Molecular phylogeny of the extinct ground sloth *Mylodon darwinii*

MATTHIAS HÖSS*, AMREI DILLING*, ANDREW CURRANT†, AND SVANTE PÄÄBO*

*Institute of Zoology, University of Munich, P.O. Box 202136, D-80021 Munich, Germany; and †Department of Palaeontology, The Natural History Museum, Cromwell Road, London SW7 5BD, United Kingdom

ABSTRACT   DNA was extracted from the remains of 35 ground sloths from various parts of North and South America. Two specimens of *Mylodon darwinii*, a species that went extinct at the end of the last glaciation, yielded amplifiable DNA. However, of the total DNA extracted, only ≈1/1000 originated from the sloth, whereas a substantial part of the remainder was of bacterial and fungal origin. In spite of this, >1100 bp of sloth mitochondrial rDNA sequences could be reconstructed from short amplification products. Phylogenetic analyses using homologous sequences from all extant edentate groups suggest that *Mylodon darwinii* was more closely related to the two-toed than the three-toed sloths and, thus, that an arboreal life-style has evolved at least twice among sloths. The divergence of *Mylodon* and the two-toed sloth furthermore allows a date for the radiation of armadillos, anteaters, and sloths to be estimated. This result shows that the edentates differ from other mammalian orders in that they contain lineages that diverged before the end of the Cretaceous Period.

Two genera containing five species of sloths currently exist in Central and South America: the three-toed sloths (*Bradypus sp.*) and the two-toed sloths (*Choloepus sp.*). They belong to the mammalian order Edentata (or Xenarthra) (1) which, in addition to the sloths, contains armadillos and anteaters. The current dearth of sloth species, as well as their limited geographic distribution, is contradictory to the situation in the late Pleistocene Epoch, when >40 genera of sloths, belonging to three families (Mylodontidae, Megatheridae, and Megalonychidae), ranged from Alaska to Tierra del Fuego. While all extant sloths are tree-living and not larger than 10 kg, many of the extinct forms were large and ground-dwelling (2). Mylondontidae, for example, were of the size of at least a black bear, and although many forms of Megatheridae and Megalonychidae were small and probably arboreal, also these families contained several large species, some the size of elephants (3). Almost all members of this vast and morphologically diverse sloth radiation went extinct at the end of the last glaciation, some 10,000 yr ago (4).

Three hypotheses exist concerning the relationship of extinct and extant sloths (Fig. 1). The first and most widely accepted one claims that the three-toed sloths are closely related to Megatheridae, while the two-toed sloths are a sister group to Megalonychidae, and that the Mylodontidae represent an earlier divergence (3, 5). According to a second hypothesis, the three-toed sloths and the two-toed sloths are monophyletic (6) and have their origin in arboreal sloths living before the divergence of Megatheridae and Megalonychidae (7), with Mylodontidae branching off even earlier. The third hypothesis, finally, argues for a close relationship between the two-toed sloths with Mylodontidae and the three-toed sloths with Megatheridae (8).

To elucidate the relationship of the late Pleistocene sloth radiation to their present descendants, we have determined over 1100 bp of mitochondrial 12S and 16S ribosomal DNA sequences from a 13,000-yr-old *Mylodon darwinii* sample, as well as all contemporary edentate groups.‡

MATERIAL AND METHODS

DNA Extraction, PCR, and Sequencing. Samples from extant edentates were obtained from the Museum of Vertebrate Zoology, Berkeley, CA, and from the Zoological Garden "Hellabrunn," Munich. Modern DNA was extracted from cardiac muscle, liver, blood, or hair using a high salt method (9). Ancient DNA was extracted from bone, teeth, and coprolites using described procedures (10). Samples used were as follows: The American Museum of Natural History, New York: 17711N, 177110, 17716-15, 17716, 11271, 11270, MB Ma 33480, 94739, 9237, 8640, FLA91-1671, FLA103-1986, FLA105-1985, FLA102-1927, MB Ma 35588, MB Ma 33621, as well as unnumbered samples of a coprolite from Nevada, two Megalonyx ribs from Florida, and a Mesocnus vertebra without provenience; The Natural History Museum, London: BM(NH)M 8758 and an unnumbered *M. darwinii* long bone from Patagonia; The U.S. Department of the Interior, National Park Service, Twin Falls, ID: ROM 44480, ROM 44481, ROM 2143, ROM 11940; The Academy of Natural Sciences of Philadelphia: ANSP 15193, ANSP 19603; The Natural History Museum of Los Angeles County: LACMHC 46986, LACMHC 46987, LACMHC 80401; The Smithsonian Institution, Washington, DC: a coprolite and metatarsus of *Nothrotheriops sp.* from Arizona; The University of California, Los Angeles: UCLA UP 25; The Peabody Museum, New Haven, CT: 33 (13198).

