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SPIRIT EYE CAVE: REESTABLISHING PROVENIENCE OF TRAFFICKED
PREHISTORIC HUMAN REMAINS USING A COMPOSITE COLLECTION-BASED
ANCIENT DNA APPROACH

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Professional Paper

presented in partial fulfillment of the requirements
for the degree of

Master of Arts
in Anthropology

The University of Montana
Missoula, MT

Official Graduation Date: November 2020

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Spirit Eye Cave: Reestablishing Provenience of Trafficked Prehistoric Human Remains Using a Composite Collection-Based Ancient DNA Approach.

Chairperson: Dr. Meradeth Snow

Abstract

Spirit Eye Cave, located on private land in west Texas near the US/Mexico border, contains as many as four human interments removed by pay-to-dig collectors in the 1950-60s. The relocated remains provide initial DNA results from a region peripheral to both the Southwest and Plains, and the bone collagen 14C dates are coeval with a period of presumed multiethnic migration. The mitochondrial DNA results from two individuals indicate a maternal relationship between each interment. Considered together, these data indicate both a familiarity with the region and a stability of land use by foraging groups during a period of reputed instability. The identification of the B2a4a1 haplogroup in both individuals ties the region to indigenous groups in present-day Mexico, Texas, and the prehistoric site of Paquimé, in Chihuahua, Mexico. These results demonstrate the utility of a collaborative collection based aDNA approach for looted and heavily collected sheltered sites.

Significance Statement

Two rediscovered human remains from at Spirit Eye cave in west Texas situated on the US/Mexico border were radiocarbon dated and sampled for mitochondrial DNA. Both burials belong to the same B2a4a1 mitochondrial haplogroup and were dated to a period of dramatic cultural change in the region. Our results indicate a group of related foraging groups repeatedly used the cave as a mortuary site over several generations. This study also illustrates the utility of pursuing collection-based research from heavily impacted archaeological sites.

Introduction

Genetic research at Spirit Eye Cave (41PS25) located on the border of US-Mexico has proven to be an important method for reestablishing the research potential at a severely looted sheltered site. The privately owned cave was a pay-to-dig site in the past and collectors recovered thousands of perishable artifacts as well as at least four sets of desiccated human remains from their primary burial locations. The ongoing professional work at Spirit Eye Cave uses genetics as part of a larger effort to reconnect looted and trafficked remains with their original interment locations, native land, and direct descendants. We argue this will become a valuable application of DNA research, especially considering the amount of destroyed interment sites, and despite the understandable issue of destructive analysis (well summarized in Bardill et al., 2018). This is a problem that is severe in arid regions of the American Southwest in general, and acute in west Texas, where thousands of sites with dry-preservation, large private land holdings, and pay-to-dig access create a set of ideal conditions for the destruction and trafficking of perishable artifacts, and in extreme cases like Spirit Eye Cave, human remains (Schroeder 2017).

The focus of renewed research at Spirit Eye Cave was to develop a collection-based research design, AMS dating and reestablishing the provenience of perishable artifacts held in private collections in order to build an occupational chronology. This is possible because records associated with the site trinominal helped uncover decades of written correspondence between individuals associated with the pay-to-dig history of the cave and tied numerous large perishable collections to the site. However, these letters also revealed as many as four burials were removed during the 1950s and 1960s, all with permission of the landowner. The location and condition of these remains were unknown, and the research focus shifted to relocating these remains. Communication with authors of the letters revealed the first two burials (Burial 1 and 2) were removed in the 1950s. Burial 1 was rumored to have been sent to the Smithsonian Institute, while the other (Burial 2) was displayed around hotel lobbies in the small town of Marfa, Texas. The Smithsonian does not have Burial 1, and Burial 2 is now in a private collection and the owner denied any request to view the materials. The third burial (Burial 3) was disinterred in the 1960s and taken to a small privately funded museum in Texas where they remained on display until the late 1980s. The remains were taken down and thought to be lost but were rediscovered as a part of this research and sampled with the consent of the current steward of the remains. The final set (Burial 4) was sold to a private buyer in California. These remains were returned to the University of Texas-Austin after a California Fish and Game animal trafficking bust found the remains in a private residence. They are now held at the Texas Archeological Research Laboratory at the University of Texas-Austin and have been through NAGPRA consultation with no claimants (Burial 4 was sampled under staff supervision).

Of the four burials, two were relocated and used for this analysis, the available notes and letters indicate that Burial 3 was found in a flexed seated position with no mention of

associated grave goods or artifacts. Burial 4 was also found in a flexed seated position, and was capped with a metate. The associated grave goods that were included in notes include two bone awls and a large piece of limonite. There is no mention of associated diagnostic or additional materials with either burial in available written accounts from the collectors.

Unfortunately, the Spirit Eye Cave example is not unique; human remains have been looted and scattered across the United States and the world, and the ability to tie them securely back to the appropriate sites, native land, and descendants is important for establishing and building relationships with descendant communities. Such actions will hopefully aid in allowing for further scientific research in the future, as this part of North America has a rich and fascinating history. Using both the written letters and conversations with pay-to-dig collectors, the provenance of the remains with the cave was reestablished. The relocated burials were sampled for DNA, stable isotopes, and AMS dating (Table 1). They are among the first widely reported results from the Big Bend of the Rio Grande and will help address relationships with populations to the South and West through the shared haplotype found throughout the region. Furthermore, because some of the remains are in private collections, the DNA results could be used to identify a direct descendant community and build a dialogue between the present owners and descendants who could take possession of their ancestors and appropriately lay them to rest.

Lacking stratigraphic data to date the interments, Burial 3 and 4 were AMS dated (\bar{x} =715 & \bar{x} =853calBP; Table 2). The results are coeval with an influx of new cultural materials and settlement patterns that have been posited to be the result of an interpreted multiethnic migration into the west Texas region (Seebach 2007). El Paso Phase Jornada Pueblo IV-like structures and pithouses established at the confluence of the Rio Concho and Rio Grande (La Junta de los Rios) as early as 800 calBP mark an influx of horticultural groups (Kenmotsu 2018). Ceramics found at these sites suggest an initial colonization by Jornada-Mogollon groups followed by periods of village fissioning and further colonization possibly from Paquimé migrants (Kelley 1990; Kenmotsu 1994). Coeval aceramic hunter-gathering populations occupied stacked stone-based wickiup structures in the region and may have developed a mutualistic relationship with La Junta villagers (Mallouf 1999). The origin of these populations, as well as the degree of admixture between them, is a major point of scientific speculation.

The sampled remains from Spirit Eye Cave provide results that address local and regional research themes. The site lies in a part of Texas on the boundary of the Southwest and Plains physiographic regions, where little is known of the prehistoric demographic histories. It also provides a possible model for approaching the complexities encountered in dealing with private collections and collected sites. Creative approaches to dealing with both issues is important for “the next generations of archaeologists [who] may find themselves working in a very different environment than those of a generation ago” (Surovell et al. 2017:298). Engaging private collectors is a critical component of establishing research potential before these materials are lost.

Materials and Methods

DNA Extraction and Analysis. Samples were sent to the University of Montana Molecular Anthropology Laboratory, which houses a dedicated aDNA facility. This facility maintains the standard protocols for the analysis of aDNA, including UV lighting, positively pressurized and filtered air supply, separation from modern DNA laboratories, daily bleaching, and full-body covering for entry, among other contamination precautions. After the samples had been placed in a 50:50 household bleach diluted with water bath for approximately five minutes, they were rinsed twice with DNase free H₂O, and were allowed to air dry in a sealed container. This was followed by UV'ing the sample in a crosslinker for 15 minutes. Drilling the root of the sample was done in a sealed box using a dremel tool and dental drill bit in order to collect approximately 35mg per tooth.

The 35mg of tooth dust, collected into lobind 2mL tubes, was then soaked in 1mL EDTA (0.5M, pH 8) , and 10ul of 1 mg/mL Proteinase K was added. The samples were incubated at 55°C overnight with slow rotation at 4rpm. Following removal from incubation, the samples were extracted following the Dabney et al. (2013) protocol.

