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Phylogenetic Position of Mammoth and Steller's Sea Cow Within Tethytheria Demonstrated by Mitochondrial DNA Sequences

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Abstract. Here we report DNA sequences from mitochondrial cytochrome *b* gene segments (1,005 base pairs per species) for the extinct woolly mammoth *(Mammuthus primigenius)* and Steller's sea cow *(Hydrodamalis gigas)* and the extant Asian elephant *(Elephas maximus)*, the Western Indian manatee *(Trichechus manatus)*, and the hyrax *(Procavia capensis)*. These molecular data have allowed us to construct the phylogeny for the Tethytheria. Our molecular data resolve the trichotomy between the two species of living elephants and the mammoth and confirm that the mammoth was more closely related to the Asian elephant than to the African elephant. Our data also suggest that the sea cow–dugong divergence was likely as ancient as the dugong–manatee split, and it appears to have been much earlier (22 million years ago) than had been previously estimated (4–8 million years ago) by immunological comparison.

Key words: Cytochrome *b* — Ancient DNA — *Mammuthus primigenius* — *Hydrodamalis gigas* — Proboscidea — Sirenia — Tethytheria — Molecular phylogeny

Introduction

The mammalian orders Proboscidea and Sirenia were confined to Africa in their early history. Because of this geographic association and the possession of common morphological characters, the proboscideans and sirenians are believed to have shared a common ancestry. Simpson (1945) classified African ungulates including the Proboscidea, Sirenia, Hyracoidea, and some extinct orders in the superorder Paenungulata. McKenna (1975) grouped the Proboscidea, Sirenia, and an extinct order, Desmostylia, in the mirorder Tethytheria, an ungulate group that originated in the coastal areas of the Tethyan Sea in the Paleogene period.

The validity of these supraordinal classifications has been substantiated by subsequent morphologic (Novacek 1992; Prothero et al. 1988; Prothero 1993) and molecular studies (Czelusniak et al. 1990; De Jong et al. 1993; Irwin et al. 1991; Irwin and Wilson 1993; Mckenna 1987; Springer and Kirsch 1993). Immunological approaches have been undertaken by Lowenstein et al. (1981), Lowenstein and Scheuenstuhl (1991), and Shoshani et al. (1985) to clarify the phylogenetic relationships among extinct and extant members of the Tethytheria. In these studies immunologically reactive protein substances were extracted from the soft tissues of the extinct woolly mammoth *(Mammuthus primigenius)* and also from bones of Steller's sea cow *(Hydrodamalis gigas)* and the American mastodon *(Mammut americanum)*. Immunological comparisons among the members of the Tethytheria established that the mammoth is immunologically equidistant from the African *(Loxodonta africana)* and the Asian elephants, and that, within the sirenians, the closest resemblance occurs between Steller's sea cow and the living dugong *(Dugong dugon)*. These molecular results seem to reject the dental morphology-

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based phylogenetic hypothesis (Aguirre 1969; Maglio 1973) that the mammoth is closely related to the Asian elephant. Prediction of time of divergence of evolutionary lineages based on the immunological comparisons was also inconsistent with the paleontological inference. The paleontological estimates (Domning 1994) had put the sea cow–dugong divergence not later than late Oligocene (>25 million years ago), whereas the immunological data suggest that divergence occurred only 4–8 million years ago. Further, the paleontological estimate for the dugong–manatee split was put at 30–40 million years ago, while the immunological data points to a common ancestor that lived only 17–20 million years ago.

In this study we determined DNA sequences from mitochondrial cytochrome *b* gene segments (1,005 base pairs, bp) for two extinct animals, the woolly mammoth *(Mammuthus primigenius)* and Steller's sea cow *(Hydrodamalis gigas),* and three extant species, the Asian elephant *(Elephas maximus)* and the West Indian manatee *(Trichechus manatus)* and the hyrax *(Procavia capensis).*

Here we present the first analysis of phylogenetic relationships among the extant and some extinct members of the Tethytheria based on mitochondrial cytochrome *b* gene sequence data obtained by us and previous authors (Irwin et al. 1991; Irwin and Árnason 1994).

Materials and Methods

DNA Isolation. Total DNA was extracted from muscle tissue of the 40,000-year-old baby Magadan mammoth known as Dima generally following the method of Pääbo et al. (1988). One gram of tissue was minced with forceps in 8 ml of TEN buffer (10 mM Tris-HCl pH 8.0/2 mM EDTA/10 mM NaCl). Following this, 4 mg of collagenase (Sigma) was added and incubated at 37°C for 3 h with gentle agitation. Tissue was then lysed by adding 440 μl of 20% SDS, 80 mg DTT, and 4 mg of proteinase K at 37°C for 12 h with gentle rotating followed by two extractions with phenol chloroform and one extraction with chloroform.

Steller's sea cow DNA was obtained from a scapula collected on Bering Island, Kamschatka. Extraction followed the method of Hagelberg and Clegg (1991). The surface of the scapula bone was removed using a hand grinder to avoid contamination by extraneous DNA. Two grams of cleaned bone were powdered in an iron mortar and decalcified by suspending in 40 ml of 0.5 M EDTA incubated at room temperature with agitation for 72 h with two fresh exchanges of EDTA. Decalcified bone powder was then subjected to lysis by incubating 37°C 12 h in 20 ml of 0.5 M EDTA/100 μg ml^{-1} proteinase K/0.5% N-lauroylsarcosine with gentle rotation followed by two extractions with phenol/chloroform and one extraction with chloroform.