PCR was done as described (10) with the following primers: 16sa L02510, 5'-CGCCTGTTTATCAAAAACAT-3'; 16s9 H02708, 5'-GCTCCGTAGGGTCTTCTCGTC-3'; 16s8 L02726, 5'-AAGACGAGAAGACCCTATGGA-3'; 16s7 H02922, 5'-TTGCGCTGTTATCCCTAGGGTAACT-3'; 16s5 H02812, 5'-ATTCTCCGAGGTCACCCCAA-3'; 16s6 L02828, 5'-TTTCGGTTGGGGCGACCTCGGAG-3'; 16sb H03063, 5'-CTCCGGTTTGAACTCAGATC-3'; 12ss L00905, 5'-AATTTCGTGCCAGCCACCGCGGTCA-3'; 12st H01134, 5'-AAGCTGTTGCTAGTAGTACTCTGGC-3'; 12sa L01091, 5'-AAAAGCTTCAAACTGGGATTA-GATACCCCACTAT-3'; 12sn H01327, 5'-CCATTTCATAG-GCTACACCTTGACC-3'; 12sl L01302, 5'-CAAACCTTG-GAGAGTACCCACAGTAAGC-3'; 12sb H01478, 5'-TGACTGCAGAGGGTGACGGGCGGTGTGT-3'. Numbers refer to the positions in human mtDNA (11), and H and L refer to heavy and light strands, respectively. PCR products were either directly sequenced (10) or cloned in a vector with 5' thymidine residues (Invitrogen), and multiple clones were

‡The sequences reported in this paper have been deposited in the GenBank data base (accession nos. Z48937–Z48946).
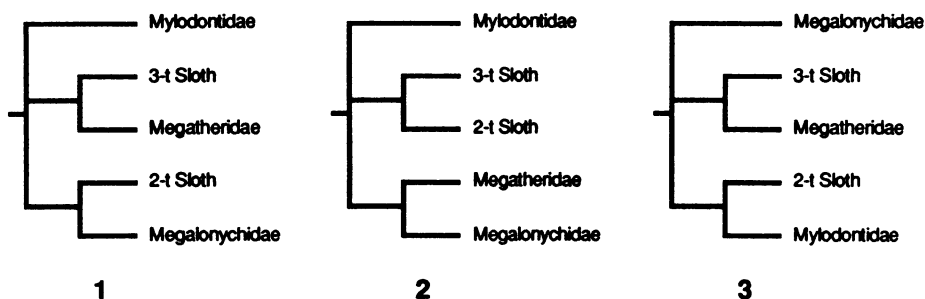
FIG. 1.   Alternative hypotheses concerning the phylogeny of extinct and extant sloths.

sequenced. Sequences of the cow (*Bos taurus*) were obtained from EMBL/GenBank (accession no. J01394).

DNA Quantification. Nucleic acids were blotted onto an uncharged nylon membrane (Hybond-N, Amersham–Buchler) using a manifold (Schleicher & Schüll). After denaturing (0.5 M NaOH/1.5 M NaCl) and neutralization (0.5 M Tris·HCl, pH 8/1.5 M NaCl), the DNA was cross-linked to the membrane by UV irradiation. Genomic DNA was labeled with [α-^{32}P]dATP by random priming (Mega prime, Amersham–Buchler) and hybridized to the filters at 68°C in 6× standard saline/citrate (SSC) over night. Subsequently, the filters were washed once in 2× SSC and once in 0.2× SSC at 68°C, followed by an overnight exposure.

DNA Sequence Analysis. Sequences were aligned by eye using secondary structure models suggested by M. Hasegawa for the 12S rRNA (12) and the 16S rRNA (M. Hasegawa, personal communication). Gaps and all positions around gaps up to the next constant position were excluded from the analyses. Phylogenetic trees inferred from ribosomal DNA sequences may be particularly sensitive to biases in base composition (12, 13). Therefore, base frequencies in the aligned parts of each of the sequences were determined and tested for homogeneity with a χ^2 test (14). In no case could the null hypothesis of a homogenous base composition be rejected on the 5% significance level. Phylogenetic trees were reconstructed using the neighbor-joining algorithm (15) with maximum-likelihood corrected distances, with a maximum-likelihood algorithm (16) using the PHYLIP package (17) and with the parsimony method using the PAUP package (18). Bootstrap tests (19) were performed for the neighbor-joining approach, whereas maximum-likelihood trees were tested by a likelihood ratio test (17).

RESULTS

DNA Extraction. DNA was extracted from 45 samples of 35 ground sloths from various localities in South and North America. The samples represented all three families of ground sloths, as well as vastly different environmental conditions, and consisted of bones, soft tissues, teeth, and coprolites. An aliquot from each extract was submitted to PCR of an ≈140-bp fragment of the 16S rRNA gene. In almost all cases, only primer artefacts were detected after 40 cycles of PCR. However, two bone samples yielded amplifiable DNA. These samples [The American Museum of Natural History, New York, catalog no. MB Ma33621 and The Natural History Museum, London, catalog no. BM(NH) M8758] stemmed from two different specimens of *M. darwinii* found in Mylodon Cave, Ultima Esperanza, Chile, and are dated to ≈13,000 before present. When the amplified fragments were sequenced, they yielded identical sequences. In subsequent experiments the sample M8758 was used.

Quantification. An extraction was performed from 0.8 g and its absorbance at 260 and 280 nm was determined. This result indicated that the extract contained ≈2.36 μg of nucleic acids. A dilution series of this *Mylodon* extract as well as a dilution series of two-toed sloth DNA of known concentration was

immobilized on a nylon filter and hybridized to radioactively labeled three-toed sloth DNA. Fig. 2 shows that ≈2 ng of *Mylodon* DNA was present in the extract. Thus, in the order of 0.1% of the total nucleic acids extracted was of *Mylodon* origin. In analyses of extracts of samples from which no PCR amplifications were possible, no signals were observed (data not shown).

DNA Sequence Determination. A total of 574 bp of the mitochondrial 12S RNA gene, as well as 555 bp of the 16S rRNA gene, were amplified and sequenced. Due to the inverse correlation of amplification efficiency with length of amplification products typical of DNA extracted from ancient remains (20), this had to be achieved by the amplifications of short overlapping products that ranged in size from around 200 bp to a maximum of 340 bp (including primers). The homologous sequences were determined from a two-toed sloth (*Choloepus didactylus*), a three-toed sloth (*Bradypus variegatus*), an anteater (*Tamandua tetradactyla*), and an armadillo (*Cabassous unicinctus*), which represent the three major groups of extant edentates. Fig. 3 shows an alignment of these sequences along with that of a cow.

