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1 Ancient Mitogenomes Shed Light on the Evolutionary 2 History and Biogeography of Sloths

3 Frédéric Delsuc,^{1,13,*} Melanie Kuch,² Gillian C. Gibb,^{1,3} Emil Karpinski,^{2,4} Dirk
4 Hackenberger,² Paul Szpak,⁵ Jorge G. Martínez,⁶ Jim I. Mead,^{7,8} H. Gregory
5 McDonald,⁹ Ross D. E. MacPhee,¹⁰ Guillaume Billet,¹¹ Lionel Hautier,^{1,12} and
6 Hendrik N. Poinar^{2,*}

7 Author list footnotes

8 ¹Institut des Sciences de l'Evolution de Montpellier (ISEM), CNRS, IRD, EPHE, Université de
9 Montpellier, Place Eugène Bataillon, 34095 Montpellier Cedex 5, France

10 ²McMaster Ancient DNA Centre, Department of Anthropology, McMaster University, 1280 Main
11 Street West, Hamilton, ON L8S 4L8, Canada

12 ³Wildlife and Ecology Group, School of Agriculture and Environment, Massey University,
13 Centennial Drive, Hokowhitu, Palmerston North 4410, New Zealand

14 ⁴Department of Biology, McMaster University, 1280 Main Street West, Hamilton, ON L8S 4L8,
15 Canada

16 ⁵Department of Anthropology, Trent University, 1600 West Bank Drive, Peterborough, ON K9L
17 0G2, Canada

18 ⁶Instituto Superior de Estudios Sociales, CONICET-Instituto de Arqueología y Museo,
19 Universidad Nacional de Tucumán, San Martín 1545, CP4000 San Miguel de Tucumán,
20 Argentina

21 ⁷The Mammoth Site, Hot Springs, Hot Springs, SD 57747, United States of America

22 ⁸East Tennessee State University Natural History Museum, 1212 Suncrest Drive, Johnson City,
23 TN 37615, United States of America

24 ⁹Bureau of Land Management, Utah State Office, 440 West 200 South #500, Salt Lake City, UT
25 84101, United States of America

26 ¹⁰Division of Vertebrate Zoology/Mammalogy, American Museum of Natural History, Central
27 Park West & 79th Street, New York, NY 10024, United States of America

28 ¹¹Centre de Recherche en Paléontologie - Paris (CR2P), UMR CNRS 7207, Sorbonne
29 Université, Muséum National d'Histoire Naturelle, 57 Rue Cuvier, 75005 Paris, France

30 ¹²Mammal Section, Life Sciences, Vertebrate Division, The Natural History Museum, Cromwell
31 Road, South Kensington, London SW7 5BD, United Kingdom

32 ¹³Lead contact

33 *Correspondence: frederic.delsuc@umontpellier.fr (F.D.), poinarh@mcmaster.ca (H.N.P.)

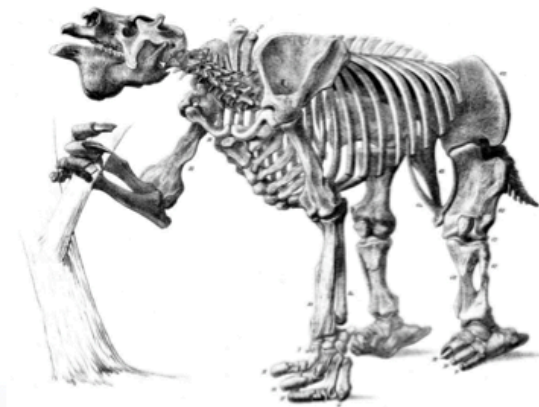
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36 Ancient DNA; Mitogenomics; Phylogenetics; Molecular dating; Extinct sloths; Morphology;
37 Convergence; Biogeography; GAARlandia.

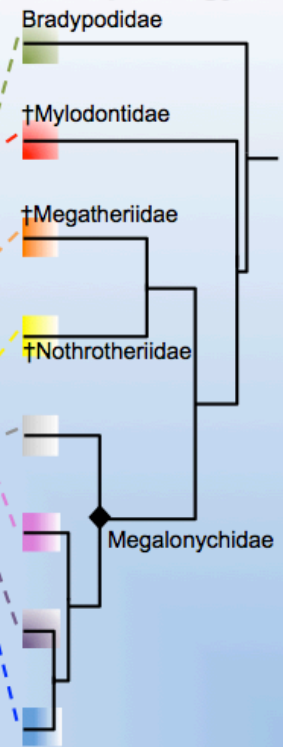
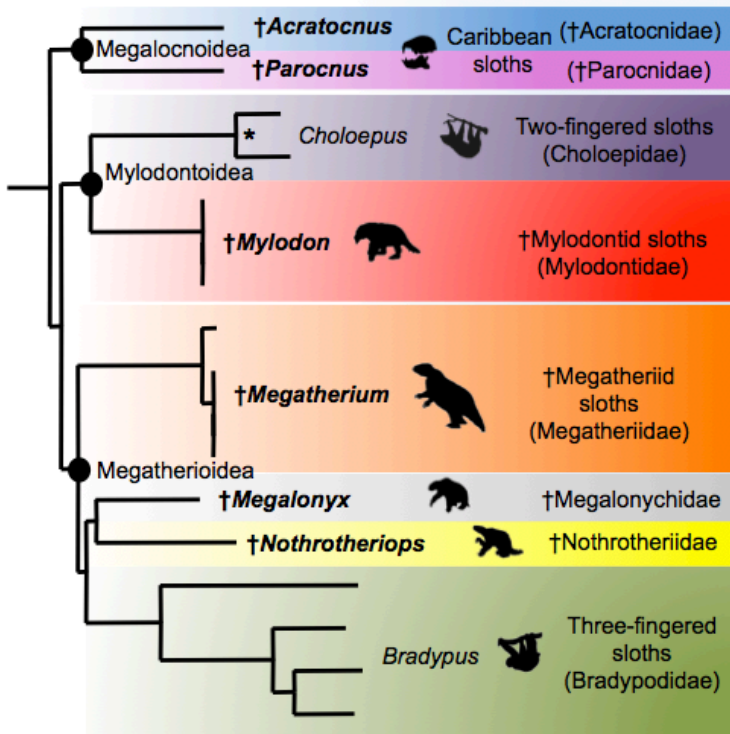
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39 GRAPHICAL ABSTRACT



Mitogenomes

Morphology



40

41

42 SUMMARY

43 Living sloths represent two distinct lineages of small-sized mammals that independently evolved
44 arboreality from terrestrial ancestors. The six extant species are the survivors of an evolutionary
45 radiation marked by the extinction of large terrestrial forms at the end of the Quaternary. Until
46 now sloth evolutionary history has mainly been reconstructed from phylogenetic analyses of
47 morphological characters. Here we used ancient DNA methods to successfully sequence 10
48 extinct sloth mitogenomes encompassing all major lineages. This includes the iconic continental
49 ground sloths *Megatherium*, *Megalonyx*, *Mylodon*, and *Nothrotheriops*, and the smaller endemic
50 Caribbean sloths *Parocnus* and *Acratocnus*. Phylogenetic analyses identify eight distinct
51 lineages grouped in three well-supported clades and whose interrelationships are markedly
52 incongruent with the currently accepted morphological topology. We show that recently extinct
53 Caribbean sloths have a single origin but comprise two highly divergent lineages that are not
54 directly related to living two-fingered sloths, which instead group with *Mylodon*. Moreover, living
55 three-fingered sloths do not represent the sister-group to all other sloths but are nested within a
56 clade of extinct ground sloths including *Megatherium*, *Megalonyx*, and *Nothrotheriops*.
57 Molecular dating also reveals that the eight newly recognized sloth families all originated
58 between 36 and 28 million years ago (Mya). The early divergence of recently extinct Caribbean
59 sloths around 35 Mya is consistent with the debated GAARlandia hypothesis postulating the
60 existence at that time of a biogeographic connection between northern South America and the
61 Greater Antilles. This new molecular phylogeny has major implications for reinterpreting sloth
62 morphological evolution, biogeography, and diversification history.

63

64 INTRODUCTION

65 Sloths (Xenarthra; Folivora) are represented today by six living species, distributed in tropical
66 forests throughout the Neotropics and conventionally placed in two genera: *Choloepus*, the two-
67 fingered sloths (two species), and *Bradypus*, the three-fingered sloths (four species). Tree sloths
68 typically weigh 4-8 kg and are strictly arboreal. However, the living species represent only a
69 small fraction of the past Cenozoic diversity of sloths. More than 100 genera of sloths have been
70 systematically described, including the large-bodied species of the Pliocene and Pleistocene
71 popularly known as ground sloths of the Ice Age. This includes the giant ground sloth
72 (*Megatherium americanum*) with an estimated body mass of more than 4000 kg, and Darwin's
73 ground sloth (*Mylodon darwini*), named for Charles Darwin who collected its first fossil remains.
74 Like their closest xenarthran relatives (anteaters and armadillos), sloths originated in South
75 America and successfully invaded Central and North America prior to the completion of the
76 Isthmus of Panama [1]. Pleistocene North American representative taxa include the Shasta
77 ground sloth (*Nothrotheriops shastensis*) and Jefferson's ground sloth (*Megalonyx jeffersonii*)
78 whose range extended up to Alaska. Late Quaternary ground sloths went extinct ~10,000 years
79 before present (yrbp) as part of the megafaunal extinction that occurred at the end of the latest
80 glaciation [2]. However, sloths also reached a number of Caribbean islands, giving rise to an
81 endemic radiation best known from Quaternary taxa (*Megalocnus*, *Neocnus*, *Acratocnus*, and
82 *Parocnus*) [3] that became extinct only shortly after the appearance of humans in the Greater
83 Antilles ~4,400 yrbp [4]. When and how sloths colonized the West Indies is still disputed. The
84 oldest accepted fossil evidence dates from the Early Miocene of Cuba [5], although discoveries
85 in Puerto Rico [6,7] demonstrate that terrestrial mammal possibly including sloths, were already
86 in the Greater Antilles by the Early Oligocene. These findings would be consistent with the
87 debated GAARlandia (GAAR: Greater Antilles + Aves Ridge) paleobiogeographic hypothesis

88 postulating the existence of a land bridge via the Aves Ridge that would have briefly emerged
89 between 35 and 33 Mya and connected northern South America to the Greater Antilles [6].

90 Until recently, the phylogenetic relationships of sloths were almost exclusively investigated
91 from analyses of morphological data. Cladistic analyses using maximum parsimony [8–11] and
92 Bayesian reconstructions [12] based predominantly on craniodental characters have
93 consistently recovered topologies defining five major sloth lineages, currently recognized as
94 families. In these phylogenetic reconstructions, modern three-fingered sloths always appear as
95 the sister-group of all other sloths and are considered to have retained a number of ancestral
96 characters [8]. Extant two-fingered sloths are also consistently found close to or nested within
97 Caribbean sloths as the sister-group of either *Acratocnus* [3] or *Neocnus* [8,12] and are
98 classified within Megalonychidae, together with other extinct sloths related to *Megalonyx*. It is
99 noteworthy, however, that there is currently no fossil that could be convincingly assigned to the
100 two independent lineages that led to extant tree sloths [13]

101 The vast majority of Quaternary sloth taxa became extinct so recently that numerous
102 remains in the form of bones, teeth, fragments of skin with hair and osteoderms, claws with their
103 keratinous sheaths, and paleofeces are still well preserved. The amount of subfossil material
104 available makes sloths an ideal group to leverage the power of ancient DNA to decipher their
105 radiation. In a pioneering study, Höss et al. [14] tested 45 samples from diverse sloth taxa, but
106 only two specimens of Darwin's ground sloth (*Myiodon darwini*) from Myiodon Cave (Chile)
107 yielded short mitochondrial ribosomal gene fragments. Recently, a bone from the same cave
108 with high endogenous DNA content allowed assembly of a high-quality complete mitogenome
109 for *Myiodon darwini* using shotgun sequencing [15]. Exceptional preservation of paleofecal
110 material of the extinct Shasta ground sloth (*Nothrotheriops shastensis*) from Gypsum Cave
111 (Nevada) enabled characterization of its diet by ancient DNA barcoding of plant remains [16,17].
112 Paleofeces from this cave also yielded short PCR-amplified mitochondrial [18] and nuclear [19]

113 sequences allowing investigation of the phylogenetic affinities among extinct and extant sloths.
114 Nowadays, DNA capture-based targeted enrichment is emerging as the method of choice in
115 ancient DNA studies. It has recently been used to reconstruct partial mitogenomes for
116 *Nothrotheriops shastensis* and *Myiodon darwinii* [20]. Moreover, it has been demonstrated that
117 baits designed from ancestral sequences reconstructed from extant xenarthran mitogenomes
118 can improve capture success from species for which there is no closely related extant taxa such
119 as the extinct glyptodont *Doedicurus* [21].

120 Both molecular [14,15,18–20] and morphological [8,9,12] phylogenetic studies have
121 supported the diphyletic origin of the two living sloth genera implying an independent evolution
122 of arboreality from terrestrial ancestors. However, molecular studies are actually in conflict with
123 morphological inferences regarding the precise phylogenetic positions of extant sloths in
124 strongly supporting a close relationship between *Choloepus* and *Myiodon* [14,15,18,20] and
125 firmly grouping *Bradypus* with *Nothrotheriops* [18–20]. In order to understand the causes of this
126 incongruence, we used ancient DNA techniques to sequence the mitogenomes of 10 extinct
127 Quaternary sloths. Phylogenetic analyses of these new mitogenomic data support a topology
128 that is markedly incongruent with the currently accepted morphological framework. Our results
129 have major implications for interpreting sloth morphological evolution and should stimulate a
130 complete rethinking of our current understanding of the evolutionary history of this group.

