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# Genetic Affiliation of Pre-Hispanic and Contemporary Mayas through Maternal Linage

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# Genetic affiliation of pre-Hispanic and contemporary Mayas through maternal lineage

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## Abstract

Maya civilization developed in Mesoamerica and encompassed all of the Yucatan Peninsula, Guatemala, Belize, part of the Mexican states of Tabasco and Chiapas, and the western parts of Honduras and El Salvador. This civilization persisted approximately three thousand years and was one of the most advanced of its time, possessing the only known full writing system, art, and sophisticated architecture, as well as mathematical and astronomical systems. This civilization reached the apex of its power and influence during the Preclassic period, from 2000 Before the Current Era (BCE)-250 Current Era (CE). Genetic variation in the pre-Hispanic Mayas from archaeological sites in the states of Yucatan, Chiapas, Quintana Roo, and Tabasco, Mexico, and their relationship with the contemporary communities in these regions have not been previously studied. Consequently, the principal aim of this study was to determine the mitochondrial DNA (mtDNA) variation in the pre-Hispanic Maya population and to assess the relationship of these individuals with contemporary Mesoamerican Maya and populations from Asia, Beringia, and North, Central, and South America. Our results revealed 1) interactions and gene flow between populations in the different archaeological sites assessed in this study; 2) the mtDNA haplogroup frequency in the pre-Hispanic Maya population (60.53%, 34.21%, and 5.26% for haplogroups A, C and D, respectively) was similar to most Mexican and Guatemalan Maya populations, with haplogroup A exhibiting the highest frequency; 3) haplogroup B most likely arrived independently and mixed with populations carrying haplogroups A and C based on its absence in the pre-Hispanic Mexican Maya populations and low frequencies in most Mexican and Guatemalan Maya populations, although this also may be due to drift; 3) Maya and Ciboneys sharing haplotype H10 belonged to haplogroup C1 and haplotype H4 of haplogroup D suggesting shared regional haplotypes indicating a shared genetic ancestry suggesting regional interaction between populations in the Circum-Caribbean region than previously demonstrated; 4) haplotype sharing between the pre-Hispanic Maya and the indigenous populations from Asia, the Aleutian islands, and North,

Central and South America provide evidence for gene flow from the ancestral Amerindian population of the pre-Hispanic Maya to Central and South America.

## **Introduction**

The Maya civilization was one of the best known classical civilizations of Mesoamerica that developed into a society equipped with astronomy, mathematics, a calendar system, hieroglyphic writing, astrology and agricultural innovations. The Maya are an indigenous people of Mexico and Central America and the descendants of the people who built the great cities are still residing on the same lands.

Historical documentation suggests that the ancestors of the Maya came to the Yucatan Peninsula through the Bering land bridge from North Asia. Eventually, these Maya immigrated south to Lake Petén, Guatemala, where they established a kingdom with their capital and sacred city of Flores Island in the lake [Muñoz et al., 2012b]. The Yucatan peninsula became the principal region of a new culture, called Toltec/Maya, which formed when Toltec migrated from the north and integrated with the Maya people.

### *Cultivation of the Maya Civilization*

Human activity in Mexico during hunter-gatherer culture sustained through corn cultivation, basic pottery and stone tools that date back to the Holocene Epoch in 7200 BCE (Before the Current Era) [Ranere et al., 2009]. Furthermore, a phytolith analysis of sediments from San Andres, Tabasco, confirmed the diffusion of maize cultivation from the tropical Mexican Gulf Coast >7,000 years ago ( $\approx$ 5,800 BCE), followed by the rapid spread of the crop to South and North America by the ancestors of the Maya [Pohl et al., 2007; da Fonseca et al., 2015; Piperno et al., 2009; Grobman et al., 2012]. This diffusion is further supported by evidence suggesting that the introduction of maize

into the Southwest United States occurred before 2,050 BCE [Merrill et al. 2009; Kemp et al., 2010; Kohler and Reese, 2014].

### *Olmec influence on Maya*

Olmec considered the oldest culture in Mesoamerica settled along the Gulf of Mexico and began building cities of stone and brick during the Pre-Classic Period. While the nature of the relationship between the Olmec and the Mayas is unknown, some archeologists have suggested that the Mayans were their descendants and/or trading partners [Benson, 1967]. Indirect evidence of the relatedness of the Olmecs and Mayans supports the notion that the Olmecs may have been the precursors of Mayans, as suggested by Arnaiz-Villena et al. [2000] based on HLA allele frequency distributions. Inomata et al. [2013] and Pringle [2013] have also documented the growth of the ceremonial space into a plaza-pyramid complex at Ceibal, Guatemala that predates the buildings at other lowland Maya sites, as well as the major occupations at the Olmec center of La Venta. These authors suggested that the development of lowland Maya civilization did not result directly from the influence of the Olmecs but from interactions with groups in the southwestern Maya lowlands, Chiapas, the Pacific Coast, and the southern Gulf Coast [Pringle, 2013; Inomata et al., 2013]. There is evidence for a settlement at La Venta between 1400 and 1150 BCE that expanded between 1150 and 800 BCE and developed into a major temple-town complex [Rust and Sharer, 1988].

### *Maya Language*

Language and culture could also have played a pivotal role in Mesoamerica during the colonization of the American continent. Proto-Mayan is the common ancestor of all modern Mayan languages today and the Classic Maya languages documented in the hieroglyphic inscriptions [Law, 2013]. According to the classification scheme by Campbell and Kaufman [1985], Proto-Mayan was split in 6 main branches: K'iche'an, Mamean, Q'anjob'alan, Ch'olan-Tzeltalan, Yuktatekan, and Wastekan, the first division occurred circa 2200 BCE, when Wastekan moved northwest along the Gulf Coast

of Mexico. Subsequently, each subgroups was spawned and at present time the Maya language family includes approximately 31 languages spoken by more than 5 million people in Guatemala, Mexico, Belize, Honduras, and in diaspora communities in the US and Canada [Law, 2013]. Three languages are now extinct: Chikomuseltek [Campbell and Kaufman 1985; Ch'olti' [Houston et al., 2000; Law, 2013] and the language of Maya hieroglyphic inscriptions recorded from approximately 20,000 terse hieroglyphic inscriptions [Law, 2013]. Linguistic and maternal genetic diversity have not been correlated in Native Mexicans [Sandoval et al., 2009]).

### *Background on Maya genetics*

Several important factors that could influence the magnitude of genetic admixture in the Maya include gene flow between different locations, including genetic exchange with people who adopted maize, other domesticates, and ceramic use but without a sedentary lifestyle for many centuries [Inomata et al., 2015], as well as gene flow introduced by rival Mayan groups. A recent study has suggested that those early migratory people contributed to the monumental constructions and public ceremonies in the lowland Maya site of Ceibal [Inomata et al., 2015]. This study supports the idea that the development of sedentism was a complex process involving and facilitating social interactions among diverse groups [Inomata et al., 2015]. Furthermore, Ragsdale et al. [2015] also suggested that trade and political relationships affected the population structure among Postclassic Mexican populations.

Several authors have used biological indicators such as dental morphology to reconstruct patterns of affinity among ancient and modern human groups [Scherer, 2007; Cucina et al., 2015, Aubry, 2009; Ragsdale et al., 2015]. The study of the population structure of the Classic period (250 BCE to 900 CE) Maya through analysis of the odontometric variation of 827 skeletons from 12 archaeological sites in Mexico, Guatemala, Belize, and Honduras indicated that the isolation by distance model is not applicable to the population structure of Classic period Maya [Scherer, 2007]. Further studies

examining dental morphology and  $^{87}\text{Sr}/^{86}\text{Sr}$  ratios indicated intense population dynamics in the peninsula during the Maya Classic period. These authors concluded that the different natures of the dental and isotopic indicators were consistent with proposed trade routes in the peninsula [Cucina et al., 2015].

### *Background Mitochondrial DNA*

From an archaeological perspective, the primary indicator of contact and the sharing of ideas or culture between populations is the presence of foreign goods or architectural patterns in archaeological sites. From a biological point of view, population interactions can be inferred through the genetic makeup of the population, in which a high diversity in both haplogroups and haplotypes can show evidence of admixture, whereas homogeneity in mtDNA lineages shows evidence of drift [Cann et al., 1987; Wallace et al., 1999; Pakendorf and Stoneking, 2005; Nesheva, 2014].

Mitochondrial DNA has been used to elucidate the maternal evolutionary history of anatomically modern humans through the reconstruction of prehistoric human dispersals [Forster et al., 1996; Goebel et al., 2008; Theyab, et al., 2012; Melton, et al., 2013; Mendisco et al., 2014] and to study human genetic predispositions to various diseases [Chen et al., 2015; Montiel-Sosa et al., 2013; Scheibye-Knudsen et al., 2013; Delgado-Sánchez et al., 2007]. These studies are possible because mtDNA (1) is transmitted as a non-recombining unit through maternal lineages, (2) provides an exceptional record of mutations over time [Ingman et al., 2000], (3) is present in numerous copies in each cell, and (4) databases of entire human mtDNA sequences are readily available for comparison [Falk et al., 2014; Shokolenko et al., 2015].

Previous studies investigating mtDNA in contemporary Mayan populations have suggested that Native Mexican populations are more closely related genetically to North American populations [González - Martín et al., 2015] and that this genetic structure is related to geography rather than to

language. Additionally, genetic differences have been identified between the populations from the northern and central regions of Mexico and Mesoamerica [Gorostiza et al., 2012]. Furthermore, the heterogeneity of the Nahuas suggests that this group is composed of several genetically distinct cultural groups that were absorbed by the Aztecs [González - Martín et al., 2015] and later admixed with the Maya populations.

These phylogenetic studies using mtDNA data suggest a demographic scenario that is compatible with moderate local endogamy and isolation of the contemporary Maya combined with episodes of gene exchange between ethnic groups and that the recent adoption of an ethnic identity in the Guatemalan Maya stems from a cultural rather than a biological basis [Söchtig et al., 2015].

Occupations in South America may have resulted from a different process involving multiple early migrations and subsequent population movements. Lewis et al. [2007] suggested that later regional isolation and differential evolutionary processes led to the formation of greater diversity estimated within western populations, and limited genetic differentiation estimated among western populations was likely attributed to larger population sizes and more extensive gene flow within this region, as well as a period of reduced population sizes and more restricted gene flow. Concurrently, the findings of de Saint Pierre [2012] indicated that the present native populations inhabiting South Chile and Argentina comprise a group with a common origin, suggesting a population break between the extreme south of South America and the more northern part of the continent. They concluded that the early colonization process was not just an expansion from north to south but also included movements across the Andes.

To date, no studies have investigated the origin and diversity of Maya maternal lineages in the Mexican region. This analysis addresses this deficiency through the use of pre-Hispanic samples from archeological sites including Xcambo, Bonampak (Group Frey and Group Quemado), Palenque (Temple XIII, Temple XV, Group B), Rey Quintana Roo, and Comalcalco (Temple V, Temple III, Tenosique, El Comal, Working areas, Dren, Peje Lagarto Pinos, Sueños de Oro,

Calicanto (Figure 1). In addition, the genetic variation in these pre-Hispanic Mayas and their relationship with contemporary Maya populations (Yucatan, Guatemala, Honduras, and Belize), Asia, Bering, North America, Central America and South America were determined to better understand their relationship and the effects of gene flow on contemporary Mayas (Suppl. Figure 2).

## **Materials and Methods**

*Archaeological sites from which bone samples for this study were obtained.*

(1) Bonampak was occupied in the earliest Classic period 250 BCE and is located in the Lacandon Jungle, 30 km to the south of Yaxchilan near the border of Mexico and Guatemala. Its maximal cultural peak at approximately 743 CE is highlighted by one of the major features of this archaeological site: the mural paintings on buildings that surround the central plaza [Flores-Gutierrez, 2007]. In this study we include 1) four samples from the “Quemado group” located 250 m to the northeast of the Gran Plaza and 2) one sample from the “Frey group” located 350 m to the north of the Acropolis (Figure 1, Table 1).

(2) The Classic Maya site of Palenque (226 BCE to 799 C.E.) is situated on a limestone shelf at the base of the Sierra de Palenque. Located near the Usumacinta River in the Mexican state of Chiapas, it lies approximately 130 km south of “Ciudad del Carmen” and 150 m above sea level. Remains from Palenque Temple XIII and Temple XV are distinguished by the naturalistic sculpture, architectural inventiveness, and detailed epigraphic record. At present, this region is inhabited by Chol-speaking Mayas [Schele, 2012]. The Mayas used old buildings to construct new temples, and an earlier structure lies beneath the Palace that was built before the construction of the galleries, suggesting a much earlier occupation of the site, probably by the same group of Mayas that

constructed the rest of the ceremonial center [Ruz Lhuillier-cited by Schele, 2012]. This study includes 9 samples from this archeological site (Figure 1, Table 1).

(3) Comalcalco (“House of Frying Pans” in Nahuatl) is one of the most significant ancient cities in Tabasco, and the only Mayan city built with bricks of baked clay instead of stone. This archaeological site is made up of three complexes: the North Square, the Great Acropolis and the Acropolis of the East. It is similar in design to Palenque, and it flourished as an agricultural center specializing in cacao. The clay bricks are decorated with iconography and/or hieroglyphs, sand and oyster shells. Comalcalco is believed to feature the earliest brick buildings found in Mesoamerica. The city was a center of the Chontal Maya people who flourished in 500 CE, and it was abandoned in approximately 1000 CE. The chemical composition of the figurines found in Comalcalco are equivalent to those found on Jaina Island [Ochoa, 2004]. Dren is also located in Comalcalco, but in the working areas. Thus, it is possible that the samples were from the common people living in this area. This study includes 6 samples from this archeological site (Figure 1, Table 1).

(4) “Sueños de Oro”, Tenosique, Calicanto Jalapa, and Peje Lagarto, Huamanguillo are small archaeological sites in Tabasco located proximal to the major archeological sites in Tabasco (Figure 1, Table 1). “Sueños de Oro” is also known as “El ceibo” and is approximately 59 km from the municipality of Tenosique on the Guatemalan border. This study includes 2 samples from Sueños de Oro, 5 from Tenosique, 1 from Calicanto and 1 from Peje Lagarto (Table 1).

(5) El Rey Quintana Roo, named Nizuctec (250-600 CE), is located in Cancun in the state of Quintana Roo—it was known as Nizuc in early Colonial times [Andrews, 2006]. This study includes 5 samples from this archaeological site (Figure 1, Table 1).

