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## RESEARCH ARTICLE

# Bioanthropological analysis of human remains from the archaic and classic period discovered in Puyil cave, Mexico

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## Abstract

**Objectives:** Determine the geographic place of origin and maternal lineage of prehistoric human skeletal remains discovered in Puyil Cave, Tabasco State, Mexico, located in a region currently populated by Olmec, Zoque and Maya populations.

**Materials and Methods:** All specimens were radiocarbon (<sup>14</sup>C) dated (beta analytic), had dental modifications classified, and had an analysis of 13 homologous reference points conducted to evaluate artificial cranial deformation (ACD). Following DNA purification, hypervariable region I (HVR-1) of the mitogenome was amplified and Sanger sequenced. Finally, Next Generation Sequencing (NGS) was performed for total DNA. Mitochondrial DNA (mtDNA) variants and haplogroups were determined using BioEdit 7.2 and IGV software and confirmed with MITOMASTER and WebHome softwares.

**Results:** Radiocarbon dating (<sup>14</sup>C) demonstrated that the inhabitants of Puyil Cave lived during the Archaic and Classic Periods and displayed tabular oblique and tabular mimetic ACD. These pre-Hispanic remains exhibited five mtDNA lineages: A, A2, C1, C1c and D4. Network analysis revealed a close genetic affinity between pre-Hispanic Puyil Cave inhabitants and contemporary Maya subpopulations from Mexico and Guatemala, as well as individuals from Bolivia, Brazil, the Dominican Republic, and China.

**Conclusions:** Our results elucidate the dispersal of pre-Hispanic Olmec and Maya ancestors and suggest that ACD practices are closely related to Olmec and Maya practices. Additionally, we conclude that ACD has likely been practiced in the region since the Middle-Archaic Period.

## KEYWORDS

ancient DNA (aDNA), artificial cranial deformation (ACD), Mesoamerica, mitochondrial DNA (mtDNA), pre-Hispanic

## 1 | INTRODUCTION

Mesoamerica (central/southern Mexico, Belize, Guatemala, El Salvador, Honduras, Nicaragua, and parts of Costa Rica) was

historically inhabited by the Olmec, Zoque, Maya, Zapotec, Teotihuacan, Toltec, and Aztec peoples (Kirchhoff, 1967). The pre-Hispanic era is divided into four periods: Archaic (10,000–2000 BCE), Pre-Classic (2000 BCE–200 CE), Classic (200–800 CE) and Post-Classic (800–

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1521 CE) (González-Martín et al., 2015; Serrano-Ursúa, 2005). The first peoples with a sedentary, agrarian lifestyle in Mesoamerica were the Olmec, whose archeological remains have been found predominantly along the Gulf Coast of Mexico, and dated to 2000–400 BCE (Campa & Winter, 2009; Childress & Foerster, 2012; Rust & Sharer, 1988). Archeological evidence also suggests that Olmec peoples lived on the Pacific coast of Mesoamerica, in present-day Guatemala and El Salvador, influencing most contemporaneous and succeeding Mesoamerican cultures (Childress & Foerster, 2012). There is both archeological and linguistic evidence that suggests that pre-Hispanic Zoque civilization is related to Olmec civilization, whose people are hypothesized to have dispersed from present-day Veracruz State, migrating to southern Tabasco between 1450 and 1350 BCE (Campbell & Kaufman, 1976; Navarrete, 1970; Pye & Clark, 2006; Terreros-Espinosa, 2006).

Tabasco possesses a great diversity of ecosystems and natural resources, representing a significant advantage for Mesoamerican populations such as the Olmec, Zoque, and Maya, that settled there (Blaha-Pfeiler et al., 2014; Wichmann et al., 2017). Tabasco's geological and climatological conditions fostered the formation of caves, which were commonly used for ceremonial purposes by pre-Hispanic cultures. Caves represented an important cosmogonic concept of the world, since they were often considered the location of life's origins or the entrance to the “underworld” (Brickley, 2018; Taube, 1986; Walker et al., 2009). In addition to Zoque culture, the Maya controlled a sprawling empire in Mesoamerica, extending, at its height, from southeastern Mexico (the states of Campeche, Chiapas, Quintana Roo, Tabasco, and Yucatán), to present-day Belize, Guatemala, Honduras and El Salvador (250–1521 CE) (Law, 2013; Muñoz et al., 2012).

The practice of artificial cranial deformation (ACD) to demonstrate membership in a given social class, ethnic affiliation, or for aesthetic purposes, has been widely practiced throughout human prehistory (Anton, 1989; Brown, 1981). In Mesoamerica in particular, the Olmec, Zoque and Maya populations (Tiesler, 2013) used this practice to indicate social status within a strict hierarchical system, sometimes accompanied by dental mutilations (Bautista-Martínez, 2010; Monte de la Paz & Linares-Villanueva, 2015), such as filings and inlays (Fastlicht, 1962). Specific types of cranial deformations were sometimes preferred, such as the annular and tabular forms (includes the formal variants tabular erect and tabular oblique) (Cottin et al., 2017). Cranial deformations were also commonly practiced throughout the prehistoric Andes (Allison et al., 1981; Torres-Rouff, 2020). The earliest cases of ACD were described by Allison et al. (1981) in the Huacho culture from Peru and the Azapa Culture from Arica, northern Chile, with radiocarbon dates of approximately 5000 BCE and 3000 BCE, respectively. Studied specimens were found to possess tabular erect and tabular oblique ACDs, depending on region (Childress & Foerster, 2012; Clark et al., 2007; Gerszten, 1993; Schijman, 2005).

Cranial deformations classified as annular and tabular (which can be tabular erect or tabular oblique) shifted through time and were largely dependent on ethnic origin (Cottin et al., 2017). Deformations can be more or less “perfect,” that is, symmetrical, as associated with one's social class (Bautista-Martínez, 2010). This “perfecting” practice

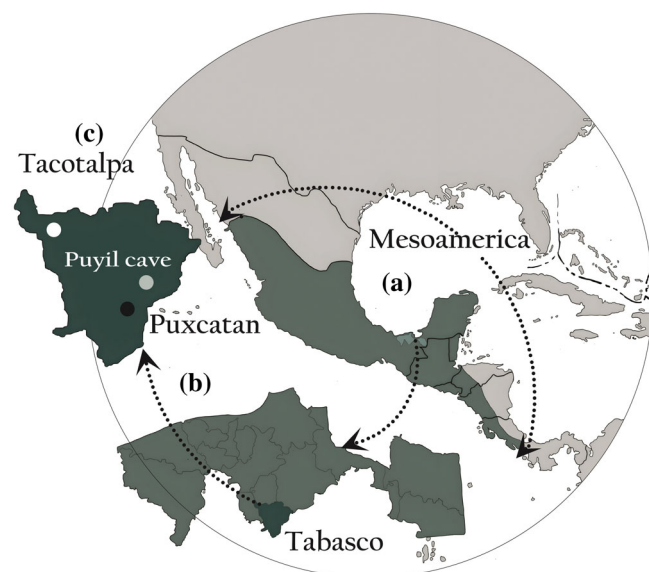
most likely began at approximately 2000 BCE, and first included simple forms of dental filing, by the end of the first millennium, becoming rather elaborate. In North America, modified teeth have been found in Indigenous American cultures in the present-day U.S. states of Arizona, Tennessee, California, and Georgia, as well as in the Caribbean (Lodder, 2008). Both male and female dental samples from Texas dated to 8000–1000 BCE display modifications, including a series of notches ground from the tooth's biting edge (Lodder, 2008). Different ACD's have been associated with specific populations, cultural practices, and periods. ACD's provide a strong affiliation of an individual with a group (Torres-Rouff & Yablonsky, 2005).

Mitochondrial DNA (mtDNA) maternal inheritance and lack of recombination have offered the opportunity to explore genealogical relationships among individuals, and to study the frequency of matrilineal clades among human populations at both continental and regional scales. Studies on mtDNA continue to be informative as a unique tool for the assessment of female-specific aspects of the demographic history of human populations.

