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A revised evolutionary history of armadillos (*Dasypus*) in North America based on ancient mitochondrial DNA

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The large, beautiful armadillo, *Dasypus bellus*, first appeared in North America about 2.5 million years ago, and was extinct across its southeastern US range by 11 thousand years ago (ka). Within the last 150 years, the much smaller nine-banded armadillo, *D. novemcinctus*, has expanded rapidly out of Mexico and colonized much of the former range of the beautiful armadillo. The high degree of morphological similarity between these two species has led to speculation that they might be a single, highly adaptable species with phenotypical responses and geographical range fluctuations resulting from environmental changes. If this is correct, then the biology and tolerance limits for *D. novemcinctus* could be directly applied to the Pleistocene species, *D. bellus*. To investigate this, we isolated ancient mitochondrial DNA from late Pleistocene-age specimens of *Dasypus* from Missouri and Florida. We identified two genetically distinct mitochondrial lineages, which most likely correspond to *D. bellus* (Missouri) and *D. novemcinctus* (Florida). Surprisingly, both lineages were isolated from large specimens that were identified previously as *D. bellus*. Our results suggest that *D. novemcinctus*, which is currently classified as an invasive species, was already present in central Florida around 10 ka, significantly earlier than previously believed.

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Xenarthra, an enigmatic superorder of mammals, first appears in the fossil record in South America during the Palaeocene (Rose *et al*. 2005), around 60 million years ago (Ma). The superorder quickly diversified into three orders: the Phyllophaga (sloths), Vermilingua (anteaters) and Cingulata (armadillos) (Gaudin & McDonald 2008). Dasypodidae, the family that includes armadillos, along with two other cingulates, Glyptodontidae and Pampatheridae, dispersed northward into North America around 3 Ma with the formation of the Panamanian isthmus (Marshall *et al*. 1982). Glyptodonts and pampatheres went extinct about 11 000 years ago (ka) (Kurtén & Anderson 1980).

Today, the Dasypodidae contains 21 extant species (Möller-Krull *et al*. 2007), seven of which are within the genus *Dasypus*, which probably diversified in South America around 7 Ma (Delsuc *et al*. 2003). One species, *Dasypus bellus* (beautiful armadillo), occurred in the USA from about 2.5 Ma (Voorhies 1987; Woodbourne 2010) until about 11 ka when it went extinct (Schubert & Graham 2000). It ranged across the southeastern USA from New Mexico and Texas to Florida, and north to Nebraska (Voorhies 1987) and Iowa across to West Virginia (Klippel & Parmalee 1984: p. 154, fig. 2). No records of the beautiful armadillo exist from South America (Schubert & Graham 2000).

The beautiful armadillo was large (~1.2 m long), about twice the size of the modern nine-banded armadillo, *D. novemcinctus*. Other than size, there appears to be a high degree of morphological similarity between the two species (Auffenberg 1957; Slaughter 1961; Voorhies 1987), although Jansinski & Wallace (2013) documented morphological differences in the calcaneus. The early evolutionary history of *D. bellus* remains unresolved. Blancan (Pliocene) forms are smaller than those of the Late Pleistocene, but it appears there is also a size reduction just before extinction (Klippel & Parmalee 1984).

There are two different hypotheses proposed to explain the origin of the modern nine-banded armadillo, *D. novemcinctus*. One hypothesis suggests that the nine-banded armadillo is directly descended from the beautiful armadillo (McNab 1980; McBee & Baker 1982). This hypothesis is supported by the fact that the body size of *D. bellus* decreased during the latest Pleistocene (Klippel & Parmalee 1984). On the other hand, the same data (Klippel & Parmalee 1984) show that *D. bellus* was extremely variable in size, with small *D. bellus* overlapping in size with large *D. novemcinctus* (e.g. band osteoderm widths varied from 13.2–6.8 mm in beautiful armadillos compared with 5.4–6.9 mm in nine-banded armadillos (Klippel & Parmalee 1984: p. 157, table 3)). Consequently, *D. bellus* and *D. novemcinctus* may not actually be different species, but rather a single, highly adaptable species that has been through a series of phenotypic changes along with range expansions and contractions in response to environmental fluctuations. This evolutionary change may have happened in Mexico or even further south with the extinction of *D. bellus*.

Today, *D. novemcinctus* is the most widespread xenarthran, with a continuous range extending from Patagonia in Argentina northward into Nebraska in the USA (McBee & Baker 1982). In fact, it is the only

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xenarthran in the USA, where the US Department of Fish and Wildlife Services consider it to be an invasive (non-native) species (Simonson *et al.* 2004). It rapidly expanded its range out of Mexico through Texas and into the mid-central southern states within the last 150 years (Humphrey 1974). Independently, in the middle 1800s, humans introduced populations of *D. novemcinctus* into Florida. Likewise, this population expanded its distribution into the southeastern USA (Humphrey 1974). In the last 15 years, *D. novemcinctus* has continued its range extension in to central Missouri and southern Illinois (Taulman & Robbins 1996). With the projection for future climate warming (IPCC 2013), expectations are that it will continue to expand northward (McBee & Baker 1982; Taulman & Robbins 1996; Fig. 1).