Phylogenetic Reconstruction. Table 1 shows the number of differences observed among the taxa analyzed. Whereas the cow differ from the edentate sequences at 133–154 positions, the anteater and armadillo are almost as divergent from each other as from the sloths (117–140 differences). In contrast, the two extant tree sloths differ at 81 positions, and the *M. darwinii* differ from the two- and three-toed sloths at 67 and 89 positions, respectively. Therefore, the different edentate groups seem to be very divergent from each other, while the extinct ground sloth seems to be as closely related to the extant tree sloths as these are to each other.

Fig. 4 shows a tree based on the sequences. Whereas the relationships of the armadillo, the anteater, and the sloths cannot be resolved, the ancient and the two modern sloths fall together in 100% of bootstrap replications. Thus, the Mylodontidae were part of the same monophyletic group as the

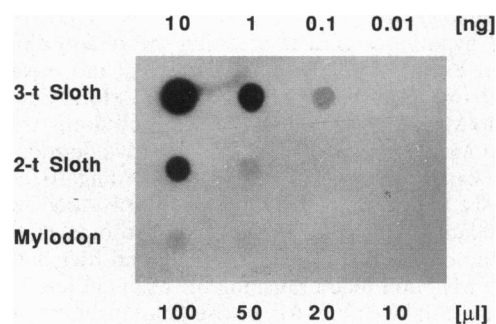FIG. 2.   Quantification of *Mylodon* DNA. Different amounts of a DNA extract from *Mylodon* bone (100, 50, 20, 10 μl; 100 μl = 0.4 g of bone material) were hybridized with ^{32}P-labeled DNA from a three-toed sloth (*Bradypus variegatus*). For comparison, different amounts of DNA (10–0.01 ng) from the three-toed sloth and the two-toed sloth (*Choloepus didactylus*) were blotted and hybridized in parallel.

```
                 10         20         30         40         50         60         70         80         90
Mylodon   TACGATTAAC CCAAATTAAT ATTCA-CCGG CGTAAAACGT GTTTAAGGAA ATCGAAC--A AATAAAGCTA AACCAAGACT AAGCCGTAAA
2-t Sloth .......... .......... .C....T... .......G.. ....G.AT. .C.C...... .......T.. ......... .G........
3-t Sloth .......... .....C.... ....CT.... A.....T.. ...G.ATT T.A....... .......T.C ......AG.. .G........
Armadillo ......G... .......... .GC.T.A... -.....G.. ...G..T. .AACT....T .......T.. ..TTTTT... .T..T.....
Anteater  .......... .......... .AC.T.T... ......GA.. ...G.AC. T.A-T..AC. .......TC. ..TTGCT... ..........
Cow       .......... ....GC...C .GGAGTA... .......... ...A..C.C CA--T...C. ....GG.T.. ..TTCTA... .....T....
                                         ●●●●●●●      ●                    ●●●    ●●●●● ●●●

                 100        110        120        130        140        150        160        170        180
Mylodon   AAGCTACAGT CAAAATAAAA TAAACA-ACG AAAGTGGCTT TAACAAACCC GAACACACGA TAGCTAAGAC CCAAACTGGG ATTAGATACC
2-t Sloth .......... .......... ..C..G...T ......A... .....CT... .......... .......... .......... ..........
3-t Sloth ........C. .......... ACCT...... ......A... ...G....T. ...T....T. ....G..T T.......... ..........
Armadillo ...CC..... T......... AT.C.CC... ......A... ..G...... .......... ....C.G... .......... ..........
Anteater  ...AC..... AT...C..G. .C........ ......A... ..G..C.T.. .......... C....G.G.. .......... ..........
Cow       ....C.TGA. T......... AT..ATG... ......A.CC ..CA.T.G.. .-..G...T. .......... .......... ..........
                                         ●●●●●●●                           ●●●

                 190        200        210        220        230        240        250        260        270
Mylodon   CCACTATGCT TAGCCCTAAA CCAAGACATT TGACAA-ACT AAAATGTTCG CCAGAGTACT ACTAGCAACA GCCTAAAACT TAAAGGACTT
2-t Sloth ........C. .......... ..C.A..... .A.TC..... .......... .....C.... .......... ..T.G..... C.........
3-t Sloth ........C. .......... ......C. .A..C.A..C ...G...CT. .......... .......T.. ...T..CA.. C.G.......
Armadillo ........C. .G........ ...A...G. .CGT....A ...C..C... .......... .......T.. ...T...... C.G.......
Anteater  ........C. ...T.G.... .AC.A.T.. CC.AC....A .G...A.... .......... .......... ..TA...... C.........
Cow       .......... .......... .AC...T.A. .ACAT.A..A ..T.A..... .......... .......... ...T...... C.........
                                         ●●●●●●●

                 280        290        300        310        320        330        340        350        360
