. m. indicus*	Vietnam	1137 bp (1–1137)	AF132526	Barriel et al. (1999)
Proboscidea	*E. m. maximus*	Sri Lanka	1126 bp (1–1126)	AF132523	Barriel et al. (1999)
Proboscidea	*Loxodonta africana*	—	1137 bp (1–1137)	D84150	Noro et al. (1998) (a)
Proboscidea	*L. africana*	—	1137 bp (1–1137)	D84151	Noro et al. (1998) (b)
Proboscidea	*L. africana*	—	1137 bp (1–1137)	D84152	Noro et al. (1998) (c)
Proboscidea	*L. a. africana*	Namibia	1128 bp (1–1128)	AF132527	Barriel et al. (1999)
Proboscidea	*L. a. africana*	South Africa	1129 bp (1–1129)	AF132528	Barriel et al. (1999) (a)
Proboscidea	*L. a. africana*	South Africa	1129 bp (1–1129)	AF132529	Barriel et al. (1999) (b)
Proboscidea	*L. a. africana*	South Africa	1137 bp (1–1137)	NC 000934	Hauf et al. (2000)
Proboscidea	*L. a. cyclotis*	Sierra-Leone	1130 bp (1–1130)	AF132530	Barriel et al. (1999)
Proboscidea	*L. a. cyclotis*	DRC	1135 bp (1–1135)	AF517566	Van Holt (1999)
Proboscidea	*Mammuthus primigenius*	Alaska	228 bp (92–319)	U23739	Yang et al. (1996) (a)
Proboscidea	***M. primigenius***	**Lyakhov**	**228 bp (92–319)**	**U23738**	**Yang et al. (1996) (b)**
Proboscidea	*M. primigenius*	NE Siberia	331 bp (98–428)	U79411	Derenko et al. (1997)
Proboscidea	*M. primigenius*	Magadan	1005 bp (1–1005)	D83047	Ozawa et al. (1997)
Proboscidea	*M. primigenius*	Taimyr	1137 bp (1–1137)	D50842	Noro et al. (1998)
Proboscidea	*M. primigenius*	Alaska	305 bp (96–402)	AF154864	Greenwood et al. (1999)
Proboscidea	*M. primigenius*	Taimyr	459 bp (81–541)	—	Thomas et al. (2000) (a)
Proboscidea	*M. primigenius*	NE Siberia	459 bp (81–541)	—	Thomas et al. (2000) (b)
Proboscidea	*M. primigenius*	NE Siberia	459 bp (81–541)	—	Thomas et al. (2000) (c)
Proboscidea	*M. primigenius*	NE Siberia	224 bp (117–342)	—	Thomas et al. (2000) (d)
Proboscidea	***M. primigenius***	**Lyakhov**	**561 bp**	**AF517567**	**This study**
Proboscidea	*Mammut americanum*	Michigan	228 bp (92–319)	U23737	Yang et al. (1996)
Sirenia	*Dugong dugon*	—	1137 bp (1–1137)	U07464	Irwin and Arnason (1994)
Sirenia	*Trichechus manatus*	—	1005 bp (1–1005)	D83050	Ozawa et al. (1997)
Sirenia	*Hydrodamalis gigas*	—	1005 bp (1–1005)	D83049	Ozawa et al. (1997)
Hyracoidea	*Procavia capensis*	—	1005 bp (1–1005)	D86909	Ozawa et al. (1997)

Note. The two sequences of the mammoth from Lyakhov are in bold characters.

always necessary to perform reliable sequencing of ancient DNA. After purification of PCR products with the QIAquick PCR purification kit (Qiagen) on 2% agarose gel, direct sequencing was performed, using the Thermo-Sequenase cycle sequencing kit US 78500 (Amersham Life Sciences) and [γ-^{33}P]dATP. One picomole of appropriate primer was used as sequencing primer (Appendix A). Sequence products were electrophoresed on 6% polyacrylamide gel with 8.3 M urea. Dry gels were exposed to X-ray films (Kodak X-OMAT) for one to three days. Each sequence was read on both strands. The program BLAST2 (Altschul et al., 1997) was used to detect contaminations. For *M. primigenius*, only fragments sequenced from at least two separate PCRs, with two different primers each, were considered.

The new sequences of *L. a. cyclotis* and of *M. primigenius* (Lyakhov) appear in the GenBank nucleotide sequence database with Accession Nos. AF517566 and AF517567 respectively.

2.4. Sequence analysis

The alignment of sequences for 36 terminals (Table 1) was performed with MUST Package version 2000 (Philippe, 1993) and was non-ambiguous. For every sequence under consideration, the open reading frame was conserved so that amplification of nuclear insert is unlikely. The data set was analyzed through three different approaches: parsimony (MP), maximum likelihood (ML), and distances (NJ). MP analyses (with equal and differential weighting) and ML analyses relevant to the

general-reversible model (Yang, 1994) were performed with PAUP* version 4.0.0d64 (Swofford, 1998). Parsimony heuristic searches were conducted with random sequence addition (five replicates each, TBR branch swapping algorithm). The calculation of Bremer support, according to Bremer's procedure (Bremer, 1994), and of MP bootstrapping (100 replicates) was also performed with PAUP*. The best-fitting evolutionary model relevant to the data for ML analyses was assessed with MODELTEST version 3.06 (Posada and Crandall, 1998) using one random MP shortest tree as a topological reference. All ML parameters of the "GTR + I + G"[1] model (Yang, 1993) were taken from MODELTEST output: proportion on invariable sites = 0.5229, base frequencies ([A] = 0.3230; [G] = 0.0987; [C] = 0.3500; [T] = 0.2283), substitution model ([A–C] = 339.7863; [A–G] = 8805.0020; [A–T] = 548.4326; [C–G] = 1074.0552; [C–T] = 9201.7617; [G–T] = 1.0000), and Gamma law distribution with α shape parameter = 1.2456 (estimated through six rate categories). The NJ analyses (pairwise-distance method; Saitou and Nei, 1987) and bootstrapping (1000 replicates) were conducted with MUST. To avoid the loss of significance of bootstrap values, only parsimony-informative sites were bootstrapped in all investigations (Carpenter, 1996).