The recent, rapid expansion of nine-banded armadillos into North America is reflected in their present-day genetic diversity. Two divergent mitochondrial lineages of nine-banded armadillos are known (Huchon *et al.* 1999; Frutos & Van den Bussche 2002; Arteaga *et al.* 2012), but only one of these has been identified in the USA (Huchon *et al.* 1999; Arteaga *et al.* 2012). In addition, in an analysis of diversity within the mitochondrial control region, which is the most rapidly evolving segment of the mitochondrial genome, only two very closely related haplotypes were observed amongst a sample of 20 nine-banded armadillos from across their US range (Huchon *et al.* 1999). By contrast, a far greater diversity of mitochondrial haplotypes is found in South and Central America, where both lineages of nine-banded armadillos are found as far north as Mexico (Huchon *et al.* 1999; Frutos & Van den Bussche 2002; Arteaga *et al.* 2012).

Here, we investigated the evolutionary relationship between the extinct *D. bellus* and extant *D. novemcinctus* using ancient mitochondrial DNA. We isolated genetic data from two North American individuals dating to the Pleistocene, one from Florida and the other from Missouri, both identified as *D. bellus*. We performed a phylogenetic analysis of these and previously isolated mitochondrial DNA data from nine-banded armadillos and from the most closely related living species to the nine-banded armadillo, the greater long-nosed armadillo (*D. kappleri*).

Material and methods

DNA extraction

We collected 11 specimens that had been identified based on morphology as beautiful armadillos from the Florida Museum of Natural History and the Illinois State Museum (Table 1). These consisted of limb bones and osteoderms from seven different sites in Florida and Missouri. The specimens were all believed to be Late Pleistocene in age, probably ranging between 40 and 10 thousand years old. We additionally attempted to extract DNA from four glyptodont and five pampathere bones and teeth (Table 1) sampled from the Florida Museum of Natural History.

From each specimen, we performed DNA extraction for poorly preserved bones as described previously (Letts & Shapiro 2012). As with all ancient DNA research, we performed DNA extraction and PCR set-up in a sterile, positive pressure clean laboratory, in this instance the ancient DNA laboratory at the Pennsylvania State University, which is physically isolated from modern molecular biology research. Prior to this project, no molecular biology research was carried out on either living or extinct armadillos in either the ancient DNA laboratory or the modern DNA laboratory in which downstream molecular biology work was performed. Workflow was always from the ancient DNA laboratory to the modern DNA laboratory, and full protective coverings were worn at all times. Negative controls were used at all steps and PCR products were cloned to characterize DNA damage and identify environmental contaminants. Prior to extraction, we removed subsamples from each specimen using a Dremel tool. We collected bone powder either by drilling directly into the interior of the bone with a drill tip, or by removing a larger piece of bone with a cutting disk and reducing this piece to powder with a Mikrodismembrator (Braun). Of those that we were able to powder (Lab ID is listed as n/a in Table 1) we collected 200–400 mg of bone powder, from which we extracted DNA using the silica-binding method described by Rohland *et al.* (2010). In the final step of the extraction, we eluted the DNA in 50 µl of TE buffer and added Tween-20 to a concentration of 0.5%, as this has been shown to prevent DNA molecules from binding to the walls of the tube in which the extract is stored (Rohland & Hofreiter 2007).

Initial results suggested that DNA was preserved in two of the 12 armadillo samples (see below). However, these samples were heavily contaminated with human DNA, probably introduced during excavation, handling and storage. We therefore performed an additional extraction from these two samples, this time including a bleach step in which coarsely powdered bone was immersed in 3% bleach for 15 min. This protocol has been shown to effectively remove nearly all exogenous DNA contamination (Kemp & Smith 2005). After the bleach step, the bone/bone powder was rinsed twice with distilled water, and the extraction was performed as described above.

DNA amplification and sequencing

We attempted to amplify short stretches of both the mitochondrial 12S rDNA locus and the more rapidly evolving mitochondrial control region (CR) separately from each DNA extract (extracts from the same

Fig. 1. The geographical range of armadillos in North America. The current range of the nine-banded armadillo is shown in dark grey and extends from South America through the southern USA. The predicted future range of the nine-banded armadillo, as determined by allowing for expansion into regions that receive at least 38 cm total annual precipitation and have January mean temperatures that are greater than –2°C, is shown in lighter grey (Taulman & Robbins 1996). Known fossil occurrences of the beautiful armadillo (Klippel & Parmalee 1984) are indicated as black dots. The Brynjulfson Cave, Missouri and Medford Cave, Florida localities, from which we were able to recover DNA, are indicated with circles, as is Miller Cave, which we highlight in the Discussion.

individual were processed separately as replicates). We selected target regions of each locus were to match previously published data from modern armadillos (Huchon *et al.* 1999). We targeted 450 base-pairs (bp) of 12S rDNA in three overlapping sequences, and 467 bp of CR in two overlapping sequences. We performed PCR amplification as described previously (Letts & Shapiro 2012). Primer sequences and PCR conditions are available from the authors on request. Amplification products were cloned using the TOPO TA cloning kit (Invitrogen) in 1/10 reactions and sequenced at the PSU Genomics Core Facility.

