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Phylogeny, diet, and habitat of an extinct ground sloth from Cuchillo Curá, Neuquén Province, southwest Argentina

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Abstract

Advancements in ancient DNA analyses now permit comparative molecular and morphological studies of extinct animal dung commonly preserved in caves of semiarid regions. These new techniques are showcased using a unique dung deposit preserved in a late glacial vizcacha (*Lagidium* sp.) midden from a limestone cave in southwestern Argentina (38.5° S). Phylogenetic analyses of the mitochondrial DNA show that the dung originated from a small ground sloth species not yet represented by skeletal material in the region, and not closely related to any of the four previously sequenced extinct and extant sloth species. Analyses of pollen and plant cuticles, as well as analyses of the chloroplast DNA, show that the Cuchillo Curá ground sloth browsed on many of the same herb, grass, and shrub genera common at the site today, and that its habitat was treeless Patagonian scrub-steppe. We envision a day when molecular analyses are used routinely to supplement morphological identifications and possibly to provide a time-lapse view of molecular diversification.

Keywords: Ancient DNA; Vizcacha; Phylogeny; Dung; Plant cuticles; Pollen; Diet; Late glacial; Extinction; Ground sloth

Introduction

Dung of herbivores that became extinct at the end of the Pleistocene is commonly preserved in cave deposits from semiarid regions, and the diets of these herbivores can be reconstructed readily from pollen and plant cuticle analyses (Laudermilk and Munz, 1934; Hansen, 1978; Moore, 1978; Mead, 1981; Thompson et al., 1980; Mead, 1981; Markgraf, 1985). Normally, such dung is assigned to species based on its morphological similarity to dung of a living species (e.g., in southeastern Utah, USA, *Mammuthus* dung at Bechan Cave closely resembles that of modern elephants; Davis et al., 1984; Mead et al., 1986; see also Harrington's mountain goat in southeastern Utah, Mead et al., 1987) or close association with other fossil remains such as bones or teeth [e.g., ground sloth remains at Cueva de Milodón, Chile (Markgraf, 1985; Borrero, 1997); Gruta del Indio, Argentina (D'Antoni, 1983; Long et al., 1998; Garcia and Lagiglia, 1999); Gypsum Cave, Nevada, USA (Harrington, 1933); Shelter Cave, Nevada, USA (Thompson et al., 1980); and Rampart Cave, Arizona, USA (Martin et al., 1961)] (see Fig. 1 for location of Cueva de Milodón and Gruta del Indio).

The advent of polymerase chain reaction (PCR) has made it possible to amplify and thus identify ancient DNA sequences from bones and plant and animal tissue (e.g., Thomas et al., 1989; Cooper et al., 1992, 2001; Höss et al., 1996). Dung usually contains abundant epithelial cells with mitochondrial DNA of the defecating animal, as well as chloroplast DNA from eaten plants, but PCR amplification of the DNA is apparently blocked by cross-links between reducing sugars and amino groups. These cross-links are

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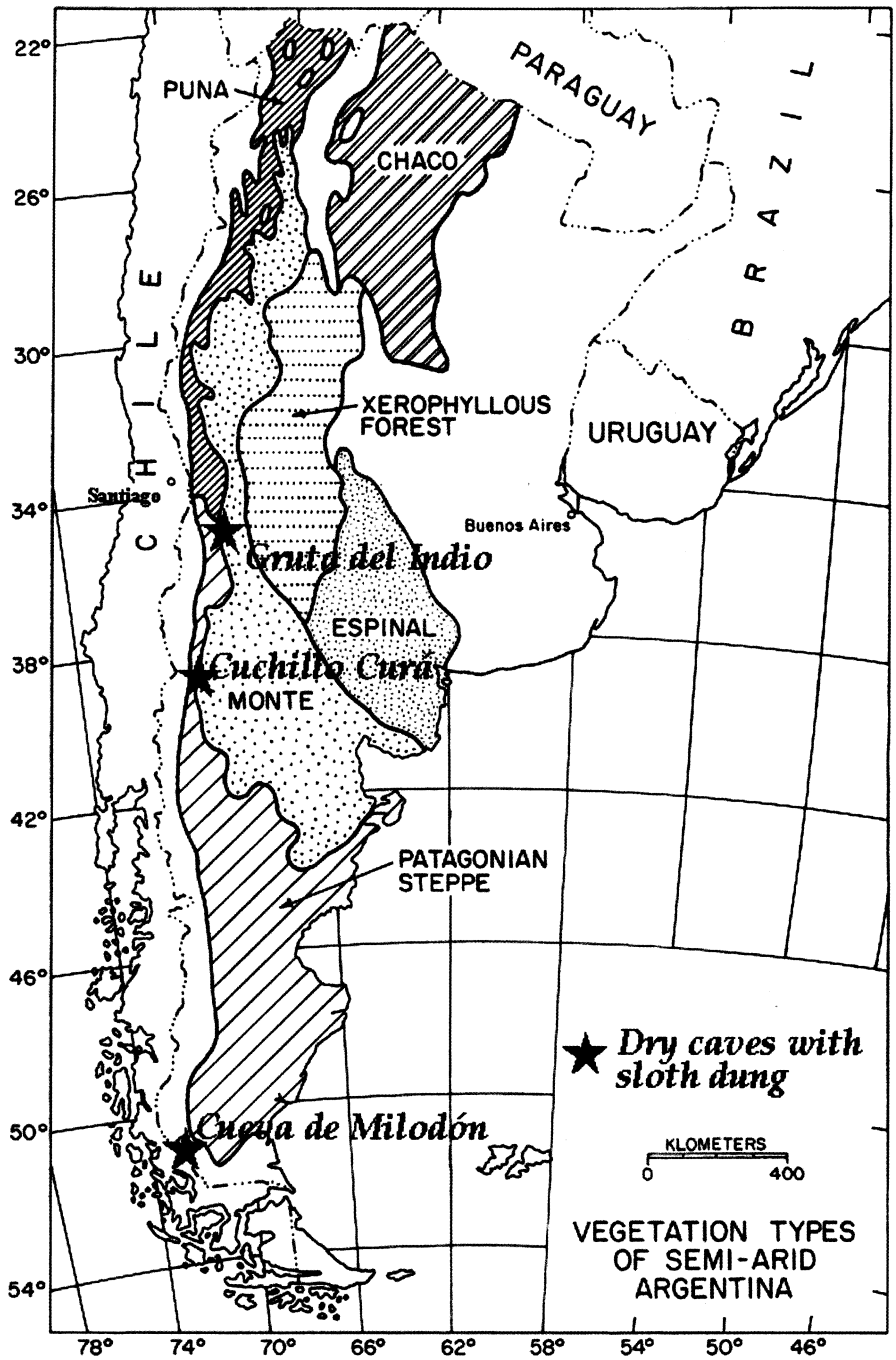

Fig. 1. Map showing location of Cuchillo Curá, Gruta del Indio, and Cueva de Milodón, three caves with sloth dung deposits.

generally the product of Maillard reactions, a series of condensation reactions that bind proteins and carbohydrates during storage of foods at room temperature, during cooking, or in long-term preservation of plant and animal tissue. Recently, it was discovered that such cross-links can be cleaved by the chemical reagent, *N*-phenacylthiazolium bromide (PTB) (Vasan et al., 1996), permitting amplification of both mitochondrial and chloroplast DNA in fossil dung (Poinar et al., 1998; Hofreiter et al., 2000; Kuch et al., 2002). Here, we report on the use of this technique in the analyses of dung from a new cave locality in the Province of Neuquén, Argentina, tentatively assigned to ground sloth based on its gross morphology. The sloth dung became incorporated and preserved in a contemporaneous, urine-cemented midden made by vizcachas (*Lagidium:* Chinchillidae, Rodentia). We amplified and sequenced 573 bp of mitochondrial 12S rDNA to identify the agent of the dung, and also analyzed ancient chloroplast DNA to supplement pollen and cuticle identifications in reconstructing the animal's diet.