(6) Xcambo is a small archaeological site located 2 km from the northern coast of the Yucatan Peninsula in the Municipality of Dzemul. The occupation of this city dates back to 150 BCE to 300 CE. Archaeological evidence suggests that Xcambo provided salt to the Mayan cities of Izamal,

Oxkintok and Ah Kim Pech [Aubry, 2009]. This study includes 2 samples from Xcambo (Figure 1, Table 1).

### *Samples*

DNA analysis was performed for 38 pre-Hispanic individuals from the following archeological sites: Xcambo, Bonampak (Group Frey and Group Quemado), Palenque (Temple XIII, Temple XV, Group B), El Rey Quintana Roo, and Comalcalco (Temple V, Temple III, El Comal, Working areas), Dren, Peje Lagarto Pinos, Sueños de Oro, and Calicanto (Figure 1 and Table 1).

Archaeological bone samples (0.5 mg) from 2 different areas of each skeleton were collected for this study. Pre-Hispanic bone samples were donated by the INAH (Instituto Nacional de Antropología e Historia, Mexico). One sample was eliminated because the DNA was damaged.

### *Ancient Sample Preparation for DNA Extraction*

DNA purification was preceded by sample decontamination to eliminate exogenous surface DNA. Each sample was washed with full-strength Clorox bleach followed by rinsing with DNA-free ddH<sub>2</sub>O and UV light irradiation of each facet for 30 min [Muñoz et al., 2012a]. Gloves, masks, hats, coats and filter pipet tips were used in all experiments to avoid sample contamination in the laboratory [Adler et al., 2011, Campos et al., 2012; Muñoz et al., 2012a].

All sample preparations, DNA extractions and PCR amplifications were completed in the UV sterilization room (positive-pressure clean room with filtered air). The clean room contains an anteroom. This room is routinely cleaned with bleach, and all containers are wiped before being placed in the laboratory. A more complete explanation of the laboratory procedures and methods utilized in this analysis is provided in Smith et al. [2009]. Furthermore, a database containing mitochondrial control region sequences is maintained for all personnel working in the laboratory and any personnel who may have come in contact with human remains prior to the DNA analysis.

## *DNA Extraction*

Bone powder was generated by grinding the skeletal material with a mortar and pestle until a fine powder was obtained. The powder (0.100 g) was transferred into a sterile 15-ml tube and suspended in 2 ml of extraction buffer (0.01 M Tris-HCl, 0.1 M EDTA and 0.2% SDS pH 8.0). The tubes were capped and sealed with Parafilm. After incubation with gentle agitation for 1 h at 37°C, 1 mg/ml proteinase K was added to the sample, followed by incubation at 50°C for 2 h. Throughout the procedure, a blank extraction treated identically to the experimental samples was included to monitor potential contamination during the DNA extraction process. Finally, the samples were centrifuged at 5,000×g for 5 min, and the supernatants were extracted using phenol-chloroform-isoamyl alcohol (24:24:1) for organic extraction [Hughes et al., 2006, Maniatis et al., 1989; Muñoz et al., 2012a]. Subsequently, the aqueous phase was concentrated by precipitation via the addition of 0.1 volumes of 3 M sodium acetate pH 5.0 and 2.5 volumes of ethanol. After mixing, the sample was incubated at -78°C overnight and centrifuged at 15,000 rpm for 10 min at 4°C. The supernatant was decanted, and the precipitate was rinsed with 70% ethanol. After drying the pellet at ambient temperature in a sterile area, the pellet was suspended in 100 µl of DNA-free sterile water. If ancient DNA was contaminated with inhibitors of polymerase DNase, the DNA was purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, to Germantown, MD., USA) as recommended by the manufacturer. Alternatively, purification of ancient genomic DNA was also performed using Magnetic Bead technology in combination with chemagic Prepito-D, following the instructions of the supplier. Extracted DNA was maintained in aliquots of 10 µl at -70 °C.

Contamination controls lacking sample were also used in every DNA extraction and PCR to detect contamination. Additionally, a series of negative controls were routinely assessed using the protocol described by Malhi et al., [2010] and Muñoz et al. [2012a]. The samples were extracted at least twice from each sample.

### *PCR and DNA sequencing*

The HVS-I of the ancient DNA samples was amplified, sequenced (corresponding to nucleotide pairs 15989 to 16236, 16159 to 16236, and 16190 to 16410) and genotyped by real-time PCR (TaqMan) for the haplogroup diagnostic markers for mitochondrial haplogroups A, C, D, and X (Applied Biosystems®). The primers used to amplify and sequence the HVS-I region of the ancient samples in this study were as follows:

L 15989 (5'-CCCAAAGCTAAGATTCTAAT-3') Gabriel et al. [2001]

L 16159 (5'-TACTTGACCACCTGTAGTAC-3') Wilson et al. [1995a]

H16236 ((5'-CTTTGGAGTTGCAGTTGATG-3') Wilson et al. [1995b]

L 16190 (5'-CCCATGCTTACAAGCAAGT-3') Gabriel et al. [2001]

H 16410 (5'-GAGGATGGTGGTCAAGGGAC-3') Gabriel et al. [2001]

Nucleotide sequencing was carried out by direct-sequencing with an ABI 3130xl Genetic Analyzer (PE Applied Biosystems, Foster City, CA, USA). To provide additional confirmation that the ancient DNA results were not derived from laboratory-specific contaminants, the samples were also verified in the Insectary Laboratory and the Genetics Laboratory at the Institute of Forensic sciences (Tribunal Superior of Justice from Distrito Federal).

If the replicate did not exhibit an identical sequence to the original, a third extraction, amplification, and sequence reaction was performed to resolve the ambiguity. Ancient DNA extraction and sequencing was replicated in the Insectary and in the INCIFO (Instituto de Ciencias Forenses). Recently, ancient DNA extraction and sequencing has been replicated in two samples from la Cueva de San Felipe Tabasco, Mexico and in the ancient DNA laboratory of Dr. Dennis O'Rourke at the University of Utah in Salt Lake City.

### *Statistical analysis*

Our analyses included a dataset of 1209 HVS-I mitochondrial sequences (38 from this study and 1173 from published sources) from North, Central, and South America, as well as Beringia and Siberia-Asia (Suppl. Table 1). The geographic location of each sample is presented in Suppl. figure 1. These sequences were aligned and compared using the revised Cambridge Reference Sequence (rCRS) [Andrews et al., 1999] with BioEdit v7.2.5 (last update 12/11/2013) [Tom Hall Ibis Biosciences, <http://www.mbio.ncsu.edu/bioedit/bioedit.html>]. The HVI region was examined between nucleotide positions 15989 and 16410 according to the rCRS modified version of the original CRS published by Anderson et al. [1981].

### *Genetic Variability*

To quantify the genetic variability of the HVI mtDNA sequences in the Maya population, standard and molecular diversity indices were estimated, namely the nucleotide diversity, mean number of pairwise differences ( $\pi$ ) [Tajima et al., 1983, Nei et al., 1987], expected diversity based on the number of pairwise differences ( $\theta\pi$ ) [Tajima, 1983], and the sequence diversity (H) [Nei et al., 1987]. These diversity parameters were computed using the ARLEQUIN program, version 3.5.2.1 [Excoffier et al., 2010].

All of the pre-Hispanic Maya samples collected in this study were pooled into 8 groups based on the archeological site and the geographic criterion (Figure 1). In addition, 3 groups of sequences were included from different ancient populations (Ciboney from Cuba; Guane from Colombia; and from the Archaeological Cemeteries of Chile). Following these same criteria, individual contemporary sequences were divided into 6 groups and named according to the geographic region: Asia, the Beringian Bridge, North, Central, and South America, and contemporary Mayas (Suppl. Figure 1, Suppl. Table 1).

### *Molecular Variance (AMOVA)*

The genetic structure of the population among and within groups was estimated by the analysis of molecular variance (AMOVA). To explore the demographic history of these pre-Hispanic populations, we analyzed the mismatch distribution as implemented in ARLEQUIN 3.5.1.2 [Excoffier and Lischer, 2010]. Next, we measured Fu's  $F_s$  and Tajima's  $D$  neutrality tests of the total number of segregating sites. All analyses were performed in ARLEQUIN 3.5.1.2 [Excoffier and Lischer, 2010]. A multidimensional scaling (MDS) plot based on pairwise distances was also constructed using R3.1.2 [<https://www.r-project.org/>].

### *Median-Joining Network analysis*

The genealogical relationships among the identified mtDNA control region haplotypes of the pre-Hispanic Maya populations from the archaeological sites were inferred by the median-joining (MJ) network [Bandelt et al., 1999] and calculated using Network 4.6.1.3 software [[http://www.fluxus-engineering.com/sharenet\\_rn.htm](http://www.fluxus-engineering.com/sharenet_rn.htm)], accounting for different rates of transitions and transversions for all mutations and setting the parameter to zero to restrict the choices of feasible links in the final network. Neighbor-joining tree clustering was constructed according to Kumar et al. [2011] and MEGA 5.2 [Tamura et al., 2013].

### *Classification of MtDNA Haplogroups*

Finally, to classify the mtDNA haplotypes into haplogroups, we adopted the nomenclature system of Starikovskaya et al. [2005], Achilli et al. [2008], Derenko et al. [2010], Gomez-Carballea et al. [2015]; Kumar et al. [2011]; and Rieux et al. [2014]. All detected polymorphic positions were confirmed with Mitomaster [<http://www.mitomap.org/bin/view.pl/MITOMASTER/WebHome>]. All new diagnostic positions indicated in these studies were taken into account by assigning the haplotypes to haplogroups/subhaplogroups. The haplogroup composition of the analyzed populations was then evaluated both on an Asian, Beringian Bridge, Amerindian scale and in the

Maya context, to gain insights into the origin of the genetic differentiation of pre-Hispanic Mexican and contemporary Maya populations.

## Results and Discussion

### *Mitochondrial DNA Diversity*

The mtDNA hypervariable segment I (HVS-1) of 38 Maya pre-Hispanic samples from different archeological sites in Mexico (Figure 1, Table 1) was sequenced to determine the haplogroup and relationship to maternal lineages of other geographic regions. All mtDNA haplotypes were in lineages A (2.6%), A2 (44.73%), A2v (13.16%), C (2.6%), C1 (15.8%), C1b14 (15.8%), and D (5.3%) (Table 2). One sequence was classified in haplogroup A based on the presence of a C>T transition at position 16223 and a G>A transition at position 16319 (G16319A) [Torrioni et al., 1993]; 17 sequences were classified as haplogroup A2 based on the presence of C>T transitions at positions 16111 (C16111T), 16223 (C16223T) and 16290 (C16290T), a G>A transition at position 16319 (G16319A) and a T>C transition at position 16362 (T16362C) [Torrioni et al., 1993; Starikovskaya et al., 1998]; 5 sequences were classified as haplogroup A2v based on the presence of the transitions observed in haplogroup A2 and a T>C transition at position 16239 (C16223T) [Kumar et al., 2011]. No haplogroup B sequences were found in this pre-Hispanic group. One additional sequence was classified as haplogroup C based on the presence of a T>C transition at position 16325 (T16325C); 6 were classified as haplogroup C1 based on the presence of as haplogroup C and a C>T transition at positions 16327 (C16327T); and 6 sequences were classified as haplogroup C1b14 based on the presence of transitions detected in haplogroup C1 and a G>A transition at position 16181 (G16181A) (Gomez-Carballea et al., 2015; Kumar et al., 2011; Rieux et al., 2014). Finally, 2 sequences corresponded to haplogroup D based on the absence of polymorphisms diagnostic of other haplogroups and of the transitions C>T (C16223T) and T>C (T16362C), as proposed by Torrioni et al. [1993] to classify HVS-1 haplogroups.

### *Distribution and Network Analysis of mtDNA haplogroups*

Analysis of the mtDNA haplogroup frequency in the pre-Hispanic populations was determined to study Maya history and population dynamics. The haplogroup frequencies are displayed in figure 1 and show the geographic distribution in each pre-Hispanic population according to the archaeological site. We also calculated the frequency distribution of pairwise differences between sequences of mtDNA for pre-Hispanic and contemporary populations to evaluate rapid population expansion. The distribution of the pairwise differences for the four haplogroups was unimodal, with main peaks at 1 for haplogroups A and 2 for haplogroups B, C, and D, indicative of population expansion (Figure 2). Supplemental figure 2 panels A and B shows the detailed mtDNA phylogenetic tree of pre-Hispanic Maya haplogroups and their immediate Siberian-Asian sister clades (panel A-D).

### *Network Analysis of Haplogroup A*

Median joining networks of haplogroup A mtDNA HVS-I sequences were analyzed to determine the pattern of substitutions in the noncoding control HVS-I region. This analysis included sequences from nucleotide 16106 to 16399 (Figure 3) in this study (pre-Hispanic Maya populations) and from the GenBank database for Maya, North Central and South America, Beringia, and Asia (Suppl. Table 1, Suppl. Figure 1). All samples/citations used for the network analysis are included in Suppl. Table 1. The results of this analysis revealed a total of 211 haplotypes in all individuals and 10 out of 23 from the archaeological sites Xcambo, Bonampak (Group Frey and Group Quemado), Palenque (Temple XIII, Temple XV, and Group B), Rey Quintana Roo, Comalcalco (Temple V, Temple III, Tenosique, and Dren), Peje Lagarto Pinos, Sueños de Oro, and Calicanto (Table 2, Figure 3). These haplotypes were subdivided into sub-clusters, depending on the presence or absence of the characteristics of the HVS-I variants (Figure 3).

This analysis revealed one main diversification center, lineage H3, encompassing the pre-Hispanic sequences from Xcambo (E9b), El Rey Quintana Roo (E23-1), Bonampak (E1GF, E2GF, E3GF, E4GF, E3GQ), and Palenque (T3PGTXV) herein and the contemporary individuals from Northern Asia, Siberia, Aleutian Islands, Canada, Native Mexicans, Maya from Mexico and Guatemala, and Chibchan from Nicaragua and Costa Rica, and Central and South America (Figure 3, Table 3). In addition, the pre-Hispanic sequences from Xcambo (E14b), Palenque (E2GB, XIII\_E3), and Comalcalco T3E9 and the contemporary individuals from Colombia (Chibchan) and Peru (Quechua) were grouped in this haplotype in a second analysis using sequences from nucleotides 16213 to 16399 (Suppl. Figure 3, Suppl. Table 1). Figure 4 and Table 3 displays the geographic distribution of this haplotype and sharing haplotypes respectively. The result is indicative of a common ancestor of the populations from North Asia that migrated through the Bering Strait Bridge, through Canada and the USA, down through Mexico and the Maya region, and from there to Central and South America.

