



HAL
open science

Resolving the phylogenetic position of Darwin's extinct ground sloth (*Myiodon darwini*) using mitogenomic and nuclear exon data

Frédéric Delsuc, Melanie Kuch, Gillian C Gibb, Jonathan Hughes, Paul Szpak, John Southon, Jacob Enk, Ana T Duggan, Hendrik N Poinar

► To cite this version:

Frédéric Delsuc, Melanie Kuch, Gillian C Gibb, Jonathan Hughes, Paul Szpak, et al.. Resolving the phylogenetic position of Darwin's extinct ground sloth (*Myiodon darwini*) using mitogenomic and nuclear exon data. *Proceedings of the Royal Society B: Biological Sciences*, 2018, 285, 10.1098/rspb.2018.0214 . hal-01879151

HAL Id: hal-01879151

<https://sde.hal.science/hal-01879151>

Submitted on 16 Nov 2018

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

**Resolving the phylogenetic position of Darwin's extinct
ground sloth (*Mylodon darwini*) using mitogenomic and
nuclear exon data**

Journal:	<i>Proceedings B</i>
Manuscript ID	RSPB-2018-0214.R1
Article Type:	Research
Date Submitted by the Author:	n/a
Complete List of Authors:	Delsuc, Frédéric; Institut des Sciences de l'Evolution, CNRS, IRD, EPHE, Université de Montpellier Kuch, Melanie; McMaster University, Department of Anthropology Gibb, Gillian; Massey University, Ecology Group, Institute of Agriculture and Environment Hughes, Jonathan; McMaster University, Department of Anthropology Szpak, Paul; Trent University, Department of Anthropology Southon, John; University of California, Earth Systems Science Department Enk, Jacob; McMaster University, Anthropology; MYcroarray Duggan, Ana; McMaster University, Department of Anthropology Poinar, Hendrik; McMaster University, 2McMaster Ancient DNA Centre, Departments of Anthropology & Pathology and Molecular Medicine
Subject:	Evolution < BIOLOGY, Genomics < BIOLOGY, Palaeontology < BIOLOGY
Keywords:	<i>Mylodon darwini</i> , Xenarthra, Ancient DNA, Mitochondrial genomes, Nuclear data, Phylogenetics
Proceedings B category:	Evolution

SCHOLARONE™
Manuscripts

1 **Resolving the phylogenetic position of Darwin's extinct**
2 **ground sloth (*Myiodon darwini*) using mitogenomic and**
3 **nuclear exon data**

4
5 **Frédéric Delsuc^{1,*}, Melanie Kuch², Gillian C. Gibb^{1,3}, Jonathan Hughes², Paul Szpak⁴,**
6 **John Southon⁵, Jacob Enk^{2,6}, Ana T. Duggan² and Hendrik N. Poinar^{2,*}**

7
8 *¹ISEM, Université de Montpellier, CNRS, IRD, EPHE, Montpellier, France.*

9 *²McMaster Ancient DNA Centre, Department of Anthropology, McMaster University, Hamilton,*
10 *ON, Canada.*

11 *³Ecology Group, Institute of Agriculture and Environment, Massey University, Palmerston North,*
12 *New Zealand.*

13 *⁴Department of Anthropology, Trent University, Peterborough, ON, Canada.*

14 *⁵Keck Carbon Cycle Accelerator Mass Spectrometer, Earth Systems Science Department,*
15 *University of California, Irvine, Irvine, CA, USA.*

16 *⁶MYcroarray, Ann Arbor, MI, USA.*

17

18 *Authors for correspondence (Frederic.Delsuc@umontpellier.fr; poinarh@mcmaster.ca).

19

20 **Running head:** The phylogenetic position of *Myiodon darwini*

21

22 **Abstract**

23 *Myiodon darwinii* is the extinct giant ground sloth named after Charles Darwin, who first
24 discovered its remains in South America. We have successfully obtained a high-quality
25 mitochondrial genome at 99-fold coverage using an Illumina shotgun sequencing of a 12,880
26 year-old bone fragment from Myiodon Cave in Chile. Low level of DNA damage showed that
27 this sample was exceptionally well preserved for an ancient sub-fossil, likely the result of the dry
28 and cold conditions prevailing within the cave. Accordingly, taxonomic assessment of our
29 shotgun metagenomic data showed a very high percentage of endogenous DNA with 22% of the
30 assembled metagenomic contigs assigned to Xenarthra. Additionally, we enriched over 15
31 kilobases of sequence data from seven nuclear exons, using target sequence capture designed
32 against a wide xenarthran dataset. Phylogenetic and dating analyses of the mitogenomic dataset
33 including all extant species of xenarthrans and the assembled nuclear supermatrix
34 unambiguously place *Myiodon darwinii* as the sister-group of modern two-fingered sloths from
35 which it diverged around 22 million years ago. These congruent results from both the
36 mitochondrial and nuclear data support the diphyly of the two modern sloths lineages, implying
37 the convergent evolution of their unique suspensory behaviour as an adaption to arboreality. Our
38 results offer promising perspectives for whole genome sequencing of this emblematic extinct
39 taxon.

40

41 **Keywords:** *Myiodon darwinii*; Xenarthra; Ancient DNA; Mitochondrial genomes; Nuclear
42 data; Phylogenetics.

43

44 1. Background

45 Darwin's extinct ground sloth (*Mylodon darwini*) was named by Richard Owen in honour of
46 Charles Darwin who discovered its early remains in South America during the Voyage of the
47 Beagle [1]. Like the vast majority of the Pleistocene megafauna, *M. darwini* went extinct at the
48 Pleistocene/Holocene boundary, approximately 10,000 years ago [2]. Numerous sub-fossils of
49 *M. darwini* have been found across the South American southern cone [3], including the famous
50 Mylodon Cave (Cueva del Milodón, Ultima Esperanza, Chile). This cave derives its name from
51 the numerous and exquisitely preserved remains of this ground sloth found inside. The constant,
52 cold, and dry conditions of the cave have enabled the exceptional preservation of *M. darwini*
53 remains in the form of paleofeces, bones, claws, and even large pieces of mummified skin
54 covered with blond fur, riddled with osteoderms [4]. These sub-fossils were the first non-human
55 samples yielding genuine ancient DNA [5]. Short overlapping mitochondrial DNA fragments of
56 12S and 16S MT-rRNA [5] and MT-CYTB [6] (550-650 base pairs (bp)), have been PCR-
57 amplified, cloned, and sequenced from *M. darwini* bones, and shorter MT-CYTB sequences of
58 150 bp have even been recovered from hairs embedded in a paleofecal sample [7].

59 Advances in sequencing technology that rely predominantly on short DNA fragments have
60 been a boon for the ancient DNA field, greatly facilitating the assembly of whole mitochondrial
61 genomes in particular [8]. Shotgun Illumina sequencing has been successfully applied to a
62 diversity of Pleistocene sub-fossil bones containing enough endogenous DNA to reconstruct
63 complete mitogenomes. These studies have helped elucidate the phylogenetic affinities of extinct
64 taxa such as Columbian and woolly mammoths [9], steppe bison [10], giant lemurs [11], and
65 South American equids [12] and camelids [13]. Shotgun sequencing of paleofeces has also
66 proven useful for reconstructing the phylogenetic position of the extinct cave hyena and

67 providing insights into its diet [14]. Recently, Slater et al. [15] have reported a partial and
68 composite *M. darwini* mitogenome reconstructed by mixing reads obtained by DNA target
69 sequence capture from a bone and a paleofeces both sampled at Mylodon Cave. However, apart
70 from endogenous retroviral sequences [15] no phylogenetically informative nuclear DNA has
71 been obtained to date for this extinct taxon.

72 Previously published mitochondrial data have suggested a close phylogenetic relationship
73 between *Mylodon* and modern two-fingered sloths of the genus *Choloepus* [5,6,15]. Here, we
74 used Illumina shotgun sequencing to obtain a high-quality, ancient mitogenome from *M.*
75 *darwini*, significantly improving upon the previously published one [15]. In addition, and
76 importantly, using target sequence capture, we assembled a complementary supermatrix of seven
77 nuclear exons totalling 15 kilobases (kb) for representatives of all xenarthran genera including
78 the extinct *Mylodon* and all modern sloth species. Our refined phylogenetic and dating analyses
79 of congruent mitogenomic and nuclear data encompassing the full diversity of modern
80 xenarthrans corroborated the close relationship of Darwin's ground sloth with two-fingered
81 sloths (*Choloepus*) from which it is estimated to have diverged some 22 million years ago.