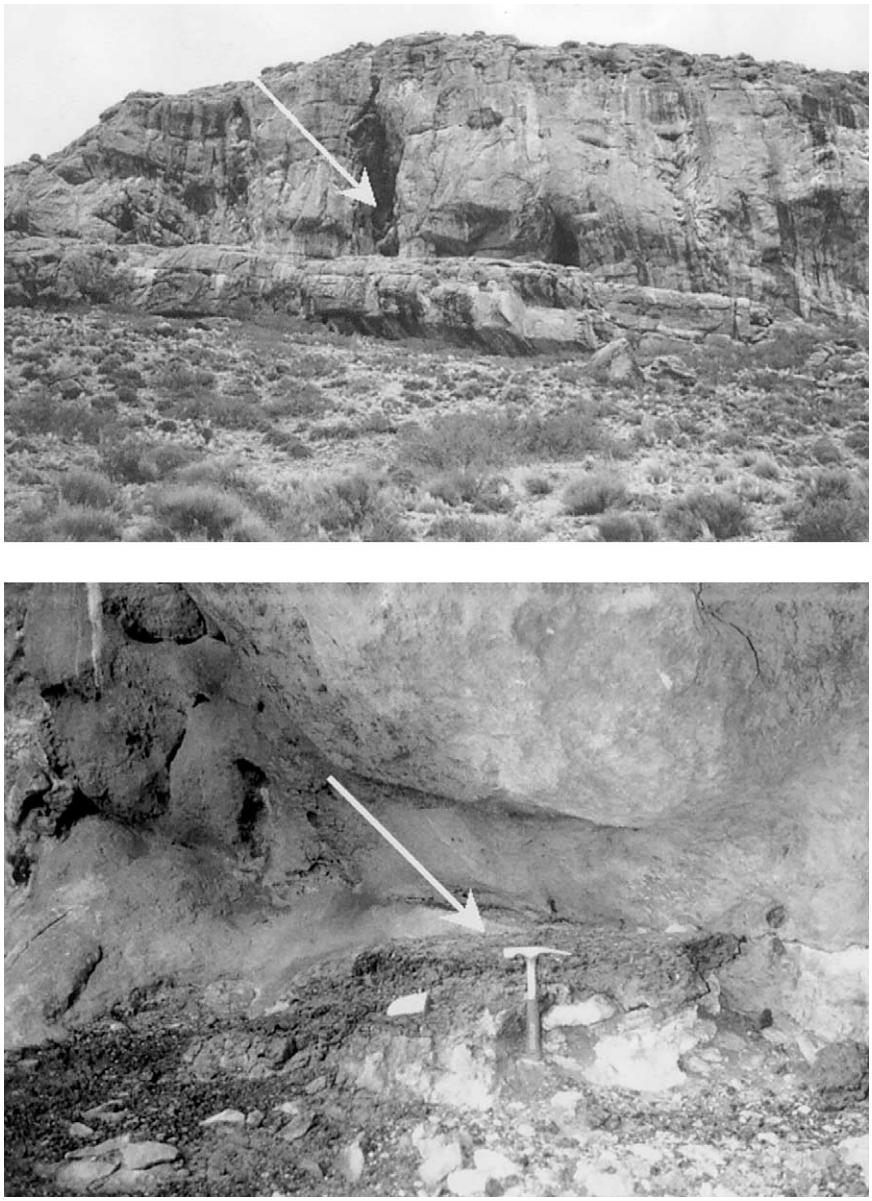

Fig. 2. Top photograph is view of north-facing limestone cliffs at Cuchillo Curá with arrow pointing to vertical chimney and cave containing vizcacha and sloth midden. Bottom photograph shows vizcacha/ground sloth midden with rock hammer for scale. This deposit contains dozens of sloth dung encased in a urine-crystallized midden made by a vizcacha.

Environmental setting of the Cuchillo Curá site

The material analyzed was collected by J.L.B. in September 1994 as part of an ongoing effort to develop the paleoecological potential of fossil rodent middens in arid South America on par with packrat middens in arid North America (Pearson and Christie, 1993; Markgraf et al., 1997; Betancourt et al., 2000; Holmgren et al., 2001; Latorre et al., 2002; Betancourt and Saavedra, 2002). The dung was found in a midden made by vizcachas, a chinchillid rodent resembling a long-tailed rabbit, near the opening to a limestone cave in northern Neuquén Province, Argentina (Fig. 1). The cave is located on the dry, north face of Cuchillo Curá (Fig. 2), an east-west trending, 2-km-long limestone ridge defining an anticline about 12 km south of the town of Las Lajas, in the valley of the Rio Agrio (the cave coordinates are 38°36.126′ S, 70°18.338 W and the elevation is ~1050 m). The primary cave-bearing unit is the Jurassic La Manga Formation, which at Cuchillo Curá contains one of the most important cave systems in Argentina (Elzerad, 1987). Four caves, El Gendarme, El Templo, El Arenal, and Los Cabritos, include more than 4 km of galleries and a unique ecosystem characterized by endemic cave fauna and flora (Anghilante, 1987; Merlin and Rodriguez, 1988; Grosso, 1995; Quaglia et al., 1999).

Cuchillo Curá is situated in the eastern foothills of the southern Andes, within the narrow ecotone between the Monte Desert to the north and east and Patagonian Steppe to

