Xenarthra (Edentata) is an extremely diverse mammalian order whose modern representatives are the armadillos, anteaters, and sloths. The phylogeny of these groups is poorly resolved. This is particularly true for the sloths (phyllophagans), originally a large and diverse group now reduced to two genera in two different families. Both morphological analyses and molecular analyses of rDNA genes of living and extinct sloths have been used with limited success to elucidate their phylogeny. In an attempt to clarify relationships among the sloths, DNA was extracted and mitochondrial cytochrome b gene sequences were determined from representatives of two extinct groups of sloths (Mylodontidae and Megatheriidae), their two living relatives (two-toed sloths [Megalonychidae], three-toed sloths [Bradytopidae]), anteaters and armadillos. A consistent feature of the latter two species was the nuclear copies of cytochrome b gene sequences. Several methods of phylogenetic reconstruction were applied to the sequences determined, and the results were compared with 12S rDNA sequences obtained in previous studies. The cytochrome b gene exhibited a phylogenetic resolving power similar to that of the 12S rDNA sequences. When both data sets were combined, they tended to support the grouping of two-toed sloths with mylodon-tids and three-toed sloths with megatheriids. The results strengthen the view that the two families of living sloths adapted independently to an arboreal lifestyle.

Key Words: xenarthrans; sloth; mitochondrial DNA; ancient DNA; nuclear insertion; phylogeny

INTRODUCTION

The phylogenetic relationships of xenarthrans (edentates, i.e., armadillos, anteaters, and sloths) are unclear. Whereas xenarthran monophyly is well supported, for example by a deletion of three consecutive amino acids in the α-crystallin gene (van Dijk et al., 1999), their relationship to other mammalian groups remains problematic. Morphological studies, for example of the reproductive system, suggest that they represent one of the earliest divergences among mammals and that they may even be a sister group to all other eutherians (Engelmann, 1985; McKenna and Bell, 1997), whereas an analysis of complete mitochondrial DNA sequences situates xenarthrans as a sister group of ferungulates or elephants, depending on the method and sequences used (Arnason et al., 1997; Waddell et al., 1999), and thus far from a basal position in an eutherian tree. Within xenarthrans, morphological analyses suggest a closer affinity of sloths and anteaters (grouped as Pilosa) relative to the armadillos (Cingulata) (McKenna and Bell, 1997). Among the xenarthrans, sloths constitute the most diverse group, with almost 100, mostly fossil, genera recorded (McKenna and Bell, 1997). A number of these forms were abundant in the Americas during the Pleistocene, but became extinct around 10,000 years ago such that only five species belonging to two genera, Choloepus (two-toed sloths) and Bradypus (three-toed sloths), survive today in Central and South America.

According to McKenna and Bell (1997), sloths are classified into two infraorders, Mylodonta and Megatheria. All mylodontans are extinct; Megatheria is subdivided into three families, Megatheriidae, Megalonychidae, and Bradytopidae. Of these, all representatives of Megatheriidae are extinct, whereas Choloepus is regarded as a megalonychid and Bradypus is placed in its own superfamily, the Bradytopoidea. This classification is, however, debated. For example, one morphological analysis suggests an association between mylodontid and megalonychid sloths (Gaudin, 1995).

We have determined mitochondrial cytochrome b gene sequences from a mylodontid (Mylodon darwinii) and a megatherid (Nothrotheriops shastensis) sloth, as well as extant xenarthran species, and used these together with the existing 12S rDNA sequences for these species in an attempt to clarify the phylogeny of this group.
Materials and Methods

Samples. Liver from a naked tail armadillo (Cabassous unicinctus), hairy ant eater (Tamandua tetradactyla), two-toed sloth (Choloepus didactylus), and three-toed sloth (Bradypus variegatus) were obtained from the University of California Museum of Vertebrate Zoology, Berkeley, California. Bone from a Mylodon darwini specimen was obtained from the Natural History Museum, London (sample number BM(NH)M8758). A coprolite, likely derived from Nothrotheriops shastensis, from Gypsum Cave, Nevada, was provided by Northern Arizona University, Flagstaff, Arizona. Bone specimens of Acratocnus odontogonus and Megalonyx sp. (AMNH Nos. 8668, 22480, and 8640) were provided by the Department of Vertebrate Paleontology of the American Museum of Natural History, New York.