Purification and Amplification. DNA solutions were concentrated using an Amicon-B15 concentrator (Amicon) to <1 ml followed by washing three times with 2 ml of deionized water using a Centricon 30 microconcentrator (Amicon) and finally preserved in 100 μl TE for use as a template. One microliter of purified total DNA was subjected to 30 or 40 cycles of PCR reactions, which were performed in 25 μl of reaction volume containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.2 μM each primer, 0.5 mg/ml BSA (Sigma), 0.5% Nonidet p-40 (Sigma), and 2 units of *Taq* polymerase (Takara). Each cycle consisted of (94°C) 45 s for denaturation, (48–50°C) 90 s for annealing, and (72°C) 120 s for extension. After the last cycles, samples were incubated for 3 min at 72°C. As a result of several trials, we succeeded in the amplification of five fragments, about 150 to 400 base pairs in length, covering 1,005 base pairs of partial mitochondrial cytochrome *b* gene as summarized below. No amplification was detected in extraction and PCR blank.

fragment 1: L14724 5′-CGAAGCTTGATATGAAAAACCATC-GTTG-3′ (Irwin et al. 1991) H15149 5′-AAACTGCAGCCCCT-CAGAATGATATTTGTCCTCA-3′ (Kocher et al. 1989)

fragment 2: L15144 5′-ATAGCCACAGC(C/A)TTCATAGG(A/C)TA(C/T)GTCCT-3′ H15347 5′-GGGTT(A/G)TT(G/T)GATCCTGTTTCGTG-3′

fragment 3: L15306 5′-CGATTCTTCGC(C/T)TTCCACTT(C/T)ATCCT(A/T/C)CCATT-3′ H15494 5′-TAGTTGTC(A/T)GGGTCTCC(G/T)A(A/G)-3′

fragment 4: L15408 5′-A(C/T)AGA(C/T)AAAAT(C/T)CC(A/C)TT(C/T)CA-3′ H15603 5′-GCTAG(G/T)AC(G/T)CCTCCTAGTTT-3′

fragment 5: L15513 5′-CTAGGAGACCC(A/T/C)GA(C/T)AA(C/T)TA-3′ H15755 5′-TCTACTGG(C/T)TG(G/T)CC(T/G/C)CC(A/G)ATTCATGT-3′

L and H refer to the sequence of light and heavy strands, respectively, and the numbers correspond to the 3′ end positions of the primers in the numbering system for human mitochondrial DNA (Anderson et al. 1981). In this numbering system the 1,005-bp sequence corresponds to the sequence from 14,747 to 15,751.

The DNA fragments were purified on a 2% Nusieve agarose gel and then subjected to asymmetric PCR (Gyllenstein and Erlich 1988) or subcloned into plasmid pUC118 after terminal polishment by klenow fragment (Frohman 1994). Direct sequencing was performed using Sequenase ver. 2.0 Kit (US Biochemical). Sequencing for plasmid clone was done by *Bca*best dideoxy sequencing Kit (Pharmacia) with A. L. F. DNA sequencer. The sequences of cloned PCR products were determined from the consensus of more than three clones. Most of the cloned molecules examined had an identical sequence. However, 10–20% of the clones exhibited sequence variation with one random mutation per 100–150 base pairs.

Extant Species. Total DNA was extracted from blood of the Asian elephant and the West Indian manatee following standard procedure. The 1,005-bp partial cytochrome *b* gene sequences were determined by direct sequencing with primer pairs cited above and H15915R: 5′-GGAATTCATCTCTCCGGTTTACAAGAC-3′ (Irwin et al. 1991).

Phylogenetic Analyses. DNA sequences were initially aligned using the ESEE program package (Cabot 1987). Neighbor-joining (Saitou and Nei 1987) and maximum parsimony analyses were performed with the PHYLIP program package (Felsenstein 1993). Distance matrices for neighbor-joining trees were estimated by DNADIST with the Kimura two-parameter option (10.0 transition/transversion ratio) and PROTDIST with the Dayhoff matrix option. The neighbor-joining tree and the maximum parsimony tree were generated by NEIGHBOR and PROTPARS, respectively. Bootstrap treatment was performed by SEQBOOT (1,000 replicates) and CONSENSE. Formerly published sequences used in the analyses (five sequences for outgroups and two sequences for ingroups) were derived from DDBJ, EMBL, and GenBank databases as follows: African elephant *(Loxodonta africana)* (Irwin et al. 1991, X56285), dugong *(Dugong dugon)* (Irwin and Árnason 1994, U07564), fallow deer *(Dama dama)* (Irwin et al. 1991, X56280), pronghorn antelope *(Antilocapra americana californica)* (Irwin et al. 1991, X56286), Grévy's zebra *(Equus grevyi)* (Irwin et al. 1991, X56282), black rhinoceros *(Diceros bicornis)* (Irwin et al. 1991,

X56283), and South American opossum *(Monodelphis domestica)* (Ma et al. 1993, X70673).

