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Nuclear Gene Sequences from a Late Pleistocene Sloth Coprolite

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Summary

The determination of nuclear DNA sequences from ancient remains would open many novel opportunities such as the resolution of phylogenies, the sexing of hominid and animal remains, and the characterization of genes involved in phenotypic traits. However, to date, single-copy nuclear DNA sequences from fossils have been determined only from bones and teeth of woolly mammoths preserved in the permafrost [1]. Since the best preserved ancient nucleic acids tend to stem from cold environments [2, 3], this has led to the assumption that nuclear DNA would be retrievable only from frozen remains. We have previously shown that Pleistocene coprolites stemming from the extinct Shasta sloth (*Nothrotheriops shastensis*, Megatheriidae) contain mitochondrial (mt) DNA from the animal that produced them as well as chloroplast (cp) DNA from the ingested plants [4]. Recent attempts to resolve the phylogeny of two families of extinct sloths by using strictly mitochondrial DNA has been inconclusive [5]. We have prepared DNA extracts from a ground sloth coprolite from Gypsum Cave, Nevada, and quantitated the number of mtDNA copies for three different fragment lengths by using real-time PCR. We amplified one multicopy and three single-copy nuclear gene fragments and used the concatenated sequence to resolve the phylogeny. These results show that ancient single-copy nuclear DNA can be recovered from warm, arid climates. Thus, nuclear DNA preservation is not restricted to cold climates.

Results and Discussion

We prepared six DNA extracts from the coprolite and quantitated the number of mitochondrial 16S rDNA copies for fragments of three different lengths by using a sloth-specific real-time PCR assay. The extracts contained an average of 7,653 ± 3,088 DNA copies per milligram of coprolite of a 114 bp DNA fragment, 79 ± 33 copies of a 252 bp fragment, and 0.5 ± 0.5 copies of a 522 bp fragment of the same gene. Thus, each gram of coprolite contained about 7 million mtDNA fragments of 100 bp in length, and there was roughly a 100-fold drop in the number of amplifiable mtDNA molecules for every doubling in amplification length.

To test for the presence of nuclear DNA in the extracts, we amplified a 74 bp (with primers) fragment of the multicopy nuclear 28S rRNA gene, cloned the product, and sequenced 14 clones. While 4 clones were of human origin and presumably represent a modern contamination [6], 10 of the 14 clones yielded a single consensus sequence (Figure S1 in the Supplemental Data available with this article online), which contained two and three differences to the extant three-toed (*Bradypus*) and two-toed (*Choloepus*) sloths, respectively. To determine whether single-copy nuclear DNA was amplifiable, we performed amplifications of six different lengths (54 bp, 76 bp, 87 bp, 109 bp, 132 bp, 214 bp with primers) of the 28th exon of the von Willebrand factor gene (*vWF*) by using a single forward and six reverse primers. Six of nine amplifications yielded products (one 132 bp and both 214 bp amplifications did not) that were cloned, and a total of 60 clones were sequenced. A total of 51 clones yielded a consensus sequence that contained 5 differences to both three-toed and two-toed sloths. It is interesting to note that the maximum amplifiable length of nuclear DNA (ca. 150 bp) appears to be similar to that of mtDNA, isolated from most other fossils to date [6, 7].

Previous attempts to resolve the relationship of extinct ground sloths with extant three-toed and two-toed sloths based on partial mitochondrial gene sequences have been inconclusive [4, 5, 8]. We therefore attempted to resolve the phylogeny of the Shasta ground sloth by amplifying fragments of two additional nuclear genes, a 120 bp fragment (with primers) of the cAMP-responsive element modulator gene (*CREM*) and a 94 bp product (with primers) of the phospholipase C, β 4 gene (*PLCB4*). A total of 14 *CREM* clones derived from two independent amplifications and 39 *PLCB4* clones derived from 4 amplifications each yielded consensus sequences with a total of 2 differences to the three-toed sloth and 5 to the two-toed sloth. Concatenation of all three single-copy nuclear genes resulted in a 227 bp fragment. The Shasta ground sloth sequence differed from the three-toed sloth sequence by 7 nucleotide substitutions, to the two-toed sloth by 10 substitutions, and by 27–29 substitutions to all other Xenarthrans (Table 1). A phylogenetic analysis (Figure 1) shows that the ground sloth and the three-toed sloth share a more recent common ancestor than do the ground sloth and the two-toed sloth. This contradicts some interpretations of morphological traits [9, 10] but agrees with others [11, 12], and it suggests that the arboreal lifestyle in extant sloths