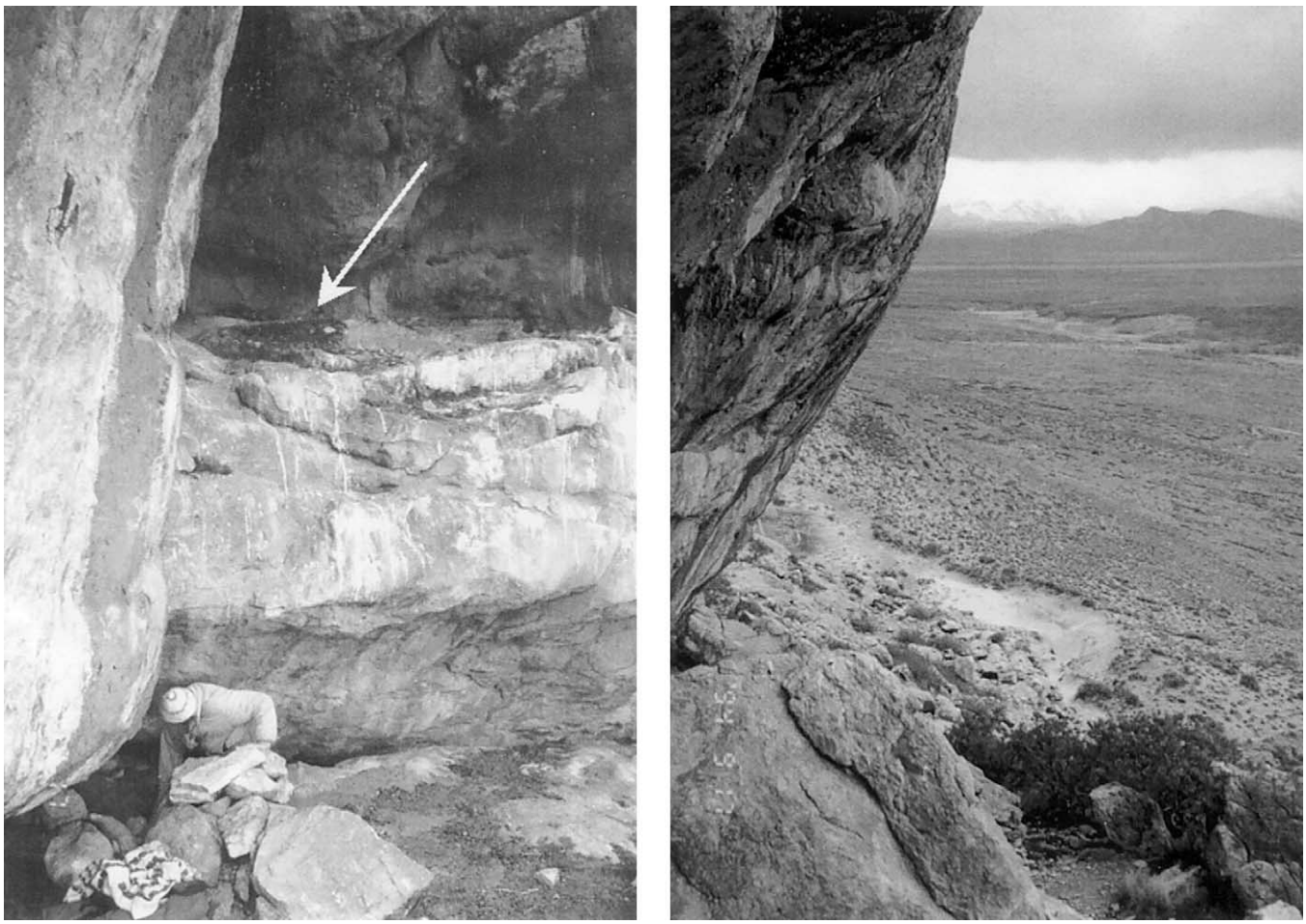

Fig. 3. Left photograph shows perched rock shelter where the vizcacha/ground sloth midden was found. Right photograph shows hillslope and valley in front of the cave, looking northeast with southern Andes in the far background.

the south (Fig. 1). Mean annual temperature at Cuchillo Curá is ~12°C, the frost-free season is about 100 days long, and mean annual precipitation is ~200 mm, falling mostly in the austral winter from May through August. The local vegetation can be described as scrub-steppe. Common plants within 100 m of the cave are *Larrea nitida* (Zygophyllaceae), *Stipa speciosa* (Poaceae), *Lycium chilense* (Solanaceae), *Schinus polygamus* (Anacardiaceae), *Acantholippia seriphioides* (Verbenaceae), *Colliguaya interregima* (Euphorbiaceae), *Mulinum spinosum* (Apiaceae), *Neosparton ephedroides*, *Junellia glauca* (Verbenaceae), *Ephedra ochreata* (Ephedraceae), *Gutierrezia solbrigii* (Asteraceae), and *Erodium cicutarium* (Geraniaceae). Other important species within a few kilometers of the site include *Happlopappus pectinatus*, *Senecio filaginoides*, *Perezia recurvata*, and *Nassauvia axilaris* (Asteraceae) (for description of regional vegetation, see Movia et al., 1992). Living vizcachas (most likely *Lagidium viscacia*) were observed at the site, as well as a spotted Geoffroy's cat (*Oncifelis geoffroyi*), which hid deep in the cave upon our arrival. Cuchillo Curá is near the northern limits and less than 500 m below the lower limits of key forest elements such as *Araucaria araucana* (Araucariaceae), *Austrocedrus chilensis* (Cuppresaceae), and *Nothofagus* spp. (Fagaceae). The closest *Araucaria* (monkey-puzzle) trees to the cave are at ~1400–1500 m elevation near Pino Hachado ~47 km to the east.

Description of deposit

The vizcacha midden (Cuchillo Curá 2B; Fig. 2), identified by both odor of the midden and morphology of the fecal pellets, is in a relatively inaccessible, open shelf in the front of the cave, perched about 4 m above the base of the cliff (Fig. 3). The midden covers an area of ~2.25 m^2 of the floor of the cave, representing a volume of ~0.7 m^3 of material. This does not include several large chunks of the midden that slid off the shelf to the base of the cliff. Numerous dung fragments from what was obviously a large, extinct herbivore were seen eroding from the edges of the vizcacha midden and were examined closely in the field. J.L.B. was familiar with extinct herbivore dung found in caves in the western United States and South America, and surmised that the material must have been produced by a

ground sloth. All of the dung segments were less than 5 cm in diameter, much smaller than segments of ground sloth dung described from Rampart Cave, Arizona, USA, and Gruta del Indio, Mendoza Province Argentina. The segments resemble some of the smaller (<5 cm diameter) dung segments from Cueva de Milodón, southernmost Chile, archived at the University of Arizona's Desert Laboratory. At Cuchillo Curá, the midden presumably formed simultaneously from the action of both the vizcacha and the extinct herbivore, with the highly concentrated urine of the viscacha imbedding the dung and other components of the midden. We sampled the in situ midden as well as several chunks that had fallen to the base of the cliff. All of the analyses were done on dung segments, vizcacha pellets, and midden matrix easily separated from the in situ midden in the field. A pooled sample of vizcacha pellets (3.6 g) and another of the herbivore dung (13 g) were sent to Geochronology Laboratories, Inc. for conventional ^{14}C dating. A third sample, a piece of the dung segment sent to the Max Planck Institute in Germany for genetic analyses, was also submitted to the Ångström Laboratory, University of Uppsala, Sweden, for TAMS dating. Plant cuticle analyses were done on vizcacha pellets, herbivore dung, and loose midden matrix. Lamentably, this particular vizcacha midden had surprisingly few identifiable plant macrofossils, which are usually the basis of rodent midden analysis. Pollen was analyzed from a single dung segment of the extinct herbivore.

Methods

Ancient DNA analyses

One of the dung segments of the presumed extinct herbivore was ground under liquid nitrogen in a freezer mill 6700 bone grinder (Spex Industries, Edison, NJ). The amount of 1.4 ml of a proteinase K buffer (0.1 M Tris-HCl, pH 7.5, 10 mM EDTA, 0.5 M LiCl, 1% LDS, 50 mM DTT, 150 μg protein-K/ml) was added to 0.2 g of powder and the sample was incubated for 24 h at 37°C under agitation. After this, 150 μl of a 0.1 M PTB solution was added (Vasan et al., 1996; Poinar et al., 1998) and the sample was incubated for another 72 h. The resulting suspension was centrifuged for 5 min at 13,000 rounds per minute (rpm) in a table centrifuge and the supernatant was transferred to a new tube. For DNA purification, the supernatant was extracted twice with chloroform/isoamylalcohol (24/1) and 400 μl of the extract was added to 1.6 ml of L6 buffer (5 M guanidinium-isothiocyanate, 0.1 M Tris, pH 8.0, 20 mM NaCl 1% Triton X-100, 20 mM EDTA; Höss and Pääbo, 1993) and 50 μl of silica solution. All subsequent steps for DNA purification were done as described in Poinar et al. (1998) and Hofreiter et al. (2000). The DNA solution obtained was used for the amplification of both animal 12S DNA and plant rbcL fragment. All extraction steps were done in a room especially dedicated for work with ancient samples, and extraction blanks as well as PCR controls were performed to monitor contamination. Polymerase chain reaction amplifications for the 12S mitochondrial DNA fragments were done using an automated hot start with AmpliTaq Gold (Perkin Elmer) to reduce secondary amplification products, using the following primers:

12S a′, 5′-CTG GGG ATT AGA TAC CCC ACT A-3′;
12S o, 5′-GTC GAT TAT AGG ACA GGT TCC TCT A-3′;
12S d, 5′-TAA AGG ACT TGG CGG TGC TTC AC-3′;
12S n, 5′-CCA TTT CAT AGG CTA CAC CTT GAC C-3′;
12S s, 5′-AAT TTC GTG CCA GCC ACC GCG GTC A-3′;
12S t, 5′-AAG CTG TTG CTA GTA GTA CTC TGG C-3′;
12S ll, 5′-GCA TAA CTA TTA CCC ATA AGT A-3′;
12S b, 5′-TGA CTG CAG AGG GTG ACG GGC GGT GTG T-3′.

For three (a′/o, ll/b, and d/n) of the four primer pairs, amplifications were done for 50 cycles, whereas for the fourth (s/t), 60 cycles were done. Cycling conditions were as described in Hofreiter et al. (2001a), with an annealing temperature of 57°C.

