Analysis of Ancient Mitochondrial DNA within the Tipu Maya Collection

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Abstract

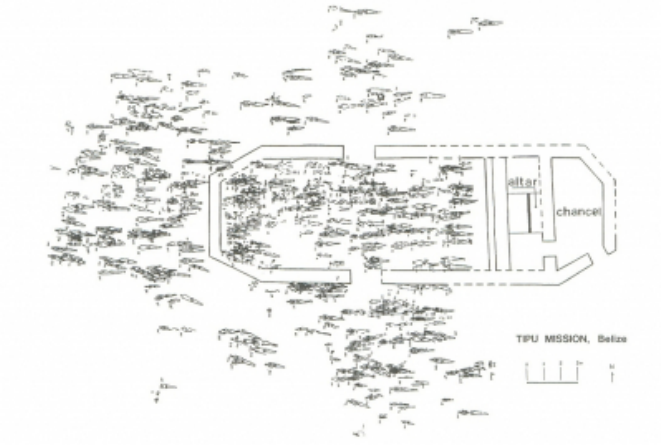
The 16th century Colonial Period Maya cemetery (ca. 585 skeletons) at Tipu, Belize is very large and well preserved, relative to Maya populations of any period. Much is known about these skeletal remains, including: gender, relative age, epigenetic dental traits, and visible pathologies. The Tipu Maya project not only continues, but it has been expanded to include ancient DNA (aDNA) analysis. The primary goal for this preliminary project was to successfully extract aDNA from bone and teeth samples to establish which protocol yielded the best quality of aDNA. Once isolated, aDNA was amplified using Polymerase Chain Reaction (PCR) in conjunction with primers that only target a 211 base pair region on the human 17th chromosome. Sequencing of the 14 base pair mitochondrial hypervariable region revealed a high frequency of rare Asian variants. Further analyses with a more targeted amplification and subsequent enzyme and sequencing application generated data that indicated all samples tested thus far have been from either haplogroups B, C, or D, three of the four apparent ancient human migrations into Latin America defined by previous aDNA analyses. This study will provide scientific knowledge to anthropological findings and will increase genetic knowledge of an ancient civilization.

INTRODUCTION

Tipu was the location of a 16th century Spanish mission among Maya on the Macal River in Belize. This site was discovered by Grant Jones through ethnohistoric documents (Graham, Pendergast and Jones 1989; Jones 1989; Jones, Kautz and Graham 1986). The cemetery located at this site was excavated from 1983 to 1988 by Dr. Mark Cohen, who led a team of archaeologists and undergraduate students from the State University of New York at Plattsburgh (SUNY Plattsburgh). The collection of 585 Maya skeletons (Figure 1) resided for some time at SUNY Plattsburgh. This collection is one of the largest excavated colonial period populations in the New World, as well as one of the largest and best preserved collections of Maya of any age.

Figure 1

Tipu Mission with burials (Jacobi, 1997 based on a site map created by Cohen).



Previous studies on the skeletons from Tipu have focused on gender, relative age, epigenetic dental traits, and visible pathologies (Cohen, 1989; Cohen et al. 1989; Jacobi, 1994; 1997). The research goal was to expand the current level of knowledge of the Tipu collection to include ancient DNA. Historically, the major problems with the isolation and analysis of ancient DNA has been sample contamination

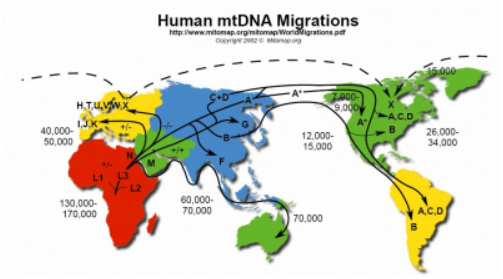
with exogenous sources of DNA (human, bacteria, fungal), in addition to problems caused by the presence of enzymatic inhibitors in the aDNA samples (O'Rourke et al., 2000). The foci of this investigation were to first successfully isolate aDNA; verify the success of the extraction protocols and then determine mitochondrial DNA (mtDNA) lineages (haplogroups), as well as analyze the 14 base pair region within the mitochondrial hypervariable region from this Tipu collection.