Ancient DNA extractions. Bone powder was produced with a Freezer/mill 6700 bone grinder (Spex Industries) or by drilling into the bone with a low-speed hand-held drill. Coprolite samples were ground to powder with a Freezer/mill 6700 bone grinder. A sample of 0.2 g of bone powder was placed in 2 ml of 0.5 M EDTA, pH 8.0, 5% sarcosyl and deaminized for 2 days. The EDTA solution was exchanged for 1 ml fresh solution containing 0.3 mg/ml proteinase K and digested for 2 days. The digested bone was extracted once with phenol, once with phenol and chloroform/isooamyl, and once with chloroform/isoamyl. The DNA was then purified using a silica-based method (Höss and Paabo, 1993; Krings et al., 1993; Poinar et al., 1998). All work with ancient DNA was carried out in a room dedicated for this purpose, where protective clothing was worn at all times. The room was under constant ultraviolet radiation when not in use.

Modern DNA extractions. Approximately 1 g of liver or 200 ml of blood was digested in 0.5–1 ml of 10 mM Tris-HCl, pH 7.5, 10 mM EDTA, pH 8.0, 50 mM NaCl, 2% SDS, and 0.6 mg/ml proteinase K. Samples were digested overnight at 37°C and concentrated to 50–100 ml using Millipore Ultrafree 30,000 NMWL columns.

PCR, cloning, and sequencing. PCR primers and combinations used are shown in Table 1. PCR of ancient DNA was performed in 30-µl reactions as described (Höss and Paabo, 1993). Products were run and visualized on 3% LMP ethidium-stained agarose gels. Where necessary, the products and relevant negative controls were reamplified for 20 cycles under the conditions used in the first amplifications but with a 60°C annealing temperature. PCR of modern DNA was performed in 100-µl reactions using 3-5 µl of extract and 67 mM Tris-HCl, pH 8.9, 4 mM MgCl2, 16 mM (NH4)2SO4, 10 mM β-mercaptoethanol, 0.125 mM each dNTP, 10 U Taq polymerase, and 30 pmol of each primer. Reactions were cycled 30 times with a 30-s 94°C denaturing step, a 1-min 50–55°C annealing step, and a 1-min 72°C extension step in a Perkin-Elmer 480 Thermocycler. Products were visualized on ethidium-stained 1.5% agarose gels and cloned in the pGEM-T vector (Promega). After electroporation into bacteria and growth under ampicillin and blue/white selection, white colonies were picked with a sterile pipette tip and added to 30-µl PCRs where the M13 forward and reverse primers were used to amplify inserts for 25 cycles using 1.5 U Taq DNA polymerase and the buffer described. PCR fragments were sequenced with FITC-labeled M13 universal primers using an PTC-200 thermal cycle (MJ Research) and analyzed on an A.L.F. DNA sequencer (Pharmacia). For radioactive sequencing, colonies were inoculated into standard LB ampicillin cultures and DNA was pre-

