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Palaeoproteomics resolves sloth relationships

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Abstract

The living tree sloths *Choloepus* and *Bradypus* are the only remaining members of Folivora, a major xenarthran radiation that occupied a wide range of habitats in many parts of the western hemisphere during the Cenozoic, including both continents and the West Indies. Ancient DNA evidence has played only a minor role in folivoran systematics, as most sloths lived in places not conducive to genomic preservation. Here we utilise collagen sequence information, both separately and in combination with published mtDNA evidence, to assess the relationships of tree sloths and their extinct relatives. Results from phylogenetic analysis of these datasets differ substantially from morphology-based concepts: *Choloepus* groups with Mylodontidae, not Megalonychidae; *Bradypus* and *Megalonyx* pair together as megatherioids, while monophyletic Antillean sloths may be sister to all other folivorans. Divergence estimates are consistent with fossil evidence for mid-Cenozoic presence of sloths in the West Indies and an early Miocene radiation in South America.

The sloths (Xenarthra, Folivora), nowadays a taxonomically narrow (6 species in 2 genera) component of the fauna of South and Central America^{1,2} were once a highly successful clade of placental mammals as measured by higher-level diversity (Fig. 1). Diverging sometime in the Palaeogene from their closest relatives, the anteaters (Vermilingua), folivorans greatly expanded their diversity and range, eventually reaching North America as well as the West Indies³⁻⁸. During the late Cenozoic sloth lineage diversity may have expanded and contracted several times⁹. Final collapse occurred in the late Quaternary (end-Pleistocene on the continents, mid-

Holocene in the West Indies), leaving only the lineages that culminated in the extant two-toed (*Choloepus*) and three-toed (*Bradypus*) tree sloths.

Radically differing from other sloth taxa in their manifold adaptations for “inverted” suspensory locomotion, tree sloths have an obscure evolutionary history¹⁰. Despite their overall similarity in body plans, tree sloths probably acquired their remarkable locomotor adaptations separately, one of many indications that the course of folivoran evolution has been marked by detailed convergences among evolutionarily distinct clades¹¹⁻¹⁹. The current consensus^{8-10,16,17} in morphology-based phylogenetic treatments is to place the three-toed sloth as sister to all other folivorans (Fig. 1, “eutardigrades”), while *Choloepus* is typically nested within the otherwise extinct family Megalonychidae, either proximate to or actually within the group that radiated in the West Indies^{3,7,11,13,16,21,22}. Although this arrangement recognizes the existence of convergence in the origins of arboreality in tree sloths, it has proven difficult to effectively test.

Sloth palaeontology is an active field of inquiry (e.g., refs 10, 17, 22-31), but the placement of a number of early Neogene clades is uncertain or disputed³² (e.g., “unallocated basal megatherioids” in Fig. 1), and the nature of their relationships with the tree sloths is accordingly indeterminate. This has an obvious impact on our ability to make macroevolutionary inferences¹⁴ (e.g., ancestral modes of locomotion) for tree sloth species, which have no known pre-Quaternary fossil record¹⁰.

Genomic evidence, now routinely used in mammalian systematic research and phylogenetic reconstruction, has so far been of limited use in evaluating these issues. Mitochondrial and at least some nuclear sequence data are available for most well-defined species of living tree sloths, but published ancient DNA (aDNA) evidence exists for only two late Pleistocene species³³⁻³⁶.

Lack of aDNA evidence is not surprising, given that the vast majority of sloth species lived in

temperate or tropical environments not conducive to aDNA preservation. Yet despite these limitations, aDNA analyses have tentatively pointed to a set of relationships between extant sloths and their extinct relatives that are very different from those implied by morphological data: the three-toed sloth is consistently recovered in association with the North American megatherioid *Nothrotheriops shastensis*^{34,38,39}, a position reflected in some older classifications^{13, 20, 21} while the two-toed sloth is firmly established as sister to the South American mylodontoid *Mylodon darwini*³⁴⁻⁴⁰. This, however, is not enough information to rigorously test, with molecular evidence, cladistic relationships established solely on morphological grounds. There is another potential source of ancient biomolecular evidence: sequence information derived from proteins⁴¹⁻⁴⁴. Because an organism's proteins are coded by its DNA, amino acid sequences in a protein are directly controlled by the gene sequences which specify them. Importantly, proteins—especially structural proteins like collagen and myosin—characteristically degrade at a slower rate than DNA⁴⁵⁻⁴⁷. Using tandem mass spectrometry coupled with high-performance liquid chromatography, it has proven possible to recover authentic collagen sequence information from mammalian fossils as old as mid-Pliocene (3.5-3.8 Ma)⁴⁸, which exceeds the current aDNA record (560–780 kyr BP) by a substantial interval^{49, 50}. Another advantage is that proteomic data can be potentially recovered from specimens from a wide range of taphonomic contexts, including ones generally inimical to aDNA preservation⁵¹. There are of course limitations. Bones and teeth are typically the only parts of vertebrate bodies that preserve as fossils, which restricts the choice of proteins to ones that occur in significant amounts in such tissues. Type 1 collagen comprises ~90% of the organic fraction of vertebrate bone⁵² and is the only bone protein⁴⁶ that is well represented in taxonomically extensive libraries such as the National Center for Biotechnology Information (NCBI). Since type 1 collagen is

coded by only 2 genes, COL 1A1 and COL 1A2, only a small fraction of a species' genome can be accessed with this probe. In the context of palaeontology, phylogenetic analyses of type 1 collagen have been shown to yield results that are highly congruent with those produced by aDNA, especially at higher taxonomic levels^{43,53}.

One such application is testing morphology-based hypotheses of higher-level relationships where there is a strong possibility that pervasive homoplasy among and between target groups has affected morphological character analysis and therefore classification, as in the case of incorrectly homologized caniniform tooth loci in living tree sloths⁵⁴. Because dental features have always played a large role in folivoran systematics,^{7, 10, 12, 13 16, 31} such fundamental reinterpretations are likely to have a significant impact. Clearly, it is desirable to use as many sources of inference as possible in reconstructing phylogeny. Also, molecular data lend themselves well to estimating divergence timing of major clades—another critical problem in folivoran systematics^{29, 34, 35}.

RESULTS

To address some of the questions raised in the previous section, as well as to add to the available molecular database for folivorans, we utilised proteomic data collected from fossil and living sloths in order to focus on three fundamental issues: (1) relationships of tree sloths to each other and to other folivorans; (2) composition of folivoran superfamilies Megatherioidea and Mylodontoidea; and (3) divergence dating of major sloth ingroups. Results were tested against datasets that additionally incorporated published genomic and phenomic information.

Samples. A total of 120 xenarthran samples comprising 24 different genus-level taxa (see Supplementary Information, Table S1 and Fig. S1) were screened for protein survival using both AAR (Amino Acid Racemization) and MALDI-ToF (Matrix-Assisted Laser Desorption/Ionization Time-of-Flight) mass spectrometry. Three additional xenarthran sequences were taken from the literature (see Methods, Proteomic Analysis). Of these, 34 or 28.3% of the total number of samples (including 31.0% of 103 folivoran samples) produced promising results for both AAR and MALDI-ToF MS. From these, the best sample per taxon was selected for LC-MS/MS (Liquid Chromatography-Tandem Mass Spectrometry) analysis to derive protein sequences, with some additions to maximize taxonomic coverage (Fig. 2, Table 1). We resampled the specimen of *Megatherium* previously utilised by ref. 44; the results presented here are *de novo*. The samples of *Neocnus dousman* and *Megalocnus zile* did not pass both MALDI-ToF and AAR screening criteria, but it was decided to analyse them because they were the best representatives of their species. However, because coverage for the *Megalocnus* sample was particularly poor, recovered sequence being mostly contaminants, it was not used in the phylogenetic analyses. To provide modern comparisons, samples of *Bradypus variegatus* (AMNH 20820) and *Choloepus hoffmanni* (AMNH 139772) were also subjected to LC-MS/MS analysis. For further details on all samples, see Supplemental Information, especially Table S1. Relevant procedures for recovering sequence information and estimating phylogenetic relationships are presented in Methods.