The extraction protocols' success was assessed based on the capability to amplify a 211 bp region through Polymerase Chain Reaction (PCR). This targeted region is a highly repetitive -satellite region (locus D17Z1) specific for human chromosome 17. Due to its high copy number of 500-1,000, this D17Z1 region can be detected with a high degree of sensitivity using PCR applications (Yang et al., 1998).

Mitochondrial DNA is very effective in investigating and constructing evolutionary routes. Studies have shown that all Native American mtDNA can be traced back to one of four maternal lineages (haplogroups); A, B, C, D (Schurr et al., 1990; Bailliet, et al., 1994; Torroni et al., 1994; Torroni and Wallace, 1995). These four well known haplogroups can be defined by three restriction fragment length polymorphisms (RFLPS), DNA sequencing and the presence or absence of a 9 bp region (González-Oliver, 2001) (Figure 2). There is also an extremely rare X migration route into the Americas (Perego, et al., 2009), if none of the aDNA samples tested matched up with one of the four haplogroups (A-D), the sample is then tested for the X migration route. A 14 base pair region located within the mitochondrial hypervariable region has also been found to be effective with its connections to specific ethnic groups and also was examined (Sato et al., 2010).

Figure 2

Human mtDNA Migration routes with major haplogroups represented. The focus of this research is on migration routes A, B, C, and D. Retrieved February 10, 2015 from http://www.mitomap.org/pub/MITOMAP/MitomapFigures/WorldMigrations.pdf



METHODS

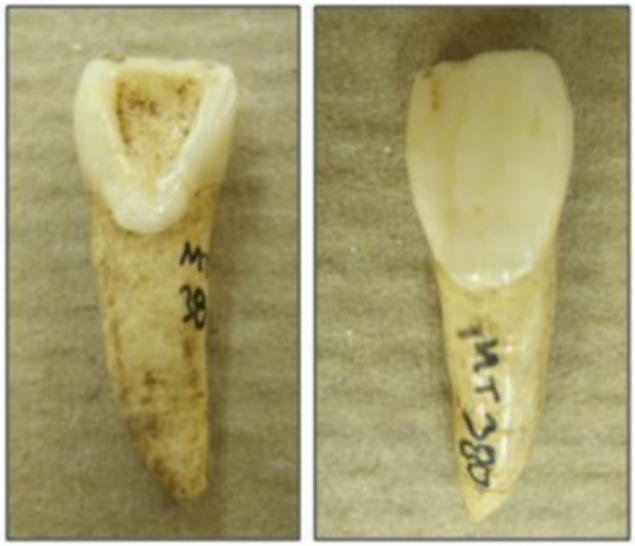
There is a designated laboratory for aDNA isolation and analysis. This laboratory has a physically isolated pre-PCR area; UV radiation capabilities; and all surfaces are constantly cleaned using 10% bleach. Throughout all procedures, great care has been taken to reduce and minimize the level of contamination with modern DNA. Protective clothing, masks, and gloves are worn when handling teeth and non-disposable equipment is decontaminated. In addition, every individual coming in contact with aDNA has had their DNA isolated and analyzed for haplogroups and the 14 base pair region. This is to ensure that findings are indeed those from the Tipu Maya samples.

DNA ISOLATION

Bones and teeth were selected, based on their absence of cracks and/or cavities. The aDNA was extracted and amplified using Yang et. al., (1998) procedures with the following exceptions: the teeth and bones were cleaned using ethanol (95%), placed in a 10% bleach solution for 10 minutes, and then exposed to UV light for forty-five minutes on each side. OD260 and OD280 readings were performed (using a Nanodrop) to determine DNA concentrations and purity. After extracting aDNA from both teeth and bone samples, aDNA concentration and purity analysis revealed that macerating teeth was more successful than drilling bone (data not shown). Dust created due to the drilling of the bone also provided more of a contamination issue. The aDNA isolated from just one tooth yielded ~ 1.2–7.5 ug aDNA per tooth (Figure 3).