Amplification products were visualized on ethidium bromide-stained agarose gels and products of correct size were isolated from the gel by cutting out a small piece of agar containing the amplification product and melting the agar in 100 μl double distilled water. All products were reamplified using 5 μl of the melted agar piece, cloned in *Escherichia coli* cells and at least five clones were sequenced. Amplification and cloning were repeated and a minimum of three clones were sequenced to verify the sequences from the first amplification. A consensus sequence was derived from the two amplifications. The complete sequence was aligned by eye to the sequences of the two living tree sloths genera [two-toed sloth (*Choelopus*) and three-toed sloth (*Bradypus*)], two extinct ground sloths (*Mylodon darwinii* and *Nothrotheriops shastensis*), three species of armadillo (*Cabassous, Chaetophractus,* and *Dasypus*), and an anteater (*Tamandua*). All sequences, except the one for *N. shastensis* (Poinar et al., 1998), were from the GenBank database (Accession Numbers: *Bradypus variegatus* Z48937; *Choelopus didactylus* Z48941; *M. darwinii* Z48943). Phylogenetic analyses were done using the Puzzle program (Strimmer and von Haeseler, 1996; http://www.tree-puzzle.de/) and by the Mega package (Kumar et al., 1993). The particular model for reconstructing Puzzle trees was chosen to account for the fact that different positions in mitochondrial sequences evolve at different rates (Meyer et al., 1999). The rate heterogeneity parameter was estimated from the data set assuming gamma-distributed rates and eight rate categories using the Puzzle program. Puzzle trees were done with the TN model (Tamura and Nei, 1993) for substitution.

The TN model takes into account the different likelihoods for different types of substitutions. A neighbor-joining tree was reconstructed using the Mega program package with Kimura-2-parameter (Kimura, 1980) corrected distances, which only assume different substitution rates for transitions and transversions. This obviates the need for more complex models for substitutions in obtaining the correct topology of a tree (Nei and Kumar, 2000). Both the Puzzle trees and the bootstrapping with MEGA were done with a thousand replications to assess the reliability of the trees.

Plant DNA was amplified using the primers rbcLZ1a (forward) and rbcL19b (reverse), as described in Hofreiter et al. (2000). Three independent amplifications were done, the products were cloned, and about 30 clones were sequenced from each amplification. All clones were aligned to the reference sequence obtained from *N. shastensis* dung in Poinar et al. (1998). Identical or highly similar plant sequences were grouped into clusters by eye and for each cluster of sequences a consensus sequence was obtained. Both consensus sequences and each individual sequence were compared to the plant sequences deposited in the sequence database GenBank by the program BlastSearch (Altschul et al., 1997). In addition, all sequences were compared to 99 herbarium sequences determined earlier (Hofreiter et al., 2000). Identifications were only accepted in cases where the sequence from GenBank (or Hofreiter et al., 2000) covered all 110 bp of our amplification product and showed no more than a single difference. If the closest match in GenBank was more than one mismatch away, the sequence was considered unidentified. Identification of sequences down to the genus level is usually hampered by the fact that the sequences, due to their shortness, match a number of genera from a family. However, in some cases it was possible to assign a genus at least tentatively. For comparison to the morphological data, genera of the closest match were also noted if not more than 4 genera matched equally close to the sequence.

Plant cuticle and pollen analysis

Plant macrofossils, normally the staple of rodent midden analysis, were scarce in this particular vizcacha midden and consisted mostly of unidentifiable twig fragments. We were able to identify one seed of *Plantago* (Plantaginaceae), a few spines (cf. *Maihuenia*, Cactaceae), a *Berberis* (Berberidaceae) leaf, and an *Adesmia* (Fabaceae) spine. We analyzed plant cuticles from the extinct herbivore dung fragment, the vizcacha fecal pellets, and midden matrix from two of the middens. Samples were generally processed according to the method used by Williams (1969), modified by Latour and Pelliza Sbriller (1981), and adapted for the different kinds of material in our study. The vizcacha pellets were soaked in 70% ethyl alcohol, ground, and sieved across 1-mm-mesh screen; the screened material was clarified with NaClO, stained with 50% safranin solution, and mounted in glycerine gel on microscope slides. The extinct herbivore dung segment and midden matrix were separated into fine (F) and coarse (C) fractions. Each fraction was also treated with alternating washes in 70% alcohol, boiling water, and NaClO. The material was then colored with a 50% safarin solution and mounted in glycerine gel on a microscopic slide. In each case 3 slides were made of the fine (F) fraction, and 5 slides of the coarse (C) fraction. For each slide, 400 microscopic fields were examined at 100× magnification under a Leitz SM LUX microscope. The identification of the epidermic elements was accomplished using a key developed for plants from the area by Latour and Pelliza Sbriller (1981). Abundance of each species was estimated as percentage of the total number of fragments identified. Microphotographs of individual cuticles were taken at 100 and 200× magnification.

Pretreatment for pollen analysis followed standard techniques and consisted of soaking the dung subsample in 5% KOH, sieving, and acetolysis (Faegri and Iversen, 1975). The residue was analyzed with a Leitz ortholux microscope under 400 and 1000 power. For pollen identification the pollen reference collection at INSTAAR, University of Colorado, and relevant pollen floras were used (Markgraf and D'Antoni, 1978; Heusser, 1971).

Results and discussion

^{14}C dates

A conventional date of 13,730 ± 1070 ^{14}C yr B.P. (GX21153; δ^{13}C of −25.3‰) was obtained for the vizcacha pellets and 13,750 ± 230 yr ^{14}C B.P. (GX21149; δ^{13}C of −27.5%‰) for dung segments of the extinct herbivore. An additional AMS date of 14,665 ± 150 yr B.P. (Ua-13871; δ^{13}C of −25.14%‰) was later obtained from the dung segment of the extinct herbivore that was also analyzed for DNA at the Max-Planck Institute. The discrepancy in the two sets of dates for the herbivore dung segments could be due to either temporal averaging over 1000 years or to differences in conventional beta counting vs AMS dating procedures at the two laboratories.

Mitochondrial DNA analyses from dung segment

In total 573 bp of the mitochondrial 12S DNA could be determined using the four overlapping primer pairs. The amplification was done using four short overlapping fragments as ancient DNA is highly fragmented and amplification of long products (> 250 bp) is in most cases not possible (Handt et al., 1994). At none of the positions determined was there a discrepancy between the first and the second amplification; therefore, unambiguous consensus sequences could be reconstructed for each fragment. Consensus sequences for each amplification product were compared to the sequences deposited in GenBank by means of the program BlastSearch (Altschul et al., 1997). Three of the

amplification products (a′/o, d/n, ll/b) yielded only one consensus sequence, which was in all three cases found by BlastSearch to be closest to one of the three genera of sloth deposited in GenBank. The fourth fragment (s/t) yielded three different consensus sequences, two of which were recovered in only one of the PCR amplifications, whereas the third was recovered in both. Whereas this latter, reproducible product was most similar to sloth, the other two were most similar to goat (*Capra hircus*, mitochondrial 12S DNA, identical over the whole fragment) and human (identical over the full length of 211 bp to a part of the X-chromosome). The somewhat surprising finding that X-chromosomal sequences can be amplified using primers specific to mammalian mitochondrial sequences is readily explained by the fact that multiple copies of mitochondrial DNA sequences exist on all nuclear chromosomes (Mourier et al., 2001). These nuclear copies of mitochondrial sequences retain sufficient sequence similarity over very long evolutionary times to be amplified using PCR primers targeting mitochondrial sequences. It is, however, noteworthy that no human mitochondrial DNA was detected, although in circumstances where nuclear DNA can be amplified, mitochondrial DNA would also be expected, as it usually occurs as a higher number of copies. However, for some tissues it was shown that nuclear insertions of the mitochondrial DNA are as prevalent as the mitochondrial DNA itself (Greenwood and Pääbo, 1999). Thus, under certain circumstances such a result might be expected.