Haplotype H30 was shared by 2 pre-Hispanic individuals from Comalcalco (T3E9) and Tenosique (E7P65) and with contemporary groups of Native Americans, Juarez, and Xochimilco in Mexico City in this network analysis of sequences from nucleotides 16106 to 16399 (Figure 3, Table 2 and 3 and Suppl. Table 1). Haplotype H208 was shared by the samples from Palenque E2GB and Palenque TXIII\_E3.

The DNA of samples from Comalcalco (T3E14 and T3E7), Xcambo (E14), Palenque (E2), El Rey (E27 and E8), Tenosique (E2P8 and Tenosique E6P6) demonstrated greater damage, and the sequences obtained were from nucleotides 16215 to 16399. Consequently, a second network analysis was performed (Suppl. Figure 2, Suppl. Table 1). Furthermore, the sequences from the archaeological site “La Purnia”, Santander, Colombia were included in this second analysis because these sequences encompassed the HVI region from nucleotides 16210 to 16364 [Casas-Vargas et al., 2011]. The results showed that haplotype H2 included the pre-Hispanic samples and

contemporary sequences containing haplotype H3 in the first analysis and Xcambo E9b, Palenque E2GB, and TXIII\_E3 individuals, the Pre-Columbian Guane and the contemporary sequences displayed in Table 3. Supplemental figure 2, Table 2 and Suppl. Table 1 show these results and figure 4 shows the geographic distribution of this haplotype. Haplotype H65 was shared by the pre-Hispanic sample from Comalcalco T3E14 and two Maya samples from La Tinta, Guatemala. Haplotypes for the pre-Hispanic samples from Paleque T5GB, El Rey Quintana Roo E27 and E8, Comalcalco T5EB and T3E7, and Calicanto E3VIP are shown in Table 2 and Suppl. figure 2. These samples did not cluster with any of the contemporary sequences, likely due to a low frequency of these haplotypes or missing haplotypes from the contemporary Native American populations.

Furthermore, the network analysis for haplogroup A revealed that among 23 samples, one contained haplogroup A (T3E14 from Comalcalco, Tabasco); haplogroup A2 was detected in all individuals from Bonampak, Palenque (T3PGTXV, E2GB, T5GB, TXIII, E2), El Rey Quintana Roo (E23-1, E27, E8), and Tenosique (E2P8 and E6P6); and haplogroup A2v that has been detected in contemporary populations from Central America [Kumar et al., 2011] was identified in 5 samples, 3 from Comalcalco (T5EBCHILD, T3E9, T3E7), 1 from Tenosique (E7P65), and 1 from Calicanto (E3VIP), all from Tabasco (Table 2).

A previous analysis of the biological relationship between Central and South American Chibchan-speaking populations using mtDNA sequences demonstrated the presence of a shared maternal genetic structure between Central American Chibchan [Melton et al., 2013], Mayan populations and northern South American Chibchan-speakers [Melton et al., 2007]. The maternal lineage affinity between the Chibchan and Maya groups was based on one contemporary Maya group from Guatemala [Boles et al., 1995, Melton et al., 2007, Melton et al., 2013]. However, this association has now been confirmed in this study analyzing the sequences of pre-Hispanic Mexican Maya and sequences from the Caribbean Islands, Colombia, and contemporary Maya groups from Mexico and Guatemala; as well as Central and South American Chibchan-speakers from previous reports (Table

3). Table 3 shows haplotype sharing between the pre-Hispanic Maya groups, contemporary Maya and Chibchan populations from central and South America.

### *Network Analysis for Haplogroup C*

Sequence analyses of 13 out of 38 pre-Hispanic Maya individuals containing haplogroup C revealed a total of 11 haplotypes (Table 2). One sample belonged to haplogroup C; 6 were found in haplogroup C1; and 6 were identified as haplogroup C1b14 (Figures 1, 4, 5, Suppl. Figure 4, and Table 2).

The median network analysis for haplogroup C, which was based on the sequences from 16153 to 16399, revealed one primary diversification center, lineage H10 (haplogroup C1), which clustered the pre-Hispanic sample from Pejelagarto\_I1 and Ciboney (CF5), Cuba, and the contemporary populations shown in Table 3 from USA, native Mexicans including Mayans, and individuals from Guatemala, Brazil, Peru, and Colombia. A second lineage, H5, encompassed sequences from Asian and North American individuals with several haplotype diversifications. The pre-Hispanic samples from Palenque T4GB and El Rey Quintana Roo E9 were found in haplotype H113, and the Comalcalco (T3AE) and Tenosique E3 samples shared haplotype H117. Haplotypes of individuals from El Rey Quintana Roo (E23), Palenque (T1GB), Comalcalco (T5I1), Tenosique (EN4P6), Sueños de Oro (E1P), and Sitio Dren (E2) are displayed in Table 2 and figure 5. Haplotypes H116, H117, H119, and H120 clustered in the same branch and were classified as belonging to haplogroup C1b14 based on the presence of a G>A transition at position 16181 [Gómez-Carballea et al., 2015; Kumar et al., 2011; Rieux et al., 2014] (Figure 5 and Table 2).

Haplogroup C1b14 observed in the prehispanic population from Tabasco (Table 2) is very infrequent in contemporary Native American populations. Specifically, this haplogroup was observed in 3 contemporary individuals, 2 Mexican Americans and 1 Zapotec [Gómez-Carballea et al., 2015; Kumar et al., 2011; Rieux et al., 2014]. Decrease of this haplogroup in the contemporary

population may be due to the reduction of the populations during the Spanish conquest. The sharing of haplogroup C1b14 may suggest common ancestry of this pre-Hispanic Maya and the 3 contemporary Mexicans.

A second network analysis was constructed because the sequences of the pre-Hispanic samples from Palenque T3AGB and Sueños de Oro E2P1 contained data from nucleotide positions 16213 to 16399 (Suppl. Figure 4). Haplotypes of these two samples and Palenque T4GB and El Rey Quintana Roo (E9, E23) are displayed in Table 2. This analysis displayed a main diversification center, lineage H9, containing sequences from pre-Hispanic individuals from Pejelargato, Comalcalco (T3AE6), Tenosique (E3), and Sitio Dren (E2) in this study, Ciboney from Cuba, and the contemporary individuals as in the first analysis containing haplotype H10 (Suppl. Figure 4, Tables 2, 3 and Suppl. Table 1). Haplotypes 84 to 89 were not observed in the database included in this study. However, Sueños de Oro E1P1 demonstrated an identity of 99% with an individual from Texas, suggesting that these haplotypes are rare and not represented in current studies, or they are not present in contemporary populations. These results support the notion that the Maya population spread to Central and South America. No haplogroup C was reported in the Pre-Columbian Guane group [Casas-Vargas et al. 2011]. The geographic distribution and shared haplotypes are shown in figure 4 and Table 3 respectively.

#### *Network Analysis for Haplogroup D*

The sequence analysis showed that of the two pre-Hispanic samples, 1 individual from Palenque E6ID and 1 from Comalcalco P3AE2, belonged to haplogroup D (Figure 6, Table 2) based on the absence of the polymorphisms diagnostic of other haplogroups and the presence of the frequent T>C and C>T transitions proposed by Torroni et al. [1993] at positions 16223 and 16362, respectively. The D1 haplogroup found throughout the Americas was absent in the samples used in

this study (Figure 6, Table 2), potentially due to the low frequency of this haplogroup in our pre-Hispanic population.

The median network haplogroup D analysis in the HV1 region, including nucleotide positions 16153 to 16399 (Figure 6), displayed three main diversification centers: haplotype H4 (haplogroup D) encompassing the individuals from the pre-Hispanic samples Palenque E6ID\_TXV and Comalcalco P3AE2 in this study plus one sequence from Ciboney, and contemporary individuals from Northern Brazil, China and Northern Asia as shown in Table 3; Haplogroups D1 of haplotypes are displayed in figure 6, and Suppl. Table 1. The geographic distribution of the pre-Hispanic and contemporary samples that shared this haplotype H4 is displayed in figure 4. These results suggest that the ancestors of the pre-Hispanic individuals containing haplotype H4 originated from China and Northern Asia and subsequently spread to Cuba and South America. The main diversification centers of the American populations were grouped in haplogroup D (H4), D1 (H34), D4h3 (H26), and D2 (H17 and H1), which included sequences from Aleuts (Bering), Eskimo, Evenk, Chukchi, and Nahua individuals (Figure 6, Suppl. Table 1). These results also supported the maternal lineage affinity between the pre-Hispanic Mexican populations in this study and the pre-Hispanic Ciboney from Cuba, suggesting shared regional haplotypes indicating a shared genetic ancestry. This may point to more regional interaction between populations in the Circum-Caribbean region than previously demonstrated.

A second analysis of haplogroup D sequences from nucleotides 16181 to 16399 was conducted including the pre-Hispanic Guane group from Colombia in the analysis to evaluate the presence of a maternal lineage relationship between the pre-Hispanic Guane from Colombia [Casas-Vargas et al., 2011] and Mexican pre-Hispanic Maya individuals (results not shown). The two sequences from the pre-Hispanic Maya did not contained the same haplotype, indicating that these individuals did not share a common ancestor.

The results of network analysis of the haplogroup A, C and D lineages in the pre-Hispanic Maya were consistent with the hypothesis that the direct ancestors of Native Americans were a hybrid of different Siberian groups that had migrated to eastern Beringia at different times and following different routes (Figure 4) [Kunz & Reanier, 1994, Starikovskaya et al., 2005].

#### *Analysis of molecular variance by timescale*

Results for AMOVA using mtDNA HVS-I data that separated Maya population into four groups: 1) Early 250-550 CE (Xcambo), 2) Middle 580-900 CE (Bonampak, Palenque, Comalcalco, Tenosique, Suenos de Oro, Calicanto, Peje Lagarto), 3) Late 1200-1500 CE (El Rey Quintana Roo), 4) Contemporary Mexican Maya base on time scale are summarized in Table 4. The lowest amount of observed variation was 1.23% for mtDNA HVS-I among-groups ( $F_{CT}=0.01234$ ), whereas within-group analysis explained 4.98% ( $F_{SC}=0.05041$ ) of variation. The majority of variation, 93.79% ( $F_{ST}=0.06213$ ), was found within-populations.

#### *Sequence diversity analysis (Demographic analysis)*

The results of the diversity indices and neutrality tests are shown in Table 5 for all contemporary and pre-Hispanic populations. Similar diversity values were obtained for all populations analyzed in this study. Fu's  $F_s$  was negative and significant for all six populations. These results suggest the presence of rare alleles at low frequencies or population expansion after a recent bottleneck.

#### *Analysis by Multidimensional Scaling (MDS)*

##### **MDS of pre-Hispanic and modern populations**

To visualize the relationships between the pre-Hispanic Mayas in this study and the contemporary populations from Asia, Beringia, and North and South America based on the HVI mtDNA sequence

data, MDS plots were constructed using the pairwise  $F_{st}$  values (Figure 7). The MDS plot revealed that the genetic distances between the pre-Hispanic and contemporary Maya populations were smaller in comparison to the Beringian, Asian, North or South American groups (Figure 7). The distance between North and South America was also shorter compared with the other groups indicating a closer affiliation. The distances between the ancient Maya population and the contemporary Mayas, Asians, Beringians, and North and South America can be explained by the shared haplotypes from haplogroups A, C, and D with the contemporary populations (Figures 3, 4, 5, and 6 and Table 3). Furthermore, the pre-Hispanic Maya populations in this study displayed a high frequency of haplogroup A (60.53%), followed by haplogroup C (34.21%) and an extremely low frequency of haplogroup D (5.26%) (Figure 1).

### **MDS of pre-Hispanic populations**

The MDS plot comparing the pre-Hispanic populations revealed that Tenosique, Comalcalco, Quintana Roo and Palenque clustered together (Figure 8), Yucatan and Bonampak were located together, whereas Tabasco, Ciboney and Chile groups clustered separately. The ancient populations from Ciboney, Cuba and Chile are expected to be separate from the other populations because of their geographic locations and high frequency of haplogroups C and D in Ciboney and B and D in Chile. Pejelagarto and Calicanto were separated from the main group because of the small sample size and geographic location (Figure 1). The clustering together of Xcambo and Bonampak can be due to Preclassic period origin of both populations. We suggest that the individuals from Xcambo (250-550 CE) migrated to Bonampak (580 to 800 CE) at that time.

### **MDS of pre-Hispanic and ancient populations**

When the ancient populations are compared with the contemporary Maya populations by the MDS plot (Figure 9), the contemporary populations from Quintana Roo, Yucatan, Guatemala, Campeche, and Tzotzil clustered together; however, Bonampak and Xcambo were also closely related;

Tenosique and Comalcalco were equidistant to the Tzotzil population; and Palenque, Pejelagarto, Calicanto, and El Rey Quintana Roo were separated from the other populations according to their geographic locations (Figures 1 and 9) and haplotype composition (Suppl. Table 1). In addition, Tojolabal was separated because it is a very different population compared with the other Maya populations, exhibiting a higher proportion of haplogroup B (58%), a lower proportion of haplogroup A (25.7%) and haplogroup D (16.2%), and the absence of haplogroup C [González - Martín et al., 2015]. The pre-Hispanic Maya groups in this study had a high frequency of haplogroup A, similar to that found in contemporary Mayas [González - Martín et al., 2015; Söchtig et al., 2015] and Aleuts from the Commander Islands [Crawford et al., 2010; Derbeneva et al., 2002].

### **MDS of pre-Hispanic and modern Mayan populations**

A comparison of all pre-Hispanic Mayan populations as one group with the contemporary Mayan populations/group by MDS plot revealed that the ancient populations were separated from the contemporary populations (Figure 10), probably due to the differences in haplotype composition resulting from recent migrations into Maya populations, consistent with the history of the Mayan populations, which suffered different invasions by other populations, such as the Nahua of Uto-Aztecan affiliation, as well as recent migrations out of Maya areas. The ancient Mayan populations in this study contained a high frequency of haplogroup A (A, A2, A2v), followed by haplogroup C (C1 and C1b14), a very low frequency of haplogroup D, and the absence of haplogroup B. Most contemporary indigenous populations from Mexico contain all four haplogroups in different proportions [Peñaloza-Espinosa et al., 2007], although haplogroup A2 exhibits the highest frequency [Santos et al., 1994, 1996; Torroni et al., 1994]. The high frequency of haplogroup A2 in the pre-Hispanic Maya populations is shared with the Asian, Siberian Eskimo, and Chukchi-Eskimo groups [Crawford et al., 2010, 2013] and is probably due to a shared common ancestor.