Modern Indigenous American populations trace their gene pool to Asian groups who colonized northeastern Siberia, including parts of Beringia, prior to the last glacial period (Farmer et al., 2023). Haplotypes of Asian ancestry were differentially preserved, and some may have been lost in Beringian enclaves. Nevertheless, novel haplotypes and alleles arose in situ in Beringia due to new hereditary mutations, which gave rise to major founder mtDNA types (Achilli et al., 2008; Fagundes et al., 2008; Perego et al., 2009; Schroeder et al., 2009; Tamm et al., 2007). The northern Asian lineages differentiated into mtDNA haplogroups A, B, C and D, and most likely diversified before the Last Glacial Maximum (LGM) in Siberia (Perego et al., 2010), giving rise to the indigenous mtDNA founder haplogroups of A2, B2, C1b, C1c, C1d, C4c, D1, and D4h3a.

In addition, mtDNA lineages D2a, D3 and X2a were restricted to northern North America, where they occur at lower frequencies (Achilli et al., 2008; Hooshiar Kashani et al., 2012; Kemp et al., 2007; Malhi et al., 2010; Perego et al., 2009; Tamm et al., 2007). MtDNA haplogroup A2, found at elevated frequency in the Americas, is also found in northeastern Asia, particularly Siberia, where it is at its greatest diversity, indicating the spread of people across Beringia into the Americas (O'Rourke & Raff, 2010; Volodko et al., 2008). All major Indigenous American mtDNA haplogroups can be traced back to Siberia/Northeastern Asia, as related lineages are also present among human population inhabiting these areas (Derenko et al., 2010; Starikovskaya et al., 2005). Early studies of mtDNA haplogroups in pre-Hispanic Maya populations using restriction enzymes identified haplogroups A and C at relatively elevated frequencies, and haplogroups B and D at relatively lower frequencies (González-Oliver et al., 2001). Recently, mtDNA haplogroups A, A2, A2v, C, C1, C1b14, and D were identified in pre-Hispanic Maya individuals from Palenque, Chiapas, and Comalcalco and Sueños de Oro, Tabasco (Muñoz-Moreno et al., 2021; Ochoa-Lugo & Muñoz, 2016).

In 2007, human skeletal specimens were discovered in a cave (Puyil Cave) located at San Felipe Mountain in the town of Puxcatán, in the municipality of Tacotalpa in southern Tabasco State, Mexico,



**FIGURE 1** Location of Puyil Cave in Mesoamerica. (a) The Mesoamerican region encompasses the meridional two-thirds of Mexico, as well as Guatemala, El Salvador, Belize, western Honduras, Nicaragua and northern Costa Rica (all in forest green). (b) Puyil Cave is located outside of Puxcatán village, in the municipality of Tacotalpa, Tabasco State, Mexico. (c) The location of Puyil Cave is represented with a gray dot, and the village of Puxcatán is represented with a black dot.

located in a geographic region currently populated by indigenous Zoque and Maya peoples (Alcalá-Castañeda, 2021). There are limited studies about these skeletons (Navarro-Romero et al., 2020; Navarro-Romero, et al., 2021) and it is known that other Tabasco caves were used by Zoque and Maya populations for ceremonial purposes (Monte de la Paz & Linares-Villanueva, 2015). To assess maternal ancestry, pattern of human cave dwelling, and the possibility of a shared genetic lineage with ancient and contemporary Mexican populations, mtDNA haplotype assignment, network analysis, craniometry, and carbon ( $^{14}\text{C}$ ) dating methodologies were employed, with findings analyzed through a demographic lens. In addition, radiocarbon ( $^{14}\text{C}$ ) dating was performed to determine if one or multiple populations historically occupied the Puyil Cave in Tabasco.

## 2 | MATERIALS AND METHODS

### 2.1 | Description of collection site

In 2007, human skeletal remains were discovered in a cave located in the municipality of Tacotalpa in southern Tabasco State, Mexico (Figure 1) ( $17^{\circ}27'38.04''$  N and  $92^{\circ}39'28.50''$  W). The geomorphological features of the Puyil Cave include calcareous stalactites and stalagmites, including those still in the process of formation, abundant sediments on the floor, and high humidity (Figure 1a\_Supp). Puyil Cave is divided into six chambers according to the conformation of the vault (Figure 2), with each chamber containing various subdivisions based on internal structures. To enter the first chamber

(dimensions:  $30\text{ m} \times 20\text{ m} \times 15\text{ m}$ ), which possesses extensive stalactite and stalagmite formation, it is necessary to descend  $\sim 10\text{ m}$  down a steep ramp (Figure 1b\_Supp). Notably, the second chamber (7 m in height) contains a cylindrical calcareous structure  $\sim 7\text{ m}$  in height (Figure 1c\_Supp). This chamber was most likely the one most commonly used for ritual activities by contemporary inhabitants, revealed by the presence of modern candle remnants. Chamber three is the first area of the cave to possess human bone fragments, though its physical description was not available at the time of this study.

Chamber four is divided into three stepped sections, the first containing highly deteriorated human bone fragments on the floor. The first and the second section both contained bone fragments inside small cavities formed by the collapse of large calcareous structures. Following a narrow corridor there is a descent of  $\sim 2\text{ m}$  to reach the third section, where four of the skulls of this study (Figure 1d\_Supp) were discovered on the surface of a  $3.5\text{ m} \times 2\text{ m}$  stone. On the floor of section three, in a westerly direction, there is a small lateral cavity of  $0.5\text{ m} \times 0.3\text{ m} \times 1.3\text{ m}$  length in the cave, which leads to a minor chamber ( $5\text{ m} \times 1.2\text{ m} \times 0.8\text{ m}$ ) that descends to the bottom of chamber five. Chamber five has the following dimensions:  $1.5\text{ m}$  length  $\times 7\text{ m}$  base, with a height that ranges from  $3.5$  to  $0.40\text{ m}$ , where it reaches chamber six. Chamber five contained bone fragments of skeleton PUXTABMEX003 (Figure 2). Chamber six has dimensions of:  $9\text{ m}$  length  $\times 3.5\text{ m}$  height, and is oriented roughly southeast to northwest, and is divided into sections one through three (Figure 2). This chamber contained individuals PUXTABMEX001 and PUXTABMEX002 (Figure 2, Table 1). Individuals PUXTABMEX001, PUXTABMEX002, PUXTABMEX003 and PUXTABMEX005 possessed no skulls. Individuals PUXTABMEX006, PUXTABMEX007, PUXTABMEX008, PUXTABMEX009, and PUXTABMEX010 corresponded to skulls with ACD. Individual PUXTABMEX004 only possessed a skull fragment (Figure 2, Table 1, and Figure 1d\_Supp).