As the sloth sequence was found in both amplifications, while the other two sequences were each found in only one of the amplifications, the sloth sequence as the only reproducible product was assumed to be the correct sequence. Several other lines of evidence also support this assignment (see below). It is notable that the fragment for which several different sequences were recovered is the longest of the four amplification products that were used. Although the primer pair 12S a′/o is also a general mammal primer pair, only sloth sequences could be amplified with this primer pair. This is consistent with previous results (Handt et al., 1994) where it was found that ancient DNA is preferably amplified when very small DNA fragments are amplified, while the amplification of contaminating DNA is favored if longer DNA fragments are amplified. A reasonable explanation for this finding lies in the fact that contamination is often (although by no means always; see Hofreiter et al., 2001b) present in smaller amounts than the endogenous ancient DNA, but the latter is fragmented to shorter pieces. Thus, if short DNA fragments are amplified, the ancient DNA may outnumber the contaminants, while with increasing length of the PCR products less and less ancient DNA fragments are long enough to be amplified. The amplification of contaminating DNA, on the other hand, is much less affected by product length, as contaminating DNA fragments are usually younger and thus often longer. Therefore by using long enough amplification products it is possible to only amplify contaminating DNA, even if endogenous ancient DNA is present (Handt et al., 1994). Thus, although it is difficult to estimate an age for the human sequence, as it could have happened any time since humans appeared in South America, a recent contamination is likely (Hofreiter et al., 2001b).

In the case of the other two sequences, it is also reasonable to assign an age. The sloth DNA is late glacial in age, whereas the goat sequence must be recent, because goats were introduced to South America by Europeans and domesticated herds are now ubiquitous in the area. In cases like this, where three sequences are found, it is not always straightforward to choose the correct one, i.e., the one that represents the defecating animal. For at least four reasons the sloth sequence most likely originates from the same animal that produced the dung. First, only the sloth sequence could be reproduced, one important criterion for authentication of ancient DNA sequences (Hofreiter et al., 2001b). Second, only for the longest PCR fragment do we find three sequences, whereas for the other three fragments we only found sloth-like sequences. Third, based on morphological grounds (i.e., the size and shape of the coprolites), both humans and goats can be excluded as the defecating animals. And finally, the age of the sample certainly predates the existence of goats in Argentina, and possibly humans, although the timing of the appearance of humans in South America remains controversial. However, our data show that one must be cautious in interpreting molecular data from samples of uncertain origin. For example, had this coprolite been contaminated with modern horse DNA, instead of goat, it would have been far more difficult to identify the origin of the dung, as the last two of the above arguments would not apply given the presence of equids in South America since the Great American Biotic Interchange. Similar problems may as well arise in the analysis of modern feces (see also Taberlet et al., 1999).

The phylogeny of sloths (Suborder Phyllophaga) is still highly controversial. Neither morphological (Patterson et al., 1992) nor molecular analyses (Höss et al., 1996; Poinar et al., 1998; Greenwood et al., 2001) have currently resolved the interrelationships of the five recognized families (Scelidotheriidae, Mylodontidae, Megatheriidae, Megalonychidae, Bradypodidae) recognized in the most recently proposed classification (McKenna and Bell, 1998). Höss et al. (1996) proposed, based on 12S and 16S mitochondrial sequences, that the extinct giant ground sloth *M. darwinii* is closer to the two-toed than to the three-toed sloth. Poinar et al. (1998) found based on 12S sequences alone that the extinct Shasta ground sloth *N. shastensis* and *M. darwinii* form a monophyletic group, although this relationship was only weakly supported, and the relationship of these two extinct species to the two extant families (two-toed and three-toed) could not be resolved in the later study.

Finally, based on 12S and complete cytochrome B sequences, Greenwood et al. (2001) proposed a sister group relationship of two-toed sloth with *M. darwinii* and the three-toed sloth was more closely related to *N. shastensis.* However, the support for this interpretation is weak, and the

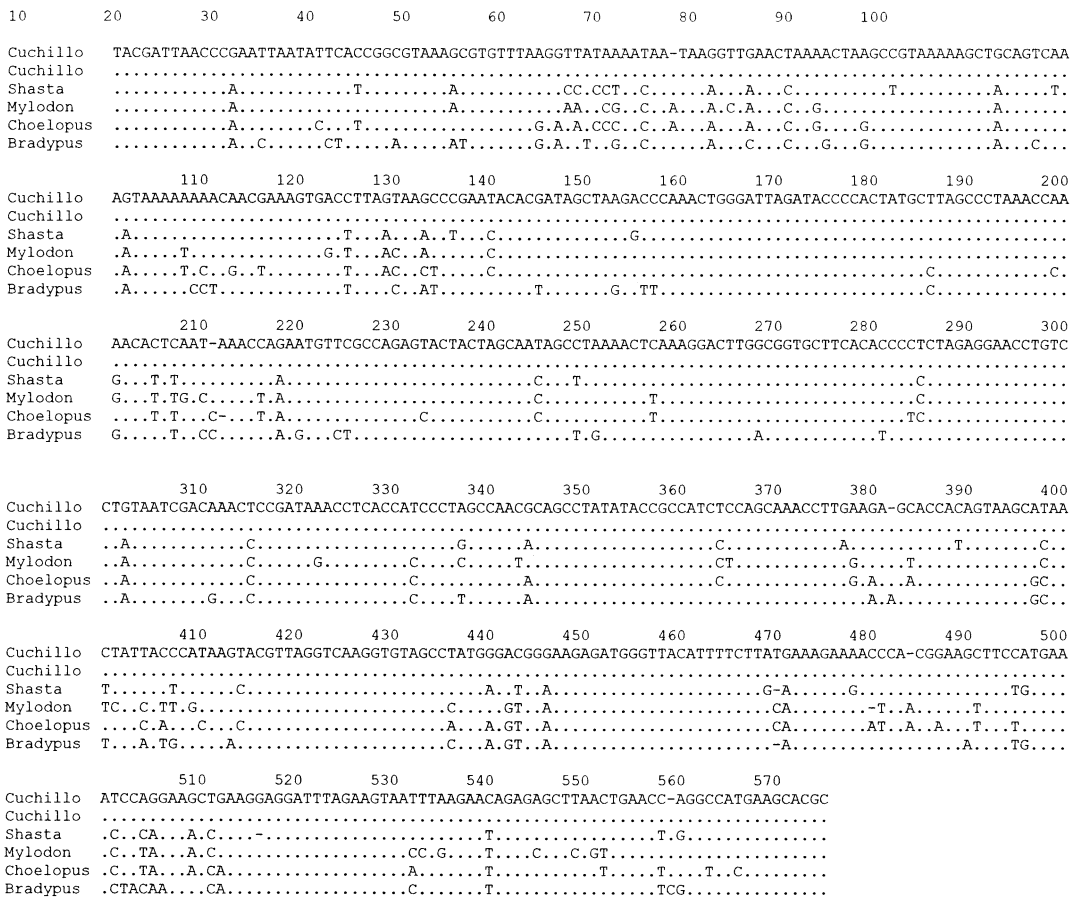

Fig. 4. Alignment of a ~575-bp fragment of the mitochondrial 12S DNA from five species of sloths. The Cuchillo Curá sequence was used as the reference. Dots indicate identity; dashes indicate gaps. Cuchillo, sequence from the sloth dung from Cuchillo Curá; Shasta, *Nothrotheriops shastensis;* Mylodon, *Mylodon darwinii* (extinct giant ground sloth); Choelopus, *Choelopus didactylus* (two-toed sloth); Bradypus, *Bradypus variegatus* (three-toed sloth).

results from the two different genes are contradictory. Overall, sloth phylogeny based on molecular data must be seen as currently unresolved, and in need of a greatly expanded database.