### *Mitochondrial haplogroup Frequencies*

In general, the mitochondrial haplogroup frequencies differed substantially between the Southwest and Mesoamerican populations; haplogroup B was very common in the Southwest populations (USA), which rarely exhibited mitochondrial haplogroup A [Kemp and Schurr, 2010]. However, there were some exceptions, such as the Tarahumara. Additionally, haplogroup B was much less common in Mesoamerican populations, in which haplogroup A predominated [Kemp et al., 2010]. However, the Nahua–Atocpan, a Mesoamerican population, exhibited slightly more haplogroup B than haplogroup A [Kemp et al., 2010], the Tojolabal Maya population exhibited a higher frequency of haplogroup B than haplogroup A [Gonzalez-Martin et al., 2015], and the ancient bones collected in a Quiché Indian village, located close to the provincial capital of Santa Cruz de Quiché, revealed the presence of 16 different mtDNA haplotypes, among which haplogroup B has the highest frequency [Boles et al., 1995].

The pre-Hispanic Maya population of this study displayed a high frequency of haplogroup A (60.53%), followed by haplogroup C (34.21%), a very low frequency of haplogroup D (5.26%), and the complete absence of haplogroup B. Similar haplogroup frequencies have also been observed in the Central American Chibchan populations Kori and Arsario [Melton et al., 2013]. Similarly, haplogroup B was apparently absent in the Fuego-Patagonians and the aboriginal groups above latitude 55 in North America and Asia, suggesting that the initial Paleoindian settlers that migrated into South America might represent an independent migration event unrelated to the Clovis people, as suggested previously [Lalueza et al., 1997]. However, Kemp et al. [2010] and Kumar et al. [2011] suggested an early coastal migration. Similarly, present-day Amerindian populations inhabiting the Caribbean region of Colombia either did not carry or had lower frequencies of haplogroup B (Kogui 0%, Arhuaco and Chimila 4.8%, Arsario 12.5% and Wayuu 17.6%), while the predominant haplogroup A was followed by haplogroup C [Yunis and Yunis, 2013]. Analysis of lower Central American Chibchan population mtDNA diversity has shown high frequencies of

haplogroup A2 and B4 [Melton et al., 2007, 2013], while those from northern South America mirror the Ancient Maya, with high frequencies of haplogroup A and moderate amounts of haplogroup C. The ancient population of Tipu from Belize has also been shown to carry haplogroup B (8%) at a low frequency, although haplogroup C (64%) was found in higher proportions compared with haplogroups D (28%) and B [Elwess et al., 2015]. Northern Colombia displayed lower frequencies of haplogroups B and D and a higher frequency of haplogroup B in western Colombia that declined towards eastern Colombia, whereas the Embed/Wounan population from Panama has been reported to contain haplogroups A, B, C and D [Kolman and Bermingham, 1997]. Similarly, the Tojolabal Maya population displayed a high frequency of haplogroup B2 (58.1%) [Gonzalez-Martin et al., 2015]. In contrast, haplogroup B was absent in the aboriginal populations of northwestern and northern Siberia [Derbeneva et al. 2002b, c], but it has been detected in populations restricted to the southwestern and south central periphery of the subcontinent [Sukernik et al. 1996; Derenko et al. 2000, 2003 Yunis and Yunis, 2013]. Haplogroup B mtDNA has also been detected in the skeletal remains exhumed from a 2000-year-old cemetery in northern Mongolia [Keyser-Tracqui et al. 2003]. Based on the results of this study and previous findings, the absence of haplogroup B in the pre-Hispanic Maya, the low frequency in most contemporary Maya, and its absence or low frequency in Colombia, may suggest that the entry of haplogroup B into the Americas occurred independently of the other mitochondrial haplogroups (A, C, and D) during a later migration process, as suggested previously [Torroni et al. 1993b, Starikovskaya et al., 1998; Lalueza et al., 1997]. Therefore, it is possible that the Maya ancestors did not carry haplogroup B or may have lost it through genetic drift, as proposed previously for the populations of Tierra del Fuego [Lalueza et al., 1997; García-Bour et al., 2004].

### *Haplotype Analysis and shared haplotypes*

The founding haplogroup A2, which was identified in the individuals in this study, has been reported to have a coalescence age of  $19.5 \pm 1.3$  kya/ $16.1 \pm 1.5$  kya, according to Kumar et al.

[2011]. These coalescence ages were calculated using the mutation rates reported by Mishmar et al. [2003] and Soares et al. [2009]. A2v has a more recent coalescent time of 9.1/9.5 kya [Kumar et al., 2011], whereas haplogroup C, C1 and C1b14 have coalescent times of 27.37 (19.55; 35.44) [Derenko et al., 2010],  $21.4 \pm 2.7$  kya/ $16.4 \pm 1.5$  [Kumar et al., 2011], and 12.4 (7.7-17.3) kya [Gómez-Carballa et al., 2015], respectively. Haplotype H3 (haplogroup A2) from the pre-Hispanic populations in this study, the pre-Columbian Guane was shared with contemporary populations from North, Central and South America, supporting the ancestral continuity of the present populations (Figures 3, 4, Suppl. Figure 2, Table 3, and Suppl. Table 1). This finding supports the ethnogenesis of these Mexican Maya populations in pre-Hispanic times on a cultural and biological basis, in contrast to the contemporary Maya populations [Söchtig et al., 2015]. Haplogroup B was not detected in the pre-Hispanic populations of this study, although it has been reported in the pre-Columbian Guane population [Casas-Vargas et al., 2011]. Therefore, to validate this finding, it will be important to pursue further studies of the pre-Hispanic populations from the Maya area because the contemporary Tojolabal Maya population displayed a high frequency of haplogroup B2 (58.1%) [Gonzalez-Martin et al., 2015]. Increasing the number of sequences from pre-Hispanic samples will be necessary to determine whether the absence of haplogroup B in the pre-Hispanic samples from the archaeological sites in this study reflects the absence or low frequency of this haplogroup.

Haplogroup D1 defined by the T>C, C>T C>T transitions at positions 16223, 12325, and 16362, respectively [Kumar et al., 2011], in the pre-Columbian Guane differed from our findings, in which only the T>C and C>T transition at positions 16223 and 16362, respectively, specific for haplogroup D were identified [Torrioni et al. 1993; Kumar et al., 2011]. This haplogroup has also been found at low frequencies in the contemporary Maya populations from Quintana Roo, Yucatan, and Campeche, Mexico [González - Martín et al., 2015] and was absent in the Mexican pre-Hispanic populations.

Bodner et al. [2012] described two new subclades of the pan-American founder haplogroup D1 that were limited to the Southern Cone of South America. These findings were based on the limited geographic dispersal, high diversity of the D1g and D1j haplogroups, and calculated coalescence ages suggestive of a coastal and rapid initial extensive trans/Andean migration that may represent the genetic heritage of the pioneer settlers of South America and apparently is preserved in the present-day Mapuche people.

Haplogroup D, with an overall estimated coalescence time of 35–37 kya, based on the mutation rate used by Derenko, et al [2010], was identified in the pre-Hispanic samples from Palenque E6ID\_TXV and Comalcalco P3AE2, which have been represented by at least three D1, D4h3a, and D4e1c branches in the Americas. The D1 haplogroup is found at a high frequency throughout the Americas, while D4h3a and D4e1c are found at low frequencies [Kumar et al., 2011]. These two samples lacked the 16325 mutation that is specific for haplogroup D1. It is not clear if this mutation has reverted in some Native American haplogroup D mtDNAs, such as the “Cayapa” haplotype BR53 [Alves-Silva et al., 2000], or if there is more than one Native American founder of this haplogroup, as suggested by Bandelt et al. [2003]. The present-day variation in the haplogroup C and D clades suggests that they expanded prior to the Last Glacial Maximum (LGM), with the oldest lineages present in eastern Asia [Derenko et al., 2010]. Our network analysis results for haplogroup D support this hypothesis because haplotype H4 contained 2 of our sequences and the sequences from eastern Asia (Figure 6, Table 3, and Suppl. Table 1). In addition, haplotype H4 also encompassed sequences from the pre-Hispanic samples from Ciboney and the contemporary individual from Brazil, supporting the dispersion of this haplotype from the Yucatan to Cuba or from Cuba to Yucatan during the pre-Hispanic era and to South America. Lalueza-Fox et al. [2003] suggested a migration of the Ciboneys from South America to the Caribbean Islands, although a migration in the opposite direction is also possible. This is also indicative of greater regional interaction among populations in the Circum-Caribbean region than previously demonstrated.

The pre-Hispanic Maya individuals from Xcambo (E9b), El Rey Quintana Roo (E23), Bonampak (E1GF, E2GF, E3GF, E4GF, E3GQ9), Palenque (T3PGTXV), Comalcalco (T3E9), and Tenosique (E7P65) all shared haplotype H3/H2 (Figures 3, Suppl. Figure 3, and Suppl. Table 1) with the pre-Hispanic individuals from Ciboney, Cuba, pre-Columbian Guane, the archaeological cemeteries from Chile, and contemporary people from Northern Asia, Siberia, the Aleutian Islands, Dogrib, and Canada, Mexican Native Americans, Maya from Mexico and Guatemala, and native groups from Central and South America (Figures 3-6, Suppl. Figures 2 and 3, and Table 3). This haplotype sharing supports the migration of the Maya ancestors from Asia across the Bering Strait Bridge, United States, and from Northern Mexico to the Maya region. It is also likely that the genetic composition of pre-Hispanic Maya populations displays continuity with contemporary Mayan populations because haplotype H3 of haplogroup A was also shared with different contemporary Maya populations. This finding also indicates that some of the contemporary Maya populations are descendants of both the original Maya population and other populations that migrated to these geographic areas because some of the haplotypes found in the pre-Hispanic individuals were also detected in different proportions in contemporary Maya populations.

The Mexican Maya in this study displayed the highest frequency of haplogroup A, similar to the ancient Post-Classic Aztecs from Tlatelolco (1450-1275 CE), Mexico [Kemp et al., 2005], the Maya from Xcaret, Mexico [González-Oliver et al., 2001], and the most modern Maya. In contrast, a 650-1200 CE Maya sample from Copán, Honduras exhibited a high frequency of haplogroup C [Merriwether et al., 1997], and the 800-1100 CE from the Tommy site, USA [Snow et al., 2010] showed a high frequency of haplogroup B, most closely resembling the ancient Anasazi, Fremont [Carlyle et al., 2000; Leblanc et al., 2007], and the modern populations of Cora, Hualapai, Huichol, Jemez, Tarahumara, Tohono O'odham and Zuni [Kemp et al., 2010], the Tojolabal Maya [Gonzalez-Martin et al., 2015], southwest (USA), and South America [Salas et al., 2009; Raff et al., 2011]. Therefore, contemporary Maya are descendants of the pre-Hispanic Maya, and the ancestral

Native Americans dispersed across North America approximately 13 thousand years ago (ka) [Raghavan et al., 2015]. Our results are consistent with other mtDNA data for the Guatemalan Maya region, demonstrating the presence of gene flow in the Mesoamerican area and a predominant unidirectional flow towards South America that most likely occurred during the Preclassic (1800 BCE-200 CE) and the Classic (200–1000 CE) eras of the Mesoamerican chronology. This pattern of gene flow is in agreement with the development of the Maya civilization [Söchtig et al., 2015]. Our results also support the expansion of maize with the human populations from the Mexican Gulf Coast to the south, as maize cultivation was dispersed from the lowland tropics of Tabasco more than 5,050 BCE to North and South America [Pohl et al., 2007, Merrill et al., 2009]. This finding is consistent with the introduction of maize into the Southwest prior to 2050 BCE [Merrill et al. 2009; Kemp et al., 2010; Kohler and Reese, 2014], according to the population expansion from the Maya area to North and South America (Figure 4) through the long-distance migration of farmers from the Mexican Gulf Coast [Malhi et al., 2003]. These results support the historically based migration theories supported by archaeological data.

#### *Genetic admixture among pre-Hispanic populations*

Figure 1 and Table 2 show HVS-I haplotype H3 (haplogroup A) being shared among the pre-Hispanic Maya from Comalcalco, Palenque, Tenosique, Sueños de Oro, Bonampak, Xcambo, and Rey Quintana Roo; haplogroup A2v among Comalcalco, Tenosique, and Calicanto; haplotype H4 (haplogroup D) between Comalcalco and Palenque; and the rare haplogroup C1b14 among Comalcalco, Tenosique, and Sueños de Oro suggesting a genetic admixture, which appears to be more pronounced in Comalcalco. These results also indicate regional gene flow among the pre-Hispanic populations and regional interaction.

## Conclusions

The results obtained in the present network analysis demonstrate the following. 1) Gene flow occurred within the Maya area, with a directional flow to South America in the Preclassic and Classic eras of the Mesoamerican chronology. 2) Historical documentation showed that the ancestors of Maya civilization entered the Yucatan Peninsula after the first movement of people from Northern Asia into the Americas, with later migration of the Maya south to Central America and the Caribbean toward the northern region of South America are supported by our analyses of contemporary and pre-Hispanic mtDNA. 3) Haplotypes H3 of haplogroup A and H4 of haplogroup D were shared among pre-Hispanic Maya, Ciboney, and Han Chinese, suggesting the migration of a common ancestor from East Asia to the Maya region of Mexico, Cuba, and Colombia. 4) Haplotype H3 of haplogroup A was shared among the pre-Hispanic Maya population and contemporary Maya from Mexico and Guatemala, suggesting maternal continuity of the pre-Hispanic Maya in the contemporary population. 5) Haplotypes of haplogroups A and C were shared among the pre-Hispanic Maya populations and native populations from Cuba (pre-Hispanic), Panama, Costa Rica, Colombia (pre-Hispanic and contemporary), Chile (archaeological cemeteries) Peru, and Brazil, that the Maya and Native American populations still carried the genetic imprint of the pre-Hispanic Maya. The results obtained for the diversity indices and neutrality tests (Table 5) for all contemporary and pre-Hispanic populations suggested the presence of rare alleles at low frequencies or population expansion after a recent bottleneck. 6) The MDS graphs displayed haplotype differences among all populations and the relationships between the pre-Hispanic Mayas in this study and the contemporary populations from Asia, Beringia, and North and South America based on HVSI mtDNA sequence data. 8) Identification of the rare haplogroup C1b14 in the Mexican Maya pre-Hispanic population and low frequency of this haplogroup in the contemporary populations may be due to genetic drift. 9) The sharing of mitochondrial haplotypes and

haplogroups among pre-Hispanic individuals also indicated regional gene flow among the pre-Hispanic populations and regional interaction.

In conclusion, the haplotype diversity observed in the pre-Hispanic and contemporary populations of Maya indicates groups that have undergone drift and lineal extinction, with periodic increases in genetic diversity through admixture with adjacent populations.

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**Table 1.** Features of samples from the pre-Hispanic Mayas.