82

83 **2. Methods**

84 **(a) Sample, DNA extraction, library preparation, and shotgun sequencing**

85 The *Mylodon* bone sample (MPI SP57) used here stems from the collection of Svante Pääbo's
86 former lab (Max Planck Institute for Evolutionary Anthropology, Leipzig, Germany) and was
87 collected by Andrew Curren (The Natural History Museum, London, UK) in Mylodon Cave
88 (Ultima Esperanza, Chile). Collagen was extracted and purified from a subsample of the
89 specimen at the University of Western Ontario and AMS radiocarbon-dated at the Keck Carbon

90 Cycle AMS facility of the University of California Irvine (USA) University of California, Irvine
91 [16].

92 All manipulations took place in the dedicated ancient DNA facilities of the McMaster
93 Ancient DNA Centre of McMaster University. Following subsampling, 300 mg of bone material
94 were reduced to small particle sizes ranging from 1-5 mm using a hammer and chisel. The
95 subsample was then demineralised with 0.5 ml of 0.5 M EDTA pH 8 at room temperature for 24
96 h with agitation, and the supernatant removed following centrifugation. The pellet was then
97 digested with 0.5 ml of a Tris-HCl-based proteinase K solution with 20 mM Tris-Cl pH 8, 0.5 %
98 sodium lauryl sarcosine (SDS, Fisher Scientific), 1 % polyvinylpyrrolidone (PVP, Fisher
99 scientific), 50 mM dithiothreitol (DTT), 2.5 mM N-phenacyl thiazolium bromide (PTB, Prime
100 Organics), 2.5 mM calcium chloride (CaCl₂), and 250 ug/ml proteinase K. Proteinase digestion
101 was performed at room temperature for 24 h, with agitation. Following centrifugation the
102 digestion supernatant was removed and pooled with the demineralization supernatant. We
103 repeated this process three more times for a total of four rounds of demineralization and
104 digestion. Organics were then extracted from the pooled supernatants using
105 phenol:chloroform:isoamyl alcohol (PCI, 25:24:1), and the resulting post centrifugation aqueous
106 solution was extracted with chloroform. We then concentrated the final aqueous phase with 10
107 kDA Amicon Ultra-4 centrifugal filters (Millipore) at 4,000 x g, with four washes using 0.1 x TE
108 buffer pH 8 to provide a concentrate of 50 ul. This concentrate was purified using the MinElute
109 PCR Purification kit (QIAGEN) with two washes of 700µL Buffer PE and eluted in 50µL Buffer
110 EB and 0.05% Tween-20. An extraction blank, which represents an aliquot of the extraction
111 buffer minus any sample, was carried alongside the *Mylodon* sample during the entire extraction
112 procedure to monitor for possible external contamination during handling.

113 We used 25 μ L of the DNA extract and of the extraction blank in the Illumina® library
114 preparation as described elsewhere [17] replacing all SPRI bead cleanups with MinElute
115 purification to 20 μ L Buffer EB. We did not heat-deactivate the Bst polymerase following the
116 fill-in step and instead purified the reaction with MinElute into 20 μ L Buffer EB. The libraries
117 were then index amplified using the common P5 and a set of unique P7 indexing primers [17] in
118 50 μ l reactions consisting of 1 PCR buffer II, 2.5 mM MgCl₂, 250 mM Deoxynucleotide
119 (dNTP) mix, 200 nM each forward (P5) and reverse (P7) primer, 2.5 U AmpliTaq Gold™ DNA
120 Polymerase (ThermoFisher Science), and 2 μ l (100 ng) of template library. Thermal cycling
121 conditions were as follows: initial denaturation at 95°C for 4 min, 12 cycles of 95°C for 30s,
122 60°C for 30s, 72°C for 30s, and a final extension at 72°C for 10 min. The amplification was
123 performed using a MJ thermocycler (BioRad). The indexed libraries were finally purified with
124 MinElute to 15 μ L Buffer EB. A qPCR using 16S MT-rRNA sloth-specific primers was
125 performed on both the *Myiodon* and the blank extract libraries, which we have previously shown
126 to be sensitive to ~10 starting copies. The *Myiodon* library had over 10,000 sloth-specific starting
127 copies whereas the blank library did not show any significant sloth-specific amplification, which
128 is not surprising given the strict conditions under which the experiments were conducted. The
129 *Myiodon* library was sequenced at McMaster Genomics Facility as part of an Illumina HiSeq
130 1500 lane using single-end 72 bp reads.

131

132 **(b) Target sequence capture and sequencing**

133 Baits for DNA sequence capture were designed using xenarthran sequences obtained in previous
134 studies [18–20] for the following seven targeted nuclear exons: ADORA3 (321 bp), APOB (2420
135 bp), BCHE (987 bp), BRCA1 (2835 bp), BRCA2 (3983 bp), RAG2 (441 bp), and TTN (4437
136 bp). For each exon, 80mer baits were generated with a 4x tiling density. This yielded

137 approximately 20 bp probe spacing, or 60 bp probe overlap. Tiling was flexible to ensure even
138 distribution of baits across the loci, as most loci were not perfect multiples of 20. All baits were
139 then BLASTed against the two-fingered sloth (*Choloepus hoffmanni*; NCBI assembly
140 GCA_000164785.2) and nine-banded armadillo (*Dasypus novemcinctus*; NCBI assembly
141 GCA_000208655.2) genome sequences. Baits with more than one hit and a T_m outside the range
142 35–40°C were conservatively excluded. This generated a final set of 4381 baits that were
143 synthesized as a myBaits kit by MYcroarray (Ann Arbor, MI, USA). Twenty-two of the modern
144 xenarthran libraries previously prepared by Gibb et al. [21], were enriched with the designed bait
145 set in order to capture target sequences representing the seven loci of interest for a representative
146 diversity of Xenarthra (electronic supplementary material, table S1). Enrichment for the *Myiodon*
147 library using the same bait set was conducted in a completely separate ancient DNA facility to
148 avoid contamination. For all libraries, we performed two rounds of enrichments using the
149 methodology previously described in Enk et al. [22].

150 In order to re-amplify the captured sequences, a LibQ Master Mix was prepared. This
151 contained 20µl of KAPA SYBR® FAST qPCR Master Mix (2x), 0.60µl Forward Primer 1469
152 (150nM) and 0.60µl Reverse Primer 1470 (150nM) per reaction. The LibQ Mix was added to the
153 18.8µl of captured template and amplified on a CFX. Amplification cycling protocols were as
154 follows: 95°C for 5 minutes; cycle 12 times through 95°C for 30 seconds, 60°C for 45 seconds;
155 finally hold at 60°C for 3 minutes. Following this, the supernatant was removed and saved,
156 yielding the captured library. This was purified using a MinElute PCR Purification Kit (Qiagen)
157 using their standard protocol, yielding a final enriched and purified library suspended in 15µl of
158 Buffer EB.

159 All libraries to be sequenced were pooled together at varying concentrations with the aim

160 of creating a single solution containing approximately 250 pM of DNA post size selection. In
161 general, each library was calculated to ideally produce one million reads for sequencing.
162 Libraries then underwent size selection to decrease the amount of non-target DNA and increase
163 sequencing efficiency. Size selection was carried out on a 2% gel (50 ml agarose/1x TAE with 2
164 μ l EtBr). Loading dye equivalent to 1/5 of the library volume was added and samples were then
165 run through the gel for 30 minutes at 100V. A 50 bp ladder was used for determining DNA
166 position and size, and the area from approximately 50 to 150 bp was excised. The excised gel
167 was then purified using a MinElute Gel Extraction Kit (Qiagen) column eluted into 60 μ l of
168 Buffer EB. Final pool concentrations prior to sequencing were verified using a 2100 Bioanalyzer
169 (Agilent). Sequencing of the enrichment set was performed at McMaster Genomics Facility on
170 an Illumina MiSeq instrument using 150 bp paired-end reads.

171

172 **(c) Mitogenome and nuclear exons assemblies**

173 Shotgun sequenced raw reads obtained from the *Mylodon* library were adapter-trimmed using
174 cutadapt v1.1 [23] with a quality score cut-off of 30. Contigs were created by *de novo* assembly
175 of the cleaned reads using ABySS 1.3.4 [24] with default parameters and a range of increasing
176 kmers. The resulting 480,662 non-redundant contigs were mapped to the *Choloepus didactylus*
177 reference mitogenome (NC_006924) using the ‘medium low sensitivity’ settings in Geneious R9
178 [25]. Iterative mapping of the reads using the more stringent ‘low sensitivity’ settings was
179 subsequently used to fill the gaps in the assembly of the 174 successfully mapped contigs. The
180 consensus sequence was called using 50% read agreement and all reads were remapped on the
181 consensus to estimate the depth of coverage. Repeating the same procedure using *Bradypus*
182 *variegatus* (NC_028501) instead of *C. didactylus* as a reference resulted in the same 174 contigs
183 being mapped and the exact same *Mylodon* mitogenome being reconstructed. The final *Mylodon*

184 mitogenome was then annotated by alignment to the *C. didactylus* reference genome.

185 Raw reads containing imperfect index combinations were discarded. Index and adapter
186 sequences were removed and overlapping pairs merged with leeHom [26], and then mapped to
187 all xenarthran reference exon sequences available with a modified version of BWA [27,28] with
188 a maximum edit distance of 0.01 (-n 0.01), allowing a maximum of two gap openings (-o 2) and
189 with seeding effectively disabled (-l 16569). Mapped reads were additionally filtered to those
190 that were either merged or properly paired [29], had unique 5' and 3' mapping coordinates [30],
191 and then restricted to reads of at least 24 bp with SAMtools [31]. The bam files were then
192 imported into Geneious for careful assessment by eye for enrichment success and selection of the
193 best assembly for each sequence depending on the most successful reference sequence.

194 Consensus sequences were called with a 50% threshold and a minimum coverage of 2x with
195 ambiguous nucleotides called at sites where the two reads disagreed and there was no third read.