In addition to mitochondrial DNA, we targeted two nuclear genes for amplification: the von Willebrand

Table 1. Bone samples collected for DNA extraction as part of this study. Bones from which we successfully amplified ancient DNA are highlighted in grey.

Lab ID	Museum ID	Genus	Species	Common name	Locality	Locality type	Sample type
BL114	UF24514	*Dasypus*	*bellus*	Beautiful armadillo	Monkey Jungle Hammock, FL	Forest	Metapodial
BL115	UF202560	″	″	″	Aucilla River 2P, FL	River	Osteoderm
BL113	UF2478	″	″	″	Medford Cave, FL	Cave	Tibia
BL121	UF2478	″	″	″	Medford Cave, FL	Cave	Osteoderm
BL203	(IL)497450	″	″	″	Brynjulfson Cave, MO	Cave	Osteoderm
BL204	(IL)495814	″	″	″	Little Beaver Cave, MO	Cave	Osteoderm
BL205	(IL)497447	″	″	″	Crankshaft Pit, MO	Cave	Osteoderm
BL207	(IL)497448	″	″	″	Crankshaft Pit, MO	Cave	Osteoderm
BL208	(IL)497449	″	″	″	Brynjulfson Cave, MO	Cave	Osteoderm
BL209	HCSW-87-1	″	″	″	Heinze Cave, MO	Cave	Osteoderm
BL210	HCSW-64-2	″	″	″	Heinze Cave, MO	Cave	Osteoderm
BL116	UF51860	*Glyptotherium*	*floridanum*	Glyptodon	LE008: Hickey Creek	River	Humerus
n/a	UF124394	″	″	″	Weika River 2, FL	River	Osteoderm
n/a	FGS6643	″	″	″	Catalina Gardens, FL	River	Osteoderm
BL068	FGS6643	″	″	″	Catalina Gardens, FL	River	Vertebra
n/a	UF217484	*Holmesina*	*septentrionalis*	Pampathere	Aucilla River 1E, FL	River	Ulna
BL117	UF217485	″	″	″	Aucilla River 1E, FL	River	Astragulus
BL118	UF103568	″	″	″	Aucilla River 3J, FL	River	Osteoderm
BL067	UF11326	″	″	″	Ichetucknee River, FL	River	Tooth
BLI-14	UF9336	″	″	″	Branford 1A, FL	River	Calcaneum

factor (vWF) and cAMP responsive element modulator (CREM), which have been used previously in xenarthran studies (Poinar *et al*. 2003). In both cases, we targeted short fragments (between 110 and 200 bp) of nucleotide sequences, as long fragments are not expected to be preserved in ancient specimens.

AMS radiocarbon dating

AMS radiocarbon dating of the Brynjulfson Cave specimen was performed by the Oxford University Radiocarbon Accelerator Unit (reference: OxA-23820) using ultrafiltration, resulting in an uncalibrated age of 33 350±50 ^{14}C years BP. We calibrated this date using the IntCal13 calibration curve (Reimer *et al*. 2013) and the online version of OxCal 4.2 (Bronk Ramsey 2009), which provided a date in the one-sigma range of 37 465 to 38 014 cal. years BP.

Phylogenetic analysis

To investigate the evolutionary relationships between both living and extinct armadillos, we obtained 10 published 12S sequences representing eight armadillo genera (Y11832; Arnason *et al*. 1997, U61080; Springer & Douzery 1996, AJ505825–AJ505829; Delsuc *et al*. 2003), a sloth (AF038022) and an anteater (AJ278153; Delsuc *et al*. 2001). In addition, we obtained 24 previously published CR sequences: three greater long-nosed armadillos (*D. kappleri*), 20 nine-banded armadillos (*D. novemcinctus*), including individuals from both North and South America (AJ010362–AJ010384; Huchon *et al*. 1999) and one large hairy armadillo (*Chaetophractus villosis*; EU100944 (Poljak *et al*. 2010) to be used as an outgroup.

To estimate a species phylogeny, we first analysed the 12S alignment using MrBayes (Huelsenbeck & Ronquist 2001), with a GTR+G model of evolution as selected by jModeltest (Posada 2008) using an algorithm implemented in PhyML (Guindon & Gascuel 2003). We ran two MCMC chains for 1 000 000 iterations, sampling from the posterior every 1000 iterations. We removed the first 10% of samples as burn-in and estimated a maximum clade credibility (MCC) consensus tree from the remaining 1800 sampled trees. We then performed a maximum likelihood (ML) analysis using RAxML (Stamatakis 2006). We performed 100 ML bootstrap replicates using the –f a command, which maps the estimated bootstrap support values onto the ML tree. Finally, we performed a maximum parsimony (MP) bootstrap analysis in PAUP v. 4.0 (Swofford 2003) with 100 bootstrap replicates and constructed a 50% majority consensus tree. We visualized and compared the resulting trees using FigTree v. 1.3.1 (Rambaut 2006).