Mylodon   GGCGGTGCTT CACACCCCCC TAGAGGAACC TGTCCTATAA TCGACAAACC CCGATAGACC TCACCACCCC TCGCCAATGC AGCCTATATA
2-t Sloth .......... .......T.. .......... .......... .......... ......A... .......... ...A...CA. ..........
3-t Sloth .A........ ..T..T... .......... .......... .....G.... ......A... .......... ...T...CA. ..........
Armadillo .......... T..T..TT.. ......G.. ...T..G... .......T.. ..T..C... .......... ..T.T...A. ..........
Anteater  .......... ..T.T.A.. ......G.. ...T...... .......T.. ......A... .......... ..T.T...A. ..........
Cow       .......... T.T.T..TT. ......G.. ...T...... .......T.. ......A... .....ATT.. ..T.T...A. ...T......

                 370        380        390        400        410        420        430        440        450
Mylodon   CCGCCATCCT CAGCAAACCT TGG-AGAGTA CCACAGTAAG CACAATCATC ATTCGTAAGT ACGTTAGGTC AAGGTGTAGC CCATGGGGTG
2-t Sloth ........C. .......... ....A..A. .......... .G...CT... .AC.AC...C .......... .......... .A...A....
3-t Sloth .......TC. .......... .AA.A..C. .......... .G...T..A .G.A...A.. .......... .......... .A........
Armadillo .......T.. ...G...C .AAC.AG.A. ...GA...G.. .G...A..- .CG.AC..AA .......... .......T... .T........
Anteater  .......TC. T.......C .AAA.AG.AG .......... ...CT..- .GC.A...AA .......... .......... .A..A.....
Cow       .......T.. ........C .AAA.AG.A. AA.A...... .GT...T..G .A.A...AA .......... .......A.. .T...AAA..
                                        ●●●                      ●

                 460        470        480        490        500        510        520        530        540
Mylodon   GAAAGAGATG GGTTACATTT TCTTACAAA- AGAAAA-TCA --ACGGAAGT TTCCATGAAA CCCTAGAAAC C-GAAGGAGG ATTTAGAAGT
2-t Sloth .......... .......... .......... .......A.. .......A.. ....T..... .......... ...A...... ..........
3-t Sloth .......... .......... .--..A .......CC.- .......AC ...TG..... .TAC.A..G. .A........ ..........
Armadillo .G....A... ..C....... .-.AG.CT .....TC.- ..AA..AC C.TA..... .A.CCTT..G .AT...C.. ......C...
Anteater  .G....A... ..C....C C.-.ATT.T .G...-..- ...A.G.. C.TTG..... G-.G..GT. T......... ......C...
Cow       .G....A... ..C....C .-...CCA .G.TCA..GC..A ATT..... .-.A.-T.. .A........ ......C...
                                        ●●●●●●●    ●●●●        ●●       ●●●●●●●●●●  ●●●

                 550        560        570        580        590        600        610        620        630
Mylodon   AACCTGAGAA TAGAGCGCTC AGTTGAA-CC AGGCCATGAA GCACGCCACC TCTAGCATAA CAACTATTAG AGGCACTGCC TGCCCAGTGA
2-t Sloth .AT.A..... .......A..T .A.......T ...T.C...... .......... .......... ..T....... .......... ..........
3-t Sloth ..T.A..... .......A..T .AC...CT.. G......... .......T.. .......... .......... .....C.... ..........
Armadillo ..G.CAG... .......A..CT ..C....... ...T..A.. .......... .......C.. ...T...... .....C.... ..........
Anteater  ..GTCA.A.. .......T..T GAC.....A .......A.. A......... ....A..T.. ...T...... .......... ..........
Cow       ..A..A.... .......T..T .......TT ........ .......... ....C...TC .C.G...G. ...T...... ..........
                                        ●●●        ●

                 640        650        660        670        680        690        700        710        720
Mylodon   CATATGTTAA ACGGCCGCGG TACCCTGACC GTGCAAAGGT AGCATAATCA TTTGTTCTTT AATTAAGGAC TTGTATGAAA GGCCCAACGA
2-t Sloth .......T.. .......... .......... .......... .......... .......C.. .......C.. .......... ..TT......
3-t Sloth .......C.. .......... .......... .......... .......... .......... .......C.. .......... ..........
Armadillo .C....C... .......... .......... .......... .......... C......C.. CC......T .......... ..........
Anteater  ..GC...T. .......A... .TT....... .......... .......... .......C.. .......G.. .A........ ..TTG.....
Cow       ..AC....T. .......... .T........ .......... .......... .......C.. .......A.. .......T ..GC....

                 730        740        750        760        770        780        790        800        810