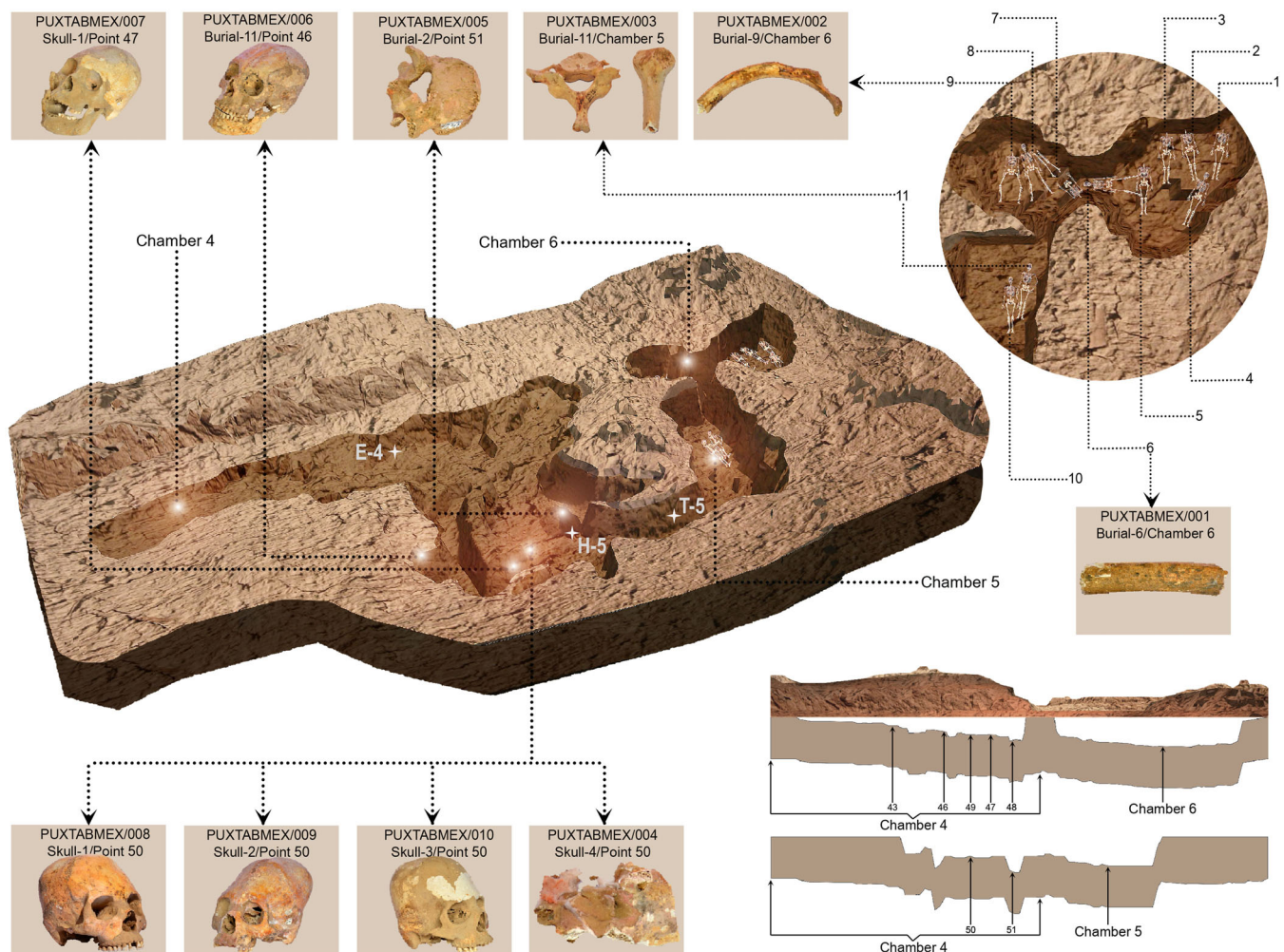
### 2.2 | Paleontology

Five human skulls and five bone fragments were collected from chambers four, five, and six (Figures 1 and 2, and Table 1). The human specimens were recovered in 2007 by a field archaeology group led by Luis Alberto Martos-López. Due to climactic and geological conditions, the archeological team did not assess ethnic origin in situ, or time of occupation by stratigraphy. Previously, sex of ten study samples were assessed by Navarro-Romero et al. (2020) using three different methods: Anthropometrics, an AMEL X / AMEL Y test, and a genetic methodology using specific amplicons of the *TTY7*, *TSPY3*, and *TTY2* genes of the human Y chromosome.

### 2.3 | Radiocarbon ( $^{14}\text{C}$ ) dating

The radiocarbon dating of seven pre-Hispanic human bone specimens was performed by Beta Analytic Testing Laboratory (Miami, FL). The process begins with the extraction of the preserved collagen proteins.





**FIGURE 2** Specimen distribution in Puyil Cave. Internal distribution of 10 bone specimens discovered collectively in chambers four, five and six of Puyil Cave and analyzed in this study. Skeletons in chambers five and six were numbered from 1 to 11 as displayed on the right side of the figure. Samples of skeletons 6 (PUXTABMEX001) and 11 (PUXTABMEX003) with crania, and 9 (PUXTABMEX00002) with no crania, were included in this study. The distribution of the 5 specimens PUXTABMEX005–PUXTABMEX010 (all in chamber four) are shown by arrows. E-4: Entrance to chamber 4; H-5: Hollow space; T-5: Tunnel leading to chamber 5. Features of all samples are displayed in Table 1.

This is the most reliable material that can be dated for non-cremated human bones. If collagen quality is satisfactory, pretreatment continues with acid-alkali-acid washes. After the initial evaluation of the collagen, it is dried for  $^{13}\text{C}/^{12}\text{C}$  ratio analysis. If the result of this analysis is satisfactory, the laboratory proceeds with accelerator mass spectrometry (AMS) dating (Linick et al., 1989).

## 2.4 | Age at death determination

Age at death was estimated using osteological features of the unearthed skeletons within the context of established forensic methodology (Zioupou et al., 2013). Age estimation was relatively straightforward and accurate ( $\pm 3$  years), using physical characteristics measured in situ, at a macroscopic level (wet & dry apparent density, porosity, organic/mineral/water fractions, collagen thermal degradation properties, and ash content).

## 2.5 | Craniometric analysis

Measurements of five skulls with noted ACD were analyzed to quantify osteological traits, assess intragroup relationships (variance of structures), and determine type of intentional cephalic modeling (Brewster et al., 2014; Dudzik & Kolatorowicz, 2016; Zaki et al., 2012). Thirteen landmarks homologous to traditional craniometric points that cover the main components of the skull (facial region, neurocranium, and base) were used (Langley et al., 2016; Romano-Pacheco, 1965; Romano-Pacheco, 2005; Serrano-Sánchez et al., 2021). This cranial evaluation system utilizes trigonometry, and allows us to glean, crania data at an individual level, including the main morphological characteristics of each skull, the intercranial relationships (covariance of structures) and the type of cephalic modeling. With the collected measurements, we performed a comparative analysis. Human skull shape was characterized using three-dimensional coordinates from the anatomical landmark coordinates acquired using a MicroScribe G2X digitizer (Reeware, Inc., Raleigh, NC).

**TABLE 1** Features of human remains found at Puyil Cave.

| Laboratory ID             | Sample code        | Chamber number | GenBank number | Ancient material    | Sample for DNA studies | Age at death | H   | Pathology                     |
|---------------------------|--------------------|----------------|----------------|---------------------|------------------------|--------------|-----|-------------------------------|
| PUXTABMEX001              | Burial-6/Cham6     | 6              | MK483648       | Skeleton            | Femur                  | 20–25        | C1  | None                          |
| PUXTABMEX002              | Burial-9/Cham6     | 6              | MK483649       | Skeleton (No Skull) | Left Rib               | 20–25        | C1c | None                          |
| PUXTABMEX003              | Burial-11/Cham5    | 5              | MK483657       | Skeleton            | Cer5 or Cer6 Vertebrae | 25–30        | A2  | None                          |
| PUXTABMEX004              | Skull-4/Point 50   | 4              | MK483650       | Skull Fragment      | Temporal Cranium       | 5–10         | C1  | None                          |
| PUXTABMEX005              | Burial-2/Point 51  | 4              | MK483651       | Skeleton            | L2 or L3 Vertebrae     | 15–20        | A2  | None                          |
| PUXTABMEX006 <sup>a</sup> | Burial-11/Point 46 | 4              | MK483652       | Skull               | Third Molar            | 25–30        | A   | Porotic Hyperostosis          |
| PUXTABMEX007              | Skull-1/Point 47   | 4              | MK483653       | Skull               | Mastoid Bone           | 25–30        | A2  | Occipitalization of the Atlas |
| PUXTABMEX008              | Skull-1/Point 50   | 4              | ND             | Skull               | Mastoid Bone           | 25–30        | ND  | Dental Caries                 |
| PUXTABMEX009              | Skull-2/Point 50   | 4              | MK483655       | Skull               | Mastoid Bone           | 25–30        | D4  | Porotic Hyperostosis          |
| PUXTABMEX010              | Skull-3/Point 50   | 4              | MK483656       | Skull               | Mastoid Bone           | 20–25        | A2  | None                          |

Note: A comprehensive accounting of the descriptors and categorizations of all study individuals/specimens.

Abbreviations: Cer, Cervical; H, Haplogroup; L, Lumbar; ND, not determined.

<sup>a</sup>The maxillary central incisors displayed two shallow occlusal grooves corresponding to the Romero type A-2 classification (Romero, 1958).

To illustrate skull differences between each ACD type, principal component analysis (PCA) was performed using Rhinoceros 5.0 software, employing the linear and angular metric information from simple transformations of the geometry of NURBS (Nonuniform rational B-splines), a mathematical model used in computer graphics (<https://www.rhino3d.com/nurbs>). The criteria considered for the layout of cranial polygons as well as the protocol for measurements were limited to the recommended strategies of deformed skulls in Romano-Pacheco, 1965 and for the characterization of normal cranial variation, according to Romano-Pacheco, 2005. Subsequently, the principal component analysis (PCA) was performed, as aforementioned. This multivariate mathematical approach was used for the purpose of reducing the dimensionality of variables and jointly analyzing changes in cranial morphology, allowing for the analysis of similarities between individuals in a multivariate manner.