The samples were prepared for sequencing the mitogenome through use of the KAPA SeqCap EZ HyperCap workflow (Roche), with minor modifications as the samples were not sheared or size-selected. The kit allowed for End-repair, ligation of adapters and indices, sample pooling based on Qubit quantification levels (Qubit HS 1X dsDNA kit by Invitrogen), LM-PCR amplification, mitogenome probe hybridization, wash of the recovered multiplex DNA sample, and another round of LM-PCR. The samples were then run on the MiSeq at the UM Genomics Core.

Sequences were analyzed via a modified pipeline based on the original from Maria Nieves-Colon (https://github.com/mnievesc/Ancient_mtDNA_Pipeline; Ozga et al 2016).

Paired-end read sequences were merged with adapter trimming using SeqPrep (<https://github.com/jstjohn/SeqPrep>). Reads <30bp in length were discarded and read quality was assessed using FASTQC

(<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Reads were mapped to the revised Cambridge Reference Sequence (rCRS, NC_012920) (Andrews et al. 1999) using Burrows Wheeler Aligner (BWA) v0.7.17 (Li and Durbin 2009) with seed disabled (-l

1000) and edit distance increased to improve mapping accuracy as recommended by Schubert et al (2012). Damage patterns were analyzed using mapDamage 2.0 to assess misincorporations and read length distributions (See figures in SI; Jonsson et al 2013) and read quality scores were modified with re-scale option accounting for post-mortem damage. SNP variants were called and reported to the level of just variant sites and those with greater than 1x coverage. MtDNA haplogroups were assigned using Haplogrep v2.0 (Kloss-Brandstatter et al 2011; van Oven, 2015).

Following the data analysis, the fasta files that were created were analyzed with others downloaded from the literature and aligned using Muscle in MEGA7 (Kumar et al. 2016). Following this, the samples were used to create a Median Joining Network in PopART (Figure 3; Leigh and Bryant 2015). Additionally, Bayesian statistics were utilized using BEAUTi and BEAST2 (Bouckaert et al., 2014) in order to create a Skyline Analysis and maximum clade credibility tree, using FigTree. This was done following the guidelines outlined in the Introduction to BEAST: Calibration and Bayesian Skyline Analyses instructions, modified so the mutation rate used for the whole mitogenome, including the D-Loop, was set to 1×10^{-8} (Gojobori et al. 2015).

Authentication of Genetic Data. Contamination is always a concern when it comes to working with ancient DNA. Beyond the laboratory methods explained above, the resulting data was also analyzed to detect signals of modern contamination. An extraction control was utilized throughout the process, through sequencing, which did result in 102 mapped unique reads, which in comparison to the average mapped read for the samples (13506.6 reads) is 0.008%, demonstrating that the amount of contamination in the samples is very low. The average read length of the samples is 82.8 base pairs, which is also considerably shorter than that of the limited reads in the extract control at 126 base pairs.

Beyond these measures, and as noted above, MapDamage (Jonsson et al. 2013) was also run on all of the samples to account for damage patterns that accumulate at the ends of strands of DNA, creating a “smile” pattern that demonstrates a higher misincorporation of thymine at the start of reads, and cytosine at the end of individual reads. Figures F7S11 demonstrate that this pattern is found in the samples, albeit to varying degrees. This is probably due to the incredibly well preserved nature of some of the samples, as supported by the fact that sample 41PS25-100 had the most pronounced “smile” and was also the sample with the lowest coverage and sample quality, including read lengths averaged 60 base pairs in length.

Radiocarbon Dating. Collagen extracted from tooth root from Burial #3 (D-AMS 033187) and a left talus of Burial #4 (D-AMS 035070) was submitted to DirectAMS for dating. Prior to submittal Burial #4 was pretreated at the University of Texas at San Antonio following the acid-base-acid procedure outlined in Mauldin et al. (2013:1374). The Burial #3 sample was directly submitted to DirectAMS for pretreatment, both samples were then combusted and reduced to graphite in sealed vials (Zoppi et al. 2007:172-173). DirecAMS measured each sample using a National Electrostatistics Corporation Model 1.5SDH-1 Pelletron Accelerator with the same level of accuracy reported in Zoppi et al. (2007).

Isotope Analysis. A single tooth root from Burial #3 and a left talus from Burial #4 were submitted for stable isotope analysis at two separate labs; Burial #3 (D-AMS 033187) was submitted to the Washington State University Stable Isotope Core Laboratory by DirectAMS after it was radiocarbon dated. Isotope Analysis for Burial #3 followed the Washington State University Stable Isotope Core Laboratory procedures wherein carbon and nitrogen isotopic analysis converted N₂ and CO₂ with an elemental analyzer (ECS 4010, Costech Analytical); the gases were separated with a 3m GC column and analyzed

with a continuous flow isotope ratio mass spectrometer (Delta PlusXP, ThermoFinnigan, Bremen). Carbon isotopic results used the NIST calibration reported in per mill relative to VPDB (Vienna Pee Dee belemnite) with NBS 19 and L-SVEC as anchor points. Nitrogen isotope ratios are reported in parts per thousand (per mill) relative to N₂ in air.

Burial #4 (D-AMS 035070) was prepared at the University of Texas at San Antonio using methods outlined by Mauldin et al. (2013:1372-1373). The prepared sample was then analyzed at the Colorado Plateau Stable Isotope Laboratory at Northern Arizona University in continuous-flow mode using a Thermo-Finnigan Delta^{plus} Advantage gas isotope-ratio mass spectrometer interfaced with a Costech Analytical ECS4010 elemental analyzer. A standard 3-meter GC column was used (set at 55°C) for peak separation, in combination with one quartz (combustion) tube filled with chromium oxide and silvered cobaltous/cobaltic oxide (set at 1020°C) and one quartz (reduction) tube filled with reduced copper (set at 650°C). Data were normalized using 4 internationally-accepted isotope reference standards (IAEA CH6, CH7, N1, and N2). External precision on these standards is $\pm 0.10\text{‰}$ or better for $\delta^{13}\text{C}$ and $\pm 0.20\text{‰}$ or better for $\delta^{15}\text{N}$. $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ data are expressed relative to VPDB for carbon, and to AIR for nitrogen.

Results

Bone collagen extracted from Burial 3 and Burial 4 returned two AMS dates, one from each interment. The date from Burial 3 is older and brackets 921–790 with a median of 853 calBP (95.4%; D-AMS 033187); Burial 4 is younger and brackets 765–680 with a median of 715 calBP (95.4%; D-AMS 035070). Using the difference function in Oxcal version 4.3, the interment of Burial 3 in the cave predates Burial 4 by as much as 220 or as little as 55 calendar years; they are not contemporaneous. Following Pestle and Colvard (2012), the atomic C:N ratio for collagen extracted from Burial 3 is 3.21 and Burial 4 is 3.186, which are both within the acceptable range for accurate AMS dates from terrestrial bone (Table 1 and 2).

Bone collagen from Burial 3 and 4 was also submitted for stable carbon and nitrogen isotopes with Colorado Plateau Stable Isotope Laboratory and the Washington State University School of Biological Sciences (Table 1). The $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ from Spirit Eye Cave samples are similar to values reported from extra-regional transitional forager groups using, but not fully reliant upon maize (Coltrain et al., 2007; Piehl 2009; Slovak and Paytan 2011). Piehl (2009) carried out a study of ten individuals from the greater Big Bend region of the Rio Grande that dated from the Late Archaic through the Formative period (2000 calBP - 500 calBP). The Late Archaic $\delta^{13}\text{C}$ collagen samples indicate a diet higher in C₄ plants compared to individuals of the same age from the Lower Pecos Canyonlands further down the Rio Grande (Bousman and Quigg 2006). Piehl (2009) also noted a lack of associated dental pathology (caries and abscesses) in the Big Bend individuals compared to the Lower Pecos mortuary population. Piehl (2009:79) suggested, given the availability of similar floral resources in both regions, Late Archaic groups in the Big Bend may have incorporated maize into their diets during the Archaic. Interestingly, the results from individuals associated with formative period

horticultural village sites that are coeval with the Spirit Eye Cave samples indicate a diet higher in C3 cool season grasses, or animals that subsist on C3 grasses, suggesting a lack of maize. Compared to the Piehl (2009) results, the Spirit Eye Cave individuals are more similar to the Late Archaic populations than to the formative period villages. The overarching implications of these dietary data suggest more studies are needed.