2.5. Tree stability

We endeavored to evaluate the homoplasy content by applying Hassanin and Douzery's procedure (1999), using a combination of consistency index "CI" (Kluge and Farris, 1969) and slope "S" of mutational saturation. An estimate of the mutational saturation impact on the topology was also carried out. It consisted first in the assignment of the variability for each site on the basis of the calculation of its retention index "RI" (Farris, 1989). Thereafter, these values were devoted to the partitioning of the initial matrix into several submatrices containing sites with RI superior to arbitrarily fixed scores. A Bremer support was then calculated within every sub-matrix for the three alternative sister groupings of elephantids: (*Mammuthus*, *Loxodonta*), (*Loxodonta*, *Elephas*), or (*Elephas*, *Mammuthus*).

The PRN (for "Pattern of Resolved Nodes") method (Lecointre et al., 1994) was applied to estimate whether different published data sets were sufficient, to establish robust and reliable relationships between mammoths and elephants. This method involves an analysis of bootstrap proportions as a function of the increasing number of nucleotides in the matrix. Thereby we focused on the variation in support (in NJ bootstrap values) for the three alternative nodes, depending on the given number of informative sites re-sampled by jackknife. This procedure was implemented for a matrix of 22 terminals displaying complete cytochrome *b* and encompassed a single outgroup (*Dugong dugon*) to rule out the re-sampling of sites, which contribute to parsimony-informative characters but when multiple rooting is performed. As reflecting most of the among-outgroup variations, these sites are of no interest for the issue we investigate. This matrix therefore comprises 124 informative sites, approximately 10% of the total number of sites (1137). Twelve spreading out sequence lengths were chosen (10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, and 120), and for each, 100 random samples of sites were drawn by jackknife with NET program (MUST). A total of 1200 subsets of sequence alignments were built. All of them were submitted to 1000 bootstrap replicates. Mean bootstrap values and standard deviation were then calculated and plotted.

3. Results

3.1. Amplification of mammoth DNA

Estimating the maximum size of amplifiable DNA had unexpected results. No positive amplification could be performed with the sample of mammoth skin, though multiple extractions were attempted. As for the bone, repeatable positive PCRs were obtained for different pairs of primers, always bearing on fragments shorter than 180 bp (primers included). Actually, we consider in our analyses a total fragment of 561 bp for cytochrome *b*, sequenced using 15 primers with overlapping domains (Appendix A). Only unambiguous sequences are reported and correspond to four fragments at positions: (14242–14590)—(14662–14746)—(14827–14869)—(14994–15082) according to the numbering of elephant mitochondrial DNA (Hauf et al., 2000).

Another domain of 128 bp of 12S rDNA was also sequenced. It was strictly identical to the same domain from the mammoth of Noro et al. (1998). It displayed but one difference towards each of the two species of elephant. We discarded this marker, assuming that it was too conservative to permit authenticating the origin of small fragments or to segregate close elephantids.

3.2. Homoplasy content and saturation

In this study, *Mammut americanum* is the closest relative to elephantids: the two lineages must have diverged 25 Ma (Shoshani and Tassy, 1996). Nevertheless, its published sequence (Yang et al., 1996) is only 228 bp long, so that it cannot be used to root the trees of the whole cytochrome *b*. Yet, we will show here how critical is the length of sequences to be considered.

[1] GTR + I + G is the shortcut used by MODELTEST for general time reversible model (GTR) with the proportion of Invariable sites (+I) and α parameter of Gamma law distribution for character variation (+G) both estimated from the data.

Table 2
Comparison of homoplasy content and resolution of the molecular matrix of 228 bp for 31 ingroup terminals, whether Sirenia and Hyracoidea or *M. americanum* are chosen as outgroup taxa

Outgroup	Terminals	CI	RI	PIS	Steps	Nodes
M. americanum	32	0.56	0.87	18	49	14
Sirenia + Hyracoidea	35	0.64	0.79	63	159	8

Note. "CI": consistency index calculated for informative sites only; "RI": retention index; "PIS": number of parsimony-informative sites; "*steps*": number of steps of the shortest trees; "*nodes*": number of nodes in the MP consensus among ingroup taxa.