Sample Code and Archeological Site	Building	Burial (E) Tomb (T)	Age	Sex	Temporality (CE)	Hap	City	Latitude/ Longitude
<b>Xcambo</b>								21.35/-89.266°
MXYUCXCAMBO_E9b_A2	Work areas	E9	Adult	M	250-550	A2	Yucatan	
MXYUCXCAMBO_E14_A2	Work areas	E14	Adult	M	250-550	A2	Yucatan	
<b>Bonampak</b>								16.704/-91.065°
MXCHBONAMPAK_E1GF_A2	Group Frey	E1	Adult	M	580-800	A2	Chiapas	
MXCHBONAMPAK_E2GF_A2	Group Frey	E2	Adult	M	580-800	A2	Chiapas	
MXCHBONAMPAK_E3GF_A2	Group Frey	E3	Adult	M	580-800	A2	Chiapas	
MXCHBONAMPAK_E4GF_A2	Group Frey	E4	Adult	M	580-800	A2	Chiapas	
<b>Bonampak</b>								
MXCHBONAMPAK_E3GQ_A2	Group Quemado	E3	Adult	M	580-800	A2	Chiapas	
<b>Palenque</b>								17.5092°/-91.982°
MXCHPALENQUE_T3PGTXV_A2	XV	T3	Adult	M	750-800	A2	Chiapas	
MXCHPALENQUE_E6ID TXV_D	XV	E6 IND-D	Adult	M	750-800	D	Chiapas	
MXCHPALENQUE_TXIII E3CHILD_A2	XIII	E3	Infant	M	750-800	A2	Chiapas	
MXCHPALENQUE_E2_A2	XIII	E2	Adult	F	750-800	A2	Chiapas	
MXCHPALENQUE_T5GB_A2	GROUP B	T5	Infant	M	750-800	A2	Chiapas	
MXCHPALENQUE_E2GB_A2	GROUP B	T2	Adult	F	750-800	A2	Chiapas	
MXCHPALENQUE_T4GB_C1	GROUP B	T4	Adult	F	750-800	C1	Chiapas	
MXCHPALENQUE_T1GB_C1	GROUP B	T1	Adult	F	750-800	C1	Chiapas	
MXCHPALENQUE_T3AGB_C1	GROUP B	T3, IND-A	Adult	F	750-800	C1	Chiapas	
<b>El Rey Quintana Roo</b>								21.061°/86.781°
MXQRELREY_E23-1_A2		E23-1	Adult		1200-1500	A2	Quintana Roo	
MXQRELREY_E23-3_C1		E23-3	Youth		1200-1500	C1	Quintana Roo	
MXQRELREY_E8_A2		E8	Adult		1200-1500	A2	Quintana Roo	
MXRELREY_E9_C1		E9	Adult		1200-1500	C1	Quintana Roo	
MXQRRELREY_E27_A2		E27	Adult		1200-1500	A2	Quintana Roo	
<b>Comalcalco</b>								18.280°/ -93.202°
MXTABCOMALCALCO_T5EBCHILD_A2v	V	T5	Infant	M	700-900	A2v	Tabasco	
MXTABCOMALCALCO_T5I1_C1b14	V	IND 1	Adult	M	700-900	C1b14	Tabasco	
MXTABCOMALCALCO_T3E9_A2v	IIIA, North Plaza	E9	Adult	M	700-900	A2v	Tabasco	
MXTABCOMALCALCO_T3E14_A	IIIA, North Plaza	E14	Adult	M	700-900	A	Tabasco	
MXTABCOMALCALCO_T3E7_A2v	IIIA, North Plaza	E7	Adult	M	700-900	A2v	Tabasco	
MXTABCOMALCALCO_T3AE6_C1b14	IIIA, North Plaza	E6	Adult	M	700-900	C1b14	Tabasco	
MXTABCOMALCALCO_P3AE2_D	IIIA, North Plaza	E2	Adult	M	700-900	D	Tabasco	

<b>Site Dren, Comalcalco</b>								
MXTABSITIODREN_E2_C1b14	Work areas	E2	Adult	M	700-900	C1b14	Tabasco	
<b>Tenosique</b>								17.466°/-91.416°
MXTABTENOSIQUE_E2P8_A2	Work areas	E2, Well 8	Adult	M	700-900	A2	Tabasco	
MXTABTENOSIQUE_E6P6_A2	Work areas	E6, Well 6 South	Adult	M	700-900	A2v	Tabasco	
MXTABTENOSIQUE_E7P65_A2v	Work areas	E7, Well 6	Adult	M	700-900	A2v	Tabasco	
MXTABTENOSIQUE_E4P6CHILD_C1b14	Work areas	E4, Well 6	Infant	ND	700-900	C1b14	Tabasco	
MXTABTENOSIQUE_E3_C1b14	Work areas	E3, Well 9	Adult	M	700-900	C1b14	Tabasco	
<b>Sueños de Oro (El Cibo), Tenosique</b>								
MXTABSUENOSDEORO_E1P1_C1b14	Work areas	E1, Well 1	Adult	M	700-900	C1b14	Tabasco	
MXTABSUENOSDEORO_E2P1_C	Work areas	E2, Well 1	Adult	M	700-900	D	Tabasco	
<b>Site Calicanto, Jalapa</b>							Tabasco	17.732°/-92.754
MXTABCALICANTO_E3VIP_A2v	Work areas	E3	Main Ind.	M	700-900	A2v	Tabasco	
<b>Peje Lagarto, Huimanguillo</b>	Los Pinos						Tabasco	17.85°/-93.383
MXTABPEJELAGARTO_I1_C1	Pipe 1	Individual 1	Adult	M	700-900	C1	Tabasco	

CE= Common Era; ND= Not determined.



Table 3. Haplotypes encountered shared at various locations through sequencing of mtDNA from pre-Hispanic and contemporary human populations.

HAPLOGROUP	Temporality (CE)	HAPLOTYPE NUMBER			
		A	C	D	
POPULATION/GEOGRAPHIC AREA		H3	H30	H10	H4
<b>Xcambo</b> (E9b_A2, E14_A2)	250-550	•			
<b>Bonampak</b> (E1GF_A2, E2GF_A2, E3GF_A2, E4GF_A2, E3GQ_A2)	580-800	•			
<b>Palenque</b> (T3PGTXV_A2)	750-800	•			
<b>El Rey Quintana Roo</b> (E23-1_A2)	1200-1500	•			
<b>Pre-Columbian Guane</b>	1090 ± 70	•			
<b>Chibchan:</b> Costa Rica (Rama), Panama (Maleku, Guaymi), and Colombia	Contemporary	•			
Ecuador (Kayapa), Bolivia (Yuracare), Uruguay (Tacuarembó), Peru (Quechua), Brazil (Xavante)	Contemporary	•			
Chile (Archaeological Cementeries)	1900*-1900	•			
Mexico City (Nahuatl Zitlala, Ixhuatlancillo)	Contemporary	•			
Mexico City (Nahuatl Xochimilco)	Contemporary	•	•		
Michoacan (Purépecha), Oaxaca (Mixe)	Contemporary	•			
<b>Maya:</b> Yucatan, Quintana Roo, Chiapas (Tzotzil, Tojolobal)	Contemporary	•		•	
Campeche ( <b>Maya</b> )	Contemporary	•			
Guatemala ( <b>Maya</b> )	Contemporary	•	•		
Guatemala (Ladino)	Contemporary	•			
Nayarit (Cora)	Contemporary	•		•	
USA (Native Americans, Hispanic)	Contemporary	•		•	
Arizona (Hualapai)	Contemporary			•	
California (Native American)	Contemporary		•	•	
Northern Asia (Mongolia, Siberia, Rusia and China)	Contemporary	•			•
Siberia (Chukchi Eskimo)	Contemporary	•			
China (Han)	Contemporary			•	•
Ojinaga, Juarez	Contemporary	•	•	•	
Aleutian archipelago (Aleut)	Contemporary	•			
<b>Comalcalco</b> (T3E9)	700-900		•		
<b>Tenosique</b> (E7P65)	700-900		•		
<b>Quechua:</b> Peru (Yancash, Yuncay, Tupe, Tayacaja, Arquipa)	Contemporary	•		•	
<b>Peje Lagarto</b> (II_C1)	700-900			•	
Cuba ( <b>Ciboney</b> ), Hualapay	40*			•	•
Sonora (Pimas), Venezuela (Guahibo, Amazon, Wayuu), Colombia (Wayuu), Bolivia (Movima), Peru (Quechua, Aymara), Brazil and Venezuela (Shamatari), Brazil (Zoró, Xikrin, Tibus), Chile (Pehuenche, Aonikenk)	Contemporary			•	
<b>Palenque</b> (E6ID TXV_D)	750-800				•
<b>Comalcalco</b> (P3AE2_D)	700-900				•
Brazil (Maranhão, SE, N),	Contemporary				•

\*BCE

Table 4. Analysis of molecular variance (AMOVA) for mitochondrial DNA control region by timescale of the following groups: 1. Early 250-550 CE (Xcambo); 2. Middle 580-900 CE (Bonampak, Palenque, Comalcalco, Tenosique, Suenos de Oro, Calicanto, Peje Lagarto); 3. Late 1200-1500 CE (El Ray Quintana Roo); and 4. Contemporary Mexican Maya populations.

Source of Variation	d.f.	Sum of Squares	Variance Components	Percentage of variation
Among groups	3	12.552	0.03295 Va	1,23
Among populations	8	52.837	0.13289 Vb	4.98
Within populations	313	783.512	2.50323 Vc	93.79
Total	324	848.902	2.66907	
Fixation Indices	FSC :	0.05041		
	FST :	0.06213		
	FCT :	0.01234		

Genetic structure test (4 groups). Distance method: Pairwise difference  
Significance tests (1023 permutations)

Vc and FST : P(rand. value < obs. value) = 0.00000  
P(rand. value = obs. value) = 0.00000  
P-value = 0.00000+-0.00000

Vb and FSC : P(rand. value > obs. value) = 0.00000  
P(rand. value = obs. value) = 0.00000  
P-value = 0.00000+-0.00000

Va and FCT : P(rand. value > obs. value) = 0.13685  
P(rand. value = obs. value) = 0.00000  
P-value = 0.13685+-0.01012

Table 5. Diversity indices and neutrality tests for 6 populations (pre-Hispanic Mayas, Contemporary Mayas, North America, South America, Bering, and Asian).

Population/City <sup>a</sup>	Ancient Maya	Contemporary Maya	North America	South America	Bering	Asian	Mean	SD
n	40	331	310	405	63	47	199.33333	166.77010
Nucleotide Diversity (Pi)	4.64103	3.97836	4.59668	5.17376	3.18382	3.80481	4.22974	0.71171
$\theta_k$ (95% CI)	21.69131 (11.77983-40.13327)	26.25052 (19.68381-34.6986)	64.64430 (50.52623-82.41044)	155.96344 (127.08540-191.28236)	13.68336 (8.09424-22.81939)	28.27285 (16.01871-50.23837)	51.75096 (38.86470-70.26374)	54.00129 (45.80205-62.63826)
$\theta_s$ (SD)	6.34765 (2.16339)	8.31005 (1.98286)	10.93126 (2.52823)	13.37413 (2.91478)	4.45633 (1.49606)	6.33959 (2.10068)	8.29317 (2.19767)	3.31960 (0.48425)
$\theta_\pi$ (SD)	4.64103 (2.58215)	3.97836 (2.20743)	4.59668 (2.50362)	5.17376 (2.77688)	3.18382 (1.85000)	3.80481 (2.16443)	4.22974 (2.34742)	0.71171 (0.33599)
Tajima's D	-0.91697	-1.49457	-1.70157	-1.79209	-0.88503	-1.33424	-1.35408	0.38570
P-value <sup>c</sup>	0.19500	0.03300	0.01300	0.00500	0.18900	0.06800	0.08383	0.08659
Fu's $F_s$	-11.51058	-25.36427	-25.13194	-24.77965	-13.05111	-20.96007	-20.13294	-6.31079
P-value <sup>c</sup>	0.00100	0.00000	0.00000	0.00000	0.00100	0.00000	0.00033	0.00052

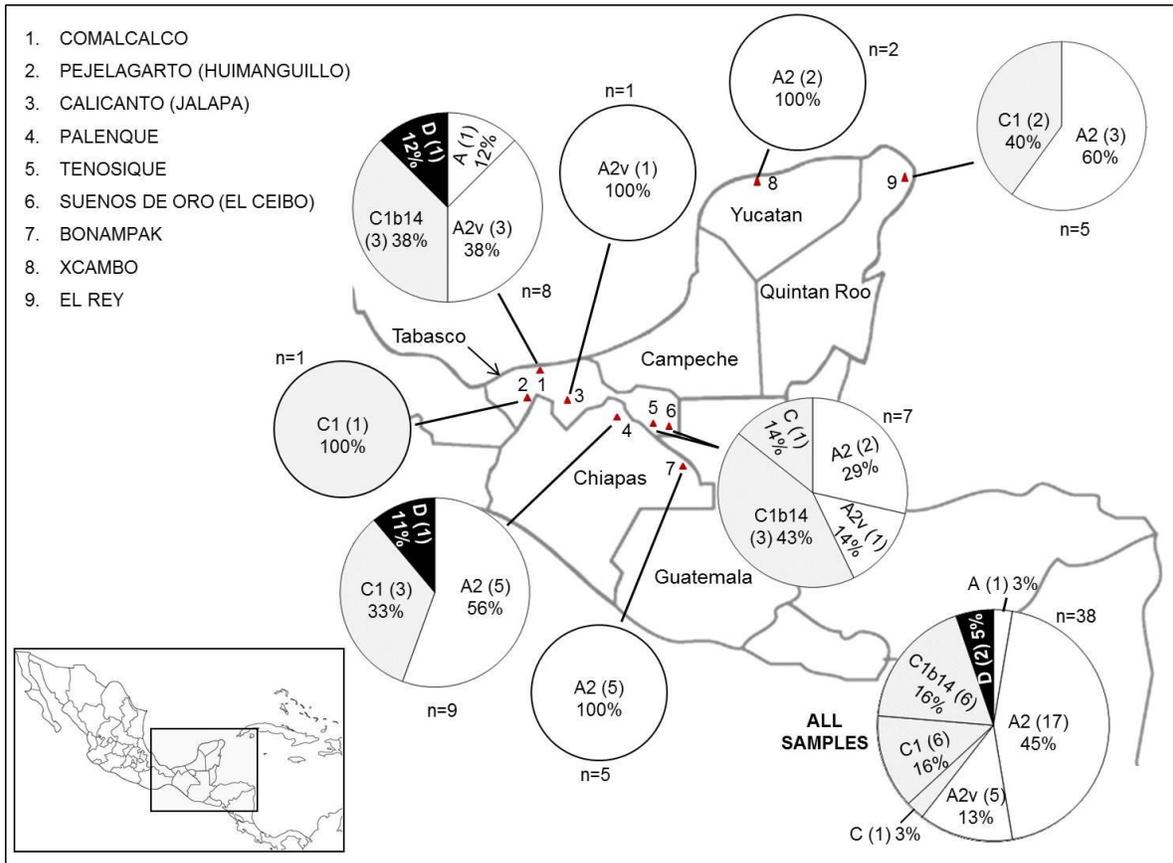
<sup>a</sup>Samples belonging to haplogroups A, B, C, and D.

n: Sample size

<sup>c</sup> Significant P-values < 0.05 (for Tajima's D) and < 0.02 (for Fu's  $F_s$ ).