## 2.6 | Classification of dental modifications

Romero (1958) examined a collection of 1212 modified teeth from the *Instituto Nacional de Antropología e Historia* in Mexico, and grouped them into seven basic “types,” lettered A through G. Each type was then divided further, resulting in a total of 59 “classifications” (Havill et al., 2006; Romero, 1958). The dental filing clearly observed only in individual PUXTABMEX006 was classified according to Romero’s classification system (1958).

## 2.7 | Specimen pretreatment

To eliminate exogenous DNA and other contaminants from the surface, each sample was rinsed and incubated for 5 min with 10%

sodium hypochlorite (bleach). The supernatant was then discarded, and the pellet was rinsed with distilled water twice, followed by incubation in distilled water for 5 min. After rinsing one more time with distilled water, the sample was incubated at 37°C overnight. All wet laboratory procedures, including DNA extraction and purification, sequence amplification via polymerase chain reaction (PCR), and preparation of libraries, were performed in an ultra-clean ancient DNA (aDNA) laboratory, with separate pre-PCR and post-PCR facilities, according to standard handling procedures (Adler et al., 2011; Campos et al., 2012). The interior of the facility and laboratory tools were routinely cleaned with bleach and ultraviolet (UV) radiation.

## 2.8 | Ancient DNA extraction and purification

Bone samples were ground into powder and total genomic DNA was extracted. The following procedure was performed in the Department of Genetics and Molecular Biology, *Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional*, Mexico City, Mexico: Twenty mg of ground bone was incubated with extraction buffer according to the Prepito DNA Tissue 10 Kit (Perkin Elmer, Waltham, MA) protocol using Magnetic Bead Technology (Perkin Elmer). Finally, DNA was eluted in 50 µL TE buffer and stored at –70°C until use.

The following procedure was performed in the Institute of Clinical Molecular Biology, Kiel University, Kiel, Germany: Fifty mg of ground bone was incubated with 500 µL of EDTA (0.5 M, pH 8) and 20 µL proteinase K (0.25 mg/mL) under gentle rotation at 37°C overnight, as described by Lee et al. (2012). The solution was then centrifuged for 3 min at 6000 RPM, and 200 µL of the supernatant was used for DNA extraction with the High Pure Viral Nucleic Acid Kit (Roche,

Basel, Switzerland). Finally, DNA was eluted in 50 µL TE buffer and stored at  $-70^{\circ}\text{C}$  until use.

## 2.9 | PCR amplification and HVR-I sequencing

Hypervariable region I (HVR-I) of the control region (nucleotide positions 16,190–16,401, size: 211 bp) was performed with the primer pair F\_16190 (5′-CCCCATGCTTACAAGCAAGT-3′) and R\_16,401 (5′-TGATTTCACGGAGGATGGTG-3′), previously designed by Gabriel et al. (2001) and Wilson et al. (1995), respectively. Platinum Taq DNA Polymerase High Fidelity Invitrogen created through a mixture of Platinum Taq DNA Polymerase and the proofreading (3′ → 5′ exonuclease activity) enzyme *Pyrococcus* species GB-D (ThermoFisher Scientific, Carthage, MO) was used for amplification, according to the following conditions:  $98^{\circ}\text{C}$  for 30 s, 35 cycles at  $98^{\circ}\text{C}$  for 10 s,  $60^{\circ}\text{C}$  for 30 s,  $72^{\circ}\text{C}$  for 20 s, then  $72^{\circ}\text{C}$  for 10 min.

The amplicon was sequenced (Sanger et al., 1977; Sanger & Coulson, 1975) using the BigDye Terminator v3.1 Cycle Sequencing Kit on an Applied Biosystems 3130/3130xl DNA Analyzer (ThermoFisher Scientific, Carthage, MO) using the primers F\_16190 and R\_16,401. Mitochondrial HVR-I sequences for all personnel in contact with the specimens during the archeological and laboratory phases were obtained as a control against contemporaneous DNA contamination. One negative control (molecular grade water) was run during the entire process, in both laboratories. None of the samples sequences from the personnel in contact with the specimens during the archeological and laboratory phases were equal to sample sequences of this study.

## 2.10 | Evaluation of aDNA quality

Before library preparation, quality control of aDNA was performed by amplifying hypervariable region II (HVR-II) (nucleotide positions 120–287; 167 bp) (Figure 2a\_Supp) and HVR-I (nucleotide positions 16,106–16,256; 150 bp) fragments using the following two primer sets: (1) F\_00120 (5′-CGCAGTATCTGTCTTTGATT-3′) and R\_00287 (5′-TTGTTATGATGTCTGTGTGG-3′), (2) F\_16106 (5′-GCCAGCCACCATGAATATTGT-3′) and R\_16256 (5′-GCTTTGGAGTTGCAGTTGATGTGT-3′), as previously designed by Lee et al. (2009). AccuPrime Pfx DNA polymerase (Thermo Fisher Scientific) was used for amplification according to the manufacturer's suggested protocol. The PCR reaction proceeded as follows: incubation at  $94^{\circ}\text{C}$  for 10 min, 42 cycles of  $94^{\circ}\text{C}$  for 30 s,  $60^{\circ}\text{C}$  for 30 s,  $72^{\circ}\text{C}$  for 30 s, then  $72^{\circ}\text{C}$  for 10 min.

## 2.11 | Library preparation

For genome-wide enrichment, additional libraries were prepared, that is, 30–50 µL aliquots of all DNA extracts were taken, according to the methods described by Meyer and Kircher (2010) and Sawyer et al. (2012), with one modification: All extracts and controls were treated with uracil-DNA glycosylase (UDG) and endonuclease VIII to avoid

potential sequencing artifacts caused by the characteristic aDNA damage profile produced by the deamination of cytosine to uracil over time (Briggs et al., 2010). Specifically, DNA libraries were prepared with DNA both untreated and treated with UDG and endonuclease VIII to remove cytosine deamination artifacts (Meyer & Kircher, 2010; Sawyer et al., 2012). Assays were performed in 50 µL final volumes, containing: 20 µL of aDNA extract, 1X NEB Buffer 2 (New England Biolabs, Ipswich, MA), 0.3 mM dNTPs, 0.25 mg/mL BSA, 0.1 mM ATP, 20 U/µL of T4 PNK (Thermo Fisher Scientific, Carthage, MO), and 3 U/µL USER Enzyme (New England Biolabs, Ipswich, MA). After incubation for 3 h at  $37^{\circ}\text{C}$ , 10 U/µL of T4 DNA polymerase (Thermo Fisher Scientific) was added and incubated at  $25^{\circ}\text{C}$  for 30 min. Isolated DNA was obtained in 18 µL of elution buffer using the MinElute Purification Kit (Qiagen, Hilden, Germany) (negative control included). Adapter ligation was performed in a 40 µL total volume, containing: 18 µL of USER enzyme treated DNA fragments, 0.06 µM adapter mix of Solexa (Illumina, CA), 1X Quick Ligase Buffer (New England Biolabs, Ipswich, MA) and 1 U/µL Quick Ligase (New England Biolabs, Ipswich, MA) final concentrations upon incubation for 20 min at room temperature ( $20^{\circ}\text{C}$ ). The products of the ligation reaction were purified using a MinElute Purification Kit (Qiagen, Hilden, Germany) in 20 µL of elution buffer.

Next, the fill-in reaction was performed with a total volume of 40 µL, containing: 20 µL DNA, 4 µL 10× ThermoPol Buffer (New England Biolabs), 0.2 µL dNTPs (25 mM), 2 µL Bsm DNA polymerase (16 U/µL) (Thermo Fisher Scientific) and 13.8 µL  $\text{H}_2\text{O}$ , with the reaction then incubated at  $37^{\circ}\text{C}$  for 20 min, then at  $80^{\circ}\text{C}$  for 20 min, to inactivate the enzyme. Sample-specific indices were added to both library adapters via amplification with two index primers (P5 and P7), performed in 50 µL total volumes, containing: 10 µL of DNA from the previous step (AccuPrimer Pfx polymerase (Thermo Fisher Scientific-Invitrogen, Carthage, MO) manufacturer's protocol) and 0.3 mM of both P5 and P7 adapter-primers. PCR reaction conditions were as follows:  $95^{\circ}\text{C}$  for 2 min, 10 cycles of  $95^{\circ}\text{C}$  for 30 s,  $60^{\circ}\text{C}$  for 30 s,  $72^{\circ}\text{C}$  for 1 min, and  $72^{\circ}\text{C}$  for 5 min.