Mitogenome Data. Burial 3 was run twice from two independent teeth and Burial 4 was run three times on independent teeth/bone. The results for Burial 3 and 4 were consistently identical from all extractions and sequences, demonstrating an identical mitochondrial haplotype for the two individuals, as well as between different teeth from the same burial. Coverage of the whole mitogenome ranged from 101X to 17X, with the average being 70X for the samples. The sample with the lowest coverage (41PS25-0-100, averaging 17X), did appear to have one minor mutational difference from the other samples from the same individual, however this is a product of low coverage at that locus. This does separate the sample out when looking at the tree created for the B2a4a1 samples (Figure 4), however examination of the data suggests this is a sequencing miscall due to low coverage.

The data obtained from the burial's mitogenome analysis was analyzed with Haplogrep (van Oven, 2015) to establish their maternal lineage, resulting in both individuals belonging to the B2a4a1 haplotype. This relatively rare lineage has been published previously in Achilli et al. (2013), where three other B2a4a1 individuals were published. These three were taken from modern individuals in Chihuahua, Jalisco, and Durango, Mexico. Unpublished data from additional modern individuals in Mexico also carried this haplotype and were collected among the Native Mexican individuals in Nayarit (a member of the Cora population), Sonora (Guarijío), Durango (Mexicanero), Guanajuato (Otomi), and San Luis Potosi (Pame) (Flores-Huacuja et al., in prep). An additional ancient DNA sample, found to belong to the haplotype, comes from the site of Paquimé in Chihuahua, Mexico (burial 17-6, coming from the Buena Fé phase house cluster). The haplogroup (B) was established in Morales-Arce et al., (2017), with the full mitogenome data newly presented here. Interestingly, burial 17-6 was classified as an extra-regional immigrant based on their oxygen isotope signature. This young adult male had a local strontium isotope range; however their oxygen values suggest that their origins are in Mexico, not the desert Southwest (Offenbecker, 2018, pg 103).

As can be seen in the Median Joining Network (Figure 3; Bandelt et al., 1999), the B2a4a1 samples form a small, roughly star-shaped cluster, speaking to the age and relatedness of the individuals, as discussed below.

Achilli et al (2013) placed the age of the haplotype at 6.1kya (95% CI 0.96k-11.42k). This haplotype derives from B2a4a, which dates to 12.68ka (95% CI 4.4k-21.34k). Utilizing these samples and their associated dates, a Bayesian skyline plot was created to look at the history of the effective population size of this lineage. As can be seen in Figure 5, there has been a gradual increase over time, with a notable uptick around 750BP, which is roughly at the time of the samples presented here, which may be influencing the analysis.

Discussion

The findings presented here demonstrate that the human remains excavated during the pay-to-dig era of Spirit Eye Cave's history have been located. Their shared matrilineal lineage also enables the two individuals to be linked to one another and aids in demonstrating the maternal occupancy of the cave bracketing a period from roughly AD 900 to AD 700. These results represent some of the first widely published aDNA findings from this region of Texas, with the hope that future investigations will aid in linking the many excavated human remains in private collections to their original place of burial, as well as those descendant communities they are most closely related to.

Since the formation of the state of Texas, Spirit Eye Cave has been on privately administered land, a history that shapes the legal responsibilities for consultation. Federal legislation like NAGPRA does not prevent a private landowner from excavating interments on their property. However, there are penalties for trespassers who traffic funerary items without the consent of the private landowner. However, these burials were removed with the permission of the landowner (at the time) and there are no state penalties for owning legally obtained human remains. At the time of this study, Burial 3 is held in a private collection, and Burial 4 has been through NAGPRA consultation with no claimants. The human remains from Burial 3 (as well as Burial 2, not sampled for this study) fall into a gray area where private land property rights are extended to the ownership of prehistoric human remains recovered from it; in such cases consultation is a self-regulated process.

In the absence of a legal framework initiating a guiding process, instances like Spirit Eye Cave place researchers in the tenuous position of balancing private property law, descendant communities' concerns, research objectives, and professional criticism. But until cultural laws in the United States include private property, the wishes of the private collector are prioritized with the hope that the results will open a productive discussion about long-term care of the collection. If the dialogue is open, it is then unclear in a multi-ethnic region like the Big Bend of the Rio Grande that defines the US/Mexico border, who to consult. Do we prioritize federal recognition over the local populations who are themselves of unrecognized mixed indigenous ancestry? The difficulty in cases like Spirit Eye Cave are that descendant communities should be invited early to the process, but the reality is that private collectors are guarded about what they show professionals; and it is difficult to gain access to a collection while also consulting with the appropriate groups about materials owned by a private party. The results of genetic analysis did identify several local living individuals belonging to the B2a4a1 haplogroup. They belong to federally non-recognized indigenous groups, and because of this research they are now part of an open dialogue with private collectors regarding the long-term care of the sampled remains. One of the main goals of this research is that through this analysis future researchers can learn how best to navigate this common, yet incredibly difficult, situation.

Genetic Data. Research at Spirit Eye cave provides some of the first DNA results from a dynamic period of multiethnic migration into the region. The results indicate stable

landscape usage by a maternally related group of foragers persisted for generations during this dynamic period throughout the region. The separation between each of the interment events by as many as eight and as little as two generations (assuming 25-year generations) suggests a stable land use pattern by a related group of maternally related foragers. Moreover, modern-day Native Mexican descendants with the same B2a4a1 haplotype were identified and future DNA work will incorporate them, with the aim of better understanding the relationship of the Spirit Eye interments and modern populations in the region.

All individuals found to belong to the B2a4a1 haplotype previous to this study have been sampled in Mexico, suggesting that this lineage may be associated with the populations there. An equally likely scenario is that sampling bias is at play due to lack of Native American samples from the Southwestern/west Texas region that abuts modern-day Mexico. Sampling bias aside, a matrilineal connection existed between the modern populations of indigenous individuals in Mexico and those in the prehistoric Paquimé and west Texas region. There has long been speculation of migration between northern Mexico and into US Southwest, bringing cultural associations of maize and the UtoAztecan language family (among many other material ties) (e.g. Casserino 2009, Di

Peso 1974, Waller 2016, Turner 1993, Mathiowetz 2011, VanPool and VanPool 2015, Hedrick 1974, Kirchhoff 1952). While the Spirit Eye individuals post-date the hypothesized entry of individuals into the region, which is thought to have coincided with the arrival of maize to the region (as early as 4000kya; Da Fonseca et al., 2015), perhaps this is a remnant of such a movement of individuals. Unpublished maize dates (2100 calBP) from Spirit Eye place a significant occupation of the cave within this early period of maize use identified throughout the US Southwest (Coaltrain and Janetski 2019), and maternal lineage use of the cave is established from the current study. Should such an occupation date back to the original migration, perhaps these interments reflect the migrating lineage to the region. Based on the current data, this is not possible to determine, however it does provide a small clue that such a migration may have occurred in the past, leading to shared maternal relationships between Mexico and the greater US Southwest.

It is also possible that what is being seen is a haplotype that spread with some of the initial settling of the region. The original maternal lineage could have spread throughout Mexico and the US Southwest/west Texas region and subsequently accumulated mutations led to the star-like distribution seen in the haplotype network (Figure 3). In the network, no region-specific mutations are present that aid in distinguishing samples from the north or south, nor do the regions share derived mutations within the haplotype. However, with the current mutation rate and shallow time depth, it is possible that not enough time has passed to allow for significant differences to have arisen within the haplotype to allow for distinguishing regions. Additional samples from the B2a4a1 haplotype would aid in determining where this haplotype arose and its link to larger regional migration hypotheses.