Mylodon   GGATTTTACT GTCTCCTGCT TCCAATCAGT GAAATTGACC TCCCCGTGAA GAGGCGGGGA TAGACCTACA AGACGAGAAG ACCCTATGGA
2-t Sloth .......... ......A. .......... .......... .......... .......A... .A...T.. .......... .......C..
3-t Sloth .......... ......A. .......... .......... C......... .......A... .AC.A.T. .......... .......C..
Armadillo ....CA.... ......AT. .......... .......... CT........ .......A.. .A.A.T. .......... ...GC.....
Anteater  ..G....... ....T..T. .......... .......... .T........ .......... .ATAA..T. .......... ...G......
Cow       ..G....... ....T.A.. .......... .......... .T........ .......A. GC..AA.T. .......... ..........

                 820        830        840        850        860        870        880        890        900
Mylodon   GCTTCAATTA ATTAGCCTAA -CTGCAATTC TCACA-AACC -AACAGGAAT TAACCACCTG CAGCCTAGCC TAACAATTTC GGTTGGGGTG
2-t Sloth .....A.... .C........ .T....CA.T ..C.CC-... C.......A A.C.-..C.. .A...G.... .G........ ..........
3-t Sloth A...T..... .C........ .TCAT..CC. AT........ T.G....G.C A.CT-TT.CA TGA....A. .G........ ..........
Armadillo .....A.... .C....C.. CAG-...CAG ..CATT-... C........C A.....AA.. .G.G...GC...T .......... ..........
Anteater  .....A...- .C.G...C. CTAT---A.. CA.TT..G.. CCT....GCC A.CA-TATAA T.AA..G.G. ..A....... ..........
Cow       ...T..C.. .CC.A..C. .AGAG...AG AT-TT..... ATTA...... A.CA-..AAT .TC.A.GAGT GGT.G..... ..........
                    ●        ●●●●●●●●●● ●●●●●●●●    ●●●●       ●●●●●●●●   ●●●●●

                 910        920        930        940        950        960        970        980        990
Mylodon   ACCTCGGAGC ACAAACAAAC CTCCGAATGG CTACATACT- CAGACCTTAC CAGTCGACGT --ACCTCAAT ACCATTAATT GATCCAAGCA
2-t Sloth .......... ......A... ..........A ....TTC... T......A. ....A..A. ...T...C G...C..... .C........
3-t Sloth ........T .....A... ..........A .C.TGC..C. .......C.. ....A... GCC...AAT. CAAC.C.... .C....T.
Armadillo .......... .A..CA..C. ..........A T-.AGC..CA ...C-...A. A....A.A.. -...ATT.. .T..C.T... .C....AA.
Anteater  .......T .A...A..T. ..........A T.TATA..C. -.....A. A....A.GT. ..GTAACTC .T..C..... .......A.
Cow       ........A .T...A..T. ......GC.A T.TT.A.GAC T.....C-.. A....A.ATC ...---A.TC. .T.GC.C... .......AA.
                                        ●         ●●       ●   ●●       ●●●●●●●●●● ●●●●●●●

                 1000       1010       1020       1030       1040       1050       1060       1070       1080
Mylodon   ATTGATCAAC GGAACAAGTT ACCCTAGGGA TAACAGCGCA ATCCTATTTC AGAGTCCATA TCGACAATAG GGTTTACGAC CTCGATGTTG
2-t Sloth .........T .........C. .......... .......... .......C.. .......... .......... .......... ..........
3-t Sloth .....C.... .........C. .......... .......... .......... .......... .......... .......... ..........
Armadillo .....C.... .......C.. .......C.. .......... .......A.. ...C...... .......... .......... ..........
Anteater  .......T .......... .......C.. .......... .......T .....T.... .......... .......... ..........
Cow       C......... .......... .......... .......... .......CA .......... .......... .......... ..........

                 1090       1100       1110       1120       1135       1140       1147
Mylodon   GATCAGGACA CCCTAATGGC GCAACCGCTA TTGATGGTTC GTTTGTTCAA CGATTAAAGT CCTACGT
2-t Sloth .......... T..C...... ...CGA.... .A.C...... .......... .......... .......
3-t Sloth .......... T.C.G.A.T .T....C. C.A.A..... .......... .......... .......
Armadillo .......... T......T ..G....... ..A.A..... .......... .......... .......
Anteater  .......... T.A....... ......A... ...AC..... .......... .......... .......
Cow       .......... T..G...T .......... CA.A...... .......... .......... .......
```
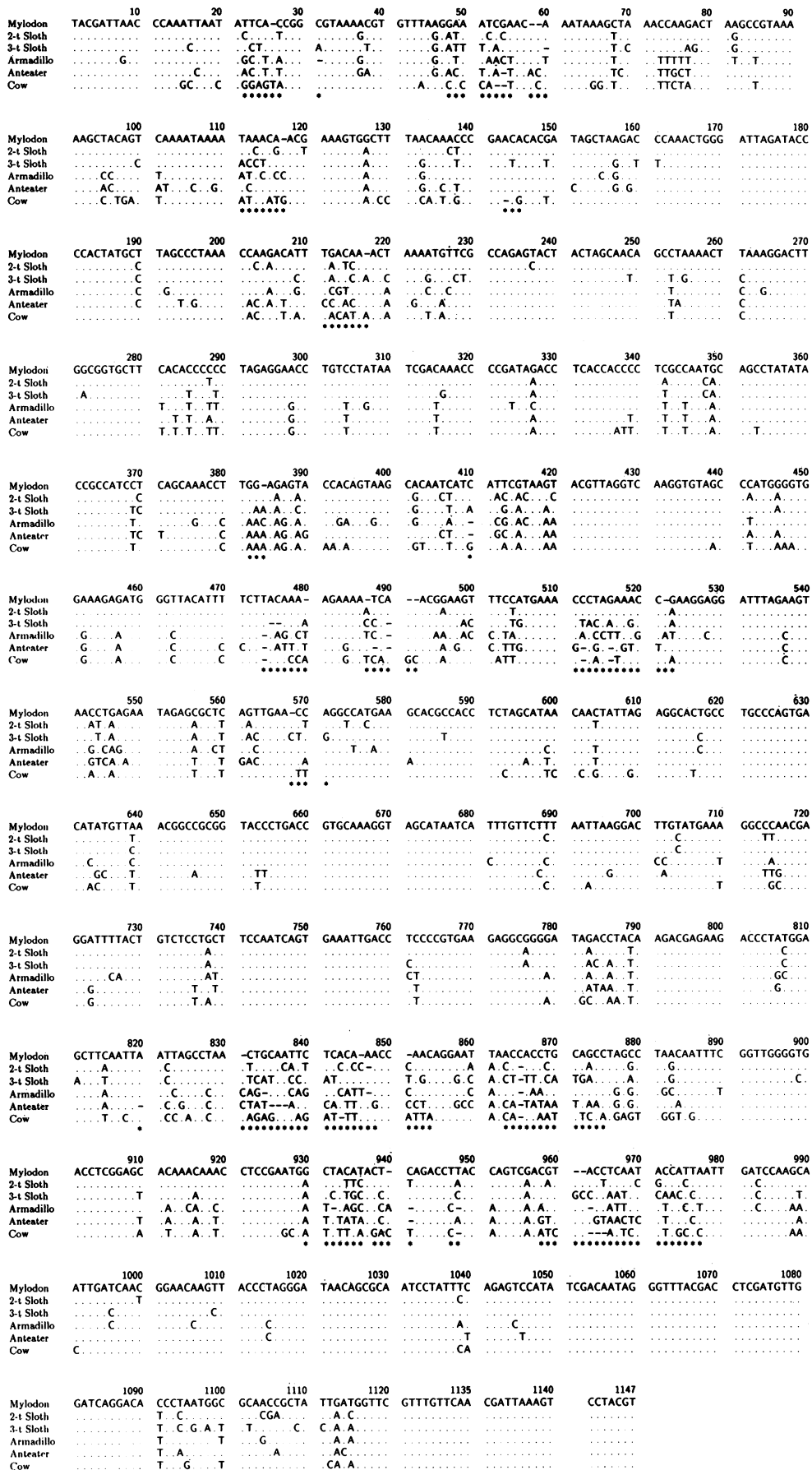
FIG. 3. Sequence alignment of *Mylodon*, two-toed and three-toed sloths (2-t sloth, 3-t sloth), armadillo, anteater, and cow. Dots represent sequence identity to the *Mylodon* sequence; dashes refer to deletions. Positions that cannot be unambiguously aligned are indicated by an asterisk.

