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DNA analysis of archaeological rabbit remains from the American Southwest

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Abstract

Ancient DNA analysis was carried out on 20 archaeological rabbit remains from an early Pueblo II period site in Colorado (circa 1000 A.D.) to explore the possibility of obtaining accurate rabbit genus and species identifications. The presence of abundant rabbit remains at archaeological sites in the American Southwest indicates the importance of rabbit species in the subsistence economy and ritual activities of early aboriginal populations. The study of these remains is hindered by the difficulty of accurate identification due to the fragmentary nature of the bones and the lack of genus- and species-specific morphological features.

A short cytochrome *b* gene fragment was amplified and sequenced to produce a genetic profile for each bone sample. At the genus level, the DNA identifications were consistent with those based on the analysis of mandible morphology for the majority of specimens. When compared to species-specific reference DNA sequences, *Lepus americanus* and *Lepus californicus* samples were easily identified. Identification of an unexpected *L. americanus* (snowshoe hare) from the remains provided new information concerning hunting ranges or exchange between groups in the region. *Sylvilagus nuttallii* and *Sylvilagus audubonii*, however, could not be confidently differentiated at this point due to the difficulty in obtaining accurate species-specific reference sequences.

The inability to obtain such reference sequences can be a serious problem for DNA species identification of non-domestic animals that lack population-level genetic data and have few sequences available in GenBank. The lack of the DNA data increases the possibility that inappropriate reference sequences could be applied, resulting in false species identification even when authentic DNA is retrieved and amplified from ancient remains.

Keywords: PCR; Ancient DNA; Species identification; Reference sequences; Rabbits; Lagomorph; mtDNA; American Southwest

1. Introduction

Throughout the American Southwest various rabbit species are found in most archaeological sites and often dominate the assemblages. Compilations of data from arid low-elevation areas in Arizona [38,39] and New Mexico [36] or moister upland environments in southern Utah and Colorado [9] consistently document rabbits as a significant component of the ancestral Puebloan diet.

The study of rabbit remains excavated from archaeological sites has provided important insights into resource exploitation and habitat modification by early aboriginal populations in the region. However, detailed studies are hindered by the inability to accurately identify these remains using morphological criteria, due to intrinsic problems associated with the lack of genus- and species-specific morphological features and the fragmentary nature of archaeological specimens.

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Most rabbit remains from the American Southwest can only be identified to the genus level based on the size of bone elements. Rabbits have distinctive morphological attributes that make separation from other small mammals relatively easy. However, the two genera, *Lepus* (jackrabbits) and *Sylvilagus* (cottontails) are morphologically quite similar, and are usually separated on the basis of size. It is assumed that larger specimens are identifiable as *Lepus*, and smaller specimens as *Sylvilagus*. Very immature specimens or highly fragmented specimens are identified only as "rabbits".

With the advent of ancient DNA techniques [10,33,37], a DNA approach has become an important alternative for accurate identification of ancient remains [3,5,30,44]. In this paper, we report a study using ancient DNA techniques to evaluate the size-based genus identification method that is currently used by zooarchaeologists to identify rabbit remains, and to explore the possibility of DNA identification. The results from 20 bone samples demonstrated the reasonable utility of genus identification based on size and the importance of DNA species identification in the study of archaeological rabbit remains. This research also showed that for some species the lack of reliable species-specific reference sequences could be a serious problem for ancient DNA species identifications.

2. Materials and methods

2.1. Archaeological rabbit bones

The archaeological specimens used in this study were excavated from an early Pueblo II community in southwest Colorado, at the northern margin of the Southwest culture area. Approximately 10,000 rabbit specimens were identified from a pit structure known as "Kiva H" at the Stix and Leaves Pueblo (5MT11555) located west of the town of Cortez in Montezuma County, Colorado [4]. The precise location of the site is not given for protection of the site. Kiva H is a rectangular, subterranean feature, lacking the masonry lining that is typical of later kivas. The fill of the kiva consisted of large amounts of ancient refuse.

Rabbit bones were initially identified to genus, mainly using size to distinguish *Lepus* from *Sylvilagus*. The identifications recorded in Fig. 1 reflect the original identifications made during the first stage of the analysis. As the analysis progressed, we became aware that size criteria were being applied somewhat subjectively. Therefore, once the analysis was complete, we tested our initial identifications with metric data [29] in order to further refine initial genus classifications. Analyses of mature long bones showed very clear differentiation into two size groups. However, measurements of mandibles (Fig. 1), revealed two problems.

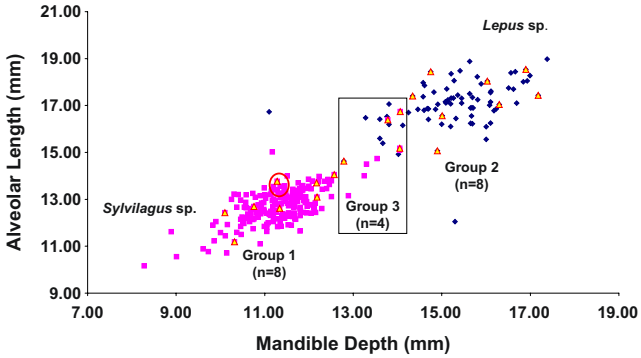

Fig. 1. Bone samples selected for ancient DNA analysis. Eight specimens were randomly selected from each of groups 1 and 2, and four samples were from an overlapping group 3. The group separation was based on alveolar length and mandible depth (see Table 1). All specimens were previously identified to the genus level based on visual examination of size: squares represent morphologically determined *Sylvilagus* sp., diamonds *Lepus* sp., triangles the samples selected for the ancient DNA analysis. The circled RB05 from group 1 was DNA-identified as *L. americanus*.