196 As expected, nuclear capture success was variable among both taxa and loci with sloths and
197 anteaters being successfully enriched for all loci, whereas armadillos presented some loci for
198 which the coverage was insufficient to confidently call a consensus sequence. The capture
199 experiment nevertheless enabled us to produce a total of 144 newly assembled xenarthran
200 sequences for the seven nuclear exons targeted (electronic supplementary material, table S1).

201

202 **(d) DNA damage and metagenomic analyses**

203 Analyses of post-mortem C to T and G to A mutations in the 53,550 reads mapping to the
204 reconstructed *Myiodon* mitogenome were conducted using mapDamage 2.0 [32]. For
205 comparisons, four modern xenarthran species from Gibb et al. [21] and the extinct glyptodont
206 *Doedicurus* [33] were also analysed. For metagenomic analyses, MEGAHIT v1.1.1 [34] was
207 used to assemble the *Myiodon* shotgun reads. The resulting 385 contigs of more than 200bp were

208 then subjected to similarity searches against the GenBank nucleotide database (version of 28
209 April 2017) using MEGABLAST followed by taxonomic assignation using MEGAN 6 with
210 default LCA parameters [35] and subsequent graphical representation with KRONA [36].

211

212 **(e) Phylogenetic and dating analyses**

213 For constructing the mitogenomic supermatrix, we chose 31 representative living xenarthran
214 species from the dataset of Gibb et al. [21] plus three afrotherian outgroups (electronic
215 supplementary material, table S1). We then added *M. darwinii* sequences (excluding the variable
216 control region) and aligned each gene with MAFFT [37] within Geneious guided by translation
217 for the protein-coding genes. We removed ambiguously aligned sites on each dataset with
218 Gblocks [38] using default relaxed parameters. The final mitogenomic matrix contained 15,222
219 sites for 35 taxa representing all living xenarthran species plus the extinct *M. darwinii*.

220 For assembling the seven nuclear exons supermatrix, the newly obtained *Myiodon* and
221 modern xenarthran sequences were added to available sequences plus the same three afrotherian
222 outgroups (electronic supplementary material, table S1). Each exon was then aligned by
223 translation with MAFFT within Geneious. We removed ambiguously aligned codons on each
224 alignment with Gblocks using relaxed default parameters. The final nuclear exons matrix
225 contained 15,216 sites for 28 taxa encompassing all living xenarthran genera and the extinct *M.*
226 *darwinii* with an overall percentage of missing data of only 16%. Importantly, *Myiodon* was
227 represented at 10,238 unambiguous sites (67.28% of the total) of the nuclear concatenated data
228 set.

229 The best-fitting partition schemes were determined for both datasets using PartitionFinder
230 v1.1.1 [39]. For the mitogenomic dataset, we used the greedy algorithm on 42 a priori partitions
231 corresponding to codon positions, 12S MT-rRNA, 16S MT-rRNA, and all tRNAs, with unlinked

232 branch lengths, and using the Bayesian Information Criterion (BIC) for model selection
233 (electronic supplementary material, table S2). For the nuclear dataset, we used the greedy
234 algorithm on 21 a priori partitions corresponding to codon positions, with linked branch lengths,
235 and the BIC for model selection (electronic supplementary material, table S3). For both datasets,
236 ML partitioned reconstruction was conducted with RAxML 8.2.8 [40] using the best-fitting
237 scheme with parameters unlinked across partitions. Maximum Likelihood bootstrap values
238 (BP_{PART}) were obtained after 100 replicates. Bayesian phylogenetic inference under a mixed
239 model was performed using the MrBayes 3.2.3 [41] using the best-fitting scheme with
240 parameters unlinked across partitions. Two independent sets of four MCMCMCs were run for
241 1,000,000 generations sampling every 1000 generations. After a burn-in of 25%, the 50%
242 majority-rule consensus tree and associated clade posterior probabilities (PP_{PART}) were computed
243 from the 1,500 trees combined in the two independent runs. Bayesian phylogenetic
244 reconstruction was also conducted under the CAT-GTR+G₄ mixture model using PhyloBayes
245 MPI 1.7b [42]. Two independent MCMCs were run for 50,000 cycles sampling every 10 cycles
246 during 2,750,000 tree generations. After a burn-in of 10%, the 50% majority-rule consensus tree
247 and associated clade posterior probabilities (PP_{CAT}) were computed from the 9000 combined
248 trees of the two runs using bpcomp.

249 Molecular dating analyses were conducted on both datasets using PhyloBayes 3.3f [43]
250 under the CAT-GTR+G₄ mixture model and a log-normal autocorrelated relaxed clock with a
251 birth–death prior on divergence times combined with soft fossil calibrations. We used the same
252 best-fitting relaxed clock model, six fossil calibrations, and priors as in Gibb et al. [21] so that
253 the divergence dates obtained could be directly compared between the two studies. Calculations
254 were conducted in each case by running two independent MCMCs for a total 50,000 cycles

255 sampling every 10 cycles. The first 500 samples (10%) were excluded as burn-in after
256 convergence diagnostics. Posterior estimates of divergence dates were computed from the
257 remaining 4500 samples of each MCMC using readdiv.

258

259 **3. Results and Discussion**

260 **(a) A new high quality *Mylodon* mitogenome**

261 The radiocarbon date we obtained for the *Mylodon* bone sample MPI SP57 is $12,880 \pm 35$ ^{14}C
262 yrBP (radiocarbon years before present), which is fully congruent with previous estimates for
263 other samples from Mylodon Cave [2]. Illumina sequencing of the *Mylodon* shotgun library
264 produced a total of 28,020,236 single-end 72 bp reads. Low, yet consistent DNA damage on
265 those reads shows that our ancient *Mylodon* sample was exceptionally well preserved and thus
266 helps support its authenticity (figure 1a, electronic supplementary material, figures S1, S2).
267 Estimates of C to T and G to A transitions caused by post-mortem mutations (up to 11% and 8%
268 respectively) were intermediate between those of the subfossil *Doedicurus* sp. osteoderm sample
269 of the same age (up to 25% and 20%) and the *Calyptophractus* museum specimen (up to 4% and
270 3%), whereas modern xenarthran tissue samples exhibit values below 1%. These values argue in
271 favour of the endogenous origin of the *Mylodon* shotgun reads and set our bone sample among
272 some of the best ancient samples analysed so far [44]. The consistently cold and dry conditions
273 encountered at Mylodon Cave [4] probably explain the exceptional preservation of samples
274 coming from this location [5].

275 Metagenomic analyses indicate that 30% of the 222 taxonomically assigned contigs (out
276 of a total of 385) are mammalian in origin: 22% are xenarthrans, with 17% matching specifically
277 to the two-fingered sloth (*C. hoffmanni*) genome assembly (figure 1b). The only 3% of contigs

278 assigned to *Homo sapiens* in fact match conserved portions of the mammalian 18S and 45S
279 ribosomal RNA genes and thus could not be considered to represent human specific
280 contamination. Moreover, 25% of the assigned contigs are fungal in origin with *Aspergillus* mold
281 representing 12%. Finally, 35% of the assigned contigs belong to Bacteria, 28% being assigned
282 to the genus *Clostridium* with dominant taxa such as *C. novyi* (13%) and *C. botulinum* (6%) that
283 are commonly found in ancient DNA samples and soil. To further quantify possible
284 contamination from humans or other mammals found in Mylodon Cave such as *Hippidion*
285 *saldiasi*, *Lama guanicoe*, *Dusicyon avus*, and *Panthera onca mesembrina* [4], we mapped our
286 shotgun reads to available mitogenomes for these species, or to those of closely related ones,
287 using the low sensitivity settings of Geneious. None of the mapping results were convincing
288 apart from a few reads, which mapped to conserved regions of the mammalian mitogenome (data
289 not shown). For instance, we had only 536 reads mapping to conserved regions of the human
290 reference mitogenome (NC_012920), again supporting the authenticity of our sample.

291 The assembly of 53,550 shotgun reads allowed for the reconstruction of a high quality
292 mitogenome for *M. darwini* at an average depth of 99x (range 1x-307x). As independent
293 replication is an important component of the ancient DNA research agenda [45], we verified that
294 our consensus sequence matched perfectly to previously obtained PCR fragments attributed to
295 *Mylodon* for the mitochondrial 12S and 16S MT-rRNAs [5] and MT-CYTb [6] genes. However,
296 a comparison with a recently published mitogenome sequence [15] revealed a number of
297 discrepancies (electronic supplementary material, figure S11). The two sequences are identical at
298 only 81% of total sites (89% when excluding ambiguous sites and the control region). The Slater
299 et al. [15] sequence (KR336794) also contains 1383 Ns and presents a substantial number of
300 substitutions in otherwise conserved regions of the sloth mitogenome when compared to *C.*

301 *didactylus*, including frameshifting substitutions causing stop codons in 11 out of the 13 coding
302 genes (electronic supplementary material, table S4). These differences most probably represent
303 errors that were spuriously incorporated into the assembly resulting from overall lower coverage
304 depth of the composite mitogenome, reconstructed from captured sequences stemming from both
305 bone and paleofecal material. Mapping the reads produced by Slater et al. [15] (SRA accession
306 SRR2007674) to our *Myiodon* mitogenome confirmed that these divergent regions correspond to
307 regions of low depth of coverage in their capture experiment (data not shown). These errors
308 likely explain an artificially inflated branch length in the phylogenetic tree, potentially impacting
309 the inference of the divergence date between the extinct *Myiodon* and modern sloths (electronic
310 supplementary material, figure S12). Based on these observations, we would recommend using
311 our fully annotated and higher quality mitogenome sequence in future analyses.