The 573-bp 12S DNA fragment from the Cuchillo Curá coprolite was aligned by eye with help of the proposed sequence alignment in Höss et al. (1996) (Fig. 4). All insertion/deletion sites were removed until the first constant base within the five sloth sequences was achieved, resulting in 545 bp. We used distance comparisons, neighbor-joining (Kumar et al., 1993) and maximum-likelihood (as implemented in the Puzzle program; Strimmer and von Haeseler, 1996) approaches to investigate the relationship of our sequences to the other four 12S sloth sequences published so far. The distance matrix (Table 1) yields little information. The comparatively small number of transversions between the three species of extinct sloth compared to the number of transversions between these and the extant sloth as well as between the extant sloths could indicate a closer relationship of the three extinct species; however, this is not reflected in the number of transitions. The high and similar number of transitions in all comparisons might indicate that most fast-evolving positions have changed, arguing for an early separation of all so far sequenced species. Thus, based on the distance matrix of the sloth sequences obtained so far it appears that they could have originated from a rapid radiation. This conclusion is further supported by the tree analyses. The bootstrap and support values for the branching within the sloth suborder are low (Fig. 5), and in the Puzzle analysis, the relationship within sloths is mostly unresolved (Fig. 6). Even the basal position of the living three-toed sloth, *Bradypus*, is

Table 1
Distance matrix showing the pairwise sequence differences between the five sloth species

	Las Lajas	Shasta	*Mylodon*	Two-toed	Three-toed
Las Lajas		52	61	61	67
Shasta	4		46	45	55
Mylodon	7	7		40	65
Two-toed	13	11	12		54
Three-toed	12	14	15	21	

Note. The distances were computed using the alignment shown in Fig. 3. Above diagonal, transitions; below diagonal, transversions.

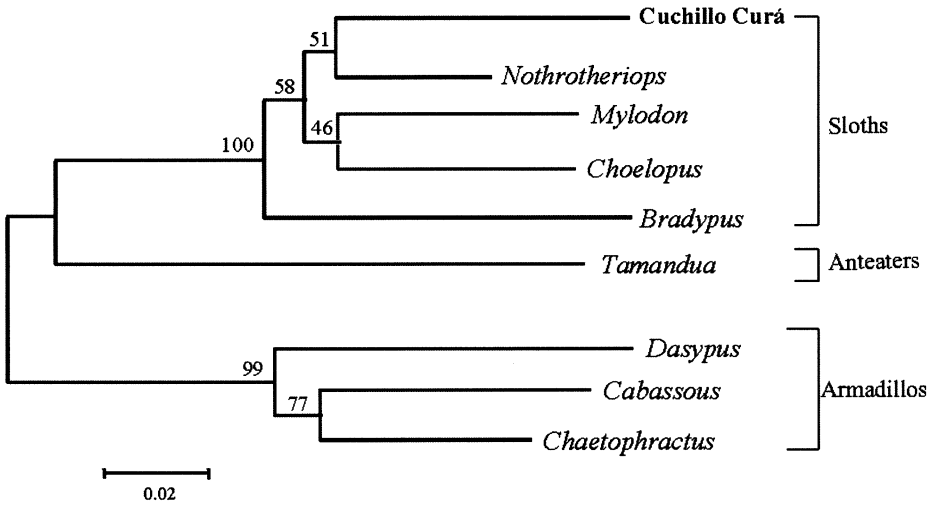

Fig. 5. Tree topology found by neighbor joining (1000 bootstrap replications). The sequence from the Cuchillo Curá coprolite is shown in bold.

only weakly supported in both analyses. Thus, it is likely that at least these five species separated very long ago, a conclusion that is also supported by the fossil record. Taken together, both the molecular and the fossil evidence indicate a rapid radiation of the suborder phyllophaga rather long ago. This situation further complicates phylogenetic reconstructions of the group. However, it should be noted that the five sloth sequences, including the new sequence from Cuchillo Curá all support the interpretation that sloths are a monophyletic group with 97 and 100% bootstrap and support values, a conclusion that is also supported morphologically by numerous derived features common to all members of the suborder.

Based on dung size alone, small nothrotheres (Infraorder Megatheria, Family Megatheriidae, Tribe Nothrotheriini; see McKenna and Bell, 1998) are the most likely candidates to have produced the Cuchillo Curá dung. There are several

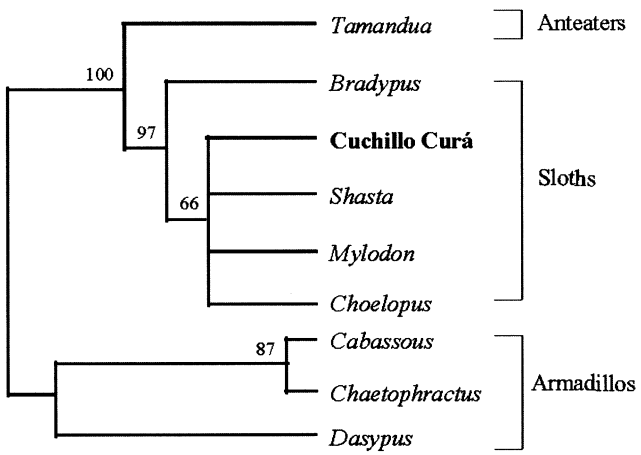

Fig. 6. Tree topology found by Puzzle (1000 replications) analysis. The sequence from the Cuchillo Curá coprolite is shown in bold.

ground sloth species from this tribe with known Pleistocene to Recent fossil records, such as *Nothrotherium maquinense* (Burmeister, 1885, 1886a, b, 1887; Cartelle and Bohorquez, 1986) and *Nothropus priscus* (Burmeister, 1882; Frailey, 1986). Although our results are inconclusive on the phylogenetic position of the Cuchillo Curá sloth, a close relationship to any of the so-far sequenced sloth species, including the single nothrothere *N. shastensis,* is unlikely. Two interpretations are possible: (1) the Tribe Nothrotheriini must represent an evolutionary group that is much older than previously thought since nothrotheres are only known from the Pliocene and Pleistocene (McDonald and Muizon, sensu stricto (2002). (2) The dung must have originated from a small sized sloth from some other tribe or even Infraorder not currently recognized in the fossil record of Argentina. Tonni et al. (1985) only included four genera and six species of ground sloth in the Lujanian (late Pleistocene) fauna of Argentina. All four genera, *Megatherium, Scelidotherium, Glossotherium,* and *Lestodon* are large taxa and would seem to have been too large to produce the dung recovered from Cuchillo Curá. Neither the smaller nothrotheres nor megalonychids were included in the faunal list of Tonni et al. (1985), although genera of both groups are known from elsewhere in South America in the late Pleistocene. The Cuchillo Curá dung may be the first record of smaller sloths in the Late Pleistocene of Argentina not yet represented by skeletal material. As such the DNA analysis of the dung provides the first indication for the presence of an extinct taxa of sloth not currently recognized based on the traditional record of skeletal remains.

Plant genetic and morphological analyses

Both genetic analysis of chloroplasts and cuticles represent plant materials that most likely were eaten by the ground sloth and vizcacha, while pollen from the dung

Table 2
Plants identified from the Cuchillo Curá coprolite using a 110-bp fagment of the chloroplast rbcL gene

Sequence	Number of clones	Amplifications	Order	Family	Genera	Mismatches	Identification
			Consensus sequences from more than 1 clone				
A	4	2	Asterales	Asteraceae	*Senecio*	0*	Senecio
B	7	2	n.m.	n.m.	n.m.	>1	n.i.
C	13	3	Sapindales	Anacardiaceae	Mangifera, Blepharocarya	1	Sapindales
D	13	3	Lamiales	various	various	0	Lamiales
E	14	3	Lamiales	Lamiaceae	*Tetraclea, Thymus, Origanum, Mentha*	0	Lamiaceae
F	2	1	Fabales	Fabaceae	various	1	Fabales
G	5	2	n.m.	n.m.	n.m.	>1	n.i.
H	3	1	Malpighiales	Euphorbiaceae	*Micrandra*	1	Malpighiales
I	4	2	Lamiales	Verbenaceae	*Junellia*	0	Junellia
J	2	2	Apiales	Apiaceae	*Steganotaenia, Daucus*	1	Apiales
K	3	2	n.m.	n.m.	n.m.	>1	n.i.
			Single clones				
L	30	2	Asterales	Asteraceae	various	0	Asteraceae
M	27	3	Apiales	Apiaceae	*Donnelsmithia*	1	Apiales

Note. The sequences were put into groups as in Hofreiter et al. (2000). n.m., no match; n.i., not identified. *Identification by comparison to Hofreiter et al. (2000). For details see main text.

material would represent primarily regional atmospheric input, either onto the dung or onto plants eaten by the animal. The very negative $\delta^{13}C$ values of −27.5, −25.14, and −25.3‰ associated with the radiocarbon dates indicate an exclusive diet of C_3 plants for both the large herbivore and the vizcacha.