First, although there was distinct clustering, there were also specimens that fell between the clusters. Second, we had probably misidentified some smaller *Lepus* as *Sylvilagus* (Fig. 1).

To validate the practice of identifying to the genus level on the basis of size, to investigate the identification of intermediate size mandibles, and also to explore the possibility of DNA species identification, a total of 20 specimens were selected for ancient DNA analysis (Figs. 1, 2 and Table 1). There were eight specimens from each group that had been confidently identified as *Sylvilagus* or *Lepus* on the basis of size. Four tentatively identified specimens of intermediate size were also selected.

Sample selection and numeration were performed by one individual (JW) within the research group while the

Fig. 2. Image showing the morphological preservation of bone sample.

Table 1
Bone samples used in this study, their morphological measurements and their morphological genus identification, materials for DNA extraction, number of sequencing (No. seq.) and their DNA species identification

Sample	G	Mandible morphology			DNA analysis			Genus match
		Meas. A	Meas. B	Genus ID	Extraction	No. seq.	Species ID	
RB1*	1	12.17	13.70	SYL	Mandible	4	*S. nuttallii ?*	Yes
RB2	1	10.10	12.43	SYL	Mandible	2	*S. audubonii ?*	Yes
RB3*	1	10.32	11.19	SYL	Mandible	3	*S. nuttallii ?*	Yes
RB4	1	10.76	12.69	SYL	Mandible	3	*S. audubonii ?*	Yes
RB5*	1	11.28	13.77	SYL	Mandible	5	*L. americanus ?*	No
RB6	1	12.57	14.05	SYL	Teeth	2	*S. audubonii ?*	Yes
RB7*	1	12.18	13.09	SYL	Mandible	4	*S. nuttallii ?*	Yes
RB8	1	11.34	12.62	SYL	Mandible	2	*S. audubonii ?*	Yes
RB9	3	14.06	16.74	LEP	Teeth	2	*L. californicus ?*	Yes
RB10	3	13.78	16.39	LEP	Teeth	2	*L. californicus*	Yes
RB11	3	12.79	14.63	SYL	Mandible	2	*L. californicus ?*	No
RB12	3	14.05	15.18	LEP	Mandible	2	*L. californicus*	Yes
RB13	2	14.34	17.40	LEP	Mandible	2	*L. californicus*	Yes
RB14	2	14.75	18.44	LEP	Mandible	2	*L. californicus*	Yes
RB15	2	15.01	16.56	LEP	Mandible	2	*L. californicus ?*	Yes
RB16	2	14.90	15.07	LEP	Mandible	3	*L. californicus*	Yes
RB17*	2	16.03	18.04	LEP	Mandible	4	*L. californicus ?*	Yes
RB18	2	16.30	17.04	LEP	Teeth	2	*L. californicus*	Yes
RB19*	2	16.90	18.54	LEP	Mandible	5	*L. californicus*	Yes
RB20	2	17.18	17.43	LEP	Teeth	2	*L. californicus ?*	Yes

Note: G is group number during sample selection (see Fig. 1 for details). * indicates the sample was extracted two times by two researchers; ? indicates uncertain species identification. Measurement A, mandible depth; measurement B, alveolar length (after Neusius and Flint [29]).

analysis was performed by another (DY), with the removal of all information in a blind test. Although complete mandibles were analysed in the morphological studies, only small pieces of mandible or the teeth were prepared and used for ancient DNA analysis (Table 1). In general, the bone samples chosen for ancient DNA analysis demonstrated good macroscopic preservation (Fig. 2).

2.2. Bone decontamination and DNA extraction

The small size of teeth and the fragile nature of mandibles prevented the application of physical surface decontamination using abrasion with sandpaper. Instead, a vigorous chemical decontamination protocol was used in an attempt to eliminate all surface contaminants [44]. A small fragment of mandible or one or two teeth (from the same mandible) were placed into a new 15 ml tube and first soaked with 10% commercial bleach solution for 5-10 min. The sample was then immersed in 1 N HCl for 1 min and subsequently in 1 N NaOH for another minute and then rinsed with ample amounts of ultra-pure water. Samples were placed in a Crosslinker for ultraviolet (UV) irradiation for 30 min on each side. The decontaminated bone sample was then ground into powder prior to DNA extraction.

A modified silica-spin column method was employed to extract DNA from the bone powder [43]. The powdered samples (0.1–0.6 g) were incubated at 50 °C overnight with 3–5 ml of lysis buffer (0.5 M EDTA pH 8.0, 0.5% SDS and 0.5 mg/ml proteinase K) in a rotating hybridization oven (to prevent the formation of sediment and ensure efficient proteinase K digestion). After centrifugation, 1.5–2.0 ml of supernatant was transferred to an Amicon centrifugal filter, Ultra-4 (Millipore, Billerica, MA), reduced to less than 100 µl, and purified through the use of QIAquick columns (Qiagen, Hilden, Germany). Approximately 100 µl of DNA solution from each sample was collected for subsequent amplifications through the polymerase chain reaction (PCR) technique. For the purposes of reproducibility tests, six of the 20 samples were repeated from bone preparation, DNA extraction to PCR amplification by a different researcher at a later time (Table 1).

2.3. PCR primer design and PCR amplification

Mitochondrial DNA (mtDNA) was chosen as the DNA marker for this study due to its high copy number per cell and its high mutation rates as compared to nuclear DNA [37]. Although the highly variable control region of the mitochondrial genome would have been preferred for this ancient DNA project, there are unfortunately only a limited number of control region reference sequences available in GenBank for the rabbit species found in the American Southwest. Therefore, the cytochrome *b* (CytB) gene portion of the mitochondrial genome was used for species identification.