Result and Discussion

Sequence Similarity

Figure 1 summarizes the alignment of 1,005-bp cytochrome *b* gene sequences of living and recently extinct paenungulates. Newly determined sequences have been deposited in DDBJ, EMBL, and GenBank under accession numbers D83047–D83050 and D86909. The matrix (Table 1) shows sequence differences and genetic distances among 1,005-bp cytochrome *b* gene sequences for the paenungulates. Pairwise sequence differences within the Elephantidae were 9.3% between the African and Asian elephants, 8.4% between the African elephant and the woolly mammoth, and 6.5% between the Asian elephant and the woolly mammoth. As to sirenians, the differences were 14.0% between the dugong and the West Indian manatee, 15.2% between the Steller's sea cow and the West Indian manatee, and 15.0% between the Steller's sea cow and the dugong. Proboscideans differed from sirenians by 24.2% on average. The hyrax was different from sirenians by 21.2% and from proboscideans by 24.2%, on average.

Molecular Phylogenetic Trees

Molecular phylogenetic trees for the seven paenungulates were constructed by the neighbor-joining (Saitou and Nei 1987) and maximum parsimony methods based on three types of data sets—namely, DNA sequences of 1 + 2 codon positions (670 bp) and inferred amino acid sequences (335 residues) with five mammals as outgroups (Fig.2).

The seven paenungulates were grouped together with bootstrap probabilities ranging from 92% in the maximum parsimony tree based on 1 + 2 codon positions to 95% in the neighbor-joining tree based on 1 + 2 codon positions in our cytochrome *b* DNA trees. The paenungulate clade was supported with higher bootstrap values in the amino acid trees; 98% in the neighbor-joining tree and 90% in the maximum parsimony trees. There is considerable disagreement about the phylogenetic position of the Hyracoidea. Some workers regard the Hyracoidea as a sister group to Tethytheria based on shared character of carpal elements and reduction of some basicranial bones (Novacek et al. 1988). Others pointed out the close relationship between Hyracoidea and some perissodactyls based on their expanded eustachian sac in the middle ear region (Prothero et al. 1988). The present molecular study strongly supports the hypothesis that Hyracoidea is more closely related to tethyteres than to perissodactyls. This conclusion is similar to that of earlier molecular studies (De Jong et al. 1981; Lowenstein and Scheuenstuhl 1991; Springer and Kirsch 1993). High bootstrap probabilities strongly support the monophyly of the proboscideans (100% in all trees) and the sirenians (94–100%). However, in our cytochrome *b* tree, the relationship among Proboscidea, Sirenia, and Hyracoidea was not clear. Some trees (NJ trees based on 1 + 2 codon positions and amino acids) showed a Proboscidea + Sirenia clade. The mitochondrial 12S ribosomal RNA gene tree of Springer and Kirsch (1993) also hypothesized a Proboscidea + Sirenia clade with a high bootstrap value.

The MP tree based on 1 + 2 codon positions showed a Proboscidea + Hyracoidea clade—a relationship also shown by IRBP gene sequences (Stanhope et al. 1996)—while the amino acid MP tree favored a Sirenia + Hyracoidea clade—a relationship also proposed in the α-crystallin tree of De Jong et al. (1981) and in the von Willebrand Factor tree of Porter et al. (1996). With respect to the phylogeny of the Elephantidae including the four genera *Palaeoloxodonta, Loxodonta, Mammuthus, and Elephas,* the morphological approach has suffered from low resolving power due to the paucity of putative synapomorphs and their mosaic distribution. These conditions often yield a polychotomous conclusion or a different view among researchers due to different methods of character evaluation (Aguirre 1969; Maglio 1973; Tassy 1994). The immunological study of Lowenstein et al. (Lowenstein et al. 1981; Lowenstein and Scheuenstuhl 1991) also resulted in a trichotomous conclusion. Recent molecular studies (Hagelberg et al. 1995; Hauf et al. 1995; Höss et al. 1994; Yang et al. 1996) based on amplified mitochondrial DNA sequences of the woolly mammoth (less than 300 base pairs) were also unable to resolve clearly which two of the Elephantid species, including the two living elephants and the woolly mammoth, share the closest kinship.

In our cytochrome *b* DNA tree (Fig.2), the Asian elephant and the mammoth are grouped together, irrespective of the tree-making method and data set used with bootstrap probabilities of 72% in the maximum parsimony and the neighbor-joining tree based on 1 + 2 codon positions. In the amino acid trees, bootstrap values increased to 90% in the neighbor-joining tree and 91% in the maximum parsimony tree. This result supports a mode of dichotomous separation within the Elephantidae, with the closest relationship between the Asian elephant and the mammoth excluding the African elephant. The emergence of *Loxodonta* species in geologic time is a few million years earlier than that of the species of *Elephas* and *Mammuthus* (Tassy 1986). Thus our molecular evidence is also concordant with fossil records.