Second, we analysed the CR data set using both MrBayes and RAxML. The larger within-species data set made it possible to assess the evolutionary relationships within *Dasypus* more closely. We performed the analysis as above, but used an HKY+G model of evolution and increased the length of the MCMC chains run in MrBayes to 2 000 000 iterations.

Results

DNA preservation

Of the 21 specimens of armadillo, glyptodont and pampathere sampled, only two armadillo specimens

yielded amplifiable mitochondrial DNA. We found a dramatic decrease in human contamination of these samples after the addition of the pre-extraction bleach step, with little influence on the pattern of damage observed in the armadillo DNA sequences. No nuclear DNA could be recovered from any ancient specimen, even after the bleach step was added.

The samples that contained amplifiable ancient mitochondrial DNA include a tibia fragment (UF 2478) from Medford Cave, a limestone cavern in central Florida. This specimen was discovered in 1956 and has been kept in the collection of the Florida Museum of Natural History since that time. Based on stratigraphy and association with extinct Pleistocene fauna, the sample is estimated to be approximately 10 to 12 000 years old. The second specimen from which mitochondrial DNA could be recovered is an imbricating (band) osteoderm from Brynjulfson Cave in north-central Missouri (ISM 497450). The osteoderm was part of a non-analogue faunal assemblage (Semken *et al.* 2010), comprising boreal animals such as stag-moose (*Cervalces*), woodland muskox (*Bootherium*) and red-backed vole (*Myodes*), and temperate species such as ground squirrel (*Spermophilis*), badger (*Taxidea*), gopher (*Geomys*), peccary (*Platygonus*) and pronghorn (*Antilocapra*) in addition to the armadillo (Parmalee & Oesch 1972; Semken *et al.* 2010). The specimen, which was destroyed entirely in this study in order to generate both the ancient DNA data and an Accelerator Mass Spectrometry (AMS) radiocarbon date, had been kept in the collection of the Illinois State Museum.

We cloned PCR products from three CR amplifications each of two DNA extractions of the Medford Cave bone (six different amplification products), and sequenced a total of 39 clones. Amongst these, 13 singleton substitutions were identified, three of which could be attributed to cytosine deamination, which is the most common form of DNA damaged observed in ancient DNA (Hofreiter *et al.* 2001). A transversion was also present (Gilbert *et al.* 2007), most probably due to a polymerase misincorporation at an apurinic/apyrimidinic site (Eckert & Kunkel 1991). Nine changes were A–>G/T–>C, which have also been shown to result from polymerase misincorporation in some ancient DNA samples, perhaps due to an unknown form of damage (Stiller *et al.* 2006). Alternatively, these differences may have represented amplification of a nuclear copy of the mitochondrial sequence, as has been observed when sequencing ancient DNA from other taxa, for example lions (Barnett *et al.* 2009). Despite the observed damage, the two extractions yielded identical consensus sequences. Amplifications from each of the three 12S primer sets were cloned for a total of 23 overlapping clones. No damage was observed in the 12S amplifications.

From the Brynjulfson Cave specimen, we cloned amplification products from three overlapping fragments of CR and sequenced 37 clones. Eleven sites, five of which were represented by singleton substitutions, could be attributed to cytosine deamination. Six singleton substitutions as above could be attributed to polymerase error, and three transversions were present, again, possibly due to polymerase error. We cloned two amplification products each for the three overlapping fragments of 12S from one extraction of the Brynjulfson Cave osteoderm, and sequenced a total of 35 clones. Five sites with damage attributable to cytosine deamination were observed, as was a single A–>G/T–>C transition that was present in two clones.

Due to the repetitive nature of the armadillo control region and associated challenges in generating these data for ancient specimens, the entire 467 bp of CR was only available for the modern nine-banded armadillos. Only 351 bp could be isolated from the Medford Cave tibia, and only 268 bp could be generated from the Brynjulfson Cave osteoderm. In addition, only 313 and 367 bp had been published previously for the long-nosed armadillos and the hairy armadillo, respectively. We coded each of these shorter fragments as containing missing data for the phylogenetic analyses.

Phylogenetic analysis

The 12S phylogenies estimated by MrBayes, RAxML and PAUP decisively placed both Pleistocene armadillos within *Dasypus* (Bayesian posterior support (BP) >99%, RAxML maximum likelihood bootstrap support (MLB) = 100%, maximum parsimony bootstrap (MPB) = 100%; Fig. 2). In addition, all analyses placed the Medford Cave specimen within the clade of modern, nine-banded armadillos, and the Brynjulfson Cave specimen outside of this clade, with high support (BP>99%, MLB = 100%, MPB = 100%). The branching order within *Dasypus* remains unresolved, with each method estimating different topologies and none providing support for a grouping of the nine-banded armadillo with either the beautiful armadillo or the long-nosed armadillo.