The sloth dung segment yielded 13 different plant DNA sequences, which were either represented by at least three clones (11 cases) or unambiguously assigned to a certain family or order (Table 2). Ten of these sequences could be assigned to one order (Lamiales) and seven families (Apiaceae, Asteraceae, Anacardiaceae, Euphorbiaceae, Fabaceae, Lamiaceae, and Verbenaceae) with two families (Asteraceae and Apiaceae) being represented by two different sequences and one order (Lamiales) by three sequences. Two sequences were assigned to genera, i.e., one of the Asteraceae sequences to the genus *Senecio*, and the Verbenaceae sequence the genus *Junellia*. We anticipate higher taxonomic resolution with further development of the GenBank database in the future, although the short length of the amplified sequences limits the resolution with which these can be identified, because often many genera from a family are identical over such a short sequence. Due to the generally short length of ancient DNA, it is unlikely that much longer fragments will be amplified in the future. An alternative strategy might be to amplify a number of short nonoverlapping fragments and cross-compare the identifications. Although a better representation of long sequences from many plant genera in the GenBank database than currently available is crucial for this approach, preliminary results using this strategy are promising (D. Serre, personal communication).

There is considerable overlap between the chloroplast DNA determinations and the pollen flora and plant cuticle remains of the dung segments, at least at the family level (Apiaceae, Asteraceae, Fabaceae, Lamiaceae, and Anacardiacea and Apiaceae, Asteraceae, and Verbenaceae, respectively) (Tables 2 and 3).

Plant cuticles (Fig. 7) in the vizcacha pellets were dominated by four taxa: *Nassauvia* (Asteraceae, 42%), grasses (Poaceae, 32%), *Mulinum* (Apiaceae, 19%), and *Perezia* (Asteraceae, 6%) (Table 3). *Nassauvia* was not registered in either sloth dung or midden matrix. Conspicuously absent from the vizcacha pellets is *Ephedra*, which was common in the other material. The midden matrix is dominated by *Mulinum*, *Perezia*, *Ephedra*, Poaceae, and *Junellia* (Verbenaceae). The sloth dung plant cuticles were dominated by *Perezia* or *Mulinum*, depending on the fine or coarse fraction, respectively, *Ephedra*, *Berberis* (Berberidaceae), *Maihuenia* (Cactaceae), Poaceae, *Tetraglochin* (Rosaceae), and *Junellia* occurred only in the fine fraction. Based on our analyses, this particular ground sloth was a browser as was the ground sloth at Gruta del Indio [Garcia and Lagiglia, 1999; JLB has also examined this dung, which contains abundant epidermis of mesquite (*Prosopis*)], whereas the one at Cueva de Milodón was primarily a grazer (Moore, 1978; Markgraf, 1985).

Plant taxa identified by the cuticles (Fig. 6) in the sloth dung, vizcacha pellets, and midden matrix were generally comparable, although proportions were different (Table 3). Only 5 taxa were identified in the vizcacha pellets compared to 9 taxa in the sloth dung. Four plant taxa dominated the assemblages: Asteraceae and Mutiseae with *Nassauvia* (42%) in the vizcacha pellets and *Perezia* (6%) in the

Table 3
Results of pollen and plant cuticle analysis from sloth dung, vizcacha pellets, and midden matrix

	Sloth dung cuticle		Sloth dung pollen	Vizcacha pellet cuticles	Midden matrix cuticle	
	Fine	Coarse			Fine	Coarse
Anacardiaceae						
Schinus	1	1			1	4
Apiaceae			9.1			
Mulinum	10	65		19	43	8
Azorella			1.6			
Asteraceae						1
Mutisieae			6.6			
Perezia	51	10		6	16	48
Nassauvia				42		
Asteroideae			2.8			
Senecio						2
Baccharis-type			6.6			
Berberidaceae						
Berberis	4					
Cactaceae						
Maihuenia	1				1	
Caryophyllaceae			0.3			
Chenopodiaceae			7.2			
Cyperaceae			1.2			
Ephedraceae						
Ephedra	14	17	9.7		19	11
Fabaceae						
Adesmia			17.0	1		
Lathyrus-type			1.6			
Prosopis					1	
Fagaceae						
Nothofagus dombeyi-type			5.6			
Nothofagus obliqua-type			0.6			
Haloragaceae						
Myriophyllym			0.3			
Hydrophyllaceae						
Phacelia-type			0.3			
Lamiaceae			0.6			
Liliaceae			0.3			
Malvaceae			0.3			
Poaceae	4		26.0	32	13	9
Podocarpaceae						
Podocarpus			6.0			
Rhamnaceae			0.6			
Rosaceae						
Tetraglochin	2					
Verbenaceae						
Verbena	6	6			6	16
Unknown			3.1			

Pollen abundance is in percentage of total assemblage. For cuticle analyses, abundance for each genus or family identified is expressed as percentage of fragments counted in 1 slide of vizcacha pellets (152 total cuticle fragments counted), 3 slides of sloth dung (224 total fragments), 3 slides of midden matrix fine fraction (135 total fragments), and 5 slides of midden matrix coarse fraction (81 total fragments).

vizcacha pellets and (51 and 10%) in the sloth dung (fine versus coarse fraction); Apiaceae with *Mulinum* (43%) in the vizcacha pellets and (10 and 65%) in the fine versus coarse sloth dung fraction; Poaceae (32%) in the vizacha pellets and 4% in sloth dung. Whereas *Ephedra* was absent in the vizcacha pellets, it registered 14 and 17%, respectively, for the fine versus coarse fraction of the sloth dung. Additional taxa encountered in the sloth dung included *Berberis, Schinus, Maihuenia, Tetraglochin,* and *Junellia.*

Pollen analysis of the sloth dung material (Table 3) yielded, in order of dominance, grasses (Poaceae: 26%), *Adesmia*-type (17%), Asteraceae (16%): Mutisiae (6.6%), Asteroideae including *Baccharis* type (9.4%), Apiaceae (10.7%) and *Ephedra* (9.7%). The presence of southern beech (*Nothofagus* sp.: 6.2%) and *Podocarpus* (6.0%) does not imply that these trees grew near Cuchillo Cura. Pollen of both taxa are easily dispersed by wind hundreds of kilometers and could have been trapped by foliage of other plant species eaten by the ground sloth.