Modern sirenians are divided into the Trichechidae *(Trichechus)* and the Dugongidae *(Dugong and Hydrodamalis)* based primarily on morphological characters of the rostrum, cheek teeth, pectoral limb, and tail fluke. In our phylogenetic analyses based on the mito-

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Asian elephant      ATG ACC CAC ACC CGA AAA TTT CAC CCC CTG TTT AAA ATC ATC AAC AAA TCC TTC ATT GAT  60
Mammoth             ... ... ... .TT ... ... .C. ... ... ..A C.. ... ... C.T ..T ... ... ... ... ...
African elephant    ... ... G.. .TT ... ... .C. ... ... T.A C.. ... ... ... ..T ... ... ... ... ...
Dugong              ... ... A.. .T. ... ... .CA ... ..A ..A A.C ... ... C.A ... ..C ... ... ... ..C
Steller's sea cow   ... ... A.. .TT ... ... ACA ..T ..A ... A.C ... ... C.G ..T G.C ..A ... ..C ..C
Manatee             ... ... A.. .TT ... ... .CA ... ..A ..A A.C ... ... T.G ..T G.C ... ... ..C ..C
Hyrax               ... ... A.. .TT ... ... AAC ... ..A ..A C.. ... .C. ... ... G.C G.. ... ... ...

Asian elephant      CTA CCC ACC CCA TCT AAC ATC TCA ACA TGA TGA AAT TTC GGC TCA CTA CTA GGA GCA TGC 120
Mammoth             ... ..T ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
African elephant    ... ..T ... ... ..C ... ..A ... ... ... ... ... ... ... ... ... ... ... ... ...
Dugong              ..C ..T ... ..C GTA ..T ... ... T.. ... ... ..C ..T ... ..C ... ..C ..G ... ...
Steller's sea cow   ..C ... ..T ... A.A ... ... ... T.. ... ..G ..C ..T ..T ..C T.. ..T ... ..G ...
Manatee             T.G ..A ..T ... A.A ... ... ... T.. ... ... ..C ..T ... ... ... ..T ..G ..G ...
Hyrax               ... ..A ..G ... ..C ... ... ... ... ... ... ..C ... ..A ..C ..C ... ... ..C ...

Asian elephant      CTA ATT ACC CAA ATC CTA ACA GGA TTA TTC CTA GCC ATA CAT TAC ACA CCT GAC ACA ATA 180
Mammoth             ... ... ... ... ... ... ... ... ..G ... ..T ... ... ... ... ..T ... ... ... ...
African elephant    ... ... ..A ... ... ... ... ... ... ... ... ... ... ... ..T ... ..C ... ... ...
Dugong              ..G ... .TT ... ..T ..C ... ... ... ... ..G ... ... ..C ... ... T.A ... ... C..
Steller's sea cow   ... ... .TT ... ..T ..C ... ... C.. ... T.G ... ... ..C ..T ..G T.C ..T ..G TC.
Manatee             ... ..C .TT ... ..T ..C ... ... ... ... ... ... ... ..C ... ... T.A ... ..G C..
Hyrax               ... ..C .T. ... G.T ... ... ..C C.. ... ... ..T ... ... ... ... T.A ..T ... ...

Asian elephant      ACT GCA TTT TCA TCT ATA TCC CAT ATC TGC CGA GAC GTC AAC TAC GGC TGA ATT ATT CGA 240
Mammoth             ... ... ... ... ... ... ... ... ... ... ... ... ..T ... ... ... ..T ... ... ...
African elephant    ... ... ... ... ... ... ... ... ... ... ..T ... ... ..T ..A ... ... ... ... ...
Dugong              ..C ... ..C ..C ..A G.. A.. ... ..T ... ..G ..T ..A ... ... ... ... ... ... ...
Steller's sea cow   ... ... ... ..C ..A G.G G.. ... ... ... ..T ... ..T ..A ... ... ... ... ... ..C ...
Manatee             ..C ... ..C ..T ..A G.. G.. ..C ..T ... ... ... ... ... ..T ..T ... ..C ... ..C
Hyrax               ..C ... ..C A.. ..A G.. A.. ..C ..T ..T ... ... ..A ... C.T ..A ... ..A ..C ...

Asian elephant      CAA CTA CAC TCA AAC GGA GCA TCT ATT TTC TTC CTC TGC CTA TAC ACA CAC ATT GGA CGA 300
Mammoth             ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
African elephant    ... ... ... ... ... ... ... ... ..C ... ... ... ... ... ... ... ... ... ... ...
Dugong              T.T ..T ... G.T ... ..G ... ..A ..A ... ... ..A ... ..C ... G.C ... ... ... ..C
Steller's sea cow   T.T ..T ... G.C ... ... ... ..A ..A ..T ... T.G ... ..C ... G.T ... ..C ... ..C
Manatee             T.C ..C ..T G.C ... ... ... ..A ..A ..T ... ..A ..T ..C ... G.C ... ... ... ..T
Hyrax               T.C T.. ..T G.C ..T ... ... ..C C.A ... ... G.A ... ..T ... G.C ... ..C ... ..G

Asian elephant      AAC ATC TAC TAT GGA TCC TAC CTA TAC TCA GAA ACC TGA AAT ACA GGC ATT ATA TTA CTA 360
Mammoth             ... ... ... ... ..G ... ... ... ... ..G ... ... ... ... ..C ... ... ... C.. ...
African elephant    ... ... ... ... ..G ... ... ... ... ..G ... ..T ... ... ..C ... ... ... ... ...
Dugong              GGA ... ..T ..C ..C ..A ... ... ..T C.. ... ... ... ... ..C .TT ..T ..C G.. C.G ...
Steller's sea cow   GGA ..T ... ..C ..C ..A ..T ... ... C.. ..G ... ... ... ..C .TT ... ..C ... C.. ...
Manatee             GGA ... ..T ..C ..C ... ... T.. ..T ... ... ... ... ... ..C .TT ..A ..C ..C C.. ...
Hyrax               GGT ..T ... ..C ... ... ... ..G ... ... ... ... ... ... .TC ..A G.. T.. C.C T..