Based on rabbit DNA reference sequences obtained from GenBank, primers for PCR amplification of degraded DNA were specifically designed for a short fragment of less than 200 bp covering one of the most variable regions of the CytB gene. To avoid potential contamination, no modern rabbit samples were used for PCR optimization. Testing of the specificity and sensitivity of the designed primers was instead heavily dependent on software such as NetprimerTM (Premier Biosoft, Palo Alto, CA) and on actual PCR experimentation with different primers. Primer F38 (5′-TTGTTAACCACTCCCTAATTGACCT-3′) and R233 (5′-AGTCAGCCGTAGTTTACRTCTCG-3′) were chosen to amplify a 195 bp CytB fragment from ancient DNA samples.

PCR amplifications were conducted in a Mastercycler Personal (Eppendorf, Hamburg, Germany) in a 25 μl reaction volume containing 50 mM KCl, 10 mM Tris–HCl, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 1.0 mg/ml BSA, 0.3 μM each primer, 2.5 μl DNA sample and 1.25 U AmpliTaq Gold™ (Applied Biosystems). PCR was run for 60 cycles at 94 °C for 30 s (denaturing), 55 °C for 30 s (annealing), and 72 °C extension for 40 s. According to the manufacturer's recommendation, an initial denaturing was performed at 95 °C for 12 min to effectively activate the polymerase. Five microlitres of PCR product were separated by electrophoresis on a 2% agarose gel and visualized using SYBR Green™ staining on a Dark Reader Box (Clare Chemical Research, Dolores, CO, USA). PCR products were purified using Qiagen's QIAquick™ or MinElut™ purification kits and were subjected to direct sequencing.

The sequencing was carried out on an ABI 3100 using ABI Big Dye version 2 (Applied Biosystem) at the Mobix Laboratory of McMaster University, Hamilton, Canada. Primers F38 and R233 were used respectively to sequence the two opposite strands and the obtained electropherograms were assembled to examine any base pair ambiguities using ChromasPro software (www.technelysium.com.au).

2.4. Analysis of modern rabbit DNA samples to obtain reference DNA sequences

A thorough GenBank search indicated there was no CytB reference sequence for *Sylvilagus nuttallii* in the database. Current biogeographic evidence strongly indicates the possible presence of this species in the region in the past [11]. Skin samples (originally collected from Wyoming, USA) from two individuals of this species were therefore sought and obtained from University of Washington Burke Museum (UWBM). To make more comparative reference DNA sequences available, skin, hair and soft tissues of three other rabbit species from UWBM were also processed (Table 2). [During the preparation of the manuscript, it was found that a new CytB sequence AY292723 (Table 2) was made available in GenBank for *Sylvilagus nuttallii*].

Table 2
Reference DNA sequences used in this study and the locations where the samples were originally collected

Reference DNA sequence	Source	Location
S. aquaticus	GenBank U58937	Louisiana, USA
S. aquaticus JR1233	UWBM 75819 /JR1233	Louisiana, USA
S. audubonii	GenBank U58938	Wyoming, USA
S. audubonii JM144	UWBM 51959 /JM144	New Mexico, USA
S. audubonii JM146	UWBM 51957 /JM146	New Mexico, USA
S. floridanus 1	GenBank U58939	Maryland, USA
S. floridanus 2	GenBank AF034257	Unknown
S. nuttallii	GenBank AY292723	Utah, USA
S. nuttallii KL95	UWBM 51985 /KL95	Wyoming, USA
S. nuttallii KL96	UWBM 51984 /KL96	Wyoming, USA
S. transitionalis	GenBank AF034256	Unknown
L. alleni 1	GenBank AF010157	Sonaro, Mexico
L. alleni 2	GenBank AF010156	Sonaro, Mexico
L. americanus 1	GenBank AF010152	Alaska, USA
L. americanus 2	GenBank U58932	Maine, USA
L. arcticus	GenBank AF010153	Greenland
L. callotis 1	GenBank AF010159	New Mexico, USA
L. callotis 2	GenBank AF010158	New Mexico, USA
L. californicus	GenBank AF010160	New Mexico, USA
L. californicus JR1234	UWBM 75820 /JR1234	Washington, USA
L. californicus JR1235	UWBM 75821 /JR1235	Washington, USA
L. othus	GenBank AF010154	Alaska, USA
L. townsendii	GenBank AF009733	Utah, USA
L. europaeus	GenBank 21425393	Sweden
L. sinensis	GenBank AJ279418	China

The UWBM samples were considered to be modern samples (originally collected from 1975 to 2000) and therefore, the DNA extraction of such samples was not conducted in the dedicated ancient DNA extraction laboratory. DNeasy Tissue Kit (Qiagen, Hilden, Germany) was used to extract DNA from skin and other soft tissues samples (1–4 mm in size) according to the manufacturer's recommendations. Due to the dry nature of the samples, overnight incubation was used to allow for complete digestions of the samples. Approximately, 200 μl of DNA solution was collected for each sample.

The PCR reaction volumes and running conditions for the modern DNA samples were the same as those used for the ancient DNA samples except for the number of cycles (50 was used for modern DNA) and the duration of the denaturing, annealing and extension times of each cycle: 1 min was used for modern DNA samples instead of 30 or 40 s used for ancient DNA samples. Modified published universal primers L14724 (5′-AGCTTGATATGAAAAACCATCGTT-3′) [32] and

H15149 (5′-CCCTCAGAATGATATTTGTCCTCA-3′) [22] were used to amplify a longer CytB fragment (474 bp) from the modern DNA samples.

2.5. Phylogenetic analysis, genus and species identifications

BLAST searches were performed against GenBank to monitor whether the obtained individual sequence would match any unexpected sequences or species. Detailed comparisons, however, were conducted through multiple alignments of all reference sequences using ClustalW [41] through BioEdit (www.mbio.ncsu.edu/BioEdit/bioedit.html).