312

313 **(b) Nuclear data corroborate the phylogenetic position of *Myiodon darwinii***

314 Phylogenetic analyses of our complete xenarthran mitogenomic dataset using Bayesian and ML
315 methods recovered the same strongly supported topology in which *Myiodon* is the sister-group of
316 modern two-fingered sloths of the genus *Choloepus* (figure 2a). The statistical support for this
317 phylogenetic position was high with the Bayesian mixture model ($PP_{\text{CAT}} = 0.95$) and maximal
318 with the Bayesian and ML partitioned models ($PP_{\text{PART}} = 1$; $BP_{\text{PART}} = 100$). All other nodes
319 within Pilosa received maximal support from all methods. These results obtained by including
320 the full species diversity of modern sloths add support to the position of *M. darwinii* originally
321 suggested by short mitochondrial PCR fragments [5,6] and recently supported by mitogenomic
322 analyses including fewer taxa [15]. The phylogenetic picture provided by the mitochondrial
323 genome alone could nevertheless be misleading in cases of mito-nuclear discordance caused by
324 factors such as adaptive introgression or past hybridization events [46]. It was thus important to

325 substantiate our mitogenomic results by nuclear data. In this case, the same phylogenetic
326 analyses applied to the supermatrix of the concatenated seven nuclear exons yield identical
327 results with maximum statistical support placing *Mylodon*, once again as the sister-group of two-
328 fingered sloths (figure 2*b*). Such perfect topological congruence between mitochondrial genomes
329 and nuclear markers provides clear and convincing evidence of the phylogenetic position of the
330 extinct Darwin's ground sloth within the evolutionary history of sloths. These phylogenetic
331 results provide further support for the diphyletic origin of modern sloths implying an
332 independent evolution of arboreality from terrestrial ancestors [47,48] and the independent
333 evolution of their unique suspensory lifestyle resulting in numerous convergent anatomical
334 adaptations [49,50].

335 Relaxed molecular clock analyses of both the mitogenomic and nuclear datasets recover
336 relatively ancient dates for the common ancestor of sloths (figure 3, table 1). The corresponding
337 divergence between the two modern sloth genera *Bradypus* (Bradypodidae) and *Choloepus*
338 (Megalonychidae) is estimated to be 25 ± 6 Mya with the mitogenomic dataset (figure 3*a*), and
339 28 ± 4 Mya with the nuclear supermatrix (figure 3*b*). These inferred dates are relatively older
340 than previous estimates based on nuclear data but with reduced taxon sampling [19,51], further
341 justifying their classification into distinct families. The difference with previous nuclear dates
342 might have to do with the CAT-GTR+G4 mixture model which allows for a better correction of
343 substitutional saturation combined with the inclusion of the internal xenarthran calibration points
344 here provided by the earliest fossil cingulate skull [52].

345 Regarding our extinct sample, our results reveal that *Mylodon* separated from two-
346 fingered sloths of the genus *Choloepus* early in sloth evolutionary history with both datasets
347 clearly agreeing upon a divergence date of about 22 Mya (figure 3, table 1). The dating estimate

348 obtained with our revised *Myiodon* mitogenome (22 ± 5 Mya; figure 3a) is somewhat
349 comparable with previous estimates proposed by Slater et al. [15] based on a different approach.
350 These authors used a recently developed dating method based on the Fossilized Birth-Death
351 (FBD) process that allows one to directly incorporate fossil taxa [53], but requires the use a
352 topological constraint including both fossil and modern taxa. Because of the uncertainty
353 associated with the phylogenetic position of some key sloth fossil taxa, Slater et al. [15] found
354 that their divergence date estimates under the FBD process were highly sensitive to the treatment
355 of ambiguous Deseadan fossil taxa as representing either stem or crown fossil folivorans. By
356 contrast using nodal calibrations and a better sampling of modern species, our results appear
357 intermediate with those obtained with the two alternative treatments. In fact, the diversification
358 of sloth lineages in the Early Miocene (25-22 Mya) corresponds with the end of the first major
359 Bolivian tectonic event, when the Andes became the principal relief of South America,
360 significantly influencing paleoclimates [54]. This period led to a major shift in South American
361 mammalian fossil communities including the Miocene radiation of ground sloths [55]. Our
362 results based on an updated mitogenomic dataset and a new nuclear supermatrix corroborate *M.*
363 *darwinii* as belonging to a distinct lineage of sloths (family Mylodontidae) originating more than
364 22 Mya and persisting until their extinction only some 10,000 years BP [56,57].

365

366

367 **4. Conclusions and Perspectives**

368 Our study provides a high quality complete mitochondrial genome as well as the first
369 phylogenetically informative nuclear loci for the extinct Darwin's ground sloth. Analyses of
370 these new data, validate the phylogenetic position of *M. darwinii* as a member of a distinct

371 lineage (Mylodontidae) and as a sister-group to modern two-fingered sloths (genus *Choloepus*;
372 Megalonychidae), originating about 22 million years ago. The exceptional preservation of these
373 cave-preserved *Myiodon* bone samples will enable complete genome sequencing of this
374 emblematic extinct taxon generating further insights into their unique features and ultimate
375 extinction.

376

377 **Data accessibility.** *M. darwinii* mitogenome: GenBank (MF061314). Nuclear exon data:
378 European Nucleotide Archive (LT852562-LT852705). *M. darwinii* shotgun Illumina reads:
379 Sequence Read Archive (ERR1958375). Additional data including bait design, alignments, and
380 trees: Dryad doi:10.5061/dryad.ft3k3.

381 **Competing interests.** Jacob Enk is an employee of MYcroarray (Ann Arbor, MI, USA).

382 **Acknowledgments.** Andrew Currant and Svante Pääbo kindly provided access to the *Myiodon*
383 bone. We thank the following individuals and institutions for tissue samples: François Catzeflis
384 (Institut des Sciences de l'Evolution, Montpellier, France), Jean-François Mauffrey, Philippe
385 Gaucher, Eric Hansen, François Ouhoud-Renoux, Jean-Christophe Vié, Philippe Cerdan, Michel
386 Blanc, and Rodolphe Paowé (French Guiana), Jorge Omar García and Rodolfo Rearte (Complejo
387 Ecológico Municipal, Presidencia Roque Sáenz Peña, Argentina), Daniel Hernández (Facultad
388 de Ciencias, Universidad de la República, Montevideo, Uruguay), John Trupkiewicz
389 (Philadelphia Zoo, USA), Darrin Lunde (National Museum of Natural History, Washington,
390 USA), Jim Patton and Yuri Leite (Museum of Vertebrate Zoology, Berkeley, USA), Gerhard
391 Haszprunar and Michael Hiermeier (Zoologische Staatssammlung München, Munich, Germany),
392 Géraldine Véron (Museum National d'Histoire Naturelle, Paris, France), Agustín Jiménez-Ruiz,

393 Nadia Moraes-Barros, and Mariella Superina. We finally thank three anonymous referees for
394 helpful comments. This is contribution ISEM 2018-041-SUD of the Institut des Sciences de
395 l'Evolution de Montpellier.

396 **Authors' contributions.** MK, GCG, JH, JE, AD carried out the molecular lab work,
397 participated in data analysis, conducted mitogenome and nuclear data assembly, participated in
398 the design of the study; PS, JS radiocarbon-dated the bone sample; FD carried out DNA damage,
399 metagenomic, sequence alignment, and phylogenetic analyses; FD, HNP conceived of the study,
400 designed the study, coordinated the study, and drafted the manuscript. All authors gave final
401 approval for publication.

402 **Funding statement.** FD: Centre National de la Recherche Scientifique (CNRS), the Scientific
403 Council of the Université Montpellier 2 (UM2), and Investissements d'Avenir managed by
404 Agence Nationale de la Recherche (CEBA, ANR-10-LABX-25-01). HNP: Natural Sciences and
405 Engineering Research Council of Canada (NSERC, # RGPIN04184-15) and Canada Research
406 Chairs program.