Subsequent to bead enrichment according to the Maricic et al. (2010) protocol, the library was purified using a MinElute Purification Kit (Qiagen, Hilden, Germany) in 50 µL of elution buffer. Moreover, in an effort to improve library quality, DNA was reamplified using 5 µL of purified DNA and 0.15 µL of both P5 and P7 primer-adapters. PCR reaction conditions were as follows:  $95^{\circ}\text{C}$  for 2 min, 10 cycles at  $95^{\circ}\text{C}$  for 15 s,  $60^{\circ}\text{C}$  for 30 s,  $68^{\circ}\text{C}$  for 70 s, and  $72^{\circ}\text{C}$  for 1 min, then  $10^{\circ}\text{C}$  hold. Amplicons were purified using a MinElute Purification Kit (Qiagen) in 50 µL of elution buffer. The quality of libraries was evaluated using a 2100 Bioanalyzer System, Agilent Technologies (Agilent, Waldbronn, Germany). Sequencing was performed using an Illumina platform at the Institute of Clinical Molecular Biology, University of Kiel, and the Department of Archaeogenetics, Max Plank Institute for the Science of Human History, Jena, Germany.

## 2.12 | Bioinformatic analysis

Library quality control was assessed using the software FASTQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Raw



sequencing reads were prepared for mapping against reference genomes. All Illumina sequencing runs were performed as paired-end, creating two raw read files for each sample: a forward FastQ file and a reverse FastQ file, which were merged to improve the accuracy of the mapping step. Adapter sequences at the end of reads were removed. The clipping of adaptors, merging of forward and reverse reads, and a subsequent quality filtering step were performed using EAGER (Efficient Ancient Genome Reconstruction) software (Peltzer et al., 2016). Mapping short reads against the revised Cambridge reference mtDNA genome using bowTie2 software (Langmead & Salzberg, 2012) was the first step of next-generation sequencing data analyses. Prior to variant calling, PCR duplicates were removed from aligned reads using SAMtools software (Cock et al., 2015; Li et al., 2009). Alignment visualizations were manually verified using Integrative Genomics Viewer (IGV) (<http://www.broadinstitute.org/igv/>) (Robinson et al., 2011, 2017; Thorvaldsdóttir et al., 2013). EAGER software was used to assemble genome sequences, remove adapter sequences, genotype samples, filter and call variants, authenticate results, and assess the quality of aDNA samples (Peltzer et al., 2016).

## 2.13 | Next generation sequencing (NGS)

Ten pre-Hispanic skeletal remain specimens found in Puyil Cave were sequenced by NGS. The damage pattern map corroborated that the sequenced DNA indeed belonged to ancient human remains (Figures 2b\_Supp and 3\_Supp). The percentage of endogenous DNA ranged from 0.063% to 1.29%, and coverage of the whole genome ranged from 0.027% to 0.316%, while the coverage of the mitogenome ranged from 1.71% to 53.72% (Table 1\_Supp). The percentage of endogenous DNA obtained is largely due to the environmental conditions of the cave and the broader climate of the region (Tiesler, 2013).

Nucleotide sequences of each sample from this study were obtained by Sanger sequencing and NGS after their alignment with BioEdit 7.2.1 and IGV software (Robinson et al., 2011, 2017; Thorvaldsdóttir et al., 2013) where they were annotated and are available in the NCBI GenBank nucleotide database with the follow accession numbers: PUXTABMEX001, MK483648; PUXTABMEX002, MK483649; PUXTABMEX003, MK483657; PUXTABMEX004, MK483650; PUXTABMEX005, MK483651; PUXTABMEX006, MK483652; PUXTABMEX007, MK483653; PUXTABMEX009, MK483655; PUXTABMEX010, MK483656.

## 2.14 | Mitochondrial DNA haplogroup classification

Haplogroups were determined based on diagnostic variants described previously (Achilli et al., 2008; Derenko et al., 2010; Gómez-Carballa et al., 2015; Kumar et al., 2011; Rieux et al., 2014; Starikovskaya et al., 2005; Torroni et al., 1993). Variants were called via sequence analysis using BioEdit 7.2.1 (sequence analysis and alignment) and IGV

software (Robinson et al., 2011, 2017; Thorvaldsdóttir et al., 2013), then confirmed twice using Mitomap (<https://www.mitomap.org/foswiki/bin/view/MITOMASTER/WebHome>) (Lott et al., 2013) and HaploGrep 2.0 (<https://haplogrep.uibk.ac.at/>) (Weissensteiner et al., 2016) software. The haplogroup of PUXTABMEX008 was not able to be determined because its DNA was found to be highly degraded, and hence, incapable of being sequenced. Haplogroup identification for each sample was also performed using TaqMan<sup>®</sup> SNP Genotyping Assays (Thermo Fisher Scientific) in triplicate: for haplogroup A (SNP A663G), C (SNP A13263G) and D (SNP C5178A) according to brand protocol. As a replicate, the aDNA was extracted, amplified, and sequenced from four samples from Puyil Cave for haplogroup identification in the aDNA Laboratory, Department of Anthropology, University of Utah, Salt Lake City, Utah. Results were the same from both laboratories.

## 2.15 | Haplotype network analysis

Phylogenetic networks were constructed using Network 5.0.0.1 (Bandelt et al., 1999; Polzin & Daneshmand, 2003; Leigh & Bryant, 2015) software from the following mitochondrial nucleotide position sections: (1) 16,000–16,569 (570 bp), 14,767–16,569 (1803 bp), and 16,224–16,569 (346 bp) for haplogroup A, (2) 16,224–16,569 (346 bp) for haplogroup C, and (3) 16,064–16,569 for haplogroup D (508 bp). The network analysis included 992 sequences (Alves-Silva et al., 2000; Arias, Barbieri, Barreto, Stoneking & Pakendorf, 2018; Barbieri et al., 2017; Bert, Corella, Gené, Pérez-Pérez & Turb, 2004; Boles, Snow & Stover, 1995; Bonilla et al., 2004; Brandini et al., 2018; Derbeneva et al., 2002; Derenko et al., 2007; Dornelles et al., 2004; García-Bour et al., 2004; Green, Derr & Knight, 2000; Ingman & Gyllensten, 2007; Ingman, Kaessmann, Pääbo & Gyllensten, 2000; Just et al., 2015; Kong et al., 2003; Lee & Merriwether, 2015; Llamas et al., 2016; Mishmar et al., 2003; Moraga, Santoro, Standen, Carvallo & Rothhammer, 2005; Rubicz, Schurr, Babb & Crawford, 2003; Santos, Ribeiro-Dos-Santos, Meyer & Zago, 1996; Starikovskaya, Sukernik, Schurr, Kogelnik & Wallace, 1998; Vona, Talchi, Moral, Calò & Varesi, 2005; Ward et al., 1996; Williams, Chagnon & Spielman, 2002; Xu et al., 2017) of contemporary indigenous populations from the Americas and East Asia, as well as ancient populations from present-day Peru, Chile, and Cuba (Table 2\_Supp).

## 3 | RESULTS

### 3.1 | Radiocarbon (<sup>14</sup>C) dating

Radiocarbon (<sup>14</sup>C) dating demonstrated that the inhabitants of Puyil Cave lived in distinct temporal periods: Middle-Archaic (PUXTABMEX004 and PUXTABMEX006), Late-Archaic (PUXTABMEX009), Middle-Classic (PUXTABMEX001), and Late-Classic (PUXTABMEX002, PUXTABMEX007, and PUXTABMEX0010). Radiocarbon dating of PUXTABMEX004, PUXTABMEX006 and PUXTABMEX009 resulted in an age estimation of 4520–4440 cal BCE, 5316–5211 cal BCE, and 2206–

2032 cal BCE, respectively (Table 3). Radiocarbon dating of the samples in the Classic period cover a temporality of ~425–774, displayed in Table 3.