<table>
<thead>
<tr>
<th>A. Primer</th>
<th>PCR primers</th>
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<tbody>
<tr>
<td>L-A1</td>
<td>ACCAATGACATGAAAAAACCACGTGGT</td>
</tr>
<tr>
<td>L-A2</td>
<td>AAAACACATCGTGGTAAATTCACATA</td>
</tr>
<tr>
<td>L-B1</td>
<td>ATGCAGCTCATCACCACTACCATCA</td>
</tr>
<tr>
<td>L-C1</td>
<td>GATACAACACCAGCGCTTTTCCTAT</td>
</tr>
<tr>
<td>L-C2</td>
<td>CACATCGACAAATCATACAGCC</td>
</tr>
<tr>
<td>L-C3</td>
<td>GGTCTACCATTTTCTTCTGCTCA</td>
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<tr>
<td>L-D1</td>
<td>GCATCAATTTTCTTCTGCTGCTT</td>
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<td>L-D2</td>
<td>GCATCAATTTTCTTCTGCTGCTT</td>
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<tr>
<td>L-E1</td>
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<tr>
<td>L-F2</td>
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</tr>
<tr>
<td>H-A1</td>
<td>TATGATTAGCATATATCTATAGGAG</td>
</tr>
<tr>
<td>H-B1</td>
<td>TTTCAGTCTCGGAGAGATGTTG</td>
</tr>
<tr>
<td>H-C1</td>
<td>GATTAGTGAAGGCCGTAGTAT</td>
</tr>
<tr>
<td>H-D1</td>
<td>AAATCGACGCCCTCCTGAGATATTGCTTCA</td>
</tr>
<tr>
<td>H-E1</td>
<td>TGGGCGAAGAAGCAGCGGTT</td>
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<tr>
<td>H-F1</td>
<td>GTATGGGGTGAGAATGGATTTT</td>
</tr>
<tr>
<td>H-G1</td>
<td>CAAGACGCCCTCCTAGTTGT</td>
</tr>
</tbody>
</table>

Note. All primers are given in the 5' to 3' orientation, where L and H represent the light and heavy strands of mtDNA replication, respectively. A. Primer sequences. B. Primer combinations used for each species. *These primers were taken from Kocher et al. (1989).
pared using the Plasmid MaxiPrep Kit (Qiagen) or an Autogene 740 robot. Radioactive sequencing was performed using \( ^{32}P \)dATP (Amersham) and the T7 Sequenase 2.0 protocol (Amersham). Sequences were resolved on 6% polyacrylamide gels and visualized by autoradiography using XAR film (Kodak). For modern sloths and Cabassous unicinctus, multiple clones were partially sequenced in both directions. For Tamandua tetradactyla, seven clones were radioactively sequenced using combinations of primers L-A1, L-G1, and H-D1 (Table 1), T7, SP6 vector primers, M13 Primers, and primers 5'-CAYCAGACACAAYCACAGCATT-3' (where Y denotes C or T), 5'-TGTCGAGACGCAACTACGGATG-3', and 5'-CGTGAGGGTAGCTTTATCTACT-3' to determine the complete clone sequence. Five or more clones were sequenced from each PCR product generated from ancient DNA, and each position in the reconstructed sequence was covered by at least two independent PCRs. Sequences obtained in this work have been deposited in GenBank under Accession Nos. AF23012 to AF23023.

Phylogenetic analyses. The cytochrome b gene sequences determined were aligned to the homologous sequence of long-nosed armadillo (Dasypus novemcinctus) extracted from GenBank (Accession No. Y11832). No gaps were necessary for the alignment. The 12S rDNA sequences were extracted from GenBank and aligned using Clustal W 1.8 (Thompson et al., 1994) with default parameters. Regions of ambiguous alignment were removed using the program Gblocks 0.7 (Castresana, 2000) with default parameters for rDNA data. This resulted in the deletion of 6% of the original 583 positions, leaving 550 positions for the analysis.
FIG. 2. *Mylodon darwini*i clones used to reconstruct the cytochrome *b* gene sequence. The reference ("ref.") is the consensus sequence of all clones. Dots represent identity to the reference, "." represents gaps, and "N" represents bases that could not be determined. The clone designations consist of a letter (A, B, C) indicating the PCR, followed by a number indicating whether it was the first or second amplification of the product, and after the period a number indicating the particular clone.
Parsimony, neighbor-joining, and maximum-likelihood trees were estimated with PAUP* 4.0b4a (Swofford, 1998). One thousand bootstrap replications (for parsimony and neighbor-joining) or 100 (for maximum-likelihood) were calculated and the 50% majority-rule consensus tree was obtained. In the parsimony analysis, transversions were weighted 5 times the transitions in the third codon positions of the cytochrome b (weights between 5 and 20 produced the same results), and searches were performed with the branch and bound algorithm.