407

408

409 **References**

- 410 1. Fernicola JC, Vizcaíno SF, De Iuliis G. 2009 The fossil mammals collected by Charles
411 Darwin in South America during his travels on board the HMS Beagle. *Rev. Asoc. Geológica*
412 *Argent.* **64**, 147–159.
- 413 2. Villavicencio NA, Lindsey EL, Martin FM, Borrero LA, Moreno PI, Marshall CR,

- 414 Barnosky AD. 2016 Combination of humans, climate, and vegetation change triggered Late
415 Quaternary megafauna extinction in the Última Esperanza region, southern Patagonia, Chile.
416 *Ecography* **39**, 125–140. (doi:10.1111/ecog.01606)
- 417 3. Varela L, Fariña RA. 2016 Co-occurrence of mylodontid sloths and insights on their
418 potential distributions during the late Pleistocene. *Quat. Res.* **85**, 66–74.
419 (doi:10.1016/j.yqres.2015.11.009)
- 420 4. Borrero LA, Martin FM. 2012 Taphonomic observations on ground sloth bone and dung
421 from Cueva del Milodón, Última Esperanza, Chile: 100 years of research history. *Quat. Int.* **278**,
422 3–11. (doi:10.1016/j.quaint.2012.04.036)
- 423 5. Höss M, Dilling A, Currant A, Pääbo S. 1996 Molecular phylogeny of the extinct ground
424 sloth *Mylodon darwini*. *Proc. Natl. Acad. Sci.* **93**, 181–185.
- 425 6. Greenwood AD, Castresana J, Feldmaier-Fuchs G, Pääbo S. 2001 A molecular
426 phylogeny of two extinct sloths. *Mol. Phylogenet. Evol.* **18**, 94–103.
427 (doi:10.1006/mpev.2000.0860)
- 428 7. Clack AA, MacPhee RDE, Poinar HN. 2012 *Mylodon darwini* DNA sequences from
429 ancient fecal hair shafts. *Ann. Anat. - Anat. Anz.* **194**, 26–30. (doi:10.1016/j.aanat.2011.05.001)
- 430 8. Paijmans JLA, Gilbert MTP, Hofreiter M. 2013 Mitogenomic analyses from ancient
431 DNA. *Mol. Phylogenet. Evol.* **69**, 404–416. (doi:10.1016/j.ympev.2012.06.002)
- 432 9. Enk J *et al.* 2011 Complete Columbian mammoth mitogenome suggests interbreeding
433 with woolly mammoths. *Genome Biol.* **12**, R51. (doi:10.1186/gb-2011-12-5-r51)
- 434 10. Marsolier-Kergoat M-C, Palacio P, Berthouaud V, Maksud F, Stafford T, Bégouën R,
435 Elalouf J-M. 2015 Hunting the extinct steppe bison (*Bison priscus*) mitochondrial genome in the
436 Trois-Frères paleolithic painted cave. *PLOS ONE* **10**, e0128267.

437 (doi:10.1371/journal.pone.0128267)

438 11. Kistler L *et al.* 2015 Comparative and population mitogenomic analyses of Madagascar's
439 extinct, giant 'subfossil' lemurs. *J. Hum. Evol.* **79**, 45–54. (doi:10.1016/j.jhevol.2014.06.016)

440 12. Der Sarkissian C *et al.* 2015 Mitochondrial genomes reveal the extinct Hippidion as an
441 outgroup to all living equids. *Biol. Lett.* **11**, 20141058–20141058. (doi:10.1098/rsbl.2014.1058)

442 13. Heintzman PD, Zazula GD, Cahill JA, Reyes AV, MacPhee RDE, Shapiro B. 2015
443 Genomic data from extinct North American Camelops revise camel evolutionary history. *Mol.*
444 *Biol. Evol.* **32**, 2433–2440. (doi:10.1093/molbev/msv128)

445 14. Bon C, Berthouaud V, Maksud F, Labadie K, Poulain J, Artiguenave F, Wincker P, Aury
446 J-M, Elalouf J-M. 2012 Coprolites as a source of information on the genome and diet of the cave
447 hyena. *Proc. R. Soc. B Biol. Sci.* **279**, 2825–2830. (doi:10.1098/rspb.2012.0358)

448 15. Slater GJ, Cui P, Forasiepi AM, Lenz D, Tsangaras K, Voirin B, de Moraes-Barros N,
449 MacPhee RDE, Greenwood AD. 2016 Evolutionary relationships among extinct and extant
450 sloths: The evidence of mitogenomes and retroviruses. *Genome Biol. Evol.* **8**, 607–621.
451 (doi:10.1093/gbe/evw023)

452 16. Beaumont W, Beverly R, Southon J, Taylor RE. 2010 Bone preparation at the KCCAMS
453 laboratory. *Nucl. Instrum. Methods Phys. Res. Sect. B Beam Interact. Mater. At.* **268**, 906–909.
454 (doi:10.1016/j.nimb.2009.10.061)

455 17. Meyer M, Kircher M. 2010 Illumina sequencing library preparation for highly
456 multiplexed target capture and sequencing. *Cold Spring Harb. Protoc.* **2010**, pdb.prot5448.
457 (doi:10.1101/pdb.prot5448)

458 18. Delsuc F, Scally M, Madsen O, Stanhope MJ, de Jong WW, Catzeflis FM, Springer MS,
459 Douzery EJP. 2002 Molecular phylogeny of living xenarthrans and the impact of character and

- 460 taxon sampling on the placental tree rooting. *Mol. Biol. Evol.* **19**, 1656–1671.
- 461 19. Meredith RW *et al.* 2011 Impacts of the Cretaceous Terrestrial Revolution and KPg
462 extinction on mammal diversification. *Science* **334**, 521–524. (doi:10.1126/science.1211028)
- 463 20. Delsuc F, Superina M, Tilak M-K, Douzery EJP, Hassanin A. 2012 Molecular
464 phylogenetics unveils the ancient evolutionary origins of the enigmatic fairy armadillos. *Mol.*
465 *Phylogenet. Evol.* **62**, 673–680. (doi:10.1016/j.ympev.2011.11.008)
- 466 21. Gibb GC, Condamine FL, Kuch M, Enk J, Moraes-Barros N, Superina M, Poinar HN,
467 Delsuc F. 2016 Shotgun Mitogenomics Provides a Reference Phylogenetic Framework and
468 Timescale for Living Xenarthrans. *Mol. Biol. Evol.* **33**, 621–642. (doi:10.1093/molbev/msv250)
- 469 22. Enk JM, Devault AM, Kuch M, Murgha YE, Rouillard J-M, Poinar HN. 2014 Ancient
470 whole genome enrichment using baits built from modern DNA. *Mol. Biol. Evol.* **31**, 1292–1294.
471 (doi:10.1093/molbev/msu074)
- 472 23. Martin M. 2011 Cutadapt removes adapter sequences from high-throughput sequencing
473 reads. *EMBnet.journal* **17**, 10–12. (doi:10.14806/ej.17.1.200)
- 474 24. Simpson JT, Wong K, Jackman SD, Schein JE, Jones SJM, Birol I. 2009 ABySS: a
475 parallel assembler for short read sequence data. *Genome Res.* **19**, 1117–1123.
476 (doi:10.1101/gr.089532.108)
- 477 25. Kearsse M *et al.* 2012 Geneious Basic: An integrated and extendable desktop software
478 platform for the organization and analysis of sequence data. *Bioinformatics* **28**, 1647–1649.
479 (doi:10.1093/bioinformatics/bts199)
- 480 26. Renaud G, Stenzel U, Kelso J. 2014 leeHom: adaptor trimming and merging for Illumina
481 sequencing reads. *Nucleic Acids Res.* **42**, e141. (doi:10.1093/nar/gku699)
- 482 27. Li H, Durbin R. 2009 Fast and accurate short read alignment with Burrows-Wheeler

- 483 transform. *Bioinforma. Oxf. Engl.* **25**, 1754–1760. (doi:10.1093/bioinformatics/btp324)
- 484 28. Stenzel U. 2017 network-aware-bwa. See <https://github.com/mpieva/network-aware-bwa>
485 (accessed on 9 May 2017).
- 486 29. Renaud G. 2015 libbam. See <https://github.com/grenaud/libbam> (accessed on 9 May
487 2017).
- 488 30. Stenzel U. 2017 biohazard. See <https://bitbucket.org/ustenzel/biohazard> (accessed on 9
489 May 2017).
- 490 31. Li H *et al.* 2009 The Sequence Alignment/Map format and SAMtools. *Bioinforma. Oxf.*
491 *Engl.* **25**, 2078–2079. (doi:10.1093/bioinformatics/btp352)
- 492 32. Jónsson H, Ginolhac A, Schubert M, Johnson PLF, Orlando L. 2013 mapDamage2.0: fast
493 approximate Bayesian estimates of ancient DNA damage parameters. *Bioinformatics* **29**, 1682–
494 1684. (doi:10.1093/bioinformatics/btt193)
- 495 33. Delsuc F *et al.* 2016 The phylogenetic affinities of the extinct glyptodonts. *Curr. Biol.* **26**,
496 R155–R156.
- 497 34. Li D, Luo R, Liu C-M, Leung C-M, Ting H-F, Sadakane K, Yamashita H, Lam T-W.
498 2016 MEGAHIT v1.0: A fast and scalable metagenome assembler driven by advanced
499 methodologies and community practices. *Methods San Diego Calif* **102**, 3–11.
500 (doi:10.1016/j.ymeth.2016.02.020)
- 501 35. Huson DH, Mitra S, Ruscheweyh H-J, Weber N, Schuster SC. 2011 Integrative analysis
502 of environmental sequences using MEGAN4. *Genome Res.* **21**, 1552–1560.
503 (doi:10.1101/gr.120618.111)
- 504 36. Ondov BD, Bergman NH, Phillippy AM. 2011 Interactive metagenomic visualization in a
505 Web browser. *BMC Bioinformatics* **12**, 385. (doi:10.1186/1471-2105-12-385)