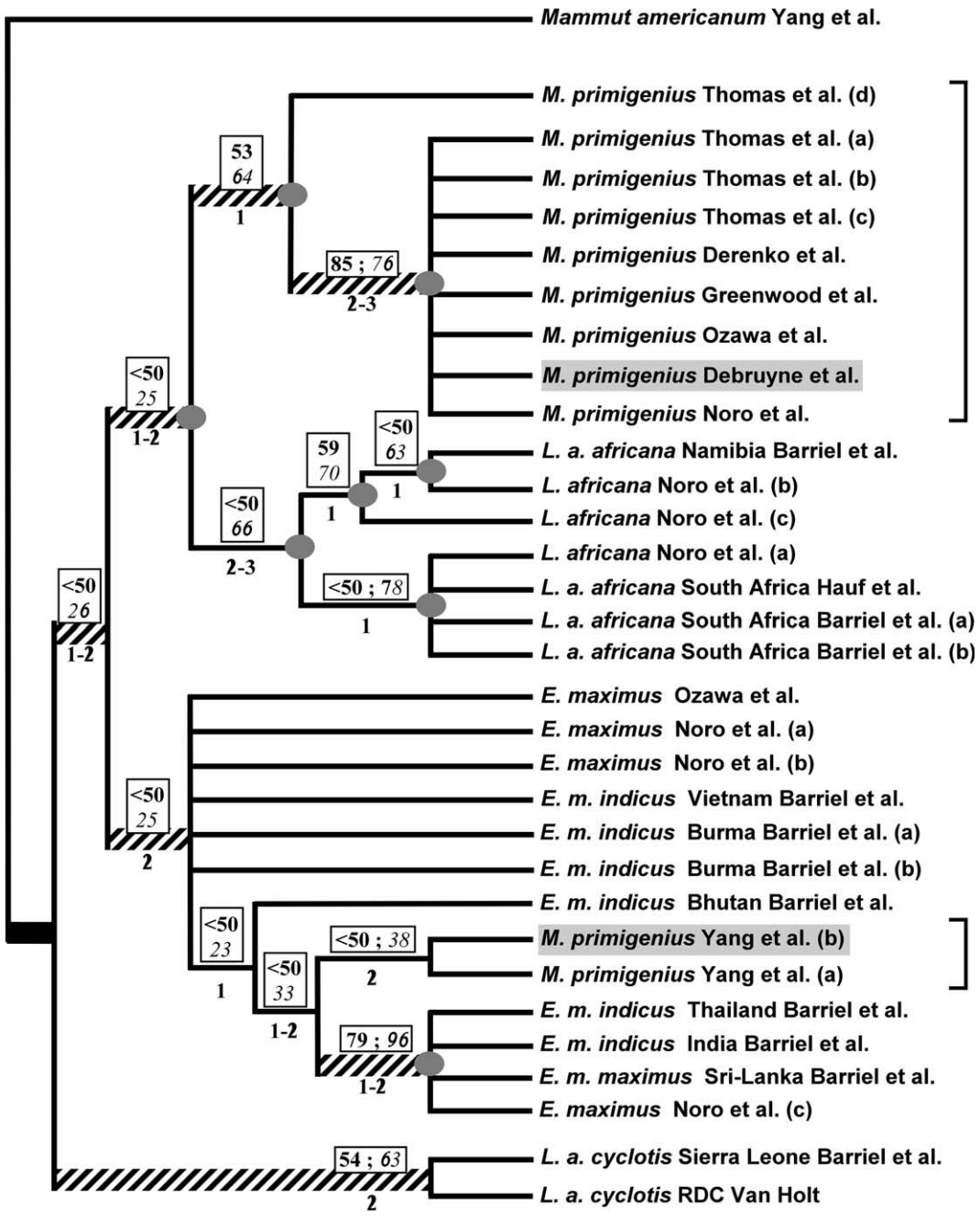

Fig. 1. Phylogeny for the partial sequences of cytochrome *b* (32 terminals; 228 bp; 18 informative characters). Majority-rule consensus (50%) of five equally parsimonious trees (unweighted parsimony; length = 49 steps; CI excluding uninformative sites = 0.56; RI = 0.87). Above branches: parsimony (boldface) and pairwise distance (italics) bootstrap values. Below branches: min and max number of apomorphies. Black stripes indicate branches present in MP/NJ and ML trees. Clades which are present when the tree is alternatively rooted with sirenians/hyrax are spotted in gray. Two clades of mammoth sequences are indicated with square brackets. Among them, the two sequences of Lyakhov mammoth are on a gray background.

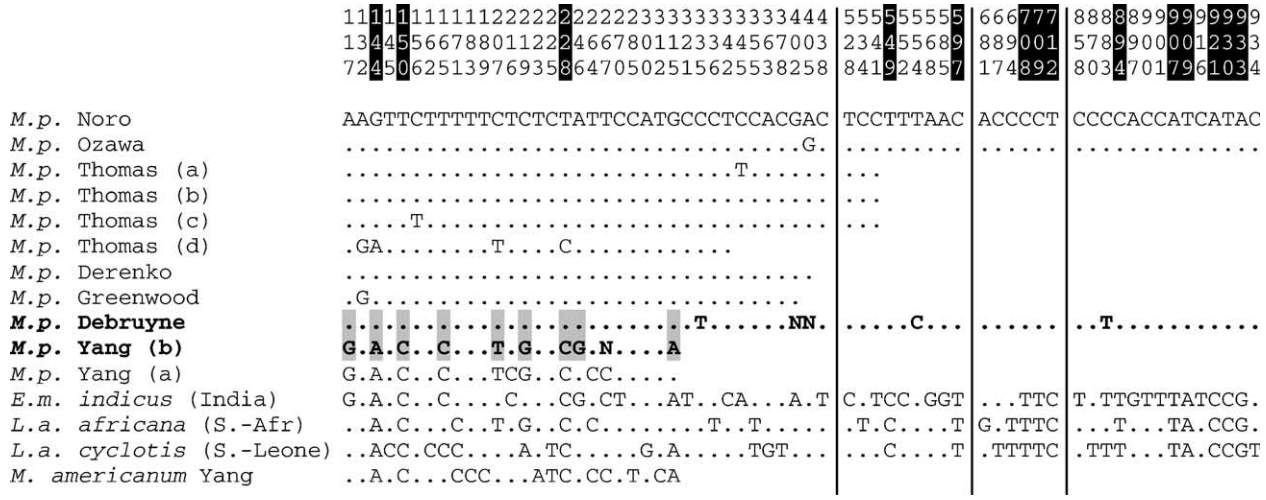

Fig. 2. Among-site variation for the different sequences of cytochrome *b* of mammoth in this study. Only sites that differ from the *Mammuthus* reference sequence (Noro et al., 1998) are shown. Sequences of elephants and mastodon are displayed for comparison. The two Lyakhov sequences are in boldface. The nine substitutions observed between these sequences are shown on a gray background. The position of mammoth-diagnosed sites (versus elephants) is indicated on a black background.

For the restricted analysis of the 228 bp shared by all Elephantidae, mastodon was used as alternative outgroup to Sirenia and Hyracoidea, which diverged from Proboscidea approximately 60 Ma, since the earliest known proboscidean is from the earliest Ypresian, circa 55 Ma (Gheerbrant et al., 2001). To test the impact of these distant outgroups, the topologies and homoplasy content were compared in both cases (features summarized in Table 2). When sirenians and hyrax are used to root the tree, the resolution of MP consensus decreases: six nodes are lost, affecting the ordering for *E. maximus*, but no original node emerges (Fig. 1). The same pattern is observed when any of the sirenians or hyrax is chosen as exclusive outgroup, except *T. manatus*, which stems from Asian elephants. On the other hand, CI and RI are not strongly affected.

For the entire cytochrome *b* gene, the analysis of the mutational saturation reveals that our data are saturated, due to the comparison with distant outgroups: the slope of the overall saturation is 0.6729 for all taxa and 0.8185 for the ingroup alone. The homoplasy content of character state transformation was evaluated for each substitution type at each codon position. It portrays a classical pattern (Table 3): a clear division is observed between frequencies of changes with C–T transitions highly saturated for each codon position (Hassanin and Douzery, 1999). The level of structuring in the substitutions is high and make a priori weighting of all transitions versus transversions inappropriate. When the product of the slope of saturation by the CI of each substitution type is implemented to weight transformations, the topology of the consensus tree remains strictly the same (with moderate changes of bootstrap values).