Parameters for the neighbor-joining and maximum-likelihood trees were calculated from an initial neighbor-joining tree obtained using a HKY distance measure (Hasegawa et al., 1985), where the transition/transversion ratio was estimated separately for each pair of taxa. Using this initial tree, the global transition/transversion ratio and the gamma shape parameters were estimated by maximum-likelihood.

Neighbor-joining trees were generated from maximum-likelihood distances with rates assumed to follow a continuous gamma distribution. Maximum-likelihood pairwise distances used for distance plots were also calculated assuming a continuous gamma distribution.

Maximum-likelihood trees were estimated using the HKY model and a discrete gamma distribution approximated with six rate categories. Branch and bound searches were carried out for the bootstrap replications. Branch lengths were estimated by maximum-likelihood from the bootstrap tree.

In addition, alignments with other outgroups external to the xenarthrans were analyzed as above, except that, when nine or more species were included, heuristic instead of branch and bound searches were done for the parsimony and maximum-likelihood bootstrap trees.

RESULTS

Contemporary species. DNA was extracted from liver tissues of a two-toed sloth (C. didactylus), a three-toed sloth (B. variegatus), a hairy anteater (T. tetradactyla), and an armadillo (C. unicinctus). A 648-bp fragment of the cytochrome b gene was amplified from the sloths and cloned; nine and eight clones from the two-toed and three-toed sloths were sequenced, respectively. Among the clones sequenced, few differences were found. In addition, an internal fragment of 307 bp (excluding primers) was amplified from the two-toed sloth and cloned, and 6 clones were sequenced. No differences were observed between the shorter and the longer fragments. Thus, these sequences were deemed to stem from the organelar sequences.

When the 648-bp cytochrome b gene fragment was amplified from the hairy anteater and nine clones were partially sequenced, seven distinct sequences that dif-
ferred substantially from each other were observed. The sequences of clones representing each of these sequences were completely determined (Fig. 1). They carried 9–132 differences from each other. In addition, one of the clones contains a stop codon and clones 2 and 5 are identical over the first 182 bp, whereas clone 5 then displayed 94 differences over the remaining 466 bp. The latter observation may represent a PCR recombination event (Paabo et al., 1990). In a tree reconstruction using cow as a more distant outgroup, two clones fall outside xenarthrans. A likely explanation for observing several widely divergent mitochondrial sequences is that some (or all) represent nuclear integrations of mitochondrial DNA fragments. Such inte-

### Table 2

<table>
<thead>
<tr>
<th>Alignment</th>
<th>Number of positions</th>
<th>Number of consant pos.</th>
<th>Number of informative pos.</th>
<th>Ti/Tv ratio</th>
<th>Gamma rate parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyt b</td>
<td>648</td>
<td>381</td>
<td>138</td>
<td>2.46</td>
<td>0.38</td>
</tr>
<tr>
<td>12S rDNA</td>
<td>550</td>
<td>363</td>
<td>91</td>
<td>4.76</td>
<td>0.32</td>
</tr>
<tr>
<td>Cyt b + 12S rDNA</td>
<td>1198</td>
<td>744</td>
<td>229</td>
<td>3.17</td>
<td>0.31</td>
</tr>
</tbody>
</table>