As was noted above, an individual with the same haplotype has also been sequenced from the archaeological site of Paquimé. The aDNA sample from Paquimé is not closely

related to those from Spirit Eye Cave, suggesting that the two lineages diverged some time before the interment of these individuals. The same can be said for the modern mitogenomes. A larger sample, as well as shared derived haplotypes, may allow for better pinpointing the closest modern descendants of these individuals, however the currently published sequences do not allow for this. Despite the genetic distance between the samples, there has been previous conjecture and analysis of the connection between west Texas and Paquimé, namely from a ceramic standpoint. Notably, El Paso Polychrome may have been a tradeware exchanged between the Jornada Mogollon and the Chihuahua populations (Burgett, 2006). Additionally, undecorated ceramics (brownwares) have been suggested to be tradeware within the region that includes west Texas and Paquimé (Hill, 2009). Among the Spirit Eye pottery assemblage, El Paso Polychrome and Paquimé trade wares were identified. Additionally, shell redistribution through Paquimé has also been suggested, with large quantities of shell artifacts moving from Paquimé to peripheral sites in the El Paso region, and possibly as far east as the Spiro Mound site in eastern Oklahoma (VanPool et al. 2005:29). These trade or migratory connections could have aided in gene flow, allowing for shared maternal haplotypes to be found in much of the region.

While the Spirit Eye and Paquimé samples do not share identical sequences (there are 4 mutational differences between them, suggesting a significant time depth between the lineages), they are close geographically and worth discussing. Interestingly, burial 17-6 was classified as an extra-regional immigrant based on their oxygen isotope signature. This young adult male had a local strontium isotope range; however their oxygen values suggest that their origins are in Mexico, not the desert Southwest (Offenbecker 2018, pg 103). If this individual is indeed non-local, and it is not a shared lineage due to the initial settling of the two regions, this would be additional support for a connection between Mexico and the US Southwest.

The ability to establish familial relationships using ancient DNA is well established (e.g.: Haawak et al., 2008; King et al., 2014; Deguilloux et al., 2014; Cui et al., 2015). The results from the B2a4a1 haplotype at Spirit Eye cave demonstrate related individuals were interred in the same location over a period of multiple decades (nearly a century-between 2 and eight generations). This suggests that the cave itself was being utilized by a maternal lineage, possibly as part of a seasonal migratory route or overwinter occupation. Additional testing of nuclear DNA could add to our understanding of the exact nature of these individual's matrilineal relationship.

The data from these two individuals builds upon limited research published in the region. To date, the only human aDNA has been presented in poster form at the AAPA meetings (Raff et al 2018), and the full results remain unpublished. When they are available, they will make for an interesting comparison. Based on the information from the poster, mtDNA haplotypes A2p and C1c were reported from two individuals (a mummy and tooth sample), along with a nuclear DNA SNP panel. The nuclear DNA, when compared with other individuals from the region, demonstrated gene flow from the Plains tribes, as well as a very close affinity to modern populations in Northern Mexico, which is upheld by our findings. These findings bode well for the potential for nuclear data from the

Spirit Eye mummies, and the potential for a more robust comparison in the future, although they are currently not comparable.

Acknowledgments

Access to Spirit Eye cave was granted by Pinto Canyon Ranch owners Jeff Fort and Kristina Van Dyke-Fort and this analysis would not have been possible without their aid. Marybeth Tomka of the Texas Archeological Research Laboratory at the University of Texas at Austin facilitated access to the Burial 4 material. Access to a private collection also made this research possible, and we thank those involved for their willingness to participate. Dr. Raymond Mauldin of the University of Texas San Antonio prepared the radiocarbon and isotope samples for Burial #4; he also reviewed and improved the methods sections for each of these analyses.

Comparative samples from Paquimé were extracted with NSF funding (award #1821483).

This article has been submitted for publication to the Journal of Archaeological Sciences (Elsevier) and acknowledges their rights to this paper as well as our rights as authors for use as a thesis paper.

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Figures and Tables

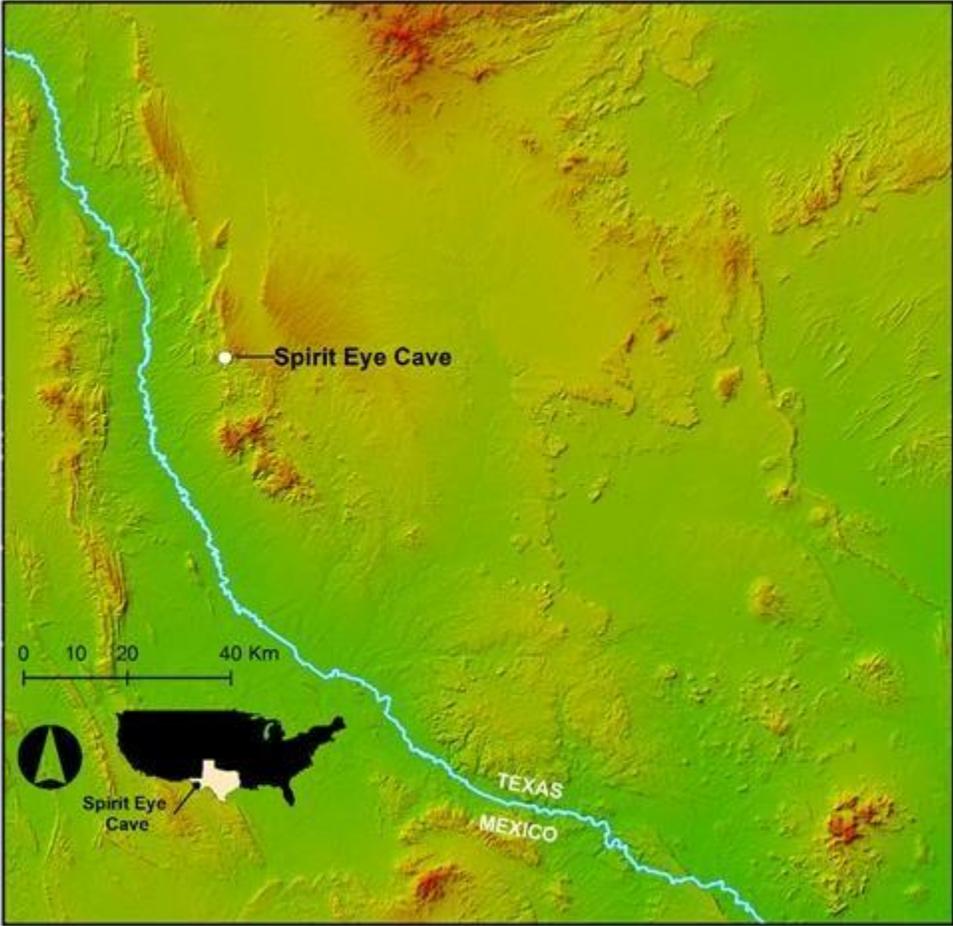


Figure 1. Location of Spirit Eye Cave (41PS25).

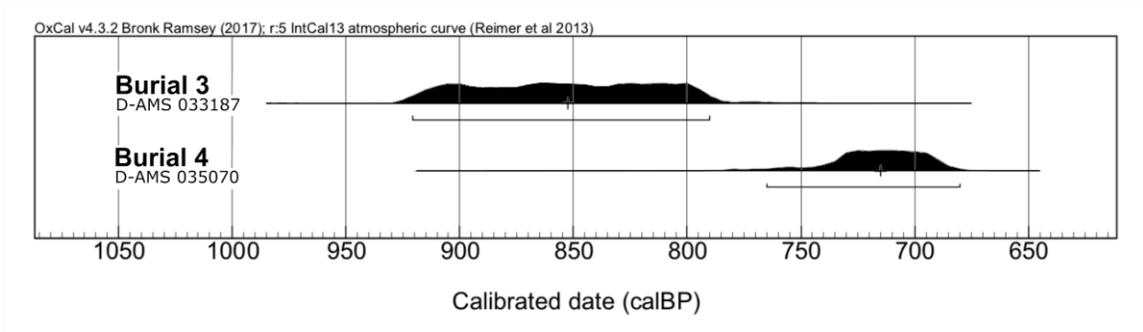


Figure 2. Calibrated multi-plot of bone collagen AMS dates from Burial 3 and 4 from Spirit Eye cave.