Phylogenetic analysis was used to assign a DNA sample to genus and species based on close phylogenetic relationship. All DNA reference sequences were first examined using MEGA2 [25] to evaluate how informative the amplified 195 bp CytB fragment was for genus and species separation. While one species, *Lepus sinensis* from China and one species *Lepus europaeus* from Europe, were included as "outgroup" species for *Lepus* species, the other reference species were all originally from North America (Table 2). Most of these reference sequences were retrieved from GenBank and the rest were extracted from modern skin and soft tissues by the authors (Table 2).

2.6. Contamination controls

The analysis of ancient DNA in this study followed strict contamination control protocols for ancient DNA [44]. For example, all ancient DNA extractions and PCR setups were conducted in the SFU dedicated ancient DNA laboratory. PCR amplification and subsequent work involving PCR products was conducted in a separate laboratory. The two laboratories are physically separated from each other and situated in two different buildings of the university. Blank DNA extractions and negative controls were undertaken during all DNA extraction and PCR setups, respectively.

To avoid the PCR products of ancient rabbit DNA mixing with modern reference DNA sequences, longer DNA fragments were targeted for the PCR amplification of modern reference DNA samples, ruling out the shorter PCR products as potential contaminant templates for the PCR amplification of modern reference rabbit DNA samples. To prevent modern reference DNA samples themselves or their PCR products from contaminating ancient DNA samples, all ancient DNA samples were extracted and amplified 6 months before modern reference samples were shipped to the university, which excludes the possibility of these reference DNA sequences as a contamination source.

3. Results

3.1. PCR amplifications and direct sequencing

Table 1 lists the number of sequencings that were carried out for each individual sample, with up to five replicate sequencings. All ancient rabbit DNA samples generated strong PCR amplifications of 195 bp fragments while both blank extraction and PCR negative controls remained negative. Direct sequencing of these PCR products resulted in generally clear electropherograms; some sequencing results occasionally showed some messy and weak signals at the beginning of the electropherogram. Reliable DNA sequences, however, were readily obtained using sequences from opposite DNA strands as well as from the repeated DNA extracts in this study.

3.2. Multiple sequence alignment and phylogenetic analysis

BLAST searches revealed that all ancient DNA and modern reference DNA sequences extracted in the laboratory matched rabbit species, clearly indicating their rabbit origin. Subsequent multiple alignments demonstrated that the amplified CytB sequences from ancient DNA samples belong to six distinct types, although two of them are separated only by a single base-pair difference (Fig. 3).

When multiple phylogenetic analytical methods including UPGMA and neighbour-joining (NJ) were employed, trees with a similar topology were obtained for the reference sequences (Fig. 4). Although the trees showed a clear separation of *Sylvilagus* and *Lepus* species, three species, *L. californicus*, *S. audubonii*, *S. nuttallii* were placed at different positions in the trees by different reference DNA sequences (from GenBank and from the authors, respectively). When our own reference sequences were removed from the dataset, the GenBank sequences generated similar trees as the originally untruncated longer sequences [13], demonstrating that the shorter CytB fragments used in this study were not significantly less informative [14,15]. Halanych et al. caution that the CytB sequence may contain limited phylogenetic information for intergeneric relationships due to substitution saturation, but they point out that the CytB is still very informative for separating intrageneric species.

The inclusion of the ancient DNA sequences in subsequent phylogenetic analyses did not change the general tree topology of the reference DNA sequences. Fig. 4 is a NJ tree with 1000 bootstrap tests using the Kimura 2-parameter model, which clearly demonstrated that all *Lepus* and *Sylvilagus* species were well separated, and *L. sinensis* and *L. europaeus* were distinct from the *Lepus* species of North America. A close examination,