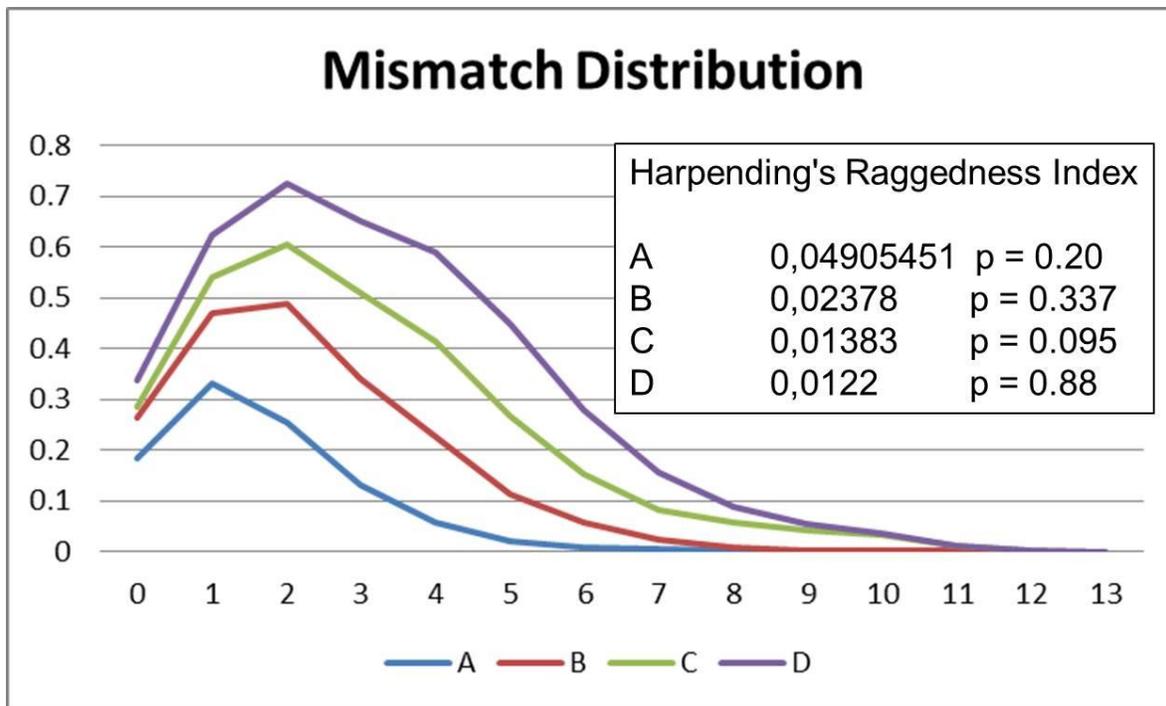
SD: Standard deviation

## Figures



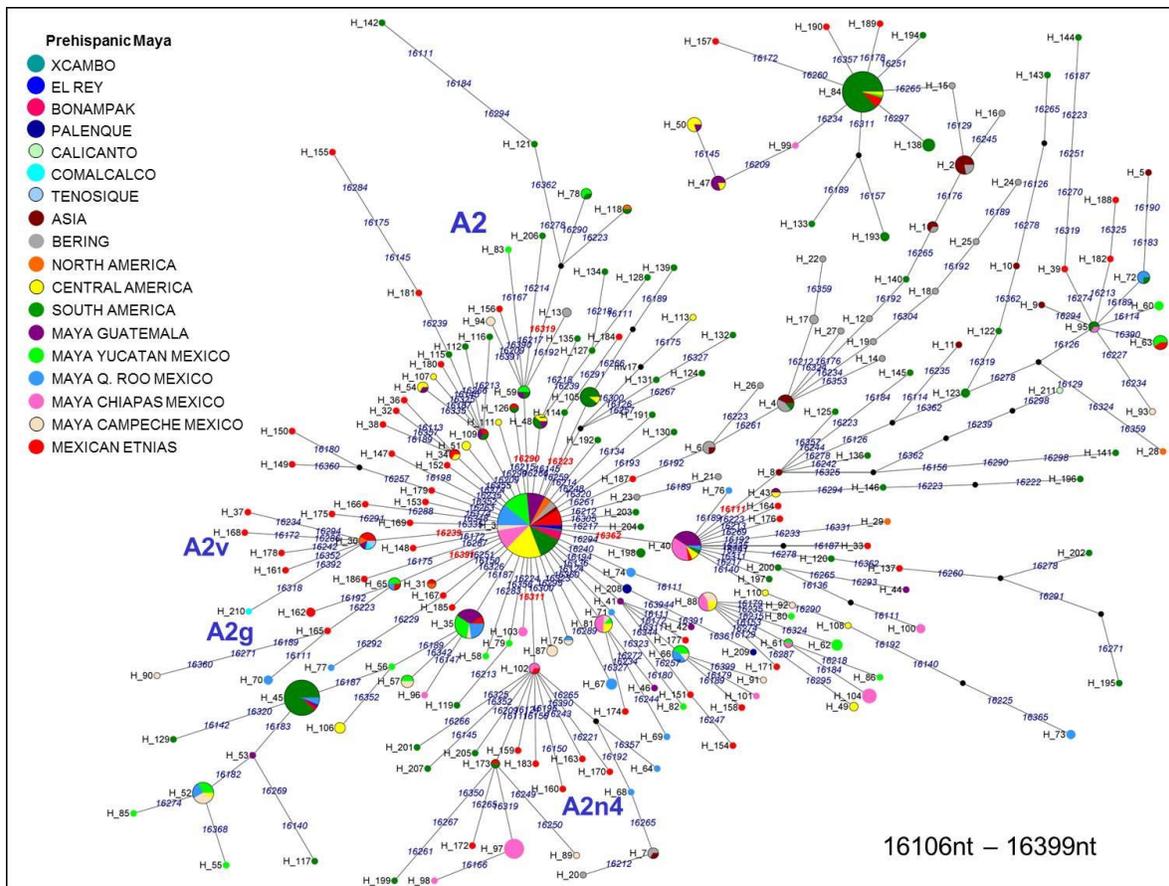
**Figure 1. Overview of the locations of archaeological Maya sites in the Yucatan, Chiapas, and Tabasco.**

The figure shows the locations and origins of the collected pre-Hispanic samples. The numbers in each pie chart section indicate the number and percentage of individuals possessing each haplogroup (pie graph). The percentage of each haplogroup in the 38 samples is also displayed on the right side of the pie chart.



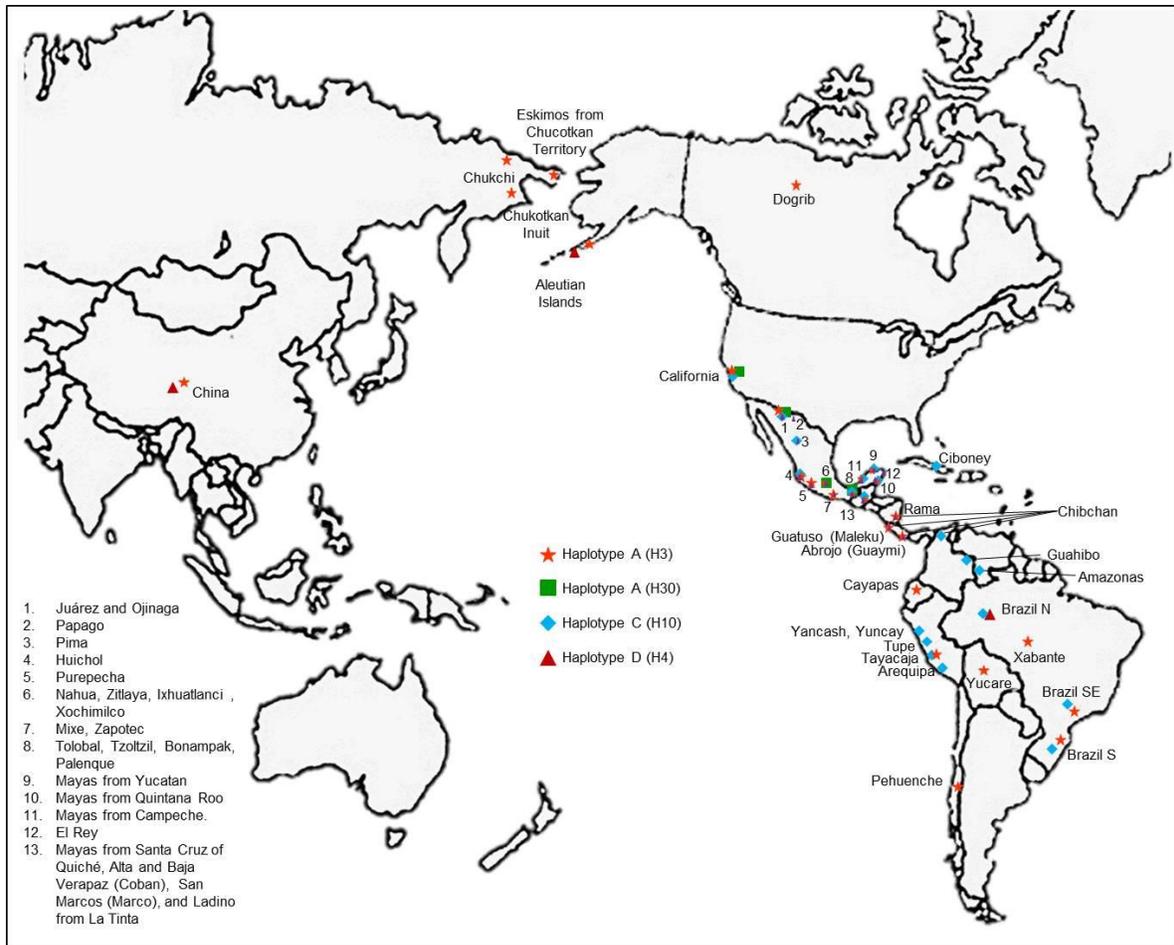
**Figure 2. Mismatch distributions for haplogroups A, B, C, and D with HVS datasets for all populations are shown.**

Demographic history inferred by mismatch distributions of the numbers of pairs of nucleotide differences among individuals within each of mtDNA haplogroups A (blue), B (red), C (green), and D (purple) for the populations from the pre-Hispanic Maya (Yucatan, Chiapas, and Tabasco) and the contemporary Maya (Yucatan, Guatemala, Honduras, and Belize); Asia, Bering Strait Bridge, North America, and South America. The observed distributions were compared using the sudden population expansion model (ARLEQUIN version 3.5.1.2) [Excoffier and Lischer, 2010]. Harpending's Raggedness Index values are also displayed in the figure.



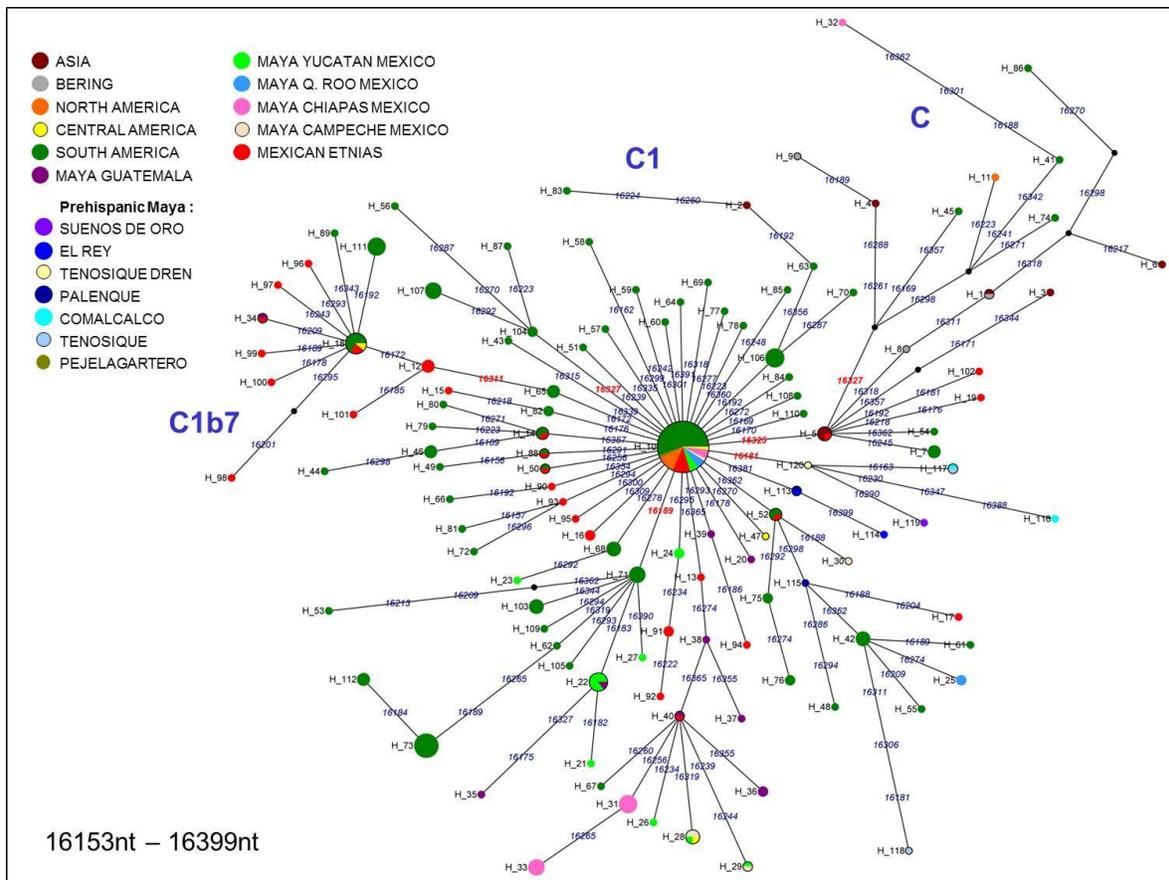
**Figure 3. Haplotype network of mtDNA haplogroup A in the pre-Hispanic Maya, contemporary Maya (Yucatan, Guatemala, Honduras, and Belize), Asia, Bering, North America, and South America.**

A phylogenetic network was constructed with the mtDNA sequences from nucleotides 16106 to 16399 using the Network 4.6.1.1 program. The size of the circle is proportional to the number of individuals in each haplotype present in the dataset (Suppl. Table 1). The distances between the circles correspond to one mutation between haplotypes; otherwise, it is indicated. Black dots on the branches represent inferred missing haplotypes (single nucleotide changes).