**FIG. 4.** Alignment of cytochrome b (cyt b), 12S rDNA, and the Concatenated Alignments.
Translocations have been observed in many species (Zhang and Hewitt, 1996) and they can sometimes dominate amplifications (Greenwood and Pääbo, 1999). To clarify whether these sequences represent nuclear insertions, an internal fragment of 307 bp was amplified (cf Table 1A, primers taken from Kocher et al., 1989), the amplification product was cloned, and four clones were sequenced. All four sequences were identical to one of the longer sequences amplified (Clone 3, Fig. 1). Since it is unlikely that two independent primer pairs would both fail to amplify organellar mitochondrial DNA sequences, which are expected to be present in larger copy numbers than any particular nuclear insertions, the sequence amplified by two primer pairs is likely to represent the mitochondrial sequence. Thus, although fresh tissue suitable for RNA isolation was not available such that the transcribed sequence could be determined (Collura et al., 1996; Greenwood and Pääbo, 1999), we assume that the DNA sequence amplified by both primer pairs represents the mitochondrial DNA sequence.

The primers used to amplify the cytochrome b gene from the anteater and sloths did not produce a product in the armadillo. Therefore, two overlapping fragments were amplified with different sets of primers to cover the same portion of the cytochrome b gene (Table 1B). From the 5' fragment, two sequences represented by seven clones and one clone, respectively, were observed, whereas the 3' fragment was represented by three different sequences seen in six, one, and one clone, respectively. The majority sequences from the 5' and 3' fragments were identical in their overlapping parts and did not carry any stop codons. One singleton sequence from the 3' fragment was identical to the majority sequence for 411 bp, after which it carried many differences (not shown). This clone may represent a PCR or cloning artifact. The second singleton 3' sequence carried two deletions of 3 and 1 bp (not shown) compared to the other clones and is thus unlikely to represent a functional cytochrome b gene. The single divergent 5' sequence was compatible with function upon translation but was not represented among the clones from the 3' fragment. Since the majority sequence was obtained in PCRs using two independent primer sets, it is regarded as the bona fide organellar sequence, whereas the other sequences are likely to represent nuclear insertions.

Extinct species. DNA was extracted from the bone of a 13,000-year-old M. darwinii specimen and from a 20,000-year-old coprolite likely to derive from N. shastensis because this sloth is the only large mammal abundantly represented in the deposit. Mitochondrial rDNA sequences have previously been determined from both of these samples (Höss et al., 1996; Taylor, 1996; Poinar et al., 1998). Based on an alignment of the modern xenarthran sequences, primers were designed to amplify fragments of 156–254 bp (including primers), as ancient DNA tends to be highly degraded (Pääbo, 1989). Also, sequencing overlapping fragments makes it possible to distinguish nuclear insertions from organellar mitochondrial sequences in ancient specimens (Handt et al., 1996). In three cases, primers that functioned for the Mylodon had to be replaced by other primers in the Nothrotheriops (Table 1B) due to sequence differences between the species. Each amplification product was cloned and several clones were sequenced (Figs. 2 and 3). Each sequence position was covered by at least two independent amplifications to assure that the sequences determined were correct (Krings et al., 1997).

For the remaining family of megatheriid sloths, the
megalonychids, bones representing two individuals of Acratocnus odontogonus from Puerto Rico and three individuals of Megalonyx sp. from North America were extracted. However, none of these samples yielded DNA from which sloth DNA could be amplified.

Phylogenetic analyses. Table 2 compares some features of the xenarthran cytochrome b gene sequences, as well as the previously determined 12S rDNA sequences (not shown). The cytochrome b gene fragment (Fig. 4) of 648 nucleotide positions contains 138 posi-

FIG. 6. Phylogenetic trees generated by parsimony, neighbor-joining, and maximum-likelihood methods from the alignments of cytochrome b gene (A), 12S rDNA (B), and after concatenating the two data sets (C). Numbers at the nodes represent bootstrap support. In maximum-likelihood trees, the scale bar represents a distance of 0.1 substitutions per site. We have chosen to place the root of the trees between armadillos and the other sequences, as supported by the use of cow as external outgroup, as well as by morphological data (McKenna and Bell, 1997).
tions that are informative in a parsimony sense, whereas the alignment of the 12S rDNA sequences of 550 positions (after removal of regions of ambiguous alignment) contained 91 informative positions. Both the estimated transition/transversion ratio and the among-site rate variation are higher in the 12S rDNA alignment than in the cytochrome b gene alignment.