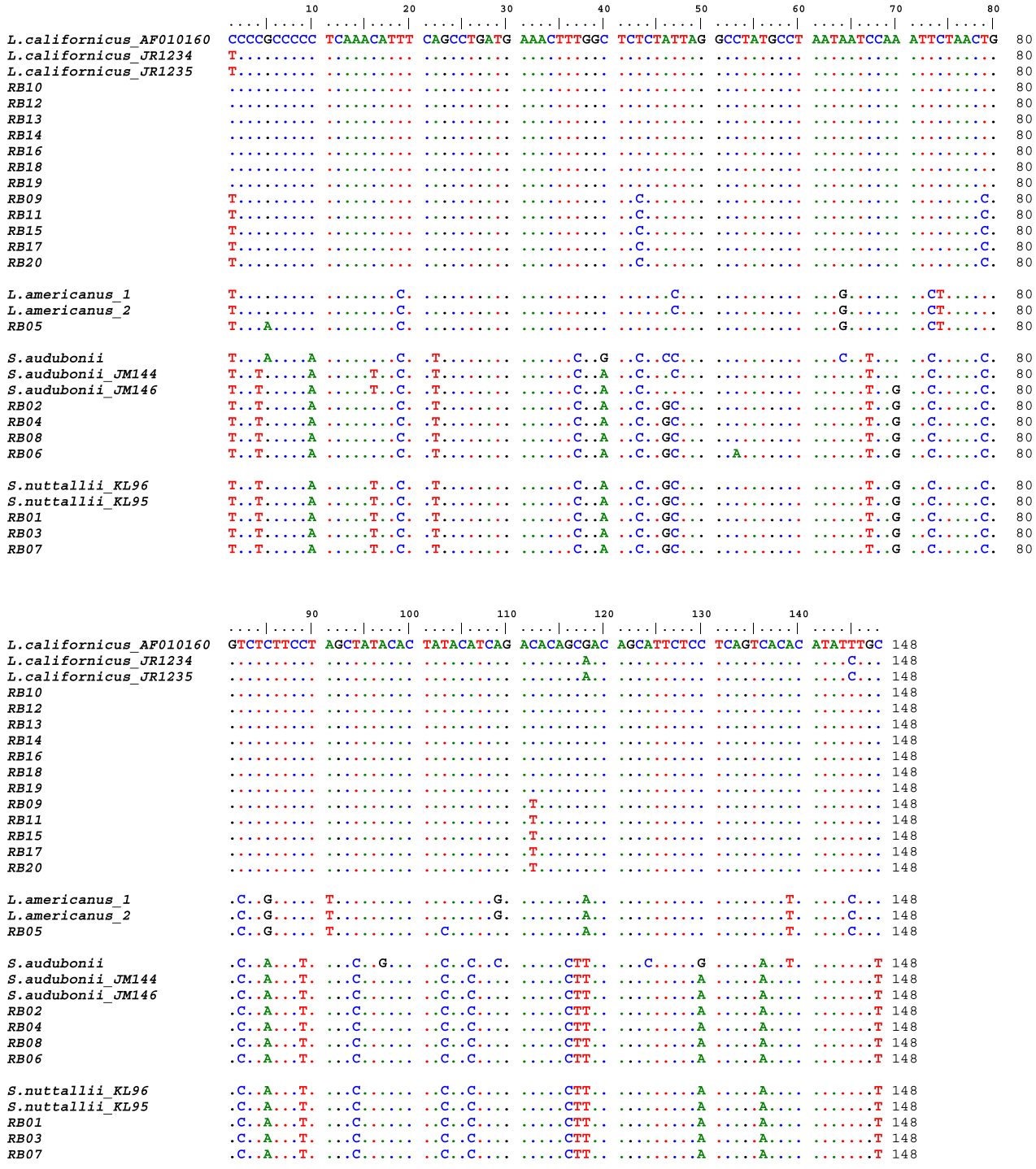

Fig. 3. Multiple alignments of the amplified ancient CytB fragments and equivalent reference sequences. See Table 2 for more information on the reference sequences: *L. californicus* sequence AF010160 (from GenBank) was arbitrarily chosen to be the alignment "reference"; dots indicate identical base pairs with AF010160.

however, revealed a complex pattern for *L. californicus* as the three reference DNA sequences were scattered over different branches and mixed with two other species, *L. callotis* and *L. alleni*. This result may reflect that the two latter species are very closely related to *L. californicus* [13] and the different *L. californicus* DNA sequences may also reflect regional variation (one from New Mexico and two from Washington State; see Table 2 for details). It seems unlikely that all of these sequences were caused by DNA template damage and PCR amplification errors since each of these *L. californicus* haplotypes was found in multiple individuals and was successfully duplicated.

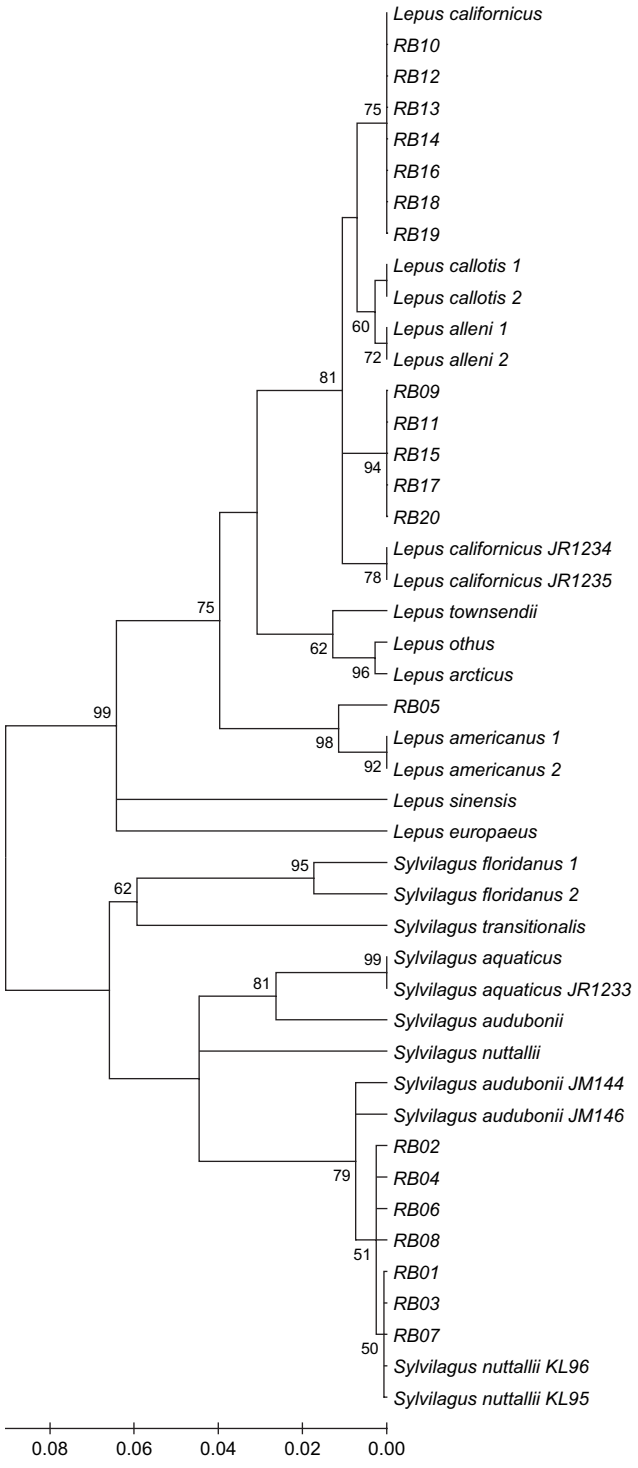

Fig. 4. Neighbour-joining tree generated using the Kimura 2 Model. Bootstrap value of 1000 iterations is shown at each node unless it is below 50%. Check Table 2 and Fig. 3 for more information on each individual sequence used in this phylogenetic analysis.