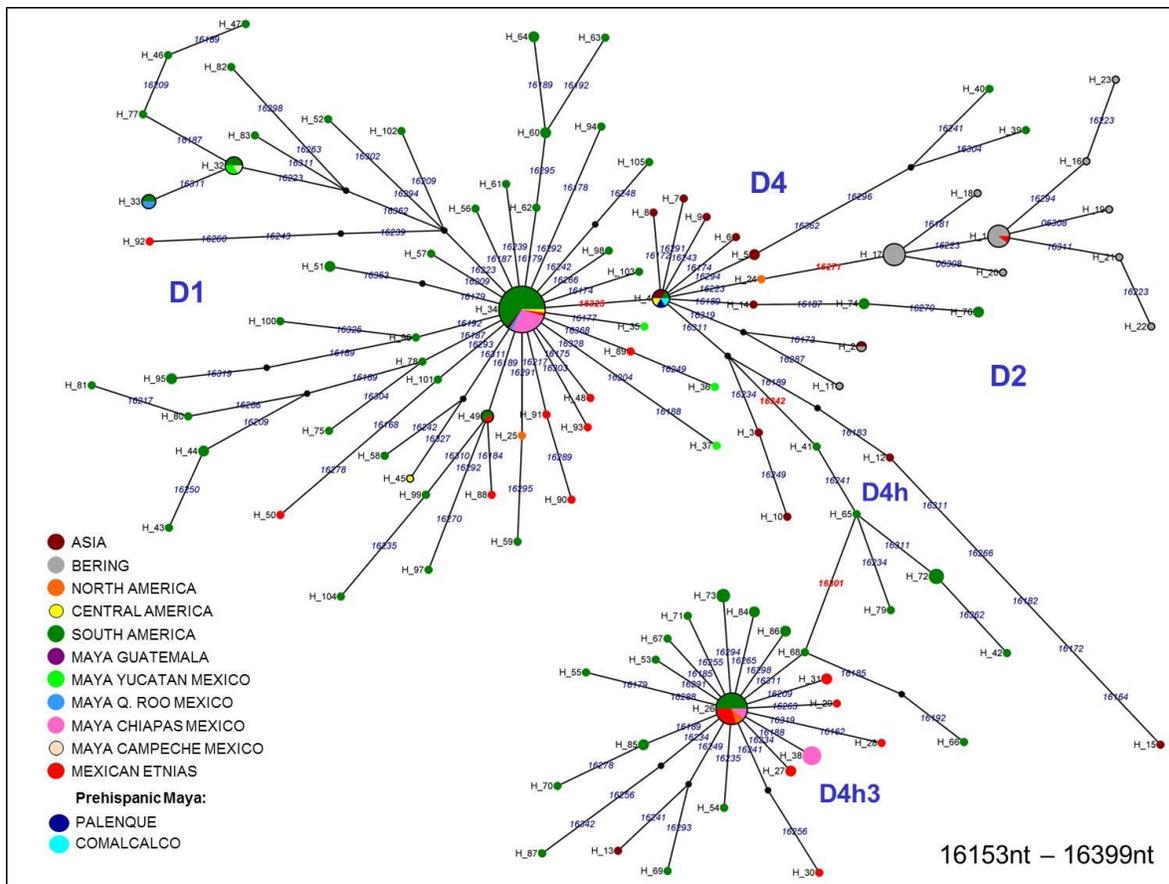
**Figure 4. Geographic distribution of shared mitochondrial DNA haplotypes between the Maya pre-Hispanic populations in this study and those reported previously for contemporary and pre-Hispanic groups (Suppl. Table 1) from Asia, Beringia, and America.**

The figure shows the geographic locations of the shared haplotypes between the pre-Hispanic Maya individuals and the pre-Hispanic and contemporary people from Asia, Beringia and America.



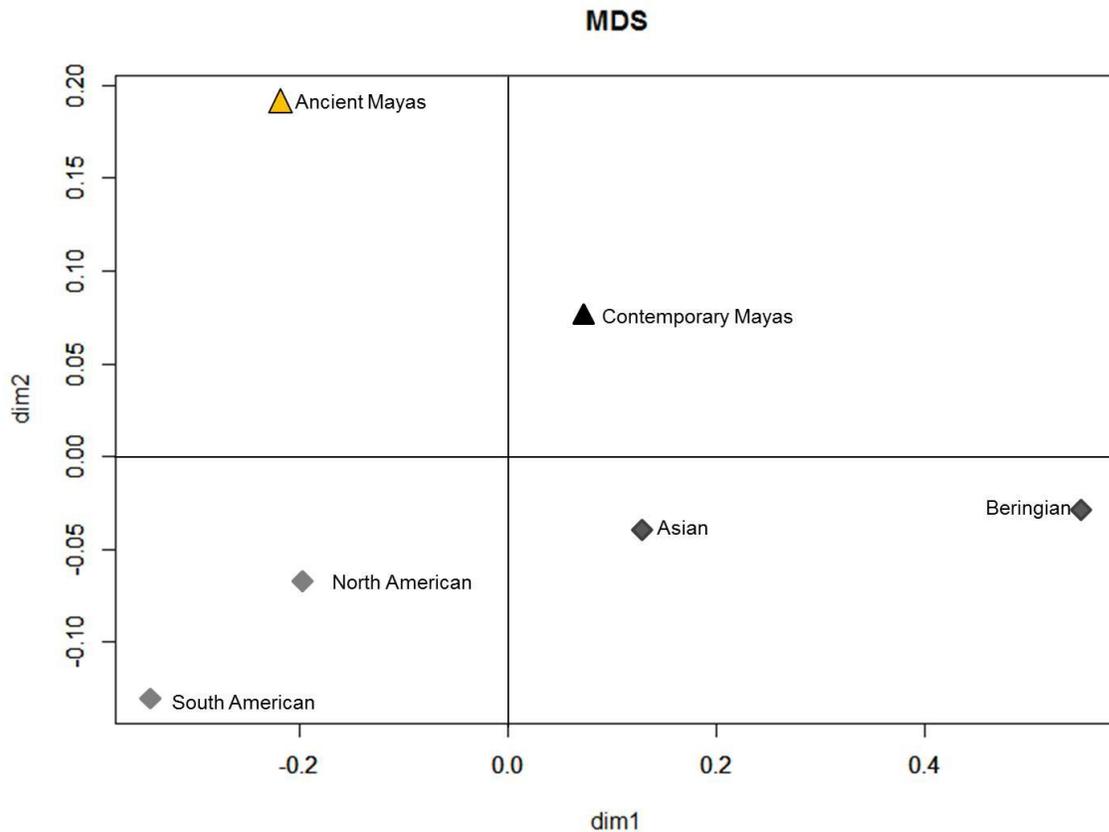
**Figure 5. Haplotype network of mtDNA haplogroup C in the pre-Hispanic Maya, contemporary Maya (Yucatan, Guatemala, Honduras, and Belize), Asia, Bering, North America, and South America.**

A phylogenetic network was constructed using the mtDNA sequences from nucleotides 16153 to 16399 with the Network 4.6.1.1 program. The size of the circle is proportional to the number of individuals in each haplotype present in the dataset (Suppl. Table 1). The distances between the circles correspond to one mutation between the haplotypes; otherwise, it is indicated. Black dots on the branches represent inferred missing haplotypes (single nucleotide changes).



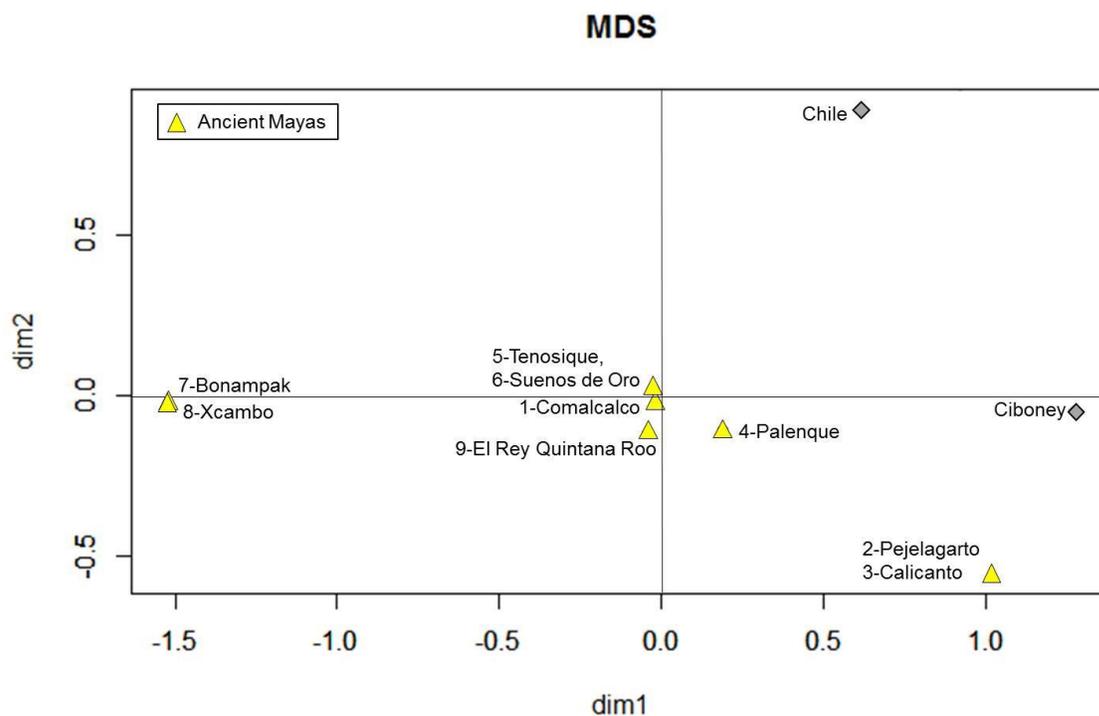
**Figure 6. Haplotype network of mtDNA haplogroup D in the pre-Hispanic Maya, contemporary Maya (Yucatan, Guatemala, Honduras, and Belize), Asia, Bering, North America, and South America.**

A phylogenetic network was constructed with the mtDNA sequences from nucleotides 16153 to 16399 using the Network 4.6.1.1 program. The size of the circle is proportional to the number of individuals in each haplotype present in the dataset (Suppl. Table 1). Distances between the circles correspond to one mutation between the haplotypes; otherwise, it is indicated. Black dots on the branches represent inferred missing haplotypes (single nucleotide changes).

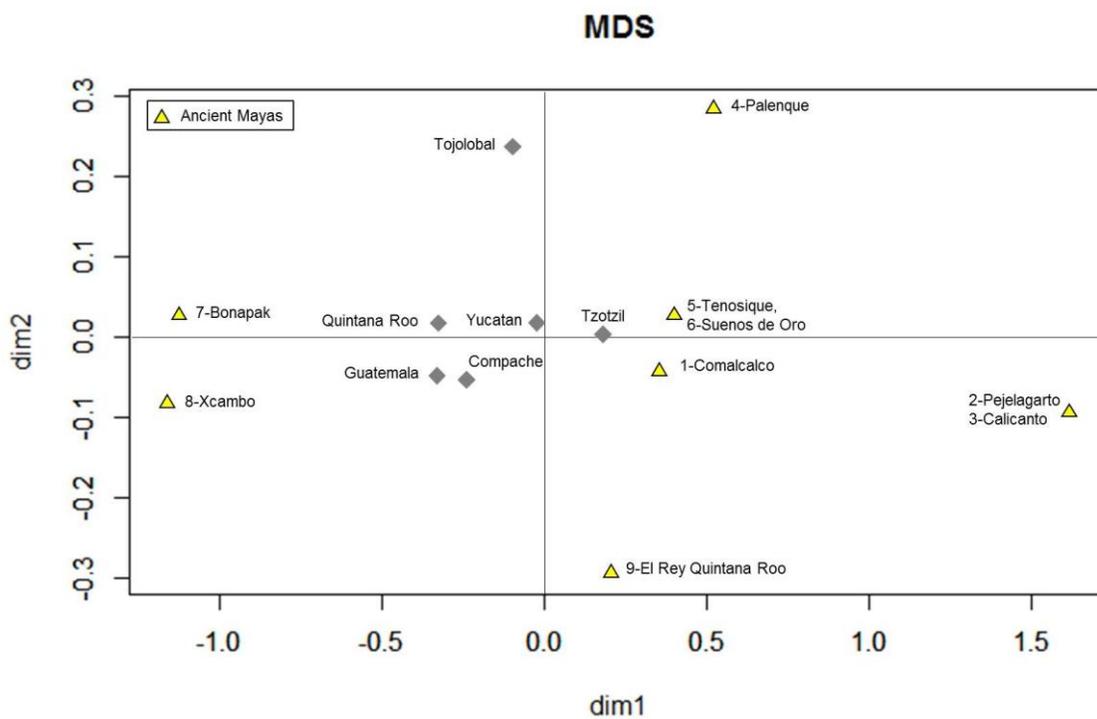


**Figure 7. Multi-dimensional scaling between pre-Hispanic Maya, contemporary Maya (Yucatan, Guatemala, Honduras, and Belize), Asia, Bering, North America, and South America.**

The genetic affinities among the pre-Hispanic Maya, contemporary Maya (Yucatan, Guatemala, Honduras, and Belize) and populations from Asia, Bering, North America, and South America (See Suppl. Table 1) were analyzed by MDS to demonstrate their genetic affinities.