Samples ranged in assigned age from late Miocene to mid-Holocene (Supplementary Information Table S1), but the 19 successfully-screened samples are all Quaternary (Table 1). Of these, 15 were selected for radiocarbon dating, and 10 returned finite ^{14}C ages (Supplementary Information Table S2). The oldest specimen that yielded sequence information, *Glossotherium*

168 *robustum* MACN-PV 2652, is catalogued as Bonaerian SALMA (South American Land
169 Mammal Age, 128-400 ka⁵⁵), but this age assignment cannot be independently confirmed.

170 To keep nomenclature manageable, we make frequent reference to the relatively simple
171 traditional taxonomic scheme presented in Fig. 1, which is in turn based on a large simultaneous
172 analysis of folivoran relationships^{8, 16}. Significant departures from traditional frameworks will be
173 denoted where necessary by an asterisk, but only for formal taxonomic names (e.g.,
174 *Mylodontoidea, i.e., clade redefined to include *Choloepus*, not a traditional member).

175 **Phylogenetic reconstruction.** Parsimony and Bayesian topology searches resulted in largely
176 congruent topologies. Bootstrap Support (BS) under parsimony was generally low, as might be
177 expected given few variable sites, while Bayesian Posterior Probabilities (PP), which make full
178 use of the data, resulted in somewhat higher clade support (Fig. 3; see Supplementary
179 Information, Fig. S2). Although Antillean sloth relationships are not meaningfully resolved,
180 other folivorans assort into two reciprocally monophyletic clades (PP = 0.99) that are consistent
181 with aDNA results^{34, 35}. The first includes the three-toed sloth and various extinct taxa
182 traditionally considered megatherioid (PP = 0.97). The sister group relationship of *Megatherium*
183 and *Nothrotheriops* (PP = 0.93) is noncontroversial (Fig. 1), but in the Bayesian consensus we
184 unexpectedly recovered a previously unreported and moderately well-supported pairing of
185 *Megalonyx* with *Bradypus* (PP = 0.89) (see Discussion). The second monophyletic clade (BS =
186 73, PP = 1.00) consists of traditional mylodontoids plus *Choloepus*. Because inclusion of
187 *Choloepus* in this group markedly contrasts with results achieved using morphological datasets,
188 we designate this clade as *Mylodontoidea. Here, *Scelidotherium* + *Scelidodon* is the earliest
189 diverging branch and *Choloepus* is recovered as part of a clade (PP = 0.83) consistent with
190 accepted mylodontid interrelationships^{16, 31, 55}.

To further interrogate the reliability of our proteomic topologies, we concatenated our collagen sequences with previously published mitochondrial genome sequences (hereafter, “proteomic + genomic data”) for all extant folivorans (2 species of *Choloepus*, 4 species of *Bradypus*), two extinct folivorans (*Myiodon darwinii* and *Nothrotheriops shastensis*) and the two extant outgroup taxa^{34, 35}. Bayesian analysis (Fig. S3) of the combined dataset yielded a nearly identical topology to that recovered using proteomic data alone, but in this instance *Megatherioidea (including *Bradypus*) and *Myiodontoidea (including *Choloepus*) were unambiguously recovered as reciprocally monophyletic clades (PP = 1). Recovery of a paraphyletic *Bradypus* (with respect to *Megalonyx*) is almost certainly due to a long genomic branch and lack of proteomic data for *B. torquatus*, combined with a comparable lack of genomic data for *Megalonyx*. As the monophyly of *Bradypus* has never been questioned and this result is based exclusively on relative branch lengths, we constrained *Bradypus* monophyly for subsequent analyses, though analyses without a constraint were not noticeably different.

Molecular clock considerations and divergence time estimates. Incorporating time as an analytical component in analysis of the combined dataset yielded a well-supported and monophyletic Antillean clade (PP > 0.99), although within-clade relationships were not satisfactorily resolved. More unexpectedly in light of traditional taxonomic concepts, BEAST placed the Antillean clade as a well-supported sister to *Megatherioidea plus *Myiodontoidea (PP = 0.97) rather than pairing it with the one or the other. Support for megatherioid (PP > 0.99) and myiodontoid (PP > 0.99) monophyly remained strong, but variable for constituent sub-clades.

The relatively permissive constraints employed for calculating divergences make it difficult to draw detailed conclusions regarding the tempo of sloth diversification, although mean ages in the

combined analysis are reasonably consistent with inferences based on genomic^{34, 35} as well as morphological²⁹ data (Fig. 4; Table 2). Posterior mean node ages suggest an early Oligocene origin for folivorans, with megatherioids and mylodontoids diverging in the middle to late Oligocene (Deseadan SALMA) and the generally-recognized families originating within the middle Miocene (Colloncuran-Laventan SALMAs). The combined analysis indicates that the last time *Choloepus* and *Bradypus* shared a common ancestor was ~ 26.9 Ma (95% HPD interval, 17.2 - 34.4), which is notably earlier than the estimate ~ 22.36 Ma (95% HPD interval, 16.87 - 28.64 Ma; Figs. S5, S6) based on proteomic evidence only and more in line with some recent morphological assessments (e.g., ref. 29).

DISCUSSION

In most respects, our higher-level results for Folivora are consistent with recently-published morphology-only phylogenies, but the few ways in which they differ are critical because they have profound implications for macroevolutionary and biogeographical inference. Harmonizing morphological and molecular datasets is complicated, as the molecular results imply that traditional clades exhibit a massive amount of unrecognized homoplasy—or equally unrecognized plesiomorphies, incorrectly interpreted as (syn)apomorphies. Molecular analyses are of course subject to the same challenges, especially in contexts like the present in which samples sizes and information content are limited. It is already widely appreciated that genomic information is exceptionally useful for testing phylogenetic hypotheses; so is proteomic information, especially when it can be shown to be highly congruent with genetic indicators of relationship⁵³. Together, as illustrated here, they provide a strong basis for formulating evolutionary hypotheses:

***Choloepus* is a mylodontoid.** That the two-toed sloth may be closer to traditional mylodontids than to megalonychids, a possibility occasionally raised in morphological studies^{16, 24, 57}, has been consistently found in recent aDNA investigations^{34, 35, 37-40}. Due to the limited number of extinct taxa included in those investigations the exact nature of their relationship has remained indeterminate. However, the multiple tests of phylogenetic relationships and broad taxonomic sampling used in the present study substantiates the conclusion that *Choloepus* is indeed a mylodontoid.

Given the recent ages of all of the taxa investigated, coupled with low rates of sequence evolution, it is unsurprising that divergence estimates based on proteins alone suggest an early/middle Miocene origin for Scelidotheriidae + Mylodontidae (including *Choloepus*). Inclusion of genomic data helps to push these estimates back to the earliest Miocene, but it should be noted that a number of mylodontoid sloths of late Oligocene to late Miocene/early Pliocene age do not fit neatly into better-defined clades. In the past, these taxa were occasionally gathered^{9, 20, 21} into the probably nonmonophyletic grouping Orophodontidae. It would be interesting to know on the basis of molecular evidence whether the inclusion of a putative orophodontid would affect the placement of *Choloepus*, possibly moving it stemward (Fig. S7) or help refine divergence time estimates at the base of *Mylodontoidea. At present there is no evidence on point; however, the youngest of these ambiguously-placed taxa, *Octodontobradys*, is late Miocene/early Pliocene in age⁵⁸—young enough to stand a chance of coming within the range of proteomic methods as these continue to improve.

Megalocnid sloths are monophyletic, and are not part of traditional Megalonychidae.