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Table 1. Distance Matrix for All Edentate DNA Sequences

	[1]	[2]	[3]	[4]	[5]	[6]	[7]
[1] Ground sloth		7 (5/2)	10 (9/1)	27 (22/5)	27 (21/6)	28 (21/7)	29 (22/7)
[2] 3-toed sloth	0.031		13 (10/3)	31 (24/7)	30 (22/8)	31 (22/9)	32 (23/9)
[3] 2-toed sloth	0.045	0.058		25 (19/6)	23 (16/7)	26 (18/8)	27 (19/8)
[4] giant anteater	0.121	0.138	0.112		6 (5/1)	31 (23/8)	30 (22/8)
[5] anteater	0.121	0.134	0.103	0.027		28 (19/9)	27 (18/9)
[6] banded armadillo	0.125	0.138	0.116	0.138	0.125		1 (1/0)
[7] hairy armadillo	0.129	0.143	0.121	0.134	0.121	0.004	

Above the diagonal are observed distances (transitions/transversions); below the diagonal are corrected distances [18].

originated twice as the result of convergent evolution [9, 12].

An interesting question is whether nuclear DNA is preserved to the same relative extent as mtDNA in ancient remains. DNA sequences of all clones from one of the four *PLCB4* amplifications contained a single consistent C to T transition when compared to the sequences of the other three amplifications (Figure S3). This PCR is therefore likely to have started from a single template molecule, perhaps containing a deaminated cytosine residue [13]. This suggests that there exists about 4×10^3 amplifiable copies of an ~100 bp single-copy nuclear DNA fragment in each gram of coprolite and roughly 2,000 mtDNA fragments for every nuclear DNA fragment. Thus, the ratio of nuclear DNA to mtDNA appears to be similar to what may exist in tissues of living organisms.

In summary, the fact that single-copy nuclear DNA sequences can be retrieved from late Pleistocene coprolites preserved in dry caves in the southwestern United States suggests that the permafrost is not the only environment suitable for long-term preservation of nuclear DNA sequences. Hydrolytic degradative processes such as depurination and deamination of DNA would also be retarded at very low levels of humidity. In fact, if the estimated average temperature over time, which may be a crucial factor for DNA preservation, as well as the chronological age are taken into account in the form of "thermal age" [14], the Gypsum Cave coprolite is some four times older than the suggested "theoretical limit" for the retrieval of nucleotide sequences. A systematic search for remains that contain mtDNA fragments in yields high enough to suggest the presence of nuclear DNA may therefore open up the possibility of studying nuclear gene sequences from Pleistocene animals and humans.

Experimental Procedures

Approximately 5 g coprolite was ground under liquid nitrogen in a freezer/mill 6700 bone grinder (Spex Industries). To 1.8 ml extraction buffer (50 mM NaH_2PO_4 [pH 7.5], 50 mM NaCl, 250 mM Tris [pH 8.0], 5% Triton X-100, 50 mM DTT, 10 mM PTB), 0.1 g sample powder was added, and the sample was incubated under agitation for 24 hr at 37°C in the dark. Samples were extracted with chloroform, and nucleic acids were purified as previously described [15]. Mock extractions were performed alongside all extractions. All extractions, amplifications, and sequencing of the extant sloth (*Bradypus*) were performed after the ground sloth data had been collected. Hot-start PCR was performed by using AmpliTaq Gold (Perkin-Elmer) and 2.5 mM $MgCl_2$, following the manufacturer's instructions, except for the addition of bovine serum albumin to a final concentration of 1 mg/ml. Cycling parameters were as follows: 94°C for an initial 3 min, followed by 92°C for 15 s, 57°C (28S, *vWF*)/51°C (*CREM*)/55°C (*PLCB4*) for 1 min, and 72°C for 1 min. This was cycled 50 (28S) or 60 (*vWF*, *CREM*, *PLCB4*) times. PCR products were cloned by using the Topo TA cloning kit (Invitrogen). Colonies were sequenced on an ABI 3700 capillary sequencer following the procedures described in [16].