- 506 37. Katoh K, Kuma K, Toh H, Miyata T. 2005 MAFFT version 5: improvement in accuracy
507 of multiple sequence alignment. *Nucleic Acids Res.* **33**, 511–518. (doi:10.1093/nar/gki198)
- 508 38. Castresana J. 2000 Selection of conserved blocks from multiple alignments for their use
509 in phylogenetic analysis. *Mol. Biol. Evol.* **17**, 540–552.
- 510 39. Lanfear R, Calcott B, Ho SYW, Guindon S. 2012 PartitionFinder: Combined selection of
511 partitioning schemes and substitution models for phylogenetic analyses. *Mol. Biol. Evol.* **29**,
512 1695–1701. (doi:10.1093/molbev/mss020)
- 513 40. Stamatakis A. 2014 RAxML version 8: a tool for phylogenetic analysis and post-analysis
514 of large phylogenies. *Bioinforma. Oxf. Engl.* **30**, 1312–1313.
515 (doi:10.1093/bioinformatics/btu033)
- 516 41. Ronquist F *et al.* 2012 MrBayes 3.2: Efficient Bayesian phylogenetic inference and
517 model choice across a large model space. *Syst. Biol.* **61**, 539–542. (doi:10.1093/sysbio/sys029)
- 518 42. Lartillot N, Rodrigue N, Stubbs D, Richer J. 2013 PhyloBayes MPI: phylogenetic
519 reconstruction with infinite mixtures of profiles in a parallel environment. *Syst. Biol.* **62**, 611–
520 615. (doi:10.1093/sysbio/syt022)
- 521 43. Lartillot N, Lepage T, Blanquart S. 2009 PhyloBayes 3: a Bayesian software package for
522 phylogenetic reconstruction and molecular dating. *Bioinforma. Oxf. Engl.* **25**, 2286–2288.
523 (doi:10.1093/bioinformatics/btp368)
- 524 44. Der Sarkissian C, Ermini L, Jónsson H, Alekseev AN, Crubezy E, Shapiro B, Orlando L.
525 2014 Shotgun microbial profiling of fossil remains. *Mol. Ecol.* **23**, 1780–1798.
526 (doi:10.1111/mec.12690)
- 527 45. Pääbo S *et al.* 2004 Genetic Analyses from Ancient DNA. *Annu. Rev. Genet.* **38**, 645–
528 679. (doi:10.1146/annurev.genet.37.110801.143214)

- 529 46. Toews DPL, Brelsford A. 2012 The biogeography of mitochondrial and nuclear
530 discordance in animals. *Mol. Ecol.* **21**, 3907–3930. (doi:10.1111/j.1365-294X.2012.05664.x)
- 531 47. Webb SD. 1985 The interrelationships of tree sloths and ground sloths. In *The evolution*
532 *and ecology of armadillos, sloths, and vermilinguas*, pp. 105–112.
- 533 48. Gaudin TJ. 2004 Phylogenetic relationships among sloths (Mammalia, Xenarthra,
534 Tardigrada): the craniodental evidence. *Zool. J. Linn. Soc.* **140**, 255–305.
- 535 49. Nyakatura JA. 2012 The convergent evolution of suspensory posture and locomotion in
536 tree sloths. *J. Mamm. Evol.* **19**, 225–234. (doi:10.1007/s10914-011-9174-x)
- 537 50. Pujos F, De Iuliis G, Cartelle C. 2017 A paleogeographic overview of tropical fossil
538 sloths: Towards an understanding of the origin of extant suspensory sloths? *J. Mamm. Evol.* **24**,
539 19–38. (doi:10.1007/s10914-016-9330-4)
- 540 51. Delsuc F, Vizcaíno SF, Douzery EJ. 2004 Influence of Tertiary paleoenvironmental
541 changes on the diversification of South American mammals: a relaxed molecular clock study
542 within xenarthrans. *BMC Evol. Biol.* **4**, 11.
- 543 52. Billet G, Hautier L, Muizon C de, Valentin X. 2011 Oldest cingulate skulls provide
544 congruence between morphological and molecular scenarios of armadillo evolution. *Proc. R.*
545 *Soc. Lond. B Biol. Sci.* **278**, 2791–2797. (doi:10.1098/rspb.2010.2443)
- 546 53. Heath TA, Huelsenbeck JP, Stadler T. 2014 The fossilized birth–death process for
547 coherent calibration of divergence-time estimates. *Proc. Natl. Acad. Sci.* **111**, E2957–E2966.
548 (doi:10.1073/pnas.1319091111)
- 549 54. Marshall LG, Sempere T. 1993 Evolution of the Neotropical Cenozoic land mammal
550 fauna in its geochronologic, stratigraphic, and tectonic context. In *Biological relationships*
551 *between Africa and South America*, pp. 329–392.

- 552 55. Patterson B, Pascual R. 1968 The Fossil Mammal Fauna of South America. *Q. Rev. Biol.*
553 **43**, 409–451. (doi:10.1086/405916)
- 554 56. Martin PS, Klein RG. 1989 *Quaternary Extinctions: A Prehistoric Revolution*. University
555 of Arizona Press.
- 556 57. Fariña RA, Vizcaíno SF, Iuliis GD. 2013 *Megafauna: Giant Beasts of Pleistocene South*
557 *America*. Indiana University Press.
- 558
- 559

560 **Figure legends**

561 **Figure 1.** (a) DNA damage profiles from the *Myiodon* bone sample ($12,880 \pm 35$ ^{14}C yrpb)
562 compared to a fossil glyptodont sample (*Doedicurus* sp.) dated at ($12,015 \pm 50$ ^{14}C yrpb), a 40-
563 year-old museum specimen of greater fairy armadillo (*Calyptophractus retusus*), and three
564 modern xenarthran samples. (b) Taxonomic assignation of 223 contigs assembled from *Myiodon*
565 shotgun reads represented with Krona.

566

567 **Figure 2.** Bayesian consensus phylograms of *Pilosa* obtained under the site-heterogeneous CAT-
568 GTR+G₄ mixture model for the mitogenomic (a) and nuclear (b) datasets. Values at nodes
569 represent clade posterior probabilities under the CAT model (PP_{CAT}), mixed model (PP_{PART}), and
570 maximum likelihood bootstrap percentages under a partitioned model (BP_{ML}). Asterisks indicate
571 maximum support from all statistical indices. The complete phylograms are available in the
572 electronic supplementary material (electronic supplementary material, figures S3-S8). Graphical
573 representation and taxon images derive from Gibb et al. [21].

574

575 **Figure 3.** Bayesian chronograms of *Pilosa* obtained using a rate-autocorrelated log-normal
576 relaxed molecular clock model under the CAT-GTR+G₄ mixture model with a birth death prior
577 on the diversification process, and six soft calibration constraints for the mitogenomic (a) and
578 nuclear (b) datasets. Mean divergence dates and associated 95% credibility intervals are
579 represented as node bars. Plain black node bars indicated calibration constraints. The main
580 geological periods follow Geological Time Scale of the Geological Society of America (E =
581 Early, M = Middle, L = Late; Paleo. = Paleocene, Pli. = Pliocene, P. = Pleistocene). The
582 complete chronograms are available in the electronic supplementary material (electronic

583 supplementary material, figures S9, S10). Graphical representation and taxon images derive from
584 Gibb et al. [21].
585

586 **Tables**

587

588 **Table 1.** Divergence time estimates for the main xenarthran nodes inferred using the site-heterogeneous CAT-GTR+G₄
 589 substitution model and an autocorrelated log-normal (LN) relaxed molecular clock model. Mean posterior estimates, associated
 590 standard errors, and 95% credibility intervals are expressed in million years ago (Mean date \pm SD [95% CredI]).

Nodes	Mitogenomes	Nuclear exons
XENARTHRA*	67.3 \pm 3.2 [59.9 – 71.4]	69.3 \pm 2.2 [63.5 – 71.8]
PILOSA MRCA* (anteaters + sloths)	55.6 \pm 4.4 [46.4 – 63.2]	61.2 \pm 2.5 [55.4 – 65.0]
Folivora MRCA* (sloths)	24.8 \pm 6.2 [15.8 – 37.6]	27.7 \pm 4.0 [19.6 – 35.9]
Mylodondidae + Megalonychidae (two-fingered sloths)	21.9 \pm 5.7 [13.3 – 33.9]	22.5 \pm 3.7 [15.5 – 30.6]
Megalonychidae MRCA (two-fingered sloths)	4.7 \pm 1.5 [2.5 – 8.3]	3.8 \pm 1.1 [2.2 – 6.5]
Bradypodidae MRCA (three-fingered sloths)	14.3 \pm 4.1 [8.4 – 23.1]	12.1 \pm 2.4 [7.8 – 17.5]
<i>B. pygmaeus</i> / others	5.4 \pm 1.8 [2.8 – 9.6]	4.5 \pm 1.1 [2.7 – 7.0]
<i>B. tridactylus</i> / <i>B. variegatus</i>	4.2 \pm 1.5 [2.2 – 7.7]	3.9 \pm 1.0 [2.3 – 6.2]
Vermilingua MRCA* (anteaters)	34.2 \pm 5.1 [23.9 – 44.2]	43.7 \pm 3.2 [36.6 – 49.7]
Myrmecophaga / Tamandua	10.7 \pm 3.0 [5.6 – 17.6]	11.9 \pm 2.1 [8.4 – 16.7]