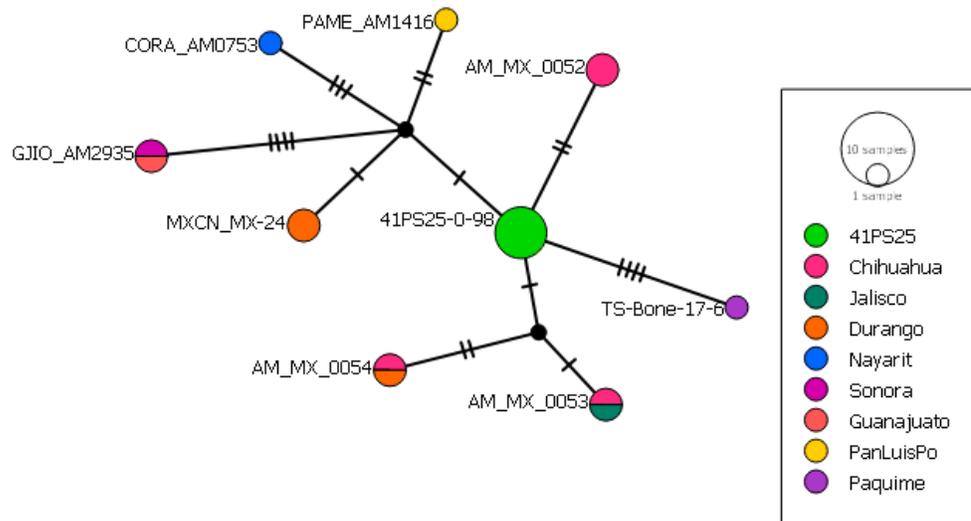


Figure 3. Median Joining Network of B2a4a1 samples (Bandelt et al., 1999). Green node containing all samples from 41PS25 (including repeats), hence the larger size. Figure created using PopART (Leigh and Bryant, 2015). Samples are labeled as follows: AMMX_0052-0054 (Chihuahua samples from Achilli et al., 2013), CK711034-36 (Chihuahua, Jalisco, and Durango respectively from Achilli et al., 2013), Cora_AM0753 (from the Cora population in Nayarit; Flores-Huacuja et al., in press), GJIO_AM2935 (from the Guarijío population in Sonora; Flores-Huacuja et al., in press), MXCN_MX-24 and MXCN_MX-55 (from the Mexicanero populations in Durango; Flores-Huacuja et al., in press), Otomi_Mex_DM1057 (from the Otomi population in Guanajuato; Flores-Huacuja et al., in press), and Pame (from the Pame population in San Luis Potosi; Flores-Huacuja et al., in press).

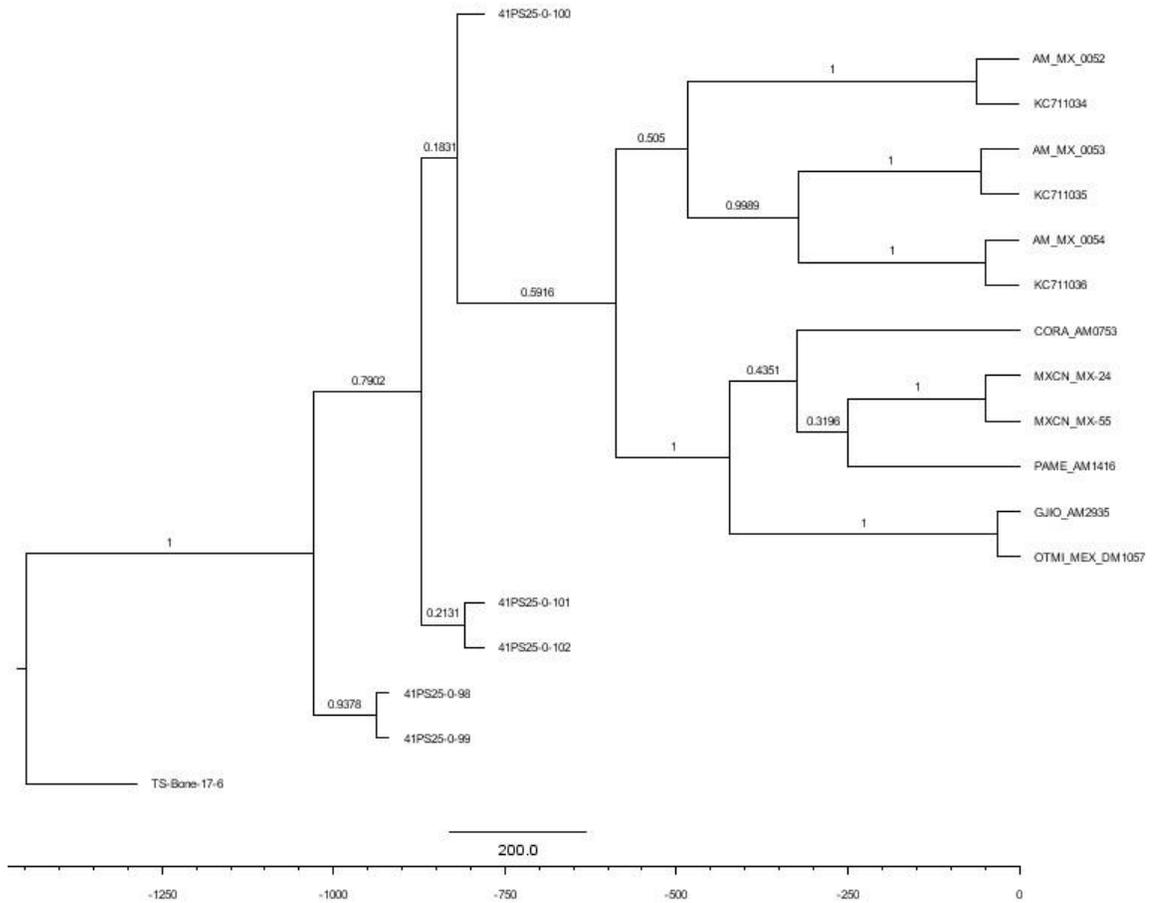


Figure 4. Maximum clade credibility (MCC) tree created in FigTree (part of the BEAST Bayesian statistical family of programs) utilizing the dates of collection or C14 dates on the respective samples. Tree dates are Before Present, and clades are presented with posterior probabilities, and those with higher probabilities (closer to 1) are more strongly supported. The low confidence on the split between the three samples from the same individual (41PS25-0-100/101/102) supports that they are from the same individuals, but as noted in the text, #101 is of lower quality. Sample names are the same as those in Figure 3.

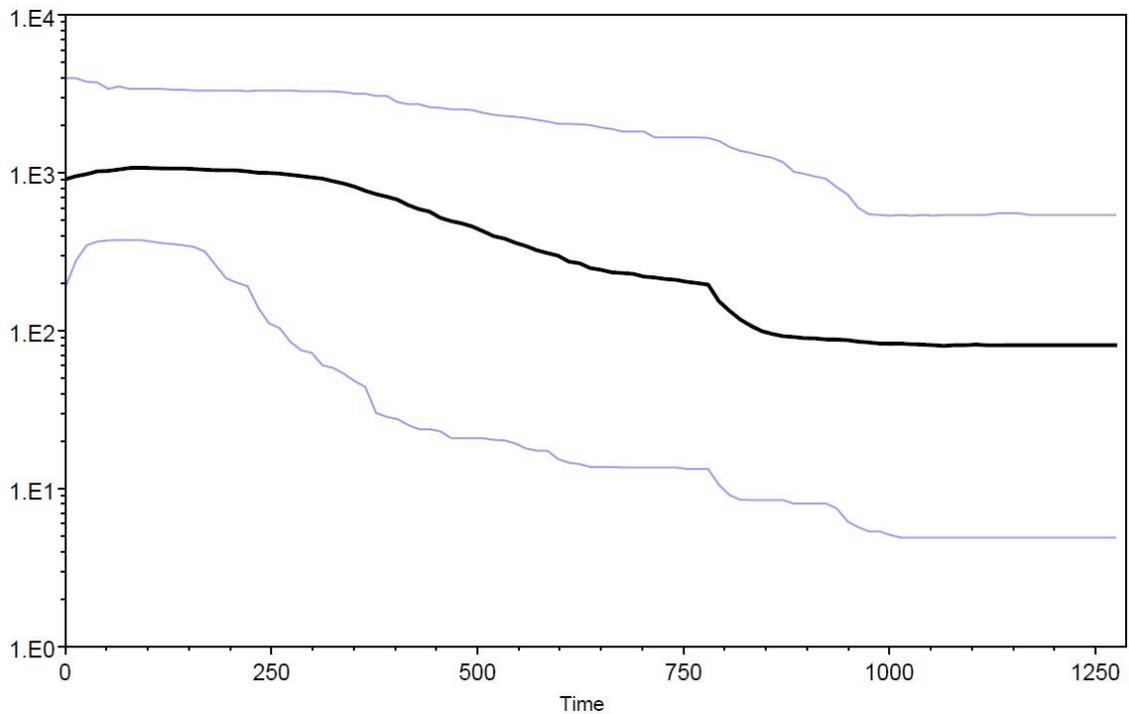


Figure 5. Bayesian skyline plot created using Tracer. 95% HPD also noted.