131

132 **RESULTS**

133 **Ten new ancient sloth mitogenomes**

134 Using capture baits designed from ancestral sequences inferred using available xenarthran
135 mitogenomes [21], we successfully captured, sequenced, and assembled nearly complete

136 mitogenomes for 10 ancient sloth samples representing the six extinct genera *Myiodon*,
137 *Megatherium*, *Megalonyx*, *Nothrotheriops*, *Parocnus*, and *Acratocnus*, and encompassing all
138 major late Quaternary sloth lineages (Table 1). Radiocarbon dates for these samples ranged
139 between $10,395 \pm 40$ radiocarbon years before present (^{14}C yrbp) for *Acratocnus ye* and $45,800$
140 ± 2000 ^{14}C yrbp for *Megalonyx jeffersonii*. Samples stemmed from diverse locations including
141 temperate and tropical regions of the continental Americas and the Greater Antilles, and from
142 different sources with osteological material and paleofeces. For five of the 10 samples, de novo
143 assembly of captured reads reconstructed a single contig covering the targeted mitogenome. To
144 ensure that our results were reproducible between experiments, we attempted capture using the
145 ancestrally designed baits on a *Myiodon darwinii* sample (Lib67) and succeeded in replicating
146 the identical mitogenome previously assembled from the same sample, but via shotgun
147 sequencing [15]. Moreover, mitogenomes from three different paleofecal samples, attributed to
148 an undetermined Megatheriinae from Peñas de las Trampas (Argentina) dated between 19,610-
149 $12,510$ ^{14}C yrbp [22,23] yielded nearly identical sequences (99.9% identity). The mitogenomes
150 from these three samples were 97% identical to one obtained from a bone of the extinct giant
151 ground sloth *Megatherium americanum*. This level of mitochondrial sequence divergence
152 typically falls within the intraspecific diversity of extant sloths [24] and implies that these
153 paleofeces likely came from *Megatherium americanum*.

154 To assess the authenticity of our ancient sloth mitogenomes, we examined the fragment
155 length distributions and the presence of DNA damage in all mapped reads. As expected, reads
156 were short (Table 1) and showed expected DNA damage patterns (Figure 1). Damage patterns
157 differed between osteological material and paleofeces, with osteological samples showing
158 higher levels of DNA damage with up to 41% cytosine deamination on the oldest bone sample,
159 *Megalonyx jeffersonii* ($45,800$ ^{14}C yrbp). Our youngest Caribbean sloth samples from the
160 Republic of Haiti also showed substantial levels of deamination (up to 33% for *Acratocnus ye*

161 and up to 35% for *Parocnus serus*). The mapped reads from the three *Megatherium*
162 *americanum* paleofecal samples from Peñas de las Trampas in the extremely arid Argentinean
163 Puna, and the paleofeces of the Shasta ground sloth (*Nothrotheriops shastensis*) from Rampart
164 cave exhibited the lowest levels of post-mortem damage (up to only 7% for *Megatherium*
165 *americanum* Lib_X18) and the highest average read lengths (Table 1). However, this seemingly
166 better preservation may be due the Uracil-DNA glycosylase and Endonuclease VIII treatment
167 used during library preparation from paleofeces [25]. The well-preserved *Myiodon darwinii* bone
168 found in Myiodon cave showed an intermediate level of DNA damage (up to 15%). In contrast,
169 the *Myiodon darwinii* osteoderm sample from the same cave presented a higher DNA damage
170 pattern similar to other osteological samples (up to 36%). Such patterns of post-mortem
171 mutations and short read lengths typical of ancient DNA molecules support the endogenous
172 origin of the reads captured from our ancient samples.

173

174 **Mitogenomic phylogeny of living and extinct sloths**

175 Phylogenetic analyses of our dataset using both maximum likelihood and Bayesian approaches
176 resulted in a topology that was markedly incongruent with the morphological tree (Figure 2). The
177 molecular phylogeny identified eight major lineages belonging to three strongly supported
178 clades, with interrelationships (Figure 2A) that are in strong conflict with morphological analyses
179 (Figure 2B). In particular, the family Megalonychidae as currently conceived was polyphyletic,
180 with three independent origins recovered for its constitutive members (extinct Jefferson's ground
181 sloth *Megalonyx jeffersonii*, extinct Caribbean sloths, and extant two-fingered sloths). While the
182 Caribbean sloth group was unambiguously monophyletic ($BP_{\text{RAxML}} = 100 / BP_{\text{IQ-TREE}} = 100 /$
183 $PP_{\text{MrBayes}} = 1.0 / PP_{\text{PhyloBayes}} = 1.0$), *Parocnus serus* and *Acratocnus ye* nevertheless belonged to
184 two deeply divergent lineages. However, this Caribbean clade was not closely related to modern
185 two-fingered sloths, nor to Jefferson's ground sloth, which is in sharp contrast to morphological

186 inferences (Figure 2B). In fact, Caribbean sloths appeared to represent the sister-group to all
187 other sloths, even though this position remained statistically uncertain ($BP_{\text{RAxML}} = 30 / BP_{\text{IQ-TREE}}$
188 $= 41 / PP_{\text{MrBayes}} = 0.68$). Extant two-fingered sloths (*Choloepus* spp.) were closely related to
189 extinct Darwin's ground sloth (*Mylodon darwini*) with strong statistical support from all methods
190 ($BP_{\text{RAxML}} = 98 / BP_{\text{IQ-TREE}} = 100 / PP_{\text{MrBayes}} = 1.0 / PP_{\text{PhyloBayes}} = 1.0$). Most phylogenetic
191 reconstruction methods also supported the grouping of Jefferson's ground sloth (*Megalonyx*
192 *jeffersonii*) with the Shasta ground sloth (*Nothrotheriops shastensis*) ($BP_{\text{RAxML}} = 74 / BP_{\text{IQ-TREE}} =$
193 $78 / PP_{\text{MrBayes}} = 1.0$). These two extinct lineages were the sister-group of modern three-fingered
194 sloths (*Bradypus* spp.) with good support ($BP_{\text{RAxML}} = 75 / BP_{\text{IQ-TREE}} = 89 / PP_{\text{MrBayes}} = 1.0 /$
195 $PP_{\text{PhyloBayes}} = 1.0$). Three-fingered sloths thus did not represent the sister-group of all other sloth
196 species as had been concluded by morphological studies (Figure 2B). Instead, they were firmly
197 nested within a strongly supported clade composed of the extinct giant ground sloth
198 *Megatherium* together with *Megalonyx* and *Nothrotheriops* ($BP_{\text{RAxML}} = 85 / BP_{\text{IQ-TREE}} = 94 /$
199 $PP_{\text{MrBayes}} = 1.0 / PP_{\text{PhyloBayes}} = 1.0$).

200

201 **Molecular dating of the sloth radiation**

202 The molecular chronogram obtained under the autocorrelated lognormal (LN) relaxed clock
203 model (Figure 3A) revealed an ancient origin of the eight newly identified sloth lineages. Their
204 rapid diversification occurred in a narrow time window of less than 10 million years (Myr), in the
205 Late Eocene / Early Oligocene, between approximately 36 and 28 Mya. The two earliest
206 divergences within the sloth radiation almost perfectly coincided with the Eocene / Oligocene
207 boundary (33.9 Mya). The early emergence of Caribbean sloths (Node 1) was estimated at $35 \pm$
208 5 Mya and the separation of the two other major clades of sloths (Node 4) at 34 ± 5 Mya. The
209 ancient monophyletic origin of Caribbean sloths was compatible with the GAARlandia

210 hypothesis (35-33 Mya). The ancient divergence between the two Caribbean sloths (Node 2)
211 was estimated to 29 ± 5 Mya. Within the second major sloth clade (Node 3), modern two-
212 fingered sloths (*Choloepus* spp.) and the extinct Darwin's ground sloth (*Mylodon darwini*) also
213 diverged 29 ± 5 Mya. Within the third major sloth clade (Node 5), the extinct giant ground sloth
214 (*Megatherium americanum*) split from the other three lineages at 31 ± 5 Mya, modern three-
215 fingered sloths diverged from the extinct Jefferson's ground sloth (*Megalonyx jeffersonii*) and
216 Shasta ground sloth (*Nothrotheriops shastensis*) at 29 ± 5 Mya (Node 7), which in turn
217 separated at 28 ± 5 Mya (Node 6). Posterior density distributions of mean divergence times
218 illustrated the synchronicity of many divergences among the eight sloth lineages (Figure 3B).
219 Very similar distributions centering on the Early to Late Oligocene transition at 29 Mya were
220 obtained for the divergences between *Parocnus* and *Acratocnus* (Node 2), *Choloepus* and
221 *Mylodon* (Node 3), and *Bradypus* versus *Megalonyx* + *Nothrotheriops* (Node 7). Similarly, the
222 age distributions of the two earliest splits (Nodes 1 and 4) were centered on the Eocene /
223 Oligocene boundary and contemporaneous with the proposed GAARlandia land bridge.

224

225 **Reconstruction of the ancestral sloth dental formula**

226 The sloth dentition in most taxa shows a morpho-functional distinction between an anteriorly
227 located caniniform and the molariforms that form the tooth row (Figure 4A). In order to
228 reinterpret dental character evolution on a sloth phylogeny including most available fossils, we
229 used our newly inferred molecular topology as a backbone in maximum likelihood and
230 parsimony reconstructions of ancestral character states performed on the morphological matrix
231 of Varela et al. [12]. Both methodologies retrieved consistent results, but reconstructions of the
232 sloth ancestral dental formula differed depending on whether the molecular backbone was
233 enforced or not (Figure 4B). All reconstructions proposed an ancestral dental formula of five

234 upper and four lower teeth for sloths, in association with the absence of diastema, and the
235 caniniform shape of the anterior most teeth (Figure 4; characters 2(0), 6(0), 19(1), and 21(0)).
236 The main differences involved the size of the upper (Cf) and lower (cf) caniniforms. When
237 considering the topology of the unconstrained morphological analyses, the reduced condition of
238 the caniniforms (characters 13(0) and 14(0)) was reconstructed as ancestral, while
239 reconstructions using a molecularly constrained topology retrieved large caniniforms (characters
240 13(1) and 14(1)) as the ancestral state.

241

242 **DISCUSSION**

243 **A revised phylogeny and taxonomy for living and extinct sloths**

244 Our mitogenomic tree revisits the phylogenetic relationships among living and extinct sloths
245 compared to the currently accepted morphological picture. Mitochondrial genomes have
246 limitations as phylogenetic markers with cases of mito-nuclear discordance resulting from
247 ancient hybridization events reported in mammals [26,27]. The relatively short internal branches
248 might also reflect the occurrence of incomplete lineage sorting. However, a parallel study of
249 sloth phylogeny based on ancient nuclear collagen proteins independently corroborates our
250 mitogenomic results [28]. The high congruence observed between the mitochondrial and
251 nuclear genome results provides substantial evidence for the newly proposed sloth phylogeny.

252 Based on this extensively revised phylogeny and reevaluated timescale, we propose a new
253 taxonomic framework for sloths (Folivora), in which the eight molecularly identified lineages are
254 recognized as distinct families (Figure 2A). Some of these molecular lineages correspond to
255 traditional families: Bradypodidae, Mylodontidae, Megatheriidae, and Nothrotheriidae. However,
256 Megalonychidae as classically defined is polyphyletic and should be divided into distinct

257 families. We propose that the family Megalonychidae be restricted to genus *Megalonyx* and
258 meaningfully related genera, and to classify extant two-fingered sloths of the genus *Choloepus*
259 in the monotypic family Choloepodidae. As the two distinct lineages of Caribbean sloths
260 diverged at about the same time as the other newly defined families, we propose respectively
261 elevating the Acratocnini and Parocnini tribes [3] to family level into Acratocnidae and
262 Parocnidae. Finally, we recommend reorganizing sloth superfamily names and content so that
263 they correspond to the three strongly supported main clades recovered in all our analyses
264 (Figures 2A): Megalocnoidea (Acratocnidae and Parocnidae), Mylodontoidea (Mylodontidae and
265 Choloepidae), and Megatherioidea (Megatheriidae, Megalonychidae, Nothrotheriidae, and
266 Bradypodidae). This newly proposed taxonomic framework would hopefully be adopted in
267 systematic paleontological studies to reassess the numerous Cenozoic fossil taxa for which
268 molecular data are inaccessible. Such a reassessment is needed to make sense of the rich
269 sloth fossil record in light of available molecular data.

270

271 **Reinterpreting sloth evolution in light of the new molecular phylogeny**

272 The new molecular results are in strong conflict with cladistic [3,8,10,29] and Bayesian [12]
273 analyses of morphological characters (Figure 2). However, in the details, analyses of
274 morphological characters provide only limited statistical support for most proposed suprafamilial
275 relationships. Gaudin [8] recognized that alternative hypotheses respectively placing *Bradypus*
276 with Megatheriidae and *Choloepus* with Mylodontidae, as suggested by early molecular studies
277 [14,18] and confirmed by our analyses, could not be statistically rejected. The Bayesian analysis
278 of Varela et al. [12] also provides a tenuous phylogenetic signal as indicated by the large
279 proportion of nodes receiving posterior probability < 0.95. These observations illustrate the

280 limited power of existing morphological matrices for resolving higher-level phylogenetic
281 relationships within sloths.

282 Such an apparently high level of incongruence between morphology and molecules is
283 reminiscent of the case of placental mammals until molecular studies [30] revealed an
284 unsuspected high level of morphological homoplasy [31]. Our new molecular phylogenetic
285 framework likewise suggests that numerous morphological characters used to reconstruct sloth
286 interfamilial relationships must have evolved convergently. The most striking example of
287 morphological convergence in sloths concerns Megalonychidae. The molecular evidence
288 demonstrates that, as currently defined, Megalonychidae is polyphyletic, with three independent
289 origins for the lineages represented by *Megalonyx*, *Choloepus*, and the Caribbean sloths. Yet
290 the monophyly of this clade has been consistently retrieved in morphological studies [3,8,10,12].
291 Gaudin [8], for example, recovered 20 unequivocal synapomorphies supporting
292 Megalonychidae, most of which were related to features of the trenchant caniniforms (Figure 4).
293 The strength of this argument depends on the validity of the assumption that tooth row structure
294 as seen in *Bradypus* is ancestral, while that of *Choloepus* is derived, which was ultimately
295 influenced by the early branching position of *Bradypus* on the sloth morphological phylogeny [8].
296 The dental formula of extinct and extant sloths is surprisingly conservative, as it never exceeds
297 five upper and four lower teeth (Figure 4). However, the homology between the upper and lower
298 caniniforms in *Choloepus* and *Bradypus* has recently been reinterpreted based on
299 developmental data. Hautier et al. [32] showed that the dental pattern of *Bradypus* might
300 represent a neotenic condition with the retention of a deciduous caniniform and the absence of a
301 functional caniniform in adults. They suggested that a large permanent caniniform as observed
302 in *Choloepus* could represent the ancestral condition for sloths. Our ancestral reconstruction
303 under the molecular constraint indicating large caniniforms as the most likely ancestral state for
304 sloths is in line with this developmental scenario, as well as with the presence of a large

305 caniniform in *Pseudoglyptodon*, considered to be the earliest fossil sloth [33]. This finding that
306 dental homologies have been misinterpreted between the two living sloth genera mitigates the
307 potential weight of dental features related to the size and shape of the caniniforms in
308 phylogenetic and systematic studies. In all cases, the utmost caution should be used when
309 coding dental features that are prone to functional convergence.