We used the following primers for PCR amplifications of the 28S rDNA, the *vWF* gene, the *CREM* gene, and the *PCLB4* gene: 28S L2638, 5′-CCTCAGGATAGCTGGCGCTCT-3′; 28S 2639H, 5′-TCTAA TCATTCGCTTTACCGGAT-3′; *vWF* 85F, 5′-CACCACCCCATATGT GGAG-3′; *vWF* 138R, 5′-CAGAAGAAGTCGTGCAGGG-3′; *vWF* 160R, 5′- GGCCAGGTCTAGCAGCTTG-3′; *vWF* 171R, 5′-TCCAGCA GGAAGGCCAG-3′; *vWF* 193R, 5′-CTCCGACAGCTTGTTGGA-3′; *vWF* 216R, 5′-GCCTTCAGGGCTTCAAACT-3′; *vWF* 298R, 5′-GGAG CCATCGTGGTATTCC-3′; *CREM* F2, 5′-CCAGTTTGGCTTTAGTGT TTG-3′; *CREM* R2, 5′-CATTTTTTTGCATGCTACTGAC-3′; *PLCB4* F2a, 5′-GCAGACCAGCAACAGTGGT-3′; *PLCB4* R2, 5′-CTGTGT GCAGAAGTTTGGAA-3′.

The real-time PCR set up was performed as previously described [17]; cycling conditions included a 95°C denaturing step for 10 min, followed by 50 cycles of 92°C for 15 s, 59°C for 1 min, and a 1 min ramp time to 72°C, which had a final extension of 1 min. The primers and probe were selected by using the software program PRIMER EXPRESS (PE Biosystems). The probe was synthesized with a 5′-VIC reporter dye and a 3′-TAMRA quencher dye (5′-VICAACATCAC CTCTAGCATAACAACTATTAGAGGCATAMRA-3′; PE Biosystems). The primers used were: 16S TM F1, 5′-AAATCCCGCCTGTTTAC CAA-3′; 16S TM R1, 5′-TGACCGTGCAAAGGTAGCA-3′; 16S TM R2, 5′-GACGAGAAGACCCTATGGAGC-3′; 16S TM R243, 5′-GACGA GAAGACCCTACGGAAC-3′; 16S TM R4, 5′-CGACCTCGATGTTG GATCAG-3′.

We estimated the rough number of ~100 bp single-copy nuclear DNA assuming that a consistent change in the clones results from amplification of a single DNA molecule, as has been shown pre-

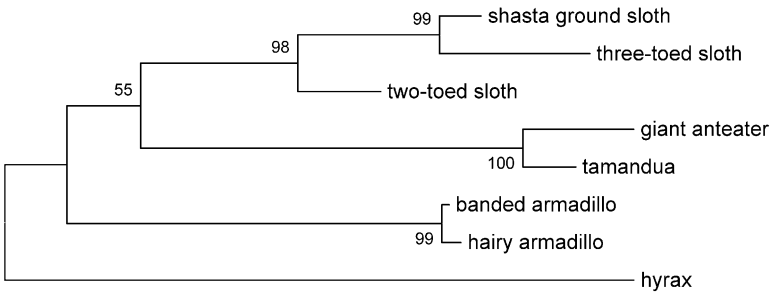

Figure 1. Phylogenetic Tree of Extant and Extinct Xenarthran

Neighbor joining tree relating the Shasta ground sloth to extant sloths, anteaters, and armadillos by using the hyrax as an outgroup.

viously [13]. As our extract was diluted 1:10 and 5 μl was used for PCR, then 200 template molecules are found in 50 mg coprolite and 4×10^3 copies of a ~100 bp single-copy nuclear DNA fragment exist in each gram of coprolite. The maximum amplicon length from our coprolite sample is roughly 130 bp from 5 μl of our DNA extract. Thus, from a 100 μl extract, prepared from 100 mg of sloth coprolite, it should be possible to obtain ~2500 bp of single-copy nuclear DNA.

We estimated a neighbor-joining tree with the program MEGA by using 1,000 bootstrap replicates and the Tamura-Nei substitution model [18]. An identical tree was produced by using the maximum likelihood method from the program PUZZLE with γ-distributed rates and an α of 2.17 estimated by the program [18]. Bootstrap values for all nodes within sloths were above 98%, with 1,000 replicates.

As was done previously, we calculated the thermal age, which is the time taken to produce a given degree of DNA degradation (based on the maximum amplifiable length of DNA in the sample) given that the average temperature was normalized to 10°C over the entire period of burial [17].

Supplemental Data

Supplemental Data including sequences of clones from all PCR products are available at http://www.current-biology.com/content/supplemental.

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Accession Numbers

Consensus sequences presented in this paper can be retrieved at GenBank under the accession numbers AY240932–0936.