Table 1. Stable Carbon and Nitrogen Isotopes from Spirit Eye Cave Burials.

Spirit Eye Cave	$\delta^{13}\text{C}$	$\delta^{15}\text{N}$	wt %C	wt %N	Atomic C:N
Burial 3 (033187)	-10.8	9.62	42.314	15.37	3.210
Burial 4 (035070)	-11.1	8.54	42.34	15.49	3.186

Table 2. Sampled Spirit Eye Cave materials with associated context and chronometric data.

Sample		Haplogroup	Location of Materials	Collection History	14C Dates
Burial 4	41PS25-0-100 Canine (Rdc ₁)	B2a4a1	Housed at TARL/UT-Austin	Collected in 1968, sold on black market to buyer in California, confiscated in 1990s, Returned to UT-Austin	765–680 calBP; \bar{x} = 715 (95.4%; D-AMS 035070)
	41PS25-0-101 Left Calcaneus				
	41PS25-0-102 Left Talus				
Burial 3	41PS25-0-98 Molar (RM ₁)	B2a4a1	In private collection	Collected in 1960s maintained in private collection	921–790 calBP; \bar{x} = 853 (95.4%; D-AMS 033187)
	41PS25-0-99 Canine (Rdc ₁)				

Supplemental Information

Please see the following figures for information on the quality and quantity of reads from each of the samples referenced in the article.

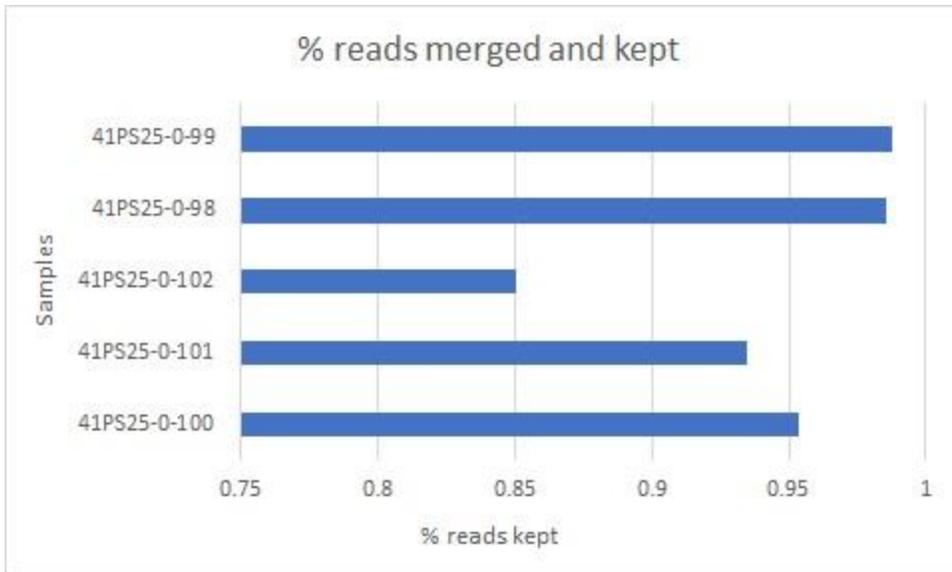


Figure S1. Percent of raw reads merged and kept from SeqPrep (<https://github.com/jstjohn/SeqPrep>).

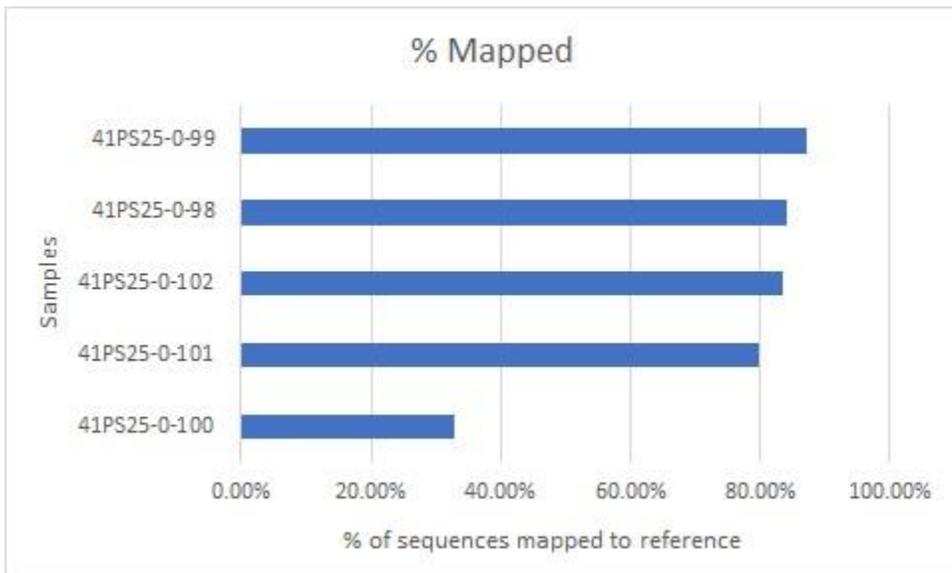


Figure S2: Percent of raw reads mapped to rCRS.