Only one ancient DNA sequence, RB05, clustered with snowshoe hare, *L. americanus*. This affiliation is supported by the bootstrap test with a high value of 98%, demonstrating the high statistical significance of the connection.

For the *Sylvilagus* species, the tree showed inconsistent placements for *S. audubonii* and *S. nuttallii*. Although the sequence of *Sylvilagus aquaticus* JR1233 from the authors was clearly clustered with that of the same species from GenBank, two *S. audubonii* and two *S. nuttallii* sequences collected by the authors showed a significant difference from those retrieved from GenBank. For example, for *S. audubonii*, the two reference sequences collected by the authors differed by only two base pairs within the 148 bp fragment, while 12 base pairs differences were observed between the author's sequences and those published in GenBank (Fig. 3). Interestingly, the modern *S. audubonii* reference sequences JM144 and JM146 and *S. nuttallii* KL95 and KL96 were clustered together with RB02, RB04, RB06, RB08, RB01, RB03 and RB07. Within this unique branch, three ancient DNA samples RB01, RB03 and RB07 were grouped with *S. nuttallii* (although this was only supported by 50% in the bootstrap test) while RB02, RB04, RB06 and RB08 were placed between *S. nuttallii* and *S. audubonii*. Clearly, the discrepancy would cause some problems for the subsequent species identification.

3.3. Genus identification

Due to significant DNA sequence differences between *Lepus* and *Sylvilagus* (Figs. 3 and 4), DNA genus identity can be confidently assigned to all 20 samples. Table 1 lists the match or mismatch between DNA and metric genus identifications of each individual sample in this study. A high match rate 90% (18/20) was observed although a higher rate (100%, 8/8) was seen for those typical "large" *Lepus* samples, a slightly lower rate (88%, 7/8) for "small" *Sylvilagus* individuals and a lower rate (75%, 3/4) for those of intermediate size. When comparing with the results from the visual identification (Fig. 1), the match rates remain the same as in the metric identifications for the "large" and "small" samples but a much lower match rate was observed for those of intermediate size (25%, 1/4).

The results demonstrated the usefulness of size in separating these two genera, but also indicated the potential for misidentification if based on size criteria alone. Although errors occurred with the *Lepus* specimens of intermediate size, the misidentification of a snowshoe hare could also occur within the "typical" group (Fig. 1). It seems that smaller *Lepus* specimens could be potentially misidentified as *Sylvilagus* (Table 1). It is not clear if this observation can also be applied to rabbit remains from other archaeological sites, because zooarchaeologists rarely publish the size criteria used to separate the two genera.

3.4. Species identification

All remains were assigned to species (Table 1) based on their DNA sequence and their proximity to reference DNA sequences in the phylogenetic tree (Figs. 3 and 4). Seven samples were confidently assigned to *L. californicus*, with a further five samples tentatively assigned to the same species, one sample was confidently assigned to *L. americanus*, three samples tentatively to *S. nuttallii* and four samples tentatively to *S. audubonii*.

Identification of *L. californicus* was based on the observation that 12 ancient DNA samples were clustered within the *L. californicus* branch (Fig. 4). Seven samples were closely clustered with one reference sequence of *L. californicus*, clearly indicating the species identity. Five other samples could be also assigned to *L. californicus* based on the following reasons: (1) they are quite different from the sequences of the two other species, *L. callotis* or *L. alleni*, grouped in the same cluster; and (2) the archaeological site is far away from the current distribution of *L. callotis* or *L. alleni*. However, the current distributions *L. callotis* or *L. alleni* might be different from those of 1000 years ago when these archaeological remains were deposited. Due to the nature of ancient DNA, these samples can be confidently identified as the genus *Lepus* while the species identity remains tentative.

The species identity of snowshoe hare *L. americanus* was assigned to RB05 due to its closeness to the species in the phylogenetic tree (Fig. 4).

Determination of *Sylvilagus* species was challenging and results could only be considered tentative since the phylogenetic trees could not provide clear species identity for the samples (Fig. 4). This problem was seemingly caused by the use of different reference DNA sequences (of the same respective species). For example, if the GenBank sequences were discarded, samples RB01, RB03 and RB07 might be identified as *S. nuttallii* while the others might be considered as possible *S. audubonii*. If the reference sequences obtained from our own laboratory were discarded from the analysis, no species identity could be assigned easily to those specimens due to the large genetic distance (Fig. 4).

Unfortunately, DNA determination of species from the remains could not be directly compared with that of morphological examinations due to the lack of reliable morphological methods for species identification of rabbit remains.

4. Discussion

4.1. Authenticity of the ancient rabbit DNA

As in any ancient DNA studies [21,23], the authenticity of the ancient rabbit DNA needs to be examined carefully and the possibility of contamination with modern rabbit DNA or previously amplified PCR products should be excluded before any identifications can be accepted [42,44]. Due to the careful research design, the authenticity of the obtained DNA sequences can be assessed through the following multiple means of examination.

4.1.1. Strict contamination controls

A dedicated ancient DNA laboratory and a separated post-PCR laboratory were used in this study and strict contamination controls were exercised throughout all stages of pre-PCR lab work [7,31]. No systematic contamination was observed since the blank extracts or PCR negative controls remained negative.