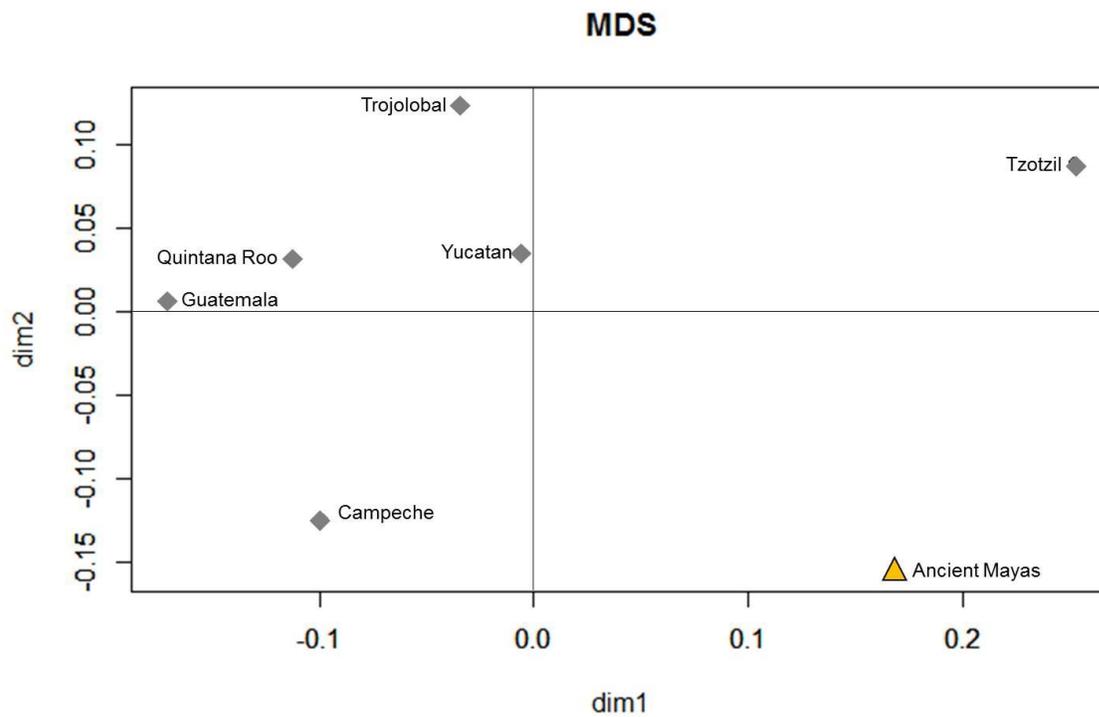


**Figure 8. Multi-dimensional scaling among ancient Maya populations.** The genetic affinities among the pre-Hispanic Maya and ancient populations from Chile and Cuba (See Suppl. Table 1) were analyzed by MDS to demonstrate their genetic affinities.



**Figure 9. Multi-dimensional scaling between the pre-Hispanic Maya and contemporary Maya (Mexico, Guatemala, Honduras, and Belize).**

The genetic affinities between the pre-Hispanic Maya and contemporary Maya (Yucatan, Guatemala, Honduras, and Belize) (See Suppl. Table 1) were analyzed by MDS to demonstrate their genetic affinities.



**Figure 10. Multi-dimensional scaling between all ancient Maya populations as a group and the contemporary Maya populations by city.** The genetic affinities between the pre-Hispanic Maya and Maya from each city (see Suppl. Table 1) were analyzed by MDS to demonstrate their genetic affinities.

## Supplemental data

### Supplemental Figure Legends



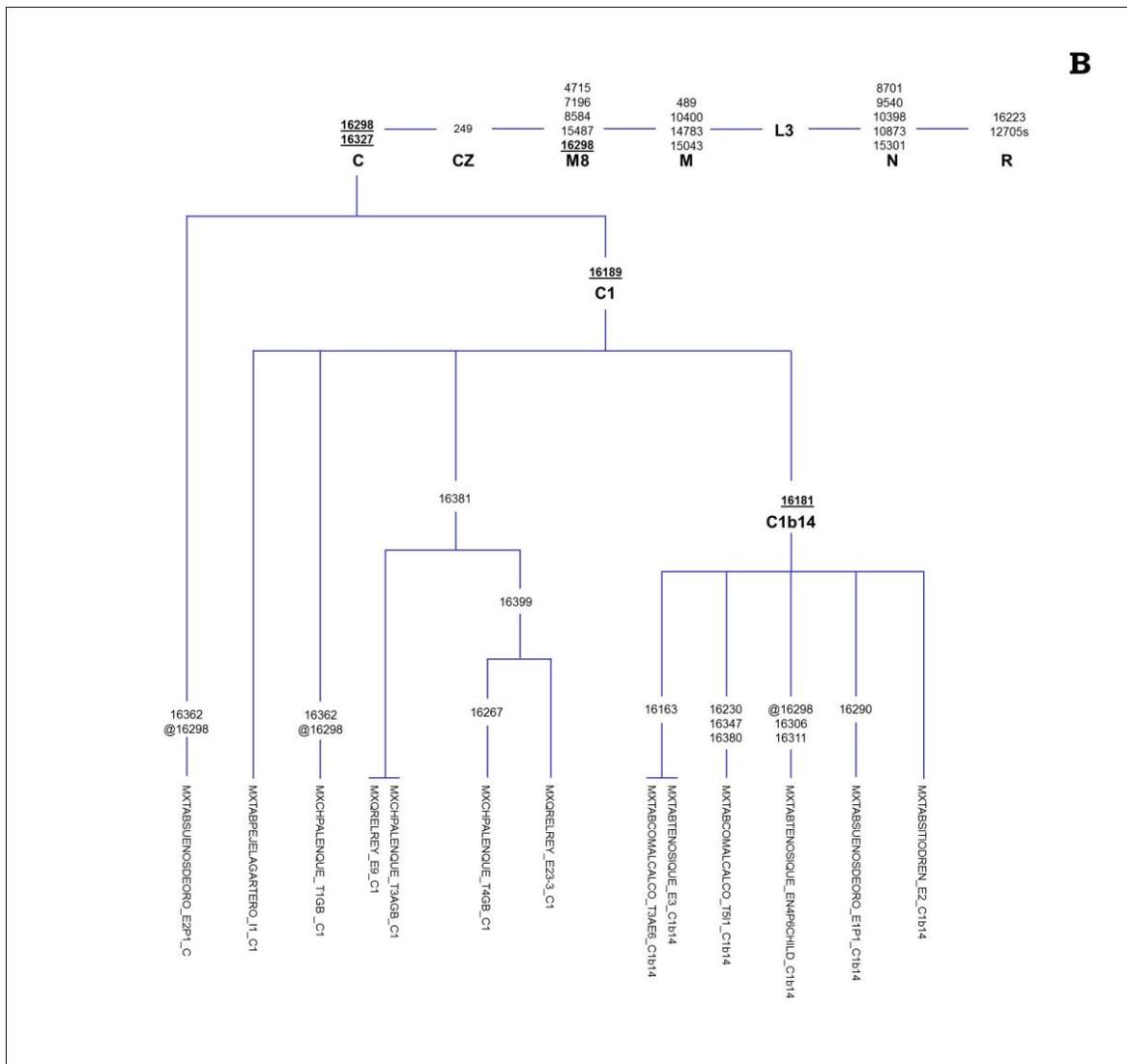
**Figure S1. Geographic locations of the sequences analyzed in this study.**

The figure shows the locations of all samples from ancient and contemporary individuals described in Table 1 and Suppl. Table 1.

**Table S1.** Please visit the following web address to view Supplementary Table S1:

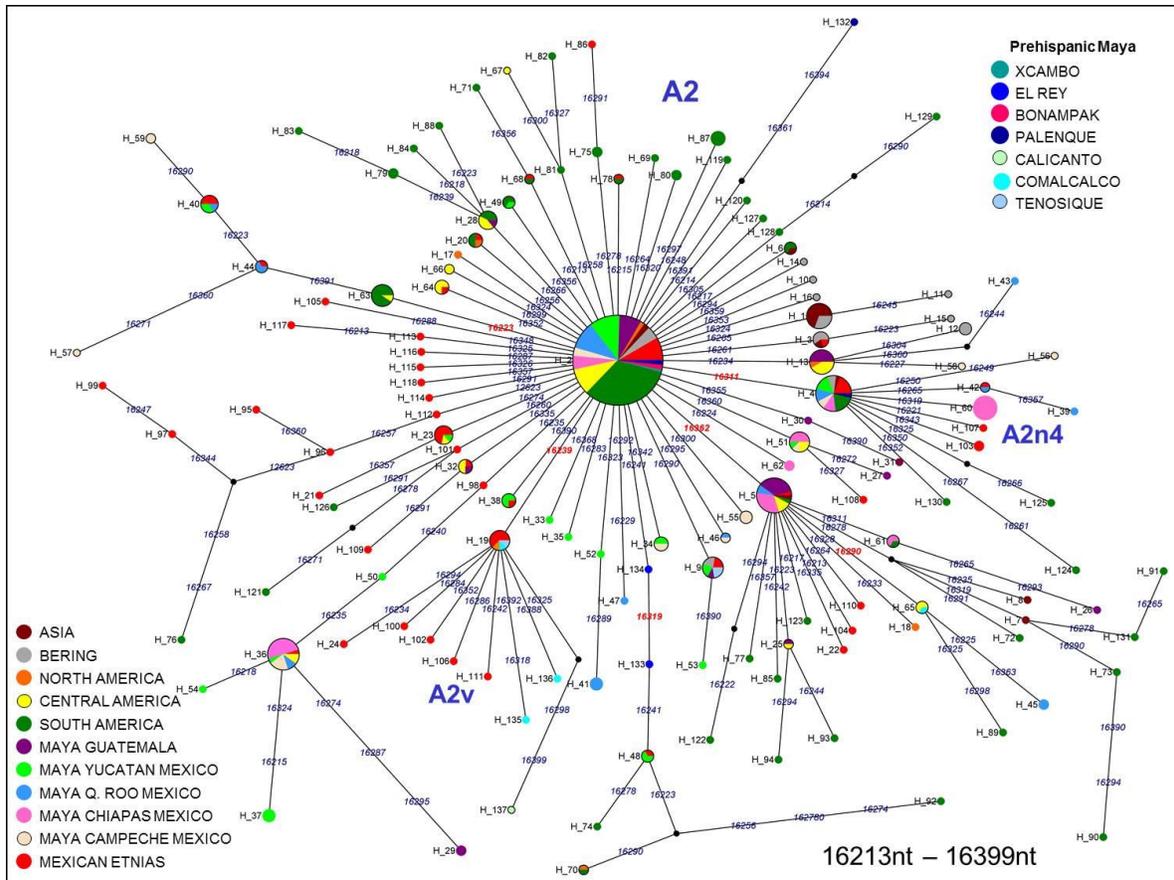
<http://digitalcommons.wayne.edu/cgi/viewcontent.cgi?filename=21&article=2574&context=humbiol&type=additional>.



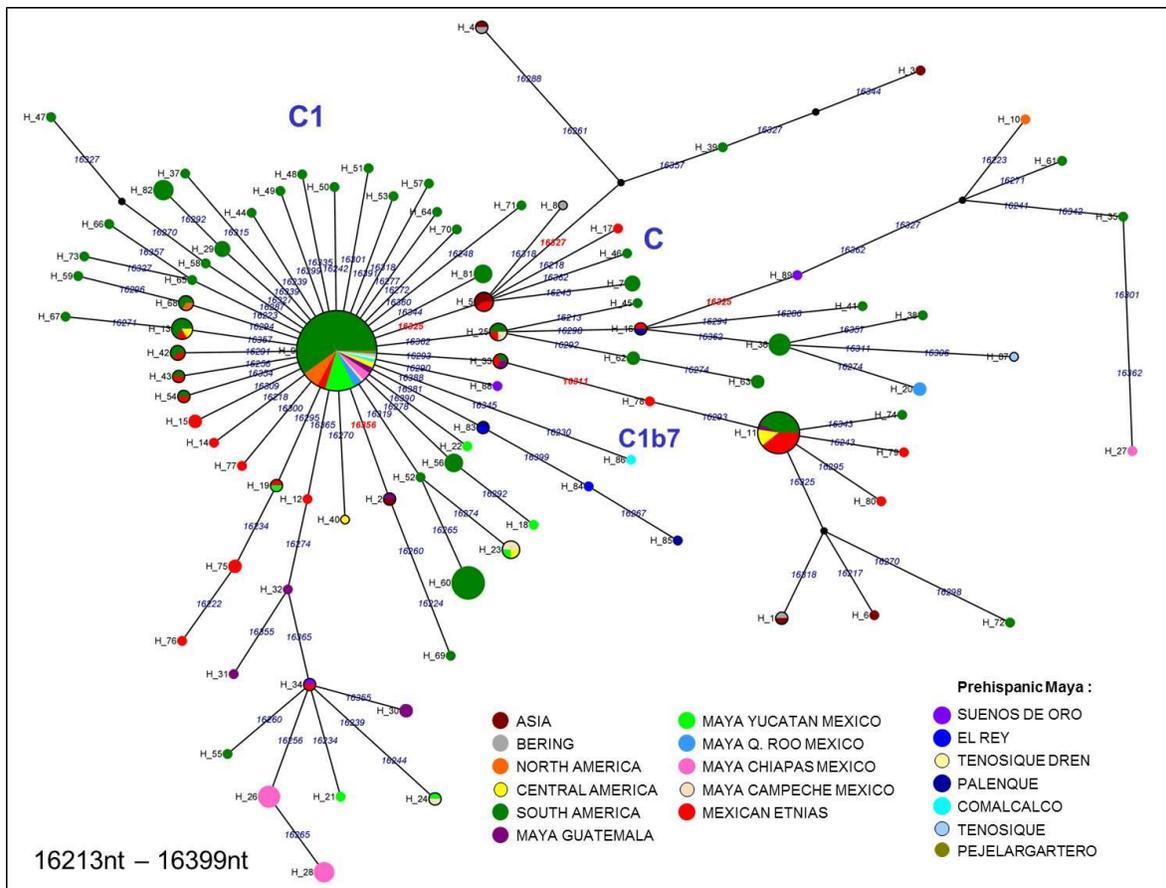


**Figure S2A and S2B. Haplotype network of mtDNA haplogroup A in the pre-Hispanic Maya, contemporary Maya (Yucatan, Guatemala, Honduras, and Belize), Asia, Bering, North America, and South America.**

A phylogenetic network was constructed using the mtDNA sequences from nucleotides 16215 to 16399 using the Network 4.6.1.1 program as described in figure 3.



**Figure S3. Mitochondrial DNA phylogenetic tree of the pre-Hispanic haplogroups A and D (panel A) and C (panel B).** The file contains figures showing the phylogenetic reconstruction of 38 HVS-I mitochondrial DNA sequences belonging to the pre-Hispanic Maya haplogroup clades presented in panel A) haplogroups A and D and panel B) haplogroup C.



**Figure S4. Haplotype network of mtDNA haplogroup C in the pre-Hispanic Maya, contemporary Maya (Yucatan, Guatemala, Honduras, and Belize), Asia, Bering, North America, and South America.**

A phylogenetic network was constructed using the mtDNA sequences from nucleotides 16215 to 16399 with the Network 4.6.1.1 program as described in figure 5.