310 An unexpected outcome of our molecular investigation is that the endemic Caribbean sloths
311 are not closely related to extant two-fingered sloths of the genus *Choloepus*, but instead
312 represent one of the three main clades of the sloth radiation. This is a radical departure from the
313 prevailing morphological consensus that has prevailed for decades [3,10,34]. Gaudin [8],
314 however, also noted that *Choloepus* shares a number of craniodental characters with
315 Mylodontidae that he interpreted as convergences. In light of our results confirming the close
316 relationship between *Choloepus* and *Mylodon* revealed by previous molecular studies
317 [14,15,18,20], these characters might in fact constitute true synapomorphies for this clade as
318 originally intuited in pre-cladistic studies of comparative anatomy [35,36]. Moreover, our results
319 challenge the position of living three-fingered sloths of the genus *Bradypus* as the sister-group
320 to all other sloths retrieved in most morphological studies [8–10,12]. Instead, we found strong
321 support for *Bradypus* being nested within a clade of extinct ground sloths, including the Shasta
322 ground sloth *Nothrotheriops* as proposed by previous molecular studies [18–20], but also the
323 giant ground sloth *Megatherium americanum*, and Jefferson's ground sloth *Megalonyx*
324 *jeffersonii* (Figure 2A). Here also Gaudin [8] noticed a number of seemingly convergent
325 morphological features between *Bradypus* and Megatheriidae, which ought to be re-evaluated
326 as signatures of common ancestry as suggested by early anatomical studies [35–37].

327

328

329 **A new timescale for sloth evolution and biogeography**

330 Our molecular dating results unveil a rapid diversification at the base of the sloth radiation with
331 an almost synchronous origin of the three main clades at the Eocene / Oligocene boundary ~35
332 Mya followed by the divergence of all eight major lineages in a narrow time window framing the
333 Early Oligocene between 31 and 28 Mya (Figure 3). This time period corresponds to a global
334 glacial maximum characterized by the formation of the Antarctic ice sheet and the set up of the
335 circum-Antarctic oceanic current following the abrupt decrease in terrestrial temperature at the
336 Eocene-Oligocene transition [38]. In South America, this prompted the transition from humid
337 tropical forest environments to drier and more open habitats [39]. According to our molecular
338 estimates these environmental changes might have triggered the diversification of sloth families
339 among other mammalian herbivore communities. The fossil record nevertheless implies at most
340 an Early Miocene origin for most sloth families [40]. Our results favor a long-fuse model of sloth
341 diversification, with molecular estimates of interfamilial divergences predating their
342 paleontological origin by more than 10 Myr. This model invites a reconsideration of the
343 taxonomic status of Oligocene sloth fossils with uncertain relationships, such as *Orophodon*,
344 *Octodontotherium*, and *Deseadognathus*, in light of the apparent antiquity of the newly defined
345 families.

346 Unsurprisingly, given the major differences between morphological and molecular
347 topologies, our mitogenomic timescale markedly contrasts with the one recently obtained by
348 Varela et al. [12] using a Bayesian morphological clock model combined with tip-dating. This
349 directly affects the timing of the origins of the two living sloth lineages given their revised
350 phylogenetic positions. With regard to three-fingered sloths, their divergence from all other
351 sloths was estimated at ~40 Mya with morphological data [12], whereas our estimate places the
352 separation of *Bradypus* from its relatives *Nothrotheriops* and *Megalonyx* at ~29 Mya (Figure 3).
353 However, the most notable inconsistency between morphological and molecular estimates

354 concerns the timing of the Caribbean sloth radiation, formerly thought to include extant two-
355 fingered sloths (*Choloepus*) based on morphological data. The morphological clock results place
356 the divergence between *Acratocnus* and *Parocnus* at only ~8 Mya and the divergence between
357 *Parocnus* and *Choloepus* at ~5 Mya [12]. In striking contrast, our molecular timescale indicates
358 that the two monophyletic Caribbean sloth genera diverged ~29 Mya, which is almost identical
359 to our dating of the separation of *Choloepus* and *Myiodon* (Figure 3). So ancient a divergence
360 between the species *Acratocnus ye* and *Parocnus serus*, both endemic to Hispaniola [3], implies
361 an early diversification of insular sloths within the West Indies. The megalocnoids subsequently
362 diversified (Figure 5), likely in part through island-island vicariance as the land masses
363 comprising present day Cuba-Hispaniola-Puerto Rico drifted apart in the Miocene [41]. The
364 early fossil record for the diversification of Caribbean sloths is, however, very limited. A partial
365 femur of uncertain affinities found in the Early Oligocene of Puerto Rico was tentatively
366 attributed to “Megalonychidae” (species A in Figure 5; [6]). The only other non-Quaternary fossil
367 is *Imagocnus zazaе* from the Early Miocene of Cuba, which has clear folivoran affinities (Figure
368 5; [42]). Given the deep divergence between Parocnidae and Acratocnidae, it is likely that other
369 ancient sloth fossils remain to be found in the Greater Antilles. Overall, our molecular dating
370 results show that recent Quaternary extinctions wiped out six of the eight newly identified sloth
371 families that originated in the Early Oligocene more than 28 Mya, including two ancient endemic
372 Caribbean sloth lineages.

373 From the biogeographical point of view, the rapid radiation of the three major sloth lineages,
374 including the Caribbean clade, is consistent with a single colonization of the Caribbean islands
375 taking place around 35 Mya. This estimation would be compatible with the debated GAARlandia
376 hypothesis, which postulates the brief existence 33-35 Mya of a land bridge that subaerially
377 united northernmost South America and the Greater Antilles-Aves Rise magmatic arc [6,41]
378 (Figure 5). This landspan is thought to correspond to the uplift of the Aves Ridge, a paleo-island

379 arc that is now submerged in the Caribbean Sea, west of the current Lesser Antilles. As
380 originally conceived, the GAARlandia hypothesis was based on mammal distributions, and
381 attempted to explain how several South American groups might have managed to reach the
382 islands without invoking overwater dispersal. More recently, molecular phylogenies obtained for
383 other terrestrial Caribbean mammals have mostly rejected the hypothesis, because the origin of
384 the investigated taxon was either too ancient in the case of solenodontids [43,44] or too recent
385 for capromyid [45] and sigmodontine [46] rodents, and primates [47]. Sloths are thus the first
386 Caribbean mammalian group for which molecular dating based on mitogenomics provides
387 support for GAARlandia. The dispersal of other terrestrial Caribbean taxa may have been
388 enabled by this temporary dispersal corridor, including a genus of toads [48] and three different
389 groups of spiders [49–51]. The existence of this dispersal corridor would also explain the
390 presence of caviomorph rodent fossils of South American origin in the Greater Antilles by the
391 Early Oligocene [7].

392 Overall, our new molecular phylogenetic framework and timescale tell a story of sloth
393 evolution very different from that of the one previously told by morphology alone. Our results
394 have important implications for reinterpreting many aspects of sloth evolution that have been
395 previously based on the morphological phylogenetic picture such as morpho-functional
396 adaptations [9], body size evolution [52,53], and macroevolutionary patterns [12]. We hope our
397 study will stimulate a complete rethinking of the evolutionary history of sloths with reassessment
398 of morphological characters in light of the significant amount of convergence revealed by the
399 new molecular framework.

400

401

402

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417

418 **AUTHOR CONTRIBUTIONS**

419 Conceptualization: F.D., H.N.P.; Data curation: M.K., E.K., H.N.P.; Formal analysis: F.D., L.H.,
420 G.B.; Funding acquisition: F.D., L.H., H.N.P.; Investigation: M.K., G.C.G., E.K., D.H., P.S.,
421 H.N.P.; Resources: F.D., J.G.M., J.I.M., H.G.M., R.D.E.M., H.N.P.; Supervision: F.D., H.N.P.;
422 Validation: F.D., H.N.P.; Visualization: F.D., L.H.; Writing – original draft: F.D., L.H.; Writing –
423 review & editing: F.D., M.K., G.C.G., E.K., J.G.M., H.G.M., R.D.E.M., G.B., L.H., H.N.P.

424

425 **DECLARATION OF INTERESTS**

426 The authors declare no competing interests.

427

428 **FIGURE LEGENDS**

429 **Figure 1. DNA damage profiles of mapped mitochondrial reads for the 10 different**
430 **libraries.** The fragment misincorporation plots represent the frequency of cytosine deamination
431 per position at both strands of mapped sequence reads (5' C=>T and 3' G=>A).

432

433 **Figure 2. Mitogenomic versus morphological phylogenies of living and extinct sloths.** (A)
434 Maximum likelihood phylogram obtained with RAxML under the best partition model for sloth
435 mitogenomes. Values at nodes represent maximum-likelihood bootstrap percentages under the
436 best partition model using RAxML (BP_{RAxML}) and IQ-TREE ($BP_{\text{IQ-TREE}}$), and clade posterior
437 probabilities under the best partition model using MrBayes (PP_{MrBayes}) and the CAT-GTR mixture
438 model using PhyloBayes ($PP_{\text{PhyloBayes}}$). An asterisk (*) indicates strong support from all statistical
439 indices ($BP \geq 95$ and $PP \geq 0.99$) whereas a dash (-) indicates that the node was not recovered
440 with the corresponding method. Taxa in bold are those sequenced in this study. Colors highlight
441 the eight newly proposed families and bullets (●) the three new superfamilies. Complete
442 phylograms are available as Figures S1-S4. See also Tables S1-S3. (B) Time-calibrated
443 phylogenetic relationships among the main sloth lineages as reconstructed from morphological
444 data showing the five currently recognized families: Bradypodidae (limited to the extant three-
445 fingered sloths in the genus *Bradypus*), Mylodontidae (extinct sloths related to *Mylodon*),
446 Megatheriidae (extinct sloths related to *Megatherium*), Nothrotheriidae (extinct sloths related to

447 *Nothrotheriops*), and Megalonychidae (including extinct sloths related to *Megalonyx*, extinct
448 Caribbean sloths, and extant two-fingered sloths of the genus *Choloepus*) (modified from [12]).
449 Dash lines highlight the incongruence between the molecular and the morphological topologies.
450 Silhouettes are from phylopic.org.

451

452 **Figure 3. Time-calibrated phylogeny of modern and ancient sloths based on complete**

453 **mitogenomes.** (A) Bayesian chronogram obtained using PhyloBayes under the CAT-GTR+G4
454 mixture model and the best-fitting autocorrelated lognormal (LN) relaxed molecular clock model.
455 Colors highlight the eight newly proposed families. The complete chronogram with 95%
456 credibility intervals is available as Figure S5. (B) Bayesian posterior density distributions of
457 divergence dates for the seven numbered nodes representing the diversification of the eight
458 newly recognized sloth families. The main geological periods follow the geological time scale of
459 the Geological Society of America (E, early; M, middle; L, late; Paleo., Paleocene; Pli., Pliocene;
460 P., Pleistocene). Silhouettes are from phylopic.org.

461

462 **Figure 4. Reinterpretation of dental evolution in sloths under the new phylogenetic**

463 **framework.** (A) 3D reconstructions of the skulls of a two-fingered sloth (*Choloepus didactylus*
464 UM 789N; left) and a three-fingered sloth (*Bradypus tridactylus* MZS 03557; right) showing the
465 six characters used for reconstructing the sloth ancestral dental features with states illustrated
466 following Varela et al. [12]: character #6, diastema [(0) absent or rudimentary; (1) elongate];
467 #13, size of upper caniniform (Cf) [(0) smallest tooth; (1) greatly enlarged; (2) neither the
468 smallest nor enlarged]; #14, size of lower caniniform (cf) [(0) smallest tooth; (1) greatly enlarged;
469 (2) neither the smallest nor enlarged]; #19, morphology of Cf/cf [(0) molariform; (2) caniniform;
470 (3) incisiform]; #21, position of Cf relative to the anterior edge of the maxilla [(0) right at the

471 edge; (1) near the edge; (2) well-separated from the anterior edge]; #23, fossa on palatal
472 surface of maxilla posterior to Cf [(0) absent; (1) present]. (B) Schematic representations of the
473 upper and lower tooth rows in *Choloepus* (left) and *Bradypus* (right), and maximum likelihood
474 reconstructions of the sloth ancestral dental morphotype based respectively on the
475 unconstrained (left) and constrained (right) ML topologies using a molecular backbone inferred
476 from the morphological character matrix of Varela et al. [12].

477

478 **Figure 5. Biogeographical context of the extinct Caribbean sloth radiation.** Distribution of
479 sloth fossil remains in the Greater and Lesser Antilles with recent Quaternary extinct species (†)
480 and Tertiary fossils (*) (adapted from [3]). Species sequenced in this study are shown in bold.
481 Species A corresponds to a small femur found in the Oligocene of Puerto Rico with uncertain
482 sloth affinities [6]. The Aves Ridge is an ancient volcanic arc that is now entirely submerged in
483 the Caribbean Sea. The dashed arrow indicates the hypothesized GAARlandia land bridge
484 linking northern South America to the Greater Antilles around the Eocene-Oligocene transition
485 (33-35 Mya) resulting from the uplift of the Aves Ridge at that time. Bathymetric map courtesy of
486 NOAA National Centers for Environmental Information.

487

488 **TABLE LEGEND**

489 **Table 1:** Sample origins, radiocarbon dates, and mitogenome assembly statistics.

490

491

492 **STAR * METHODS**

- 493 • Key resources table
- 494 • Contact for resource sharing
- 495 • Experimental model and subject details
- 496 • Method details
- 497 • Data and software availability

498

499 **CONTACT FOR RESOURCE SHARING**

500 Further information and requests for resources should be directed to, and will be fulfilled by the
501 lead contact author Frédéric Delsuc (Frederic.Delsuc@umontpellier.fr).