Antillean sloths have had a complex taxonomic history⁷. In the past, this geographical grouping of folivorans was sometimes regarded as diphyletic, with different island taxa having diverged

from different mainland antecedents^{3, 7, 20, 58}. Diphyley now seems unlikely on the basis of our molecular clock results (Fig. 4; see also Supplemental Information, Figs. S5, S6) as well as recent morphology-based studies^{16, 25}. Although within-clade relationships are poorly resolved (cf. paraphyletic *Neocnus*), the Antillean clade as a whole resolves as strongly monophyletic (PP >0.99). In light of this fact, as well as clade antiquity, it is appropriate to remove Megalocninae from traditional Megalonychidae and raise it to family level (*Megalocnidae).

***Megalonyx* and *Bradypus* are megatherioids.** Although recent morphology-oriented cladistic studies have usually recovered *Bradypus* as sister to all other folivorans^{8-10, 16}, genomic approaches^{34, 35, 39} have consistently paired the three-toed sloth with the extinct North American Pleistocene megatherioid *Nothrotheriops*. On this point the proteomic data presented here are fully compliant with the genomic evidence and support rejection of the inference^{9, 16} that Bradypodoidea (i.e., *Bradypus*) is sister to traditional Megatherioidea + Mylodontoidea, as tested by both parsimony (13 additional steps) and Bayesian inference (2*lnBayes Factor = 6.72, support = Strong). Equally controversial is the sister group relationship detected between *Bradypus* + *Megalonyx* (PP = 0.89 - 0.98; Fig. 4; see Supplementary Information, Fig. S3). Although well supported in analyses of both collagen-only and combined proteomic + genomic data, this remains a surprising finding, inasmuch as such an association has never been reported in any taxon-rich phylogenetic study emphasizing morphology. While both the three-toed sloth and *Megalonyx* are likely to be megatherioids cladistically, settling their deeper relationships will require substantially more data than is currently available.

That none of the Antillean sloths used in this study showed any proteomic affinity for *Megalonyx* is also surprising, because much of what has been understood to morphologically characterize non-South American Megalonychidae was based on Antillean species, the fossils of which tend

to be far more complete than those of most other taxa conventionally included in this family^{12, 16,}
¹⁷. To resolve this conflict, additional high-quality data will be required, genomic and proteomic
as well as phenomic. The only certainty at present is that, if *Choloepus* is excluded,
Megalonychidae must now be relegated to the list of formerly diverse but now completely
extinct folivoran families.

The West Indies may have been colonized early. An early appearance of megalocnid sloths in
the West Indies has been proposed on general palaeobiogeographical grounds^{3, 10, 17, 24, 60}, but at
present the only pre-Quaternary fossil evidence for Antillean folivorans consists of a
morphologically inconclusive partial femur from the early Oligocene (~31 Ma) Yauco Formation
of Puerto Rico⁶¹ and unassociated remains attributable to a folivoran, *Imagocnus zazae*, from the
late early Miocene (~17.5 Ma) Lagunitas Formation of Cuba⁵. Although “megalonychid”
affinities have been assumed for both on biogeographical grounds, now no longer applicable,
neither has been included in formal phylogenetic analyses and their placement within Folivora
remains uncertain.

The presence of sloths in the West Indies at least as early as the early Miocene is congruent with
our mean age estimate (31.2 Ma; Fig. 4, Table 2) for the last common ancestor of sloths sampled
in this study. This inference is also roughly consistent with the GAARlandia dispersal
hypothesis^{5, 62}, which holds that northwestern South America and the Greater Antilles were
briefly in land connection during the Eocene-Oligocene transition. Without going beyond the
very slim body of molecular evidence currently available, there is now at least some basis for
hypothesizing that *Megalocnidae might represent an *in situ* Antillean radiation that was
emplaced on the islands during the earliest phases of the evolution of the folivoran crown-
group—much earlier than previously thought and inconsistent with the hypothesis of a

306 Patagonian origin for Folivora as a whole⁹. If it proves possible to acquire genomic information
307 from Greater Antillean sloth taxa known to have survived into the mid-Holocene⁶³, we may
308 expect more light to be shed on megalocnid origins.

309 Systematic repositioning of *Bradypus*, *Choloepus* and megalocnid sloths also permits a better
310 understanding of how often “extreme” arboreality arose during folivoran evolution. The living
311 tree sloths are uniquely defined among extant vertebrates by a combination of relatively rigid
312 hooklike hands and feet, marked limb mobility, extremely long arms, and powerful flexion
313 capabilities in proximal limb joints¹⁹. None of the West Indian sloths possessed all of these
314 osteological traits, but, importantly, some came close—notably the Puerco Rican species
315 *Acratocnus odontrigonus*, which may have been technically capable of hand- and foot-
316 suspension but probably did not perform the “upside-down” form of locomotion characteristic of
317 extant sloths^{7,14}. Remains assigned to the early Miocene Patagonian sloth *Eucholoeops*, possibly
318 part of a clade ancestral to the Antillean radiation, also display many features consistent with
319 highly-developed arboreality^{14, 18}. Our phylogenetic results suggest that evolutionary
320 experiments connected with life in the trees probably occurred multiple times, and early on, in
321 folivore evolution. If so, it is puzzling that small-bodied sloths with highly mobile limbs and
322 other arboreal adaptations are as yet unknown for the interval between the early Miocene (e.g.,
323 *Eucholoeops*) and the Quaternary (e.g., *Diabolotherium*)¹⁸. It is possible that their absence is
324 only apparent, if they lived in heavily forested tropical environments that do not favour
325 fossilization (e.g., mid-Cenozoic proto-Amaozonia^{64, 65}).

326 The advent of molecular resources providing novel information on both extinct and extant
327 species offers new ways of testing hypotheses about relationships that, in the past, were by
328 necessity based on morphological data alone. Thanks to ongoing improvements in

instrumentation and applicable software, the future for palaeoproteomics should be bright if it can continue to make significant contributions to solving difficult questions like the ones explored here.

A new aDNA study⁸⁷ of folivoran phylogeny, published as this paper was going to press, reaches conclusions almost identical to ours regarding the evolutionary relationships of living tree sloths and the phylogenetic distinctiveness of the West Indian radiation. Because the taxonomic distribution of sampled species is not identical in the two studies, there are some minor differences in lower-level relationships and estimated divergence times. However, their detailed agreement overall supports the argument that high-quality protein sequence information is a reliable source of evidence for reconstructing phylogenetic relationships.

METHODS

Proteomic Analyses

The 5-number codes following taxon names in this section refer to lab sample ID numbers referenced in Table 1.

AAR. Samples were prepared using a slightly modified version of the protocol in ref. 66. A small sub-sample of bone (~1 mg) was hydrolysed in 7M HCL (100 µl per mg) under N₂ for 18 hours at 110°C. After hydrolysis, the samples were dried down overnight before being re-hydrated in 0.01mM L-homo-arginine as an internal standard. The samples were analysed using reversed phase high pressure liquid chromatography (RP-HPLC) following a slightly modified

version of the protocol developed by ref. 67. Amino acid composition and extent of racemization was used to assess promising samples for sequencing.

Sample preparation for MS. The majority of samples (see Supplementary Information, Table S1) were prepared using a slightly modified version of the ZooMS protocol for bone reported by ref. 43. Bone samples (15-30 mg) were demineralized in 250 µl 0.6M HCl for a minimum of 3 weeks at -20°C. This allowed for a gentler demineralization and helped to protect any remaining collagen. After demineralization, the samples were rinsed once in 200 µl 0.01M NaOH, and three times in 200 µl 50mM ammonium bicarbonate (Ambic). The samples were gelatinized by being resuspended in 100 µl 50mM Ambic and heated at 65°C for 1 hour before being digested overnight at 37°C; 50 µl of the heated sample was digested using 1 µl of 0.5 µg/µl porcine trypsin in trypsin resuspension buffer (Promega, UK) and the other 50 µl was dried down and resuspended in 50 µl 100mM Tris solution to be digested with elastase (Worthington; USA) at the same concentration in 10% Tris solution. Two different enzymes were used to increase the protein sequence coverage for LC-MS/MS^{43, 68}. Digestion was stopped by the addition of trifluoroacetic acid (TFA) at a concentration of 0.5-1% of the total solution. Peptides were desalted using zip-tips⁶⁴ and eluted in 100 µl of 50% acetonitrile (ACN)/0.1% TFA (v/v).