These results lead us to the conclusion that Sirenia and Hyracoidea used as outgroups weaken the resolution within Elephantidae because of mutational saturation, which hinders the assessment of ancestral character states. However, the analyses reveal that the remaining nodes are no topological artifacts but repeatable clusters. Hence, for want of closer extant relative, Sirenia and Hyracoidea can be used as convenient outgroups to Elephantidae.

3.3. The Lyakhov mammoth and the pattern of mammoth sequences

The result of the restricted analysis for the 228 bp shared by 32 terminals, when rooted with the American mastodon, is displayed in Fig. 1. The same pattern is repeated on parsimony, NJ, and poorly resolved ML trees. The two Lyakhov sequences (which belong to the same individual: dry skin, Yang et al., 1996; bone, this study) are at odds. One (Yang et al., 1996) is closely related to *E. maximus* whereas ours clusters with other mammoth sequences linked to *L. a. africana*. The display of the variable sites involved is shown in Fig. 2. Nine substitutions (on a gray background) can be found between the overlapping domain of the two Lyakhov sequences. For these sites, our sequence always displays the more common state among mammoth sequences, especially most complete sequences (Noro et al., 1998; Ozawa et al., 1997). On the contrary, the sequence from Yang et al. (1996) rather exhibits Indian elephant states of characters. Position 219 shows the only transversion (C–G/A) among elephantids along this fragment (Fig. 2). This character is identical for all mammoth sequences (cytosine), except the two from Yang et al. (1996), which display a guanine that has been observed only elsewhere in bush African elephants.

In fact, the two mammoths sequenced by Yang et al. (1996) are deemed composite sequences: both of them

Table 3
Homoplasy content for each type of substitution at each codon position

	Informative sites	Amount of homoplasy (CI)	Slope of saturation (S)	Product (CI * S)
First position				
C–T	31	**0.689**	**0.8627**	**0.594**
A–G	22	0.759	0.9009	0.685
A–T	11	0.917	0.9991	0.917
A–C	13	0.929	0.9702	0.901
G–C	6	1.000	1.0000	1.000
G–T	6	1.000	1.0000	1.000
Second position				
C–T	22	**0.733**	**0.9068**	**0.665**
A–G	4	1.000	1.0000	1.000
A–T	0	1.000	1.0000	1.000
A–C	1	1.000	1.0000	1.000
G–C	4	1.000	1.0000	1.000
G–T	2	1.000	1.0000	1.000
Third position				
C–T	127	**0.510**	**0.7653**	**0.390**
A–G	43	0.541	0.8483	0.459
A–T	32	0.914	0.9987	0.913
A–C	67	0.827	0.9603	0.794
G–C	9	1.000	1.0000	1.000
G–T	4	1.000	1.0000	1.000

Note. The number of informative sites, the consistency index excluding uninformative sites (Farris, 1989), the slope of linear regression for saturation, and the product (CI * S) are given. The lowest values of CI, S, and CI * S are indicated in boldface.

display typical features of Indian and African elephants. Conversely, they share only symplesiomorphies with mammoths, whereas the three mammoth-diagnosed apomorphies among 25 variable sites are lacking (positions numbered on black background, Fig. 2). This result casts doubt on the nature of the sequences (see further) obtained by Yang et al. (1996).

Otherwise, Fig. 1 shows that these 228 bp are insufficient to retrieve the African elephant clade shown elsewhere on the same molecular grounds (Barriel et al., 1999): here, the bush and forest elephants are kept separate because forest elephants emerge earlier in the tree. The Asian elephants are not monophyletic because they are closely associated with the two mammoth sequences from Yang et al. (1996). The latter two are distant from all the other mammoths, which form a clade of nine sequences from six different studies. The eight longer sequences of this clade (including ours) make one of the two well-supported branches in this tree (MP Bootstrap score: 85). This analysis betrays how questionable phylogenetic inferences made through short fragments may be: nearly all nodes are weakly supported (nine nodes have MP bootstrap values below 50%) because of extreme shortness of internal branches (Fig. 1).

3.4. Sister group relationships between Mammuthus and Loxodonta

To establish the affinities of mammoth sequences with other elephantids on reliable grounds, the analysis of the whole cytochrome *b* was performed. In general structure, MP and NJ trees conform to the previous results with greater support and resolution (Fig. 3). The monophyly of the three longest sequences of mammoth (Noro et al., 1998: 1137 bp; Ozawa et al., 1997: 1105 bp; ours: 561 bp) is recovered and highly supported in MP analysis (Bremer support = 9; Bootstrap value = 100). For distance analysis, our sequence was not taken into account because of the numerous missing positions it contains, which preclude any calculation of global similarity. Yet, the two other sequences of mammoth cluster with a high bootstrap value (100). In both cases, the mammoths are the sister group of monophyletic African elephants, although this node is not extremely robust (Bremer Support = 3; bootstrap value of 86 for NJ but only 63 for MP). The monophyly of the Asian elephants is highly supported.

The inclusion of the two forest African elephant sequences *L. a. cyclotis* has important impact on the optimization of characters. Table 4 shows that the length of the internal branch for *Loxodonta* is greatly affected as well as its robustness. It confirms previous studies (Barriel et al., 1999; Groves and Grubb, 2000; Roca et al., 2001) on the deep division between the two lineages of African elephants.

ML analyses are less conclusive with regard to the affinities of *Mammuthus* and *Loxodonta*. It is tricky to evaluate the fitness of ML parameters because of the extreme divergence between elephantids and outgoup taxa. Likelihood scores for the main alternative ingroup topologies are similar (Table 5). The Kishino and Hasegawa