### 3.2 | Age at death

Age at death is vital to the reconstruction of past populations and societies because it is necessary for calculating mortality rate and demographic profiles. Table 1 displays the calculation for age at death for all skeletal remains. The age at death for (1) PUXTABMEX004 was ~5–10 years old, (2) PUXTABMEX005 ~15–20 years old, (3) PUXTABMEX001, PUXTABMEX002, and PUXTABMEX010 ~20–25 years old, and (4) PUXTABMEX003 and PUXTABMEX006 through PUXTABMEX009, ~25–30 years old.

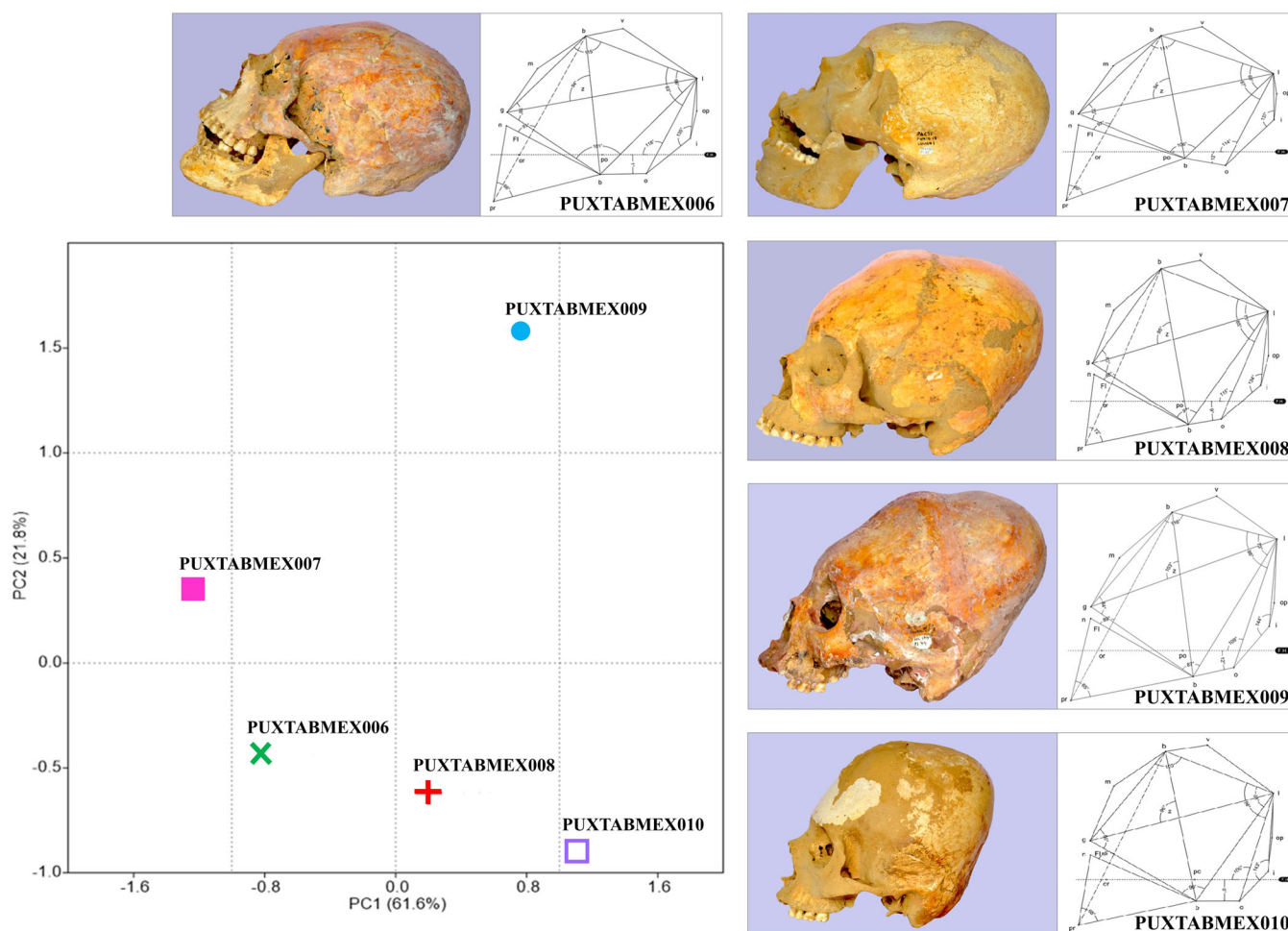
### 3.3 | Craniometric analysis

ACD was assessed in the process of quantifying cranial variation. Craniometry and trigonometric analyses suggest that specimens

PUXTABMEX006, PUXTABMEX007, and PUXTABMEX009 are the product of tabular oblique ACD (Figure 3). Skull specimen PUXTABMEX008 displayed characteristics associated with both tabular erect and oblique ACD, also known as tabular mimetic ACD (Dembo & Imbeloni, 1938). In addition, this skull possessed dental cavities and a deviated nasal septum, probably due to receiving a blow that may have resulted in death. Specimen PUXTABMEX010 was confidently classified as possessing tabular erect ACD (Figure 3). Additionally, skull specimens PUXTABMEX006 and PUXTABMEX008 displayed imperfect deformations (asymmetrical head shape). Skull specimens PUXTABMEX006 and PUXTABMEX007 both displayed post-lambdoid occipital bands, producing a pseudo-annular appearance (Figure 3).

### 3.4 | Principal component analysis of ACD traits

To statistically and visually contrast modified with unmodified crania, and to assess craniometric alteration standards within Puyil Cave and related populations, PCA was employed (Figure 3). Results included a variance of 83.4% in overall skull morphology, where positive values



**FIGURE 3** Craniometric modeling of crania. Analysis of ACD in five individuals using 14 surface landmarks. Principal component 1 (PC1) accounted for 61.6% of measured variability and PC2 for 21.8% of variability in cranial morphology. Cephalometric landmarks and abbreviations of the cranial polygons are as follow: Prosthion (pr), nasion (n), glabella (g), metopion (m), bregma (b), vertex (v), lambda (l), Opisthocranium (op), inion (i), opistion (o), basion (ba), porion (po) y orbitale (or).

(PC 1) positioned the crania of PUXTABMEX006 and PUXTABMEX007 among those with less obliquity, shorter neurocranial length, and greater height and prognathism. Skull specimens PUXTABMEX008, PUXTABMEX009, and PUXTABMEX010 were grouped toward possessing greater obliquity and neurocranial length, and lower height and prognathism. The second component (PC 2) displayed greater variation in the frontal obliquity observed in skulls PUXTABMEX007 and PUXTABMEX009 (Figure 3).

### 3.5 | Dental modification analysis

Dental filing marks were identified on specimen PUXTABMEX006. The maxillary central incisors each displayed two shallow occlusal grooves corresponding to modification type A-2 (Romero, 1958) (Figure 4). Modification category A (upper incisors worked on their cutting edge) was the most common alteration made during the Pre-Classic period of Mesoamerica, particularly among Maya peoples (Romero, 1958).

### 3.6 | Mitochondrial DNA haplogroup assignment

The mitochondrial DNA of 10 pre-Hispanic bone specimens (Figure 2; Table 2) were analyzed using NGS and Sanger sequencing (Sanger et al., 1977; Sanger & Coulson, 1975). Variants obtained by alignment with the revised Cambridge reference sequence, human\_hg38 (NCBI Reference Sequence: NC\_012920.1), are shown in Table 2. Haplogroups A, A2, C1, C1c, and D4 were identified using the Kumar classification (Kumar et al., 2011), and Mitomaster (Brandon et al., 2009) and HaploGrep2 (Weissensteiner et al., 2016) software.



**FIGURE 4** Intentional dental modification. Dental modification, specifically the filing of the maxillary central incisors, in specimen PUXTABMEX006.

Haplogroup B, a haplogroup indigenous to the Americas, was not identified in any of the study samples. Sequence identities using both methods authenticated our results. Haplogroups A, B, C, and D were also confirmed by qPCR.