The CR analyses also recovered the Medford Cave armadillo within the genetic diversity of nine-banded armadillos; its sequence is identical to one of two haplotypes found in North America in the present day. This analysis revealed a distinct haplotype for the Brynjulfson Cave armadillo, outside of the diversity of all nine-banded armadillos and the great long-nosed armadillo. However, these analyses were also inconclusive with regard to the branching order within *Dasypus* (Fig. 3) and the evolutionary relationship between the beautiful armadillo and the nine-banded armadillo. Both MrBayes and RAxML recovered the nine-banded armadillos as monophyletic with respect to both the beautiful armadillo from Brynjulfson Cave and the great long-nosed armadillos from South America, although neither did so with any support (BP = 64%,

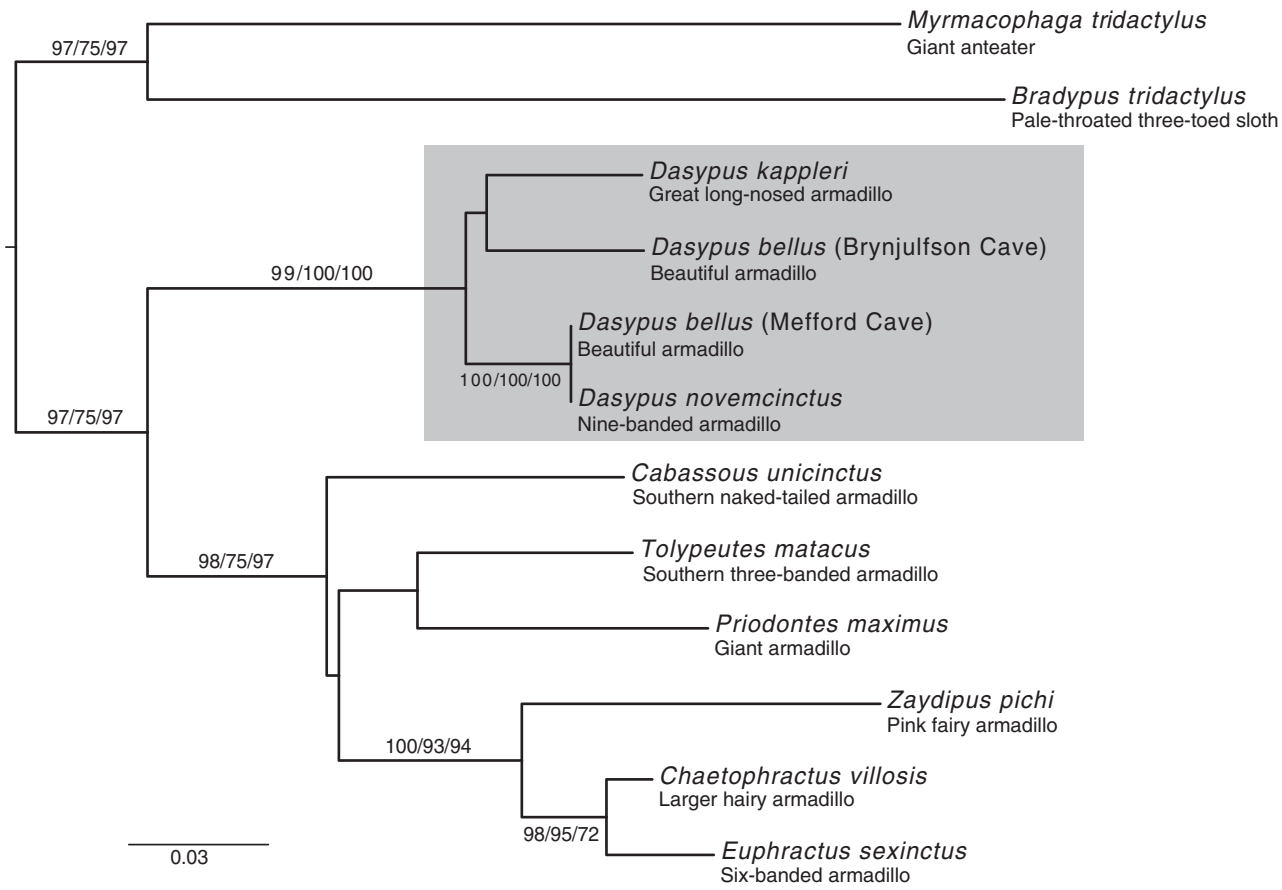

Fig. 2. Maximum likelihood (ML) phylogeny of 12S rDNA isolated from armadillos, sloths and anteaters generated using RAxML. Support values are indicated above or below the branches leading to the indicated nodes. Support values are provided as Bayesian posterior support (BP)/RAxML ML bootstrap support (MLB)/PAUP maximum parsimony bootstrap support (MPB). *Dasypus* is highlighted.