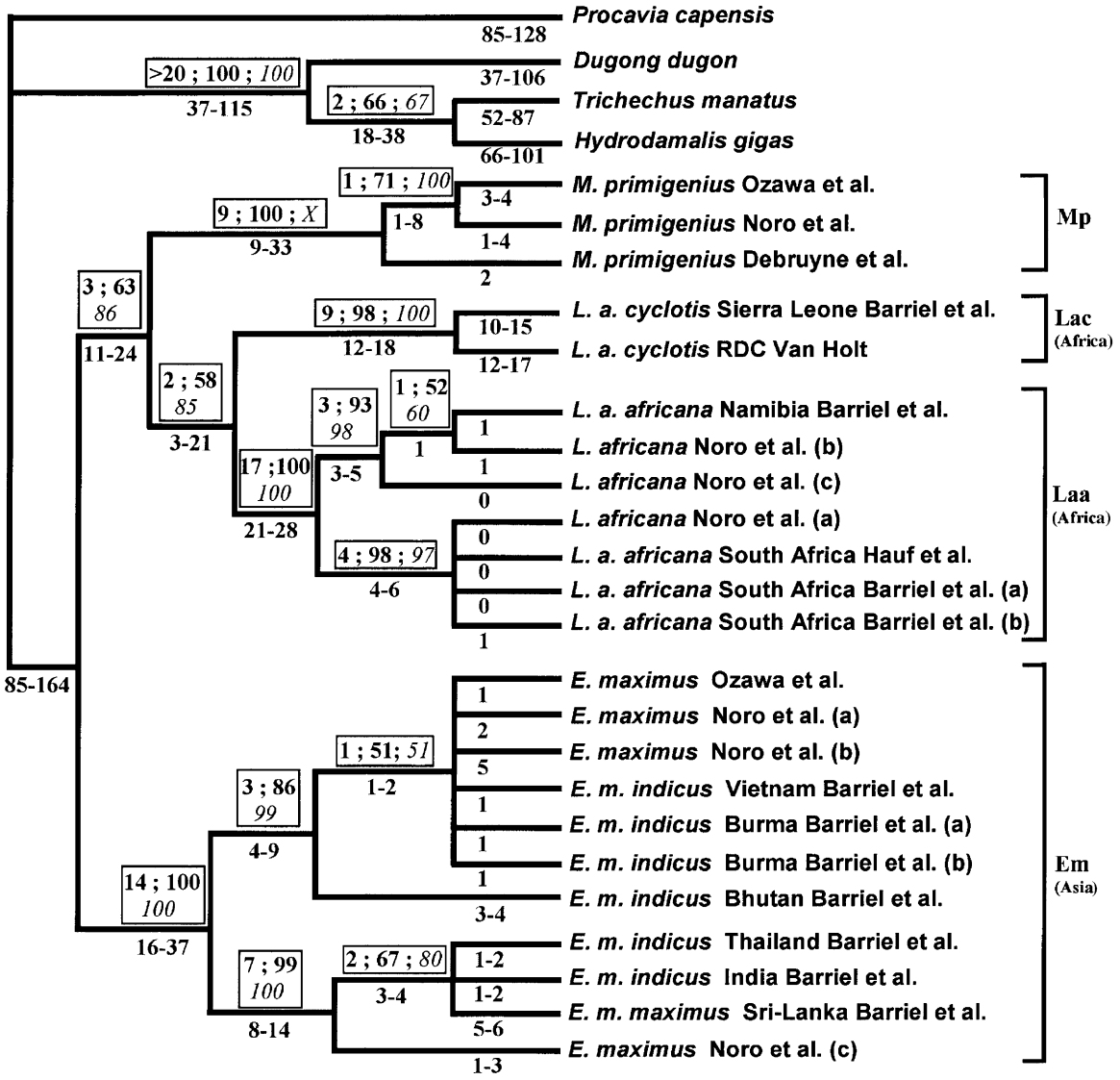

Fig. 3. Phylogeny for the matrix of entire sequences of cytochrome *b* (27 terminals; 1037 bp; 312 informative sites). Strict consensus tree of nine equally parsimonious trees (unweighted parsimony; length = 767; CI excluding uninformative characters = 0.65; RI = 0.86). Above branches: Bremer support (boldface), parsimony (boldface), and pairwise distance (italics) bootstrap values. Below branches: minimum and maximum number of apomorphies. Calculation of pairwise-distance bootstrapping is applied to 26 terminals (our sequence of *Mammuthus primigenius* was removed). Thus, an "X" is displayed for the bootstrap value of the monophyly of the three longer mammoth sequences, which could not be calculated.

Test (Kishino and Hasegawa, 1989) thus detects that the nine most parsimonious trees are not significantly different (at 95% confidence level) from the most likely tree where mammoths are sister group of Asian elephants. If a consensus of the non significantly different trees is built, ingroup topology is thoroughly unresolved.

3.5. Robust branches and reliable associations

The differential weighting of substitutions does not affect the position of mammoth sequences in MP analyses. To test further the robustness of the mammoth clade, we investigated the evolution of the Bremer

Table 4
Impact of the inclusion of African forest elephants (*L. africana cyclotis*) on the topology

Data set analysed	Terminals	(*Loxodonta*) branch			(*Mammuthus*, *Loxodonta*) branch		
		Length	Bootstrap **MP**; *NJ*	BS	Length	Bootstrap **MP**; *NJ*	BS
L. a. cyclotis excluded	25	19–39	**100**; *100*	+18	11–24	**60**; *89*	+2
L. a. cyclotis included	27	3–21	**58**; *85*	+2	12–25	**63**; *88*	+3

Note. Bootstrap scores (for MP and NJ analyses), Bremer support ("BS") as well as minimum and maximum lengths of the branches are shown.

Table 5
Results of K–H test for trees of interest

Method	Topology	−LnL	ΔLnL	SE	ΔLnL/SE	*P*
ML	Best: (((Em, Mp), Laa), Lac)	4339.39591	[best tree]			
MP	Tree1-9: (((Laa, Lac), Mp), Em)	4340.96964	1.57374	1.39322	1.1296	0.259
—	(((Laa, Lac), Em), Mp)	4340.62604	1.23013	1.66785	0.7376	0.461

Note. The column "method" indicates in which analysis(es) the corresponding tree is retrieved."−LnL": log likelihood of the topology in ML analysis; "ΔLnL": log-likelihood difference between the chosen tree and the best ML tree; "SE": standard error of log-likelihood difference; "P": probability of getting a more extreme ΔLnL/SE value under the null hypothesis that topologies are not different.

support for this node according to the variability of sites conserved in the matrix (Fig. 4). When the sites with lowest RI are dismissed, the score of the (*Mammuthus*, *Loxodonta*) clade regularly increases up to 9 while the support of the two alternative nodes collapses. When more than 126 sites are rejected, all supports converge to zero, because of the mere removal of the phylogenetic signal contained in the data set.