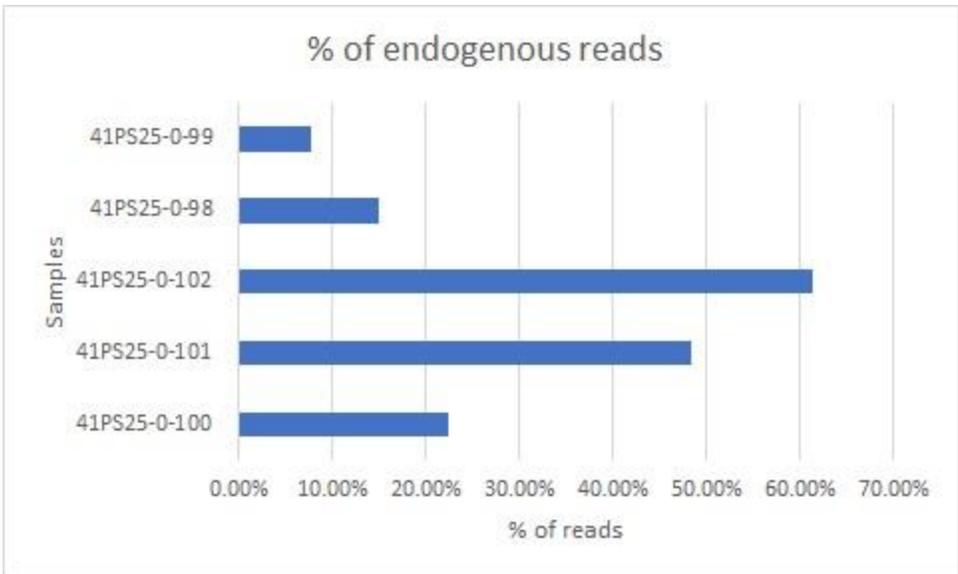


Figure S3: Percent of endogenous reads

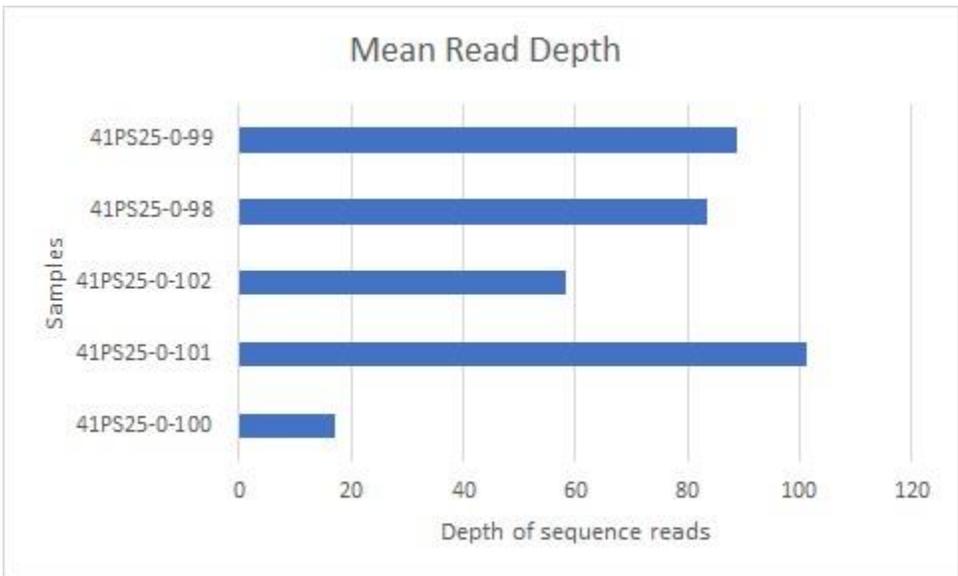


Figure S4: Mean read depth of sequencing reads

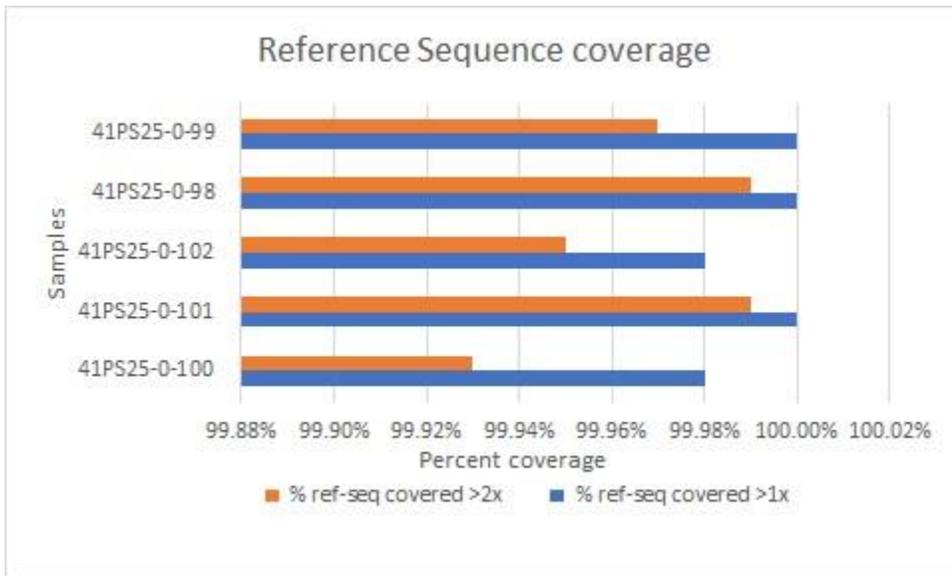


Figure S5: Coverage of reference sequence per sample at either greater than 2x and/or greater than 1x coverage.

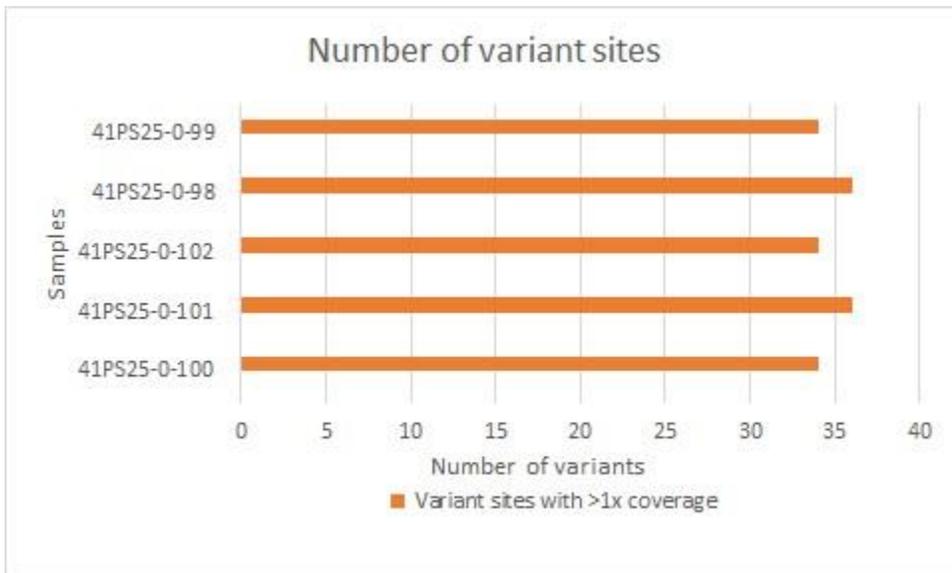


Figure S6: Number of variant sites with greater than 1x coverage

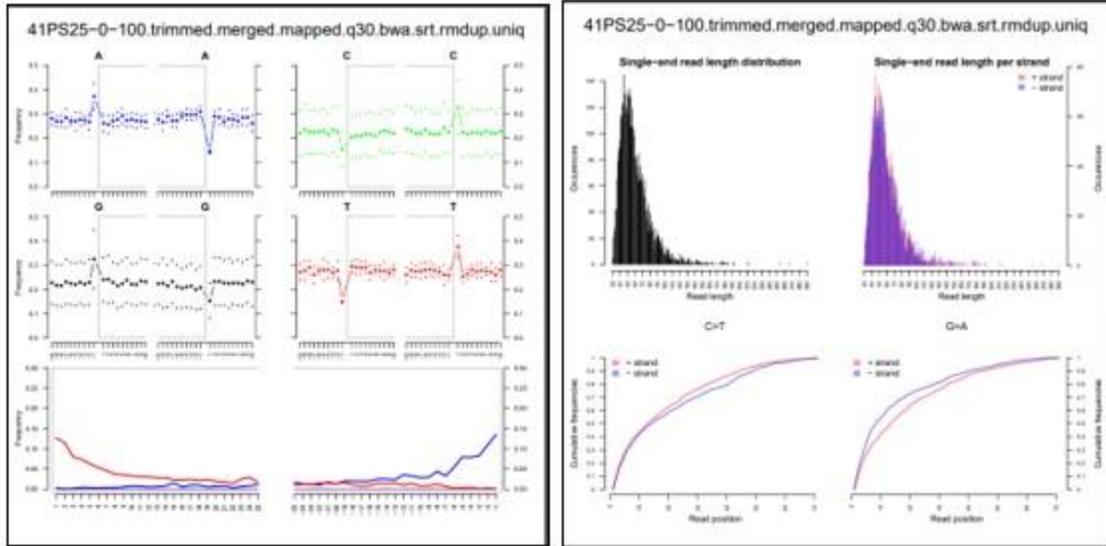


Figure S7: mapDamage 2.0 analysis indicating ancient authentic sequences by plotting of C>T (red lines) and G>A (blue lines) transitions.