SDS-PAGE. Selected samples were analysed using SDS PAGE (Table 1). This method was used on certain samples as the standard ZooMS protocol had not yielded positive results on certain samples that were deemed potentially important phylogenetically. Bone samples were crushed to ~1 µm sized particles using a Retsch PM100 ball mill cooled with liquid nitrogen. The ball mill was cleaned with distilled water and methanol before and after each sample⁶⁹. Nanoscale crushing allowed for the highest potential retrieval of proteomic information. 50 mg of powdered sample was heated at 70°C for 10 minutes in 200 µl SDS solubilizing buffer (0.5M Tris base, 5%

373 SDS, 130mM DTT), cysteines were alkylated by the addition of 6 μ l 1M IAA at room
374 temperature in the dark for 30 minutes before the addition of 200 μ l of dye solution (0.05%
375 bromophenol blue, 5% glycerol). 20 μ l of the samples were run on a Bis-Tris gel (NuPAGE) for
376 10 minutes to concentrate the samples into a gel plug which was briefly washed in a fixing
377 solution (16% methanol, 10% acetic acid), before being washed twice in boiling water. The gel
378 was stained using Coomassie stain.

379 The gel plug was cut into approximately 1mm sized cubes in a fume hood with a scalpel and the
380 gel cubes for each sample placed in a separate Eppendorf. The gel pieces were washed in a de-
381 staining solution (66% ammonium bicarbonate 33% acetonitrile) until no more dye could be seen
382 before being washed in the following solvents for 10 minutes per solvent; ACN, HPLC grade
383 water, ACN and 50mM ammonium bicarbonate⁷⁰. The samples were digested overnight with 100
384 μ l 3.125 μ g/ μ l trypsin in 50mM ammonium bicarbonate at 37°C and then the tryptic digest was
385 pipetted into a cleaned Eppendorf tube. 100 μ l of 70%ACN/1.7% formic acid/0.1% TFA was
386 added to the gel pieces and the gel was heated at 37°C for 1 hour with the supernatant being
387 collected and added to the tryptic digest. This step was repeated sequentially with 100mM
388 triethyl ammonium bicarbonate (TEAB) and ACN. The extracted peptides were dried down and
389 then resuspended in 5% Formic acid/0.1% TFA desalted and purified on C18 membranes
390 (Empore) before being eluted in 80% ACN/0.5% acetic acid. The purified peptides were spun to
391 dryness ready for LC-MS/MS analysis.

392 **MALDI-ToF MS.** 1 μ l of sample was spotted in triplicate onto an MTP384 Bruker ground steel
393 MALDI target plate. 1 μ l of α -cyano-4-hydroxycinnamic acid matrix solution (1% in 50%
394 Acetonitrile/0.1% Trifluoroacetic acid (v/v/v)) was added to each sample spot and mixed with

the sample⁴³. All samples were analysed on a Bruker Ultraflex MALDI-ToF mass spectrometer in triplicate.

LC-MS/MS. Most samples were analysed at the Discovery Proteomic Facility (DPF) at Oxford (Table 1). *Choloepus* 17009 and *Myiodon* 16222 were analysed at the Novo Nordisk Foundation Centre for Protein Research (NNFCPR), University of Copenhagen. The *Megalonyx* sample (ID 16849) was run at the Laboratory of Mass Spectrometry and Gaseous Ion Chemistry, Rockefeller University.

At DPF, sample batches were analysed on an Orbitrap Fusion Lumos or Q-Exactive with identical front-end separation, employing an Easyspray column (ES803, 500mmx75µm, Thermo) and a gradient of 2%-35% ACN in 0.1% FA/5%DMSO over 60 minutes. On the Fusion Lumos, MS1 resolution was set to 120,000 with an AGC target of 400,000. MS2 spectra were acquired in TopSpeed mode (3 seconds duty cycle) in the linear ion trap (rapid scan mode) for up to 250ms, with an AGC target of 4,000 and fragmentation in CID mode (35% normalized collision energy). The MS1 resolution on the Q-Exactive was set to 70,000 with an AGC target of 3E6. MS2 spectra for up to 15 precursors were acquired with a resolution of 17,500 and an AGC target of 1E5 for up to 128ms and 28% normalized collision energy (higher-energy collision dissociation). On both instruments, precursors were excluded for 27 seconds from re-selection.

At NNFCPR, dried peptides were resuspended in 50µl of 80% ACN and 0.1% formic acid before being transferred to a 96 well plate and placed in a vacuum centrifuge at 40°C until approximately 3 µL of solution was left. The samples were rehydrated with 5 or 10 µL (*Myiodon* 16222 and *Choloepus* 17009 respectively) of 0.1% TFA, 5% ACN. Samples were separated on a

417 15 cm column (75 μ m inner diameter) in-house laser pulled and packed with 1.9 μ m C18 beads
418 (Dr. Maisch, Germany) on an EASY-nLC 1000 (Proxeon, Odense, Denmark) connected to a Q-
419 Exactive HF (Thermo Scientific, Bremen, Germany) on a 77 min gradient. 5 μ l of sample was
420 injected. Buffer A was milliQ water. The peptides were separated with increasing buffer B (80%
421 ACN and 0.1% formic acid), going from 5% to 80% over an 80 minute gradient and a flow rate
422 of 250 nL/min. In addition, a wash-blank injecting 2 μ l 0.1% TFA, 5% ACN was run in-between
423 each sample to hinder cross-contamination.

424 The Q-Exactive HF was operated in data dependent top 10 mode. Full scan mass spectra (350-
425 1400 m/z) were recorded at a resolution of 120,000 at m/z 200 with a target value of 3e6 and a
426 maximum injection time of 25 ms for *Choloepus* 17009 and 45ms for *Myiodon* 16222. Fragment
427 ions were recorded with a maximum ion injection time set to 108 ms and a target value set to 2e5
428 and recorded at a resolution of 60,000 for *Choloepus* 17009 and 30,000 for *Myiodon* 16222.
429 Normalized collision energy was set at 28% and the isolation window was 1.2 m/z with the
430 dynamic exclusion set to 20 s.

431 At Rockefeller University, peptides were resuspended in 20 μ L 5% methanol, 0.2% formic acid.
432 10 μ L were loaded onto an EASY-Spray column (Thermo Fisher Scientific ES800: 15 cm $\times$ 75
433 μ m ID, PepMap C18, 3 μ m) via an EASY-nLC 1200 and separated over a 120 minute gradient of
434 2-32% Solvent B (Solvent A = 0.1% formic acid in water, Solvent B = 0.1% formic acid, 95%
435 acetonitrile) during online ESI-MS and MS/MS analyses with a Q Exactive Plus mass
436 spectrometer (Thermo Fisher Scientific). MS/MS analyses of the top 25 precursors in each full
437 scan (300 to 1700 m/z) used the following parameters: resolution: 17,500 (at 200 Th); AGC
438 target: 2×10^5 ; maximum injection time: 200ms; isolation width: 2.0 m/z; normalized collision
439 energy: 24%.

Protein sequence analysis. The LC-MS/MS raw files were converted to MGF files using Proteowizard⁷¹ and searched against a mammal collagen database which included common contaminants (<http://www.thegpm.org/crap/>) in PEAKS v7.5. Mass tolerances were set at 0.5Da for the fragment ions and 10ppm for precursor ions and up to 3 missed cleavages were permitted. Searches allowed various post translational modifications (PTMs) including oxidation (MHW) and hydroxylation of proline (both +15.99), deamidation (NQ; +0.98) and pyro-glu from E (-18.01) as well as a fixed PTM of carbamidomethylation (+57.02) which occurs as part of the sample preparation. A maximum of 3 PTMs were allowed per peptide. Protein tolerances were set at 0.5% false discovery rate (FDR), >50% average local confidence (ALC; *de novo* only) and -10lgP score ≥ 20 .