The randomization and partitioning of informative sites through the PRN method demonstrate that the accuracy of the phylogenetic assumptions is highly dependent on the size of the data set. For fragments equivalent to 100 up to 200 bp (considering a 10% informative site proportion as explained above), the support of the critical node within Elephantidae is greatly unstable (Fig. 5). Any of the three possibilities: (*Mammuthus*, *Loxodonta*), (*Mammuthus*, *Elephas*) or (*Loxodonta*, *Elephas*) can be highly supported according to jackknifed sites, although mean bootstrap proportions (BP) are favorable to the first grouping (Fig. 5A).

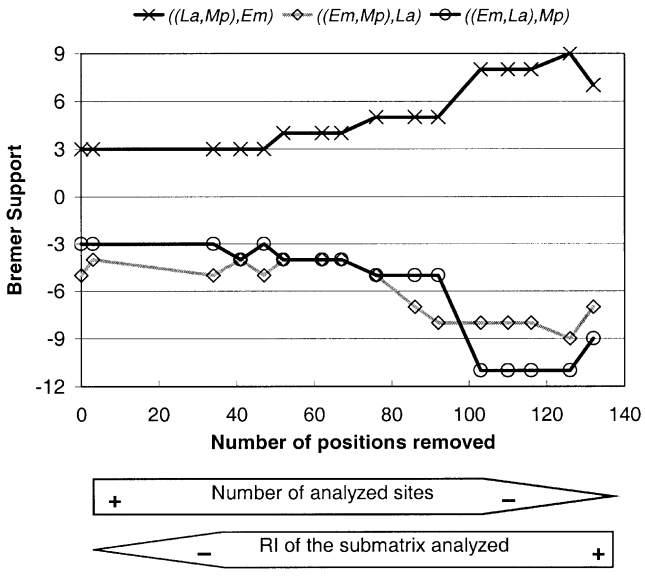

Fig. 4. Evolution of the Bremer support for the three alternative nodes about relationships between Elephantidae, as a function of an RI-dependent partitioning of the entire matrix of cytochrome *b*. In abscissa, the number of sites removed from the analysis (according to their RI value) is given. In ordinate, the Bremer support is displayed.

However, when longer sequences are available, the pattern is clearcut: the only supported cluster is the (*Mammuthus*, *Loxodonta*) clade. Its mean BP increases at each step while the related standard deviation decreases. This latter observation is partially an artifact due to resampling of identical sites within independent bootstrap procedures. The partition of the BPs of the (*Mammuthus*, *Loxodonta*) clade fits to the PRN equation: $f(x) = 100(1 - e^{-b(x-x')})$, with x being the number of informative sites sampled and $x' = -10$ and $b = 0.019$ parameters estimated by non-linear regression. According to these parameters, the number of informative sites necessary to assure (*Mammuthus*, *Loxodonta*) can be estimated. For a mean BP of 70%, regarded as a probability $\geqslant$95% to retrieve historical lineages (Hillis and Bull, 1993), the relevant number of informative sites is 53. For a BP of 90%, it reaches 147, not far from the total number of informative sites in the actual matrix.

4. Discussion

4.1. Discrepancies between the two sequences of M. primigenius from Lyakhov Island

No positive extraction was obtained on the same material than that previously used (Yang et al., 1996): the skin which has been air-dried for more than 90 years appears to have DNA of poor quality. On the other hand, the sesamoid bone of the hind-limb gave positive results. However, the former study on the DNA of this mammoth had indicated that quite-long amplifications could be obtained with DNA extracted from its skin: Fragments up to 420 bp had been amplified according to Yang et al. (1996). Unfortunately, we were unable to reproduce such results in spite of numerous attempts.

Other data showed likewise that, for a much recent mammoth (less than 14,000 years old), the largest size of successful amplification for mitochondrial DNA comprised between 239 and 376 bp (Greenwood et al., 1999). Yet, extracts had been performed from bone, which is generally regarded as the best substrate for preserving DNA through time (Austin et al., 1997; Colson et al., 1997; Hagelberg, 1994). In addition, we tested a continuous range of sizes for amplifications that proved that