MLB = 12%). Both methods also recovered separate clades comprising nine-banded armadillos from North and South America, but again without strong support (BP for the North American clade = 93% and South American clade = 60%; MLB for the North American clade = 66%, and South American clade = 81%).

Discussion

Pleistocene armadillos in North America

Although both Pleistocene armadillos from which we could recover DNA had been identified morphologically as beautiful armadillos, mitochondrial DNA isolated from the Medford Cave tibia unambiguously groups this specimen with the nine-banded armadillos. In the rapidly evolving control region, the Medford Cave specimen is identical to one of two haplotypes currently found in North America. The sequence isolated from the Brynjulfson Cave armadillo, however, does not cluster with any extant armadillos, and is likely to represent the mitochondrial linage of the extinct *D. bellus*.

Two conclusions can be drawn from these results. First, although only mitochondrial data are available and the sample size is small, our data strongly suggest that the nine-banded and beautiful armadillos are genetically distinct and appropriately subdivided into different species. Our data also indicate strongly that the Medford Cave armadillo was incorrectly identified as a beautiful armadillo. In a detailed description of this specimen, Auffenberg (1957) wrote that the majority of the features of the Medford Cave specimen were more similar to those observed in the modern nine-banded armadillo than to other beautiful armadillo specimens, including the holotype beautiful armadillo from Seminole Field, Florida. Specifically, the Medford Cave specimen differed from other beautiful armadillos in the number of follicles in its osteoderms, leading Auffenberg (1957) to conclude that, with the exception of being nearly 1.2 m in length, the specimen was morphologically identical to the modern nine-banded armadillo (Auffenberg 1957). An alternative but less likely explanation is that this sample contained no preserved DNA, and became somehow contaminated with DNA from a nine-banded armadillo during storage or handling. We find this explanation less