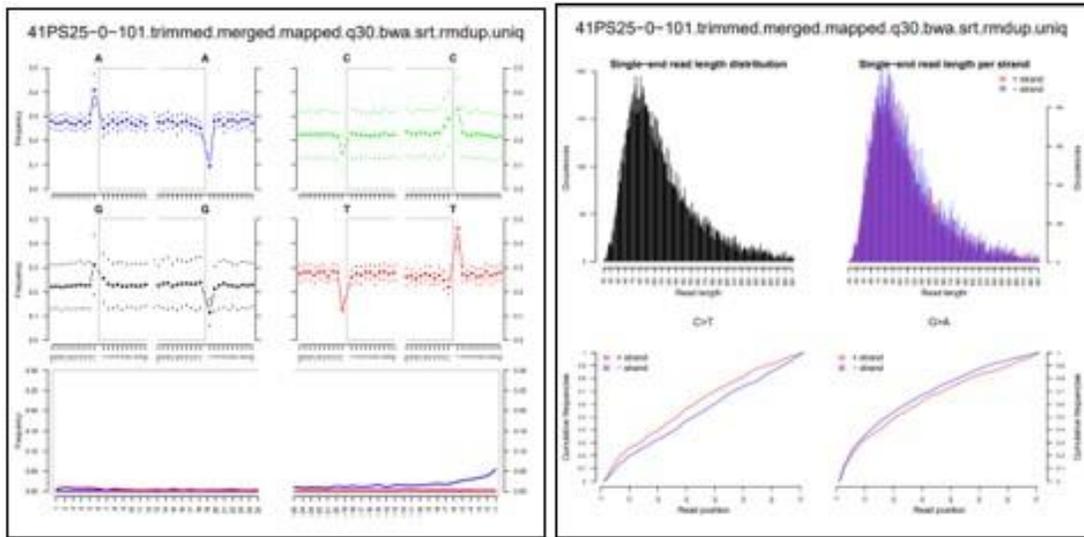


Figure S8: mapDamage 2.0 analysis indicating ancient authentic sequences by plotting of C>T and G>A transitions. While the pattern for this sample does not fully align with what is typically looked for in aDNA samples in terms of damage (the smiling damage pattern), it is from the same individual as pictured in figures S7 and S9. It would seem that the DNA preservation in this sample (taken from a calcaneus bone) is actually quite good, suggesting that this skeletal feature may be a good place to sample DNA from.

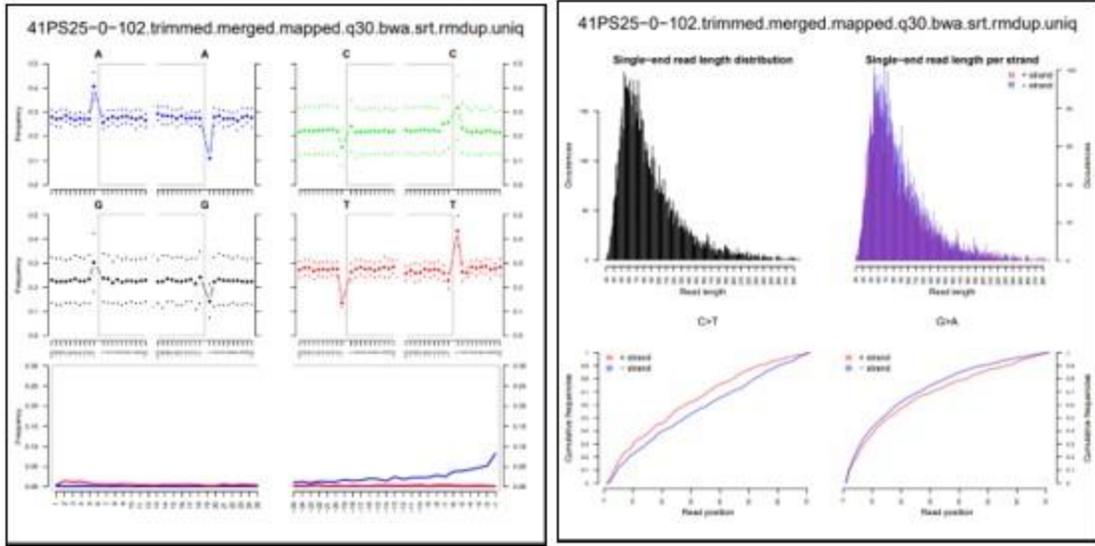


Figure S9: mapDamage 2.0 analysis indicating ancient authentic sequences by plotting of C>T and G>A transitions. See discussion on Figure S8 regarding the damage pattern.

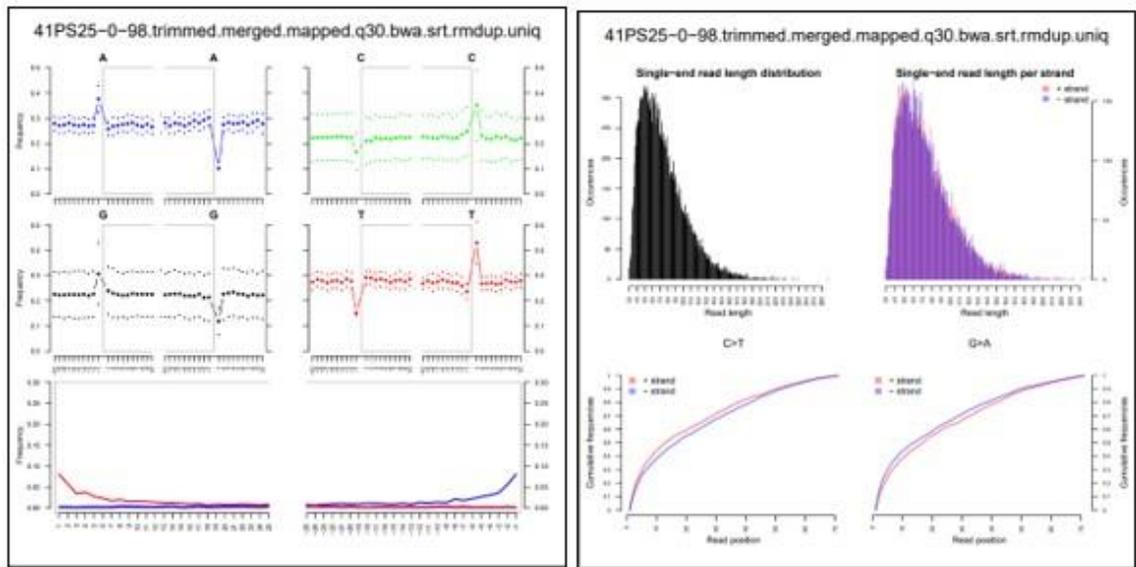


Figure S10: mapDamage 2.0 analysis indicating ancient authentic sequences by plotting of C>T and G>A transitions.

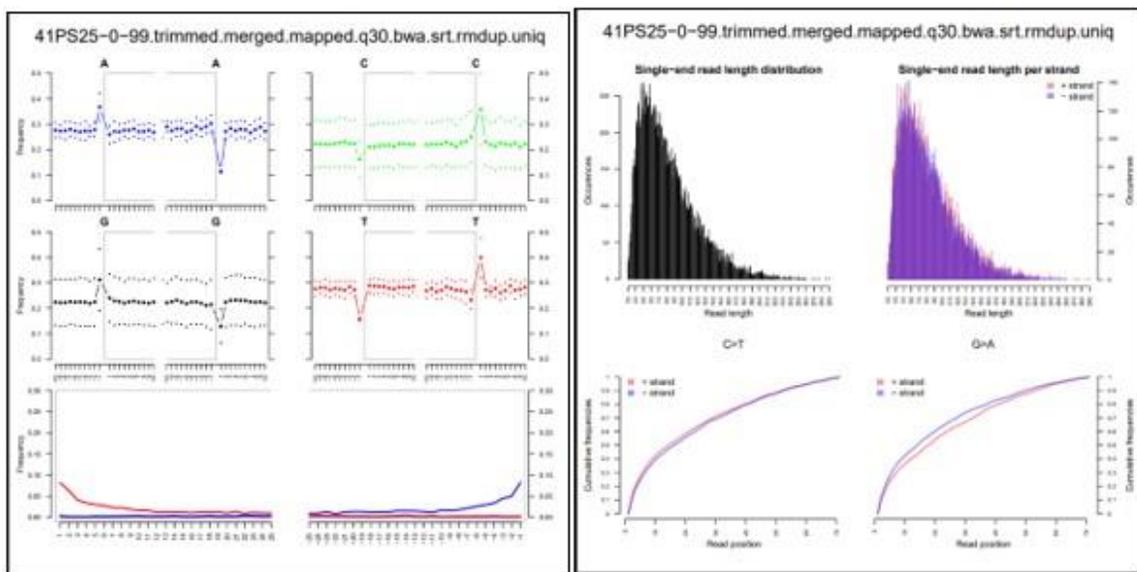


Figure S11: mapDamage 2.0 analysis indicating ancient authentic sequences by plotting of C>T and G>A transitions.