Figure 3

Example of Maya teeth which provided the ancient DNA. Photos from Joshua Coons.



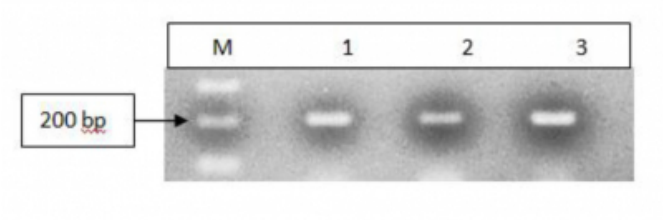
AMPLIFICATION OF REPETITIVE -SATELLITE (D17Z1) DNA ON CHROMOSOME 17

Amplification was conducted using the pair of primers described in Table 1; 2.0uM of each primer was added along with 100 to 200 ng of aDNA. These were placed in PuReTaqTM Ready-To-Go PCR bead tubes (GE Healthcare) and brought up to a 25uL volume with sterile, distilled water.

Samples were amplified for a total of 35 cycles. Each cycle was comprised of 30 seconds at 94o C, 30 seconds at 54 o C, and 45 seconds at 72 o C. To confirm amplification, each sample was run on a 1.5% gel using DNA electrophoresis (Figure 4).

Figure 4

aDNA isolated from skeletons 314, 366, and 275, lanes 1-3 respectively were amplified with primers that targeted a region of the 17th chromosome and were analyzed using electrophoresis in a 1.5% agarose gel. Expected PCR products ~211 bp. M= 100 base pair marker.



AMPLIFICATION AND DIGESTION OF DNA FOR HAPLOTYPING LINEAGES VIA MITOCHONDRIAL DNA

All aDNA samples amplified for haplogrouping (N=25) followed the protocol outlined in Gonzalez-Oliver et. al., 2001 (Table 1). The twenty five samples chosen were from skeletons located within the temple and from each side where skeletons were located (Figures 1 & 5). A total volume of 25uL for each reaction was used. In addition, all samples were done in duplicate for the primers that targeted restriction enzyme sites (Hae III, Alu I, Hinc II).

Table 1

Haplogroup A, B, C, and D determination

Haplogroups	Haplogroup Determination Criteria
A	Gain of *Hae III* cut site at 663 nucleotide position (np)
B	Presence of a 9 base pair deletion between 8196-8316 np
C	Combined *Hinc II* 13259 np site loss and *Alu*I 13262 site gain
D	*Alu I* 5176 np site loss

Lineage A is defined by the presence of a Hae III restriction cut site at 663 base pairs, according to Anderson et al. (1981). The presence of a 9-bp deletion located between the cytochrome oxidase II and lysine tRNA genes determines lineage B. The loss of the restriction enzyme site Hinc II at 13,259 base pair and the gain of an Alu I restriction cut site at 13,262 base pair defines lineage C. Finally, lineage D is determined by the loss of the Alu I restriction enzyme cut site (Torroni et al. 1992). In addition to the testing the PCR products with their respective restriction enzymes, each sample was sequenced to determine the presence of each of these restriction enzyme cut sites. The amplified samples were run on a 1.5% agarose gel; each band was excised from the gel.

MITOCHONDRIAL 14 BASE PAIR HYPERVARIABLE REGION

All samples were amplified using 2.0 µM primers (Table 2) with 100-200ng of aDNA in PuReTaqTM Ready-To-Go PCR bead tubes (GE Healthcare) and brought up to a 25uL volume with sterile, distilled water. Samples were amplified in the thermocycler for 35 cycles. Each cycle was comprised of 30 seconds at 94o C, 45 seconds at 52 o C, and 1 minute at 72 o C. Samples were confirmed using a Bioanalyzer , once samples were confirmed, they were run on a 1.5% gel using DNA electrophoresis; each sample was then excised from the gel for DNA sequencing.