Sequences of both COL 1A1 and COL 1A2 were concatenated using previously published mammal collagen consensus sequences taken from NCBI, including sequences for the xenarthrans *Dasypus novemcinctus* (nine-banded armadillo; GenBank: XP_004470764), *Cyclopes didactylus* (silky anteater; Uniprot: COHJP1/COHJP2), and *Lestodon armatus* (extinct mylodontoid sloth, ref. 44). Telopeptides very rarely survive in fossil samples and so these were removed from all sequences. Isoleucine and leucine cannot be differentiated using low energy tandem mass spectrometry and *de novo* sequencing as both amino acids are isobaric. Therefore, the identification of leucine/isoleucine was consistent throughout the sequence analyses concatenated in this study. Our approach is in line with previous phylogenetic studies using collagen as probe⁴³, under the assumption that MS/MS sequence variation was not interpreted as significant phylogenetic change (see below, Phylogenetic Analyses).

Once a potential collagen sequence was compiled for a given sloth taxon, the sequence was added to the collagen database and the sample was re-run through PEAKS to check for coverage

and sequence substitutions. Any differences noted in either the consensus sequences or between different species of sloths were inspected manually. In order for a difference to be considered authentic, it had to occur in more than 1 product ion spectrum and be covered by both b and y ions. For additional discussion, see Supplemental Information and Table S4.

Phylogenetic Analyses

Sequences developed from the MS/MS analyses were aligned in Geneious v. 9.1.7⁷² using the MUSCLE algorithm⁷³ with default settings and then checked by eye. Mitochondrial sequence data for extant folivorans and *Mylodon darwini* were obtained from ref. 35 and supplemented with protein coding sequences for *Nothrotheriops shastensis* from ref. 34. Because the order of genes differs between these two alignments, we extracted and aligned genes for *Nothrotheriops* individually using MUSCLE in Geneious, checking each by eye to ensure accuracy. Of the 2096 amino acids in our alignment of the type 1 collagen molecule, 134 (6.4%) were variable and 76 (56 % of variable sites, 3.6% of total) were parsimony informative for the taxa represented.

We conducted three sets of phylogenetic analyses on the resulting protein alignment (see Results). We first performed a Strict Parsimony (SP) analysis using PAUP v. 4.0a (build 157)⁷⁴. We employed a branch and bound search with all sites treated as unordered and equally weighted. To assess clade support, we performed 10,000 bootstrap replicates using full heuristic tree searches and generated a weighted 50% majority rule (MR) consensus tree from the resulting sample of most-parsimonious bootstrapped trees.

We performed two forms of model-based phylogenetic analyses, both in a Bayesian framework. We used PartitionFinder v. 2.1.1^{75, 76} to determine the most appropriate model(s) of amino-acid

substitution and partitioning scheme for our concatenated alignment, resulting in selection of separate Dayhoff models⁷⁷ with gamma-distributed rates for COL 1A1 and COL 1A2. The first set of Bayesian phylogenetic analyses used MrBayes v 3.2.5⁷⁸. We performed two Markov Chain Monte Carlo (MCMC) runs, each of four chains (one cold, three heated), for 10,000,000 generations, sampling from the chain every 5000 generations. After checking for convergence of the two chains based on Gelman-Rubin statistics and ensuring that effective sample sizes for all parameters were sufficient (> 200), we discarded the first 50% of each chain as burn-in, combined the remaining posterior samples and summarized them as a 50% majority rule consensus tree, with clade frequencies interpreted as posterior probabilities for a given clade. To determine whether our unconstrained topology provided a better explanation of the data than a previously proposed morphological topology¹⁶ in which *Bradypus* is the sister lineage to all other folivorans and *Choloepus*, Megalocnidae, and *Megalonyx* form a monophyletic Megalonychidae (including other taxa not referenced here), we estimated the marginal likelihood of the data on unconstrained and constrained topologies using the stepping stone algorithm in MrBayes. We performed two runs, each with four chains (three heated, one cold) for 10,000,000 generations over 50 steps, with default settings for the Alpha parameter of the Beta distribution (0.4) and burn-in (-1). We calculated $2 * \ln(\ln Lk_{unconstrained} - \ln Lk_{constrained})$ from the resulting estimates and assessed support using the scale in ref. 79.

The fact that we cannot differentiate between isoleucine and leucine using low energy tandem mass spectrometry creates a unique problem for model-based phylogenetic inference procedures. The standard approach in ancient protein studies⁴³ is to designate all sites with a molecular mass of 131.17 g/mol as leucine, but this has the potential to bias estimates of the instantaneous rate matrix, branch lengths and, possibly, topology by entirely excluding one amino acid. We

investigated this by replacing all peptides coded as leucine with ambiguous codings {IL} and repeating Bayesian estimation of topology and branch lengths using MrBayes. The resulting 50% majority rule consensus tree was identical across coding schemes, and comparison of branch length estimates among analyses show no significant deviation from 1:1 ($\text{branch length}_{[\text{Leucine}]} = -0.00009 + \text{branch length}_{[\text{ambiguous}]} * 0.96$, $R^2 = 0.995$, $p < 0.001$), indicating that the use of leucine is appropriate. We repeated Bayesian analyses of the combined proteomic + genomic dataset using the same settings but with partitioning schemes and substitution models for genetic data following ref. 35.

We attempted to integrate our combined molecular dataset with a large, recently-published morphological dataset (ref. 9). The resulting majority rule consensus tree (Fig S7) is congruent in some respects with our molecular topologies (e.g., *Choloepus* was recovered as a mylodontoid and *Bradypus* as a megatherioid) but other results repeatedly found in molecular analyses were not obtained. In particular, we recovered a strong (PP = 1.0) traditional Megalonychidae nested within Megatherioidea that included Antillean sloths minus *Choloepus*. Although the Antillean species were represented in the total dataset by proteomic sequences, genomic data were unavailable. This result suggests that the large number of morphological characters, some known to be highly homoplastic⁵⁴, were able to swamp the signal arising from the smaller proteomic dataset. While combined analysis of morphological and molecular data will ultimately be necessary to fully resolve folivoran phylogeny, this exercise suggests that it is premature to consider such simultaneous analyses reliable at this point in time.

Our MrBayes analyses sample tree topologies with branch lengths in units of substitutions per site and so ignore temporal information inherent in phylogenetic analysis of non-contemporaneous tips or external information about relative branch lengths that can be provided

by the fossil record. We therefore also performed a series of Bayesian tree searches assuming a molecular clock under the fossilized birth-death framework⁸⁰⁻⁸², as implemented in BEAST v2.5.1⁸³. Briefly, this framework allowed us to sample from the posterior distribution of time-scaled trees for taxa in our proteomic dataset, inferred using their sequences and stratigraphic ages, while using phylogenetically constrained fossil taxa that lack amino acid data to provide additional information on relative branch lengths and divergence times. Our choice of fossil taxa and topological constraints broadly followed the approach undertaken in ref. 34 for sloth mitogenomes. However, our proteomic topologies raise questions about the phylogenetic positioning of some fossil folivorans that have previously been considered on morphological grounds as early representatives of Pleistocene and Holocene families. For example, some extinct folivorans, such as the Huayquerian nothrotheriid *Mionothropus*⁸⁴ can be plausibly assigned to a specific terminal branch in our proteomic topology. Others, however, are customarily assigned to clades that we failed to recover. This applies to the Santacrucian taxon *Eucholoeops*, usually interpreted as a basal megalonychid^{24, 85, 86} and therefore as a member of a clade not found to be monophyletic in our analyses. Such issues inevitably affect efforts to calibrate the proteomic + genomic data clock and to infer divergence times. Acknowledging this, we employed a minimal set of constraints (see Supplemental information, Fig. S4) on the positioning of fossil folivorans in our Bayesian estimation of topology and divergence times, integrating over all possible placements of phylogenetically uncertain fossils using stratigraphic context alone when necessary. We performed analyses with and without a monophyly constrain on *Bradypus* and results did not differ at unaffected nodes.

