Correspondence

The phylogenetic affinities of the extinct glyptodonts

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Among the fossils of hitherto unknown mammals that Darwin collected in South America between 1832 and 1833 during the Beagle expedition [1] were examples of the large, heavily armored herbivores later known as glyptodonts. Ever since, glyptodonts have fascinated evolutionary biologists because of their remarkable skeletal adaptations and seemingly isolated phylogenetic position even within their natural group, the cingulate xenarthrans (armadillos and their allies [2]). In possessing a carapace comprised of fused osteoderms, the glyptodonts were clearly related to other cingulates, but their precise phylogenetic position as suggested by morphology remains unresolved [3,4]. To provide a molecular perspective on this issue, we designed sequence-capture baits using *in silico* reconstructed ancestral sequences and successfully assembled the complete mitochondrial genome of *Doedicurus* sp., one of the largest glyptodonts. Our phylogenetic reconstructions establish that glyptodonts are in fact deeply nested within the armadillo crown-group, representing a distinct subfamily (Glyptodontinae) within family Chlamyphoridae [5]. Molecular dating suggests that glyptodonts diverged no earlier than around 35 million years ago, in good agreement with their fossil record. Our results highlight the derived nature of the glyptodont morphotype, one aspect of which is a spectacular increase in body size until their extinction at the end of the last ice age.

Although the phylogenetic unity of order Cingulata has never been seriously questioned, how its three constituent groups (armadillos, glyptodonts, and pampatheres) are related to one another has been difficult to resolve in fi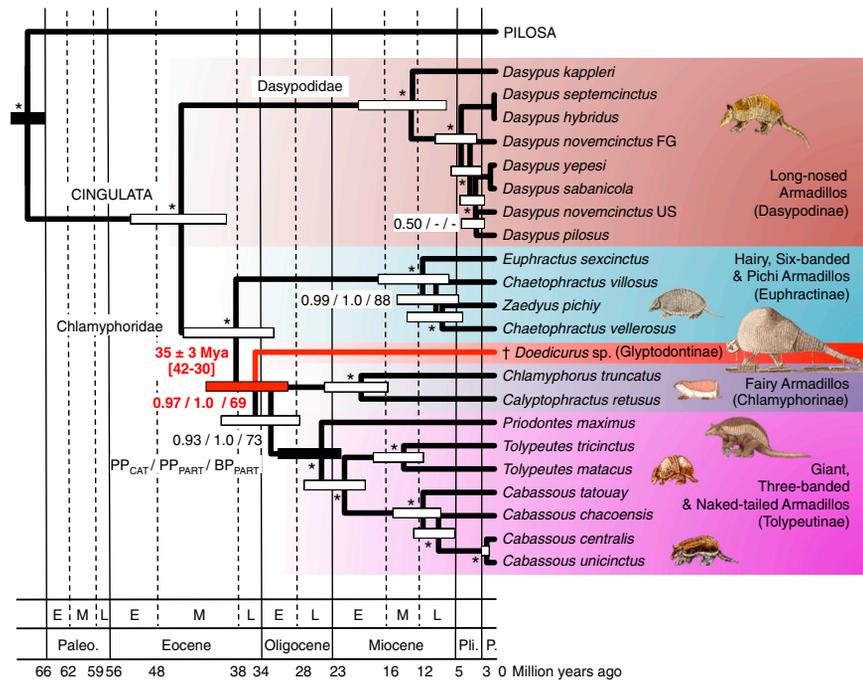ne detail. Of special interest in this regard is the recent proposal that, despite numerous differences in body size and carapace structure, glyptodonts do not constitute a sister-group to armadillos, as traditionally assumed [2], but are instead nested within them [4,6]. This hypothesis is, however, based on a restricted set of cranio-dental characters. Here, we put this proposition to the test by analyzing the mitochondrial genome of a specimen of the late surviving glyptodont *Doedicurus*. One of the largest members of its clade, with an estimated body mass of ~1.5 tons [7], *Doedicurus* exhibited numerous distinctive characters, famously including a club-shaped, armored tail adorned with spikes, presumably used in intraspecific combat.

Using ancient DNA (aDNA) extraction techniques, we recovered endogenous DNA from a carapace fragment (MACN Pv 6744) dated to 12,015 ± 50 ^{14}C radiocarbon years before present (Supplemental information). Utilizing a recently assembled dataset encompassing all modern xenarthran species [5], we reconstructed, *in silico*, a set of ancestral mitogenomic sequences, which permitted the synthesis of a set of target capture RNA baits. Baits constructed in this way may allow for a more specific sequence capture of phylogenetically distant ancient specimens than baits based solely on available modern sequences. This permitted the reconstruction of a nearly complete mitochondrial genome of *Doedicurus* at 76x coverage. Illumina reads mapping to the newly assembled *Doedicurus* mitogenome were 45 base pairs on average and displayed C-to-T damage patterns at both 3' and 5' ends, characteristic of authentic aDNA. We have ruled out the possibility of the inadvertent enrichment of nuclear copies of mitochondrial origin (NUMTs) by performing additional phylogenetic controls (Supplemental information).