In addition to the dominance of grasses in the late glacial

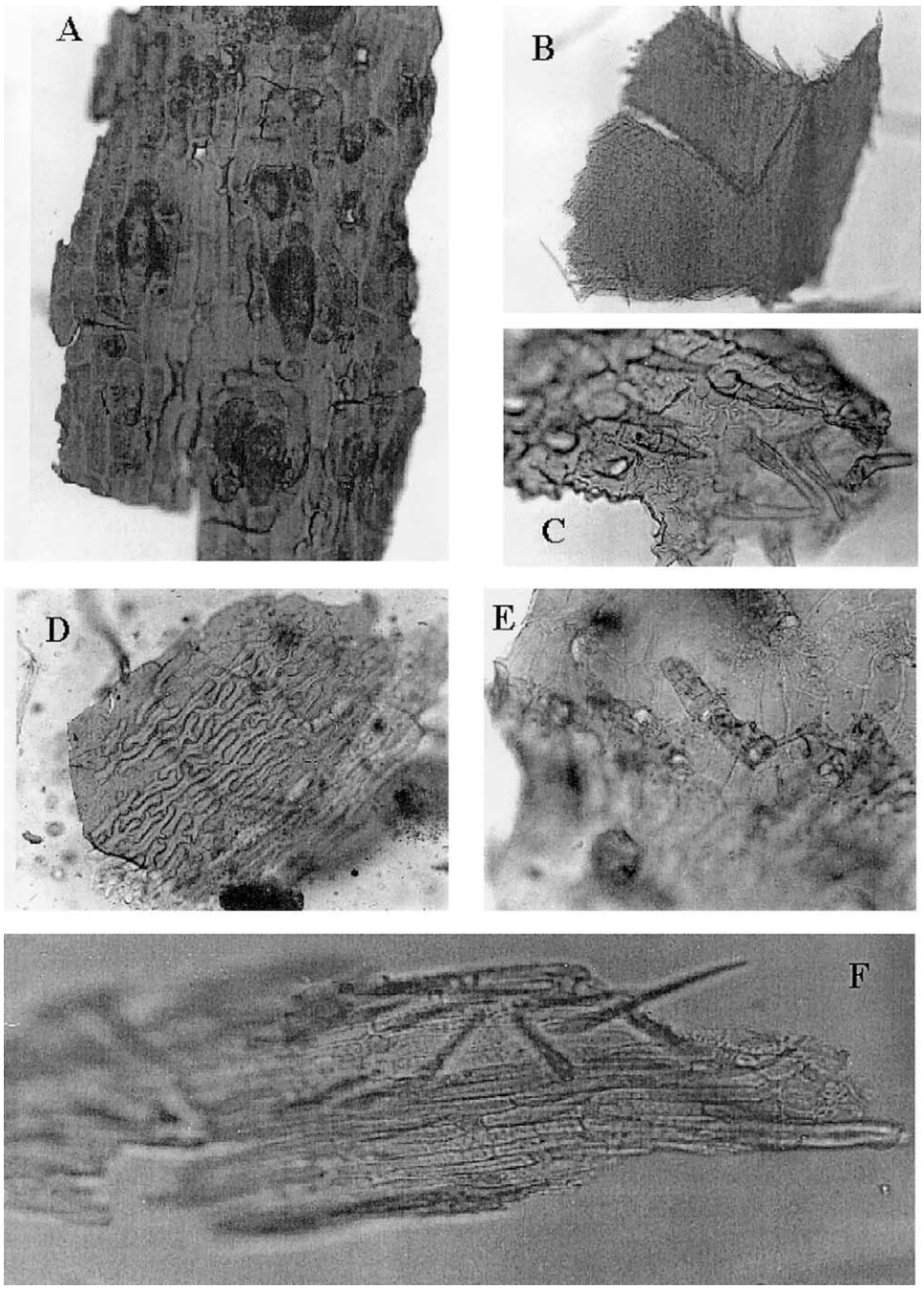

Fig. 7. Example of plant cuticles identified from the vizcacha pellets, sloth dung, and midden matrix: (A) *Ephedra* twig (100×) from midden matrix, (B) *Mulinum spinosum* fruit, (C) *Junellia* leaf, (D) *Perezia recurvata* leaf from midden matrix, (E) *Perezia recurvata* leaf with hairs from midden matrix, (F) *Nassauvia* sp. leaf (200×) from vizcacha pellets.

sample, the major difference between late glacial and modern vegetation is the absence of *Larrea*, a dominant shrub at the site today. The dominance of *Nassauvia* in the vizcacha pellets is understandable; this plant tends to be most abundant in rocky areas such as the cliff and talus community at Cuchillo Curá. *Perezia recurvata* probably grew as low evergreen mats on steep, dry sunny slopes, as it does near the site today. Overall, the pollen assemblage from Cuchillo Curá compares well with the late glacial pollen assemblages in dung and cave sediment from Gruta del Indio (D'Antoni, 1983), a site in the well-developed Monte Desert of northern Mendoza Province. More recent cuticle analyses of the sloth dung from Gruta del Indio, however, indicate the glacial presence of several Monte elements including *Prosopis flexuosa* var. *depressa*, *Capparis atamisquea*, and *Cercidium praecox* ssp. *glaucum* (Garcia and Lagiglia, 1999). This suggests minimal glacial-age displacement of vegetation not only at Cuchillo Curá but also at Gruta del Indio.

This scenario is very different than the dramatic vegetation changes recorded in either the Atacama Desert of northern Chile (Betancourt et al., 2000; Latorre et al., 2002) or in the North American deserts (Betancourt et al., 1990).

Conclusions

The molecular analysis unambiguously indicates a sloth (suborder Phyllophaga) as the animal that produced the late glacial herbivore dung at Cuchillo Curá. However, no close relative could be found among the sloth species sequenced to date, and assignment to any of the currently recognized groups within the ground sloth clade awaits further study. This is not surprising given the diversity of ground sloths present in South America during the late Quaternary. Although the dung cannot be assigned to any sloth taxon currently known from skeletal remains, once sloth skeletal remains are recovered from the area, the DNA sequence documented here will be critical to establish a link between the dung and the skeleton to establish that they represent the same taxon. It is possible that the dung originates from an already described sloth taxon for which we do not currently have a DNA sequence and the record from Cuchillo Curá is simply a range extension. It is also possible that the dung originated from a new taxon not currently represented by skeletal material. The recovery of a sloth DNA sequence from the dung that is not currently known from any other source underscores the need to expand the limited database of known DNA sequences for extinct sloths. More importantly this record demonstrates that, despite extensive studies of the extinct Pleistocene mammalian fauna in South America, there are still major gaps in our knowledge. The use of DNA analysis as a method of identification can fill some of these gaps. DNA analyses can also serve as a bridge between body and trace fossils to improve our understanding of the ecology and dietary habits of extinct species. This linkage will in turn permit a better understanding of possible causes of Pleistocene extinction. Though we cannot assign the Cuchillo Curá dung to a specific sloth at this time, our data do provide additional support to the conclusions of previous studies, both morphological and genetic, that sloths represent an extremely rapid and very early radiation within the eutherians. Furthermore, chloroplast DNA, pollen, and cuticle analyses suggest that the late glacial environment at Cuchillo Curá was similar to the present, and it is unlikely that changing diet could have caused local extirpation of ground sloths.

The late glacial plant assemblage from Cuchillo Curá suggests the absence of trees, and hence it is certain that the sloth species investigated was a ground sloth and most certainly a browser rather than a grazer. The pollen, plant cuticle, and chloroplast DNA analyses indicate that both vizcachas and sloths were feeding on plants similar to those that occur today at Cuchillo Curá, and that they were living in Patagonian scrub-steppe. The slightly different proportions of the plant taxa, showing higher amounts of grasses, suggest climatic conditions perhaps slightly cooler than the present. This interpretation was also proposed by the pollen analysis of the Gruta del Indio (Mendoza) record, which also includes analysis of sloth dung (D'Antoni, 1983). The diets of the sloths at Cuchillo Curá and Gruta del Indio are markedly different from that in the high latitude Cueva del Milodón site, where pollen analysis suggested that the animal fed primarily on grasses and sedges (Moore, 1978; Markgraf, 1985).

Although ancient DNA analyses are hardly novel (see bibliography in http://www.comic.sbg.ac.at/staff/jan/ancient/references.htm), genetic studies are just starting to tap the rich store of plant and animal remains that are mummified in caves and rock shelters of arid lands (Poinar et al., 1998; Hofreiter et al., 2000; Kuch et al., 2002). Such studies could add significantly to the roster of mammals lost to extinction in the late Quaternary, like at Cuchillo Curá. Additionally there are other potential contributions because DNA analyses of fossil material can supplement identifications based on morphology, and could ultimately call into question the set of taxonomic assumptions currently made to develop response functions and paleoclimatic inferences. For well-represented species such as the rodents that produce fossil middens, a time-lapse view of molecular diversification eventually may be obtainable that reveals the dynamics of datable range shifts and the timing of origination of phylogeographic structure observed today (e.g., Kuch et al., 2002). Such a comparative, historical effort could also furnish a set of empirical tests for the analytical methodology of population genetics.

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