Asian elephant      CTA ATT ACC ATA GCC ACC GCC TTC ATA GGA TAT GTC CTT CCA TGA GGA CAA ATA TCA TTC 420
Mammoth             ... ..C ... ... ... ... ... ... ... ... ... ... ... ..G ..G ... ... ... ... ...
African elephant    ... ..C ... ... ... ... ... ... ... ... ... ... ... ..G ... ... ... ... ... ...
Dugong              ..C .CA GTT ... ..T ..T ... ... ... ..G ..C ... ..C ... ... ... ... ... ... ...
Steller's sea cow   ..C .CA GTG ... ... ..T ..T ... ... ... ..C ... ..C ..G ... ... ... ... ... ...
Manatee             ..C .CA .TT ... ... ... ..T ... ... ... ..C ... ..C ... ..G ... ..G ... ... ...
Hyrax               ... .CA ..A ... ..A ..A ..A ..T T.. ... ..C ... ..A ... ..G ... ... ... ... ...

Asian elephant      TGA GGG GCA ACC GTA ATT ACT AAC CTC TTC TCA GCA ATT CCC TAC ATC GGC ACA AAC CTA 480
Mammoth             ... ... ... ... ... ..C ... ... ... ... ... ... ... ... ... ... ... ... G.. ...
African elephant    ... ... ... ... ... ..C ... ... ..T C.. ... ... ..C ..T ..T ... ... ... ... ...
Dugong              ... ..A ... ... ..T ... ... ... ... C.G ... ..T ..C ... ... ... ... ..C ... ...
Steller's sea cow   ... ... ..T ... ... ... ... ... ..T C.G ..T ..T ... ... ..T ... ... ..C ..T ..G
Manatee             ... ... ... ..T ... ..C ..C ... ..T C.A ... ..C ..C ... ... ... ... ..C G.. ...
Hyrax               ... ..A ... ..A ... ... ..A ... ..A C.T ... ..C C.A ... ... ..T ... ..C G.. ..T

Asian elephant      GTA GAA TGA ATT TGA GGA GGC TTT TCG GTA GAT AAA GCA ACC TTA AAC CGA TTC TTC GCC 540
Mammoth             ... ... ... ..C ... ... ... ... ... ... ... ... ... ... ... ..T ... ... ... ...
African elephant    ... ... ... ..C ... ... ... ... ... ..A ... ..C ... ... ... ... ..T ... ..T ... ...
Dugong              ..C ... ... G.. ... ..G ..A ..C ..A ... ..C ... ..C ... C.C .C. ... ... ... ...
Steller's sea cow   ... ..G ... G.C ... ..T ..A ..C ... ..G ..C ... ..C ... C.T .C. ... ... ... ..T
Manatee             ... ... ... ..C ... ..T ..A ..C ..A ... ..C ... ..T ... C.C .C. ... ... ..T ... .TG
Hyrax               ... ... ... ..C ... ... ..A ..C ..A ..G ..C ... ..C ... ..G .C. ... ... ... ...
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Fig. 1. Alignment of 1,005-bp cytochrome *b* gene sequences of seven paenungulates. A *dot* (.) denotes that the nucleotide at that position is identical to that of Asian elephant. A *dash* (-) represents a gap.

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Asian elephant      TTC CAT TTC ATC CTT CCA TTT ACT ATA GTT GCA CTA GCA GGA GTG CAC CTA ACC TTT CTT  600
Mammoth             C.. ... ..T ..T ... ... ... ... ... A.. ... ... ... ... ... ..A ... ... ... ..C ...
African elephant    C.. ... ... ..T ... ... ... ... ... A.. ... ... ... ... ... ..A ... ... ... ... ...
Dugong              C.A ..C ... ... ..A ..C ..C .TC G.. ACC ..C ... .T. AT. ..C ... T.. CTA ..C ..C
Steller's sea cow   ..G ..C ... ... ..A ... ..C .TC G.. ACC ..C ... .T. .C. ..T ..T ... TTA ..C ...
Manatee             ..A ..C ... .C. ..A ..T ..C GTC G.. ACC ..C ... .T. .CC ..C ..T ..C CTA ..C ...
Hyrax               ..T ..C ... ... A.A ... ... .TC ..T A.A ..C ... ... AT. ..C ... ..T CTA ..C T.A

Asian elephant      CAC GAA ACA GGC TCA AAC AAC CCA CTA GGT CTC ACT TCA GAC TCA GAC AAA ATT CCC TTT  660
Mammoth             ... ... ... ... ... ... ..T ..T ... ... ..C ... ... ... ... ... ... ... G.C ... ...
African elephant    ... ... ... ... ... ... ... ..T ... ..G ..C ... ... ... ... ... ... ... ..C ... ...
Dugong              ... ... ... ... ..C ... ... ..C ACG ..A ..G .TC ..C ... ... ... ... ..C ..A ..C
Steller's sea cow   ... ... ... ..A ..C ... ..T ..T AC. ..C T.A GTC ..C A.. ... ... ..G ..C ..A ..C
Manatee             ... ..G ... ..G ..C ... ... ..C AC. ..C ..A .TC ..C ..T ..C ... ... ... ..A ...
Hyrax               ... ... ... ... ..T ... ... ... ... ..A ..A .TC ..C A.. G.C ... ... ..C ..A ...