<i>T. mexicana</i> / <i>T. tetradactyla</i>	0.8 ± 0.3 [0.4 – 1.5]	2.0 ± 0.5 [1.2 – 3.2]
CINGULATA MRCA (armadillos)	44.2 ± 3.5 [37.9 – 51.5]	42.3 ± 2.4 [37.7 – 47.2]
Dasypodidae MRCA (Long-nosed armadillos)	11.5 ± 3.4 [7.2 – 20.4]	8.7 ± 1.6 [6.3 – 12.4]
Chlamyphoridae MRCA	36.6 ± 3.3 [31.2 – 44.1]	33.4 ± 2.1 [29.7 – 38.2]
Euphractinae MRCA (Hairy armadillos)	10.3 ± 2.7 [6.4 – 16.6]	6.1 ± 1.3 [4.0 – 9.2]
Chlamyphorinae / Tolypeutinae	32.4 ± 3.1 [27.8 – 39.7]	31.5 ± 2.0 [28.0 – 36.0]
Chlamyphorinae MRCA (Fairy armadillos)	19.7 ± 2.7 [15.5 – 26.3]	14.8 ± 2.4 [10.4 – 19.9]
Tolypeutinae MRCA*	25.8 ± 2.6 [22.5 – 32.5]	23.7 ± 1.3 [21.7 – 27.1]
<i>Tolypeutes</i> / <i>Cabassous</i>	22.5 ± 2.5 [19.1 – 28.8]	21.1 ± 1.5 [18.6 – 24.6]
<i>Tolypeutes</i> MRCA	13.7 ± 2.0 [10.7 – 18.4]	11.9 ± 1.5 [9.2 – 15.2]
<i>Cabassous chacoensis</i> / <i>C. unicinctus</i>	8.4 ± 1.5 [5.9 – 11.9]	6.5 ± 1.4 [4.1 – 9.4]

591

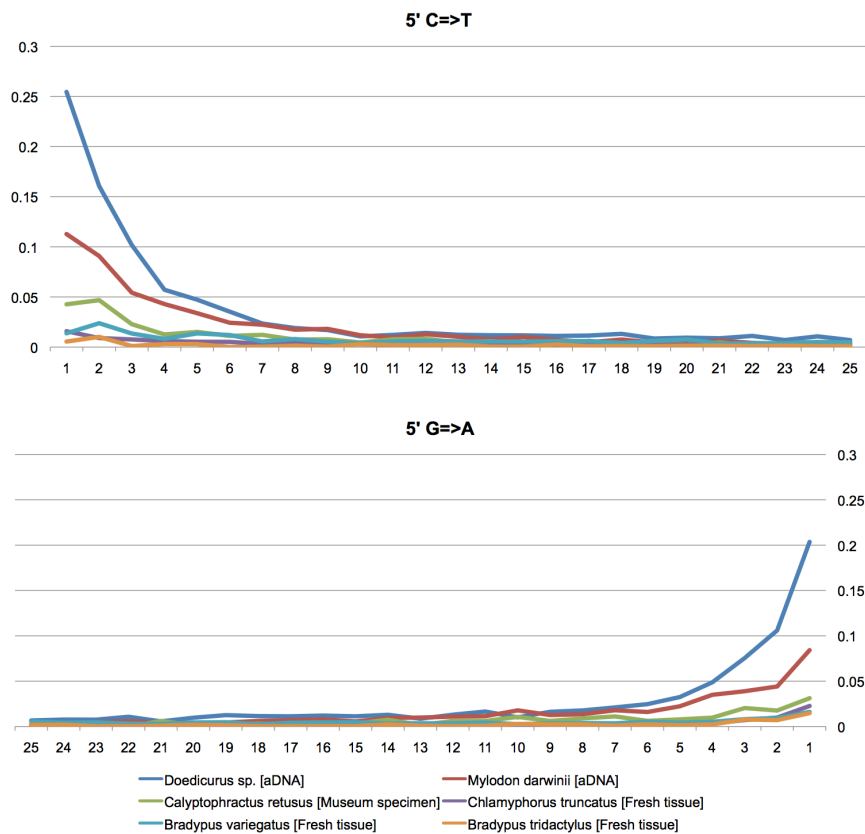
592 Notes -- SD: Standard Deviation; 95% CredI: 95% Credibility Interval; *: Used as a priori calibration constraints; MRCA: Most

593 Recent Common Ancestor.

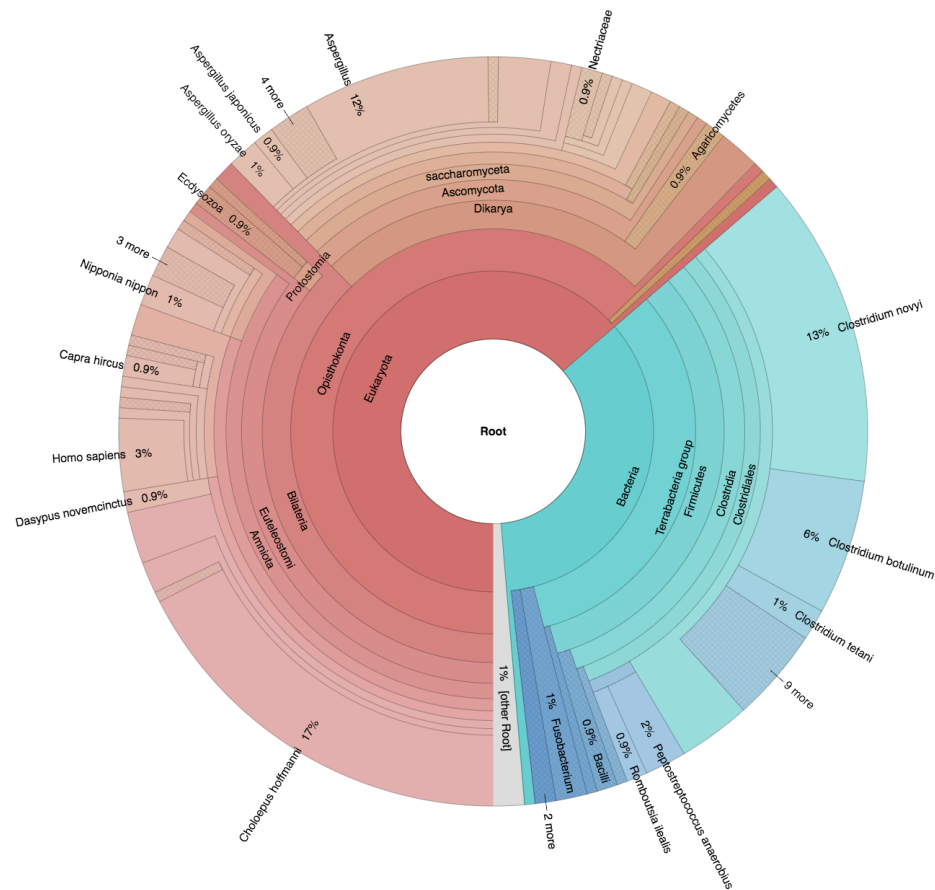
594

595

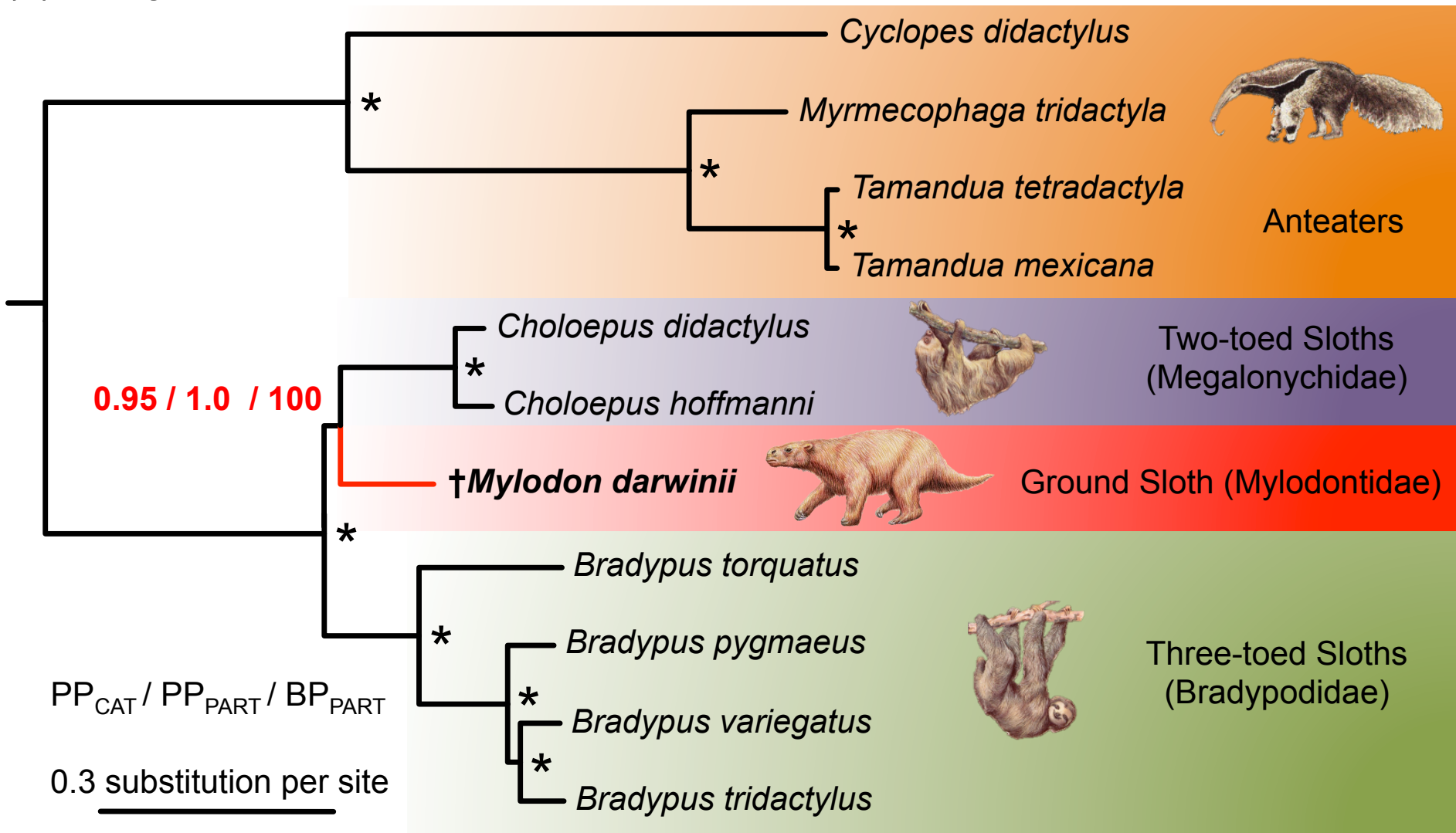
(a)