Cross-contaminations between ancient and modern DNA could be excluded in this study. Due to timing of the analyses of ancient DNA and modern rabbit DNA samples, it is impossible that the obtained ancient rabbit DNA sequences could be the result of contaminant modern reference DNA samples. Although the possibility of cross-contamination with comparative rabbit skeletons during the morphological analysis could not be completely ruled out, such contamination, if it occurred, could only be minimal and sporadic. The comparative collection in the zooarchaeology laboratory includes *S. floridanus* but this species was not identified from these 20 samples. Nevertheless, the potential risk of such cross-sample contamination was dealt with through the application of extremely rigorous chemical decontamination measures [44], in spite of the potential damage these protocols may effect on authentic DNA in ancient remains.

4.1.2. DNA sequence analyses

One of the intrinsic advantages associated with ancient faunal DNA studies is that the obtained sequences themselves could be indicative of their authenticity [44]. Two genera and four possible species of rabbit were determined from the same set of archaeological remains, theoretically excluding the possibility that these results were derived from a single contaminant source. The identified species are among those candidate species inferred from ethnographic evidence and current habitat distribution of these species; no species from outside the American Southwest were detected in the remains.

4.1.3. DNA preservation

The good morphological condition of most bone samples (Fig. 2) and the recent antiquity of 1000 years of the site should be considered favourable factors for good quality DNA preservation in the studied samples [20,26,27]. The dry climate of the region should also aid in the preservation of DNA by slowing DNA degradation.

In addition, ancient DNA has been reportedly extracted from ancient rabbit remains at much older ages in Europe [17–19].

4.1.4. Reproducibility test

If an obtained DNA sequence is from authentic ancient DNA, multiple extractions and amplifications of the same bone sample should generate the same DNA sequence [2,24,34]. In this study, the same result was successfully replicated multiple times: (1) almost one-third of the samples were replicated from the sample preparation through bone powders to sequencing of PCR products; (2) the repeat was carried out by two researchers and at different times; and (3) multiple PCR amplifications and two strands sequencings (forward and reverse) resulted in the same DNA sequence.

4.1.5. Blind test and morphological examination

Other independent evidence supporting the authenticity of the ancient DNA came from the results of the blind test in this study [44,45]. Based on mandible measurements, groups 1 and 2 (Table 1) were classified as *Sylvilagus* and *Lepus*, which was positively confirmed by ancient DNA analysis with 100% and 88% match (Table 1 and Fig. 2). If any contamination took place in this study, the contaminant DNA had to affect only bone samples of the same genus. In other words, all contamination of *Lepus* specimens would be from modern *Lepus* DNA and all contamination of *Sylvilagus* would have to be from modern *Sylvilagus* DNA. Although, this could conceivably take place with one sample, it is doubtful that all 16 samples in the two clusters could have been contaminated in this manner.

4.2. DNA identification of archaeological rabbit remains

This study demonstrated that size is a valid criterion for most ancient *Sylvilagus* and *Lepus* remains. If research questions only require accurate genus identifications, as a non-destructive and inexpensive approach, morphological identification should be employed, ideally in combination with ancient DNA analysis to delineate the boundary lines between the *Sylvilagus* and *Lepus* size groups. The combination of DNA and morphology will allow more samples to be analysed for accurate genus identifications. It should be pointed out that an ancient DNA analysis might be the only method available if bone samples are very fragmentary and juvenile.

This study also showed that ancient DNA analysis can be used for species identification of certain archaeological rabbit remains. The identification of snowshoe hare is a good example of DNA's utility in accurately determining some less common species. However, the need for the confirmation of some *Sylvilagus* reference DNA sequences clearly indicates that DNA species identification of ancient remains is not always a simple and straightforward application of DNA analysis.

4.3. Species determination from DNA sequence comparison

Theoretically, species identification can be obtained when DNA is retrieved from the studied samples and relevant reference DNA sequences are available from on-line databases [44]. However, this study demonstrated that ambiguity could still appear if the reliability of on-line reference sequences is in question.

In the case of *L. californicus*, although the three sequences used in this study were clustered together (Fig. 4), they were also clustered with two other species, *L. callotis* and *L. alleni*. This may be caused by species-level paraphyly or polyphyly [12]. While some ancient DNA sequences matched perfectly with one of the reference sequences, the others still exhibited some difference. A population-level genetic study of the species will be needed to show the genetic variability and to provide more comparative reference DNA sequences, thus yielding closer and less ambiguous matches in species identifications.

For species identifications of *S. audubonii* and *S. nuttalii*, unknown but potentially high genetic variability of these two species might have affected our ability to reveal species identity, a situation that can be seen with *L. californicus* samples. However, the observed genetic distances between the two types of the reference sequence seemed to be unreasonably high for *S. audubonii* and *S. nuttalii* (over 0.04 compared to approximately 0.01 for *L. californicus*) (Fig. 4). Difference in sample locations (Wyoming and New Mexico) probably could not account for such large genetic distance. We therefore propose that misidentified specimens or species-hybrid samples might have been used to generate reference sequences. Studies have demonstrated that these two *Sylvilagus* species are phylogenetically the most closely related [28,35]. Such phylogenetic closeness could be supported by our sequence data (JM144/JM146 and KL95/KL96) but not by the GenBank sequences that put *S. audubonii* closer to *S. aquaticus*. Unfortunately, we could not rule out another possibility that JM46/JM166 and KL95/KL96 might come from the same species. For these closely related species, a more comprehensive population genetic study is needed to clarify the aforementioned issues [12], especially in view of the difficulty of distinguishing living specimens of cottontail species that occur sympatrically [11]. Alternatively, population studies may also reveal the possibility that *Sylvilagus* species-level identifications are unachievable due to a genuine lack of differentiation between the two species themselves.