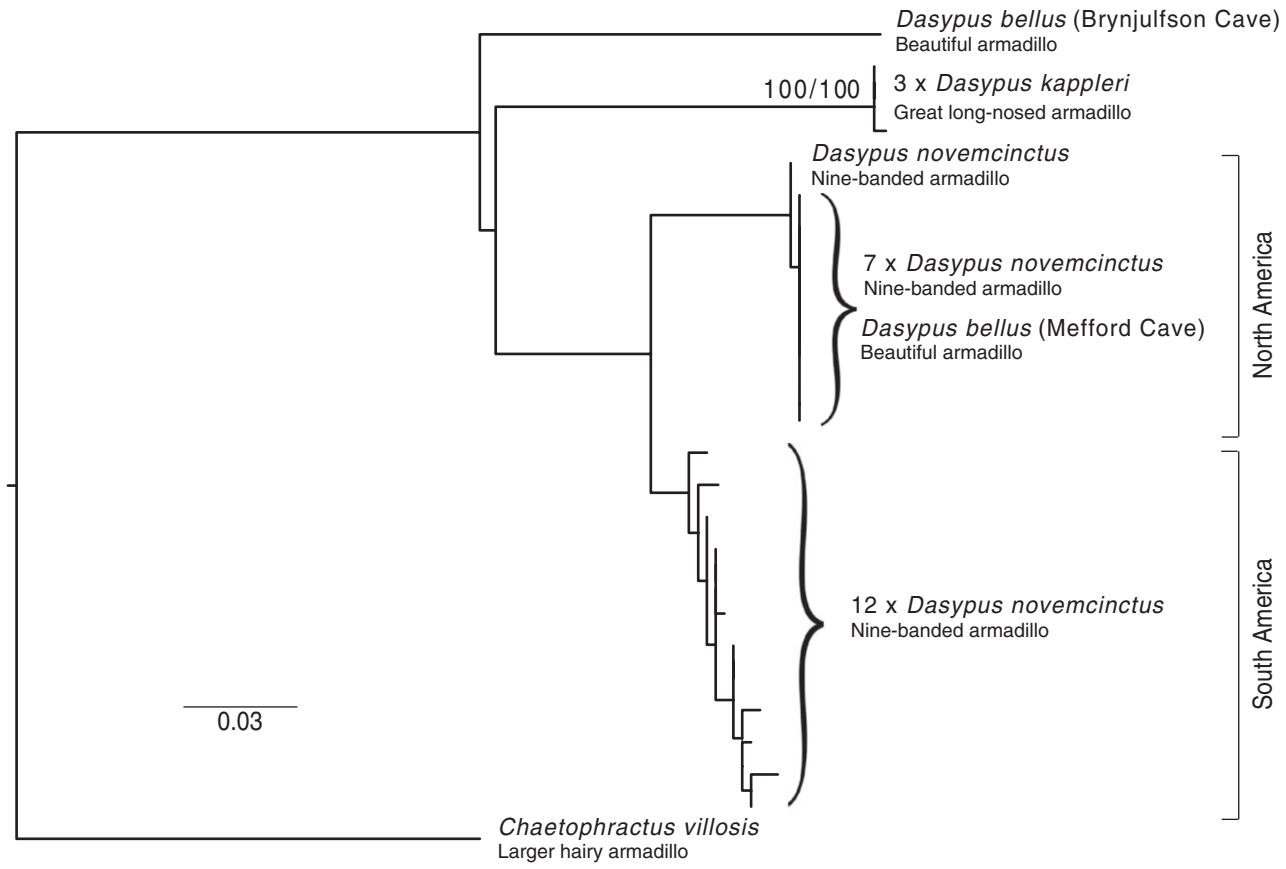

Fig. 3. Maximum likelihood (ML) phylogeny of *Dasypus* control region sequences generated using RAxML. The only well-supported node is that separating *Dasypus kappleri* (the great long-nosed armadillo) from the other armadillos, with Bayesian posterior support >99% and RAxML ML bootstrap support = 100%. North and South American clades of nine-banded armadillo are indicated. The larger hairy armadillo, *Chaetophractus villosis*, was used as an outgroup.

satisfactory, given (i) the morphological analysis of this specimen, (ii) the inability to amplify nuclear DNA, (iii) the observation of DNA damage lesions that are typical of ancient DNA, and therefore suggest that the DNA within the specimen is both old and degraded (unlike a modern contaminant).

Second, the nine-banded armadillo was present in what is now the continental USA much earlier than was known previously. The lack of mitochondrial genetic diversity in North American nine-banded armadillos has been attributed to a founder effect followed by rapid expansion during historic times (Huchon *et al.* 1999). Although our results do not question the validity of this interpretation, they do raise the possibility of multiple expansions followed by local extinctions throughout the Late Pleistocene and Holocene.

Armadillo remains from Miller's Cave (Patton 1963) in central Texas may support this contention. The remains of *D. bellus* were found in a basal travertine deposit that contained other extinct Pleistocene taxa. An uncalibrated radiocarbon date of 7200±300 (A326), which provides a calibrated date of 7756 to 8330 cal. a BP in the one-sigma range, was obtained on bone, not from the armadillo, but from the same deposit. Frequently, radiocarbon dates on bone are too young because of contamination of the bone with younger carbon from fulvic and humic acids. Since the mid-1980s, methods have been developed to provide accurate dates on bone if the collagen has been purified by removing the contaminants at a molecular level (Stafford *et al.* 1991). Since the Miller's Cave date was carried out in the early 1960s, contaminants were not removed and the date can be rejected as inaccurate. Even though there is not a reliable radiocarbon date on the material from the basal travertine, a Pleistocene age can be assumed because of the association of boreal and eastern taxa that were extirpated at the end of the Pleistocene, after 11 ka.

A band scute of *D. novemcinctus* was found in the younger overlying brown clay unit at Miller's Cave (Patton 1963). Although the scute was not dated, charcoal from the base of the brown clay unit thought to be in association with it has yielded a radiocarbon date of 3008±410 (SM 596) (Patton 1963). Charcoal is not as susceptible to molecular contamination as is bone so that date can be assumed to be reasonably accurate even

though it was processed in the early days of radiocarbon dating. Taken at face value, this record would suggest that *D. bellus* went extinct at the end of the Pleistocene and was replaced by *D. novemcinctus* in the late Holocene at Miller's Cave. The source of the late Holocene population is unknown. It could have resulted from an invasion from Mexico or it could have been derived from small populations that persisted in the USA from the Late Pleistocene (e.g. Medford Cave).

However, this record must be regarded with some caution. Unfortunately, Patton (1963) did not discuss where the *D. novemcinctus* specimen was actually located within the brown clay. It is possible that it could represent contamination of a younger (historic?) specimen that intruded into older sediments. Also, as the specimen was not directly dated, there is a chance that the dated charcoal and scute are not the same age, especially because the radiocarbon date is from the base of the brown clay deposit. Furthermore, studies of Pleistocene deposits in caves, even those carefully excavated in 5–10 cm levels, can be time-averaged by thousands of years (Semken *et al*. 2010). A radiocarbon date on the actual specimen itself, if it contains enough collagen, would be the best test of its age.

In their review of habitat usage by *D. novemcinctus*, Loughry & McDonough (2013) indicated that there are first-order, second-order and third-order variables that must be considered as originally outlined by Johnson (1980). First-order selection is a function of particular geographical areas and is probably controlled by climatic factors such as temperature and moisture. *Dasypus novemcinctus* prefers moist environments with relatively warm winters (Taulman & Robbins 1996; Bond *et al*. 2002). They do not do well in dry environments (e.g. deserts and some grasslands) and areas with severe winter temperature extremes (Loughry & McDonough 2013). Particular habitat types within the geographical range form the second-order parameters. Riparian, bottomland, hardwood forests appear to be one of the major habitats preferred by *D. novemcinctus* throughout its range as determined by Loughry & McDonough's (2013) summary of the literature. However, they cautioned that second-order habitat types may be broader, because Gammons *et al*. (2009) failed to find any evidence for habitat preferences in a radiotelemetry study of *D. novemcinctus* in southwestern Georgia. Third-order factors, preferences within a specific habitat type, are poorly known for armadillos (Loughry & McDonough 2013), as well as most mammal species.