Table 1.   Sequence differences among extant edentates and
M. darwinii

	1	2	3	4	5	6
1. Mylodon	—	70	94	147	150	164
2. Two-toed sloth	67	—	86	138	128	164
3. Three-toed sloth	89	81	—	142	155	174
4. Armadillo	133	125	129	—	142	148
5. Anteater	135	117	140	128	—	150
6. Cow	146	145	154	133	134	—

Observed sequence differences among aligned parts of edentate rDNAs (Fig. 3) are given below the diagonal and maximum-likelihood corrected distances above the diagonal.

current tree-living forms. Within this group, *M. darwinii* tends to fall together with the two-toed sloth to the exclusion of the three-toed sloth. This result is supported by 76.1% of bootstrap replications.

To further resolve the relationship of the sloth species, all taxa except the sloths and their closest relative in the initial analysis, the armadillo, were excluded from the data set. This allowed 119 additional positions to be included in the alignment. With this enlarged data set, the sister group status between *M. darwinii* and the two-toed sloth is supported by 93.7% of bootstrap replications, as well as by three phylogenetically informative insertion/deletion events (Fig. 3, positions 384, 480, and 490). No such event supports the association of *M. darwinii* with the three-toed sloth or of the extant sloths with each other. Thus, the phylogenetic analyses show that *M. darwinii* belonged to the same phylogenetic group as the extant sloths and furthermore indicate that it was more closely related to the extant two-toed than the three-toed sloths.

DISCUSSION

Authenticity. Contamination is the major problem in the retrieval of ancient DNA sequences (21–23). To assure, to the greatest extent possible, the authenticity of the ancient DNA sequences determined, bone processing, DNA extraction, and the setup of the PCR were done in a laboratory dedicated exclusively for these purposes. Protective clothing, separate
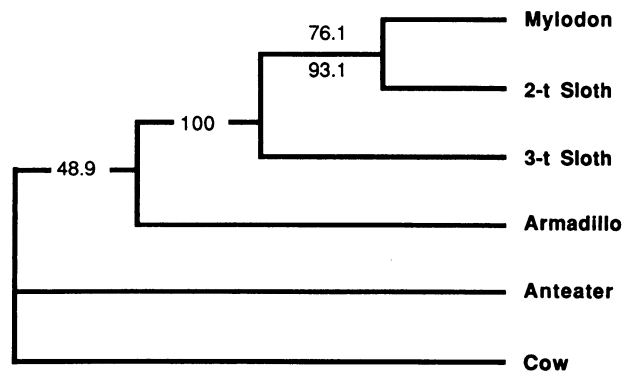
FIG. 4.   Phylogeny of extant edentates and the extinct *M. darwinii*. The tree was inferred using a neighbor-joining algorithm, and the 998 nucleotide positions are aligned in Fig. 2. Numbers at internal edges refer to percentages of 1000 bootstrap replications. For the internal edge separating *Mylodon* and two-toed sloth (2-t sloth) from the three-toed sloth (3-t sloth), the upper bootstrap value refers to the alignment shown in Fig. 2, whereas the bottom bootstrap value refers to an alignment of only sloths and armadillo. Parsimony and maximum-likelihood methods yielded the same tree topology. In a likelihood ratio test, the tree shown is 1.04 (all species included) and 1.85 (sloths and armadillo) SDs better than a tree where two-toed and three-toed sloths are sister taxa.

laboratory ware and chemicals, as well as other measures to avoid contamination were used (21). Appropriate extraction and PCR controls (24) showed no specific amplification products. From the two *Mylodon* samples that yielded sequences, several extractions from different parts of the specimens were performed. These consistently yielded the expected products, whereas extracts from other samples yielded no products of the expected sizes. All amplification products sequenced yielded identical sequences. Further support for the authenticity of the sequences stems from a sample removed independently from one of the specimens. When a 92-bp segment of the 16S rDNA was amplified and sequenced from this sample in another laboratory, a sequence identical to the one presented in Fig. 3 was obtained (34).