To study the effect of multiple substitutions, plots of observed differences versus maximum-likelihood distances were generated (Fig. 5). This indicated that a higher number of multiple substitutions affects both sets of sequences. However, for the cytochrome b sequences, this effect is less extreme than that for the 12S rDNA sequences.

Phylogenetic trees obtained from the cytochrome b alignment by parsimony, neighbor-joining, and maximum-likelihood are shown in Fig. 6A. All methods fail to recover a well-supported phylogenetic tree probably due to the high number of multiple substitutions. In addition, neighbor-joining produces a conflicting topology. The phylogenetic performance of the 12S rDNA alignment (Fig. 6B) is similarly poor, with parsimony producing a tree different from those of the other methods. When both alignments are concatenated (Fig. 6C) the topologies obtained with all methods are identical, favoring the association of the two-toed sloth with M. darwini and the three-toed sloth with N. shastensis, albeit not in a statistically supported way. In addition, the analyses were repeated using different outgroups with the concatenated alignments: only anteater, cow + anteater + armadillos, hyrax (Procavia capensis) + anteater + armadillos, cow + hyrax + anteater + armadillos, and elephant (Loxodonta africana) + hyrax + cow + anteater + armadillos. In all cases, the same grouping of sloths (two-toed sloth with M. darwini and three-toed sloth with N. shastensis) was found with neighbor-joining and maximum-likelihood, although parsimony produced other topologies. However, the relative grouping of sloths, anteater, and armadillos was different for the different outgroups: whereas the use of cow shows the anteater to be a sister group of sloths with neighbor-joining and maximum-likelihood, the choice of other outgroup combinations that include elephant or hyrax produced a tree in which the anteater is closer to the armadillos with all three reconstruction methods. Thus, the choice of different outgroups seems to influence the phylogeny of the major xenarthran groups but not the relationships within sloths. Although recent analysis of complete mtDNA data shows that elephants could be the sister group of xenarthrans (Waddell et al., 1999), the mitochondrial sequences of elephants (and, to a lesser extent, those of the close relative hyrax) are quite divergent, which may weaken their performance as an outgroup in our data set, whereas the sequences of cow, which is also close to xenarthrans (Arnason et al., 1997; Waddell et al., 1999), are more slowly evolving (not shown).

**DISCUSSION**

The present study doubles the amount of DNA sequence information available from extinct xenarthrans. In view of this, it may seem disappointing that the phylogenies produced fail to resolve the relationship of all taxa. However, it is clear that xenarthran groups are extremely old. Immunological data suggest that two-toed and three-toed sloths diverged 35 MYA (Sarich, 1985) and this is supported by recent fossil discoveries, for example a 30 MYA megalonychid in Puerto Rico and other equally old megalonychids from southern South America (MacPhee and Iturralde, 1995). Major sloth groups probably diverged during a short period of time in the mid-Oligocene and thus it may not be unexpected that these relatively short sequences are unable to resolve the phylogenies in a decisive way.

The phylogenetic analyses of the concatenated alignment support the association of mylodontid sloths with two-toed sloths and megatherid sloths with three-toed sloths. This is in agreement with previous molecular analysis (Höss et al., 1996) and, to a large extent, with some morphological analysis (Gaudin, 1995). However, the association of mylodontid sloths with two-toed sloths is unexpected since they have recently been classified in two different infraorders (McKenna and Bell, 1997). In any case, these results reinforce the hypothesis that the arboreal life-style of two-toed and three-toed sloths evolved independently (Webb, 1985; Höss et al., 1996). Consequently, their adaptations to life in tree canopies is probably due to convergence.

It is hoped that the determination of further mitochondrial sequences, as well as more slowly evolving sequences in the nuclear genome (Greenwood et al., 1999), will eventually more definitively resolve the relationships of these groups of animals.

**ACKNOWLEDGMENTS**

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