Applying a direct analogy of habitat preferences for the extinct *D. bellus* from the modern *D. novemcinctus* may be misleading. For instance, tapirs (*Tapirus* spp.) ranged from South America throughout the southern USA to locations as far north as Nebraska, Pennsylvania and New York during the Pleistocene, and were extinct in the USA and northern Mexico by 11 ka (Graham 2003). The range of Pleistocene tapirs in the USA is very similar to the range of *D. bellus*. Some of the Pleistocene tapirs as well as *D. bellus* in the USA were associated with non-analogue biotas containing boreal rodents and vegetation (Stafford *et al*. 1999; Semken *et al*. 2010). Based upon these associations, Graham (2003) felt that the Pleistocene tapirs in the USA may have been adapted to colder climates like the modern Andean tapir (*T. pinchequa*) that lives at elevations greater than 2000 m and ranges up to 4500 m (Nowak 1983). It is adapted to colder climates. The larger *D. bellus* may have been similar in that it had colder tolerance limits than the living *D. novemcinctus*. *Megalonyx jeffersonii*, Jefferson's ground sloth, is another example of a South American-derived taxon that has been found in direct association with boreal environments in the USA during the late Pleistocene (Schubert *et al*. 2004).

Its range expansion and contraction are therefore more likely to have been driven by changes in temperature and perhaps habitat. Radiocarbon dates on scutes from Missouri and Arkansas caves suggest that *D. bellus* dispersed southward from Missouri, and perhaps Arkansas, during the cold full glacial (18–15 ka) and then reinvaded Arkansas (~14 ka) and Missouri (~11 ka) in the warmer late glacial (Schubert & Graham 2000).

It remains unclear why the nine-banded armadillo did not re-expand northward as the climate warmed in the Holocene. One possibility is that humans played a role in limiting what habitat was available to armadillos (Taulman & Robbins 1996). The Coahuiltecan tribes of northern Mexico and southern Texas hunted the nine-banded armadillo, and brushfires prevented the growth of potential armadillo habitat (Taulman & Robbins 1996). In addition, the Rio Grande probably presented a barrier to expansion (Taber 1939). When the Coahuiltecan tribes were displaced by European settlement in the 19th century and a suppression of fire by settlers began, the vegetation returned, and so did the nine-banded armadillo. Armadillo expansion has also been linked to corridors such as creeks, rivers and roads (Taulman & Robbins 1996), which have been increasing in number and even crossing the Rio Grande River since the mid-19th century (Taulman & Robbins 1996).

We acknowledge that our sample size is small; our low success rate in extracting and sequencing DNA from preserved armadillo remains reflects the preference of this species for climates that are less than ideal for long-term DNA preservation. Despite this small sample size, we are able to confirm that two genetically distinct armadillo lineages were present in North America during the Late Pleistocene and early Holocene. Additional samples will be required in order to evaluate the extent of genetic diversity that existed between these armadillo lineages and to evaluate how geographically widespread each lineage was.

Although our conclusions are well supported by the mitochondrial genetic data, it should be noted that mitochondrial genetic data can only be used to recover part of the evolutionary history, specifically the maternal evolutionary history, of a species. It has been shown that mitochondria can experience evolutionary histories that differ from many nuclear loci, including movement between closely related species via hybridization and introgression (Edwards *et al*. 2011). Although the amplification and analysis of nuclear loci would strengthen the results of this analysis, it was unfortunately not possible to recover nuclear DNA from any specimen that we were able to sample. Future identification and analysis of better preserved beautiful armadillos, for example from caves located in more temperate regions of either North or South America, would be useful to confirm the results presented here.

Conclusions

According to the mitochondrial data, at least one fossil skeleton attributed to the beautiful armadillo in North America has been misidentified, and is instead a large nine-banded armadillo. Unfortunately none of the other 11 armadillos sampled from the collection at the Florida Museum of Natural History were sufficiently well preserved to contribute data to this study. Recent advances in ancient DNA technologies are likely to improve chances of recovering DNA from Pleistocene-aged specimens from warm, wet environments such as Florida (Shapiro & Hofreiter 2014). These technologies, in combination with increasingly available genomic resources from living xenarthrans, will make it possible to return to these specimens in future, and use them to better characterize the diversity of armadillos in Late Pleistocene and early Holocene Florida.

In support of the genetic data, osteoderm measurements show that the beautiful armadillo ranged in size considerably and that large, modern nine-banded armadillos may overlap in size with the smallest beautiful armadillos (Klippel & Parmalee 1984). Given our results, it is possible that the large size variation observed in beautiful armadillos may be an artefact of the misidentification of large, Pleistocene nine-banded armadillos as small beautiful armadillos, as has been suggested previously (Schubert & Graham 2000). Care should therefore be taken when using these identifications, for example to predict species ranges or to infer palaeoenvironments. Further work, including both morphological and genetic analyses, will be necessary to understand the extent of misidentification in the fossil record.

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