The use of a Bayesian approach requires the specification of prior probabilities on model parameters. We used default priors on substitution model parameters but specified the following:

net diversification $\sim \text{Exp}(1)$, yielding a broad, vague prior; turnover $\sim \text{beta}(2,1)$, yielding high prior weight on extinction $\cong$ speciation; sampling probability $\sim \text{beta}(2,2)$ yielding a humped distribution that placed most prior weight on sampling probabilities of 0.5; origin $\sim \text{U}(61.5, 150)$ yielding a flat prior on ages older than 61.5 Ma up to 150 Ma. In addition, the analysis was conditioned on the number of extant taxa sampled ($\rho = 0.129$ in the xenarthran proteomic analyses, $\rho = 0.333$ in the folivoran proteomic analyses, $\rho = 0.266$ in the combined analyses). Based on comparisons of marginal likelihoods computed via Path Sampling (see Supplementary Information, Table S3), we employed a relaxed uncorrelated clock with log-normally distributed rates for proteomic and combined analyses, with an exponential prior (mean=0.1) placed on the mean of log-normal distribution and the default gamma $\Gamma(0.5396, 0.3819)$ on the standard deviation. Two MCMC analysis were run for 10 million generations each, sampling every 1000 generations, after which fossils without data were pruned from the trees, the first 20% of the retained samples were discarded as burn-in, the samples combined, and maximum clade credibility trees constructed using the tree annotator software accompanying the BEAST suite. Runs from the prior using a fixed topology (the maximum clade credibility tree based on the pre-pruning sample) were used to confirm that divergence time estimates were not simply returning the prior.

Data availability Mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD012859. Collagen sequences are available on the Uniprot website (<https://www.uniprot.org/>); the complete list can be found in Supplemental Information, Table S5. Phylogenetic datasets have been deposited on DataDryad (doi:10.5061/dryad.7dd64gs).

578 **Supplementary Information**

579 [\[URL tk\]](#)

580

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Author contributions

RDEM, MC, and SP conceived the project. SP undertook AAR and proteomic analysis and concatenated collagen sequences, with laboratory and technical assistance from RF, JO, KM, MM, MC, KP, and BC. GJS conducted phylogenetic analyses. FP and AMF supplied paleontological information. AK, MT, FS, ML, AH, AI, RF, JB, JLL, FMM, RSG, MR, AG, CdM, and GB supplied fossil samples, locality information, species identifications, and commentary on the manuscript. RDEM, SP, and GJS wrote the manuscript, with input from all authors.

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940

941 **FIGURE AND TABLE LEGENDS (MAIN TEXT)**

942 **Figure 1:** Phylogenetic relationships among major folivoran taxa based on morphological
 943 evidence (mostly after ref. 8, 16), with existence of unallocated taxa acknowledged. In this
 944 framework, the three-toed tree sloth *Bradypus* is sister to other sloths (grouped here as
 945 Eutardigrada), while the two-toed tree sloth *Choloepus* is included within Megalonychidae.

946

947 **Figure 2:** Geographical locations of sequenced samples. Sequences for *Cyclopes* and *Lestodon*
 948 (**in bold**) taken from the literature; others, this paper (Table 1 and Supplementary Information,
 949 Fig. S1).

950

Figure 3: 50% majority rule consensus tree from Bayesian analysis of the proteomic data without temporal information, as performed in MrBayes. Values below nodes are posterior probabilities for the descendant clade (see Results). Values above nodes are bootstrap support derived from 10,000 bootstrap replicates. A dash (–) indicates that a node was not represented in the 50% majority rule bootstrap consensus. Extant *Dasypus* and extinct *Doedicurus* and *Glyptodon* are members of the order Cingulata; extant *Cyclopes* is a representative of Vermilingua, which together with Folivora comprise order Pilosa. Cingulates and pilosans together comprise superorder Xenarthra (see also Fig. 4).

Figure 4: Time scaled maximum clade credibility tree from BEAST analysis of 24 extant and extinct xenarthran collagen sequences plus published mitochondrial genomes (see text). Branch lengths are the mean values from the retained posterior sample, while blue bars represent 95% highest posterior density intervals. Values at nodes are posterior probabilities (note that the monophyly of *Bradypus* is constrained here). Vertical shaded bars correspond to South American land mammal ages (SALMAs), two of which are emphasized: Deseadan (**), 29–21 Ma, during which the first generally-accepted representatives of traditional Megatherioidea and Mylodontoidea appear paleontologically; and the Santacrucian (*), 17.5–16.3 Ma, the SALMA during which mylodontids maintained substantial taxonomic diversity but megalonychids and megatheriids declined⁹. On the right (grey boxes), folivoran species used in analyses are associated with their traditional family names, but with superfamily contents organized according to phylogenetic conclusions in text. Megalocnidae is placed outside traditional superfamily structure in its own (unnamed) box. The tree implies that the fundamental split within Folivora is not between Megatherioidea and Mylodontoidea vs. Bradypodoidea as

974 classically understood, but instead between redefined *Megatherioidea and *Mylodontoidea vs.
975 Megalocnidae.

976

977 **Table 1:** Collagen peptides and per cent coverage of the sequenced ancient and modern samples.

978

979 **Table 2:** Selected divergence time estimates from BEAST analyses using different combinations

980 of taxa and data (see Results and Supplementary Information). Note that, although consistently

981 recovered as monophyletic, the position of Megalocnidae shifted among analyses, falling

982 alternately as sister to all other Folivora (Xenarthra) or Megatherioidea (Folivora).