Asian elephant      CAC CCG TAC TAT ACT ATC AAA GAC TTC CTA GGG CTA CTT ATC CTA ATT TTA CTC CTT CTA  720
Mammoth             ... ... ... ... ..C ... ... ... ... ... ..A ... ... ... ... ..C C.. T.. ... ...
African elephant    ... ..A ... ... ..C ..T ..G ... ... ... ..A T.. ... ... ... ... ..T ... ... ...
Dugong              ... ..A ..T ... T.A G.. ... ... C.. ... ..C ... T.C C.. ..C ... C.. G.. T.A ..C
Steller's sea cow   ... ..A ... ..C T.. .C. ... ..T ... ... ..C ... T.. C.. ..C ..C C.. G.. ... ..C
Manatee             ..T ..A ... ..C T.A ... ... ... C.. ... ..C ... T.. C.. ..C ..C C.. A.. ..A ..C
Hyrax               ... ..C ..T ... ..C ..A ... ... C.A GC. ..A ... G.A C.A ..C .CC C.. ACA ..C ...

Asian elephant      CTC TTA GCC CTA CTA TCT CCA GAC ATA CTA GGA GAC CCC GAC AAC TAC ATA CCA GCT GAT  780
Mammoth             ... ... ... ... ... ... ... ..T ... ... ... ... ... ... ... ... ... ... ... ...
African elephant    ... C.. ... ... ... ... ... ..T ... CAT ... ... ... ..T ... ... ... .CC .TG ..C A.C
Dugong              ..A C.. A.. ..G T.C ..C ..G ... ... ..G ... ... ..A ... ... ... .C. ... ..C A.C
Steller's sea cow   ... C.G A.. ... T.C ..C ..G ..T ..G ... ... ... ... ..G ... ..T ... .C. ..T ..C A.C
Manatee             ..A C.G A.. T.. T.C ..C ... ... ..G T.. ... ... ..A ... ... ... .C. ..C ..A A..
Hyrax               ACA C.. A.T ... T.C .TC ... ... ... ... ... ... ... ..T ... ..T .C. ..C ..C A.C

Asian elephant      CCA CTA AAT ACT CCC CTA CAC ATC AAA CCA GAG TGA TAC TTC CTT TTT GCT TAC GCC ATT  840
Mammoth             ... ..T ... ... ... ... ... ... ... ... ... ... ... ... ..C ... ... ... ..A ..C
African elephant    ..C T.. ... .AC ... .CT ..T ... ..G ... ... ... ..T ..T ..C ... ... ... ... ..C
Dugong              ... ... ..C ..C ..T .CC ... ..T ... ... ..A ... ... ..T ..A ..C CGA ... ..T ..C
Steller's sea cow   ... ... .GC ..C ..A .CC ... ... ... ... ..A ..G ... ... ..A ..C ..G ... ..T ..C
Manatee             ... ... .GC ..C ..A .CC ... ..T ..G ... ..A ..G ... ... ..A ... ..A ... ... ..C
Hyrax               ..C ..C ..C ..C ..T .C. ... ... ... ..C ..A ... ... ... ..A ..C ..A ... ..A ..C

Asian elephant      CTA CGA TCT GTA CCA AAC AAA CTA GGA GGC GTC CTA GCC CTA TTT CTA TCA ATC CTG ATT  900
Mammoth             ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... C.C ... ... ... ..A ..C
African elephant    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... C.C ... ... ..T ..A ..C
Dugong              ..C ... ... A.C ..T ..T ... ... ..C ... ..G T.. ... ..C G.A ..C ..C ... ..A ..C
Steller's sea cow   ..T ... ..C A.C ..C ... ... T.. ..T ..T ..A ... ... ..T A.C ... ..C ... T.A ..C
Manatee             ..T ... ..C A.C ..T ... ... ... ... ..T ..G ... T.. ... ... G.. ..C ..T ... ..A ...
Hyrax               ... ... ..C A.T ... ..T ... ... ... ..A ..A A.T ... ... A.C A.. ... ..T ..C ...

Asian elephant      TTA GGA TTA ATA CCA TTT CTC CAT ACA TCC AAG CAC CGA AGT ATA ATA CTC CGA CCT CTC  960
Mammoth             C.. ... A.T ... ... C.. ..A ... ... ..T ..A ... ... ... ..G ... ..T ... ... ..T
African elephant    C.. ... ... ... ... C.. ... ... ... ... ... ... ... ..C ... ... ... ... ... ..T
Dugong              C.. .CG C.C C.C ... C.C ... ..C ... ... ..A ..A ... ..C C.. TC. T.. ... ... ..A
Steller's sea cow   C.. .CG C.C C.T ... C.. ... ... ... ... ..A ..A ... ..C C.G TC. T.T ..C ..C ...
Manatee             C.. .CC C.C C.C ..T C.C ... ..C ... ... ..A ..A ..C ..C C.. T.G T.. ..G ..C ..A
Hyrax               C.. .C. C.C C.C ..C C.A ... ..C ... ..A ..A ..G ... ..C C.. ... T.T ..T ..A ...