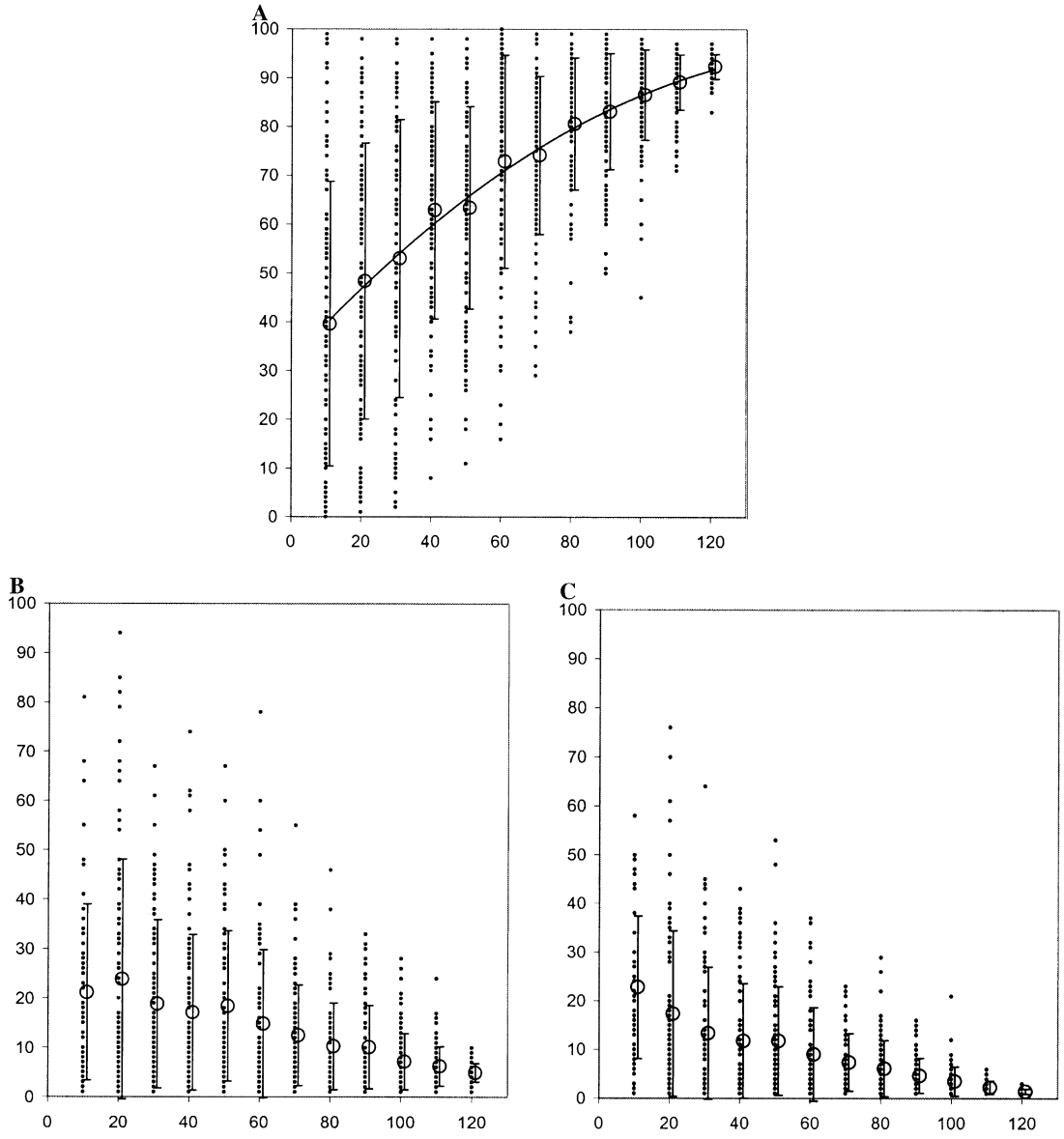

Fig. 5. Application of the PRN method (Lecointre et al., 1994) to the matrix of entire sequences of cytochrome *b*. *Dugong dugon* was used as outgroup. In ordinate: pairwise distance bootstrap proportion (BP). Mean values and standard deviation are displayed. In abscissa: the number of informative sites re-sampled by jackknife. (A) support of the (*Mammuthus*, *Loxodonta*) node. (B) support of the (*Elephas*, *Mammuthus*) node. (C) support of the (*Elephas*, *Loxodonta*) node.

no fragments longer than 180 bp could be amplified from Lyakhov mammoth, that is to say, far less than what Yang et al. (1996) obtained.

Furthermore, the phylogenetic analyses depict a deep division between the two sequences of mammoths from Yang et al. (1996) and all other mammoth sequences under consideration (Fig. 1) with which they share no apomorphy (Fig. 2). On the other hand, our own sequence of the same specimen from Lyakhov Island displays a typical mammoth sequence that clusters with others in a highly supported clade.

All these features led us to the conclusion that the sequences of mammoths obtained by Yang et al. (1996) likely derived from crossed contaminations by exogenous DNA: a chimera of African and Asian elephant sequences is foreseen.

In 1997, Yang et al. proposed a blind testing to authenticate sequences of ancient DNA. They were forced to recognize that this solution is but an alternative possibility "when independent testing by two laboratories is not available" (Yang et al., 1997, p. 261). This procedure keeps the experimenter unaware of the nature of the material he sequences: the phylogenetic consistency of sequences produced is examined a posteriori to determine the reliability of ancient DNA. It appears now that their mammoth sequences (Yang et al., 1996)

do not fit the consistency criterion. Woolly mammoths belong to a remarkable taxon which can rely on various sources of molecular data, so that the phylogenetic analyses "should make phylogenetic sense" (Austin et al., 1997, p. 304) as seen in this study. But the reproducibility of the sequences in different laboratories should still be preferred as a criterion of authenticity for ancient DNA (Lindahl, 1993; Taylor, 1996).

4.2. Far from the outgroup

The greatest problem in the phylogenetic analysis of DNA aligned sequences from close relatives like mammoths and elephants deals with the choice of appropriate outgroup (Nixon and Carpenter, 1993; Yang et al., 1996). In the present case, this choice is hindered by the lack of extant taxa related to Elephantidae, because major groups of Proboscidea (i.e., mammutids, gomphotheres, and deinotheres) are all extinct, the very last members of these taxa disappearing during the Pleistocene. One mammutid, the American mastodon whose last representatives met Amerindians 10,000 years ago, was used to root the tree of Elephantidae by Yang et al. (1996). However, our analyses attest that there is no incongruence in MP topologies whenever the mastodon is replaced by hyracoid and sirenians. The saturation produced by this alternative rooting of the tree causes the lack of resolution of ML trees but does not completely overwhelm the phylogenetic signal with regard to the interrelationships of elephantids. This is evidenced by the unrooted tree of the elephantids alone which displays the same pattern as the rooted MP consensus shown in Fig. 3 (data not shown).

4.3. No more conflict in molecular results

When the two dubious sequences of Yang et al. (1996) are removed, the affinities of mammoths can be identified: they form a clade with African elephants. This result was not retrieved with the partial matrix (Fig. 1) but we demonstrated with the PRN method that the amount of phylogenetic signal in such short fragments was insufficient to establish a reliable pattern for nodes lying on short branches. The PRN method also indicates that a mean of 53 informative sites (when only one sequence is chosen as outgroup) is necessary to display a BP of at least 70% for the (*Mammuthus*, *Loxodonta*) clade. This conclusion validates the recent approaches of mammoth molecular phylogeny (Noro et al., 1998; Ozawa et al., 1997; Thomas et al., 2000), which seek after sequences longer than those previously published. To conclude, our successive treatments depict that: (1) the fast-evolving cytochrome *b* shows limited variation among elephantids, which leads to poorly resolved trees and (2) the longest fragments as possible are needed to reach robust topologies and diagnostic apomorphies between *Loxodonta* and *Mammuthus*.