Table 2

Primers used for Maya aDNA study

Site	Primer Sequence	Fragment size(bp)
*Hae*III mtDNA	F 5'-ACCTCCTCAAAGCAATACACTG-3' R 5'-GTGCTTGATGCTTGTTCCTTTTG-3' (Gonzalez et. al., 2001)	176
9-bp deletion mtDNA	F 5'- ACAGTTTCATGCCCATCGTC-3' R 5'- ATGCTAAGTTAGCTTTACAGTG-3' (Gonzalez et. al., 2001)	112 or 121
*Hinc*II mtDNA	F 5'- CGCTATCACCACTCTGTTCGC- 3' R 5'- CAGATGTGCAGGAATGCTAGG-3' (Gonzalez et. al., 2001)	147
*Alu*I mtDNA	F 5'- TAACTACTACCGCATTCCTACT-3' R 5'- AAAGCCGGTTAGCGGGGGCA-3' (Gonzalez et. al., 2001)	149
17th Chromosome	F 5'-CAAATCCCCGAGTTGAACTT-3' R 5'-AAAACTGCGCTCTCAAAAGG-3' (Yang et. Al, 1998)	211
14 bp hypervariable region	F 5'- AAACTATTCTCTGTTCTTTC-3' R 5'- GGTTGATTGCTGTACTTGCT-3' (Elwess, 2009)	212

DNA GEL EXTRACTION AND SEQUENCING

DNA bands for sequencing were excised from the agarose gels. Protocols for isolation and purification of DNA were followed as stated in Qiagen's Qiaquick Gel Extraction Kit. Once each DNA sample was isolated and purified, 10 ng of DNA with 5pm of the respective primer was sent to the Clemson University Genomics Institute for sequencing.

Table 3

Individual Results for Haplogroups

Sample	Sex	Age	Burial Description	Migration route
6	Unknown	7-9	Primary burial	B
8	Male	30-35	Primary burial; of church	D
11	Male	35-40	Primary burial; Arrow heads next to skeletons head	D
19	Male	20-30	Secondary burial; outside church; buried next to skeleton #16	C
21	Unknown		Skull only lying upside down a top burial #16	B
23	Male	30-40	Secondary burial; outside of church	C
78	Male?	40-50	Secondary burial; inside church	C
82	Male	20-30	Secondary burial; inside church	C
85A	Male	20-30	Secondary burial	C
86	Female	30-40	Primary Burial; Inside of church (nave)	C
101	Female	25-30	Primary burial	C
108	Male	18-25	Primary burial	C
115	Female	35-40	Primary burial	D
161	Male	18-25	Secondary burial; inside church	D
175	Female (Juvenile)	12-15	Primary burial	C
176	Female (Juvenile)	11-13	Outside of church to the west; pin or needle on feet	C
186	Female	16-20	Primary burial	D
230	Unknown	Adult	Primary burial	C
275	Male	18-26	Primary burial; Buried with other skeletons	C
314	Female	18-24	Primary burial; Buried with other skeletons	C
355	Male	25-30	Primary burial	C
366	Male	20-25	Primary burial	C
432	Male	18-25	Primary burial	D
442	Unknown		No information	D
515	Unknown		No information	C
Totals : B= 2, C=16, D=7 N = 25				

RESULTS

The aDNA was successfully extracted for twenty five individuals. The samples were amplified using the primers that target a 211 base pair region of the 17th human chromosome (Figure 4). All isolated samples were haplogrouped using the specific primer-restriction enzyme combination (Table 1), and thus far belong to haplogroups B, C and D (Tables 3 & 4, Figure 5). The results were 0% haplogroup A, 8% haplogroup B, 64% haplogroup C and 28% haplogroup D. None of the researchers working on the haplogroup testing had any of these haplogroups, the only exception was the Principle Investigator, who has an A haplogroup (which was not present in the results).