Asian elephant      AGC CAG GTT CTA TTC --- TGA ACT CTA ACA ATA GAT TTA CTA ACA CTT                1005
Mammoth             ... ..A ..C ... ... --- ... ... ... G.. .C. ... C.. ... .T. ...
African elephant    ... .TA TG. GCC .AT TGC ... ... ... ... ... ... ... ... ... ...
Dugong              ... ..A TGC ..C ..T --- ... .T. ..G GT. GCC ..C C.G A.C ... ..C
Steller's sea cow   ... ..A TGC ..C ... --- ... .T. ... GT. GCC ..C C.. A.T ... ...
Manatee             ... ..A TGC ... ... --- ... .TC ... GT. .CC ..C C.. A.C ... ...
Hyrax               ... ..A TGC ... ... --- ... CT. ... GT. GCT ..C C.T A.C ... ..A
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Fig. 1 Continued.

chondrial cytochrome *b* sequence data, the relationships among the three sirenian lineages are not well resolved due to the trichotomous relationship. The *Dugong* + *Hydrodamalis* grouping (Dugongidae) occurred in the neighbor-joining tree based on amino acids and the maximum parsimony tree based on amino acids. In two trees the bootstrap value were rather low, 69 and 61% respectively. In other trees, alternative topologies appeared. Such an old divergence within the Sirenia was not shown in the immunological work of Lowenstein and Scheuenstuhl (1991). In this study, divergence between the Trichechidae and the Dugongidae was three times as much as the divergence between *Dugong* and *Hydrodamalis*. The conflicting topology of the molecular-based

Table 1. Sequence differences and genetic distances among 1,005-bp cytochrome *b* gene sequences for seven paenungulates[a]

	1	2	3	4	5	6	7
1. Asian elephant		58/7	74/19	136/101	152/97	138/104	124/117
2. Mammoth	0.069		62/22	137/102	141/98	134/105	119/118
3. African elephant	0.102	0.092		134/106	149/102	139/113	126/124
4. Dugong	0.346	0.350	0.355		127/24	110/31	99/110
5. Steller's sea cow	0.367	0.348	0.375	0.177		120/33	121/104
6. Manatee	0.358	0.352	0.385	0.165	0.182		98/107
7. Hyrax	0.364	0.356	0.388	0.297	0.324	0.288	

[a] Number of transitions and transversions (above the diagonal) and Kimura's two-parameter distances with a transition/transversion ratio of 10.0 (below the diagonal)