The only point of disagreement which remains to be discussed is the position of the mammoth sequence by Ozawa et al. (1997): in their study, the authors found a classical grouping with Asian elephants, although we always find this sequence clustering with other mammoths as sister group of African elephants. This difference bears on their unsuitable sampling for *Loxodonta* representatives. Ozawa et al. (1997) chose the sequence published by Irwin and Arnason (1994) as the only representative of this genus. Strikingly, when replaced by any other published African elephant sequence, the topology changes to grouping mammoths with loxodonts instead. Likewise, we investigated the phylogenetic disturbance, caused by encompassing Irwin's sequence in our analyses. As formerly reported by Barriel et al. (1999), it does not affect the topology and rather clusters within savanna African elephants (precisely as the sister group of South-African haplotypes). This intriguing case originates from the divergence exhibited by Irwin's haplotype. It is compounded by exclusive features—especially near 3′ end of the sequence—among which a supernumerary codon must be noted, that are responsible for its remote relationship with mammoths when used as the only African sequence. Nevertheless, it shows enough commonalities with other African sequences to warrant its inclusion within *Loxodonta* clade in broader taxonomic array. Sequencing errors (Noro et al., 1998) and even nuclear insertion of mitochondrial DNA (Greenwood, 2001) have been advocated to invalidate this reference. Accordingly, this bias accounts for dismissing this haplotype as assumed by other recent publications (Barriel et al., 1999; Noro et al., 1998; Thomas et al., 2000).

4.4. Confrontation of molecular and morphological results

The clustering of *Mammuthus* and *Loxodonta* is clearly the best supported grouping within Elephantidae on molecular grounds (Noro et al., 1998; Thomas et al., 2000; this study). This result conflicts with the morphological scheme, which establishes *Mammuthus* and *Elephas* as sister groups (Shoshani and Tassy, 1996). Thomas et al. (2000) question this point of view by review of the morphological characters of elephantids. Nevertheless, their discussion does not mention characters that support unambiguously sister group relationships between *Mammuthus* and *Loxodonta*. It rather demonstrates that most characters that support an (*Elephas*, *Mammuthus*) clade, several being known for a long time (summary by Tassy and Shoshani, 1988), are variable and affected by convergence. Yet, some of them, as the large dorsal parietal bulges and concave fronto-parietal region, although evolving in parallel are

already more comparable in early *Mammuthus* and *Elephas* species, known so far. Yet, because the skulls of earliest African mammoths such as *Mammuthus subplanifrons* and *Mammuthus africanavus* are still poorly known, the debate is not closed. It can also be noted that, according to Tassy (1995), a partial skull of cf. *Primelephas* from the late Miocene of Uganda exhibits characters present in *Elephas* and *Mammuthus* and not *Loxodonta*, especially a concave frontal along the linea temporalis, with this character reminiscent of the condition seen in a late Miocene gomphothere *Paratetralophodon hasnotensis*. This species is a gomphothere of tetralophodont grade, a grade usually understood as the stem group of elephantids. Such traits could be plesiomorphic traits for Elephantinae retained in both *Mammuthus* and *Elephas* but transformed in *Loxodonta*, in connection with the "globular skull" considered autapomorphic by Kalb and Mebrate (1993). These traits would then be compatible with a (*Mammuthus*, *Loxodonta*) clade. Yet, while molecular data seem to converge unambiguously, more is needed to conclude on the basis of morphology.

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Appendix A

Primers used for sequencing of *M. primigenius*. *The letters L and H refer to the sequence of light and heavy strands, respectively. Numbers correspond to 5′ end position of the primers in the complete African elephant mitochondrial sequence (Hauf et al., 2000). The suffixes E, M refer to the specificity of the primer: Elephantidae or Mammoth only. A terminal "*n*" was added to primers taken or modified from Noro et al. (1998)

Primer*	Sequence	Sequencing primer
L14096	5′-GCTTGATATGAAAAACCATCGTT-3′	−
L14147 E	5′-ATGACCCACAYYCGAAAATCTCA-3′	−
L14160 M	5′-GAAAATCTCACCCCCTACTTA-3′	−
L14241 M	5′-ATTTCGGCTCACTACTAGGAG-3′	+
L14283 E	5′-TAACAGGATTATTCCTAGCCA-3′	−
H14306 M	5′-GTATGGCTAGAAATAACCCTGTTAG-3′	+
L14310 E	5′-ATACACCTGACACAATAACTGC-3′	+
L14328 En	5′-CTGCATTTTCATCTATATCCCAT-3′	+
H14349 E	5′-TGGGATATAGATGAAAATGCA-3′	+
L14421 E	5′-TCTGCCTATACACACACATTGGA-3′	−
L14442 Mn	5′-GACGAAACATCTACTATGGGTCC-3′	+
H14452 E	5′-GATGTTCCGTCCAATGTGTG-3′	+
H14491 E	5′-GGTATTTCAGGTTTCCGAGTAT-3′	+
L14558 En	5′-ATATCATTCTGAGGGGCAACC-3′	−
H14613 En	5′-GATATAGGGAATTGCTGAGAAGAG-3′	+
L14639 E	5′-TGAGGAGGCTTTTCRGTAGATAA-3′	+
H14709 Mn	5′-GTAAATGGAAGAATAAAATGGAG-3′	−
H14769 E	5′-GAATTGTTTGAGCCTGTTTCGTG-3′	+
L14807 M	5′-CACCCGTACTATACCATTAAA-3′	+
H14875 E	5′-GGCTAGGAGTAGAAGAAGTAA-3′	−
L14899 E	5′-AGACCCTGACCACTACATACC-3′	−
H14946 E	5′-TGTAGGGGRGTATTTAGTGG-3′	+
L14981 E	5′-GCCATCCTACGATCTGTACCA-3′	+
H15038 E	5′-TTGATAGGAGTAGGGCTAGGA-3′	−
L15074 E	5′-TCTAAACACCGAAGTATGATAC-3′	−
H15103 E	5′-AGGTCGGAGTATTATGCTTCG-3′	+
L15151 E	5′-TACATGAATTGGCAGTCAACC-3′	−
H15171 E	5′-GGTTGGCTGCCAATTCATGT-3′	−

Appendix A (*continued*)

Primer*	Sequence	Sequencing primer
H15199 E	5′-GCCGATAATGATGTAGGGATA-3′	−
H15283 E	5′-TTACTTAATGAGGTAGTTTTCG-3′	−
H15333 E	5′-GCTTTCATTTATGGCTTACA-3′	−
L00521 En	5′-GCCCTAAACTTTGATAGCTACCTTT-3′	+
H00699 En	5′-GAAGATGGTGGTATATGGACTGAATT-3′	+

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