This study raises an important question regarding the quality, reliability and utility of reference DNA sequences retrievable from GenBank and other databases for DNA species identification. Some GenBank sequences might not be replicated and validated in the same way as ancient DNA sequences before they were submitted to GenBank. Furthermore, most phylogenetic analyses only use one specimen to represent a species [12]. This sampling strategy not only fails to reveal intra-specific variations but also makes it difficult to detect the use of misidentified specimens. If unreliable reference DNA sequences are used, false species identifications can occur even though authentic ancient DNA is retrieved. Therefore, extra care should be taken to ensure the reliability, accuracy and representativeness of the reference DNA sequences for individual species. While large-scale population DNA studies will provide an excellent opportunity to cross-examine existing reference DNA sequences, it may take time to see such research data become available. Alternatively, extra efforts could be made to secure morphologically correctly identified specimens for generating needed reference DNA sequence data. It should be kept in mind that introgression and hybridization between species [1] can also take place, which can further complicate the efforts to obtain authentic species-specific DNA sequences.

Theoretically, differences in the DNA sequence should be expected between ancient and present samples due to normal evolutionary changes. However, this raises the fundamental question of whether ancient species boundaries were the same as those of modern populations. Unfortunately, this possibility cannot be evaluated in this study because species boundaries cannot be reliably determined for these two modern *Sylvilagus* species.

4.4. Implications for archaeological and palaeoenvironmental studies

The study of rabbit remains from archaeological sites provides an opportunity to reconstruct the dynamic interaction between humans and various rabbit species, and to observe the consequences of environmental or societal change in the region. Varying habitat adaptations and population ecology of each individual species could affect human hunting strategies; alternatively, habitat alteration caused by farming practices could change the relative abundance of rabbit species.

Distributions of rabbit species may not have remained the same over the last few thousand years [6,16]. Modern studies demonstrate that ranges of rabbits are not stable, and have fluctuated in historic times as a result of factors such as varying land-use, over-grazing, and deliberate introductions. For example, a recent study documents changing ratios of *Lepus* species in response to humanly induced vegetation change [8]. Accurate genus and species identifications of ancient remains will allow accurate reconstruction of such dynamic interaction between humans and the rabbit species.

Accurate genus level identification of rabbits is necessary to address important archaeological questions in this region. For example, when considering human hunting and trapping behaviour, all *Sylvilagus* species behave in similar ways and were probably hunted using techniques adapted to those characteristics. Similarly, all *Lepus* species behave in similar ways, and hunting methods were probably the same for all species. Cottontails and jackrabbits have different habitat preferences; zooarchaeologists have used the *Lepus* to *Sylvilagus* ratio in archaeological assemblages to reconstruct the degree of vegetation clearance associated with agriculture or the kind of hunting methods used to procure prey [36,39,40].

Accurate species level identifications of rabbit remains, if all succeed, will enable archaeologists to address the same questions but on a more refined scale. DNA identification of specimens from site 5MT11555 showed some samples are from *L. californicus*, which is in agreement with the species in the region today. Moreover, DNA data also indicated that there might be another maternal lineage of *L. californicus* or another unknown but closely related species present in the remains. The presence of these two lineages or another unknown species certainly deserves further investigations.

DNA identification of *L. americanus* within the sample showed that DNA analysis can be a precise means for species identification since metrical data and ecological distribution could be insufficient to identify the presence of this species within the assemblage. Snowshoe hare was not expected, given that the archaeological site lies at least 40 km away from the nearest modern population, and is in an ecological zone not normally occupied by the species. Further studies of the relative abundance of this species in the remains will allow for a better understanding of its implications for hunting activity. Some possible interpretations are that this specimen was obtained on a long-range hunting trip to mountains about 40 km east of the site, or through exchange with another group. Unfortunately, we could not differentiate *S. audubonii* and *S. nuttallii*, hampering our ability to use the ratio between these two species to reconstruct palaeo-environmental conditions and human subsistence practices in the region.

Future studies will focus on the following three areas: (1) to process more identification-secured samples to conduct population-level DNA studies to determine the reference CytB DNA sequences of *S. audubonii* and *S. nuttallii*; (2) to collect reference sequences of other mtDNA fragments such as 12S rDNA [14] and control

region to examine the utility of these DNA fragments for rabbit species identification and population separations; and (3) to increase the sample size to study the long-term changes of early human activities and palaeoenvironments in the region.

5. Conclusions

Ancient DNA analysis of 20 archaeological rabbit remains demonstrated that ancient DNA was still present in 1000-year-old archaeological bone samples from the American Southwest.

1. Ancient DNA was successfully extracted and analysed from archaeological faunal remains using strict contamination controls and decontamination measures. The blind test and the replicated results all strongly support the authenticity of ancient DNA samples in this study.
2. Through phylogenetic analysis of a short CytB mtDNA fragment, two genera (*Sylvilagus* and *Lepus*) and two species (*L. californicus* and *L. americanus*) were determined from the remains while confident identifications of *S. audubonii* and *S. nuttallii* could not be made due to the difficulty in determining accurate species-specific reference sequences.
3. The DNA identification confirmed the morphologically based genus-level identifications of *Sylvilagus* and *Lepus* to small and large remains, respectively. Results also demonstrated that smaller *Lepus* could be mistakenly identified as *Sylvilagus*, and that *L. americanus* could not be detected using morphological methods alone due to its overlapping size range with *Sylvilagus*.
4. If genus level identification is adequate to address questions being asked, a combination of a size-based morphological method and a DNA-based molecular method would enable archaeologists to analyse large sample sizes to obtain more meaningful comparative results. However, ancient DNA analysis might be the only suitable approach if bone samples are fragmentary or juvenile.
5. The inability to obtain reference sequences can be a serious problem for DNA species identifications of non-domestic animals that lack population sequence data and have few sequences available in GenBank.

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