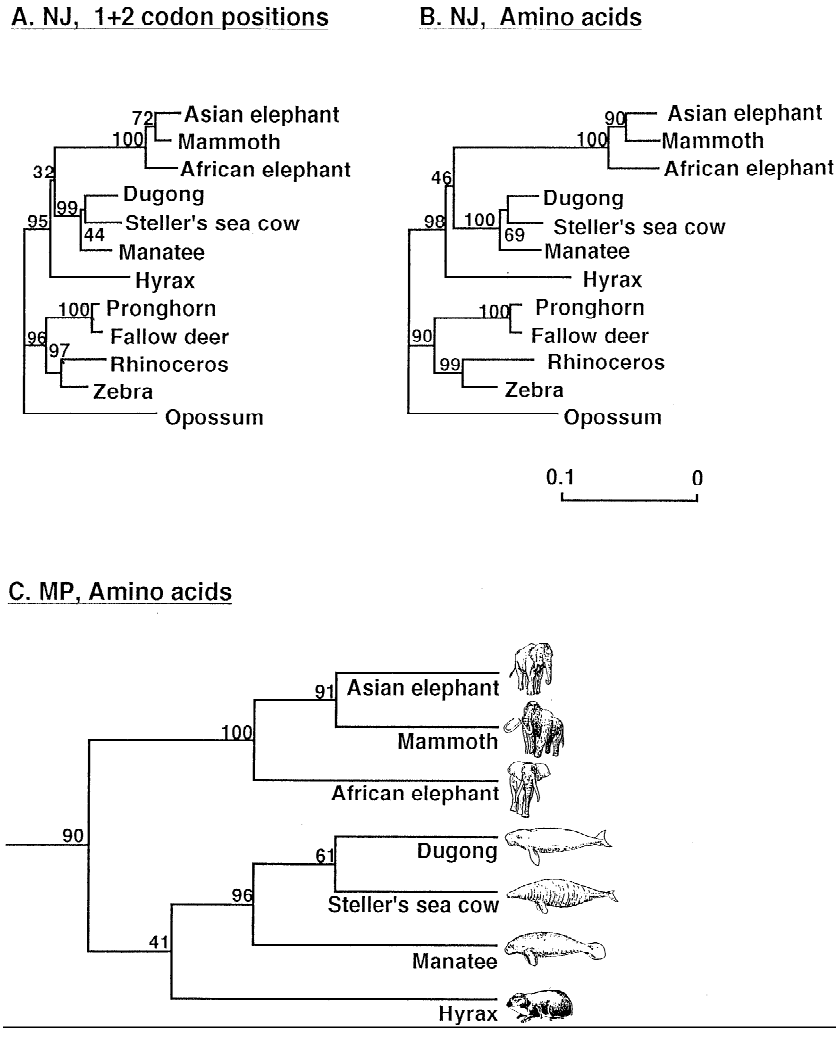

Fig. 2. Two neighbor-joining trees (**A** and **B**) and a maximum parsimony tree (**C**) of seven paenungulates using five mammalian species as outgroups. They were constructed based on data from first and second codon positions **(A)** and deduced amino acid sequences (**B** and **C**) of 1,005 bp from the cytochrome *b* gene. The neighbor-joining trees are depicted inclusive of all outgroups; as to the maximum parsimony tree, outgroups are excluded. The *numbers above or below the nodes* in the trees are bootstrap probabilities (%) based on 1,000 resamplings. The *scale bar* represents tree length (substitutions per site) for the neighbor-joining trees.

phylogenetic trees implies that the previously recognized first (between Trichechidae and Dugongidae) and second divergences (between *Dugong* and *Hydrodamalis*) of modern sirenians occurred closely together in time. Historically, it had been believed that the trichechid and dugongid lineages diverged in Oligocene time and that the hydrodamaline–dugongine divergence occurred in the early Miocene, based on the first appearance of *Metaxytherium* (Domning 1994). In a recent cladistic analysis based on cranial and dental characters, Domning (1994) provided a new hypothesis of sirenian evolutionary history. In his cladogram, Eocene sirenians were placed as outgroups to modern sirenians and their direct ancestors. This analysis further showed the proximity of two branching points (Trichechidae–Dugongidae and *Dugong–Hydrodamalis*) in time and offered a good explanation for the trichotomous relationship of sirenians.

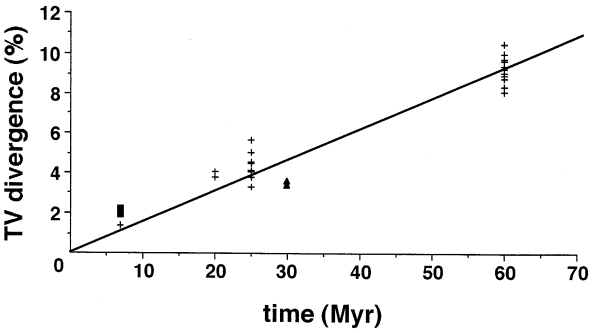

Fig. 3. Transversional divergence (%) between artiodactyls (+), proboscideans (■), and sirenians (▲) is plotted against divergence time. All artiodactyls and African elephant data are from Irwin et al. (1991). The used divergence times are as in Table 2.

Evolutionary Rates and Divergence Times

Figure 3 shows the relationship between transversional divergence corrected by Kimura's two-parameter method (Kimura 1980) and divergence time estimated from the fossil records with respect to artiodactyls, proboscideans, and sirenians. Transversional change is expected to be proportional to time as suggested in early studies although it varies among lineages. Table 2 shows transversional divergence rates in the three groups. The rate in the artiodactyls ranges from 0.15% to 0.20% per million years. This contrasts with a rather higher divergence rate of 0.30% per million years in the elephant clade (*Loxodonta* vs *Elephas/Mammuthus*) as suggested in Irwin et al. (1991). A much lower divergence rate of 0.11% per million years was observed in the sirenian clade (*Trichechus* vs *Dugong/Hydrodamalis*). These are comparable to the highest and lowest rates observed in artiodactyls, respectively.

Assuming that transversional substitution rate has been clock-like within the sirenian lineage, the *Dugong–Hydrodamalis* divergence can be dated as 22 million years ago, taking 30 million years ago as a reference date for the trichechid–dugongid divergence. The *Mammuthus–Elephas* split can be dated as 4 million years ago using 7 million years ago for the first appearance of *Loxodonta* species as a reference date (calculated using total divergence excluding the contribution of transition at third codon positions, because transversion clock analyses tend to underestimate divergence times within more recent dates). These estimated dates are essentially concordant with fossil records.

In the future, additional sequences will be needed for a more robust molecular tree of Tethytheria. Sequence data from two other species of manatees will provide new information for the evaluation of the sirenian divergence rate.

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Table 2. Transversional (TV) divergence rate of artiodactyl, proboscidean, and sirenian lineages

Compared groups	TV divergence[a] (%)	Divergence time[b] (Myr)	Rate (%/Myr)
Goat vs sheep	1.4	7	0.20
Cow vs goat/sheep	3.9	20	0.20
Bovidae vs giraffe/deer	4.3	25	0.17
Ruminantia vs suiidae	9.1	60	0.15
Proboscideans[c]	2.1	7	0.30
Sirenians[d]	3.3	30	0.11

[a] Averages of possible pairwise transversional divergence between representative species of each compared groups. Species used in each category for rate analyses are as follows, Bovidae (cow, goat, sheep), deers (fallow deer and black-tailed deer), Ruminantia (cow, sheep, goat, pronghorn antelope, giraffe, fallow deer, black-tailed deer, and chevrotain), and Suiidae (pig and peccary). Sequence data are derived from Anderson et al. (1982) and Irwin et al. (1991)

[b] Divergence dates are taken from Domning (1994), Tassy (1986), and Savage and Russell (1983)

[c] African elephant vs Asian elephant/Mammoth

[d] Manatee vs dugong/Steller's sea cow

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