DNA Preservation. Extractions from 35 specimens, representing all three families of extinct sloths, from various localities in mainland South and North America, as well as Cuba, were performed. In only two cases could amplifications of 140 bp of mitochondrial DNA be achieved, in spite of the fact that the primers and conditions used allow the amplification of single template molecules. Thus, only a minority of samples yield amplifiable DNA, a situation that is typical of bones and other tissues retrieved at archaeological excavations. However, it is noteworthy that two other samples from which Pleistocene DNA sequences have been determined stem from an Alaskan horse (10) and Siberian mammoths (25, 26, 35). Because the Mylodon Cave is located in dry and cold, subantarctic conditions in Southern Chile, low temperatures may be one condition that is critical for the survival of DNA over long time periods. Presumably, the reason is the decreased rate of chemical processes that degrade nucleic acids, such as depurination (27, 28), brought about by low temperatures.

DNA Quantification. Extracts from the remains of ancient animals and plants may contain not only DNA from the organism of study but also from microorganisms that colonized the tissues after the death of the organism. To determine the amount of ancient sloth DNA in the extracts, three-toed sloth DNA, which is phylogenetically equally distant to *Mylodon* as to the two-toed sloth (Fig. 4), was used as a probe to quantitate dilution series of a *Mylodon* extract and two-toed sloth DNA (Fig. 1) by hybridization. Approximately 2.5 ng of *Mylodon* DNA per g of *Mylodon* bone was found to be intact enough to hybridize to the three-toed sloth probe. This was in stark contrast to the results obtained when the absorbance at 260 nm was determined, which indicated that about three orders of magnitude more DNA existed in the sample. Thus, the vast majority of the DNA extracted is not of *Mylodon* origin or is too damaged to hybridize to a sloth probe.

To obtain a qualitative assessment of other sources of DNA in the extract, PCR was performed with primers specific for eubacterial, as well as fungal, ribosomal genes. In both cases, strong amplifications resulted (data not shown). Thus, a substantial proportion of the extracts are composed of bacterial and fungal DNA. However, because the bacterial and fungal genera present are not known, a quantitative determination of the microbial DNA was not feasible. However, it is noteworthy that when amplifications of different length were done, an inverse correlation of amplification efficiency with length of the amplification product could not be observed for the fungal and bacterial amplifications. This is in contrast to the sloth amplifications, where the efficiency of amplification decreased drastically with length such that no amplification longer than ≈340 bp was possible. The fact that the microbial DNA is better preserved than the *Mylodon* DNA could have several reasons. Either the DNA stems from microorganisms that colonized the ancient tissues in recent times or the microbial DNA is of Pleistocene origin but has been better protected from damage than the vertebrate DNA (29) and/or has survived better due to its high abundance in the tissues in the immediate *post mortem* period.

Evolution: Höss *et al.*

Proc. Natl. Acad. Sci. USA 93 (1996)   185

Age and Biogeography of Edentates. To elucidate whether the ribosomal DNA sequences can be used to estimate the age of the tree sloths, as well as edentates in general, maximum-likelihood trees were constructed with, as well as without, a clock assumption (data not shown). By using the likelihood ratio test, the hypothesis of a molecular clock for the edentate rDNA could not be rejected. These sequences can therefore be used to estimate the age of taxa within this group, if a calibration point can be found. Such a point is provided by the *M. darwinii* sequence.

Mylodontidae, to which *M. darwinii* belong, is a well-supported monophyletic group (3), which appeared in the late Eocene ≈40 million yr ago (7). Thus, the divergence between *M. darwinii* and the two-toed sloth goes back at least 40 million yr. By using this as a calibration point, the corrected distance data (Table 1) indicate that the divergences among anteaters, armadillos, and the sloths date back ≈80 (range 73–88) million yr—i.e., well into the Cretaceous Period. This is in agreement with immunological comparisons between albumins from sloths, anteaters, and armadillos (30). Thus, whereas in other mammalian groups, such as rodents and primates, the lineages leading to these groups predate the Cretaceous–Tertiary boundary (31), the edentates are unique in that the divergences within the group go back to the Cretaceous Period. This is compatible with the fossil record in that armadillo scutes appear in South America during the Upper Palaeocene Epoch ≈60 million yr ago (7). It furthermore throws light on the enigmatic occurrence of an anteater (*Eurotamandua joresi*) in Germany during the Middle Eocene Epoch ≈45 million yr ago (32). Because the molecular data show that the phylogenetic lineages leading to armadillos and anteaters diverged before the complete separation of South America and Europe/Africa, some 65 million yr ago, the Eocene anteaters in Europe could be derived from the same lineage as the ones that currently exist in the New World.

Evolutionary Relationships and Extinction. The phylogenetic tree (Fig. 4) indicates that the Mylondontidae were closer related to the two-toed sloths than to the three-toed sloths. Thus, the widely favored hypotheses that the Mylodontidae represented an outgroup to the two extant sloth genera are not supported by the molecular data. However, it agrees with two morphological views (8, 36) as well as with two studies of the extant sloths using amino acid sequences of eye-lens protein (33) and serum proteins (30). In both cases a relatively distant relationship of the two- and three-toed sloths was found, arguing against a recent common origin of the two living sloth genera. Sloths are believed to have been derived from terrestrial ancestors (3). Because all Mylodontidae, and certainly *M. darwinii*, were ground-dwelling, the molecular data thus indicate that the arboreal life-style emerged at least twice among ground-living ancestral forms of sloths.