983

Fig. 1

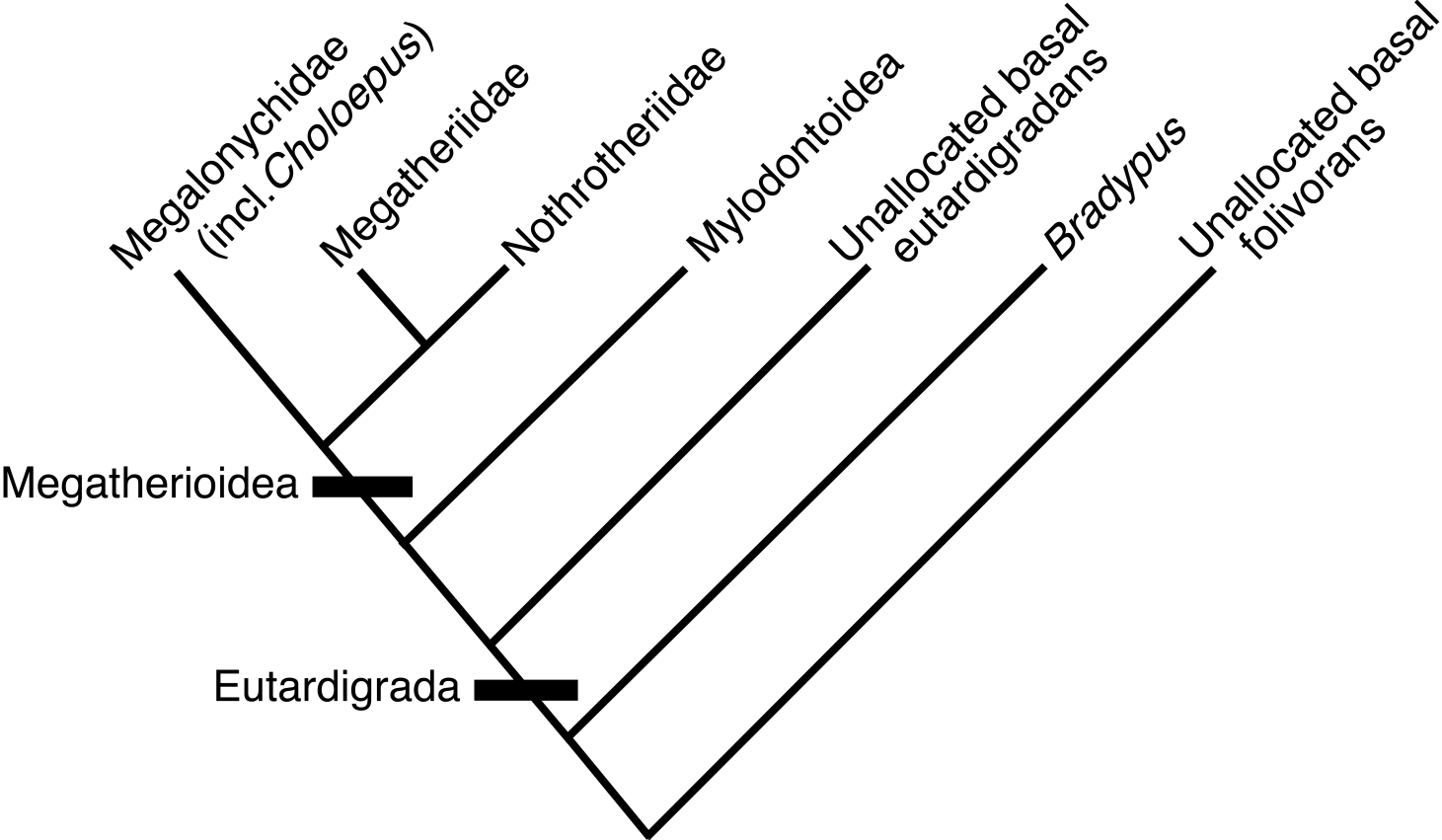
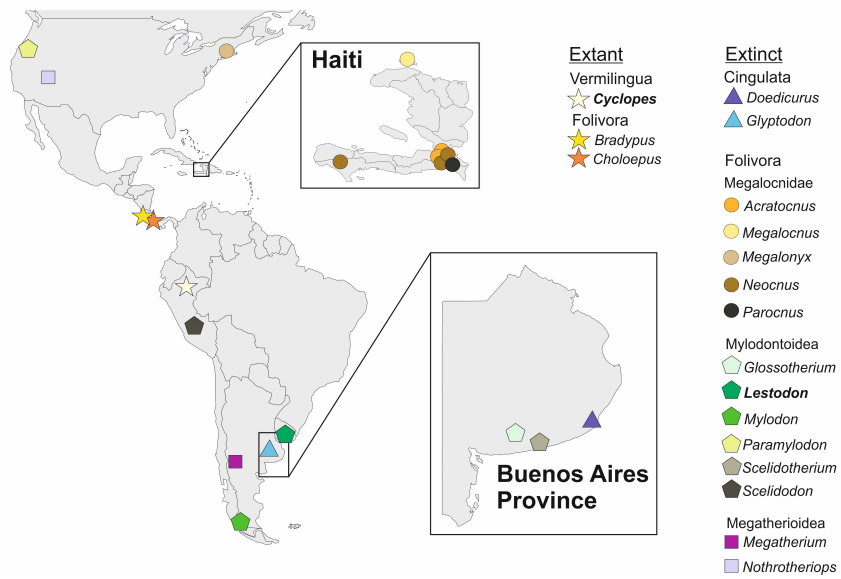