### 3.7 | Network analysis

A haplotype network analysis was performed to elucidate the relationship between pre-Hispanic Puyil Cave specimens and populations with different spatiotemporal dimensions (Table 2\_Supp). Five Puyil Cave samples (PUXTABMEX003, PUXTABMEX005, PUXTABMEX006, PUXTABMEX007, and (Table 2, Figures 5, 4\_Supp and 5\_Supp). Samples PUXTABMEX003 and PUXTABMEX007 were shown to be related primarily to contemporary Maya populations (Maya of Yucatán and Tzotzil, Mexico). Subsequently, node H8 was directly connected to node H4, which grouped the aforementioned samples with the Kiche Maya from Guatemala, Tzotzil Maya from Quintana Roo, Mexico, Putumayo from Colombia, Brazilian Amazonians (Arara, Awa-Guaja, Katuena, Kayapo, Poturujara, Tiryo, Wayampi, and Yanomama), and Han Chinese. Sample PUXTABMEX006 was only three mutations away from the sequence of contemporary populations from Mongolia, and Maya from Mexico (Tzotzil) and Guatemala (Ladino, Verapaza, and La Tinta). Although the three-network analysis of samples have different lengths, they were localized in the same cluster of haplotype A2. The reconstruction of median joining haplotype network analysis using Network 10 revealed a similar haplotype profile. It is important to mention that to better determine the relationship among haplotypes, longer sequences or complete mitogenomes should be amplified, and sequencing of the regions not included in this study should occur.

Samples PUXTABMEX001 and PUXTABMEX004 were assigned to mtDNA subclade C1. Sample PUXTABMEX002 was assigned to mtDNA subclade C1c (Figure 6a). Samples PUXTABMEX002 and PUXTABMEX004 branched from node H\_83 (PUXTABMEX001), and this node, in turn, branched from node H\_34, that encompasses the Movima population of Bolivia, and then node H\_45 (that encompasses the Maya from Yucatán, Campeche, and Quintana Roo, Mexico as well as populations from the Dominican Republic). One specimen, derived from PUXTABMEX009, was assigned to mtDNA haplogroup D4 (Figure 6b). This individual was, according to a network analysis, placed at node H\_133, which branched with node H\_119 (Han Chinese), and then node H\_2 (populations from northern Brazil (*mestizos* from Pará, *Rondônia*, and Acre) and northern Asia (Chukchi, Chukotkan Inuit, Kazakh, and Kchanti populations).

## 4 | DISCUSSION

Caves were utilized as ceremonial centers or for shelter by numerous Mesoamerican populations, which make them of great interest for comparative studies of many types. Many caves in Mesoamerica were employed for shelter, and some of them became ritual sites. It has

**TABLE 2** Polymorphic sites in mtDNA sequences.

[illegible]

Note: Distribution of polymorphisms for haplogroups A, C, and D of pre-Hispanic samples from Puyil Cave. Hyphens (–) represent uncovered regions and dots (•) represent covered invariant regions in the sequence. Nucleotides in black of the rCRS correspond to the D-Loop Region. Nucleotides in black in samples sequences correspond to variants of haplogroup determination.

<sup>a</sup>Phylogeny and haplotype determination utilized Kumar et al. (2011) and PhyloTree data (<https://www.phyloree.org/tree/index.htm>).







been shown that nomadic foragers used caves for temporary habitation at the close of the last glacial maximum and that populations including the Coxcatlan and Guila Naquitz of Mexico, Pikimachay and Guitarrero of Peru (Brady & Prufer, 2005a). Studies of Mesoamerican caves have combined archaeology, ethnography, ethnohistory, epigraphy, and osteology. Such studies have shown that current, general anthropological studies of caves can provide extraordinary insights into early Mesoamerican customs, ceremonies, and beliefs, also indicating that caves played a significant role in religion, suggesting that some caves were perceived as vital aspects of a sacred landscape with connections to a supernatural underworld. The first studies in the Maya geographic area suggested that caves were used as temporary or permanent residences, however, the most recent investigations have shown many evidences, demonstrating that caves played important roles mostly as ceremonial centers in pre-Hispanic populations, and Maya peoples specifically, and that they continue to be used for similar purposes by contemporary Maya groups (Brady & Prufer, 2005a, 2005b; Healy, 2007).

There are several types of evidence to suggest that Puyil Cave was a ceremonial center. In fact, the continuity of Indigenous ritual practices in caves remains into the present. This interdisciplinary study, which includes radiocarbon ( $^{14}\text{C}$ ) dating, dental modification classification, evaluation of artificial cranial deformation, mtDNA variant identification, maternal lineage assignment by haplogroup identification, and network haplotype analysis allowed us to comprehensively elucidate cultural affiliation and suggest the ancestral origins of the pre-Hispanic remains. More than ~30 human remains were discovered in Puyil Cave, including three near-complete skeletons, three bone fragments, one skull fragment, and five crania exhibiting differential deformations. Radiocarbon dates from seven of the remains indicate that Puyil Cave was continuously inhabited or used for ritual practices throughout the Middle-Archaic (PUXTABMEX004 and PUXTABMEX006), Late-Archaic (PUXTABMEX009), Middle-Classic (PUXTABMEX001), and Late-Classic (PUXTABMEX002, PUXTABMEX007, and PUXTABMEX0010) periods. This finding is fundamental to understanding possible ancestral origin(s), since, because the temporality of samples varies from the Archaic period to the Late-Classic period, we can follow a timeline of occupation in the cave. There is no information about Olmecs or Zoques who inhabited the state of Tabasco during these different periods or a mtDNA lineage of contemporary Olmecs/Zoques, therefore, some of the samples could be related to Olmecs or Zoques, but currently there is no clear evidence. Previously the sex of 10 study samples were determined as male (Navarro-Romero et al., 2020), suggesting a role for men in religious offerings.

Over the past 40 years, archeologists have proposed that the ancient Maya buried sacrificed individuals in caves, typically children

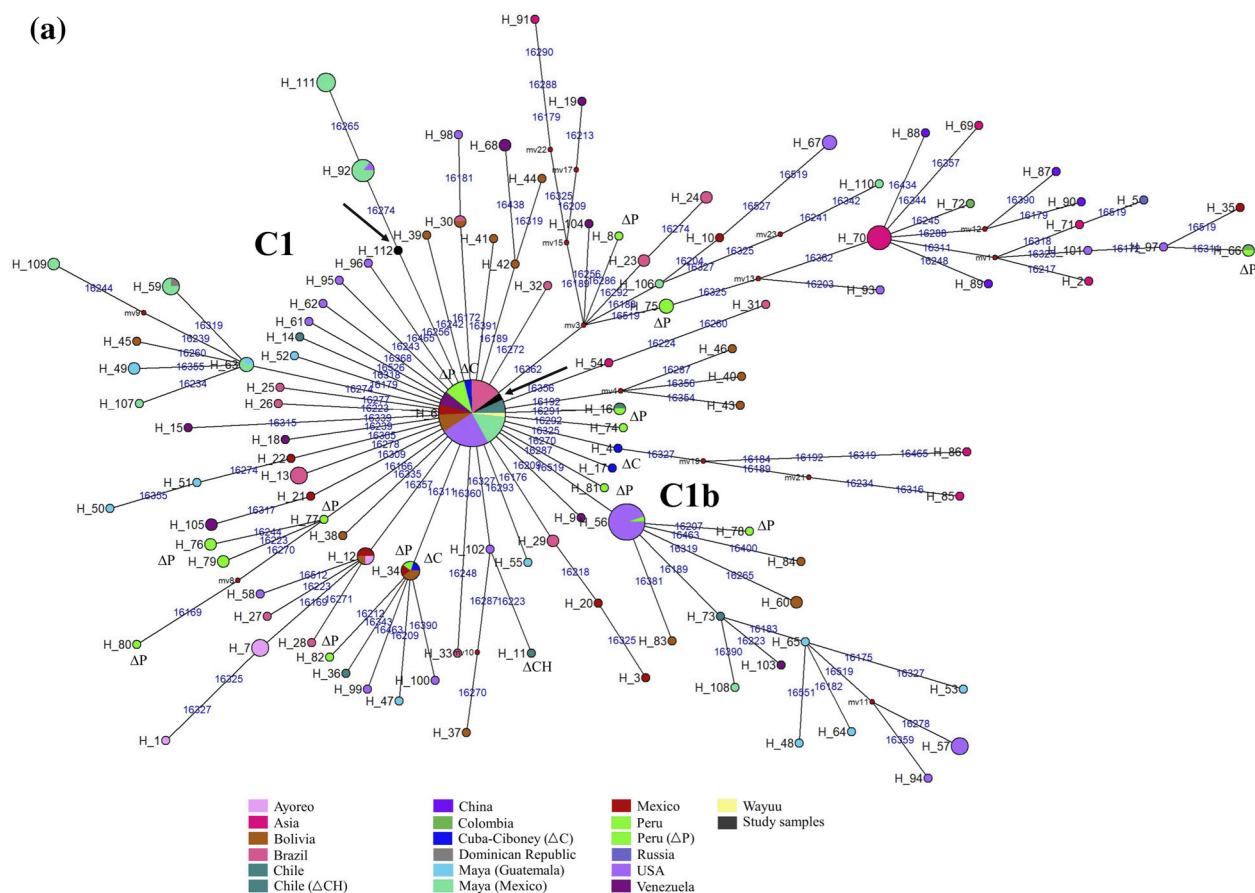
and young adults, suggested to be favored as sacrificial victims in Mesoamerican societies (Macleod & Puleston, 1978). Comparing the distribution of age groups from Barton Creek Cave and three other caves in western Belize, approximately half of the individuals identified were either mail infants (<3 years) or children (3–12 years) (Owen, 2005). The unnatural position of some adult individuals in the Barton Creek Cave suggests that these individuals were sacrificial victims (Owen, 2005). In Puyil Cave there were eight adults, one young adult, and one child (Table 1) found, suggesting burials or sacrifice events.