It is interesting that, in spite of not being closely related, two arboreal genera survived the mass extinction of sloths at the end of the last glaciation, whereas all ground-dwelling sloths, including those more closely related to the tree-living forms and existing in the same areas as these, disappeared. One may ask what caused specifically the tree-living forms to survive. An attractive hypothesis would be that humans colonizing the Americas in the late Pleistocene Epoch were responsible for the demise of the sloth radiation, a fate that only arboreal forms were able to avoid.

1. Wetzel, R. M. (1985) in *The Evolution and Ecology of Armadillos, Sloths, and Vermilinguas*, ed. Montgomery, G. G. (Smithsonian Institution, Washington, DC), pp. 5–21.
2. Janzen, D. H. & Martin, P. S. (1982) *Science* **215**, 19–27.
3. Webb, S. D. (1985) in *The Evolution and Ecology of Armadillos, Sloths, and Vermilinguas*, ed. Montgomery, G. G. (Smithsonian Institution, Washington, DC), pp. 105–112.
4. Long, A. & Martin, P. S. (1974) *Science* **186**, 638–640.
5. Patterson, B. & Pascual, R. (1968) *Q. Rev. Biol.* **43**, 409–451.
6. Simpson, G. G. (1945) *Bull. Am. Mus. Nat. Hist.* **85**, 1–350.
7. Carroll, R. L. (1988) *Vertebrate Paleontology and Evolution* (Freeman, New York).
8. Guth, C. (1961) *La Région Temporale des Edentés* (Le Puy, Haute Loire, France).
9. Miller, S. A., Dykes, D. D. & Polesky, H. F. (1988) *Nucleic Acids Res.* **16**, 1215.
10. Höss, M. & Pääbo, S. (1993) *Nucleic Acids Res.* **21**, 3913–3914.
11. Anderson, S., Bankier, A. T., Barrell, B. G., de Bruijn, M. H. L., Coulson, A. R., Drouin, J., Eperon, I. C., Nierlich, D. P., Roe, B. A., Sanger, F., Schreier, P. H., Smith, A. J. H., Staden, R. & Young, I. G. (1981) *Nature (London)* **290**, 457–465.
12. Cao, Y., Adachi, J. & Hasegawa, M. (1994) *Jpn. J. Genet.* **69**, 455–472.
13. Hasegawa, M. & Hashimoto, T. (1993) *Nature (London)* **361**, 23.
14. von Haeseler, A., Janke, A. & Pääbo, S. (1993) *Verh. Dtsch. Zool. Ges.* **86.2**, 119–129.
15. Saitou, N. & Nei, M. (1987) *Mol. Biol. Evol.* **4**, 406–425.
16. Felsenstein, J. (1981) *J. Mol. Evol.* **17**, 368–376.
17. Felsenstein, J. (1993) PHYLIP, Phylogeny Inference Package (Univ. of Washington, Seattle), Version 3.5P.
18. Swofford, D. L. (1993) PAUP: Phylogenetic Analysis Using Parsimony (Illinois Natural History Survey, Champaign), Version 3.1.1.
19. Felsenstein, J. (1985) *Evolution* **39**, 783–791.
20. Pääbo, S. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 1939–1943.
21. Handt, O., Höss, M., Krings, M. & Pääbo, S. (1994) *Experientia* **50**, 524–529.
22. Höss, M., Handt, O. & Pääbo, S. (1994) in *The Polymerase Chain Reaction*, eds. Mullis, K. B., Gibbs, R. A. & Ferré, F. (Birkhäuser, Boston), pp. 257–264.
23. Lindahl, T. (1993) *Nature (London)* **365**, 700.
24. Pääbo, S., Higuchi, R. G. & Wilson, A. C. (1989) *J. Biol. Chem.* **264**, 9709–9712.
25. Höss, M., Vereshchagin, M. K. & Pääbo, S. (1994) *Nature (London)* **370**, 333.
26. Hagelberg, E., Thomas, M. H., Cook, C. E., Jr., Sher, A. V., Baryshnikov, G. F. & Lister, A. M. (1994) *Nature (London)* **370**, 333–334.
27. Lindahl, T. & Nyberg, B. (1972) *Biochemistry* **11**, 3610–3618.
28. Pääbo, S. & Wilson, A. C. (1991) *Curr. Biol.* **1**, 45–46.
29. Lindahl, T. (1993) *Nature (London)* **362**, 709–715.
30. Sarich, V. M. (1985) in *The Evolution and Ecology of Armadillos, Sloths, and Vermilinguas*, ed. Montgomery, G. G. (Smithsonian Institution, Washington, DC), pp. 77–81.
31. Janke, A., Feldmaier-Fuchs, G., Thomas, W. K., von Haeseler, A. & Pääbo, S. (1994) *Genetics* **137**, 243–256.
32. Storch, G. (1981) *Senckenbergiana lethaea* **61**, 247–289.
33. de Jong, W. W., Zweers, A., Joysey, K. A., Gleaves, J. T. & Boulter, D. (1985) in *The Evolution and Ecology of Armadillos, Sloths, and Vermilinguas*, ed. Montgomery, G. G. (Smithsonian Institution, Washington, DC), pp. 65–76.
34. Taylor, P. G. (1996) *Mol. Biol. Evol.* **13**, in press.
35. Hauf, J., Baur, A., Chalwatzis, N., Zimmermann, F. K., Joger, U. & Lazarev, P. A. (1995) *Curr. Genet.* **27**, 486–487.
36. Gaudin, T. J. (1995) *J. Vertebr. Paleontol.* **15**, 672–705.