Fig. 2



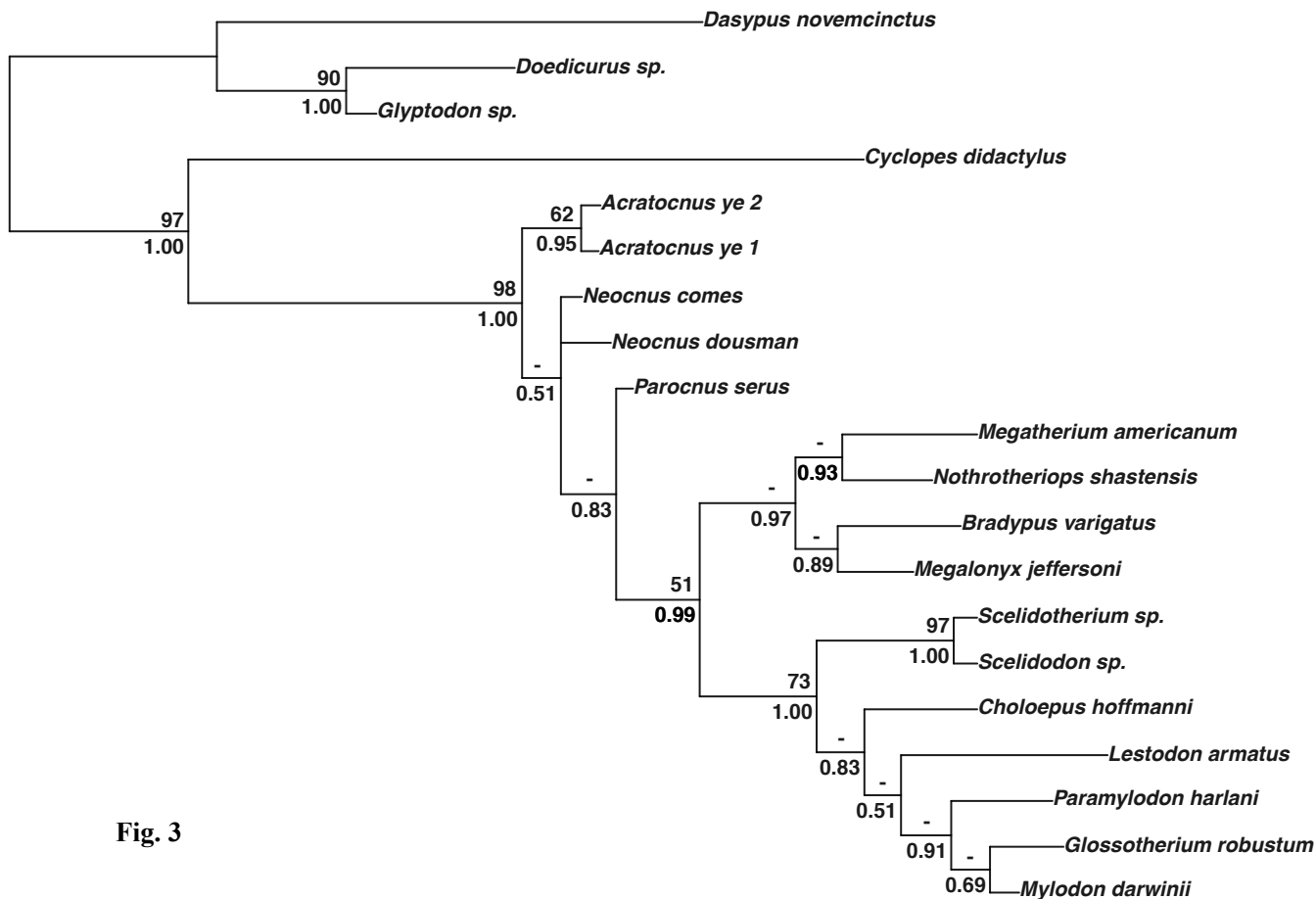


Fig. 3

Fig. 4

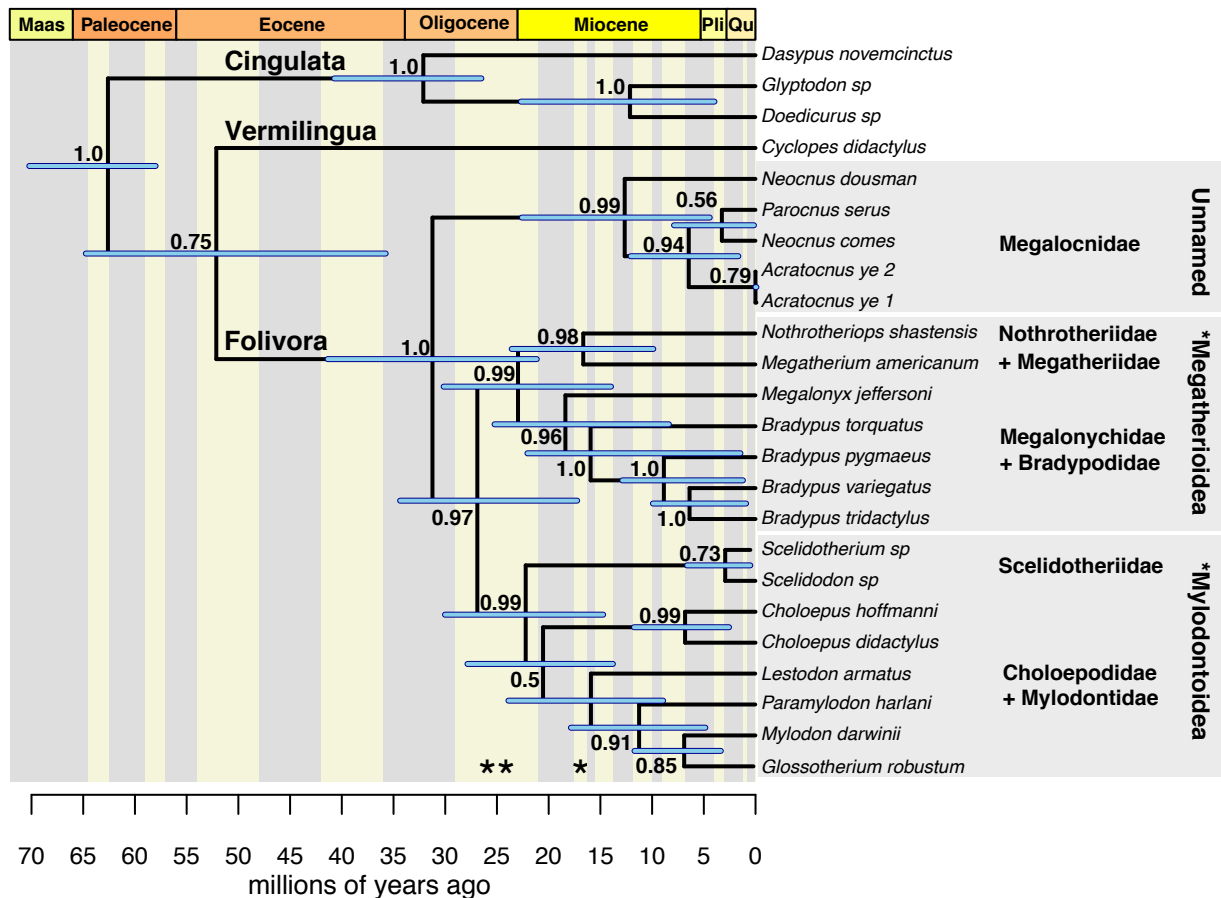


Table 1 Collagen peptides and per cent coverage

Museum reference ¹	ID	Species	# Collagen Peptides	% Coverage
MMP 5672	15191	<i>Doedicurus sp.</i>	867	90
MACN-PV 7	15194	<i>Glyptodon sp.</i>	731	84
UF 76796	15559	<i>Acratocnus ye</i>	696	86
UF 76385	15565	<i>Acratocnus ye</i>	629	87
AMNH 20820	16265	<i>Bradypus variegatus</i>	793	88
AMNH 139772	17009	<i>Choloepus hoffmanni</i>	1109	94
MACN-PV 2652	15216	<i>Glossotherium robustum</i>	837	88
UF 169931	15564	<i>Megalocnus zile</i> ²	6	6
NYSM VP-46	16849	<i>Megalonyx jeffersonii</i> ³	874	85
MAPBAR 3965	15225	<i>Megatherium americanum</i>	520	81
UMAG ah 5854	16222	<i>Myiodon darwini</i>	1371	96
UF 171347	15548	<i>Neocnus comes</i>	699	84
UF 170210	15780	<i>Neocnus comes</i>	591	84
UF 75469	15781	<i>Neocnus dousman</i>	614	74
USNM 244372	14723	<i>Nothrotheriops shastensis</i>	528	79
USNM 3000	14715	<i>Paramylodon harlani</i>	642	87
UF 75526	15556	<i>Parocnus serus</i>	575	82
MUSM 1386	17480	<i>Scelidodon sp.</i>	1324	92
MACN-PV 1791	15202	<i>Scelidotherium sp.</i>	475	76

¹Institutional acronyms:

AMNH-M, American Museum of Natural History (Mammalogy), New York, USA

MACN-PV, Museo Argentino de Ciencias Naturales "Bernardino Rivadavia", Buenos Aires, Argentina

MAPBAR, Museo de la Asociación Paleontológica Bariloche, Bariloche, Argentina

MMP, Museo Municipal de Ciencias Naturales "Lorenzo Scaglia" Mar del Plata, Buenos Aires, Argentina

MUSM, Museo de Historia Natural, Universidad Nacional Mayor de San Marcos, Lima, Peru

NYSM VP, New York State Museum (Vertebrate Paleontology), Albany, New York, USA

UF, University of Florida, Natural History Museum of Florida, Gainesville, USA

UMAG ah, Instituto de La Patagonia, Universidad de Magallanes, Punta Arenas, Chile

USNM, United States National Museum of Natural History (Paleobiology), Washington DC, USA

²Mainly contaminants; not sequenced.

³SDS/PAGE protein extraction

Table 2: Selected divergence time estimates from BEAST analyses using different combinations of taxa and data (see Results and Supplementary Information). Note that, though consistently recovered as monophyletic, the position of Megalocnidae shifted among analyses, falling alternately as sister to all other Folivora (Xenarthra) or Megatherioidea (Folivora).

Clade	Protein only		mtDNA + Protein
	Xenarthra	Folivora	Xenarthra
Crown Xenarthra	62.0 (57.6 - 62.8)	-	62.6 (58.0 - 70.2)
Pilosa	50.4 (37.4 - 62.8)	-	52.1 (35.8 - 64.8)
Folivora	26.4 (18.0 - 36.0)	23.4 (14.9 - 33.9)	31.2 (21.1-41.4)
Megalocnidae	9.9 (3.8 - 17.8)	7.7 (3.4 - 13.0)	12.7 (4.4-22.6)
Megatherioidea + Megalocnidae	-	19.4 (12.8 - 27.8)	-
Megatherioidea + Mylodontoidea	22.7 (16.1 - 31.0)	-	26.9 (17.2 - 34.4)
Megatherioidea	15.7 (10.7 - 21.8)	13.9 (9.4 - 19.4)	23.0 (14.0 - 30.1)
<i>Megalonyx</i> + <i>Bradypus</i>	11.1 (8.4 - 15.0)	10.5 (8.4 - 14.1)	18.4 (8.4 - 25.2)
<i>Bradypus</i> spp.	-	-	16.0 (1.5 - 22.1)
<i>Megatherium</i> + <i>Nothrotheriops</i>	12.3 (8.4 17.7)	10.9 (7.8 - 15.1)	16.7 (9.9 - 23.6)
Mylodontoidea	15.3 (9.8 - 21.4)	15.4 (8.9 - 23.4)	22.2 (14.7 - 30.0)
<i>Choloepus</i> + Mylodontidae	12.03 (7.3 - 17.2)	10.5 (6.2 - 15.9)	20.5 (13.8 - 27.9)
<i>Choloepus</i> spp.	-	-	6.8 (2.6 - 11.8)