502

503 **EXPERIMENTAL MODEL AND SUBJECT DETAILS**

504 The 10 extinct sloth samples used in this study come from different specimen sources and are
505 stored in natural history museums in Europe, USA, and Argentina (Table 1). For Darwin's
506 ground sloth (*Mylodon darwini*), we used two different samples both collected at Mylodon Cave
507 (Última Esperanza, Chile) in the form of a bone (NHMUK PV M8758) stored at the Natural
508 History Museum (London, UK) and a skin sample with osteoderms (MNHN 1905-4) stored at the
509 Muséum National d'Histoire Naturelle (Paris, France). The *Mylodon* bone NHMUK PV M8758
510 was previously used to obtain a complete mitogenome using shotgun sequencing [15] of the
511 same library as the one used here for sequence capture. For Jefferson's ground sloth
512 (*Megalonyx jeffersonii*), we used a bone (PMA P98.6.28) collected at Big Bone Cave (TN, USA)

513 and conserved at the Academy of Natural Sciences of Drexel University (Philadelphia, PA,
514 USA). For Shasta's ground sloth (*Nothrotheriops shastensis*), we used a paleofeces (RC L12
515 #1) collected at Rampart Cave (AZ, USA) and conserved at the Desert Lab at Arizona State
516 University and collected by the late Paul S. Martin. For the giant ground sloth (*Megatherium*
517 *americanum*), we had access to a rib bone sample (MAPB4R 3965) from Los Chaceras
518 (Argentina) conserved in the Museo de la Asociación Paleontológica (Bariloche, Río Negro,
519 Argentina). We also used three paleofeces from two different layers (C.2C_Layer 2, C.2E_Layer
520 4_1, C.2E_Layer 4_2) attributed to an undetermined Megatheriinae from Peñas de las Trampas
521 1.1 archeological site (Catamarca, Argentina) and deposited in the Institute of Archaeology and
522 Museum of the National University of Tucumán (IAM-UNT; Tucumán, Argentina). Our analyses
523 have shown that those paleofeces most likely came from the giant ground sloth (*Megatherium*
524 *americanum*). Finally, for the two Caribbean sloths *Acratocnus ye* and *Parocnus serus*, we used
525 a mandible with molars (UF 76365) and a bone (UF 75452) collected at two different localities
526 from the Département de l'Ouest of the Republic of Haiti, respectively. Both samples are stored
527 in the collections of the Florida Museum of Natural History (Gainesville, FL, USA).

528

529 **METHOD DETAILS**

530 **Radiocarbon dating**

531 Aliquots of freeze-dried ultrafiltered gelatin prepared from each sample were radiocarbon dated
532 by the Keck Carbon Cycle AMS facility of the University of California Irvine (USA).

533

534 **DNA extraction and library preparation from bone**

535 Subsampling of bones was done in a dedicated ancient DNA laboratory facility at the McMaster
536 Ancient DNA Centre for *Myiodon darwinii* MNHN 1905-4 (40 mg), *Myiodon darwinii* NHMUK PV
537 M8758 (300 mg), *Acratocnus ye* UF 76365 (360 mg), *Parocnus serus* UF 75452 (360 mg),
538 *Megalonyx jeffersonii* PMA P98.6.28 (300 mg), and *Megatherium americanum* MAPB4R 3965
539 for which three subsamples were taken from the rib cross section (187-285 mg). Each
540 subsample was further reduced to small particle sizes of 1-5 mm using a hammer and chisel.
541 The subsamples were then demineralized with 0.5 M EDTA (pH 8.0) for 24 h at room
542 temperature with agitation, and the supernatant removed following centrifugation. The pellets
543 were digested using a Tris-HCl-based (20 mM, pH 8.0) proteinase K (250 µg/ml) digestion
544 solution with 0.5% sodium lauroyl sarcosinate (Fisher Scientific), 1% polyvinylpyrrolidone (PVP,
545 Fisher scientific), 50 mM dithiothreitol (DTT), 2.5 mM N-phenacyl thiazolium bromide (PTB,
546 Prime Organics), and 5 mM calcium chloride (CaCl₂). Proteinase digestions were performed for
547 24 h at room temperature with agitation. Following centrifugation, the digestion supernatants
548 were removed and pooled with the demineralization supernatants. This process was repeated
549 three to four times, pooling supernatants with the original rounds. Organics were then extracted
550 from the pooled supernatants using phenol:chloroform:isoamyl alcohol (PCI, 25:24:1), and the
551 resulting post-centrifugation aqueous solution was again extracted with chloroform. The final
552 aqueous solution was concentrated using 10 kDA Amicon centrifuge filters (Millipore) at 4000 x
553 g or 14,000 x g depending on filter volume used (Amicon Ultra 0.5 ml or Amicon Ultra 4 ml), with
554 up to four washes of 0.1x TE buffer (pH 8) to provide a final desalted concentrate of 50 µl. For
555 *Megatherium americanum* MAPB4R 3965, demineralization and digestion were carried out
556 similarly to other bone samples, with modifications based on in-house optimization. Pooled
557 demineralization and digestion supernatants were extracted using the "Method B" extraction
558 procedure outlined in Glocke and Meyer [54], except eluted off the column in 50 µl of EBT.
559 Extraction blanks were carried alongside each sample during the entire extraction procedure to
560 monitor for possible external contamination during handling.

561 Ancient DNA extracts and extraction blanks were finally purified with a MinElute column
562 (Qiagen) to 50 µl EBT and converted to a double-stranded, Illumina sequencing library
563 according to the protocol developed by Meyer and Kircher [55] with the following modifications:
564 1) the reaction volume for blunt-end repair was reduced to 40 µl with 25ul template; 2) all SPRI
565 purification steps were substituted by spin column purification (MinElute PCR purification kit,
566 Qiagen), and 3) adapter ligation was performed overnight at 16°C. For *Megatherium*
567 *americanum* MAPB4R 3965, three libraries (L1043, L1044, and L1045) were generated from the
568 three independent subsamples of the same specimen. These libraries were constructed from 20
569 µl of each purified extract as input in 40 µl reactions as above, with modifications in the End
570 Repair step to accommodate the switch from NEBuffer 2 to NEBuffer 2.1, and the removal of
571 Uracil-DNA glycosylase and Endonuclease VIII treatment.

572 **DNA extraction and library preparation from paleofeces**

573 Subsampling of paleofeces was performed in a dedicated ancient DNA laboratory facility at the
574 McMaster Ancient DNA Centre for *Megatherium americanum* IAM-UNT C.2C_Layer2 (160 mg),
575 *Megatherium americanum* IAM-UNT C.2E_Layer4_1 (140 mg), *Megatherium americanum* IAM-
576 UNT C.2E_Layer4_2 (120 mg), and *Nothrotheriops shastensis* RC L12 #1 (130 mg). Using
577 tweezers and scalpels subsamples were further reduced to small particle sizes of 1-5 mm. Each
578 subsample was then incubated with a Guanidinium thiocyanate buffer (6 M GuSCN, 20 mM Tris
579 (pH 8.0), 0.5% sodium lauroyl sarcosinate, 8 mM DTT, 4% PVP, and 10 mM PTB for 20 h at
580 37°C with agitation, and the supernatant removed following centrifugation. 500 µl of supernatant
581 were then purified using MinElute columns eluting to a final volume of 25 µl with 0.1x TE plus
582 0.05 % Tween. Extraction blanks were carried alongside each sample during the entire
583 extraction procedure to monitor for possible external contamination during handling.

584 Ancient DNA extracts and extraction blanks were converted into Illumina blunt-ended
585 libraries as described by Meyer and Kircher [55] with the following modifications: 1) the reaction
586 volume for blunt-end repair was reduced to 50 μ l with 25 μ l template; 2) buffer Tango (10x) was
587 substituted with NE Buffer 2 (10x); 3) BSA was added to the blunt-end repair reaction at a final
588 concentration of 0.1 mg/ml; 4) T4 polynucleotide kinase was reduced to a final concentration of
589 0.4 U/ μ l; 5) Uracil-DNA glycosylase and Endonuclease VIII were added to the blunt-end repair
590 reaction at a final concentration of 0.1 U/ μ l and 0.4 U/ μ l respectively; 6) the blunt-end repair
591 reaction was incubated at 37°C for 3 h without the addition of T4 DNA polymerase and again
592 after the addition of T4 DNA polymerase at a final concentration of 0.2U/ μ l at 25°C for 15 min
593 and 12°C for 15 min; 7) all SPRI purification steps were substituted by spin column purification
594 (MinElute PCR purification kit as suggested by Kircher et al. [56]); 8) adapter concentration in
595 the ligation reaction was reduced to 0.25 μ M of each adapter as suggested by Kircher et al. [56];
596 9) adapter ligation was performed overnight at 16°C; 10) Bst polymerase was increased to a
597 final concentration of 0.4 U/ μ l; and 11) no purification step was performed after adapter fill-in
598 with Bst polymerase but instead, the enzyme was heat inactivated at 80°C for 20 min following
599 Kircher et al. [56].

600 **Library indexing, qPCR assay, target enrichment, and sequencing**

601 Constructed libraries were then double-indexed with P5 and P7 indexing primers [56] in a 50 μ l
602 reaction containing 1x Herculase II Reaction Buffer, 250 μ M each dNTP, 0.5x EvaGreen, 400
603 nM of each primer, 0.5 μ l Herculase II Fusion DNA Polymerase, and 10 μ l library. Cycling
604 conditions were 95°C for 2 min, 10 amplification cycles of (95°C for 15 sec, 60°C for 20 sec,
605 72°C for 30 sec), and a final extension of 72°C for 3 min. Amplifications were performed using a
606 MJ thermocycler (BioRad). Reactions were purified again with MinElute to 15 μ L EBT. For
607 *Megatherium americanum* MAPB4R 3965, heat-deactivated libraries were indexed using 12.5 μ l
608 of template with unique P5 and P7 indexes, with an increased primer concentration (750 nM)

609 and 1X KAPA SYBR®FAST qPCR Master Mix as this method produces less PCR artifacts than
610 Herculase II Fusion DNA Polymerase. To ensure that libraries contained endogenous DNA after
611 preparation, and that the blank extract libraries did not, each indexed library was subjected to a
612 quantitative PCR assay specifically targeting a 47 bp portion of the xenarthran mitochondrial
613 16S rRNA gene using primers Xen_16S_F2 and Xen_16S_R2 [21]. The following protocol
614 employing 1 µL of the library in a total reaction volume of 10 µl was used: 1x PCR Buffer II, 2.5
615 mM MgCl₂, 250 µM dNTP mix, 1 mg/ml BSA, 250 nM each primer, 0.5x EvaGreen, 0.5 U
616 AmpliTaq Gold.

617 To maximize the capture of mitochondrial DNA from potentially divergent extinct sloth taxa,
618 the 5207 RNA baits previously designed using ancestral sequence reconstruction from a
619 representative sample of xenarthran mitogenomes were used [21]. These baits target the whole
620 mitogenome except the control region that is too repetitive to be reliably assembled with short
621 reads and too variable to be aligned among xenarthrans. The corresponding MYbaits targeted
622 enrichment kits were synthesized by Arbor Biosciences (<https://arborbiosci.com/>). A first round
623 of enrichment at 50°C was performed, followed by a second round at 55°C using 7.47 µl of
624 indexed library for 36-39 h, following the manufacturer's protocol. Phosphate-group end-blocked
625 oligonucleotides matching one strand of the regions flanking the 7 bp indexes of the library
626 adapters were included. A quantity of 25 ng of baits per reaction was used as it has been shown
627 to be sufficient for very sensitive capture of a small target region [57]. Following hybridization,
628 the reaction was cleaned according to the suggested protocol except that we used 200 µl rather
629 than 500 µl volumes of wash buffers for each wash step, to accommodate a 96-well plate-
630 format. Hot washes were performed at 50-55°C. The enriched library was eluted and then
631 purified with MinElute to 15 µl EBT, which we then re-amplified according to the protocol above
632 and again purified this time to 10 µl EBT. For *Megatherium americanum* MAPB4R 3965,

633 enrichment was carried out using the optimized protocol outlined in Karpinski et al. [58] using 5
634 μ l of purified indexed library and 100 ng of bait set.

635 The enriched libraries were size-selected for fragment between 150 bp to 600 bp, pooled,
636 and sequenced at McMaster Genomics Facility on the Illumina HiSeq 1500 system using the
637 TruSeq Rapid (v1) chemistry with initial hybridization on the cBot. Each lane included a 1%
638 spike-in of Illumina's PhiX v3 control library. Paired-end reads of either 2 x 90 bp (*Megatherium*
639 *americanum* libraries) or 2 x 110 bp (all other libraries) were generated, along with dual 7 bp
640 indexing on both runs.

641 **Mitogenome assembly and annotation**

642 Adapter and index tag sequences were trimmed from raw sequence reads using CutAdapt
643 v1.16 [59]. Trimmed reads were then imported into Geneious Prime [60]. For each sample,
644 reads were mapped against the *Homo sapiens* reference mitogenome sequence (NC_012920)
645 using the “Low Sensitivity / Fastest” mapping strategy of Geneious Prime. Matching reads were
646 excluded as human contamination and de novo assembly of the remaining reads was then
647 performed using the metagenomic assembler MEGAHIT v1.1.1 [61]. Mitochondrial contigs were
648 then identified by mapping MEGAHIT contigs of each sample against its closest reference
649 xenarthran genome using the “High Sensitivity / Medium” mapping option of Geneious Prime. In
650 the five cases for which multiple contigs were identified, draft partial mitogenomes were created
651 by filling regions that lacked any coverage with question marks. Iterative mapping of
652 deduplicated reads was then conducted using the “Low Sensitivity / Fastest” mapping strategy
653 of Geneious Prime until there were no further improvements in extending coverage into the gap
654 regions of the consensus sequence. The resulting partial mitogenomes were scanned by eye to
655 check for the inclusion of any conflicting reads that might represent contaminants. The final
656 partial mitogenomes were annotated by manually reporting annotations after pairwise alignment

657 with their closest xenarthran reference mitogenome using MAFFT v7.388 G-INSI [62] within
658 Geneious Prime. The depth of coverage was estimated by remapping deduplicated reads to
659 each partial mitogenome using the “Low Sensitivity / Fastest” mapping strategy of Geneious
660 Prime.

661 **DNA damage analyses**

662 To check the authenticity of our newly obtained mitogenomes, we examined the patterns of
663 DNA damage caused by post-mortem mutations using mapDamage v2.0.8 [63]. We screened
664 our sequenced libraries for the presence of an excess of C-T and G-A transitions by mapping
665 non-duplicated reads against their corresponding reconstructed consensus mitogenomes.