Table 4

Comparative Results of Haplogroups

Population	N	A (%)	B (%)	C (%)	D (%)	Reference
Tipu	**25**	**0**	**8**	**64**	**28**	**Present Study**
Xcaret	25	84	4	8	0	González-Oliver et al. 2001
Copán	9	0	0	89	11	Merriwether et al. 1997
Contemporary Maya	27	51.9	22.2	14.8	7.4	Torroni et al. 1992

Twelve aDNA samples were sequenced for the 14 bp

hypervariable region. When compared against the accepted and published 14 bp hypervariable region for Native Americans, only one sample (Maya 438) matched (Table 5). The rest of the Maya samples had a cytosine in place of a thymine at the 10th base (Table 5).

Table 5

14 base pair DNA sequences

Sample	Sequence
Published conserved 14 bp	AAAACCCCCTCCCC Anderson et al., 1981 Horai et al., 1993
Maya 08	AAACCCCCCCCCCCC
Maya 21	AAAACCCCCCCCCC
Maya 23	AAAACCCCCCCCCCC
Maya 60B	AAAACCCCCCCCCCC
Maya 78	AAAACCCCCCCCCC
Maya 85	AAAACCCCCCCCCC
Maya 101	AAAACCCCCCCCCC
Maya 101A	AAAACCCCCCCCCC
Maya 108A	AAAACCCCCCCCCCCC
Maya 234	AAAACCCCCCCCCCCC
Maya 275	AAAACCCCCCCCCC
Maya 438	AAAACCCCCCTCCCC

CONCLUSIONS

The Tipu collection is one of the largest, if not the largest, of ancient Maya skeletons to date and provides a significant sample population for haplogroup testing. The previous studies performed on ancient Maya from Copán and Xcaret were limited by the collection sample size (Table 4). A larger sample size may have yielded different results, especially for the Copán results with a sample size of only nine. The results found that the majority (64%) of the Tipu Maya belong to haplogroup C. This agrees best with the Copán study (Merriwether et al. 1997) of ancient Maya haplogroups, which found the majority (89%) to belong to haplogroup C. However, when compared to results from another ancient Maya haplogroup study (González-Oliver et al., 2001) and a contemporary study (Torronei et al., 1992), it disagreed with both, due to having the majority (83% and 51.9% respectively) belonging to haplogroup A (Table 4).

Additionally, the high frequency of the A haplogroup was consistent with the presence of this lineage within different Native American populations in North and Central America (Torrini et al., 1994). This high frequency was also found in contemporary Mesoamerican populations. The location of the skeletons selected for this study had no connection to their haplogroup determination (Figure 5). As mentioned in all these studies, including this one, more samples needed to be tested. To date, seven studies were done, with the sample number ranging from nine to twenty seven; the results presented here were from twenty five samples.

{image:10}

In addition to determining ancient Tipu Maya skeletons Amerindian mtDNA lineages, this study also performed nucleotide DNA sequence analysis of a 14 base-pair region found within the noncoding region of mitochondrial DNA. Results of Native American mtDNA polymorphisms in the 14 bp hypervariable region (bp 16180-16193) from North, Central and South American populations showed a high frequency of rare Asian variants (Horai et al, 1993). Typically, most individuals exhibit the conserved sequence for the 14 bp region, which is four A's, five C's, one T, and four C's (Table 5). However, within the twelve Maya samples tested, only one matched this conserved sequence, Maya 438 (Table 5). Four variant sequences were due to nucleotide base substitutions in the regions of C's; Maya 21, 23, 60B, 78, 85, 101, 101A, 108A, 234, and 275, as well as the shortening of the number of A's, Maya 08 (Table 5). These results were consistent with sequence patterns in the 14 bp mtDNA region that revealed that as soon as the T (mtDNA bp 16189) is replaced by a C, the number of A's and C's become variable (Horai et al., 1993; Sato et al., 2010). Results have shown that the polymorphic sites within this region were commonly found between Asians and Native Americans; whereas fewer polymorphic sites were shared by other racial populations (Horai et al., 1993; Sato et al., 2010). As a result, the ancient Maya skeletons from Tipu possess similar characteristics to Asian sequences. Here again, more analysis on this region must be done.

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