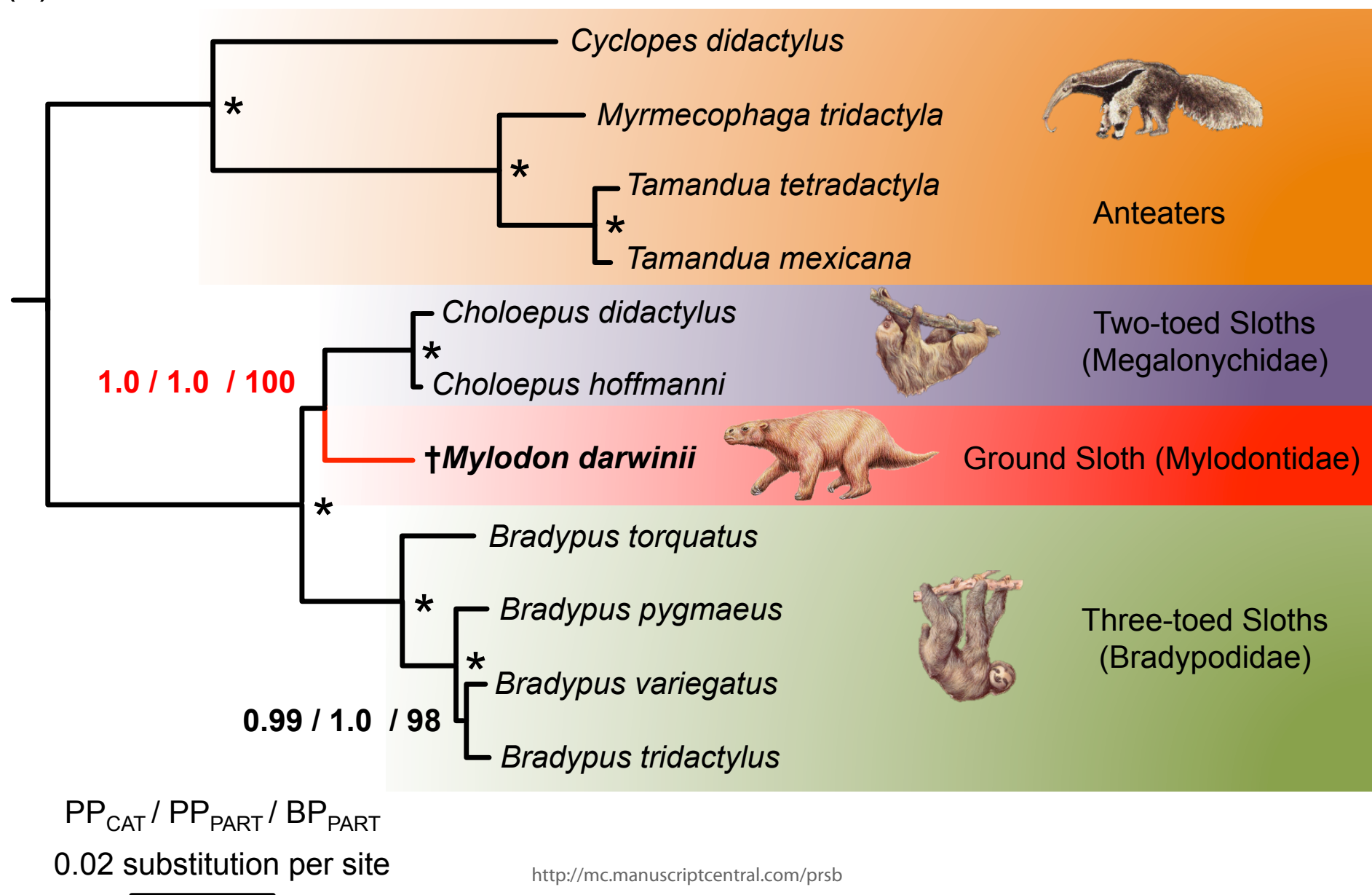
(b)



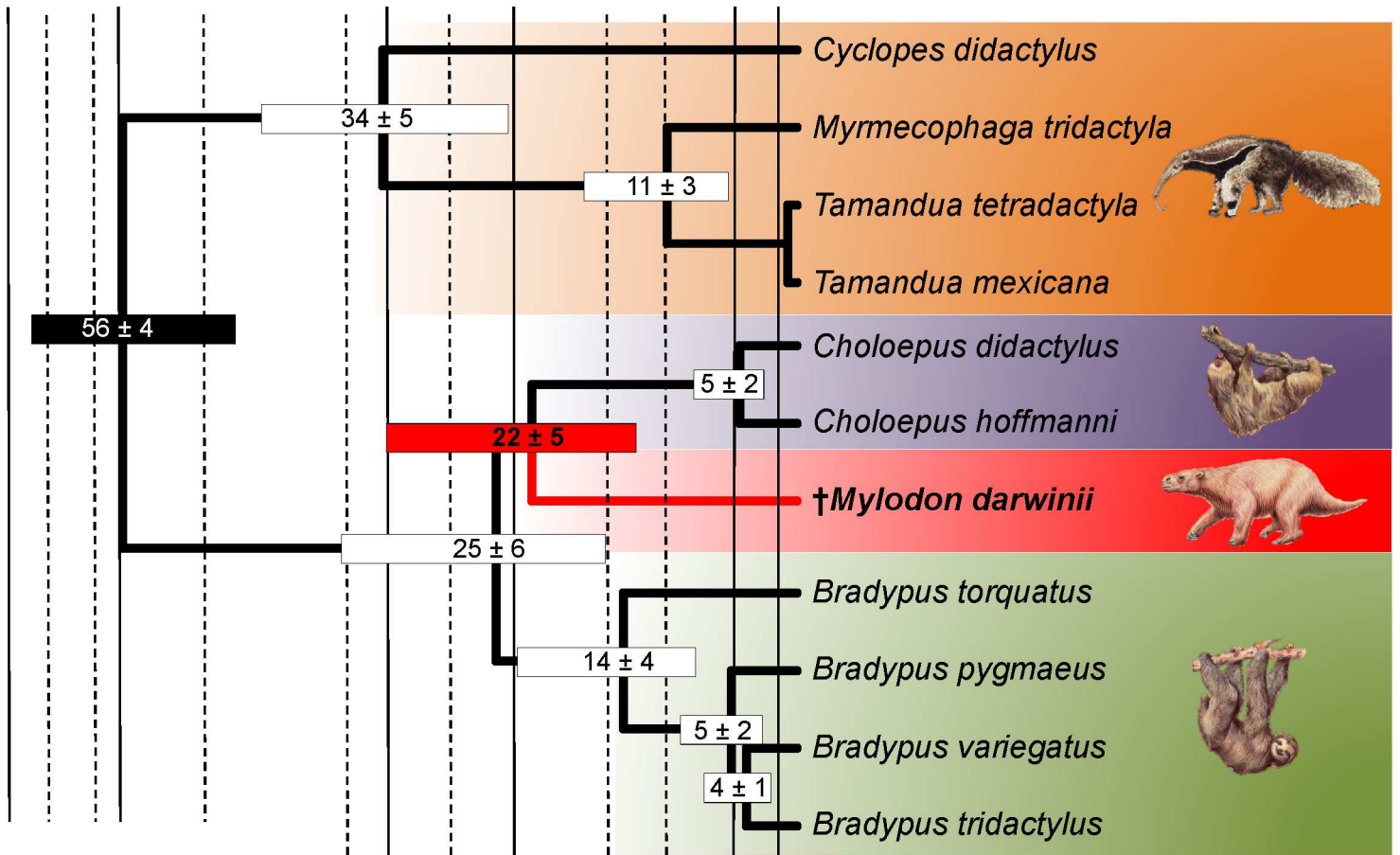
(a) mitogenomes



(b) nuclear exons



(a) mitogenomes



(b) nuclear exons