666 **Mitogenomic dataset construction**

667 We selected available mitogenomes for 25 living xenarthran species that are representative of
668 the xenarthran diversity. We then added previously obtained mitogenomes from the extinct
669 glyptodont (*Doedicurus* sp.) and extinct Darwin’s ground sloth (*Mylodon darwini*), as well as our
670 10 newly generated mitogenome sequences, and three afrotherian outgroup taxa. A careful
671 comparison between our nearly complete *Nothrotheriops shastensis* mitogenome obtained from
672 a paleofecal sample from Rampart Cave in Arizona and that of a partial mitogenome (9364 bp)
673 from Gypsum Cave in Nevada produced by Slater et al. [20] revealed a number of discrepancies
674 resulting in only 7183 identical sites between the two sequences. As most of these differences
675 are likely the result of sequencing or assembly errors in the Slater et al. [20]’s *Nothrotheriops*
676 sequence, as previously shown also for *Mylodon darwini* [15], we have used our more complete
677 and accurate sequences for these two taxa. All mitochondrial genes except the mitochondrial
678 control region, which has not been sequenced for most of the extinct taxa, were extracted from
679 the mitogenome annotations. The 24 tRNA and the two rRNA genes were then aligned at the
680 nucleotide level using MAFFT G-INSI within Geneious Prime, and the translation-align option

681 was used to align the 13 protein-coding genes based on their amino acid sequences. Selection
682 of unambiguously aligned sites was performed on each individual gene data set with Gblocks
683 v0.91b [64] using default relaxed settings and the codon option for protein-coding genes. The
684 final concatenation contained 15,157 unambiguously aligned nucleotide sites for 40 taxa.

685 **Phylogenetic reconstructions**

686 The best-fitting partition schemes and associated optimal models of sequence evolution were
687 determined using both PartitionFinder v2.1.1 [65] and ModelFinder [66]. In both cases, the
688 greedy algorithm was used starting from 42 a priori defined partitions corresponding to the three
689 codon positions of the 13 protein-coding genes ($3 \times 13 = 39$ partitions), the 12S (1) and 16S
690 rRNAs (1), and all 24 concatenated tRNAs (1). Branch lengths have been unlinked and the
691 Bayesian Information Criterion (BIC) was used for selecting the best-fitting partition scheme in
692 all cases (Tables S1-S3). Maximum Likelihood reconstructions were conducted under the best-
693 fitting partitioned models with both RAxML v8.1.22 [67] and IQ-TREE v1.6.6 [68] linking
694 branches across the best-fitting partitions. Maximum Likelihood bootstrap values (BP_{RAxML} and
695 $BP_{\text{IQ-TREE}}$) were computed by repeating the same ML heuristic search on 100 nonparametric
696 bootstrap pseudo-replicates.

697 Bayesian phylogenetic inference under the best-fitting partition model was conducted using
698 MrBayes v3.2.6 [69] with model parameters unlinked across partitions. Two independent runs of
699 four incrementally heated Metropolis Coupling Markov Chain Monte Carlo (MCMCMC) starting
700 from a random tree were performed. MCMCMC were run for 10,000,000 generations with trees
701 and associated model parameters sampled every 1000 generations. The initial 2500 trees of
702 each run were discarded as burn-in samples after convergence check as determined by
703 monitoring the average standard deviation of split frequencies (ASDSF) between the two runs
704 ($\text{ASDSF} < 0.05$) and effective sample size ($\text{ESS} > 100$) and potential scale reduction factor

705 (1.00 < PSRF < 1.02) values of the different parameters. The 50% majority-rule Bayesian
706 consensus tree and associated clade posterior probabilities ($PP_{MrBayes}$) were then computed
707 from the 15,000 combined trees sampled in the two independent runs.

708 Bayesian phylogenetic reconstruction was also conducted under the CAT-GTR+G4 mixture
709 model using PhyloBayes MPI v1.7b [70]. Two independent Markov Chain Monte Carlo (MCMC)
710 starting from a random tree were run for 18,000 cycles with trees and associated model
711 parameters sampled every cycle. The initial 1800 trees (10%) sampled in each MCMC run were
712 discarded as the burn-in after convergence checking by monitoring the ASDSF between the two
713 independent runs (<0.05) and the effective sample sizes (ESS > 1000) of the different
714 parameter values using PhyloBayes diagnostic tools *bpcomp* and *tracecomp*, respectively. The
715 50% majority-rule Bayesian consensus tree and the associated posterior probabilities
716 ($PP_{PhyloBayes}$) was then computed using *bpcomp* from the remaining combined 32,400 (2 x
717 16,200) trees.

718 **Molecular dating**

719 Dating analyses were conducted using PhyloBayes v4.1c [71] under the site-heterogeneous
720 CAT-GTR+G4 mixture model [72] and a relaxed clock model with a birth–death prior on
721 divergence times [73] combined with soft fossil calibrations [74]. As calibration priors, we used
722 five node intervals as determined from the fossil record following Gibb et al. [75]: 1)
723 Paenungulata (maximum age 71.2 Mya, minimum age 55.6 Ma); 2) Xenarthra (maximum age
724 71.2 Mya, minimum age 58.5 Mya); 3) Pilosa (maximum age 65.5 Mya, minimum age 31.5
725 Mya); 4) Vermilingua (maximum age 61.1 Mya, minimum age 15.97 Ma); and 5) Tolyteutinae
726 (maximum age 37.8 Mya, minimum age 23.0 Mya). The ancestral Folivora node was left
727 unconstrained. The prior on the root of the tree (Placentalia) was set at 100 Mya according to

728 Meredith et al. [30]. The topology was fixed to the tree previously inferred in the RAxML, IQ-
729 TREE, and MrBayes analyses under the best fitting partition model.

730 Selection of the best-fitting clock model was performed using the cross-validation procedure
731 as implemented in PhyloBayes. The autocorrelated lognormal model (LN; [76], the uncorrelated
732 gamma (UGAM) relaxed clock model [77], and a strict molecular clock (CL) model were
733 compared. The cross-validation tests were performed by dividing the original alignment in a
734 learning set of 13,642 sites and a test set of 1515 sites. The overall procedure was randomly
735 replicated 10 times for which a MCMC chain was run on the learning set for a total 1100 cycles
736 sampling posterior rates and dates every cycle. The first 100 samples of each MCMC were
737 excluded as the burn-in period for calculating the cross-validation scores averaged across the
738 10 replicates in order to determine the number of time a given model fits the data better than the
739 reference model. Cross-validation tests indicated that both the autocorrelated lognormal (LN)
740 and the uncorrelated gamma (UGAM) models offered a much better fit to our mitogenomic
741 dataset than a strict molecular clock (CL) model (LN vs. CL: 32.5 ± 7.0 ; UGAM vs. CL: $29.3 \pm$
742 6.9). Between the two relaxed clock models, LN was the best fitting model (LN vs. UGAM: $3.2 \pm$
743 2.8).

744 The final dating calculations were conducted using PhyloBayes under the best-fitting CAT-
745 GTR+G mixture model and an autocorrelated lognormal relaxed clock with a birth–death prior
746 on divergence times combined with soft fossil calibrations. We run two independent MCMC
747 chains for a total 50,000 cycles sampling parameters every 10 cycles. The first 500 samples
748 (10%) of each MCMC were excluded as the burn-in after convergence diagnostics based on
749 ESS of parameters using *tracecomp*. Posterior estimates of divergence dates were then
750 computed from the remaining 4500 samples of each MCMC using the *readdiv* subprogram.
751 Posterior density plots of mean divergence times were then computed by using the R packages

752 ape v5.0 [78] to extract mean dates from sampled chronograms and ggridges v0.5.1 [79] to plot
753 the overlapping distributions.

754

755 **Ancestral reconstructions of dental characters**

756 Maximum likelihood reconstruction of sloth phylogeny was performed on the morphological
757 matrix of Varela et al. [12] using RAxML under the MK+GAMMA model with: 1) the same
758 topological constraint that the original authors used in their Bayesian reconstructions, and 2) the
759 molecular topology used as a backbone constraint. Maximum likelihood estimation of ancestral
760 character states was then conducted for six dental characters using Mesquite v3.6 [80] using
761 the Mk model on the two ML topologies previously obtained with RAxML. A similar investigation
762 was realized using maximum parsimony for the tree search with the same matrix and
763 constraints using PAUP* v4.0b10 [81] and for the estimation of ancestral character states using
764 Mesquite. The six dental characters from Varela et al. [12] are: #6, diastema ([0] absent or
765 rudimentary; [1] elongate); #13, size of Cf ([0] smallest tooth; [1] greatly enlarged; [2] neither the
766 smallest nor enlarged); #14, size of cf ([0] smallest tooth; [1] greatly enlarged; [2] neither the
767 smallest nor enlarged); #19, morphology of Cf/cf ([0] molariform; [2] caniniform; [3] incisiform);
768 #21, position of Cf relative to the anterior edge of the maxilla ([0] right at the edge; [1] near the
769 edge; [2] well-separated from the anterior edge); #23, fossa on palatal surface of maxilla
770 posterior to Cf ([0] absent; [1] present).

771 High-resolution microtomography (microCT) of the skulls of a two-fingered sloth (*Choloepus*
772 *didactylus* UM 789N; Université de Montpellier, France) and a three-fingered sloth (*Bradypus*
773 *tridactylus* MZS 03557; Musée Zoologique de Strasbourg, France) was performed at the
774 Montpellier Rio Imaging (MRI) platform using a Microtomograph RX EasyTom 150 with X-ray

775 source 40-150 kV. The 3D reconstructions of the skulls were performed with Avizo 9.4.0
776 (Visualization Sciences Group).

777

778 **DATA AND SOFTWARE AVAILABILITY**

779 Annotated mitogenomes have been deposited in GenBank (Accession Numbers MK903494-
780 MK903503) and the corresponding raw Illumina reads in the European Nucleotide Archive
781 (PRJEB32380). Additional data, including capture bait sequences, alignments, and trees can be
782 retrieved from zenodo.org (DOI: 10.5281/zenodo.2658746).

783

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1014

Species	Common name	Family	Sample type	Radiocarbon 14C age BP	Museum	Specimen voucher	Library	Origin	Mean read length (bp)	Mean coverage	MEGAHIT mito Contigs
<i>Myiodon darwinii</i>	Darwin's ground sloth	Myiodontidae	HP1502 Skin with osteoderms	13,360 ± 40	Muséum National d'Histoire Naturelle (Paris, France)	MNHN 1905-4	Lib_16	Myiodon Cave (Última Esperanza, Chile)	44.5	567X	5
<i>Myiodon darwinii</i>	Darwin's ground sloth	Myiodontidae	HP1554 Bone	12,880 ± 35	Natural History Museum (London, UK)	NHMUK PV M8758	Lib_67	Myiodon Cave (Última Esperanza, Chile)	54.3	465X	1
<i>Megalonyx jeffersonii</i>	Jefferson's ground sloth	Megalonychidae	HP1652 Bone	45,800 ± 2000	Academy of Natural Sciences of Drexel University (Philadelphia, PA, USA)	PMA P98.6.28	Lib_69	Big Bone Cave (TN, USA)	56.2	271X	1
<i>Nothrotheriops shastensis</i>	Shasta ground sloth	Nothrotheriidae	HP1904 Paleofeces	28,460 ± 320	The Desert Lab, Arizona State University (Tempe, AZ, USA)	RC L12 #1	Lib_X32	Rampart cave (AZ, USA)	88.4	402X	1
<i>Megatherium americanum</i>	Giant ground sloth	Megatheriidae	HP3613 Rib bone	19,050 ± 80	Museo de la Asociación Paleontológica (Bariloche, Argentina)	MAPB4R 3965	Lib_1043, Lib_1044, Lib_1045	Los Chaceras (Bariloche, Argentina)	57.5	2277X	4
<i>Megatherium americanum</i>	Giant ground sloth	Megatheriidae	HP2087 Paleofeces	12,920 ± 190 - 12,510 ± 240*	Institute of Archaeology and Museum of the National University of Tucumán (Tucumán, Argentina)	C.2C_Layer 2	Lib_X18	Peñas de las Trampas 1.1 (Catamarca, Argentina)	93.9	192X	1
<i>Megatherium americanum</i>	Giant ground sloth	Megatheriidae	HP2093 Paleofeces	19,610 ± 290*	Institute of Archaeology and Museum of the National University of Tucumán (Tucumán, Argentina)	C.2E_Layer 4_1	Lib_X23	Peñas de las Trampas 1.1 (Catamarca, Argentina)	63.2	108X	7
<i>Megatherium americanum</i>	Giant ground sloth	Megatheriidae	HP2095 Paleofeces	19,610 ± 290*	Institute of Archaeology and Museum of the National University of Tucumán (Tucumán, Argentina)	C.2E_Layer 4_2	Lib_X25	Peñas de las Trampas 1.1 (Catamarca, Argentina)	81.8	335X	1
<i>Acratocnus ye</i>	Hispaniolan ground sloth	Acratocnidae	HP1655 Mandible with molar	10,395 ± 40	Florida Museum of Natural History (Gainesville, FL, USA)	UF 76365	Lib_58	Trouing de la Scierie (Département de l'Ouest, Republic of Haiti)	49.6	135X	10

<i>Parocnus serus</i>	Greater Haitian ground sloth	Parocnidae	HP1602 Bone	NA	Florida Museum of Natural History (Gainesville, FL, USA)	UF 75452	Lib_54	Trouing Marassa (Département de l'Ouest, Republic of Haiti)	55.2	66X	17
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* dated by Martínez [23]. NA : not available.