Figure 1. Phylogenetic position of glyptodonts.
Phylogeny and molecular timescale of extant armadillos including the extinct glyptodont *Doedicurus* sp. (in red). Bayesian chronogram was obtained using a rate-autocorrelated log-normal relaxed molecular clock model using PhyloBayes under the CAT-GTR-G mixture model with a birth death prior on the diversification process, and six soft calibration constraints. Mean divergence dates and associated 95% credibility intervals are represented as node bars. Plain black node bars indicated calibration constraints. The main geological periods follow Geological Time Scale of the Geological Society of America (E = Early, M = Middle, L = Late; Paleo. = Paleocene, Pli. = Pliocene, P. = Pleistocene). Statistical support values obtained from three different phylogenetic reconstruction methods (PP[CAT]: Bayesian Posterior Probability under the CAT-GTR+G mixture model; PP[PART]: Bayesian PP under the best partition model; BP[PART]: Maximum likelihood Bootstrap Percentage under the best partition model) are indicated with stars corresponding to nodes with PP > 0.95 and BP > 90. The full chronogram and phylogram are provided in Figure S2.

By comparing our ancient mitogenome to those of living xenarthrans (Figure 1), we were able to confidently place *Doedicurus* within armadillos as the sister-group of a clade composed of Chlamyphorinae (fairy armadillos) and Tolypeutinae (three-banded, naked-tailed and giant armadillos; Supplemental information). This clearly contradicts the old view that glyptodonts must have diverged from other cingulates at a very early point in their phylogenetic history, on the grounds that, for example, they possessed such features as a completely fused carapace lacking movable bands [3]. Our results are more compatible but still incongruent with recent morphological cladistic analyses [4,6] that position glyptodonts within a more inclusive but nevertheless paraphyletic Euphractinae.

To examine the consequences of this novel phylogenetic placement, we incorporated *Doedicurus* into the morphological character matrix of Billet *et al*. [6], but were unable to identify any exclusive synapomorphies justifying grouping of the former with Chlamyphorinae + Tolypeutinae. In our study, only two characters, pertaining to the shape and position of the mandibular coronoid process, might qualify as potential synapomorphies, but only under the assumption that both have reverted to ancestral states in three-banded armadillos. This analysis nevertheless revealed other morphological similarities between glyptodonts and fairy armadillos (Supplemental information).

We estimate that glyptodonts diverged from Chlamyphorinae + Tolypeutinae 35 ± 3 million years ago, close to the Eocene–Oligocene transition (Figure 1). This molecular estimate is compatible with the age of the oldest and widely accepted glyptodont remains (Mustersan *Glyptatelus* osteoderms [8], ca. 36–38 million years old [9]). Tarsal bones from the Early Eocene locality of Itaboraí (Brazil), currently dated to more than 50 Myr [9], have been interpreted as glyptodont, but the elements in question are better interpreted as belonging to indeterminate dasypodoids [10]. According to our results, they might belong to stem cingulates that evolved before basal divergences occurred within the armadillo crown group, an event we date to ca. 45 million years ago (Figure 1).

While our results are based strictly on the comparison of mitogenomes,

the global congruence observed with previous nuclear-based phylogenies as well as molecular dating analyses provides convincing evidence for the proposed xenarthran evolutionary history [5]. On this evidence, glyptodonts (Glyptodontinae) comprised a distinct, Late Paleogene lineage of chlamyphorid armadillos [5]. Such a radical repositioning of glyptodonts within the armadillo crown group has major consequences for interpreting aspects of cingulate evolution. For example, the dome-shaped, tightly-fused carapace of glyptodonts has long been thought to be fundamentally different from that of armadillos and pampatheres, in which the carapace consists of articulated sections. Our results imply that the unarticulated carapace is in fact a derived feature, which in turn provides an explanation for the apparent presence of movable bands in some Miocene glyptodonts [3].

Glyptodonts were a group of ambulatory specialized herbivores that reached giant size bracketed between two extant clades of armadillos that do not share either of these characteristics. Based on our new phylogenetic framework, we performed a statistical reconstruction of ancestral body masses. According to our analysis, the mean ancestral body mass estimate of the last common ancestor of Glytodontinae + Chlamyphorinae + Tolypeutinae was a mere 6 kg (95% credibility interval: 1–19 kg), implying a spectacular increase in glyptodont body mass during the Neogene (Supplemental Information). This inference is in line with the fossil record, which indicates that glyptodonts evolved from medium-sized forms in the Miocene (e.g., *Propalaehoplophorus*, ~80 kg) to become true megafauna in the Pleistocene (e.g., *Glyptodon clavipes*, ~2,000 kg) before disappearing with most other South American large mammals some 10,000 years ago [7].

SUPPLEMENTAL INFORMATION

Supplemental Information including results, acknowledgements, experimental procedures and two figures can be found with this article online at http://dx.doi.org/10.1016/j.cub.2016.01.039.

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