Figure 1

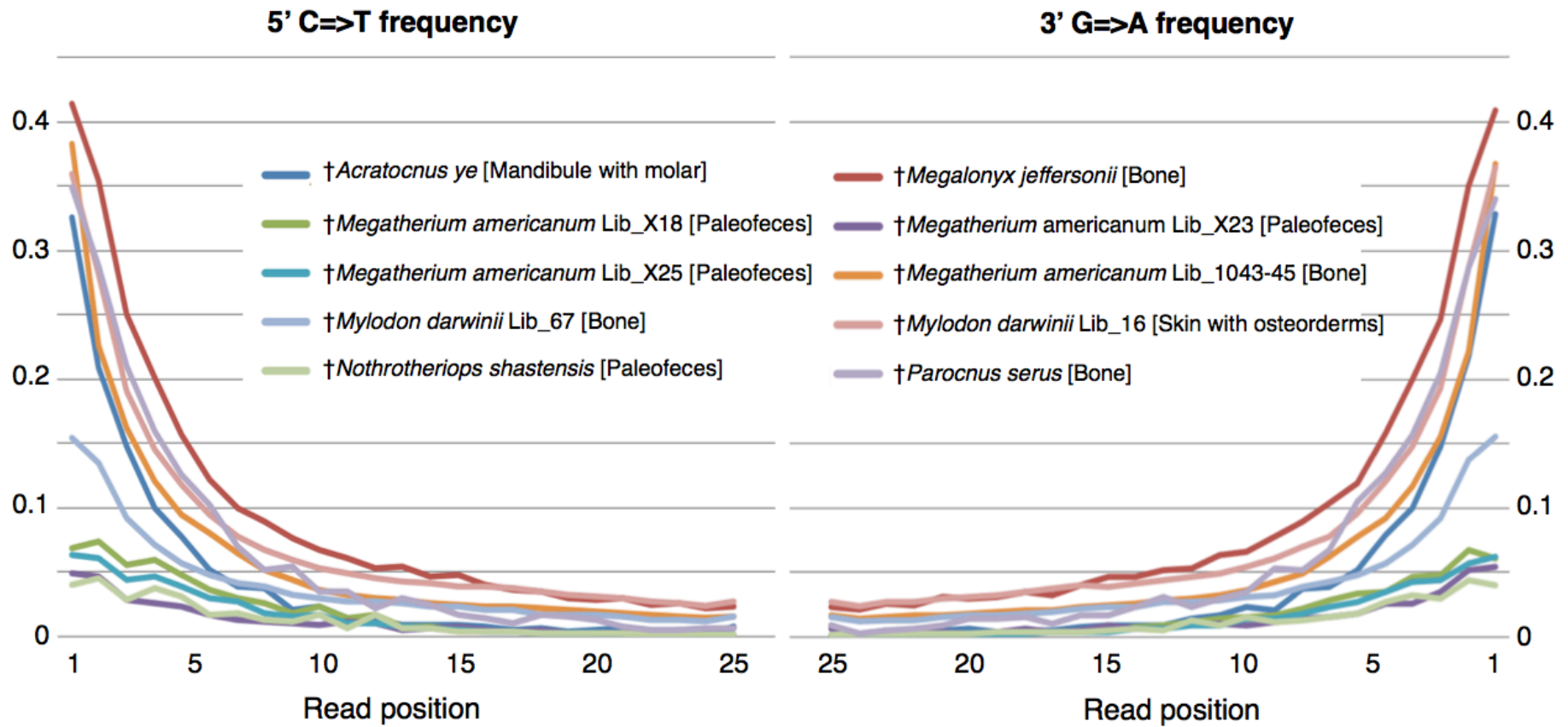


Figure 2

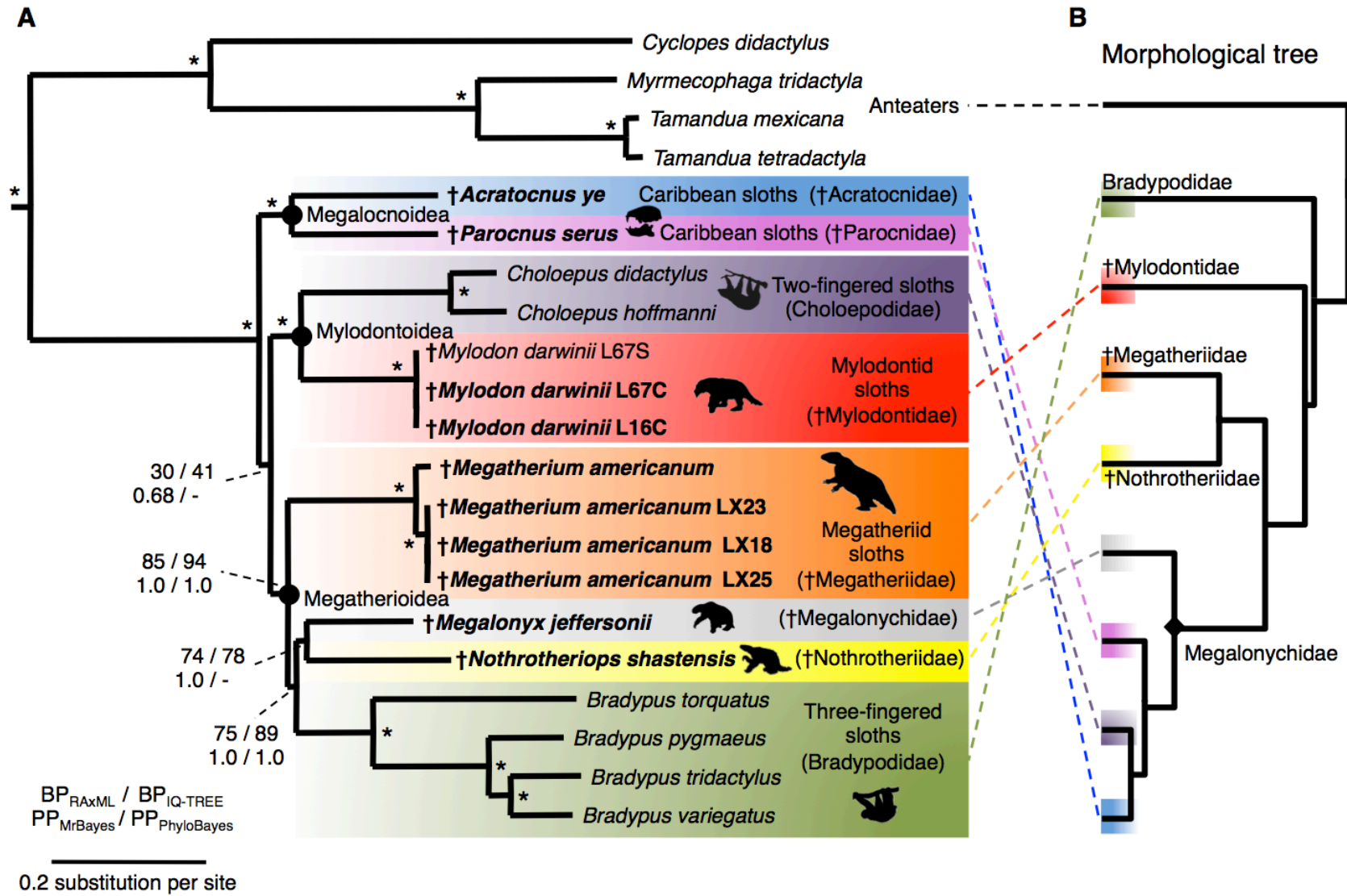


Figure 3

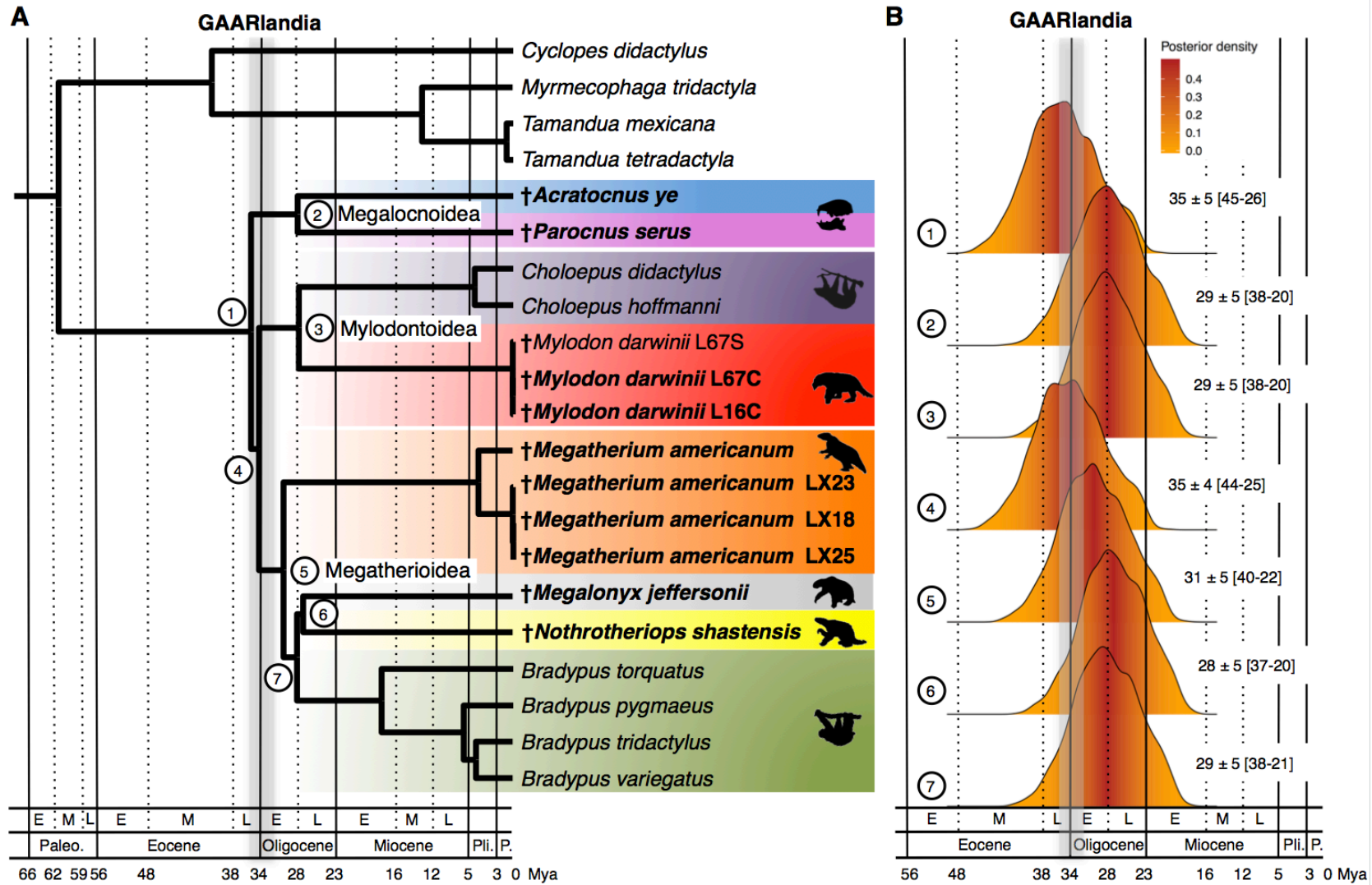


Figure 4

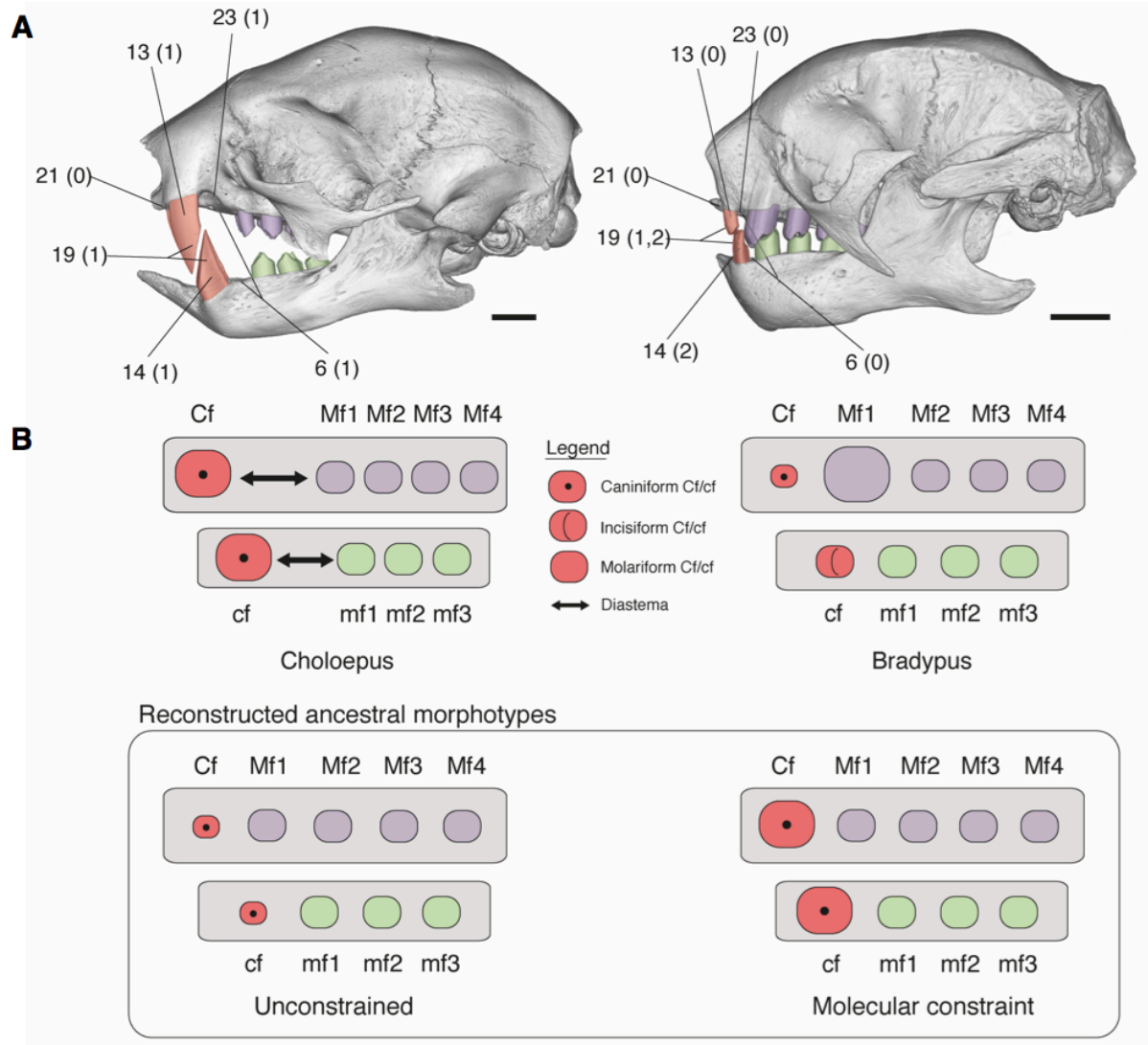
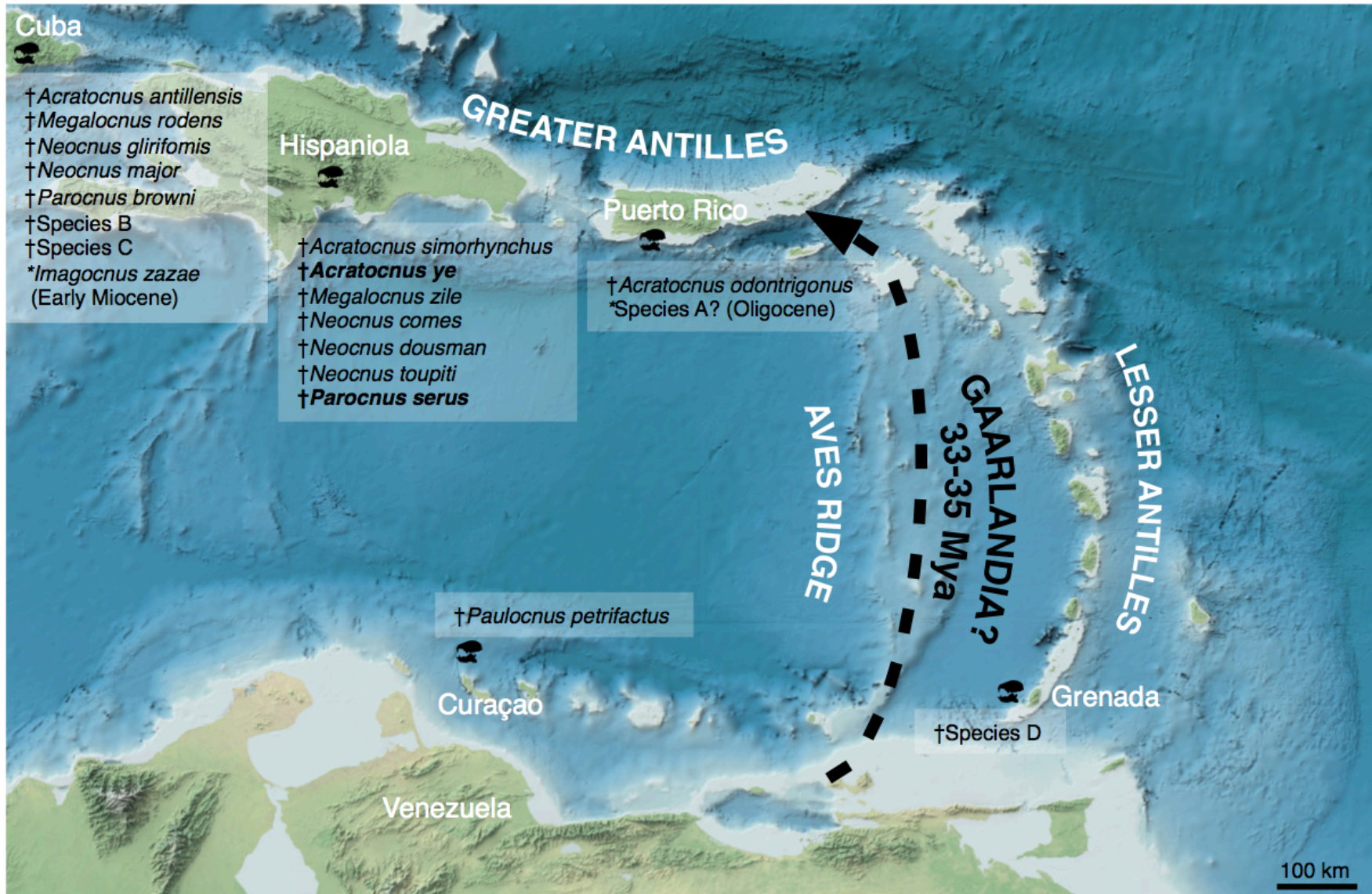


Figure 5



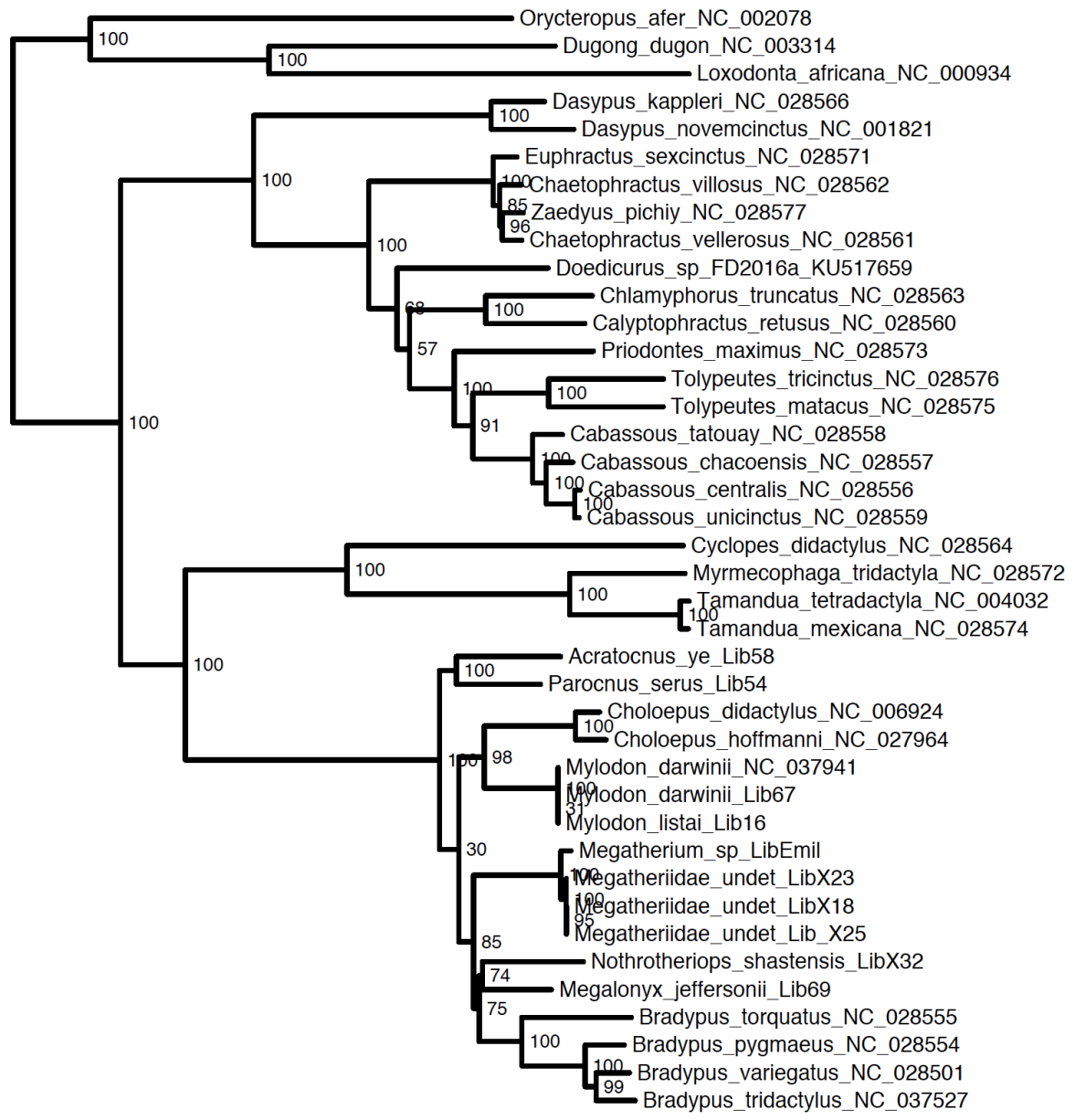
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Supplemental Information

Ancient Mitogenomes Reveal the Evolutionary

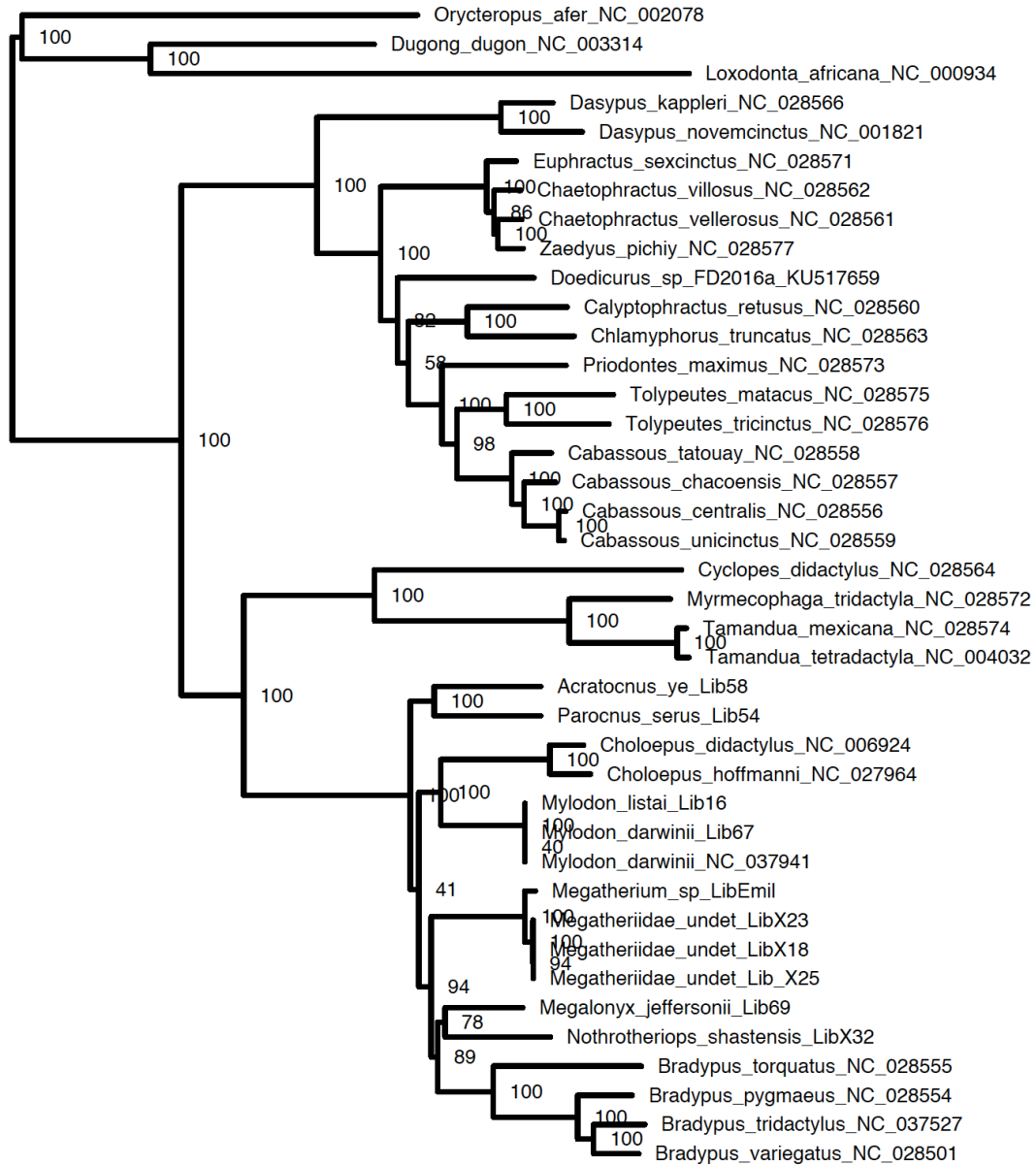
History and Biogeography of Sloths

Frédéric Delsuc, Melanie Kuch, Gillian C. Gibb, Emil Karpinski, Dirk Hackenberger, Paul Szpak, Jorge G. Martínez, Jim I. Mead, H. Gregory McDonald, Ross D.E. MacPhee, Guillaume Billet, Lionel Hautier, and Hendrik N. Poinar



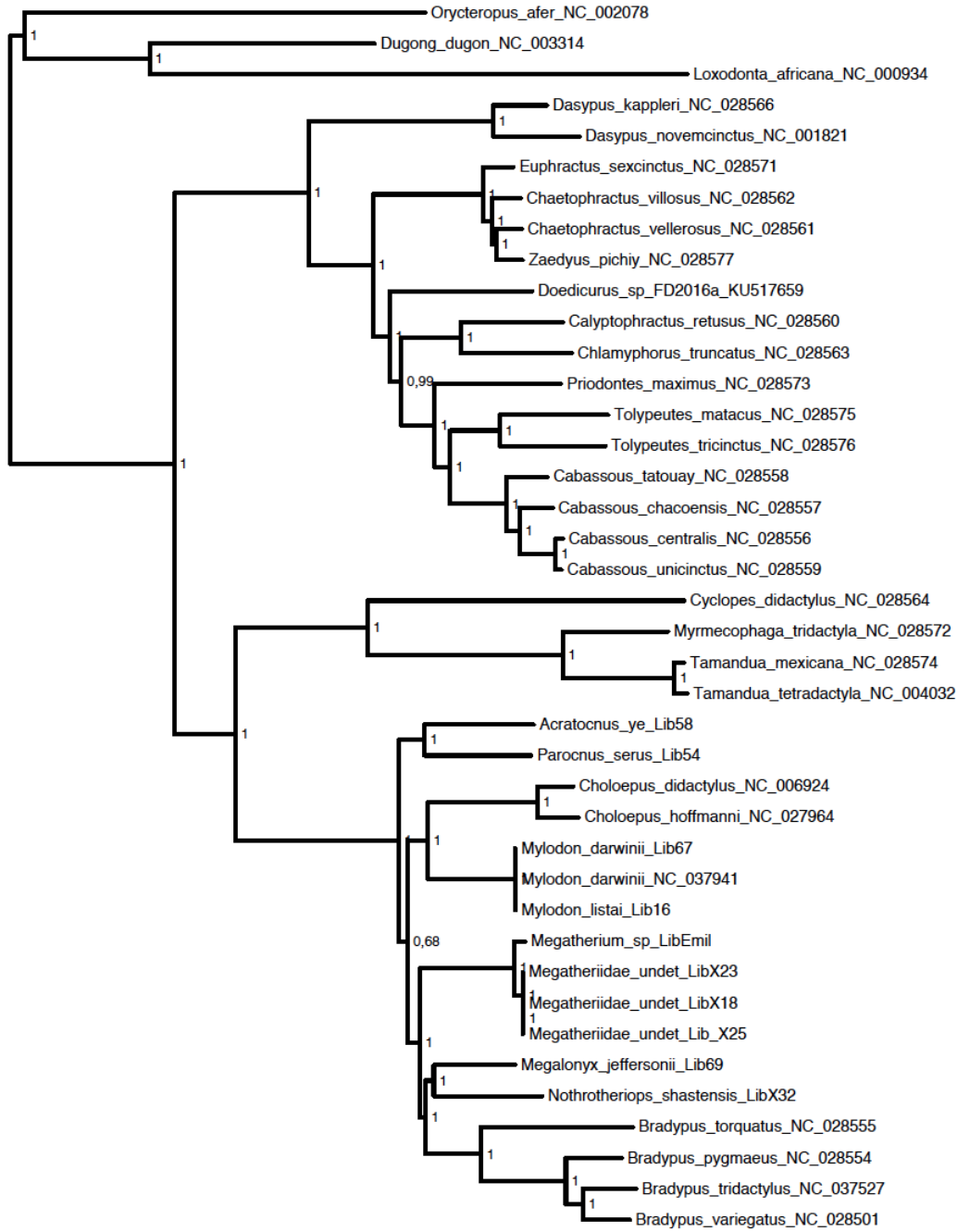
0.3

Figure S1. Maximum likelihood mitogenomic tree inferred under the best-fitting partitioned model using RAxML. Related to Figure 2. Maximum-likelihood bootstrap percentages are indicating at nodes (100 replicates). Tree is rooted on midpoint. Scale is in mean number of substitutions per site.



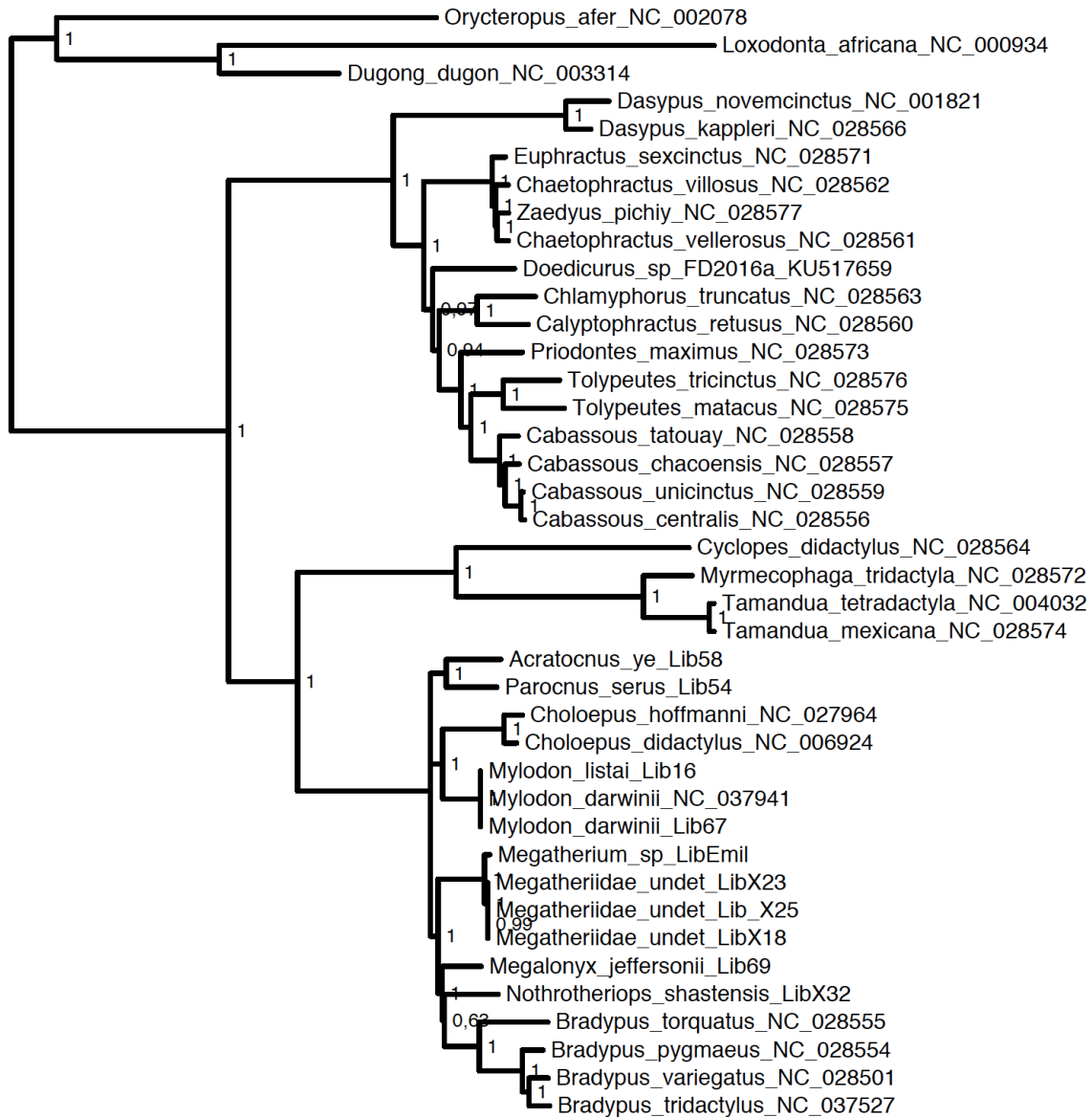
0.2

Figure S2. Maximum likelihood mitogenomic tree inferred under the best-fitting partitioned model using IQ-TREE. Related to Figure 2. Maximum-likelihood bootstrap percentages are indicating at nodes (100 replicates). Tree is rooted on midpoint. Scale is in mean number of substitutions per site.



0.2

Figure S3. Bayesian consensus mitogenomic tree inferred under the best-fitting partitioned model using MrBayes. Related to Figure 2. Clade posterior probabilities (PP) are indicated at nodes. Tree is rooted on midpoint. Scale is in mean number of substitutions per site.



0.5

Figure S4. Bayesian consensus mitogenomic tree inferred under the CAT-GTR+G₄ mixture model using PhyloBayes. Related to Figure 2. Clade posterior probabilities (PP) are indicated at nodes. Tree is rooted on midpoint. Scale is in mean number of substitutions per site.

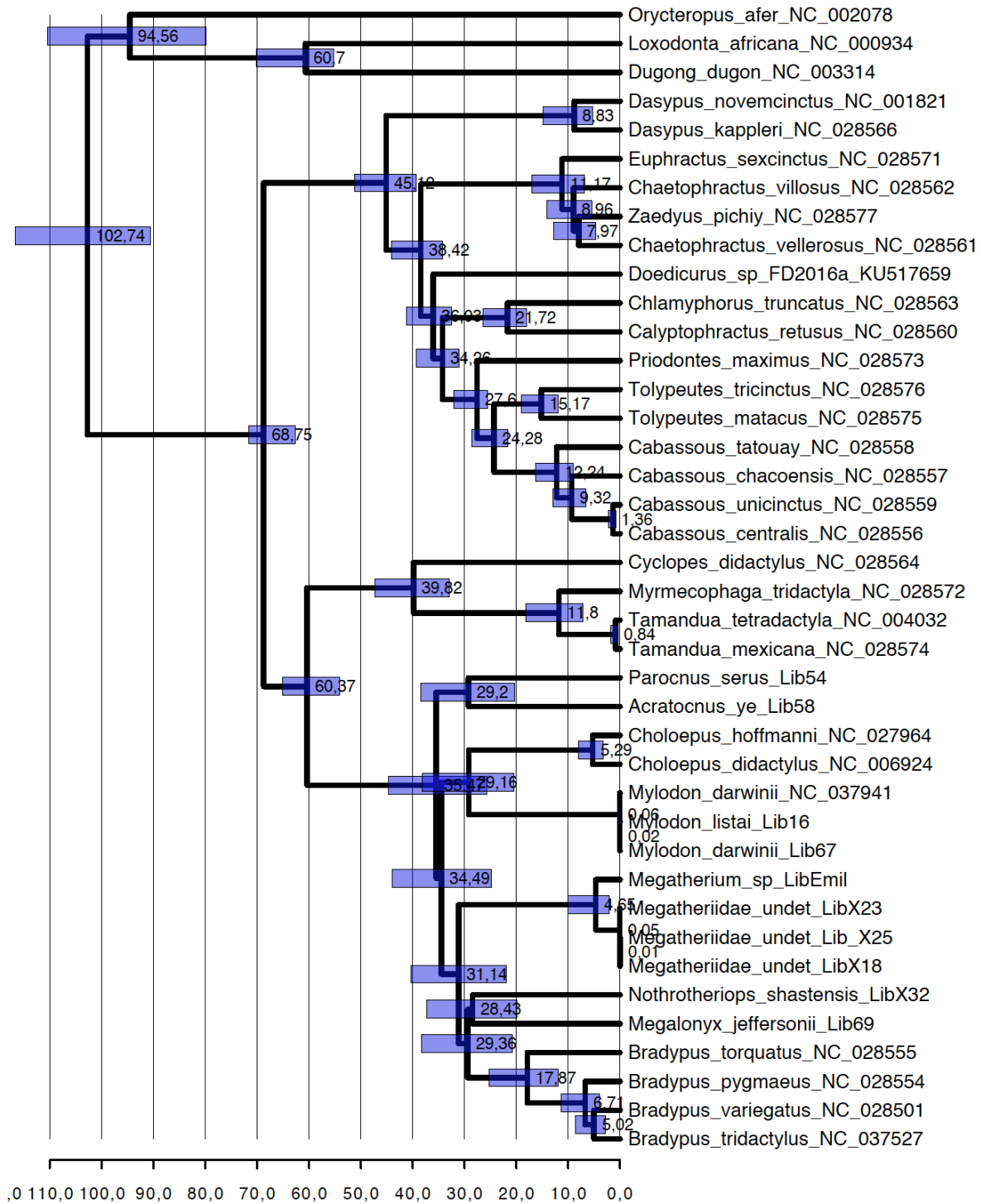


Figure S5. Bayesian mitogenomic chronogram. Related to Figure 3. This chronogram was inferred under the CAT-GTR+G₄ mixture model and an autocorrelated lognormal model of clock relaxation using PhyloBayes. Time scale is in million years.

Subset	Best Model	# Sites	Subset Partitions
1	GTR+I+G	7,875	12S, 16S, tRNAs, ND5_p1, CYTB_p1, ATP6_p1, ND1_p1, ND3_p1, ND4L_p1, ND4_p1, ND2_p1, ATP8_p3, ATP8_p2, ATP8_p1, COX1_p1, COX3_p1, COX2_p1, ND6_p1, ND6_p2
2	GTR+I+G	3,554	COX1_p2, COX2_p2, COX3_p2, CYTB_p2, ND1_p2, ATP6_p2, ND4_p2, ND5_p2, ND2_p2, ND4L_p2, ND3_p2
3	GTR+I+G	3,554	CYTB_p3, ND2_p3, ND1_p3, ND5_p3, COX1_p3, ND4_p3, ND3_p3, ND4L_p3, ATP6_p3, COX2_p3, COX3_p3
4	GTR+I+G	174	ND6_p3

* Settings: A priori partitions (42), Branch lengths (Unlinked), Models (GTR+G, GTR+I+G), Model selection (BIC), Search (Greedy).

Table S1. Detailed results of the PartitionFinder analysis for RAxML. Related to Figure 2.

Subset	Best Model	# Sites	Subset Partitions
1	GTR+F+I+G4	2,710	12S, 16S, CYTB_p1
2	GTR+F+I+G4	1,218	ATP6_p1, ND1_p1, ND3_p1, ND4L_p1, ND4_p1
3	TVM+F+I+G4	2,552	ATP6_p2, CYTB_p2, ND1_p2, ND2_p2, ND3_p2, ND4L_p2, ND4_p2, ND5_p2
4	TIM3+F+I+G4	968	ATP6_p3, COX1_p3, COX2_p3
5	TN+F+I+G4	198	ATP8_p1, ATP8_p2, ATP8_p3
6	TIM2e+I+G4	513	COX1_p1
7	TPM2+F+I+G4	1,002	COX1_p2, COX2_p2, COX3_p2
8	TIM2e+I+G4	489	COX2_p1, COX3_p1
9	TIM3+F+I+G4	2,586	COX3_p3, CYTB_p3, ND1_p3, ND2_p3, ND3_p3, ND4L_p3, ND4_p3, ND5_p3
10	TVM+F+I+G4	954	ND2_p1, ND5_p1
11	TN+F+I+G4	174	ND6_p1
12	TVM+F+G4	174	ND6_p2
13	TIM2+F+I+G4	174	ND6_p3
14	GTR+F+I+G4	1,445	tRNAs

* Settings: A priori partitions (42), Branch lengths (Unlinked), Models (All), Model selection (BIC), Search (Greedy).

Table S2. Detailed results of the ModelFinder analysis for IQ-TREE. Related to Figure 2.

Subset	Best Model	# Sites	Subset Partitions
1	GTR+I+G	7,743	12S, 16S, tRNAs, ATP8_p1, ND2_p1, ATP6_p1, ND3_p1, ND1_p1, ND5_p1, CYTB_p1, ND4L_p1, ND4_p1, COX1_p1, COX2_p1, COX3_p1, ND6_p1, ND6_p2
2	GTR+I+G	3,620	COX1_p2, COX2_p2, ATP8_p2, ND5_p2, ND2_p2, ATP6_p2, ND4_p2, ND3_p2, ND1_p2, COX3_p2, CYTB_p2, ND4L_p2
3	GTR+I+G	3,620	COX3_p3, ND4_p3, ND3_p3, ND4L_p3, CYTB_p3, ND2_p3, ND5_p3, ND1_p3, ATP8_p3, COX2_p3, ATP6_p3, COX1_p3
4	GTR+I+G	174	ND6_p3

* Settings: A priori partitions (42), Branch lengths (Unlinked), Models (JC, K80, SYM, F81, HKY, GTR, JC+G, K80+G, SYM+G, F81+G, HKY+G, GTR+G, JC+I, K80+I, SYM+I, F81+I, HKY+I, GTR+I, JC+I+G, K80+I+G, SYM+I+G, F81+I+G, HKY+I+G, GTR+I+G), Model selection (BIC), Search (Greedy).

Table S3. Detailed results of the PartitionFinder analysis for MrBayes. Related to Figure 2.