Individual PUXTABMEX001, from the Middle-Classic Period, with an age at death of ~20–25 years, was assigned to mtDNA subclade C1. The H<sub>6</sub> node where this individual is phylogenetically located (according to a network analysis) represents downstream genetic relatedness with contemporary populations from Bolivia (H<sub>34</sub> node), the Dominican Republic (H<sub>45</sub> node), and Maya populations from Mexico (H<sub>6</sub> node) and Guatemala (H<sub>6</sub> node) (Figure 6a). Individual PUXTABMEX002 from the Late-Classic Period, with an age at death of ~20–25 years, was assigned to mtDNA subclade C1c. Haplotype network analysis showed the relationship between PUXTABMEX002 (H<sub>6</sub> node) and PUXTABMEX001 (H<sub>6</sub> node) (Figure 6a), as well as their affinity with Maya people from present-day Mexico and Guatemala. Individual PUXTABMEX002 was discovered adjacent to a cultural offering (green stone ax and remnants of cinnabar, and under this, a flint knife and a green stone pectoral) dating from the Late-Classic Period, which corresponds with the radiocarbon ( $^{14}\text{C}$ ) dating of the individual. Because the crania in chamber 4 may represent a different ancestral lineage than the skeletal remains from chamber 6, we compared the maternal lineage of a fragment from individual 9 (PUXTABMEX002) to all crania from chamber 4. While the bone fragment could be assigned to mitochondrial DNA subclade C1c, all crania from chamber 4 were of either mtDNA haplogroup A or D4 (Figures 5 and 6b, respectively).

Individual PUXTABMEX003 had an age at death of ~20–25 years and was assigned to mtDNA subclade A2 (Figure 5). Network analysis placed it at the H<sub>167</sub> node. Its close proximity to the H<sub>212</sub> and H<sub>25</sub> nodes (Figure 5) (which encompass contemporary Maya populations from Mexico and Guatemala) suggests that this individual shared a recent common ancestor with these populations. Individual PUXTABMEX004, from the Middle-Archaic Period, with an age at death of ~5–10 years, possessed a mutilated skull, and was assigned to mtDNA subclade C1. Samples PUXTABMEX004 (H<sub>85</sub>) and PUXTABMEX002 (H<sub>112</sub>) branched from node H<sub>6</sub> (PUXTABMEX001), and node 92, which in turn, branched to node H<sub>11</sub>. Node H<sub>6</sub> encompasses the ancient samples from Peru, Ciboney, Maya from Yucatán, Campeche, and Quintana Roo, and Tzotzil from Mexico. This suggests that individual PUXTABMEX004 is related to Mayas.

**FIGURE 5** Haplogroup A network analysis for mtDNA HVR-I. (a) Haplogroup A network analysis from nucleotide positions 14,767–16,569 (1803 bp) of individual PUXTABMEX003 (H<sub>167</sub>), and 358 sequences reported previously. (b) Haplogroup A network analysis from nucleotide positions 16,000–16,569 (570 bp) of individual PUXTABMEX005 (H<sub>212</sub>), and 509 sequences reported previously. The delta symbol ( $\Delta$ ) prior to letters “CU,” “CH,” and “P” indicates the sequence of ancient samples from Cuba, Chile, and Peru, respectively.

(a)



(b)

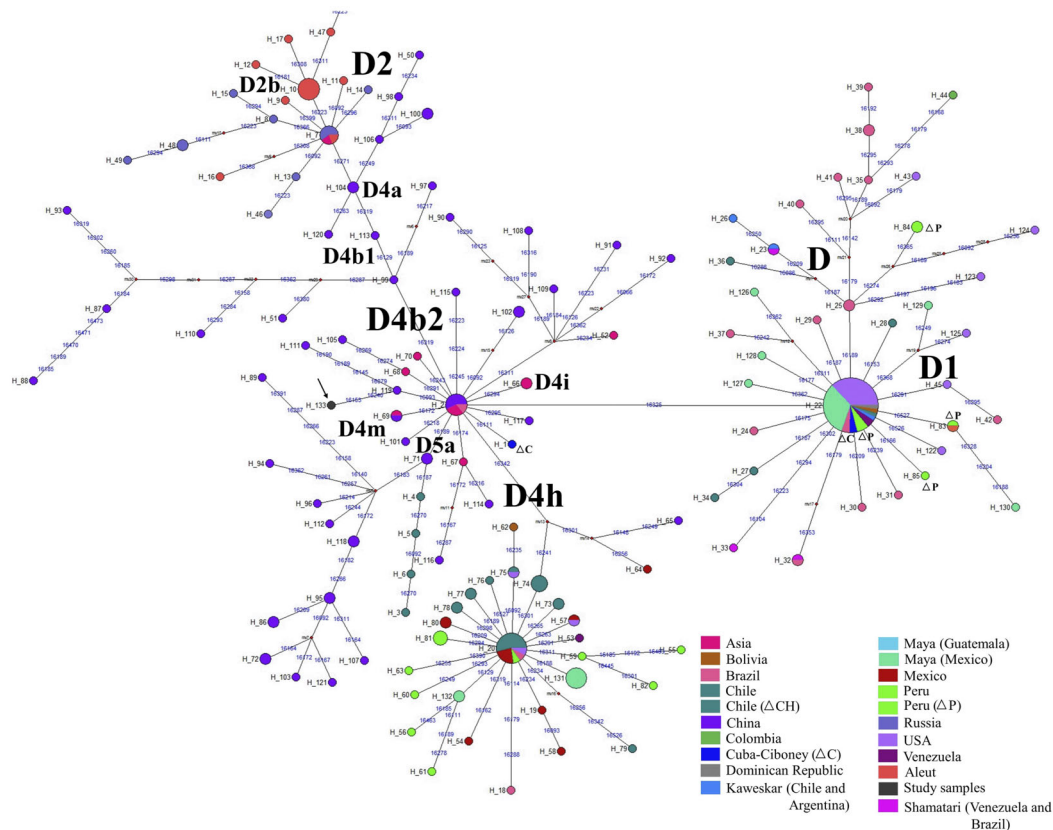


FIGURE 6 Legend on next page.

**TABLE 3** Ethnicity as suggested by specimen age and cranial deformation.

| Sample       | Conventional age | Calendar calibration | Cephalic deformation | Ethnicity <sup>a</sup> |
|--------------|------------------|----------------------|----------------------|------------------------|
| PUXTABMEX001 | 1539 ± 35 BP     | 425–595 cal CE       | ND                   | Maya                   |
| PUXTABMEX002 | 1300 ± 30 BP     | 660–730 cal CE       | ND                   | Maya                   |
| PUXTABMEX003 | ND               | ND                   | ND                   | Maya                   |
| PUXTABMEX004 | 5630 ± 30 BP     | 4520–4440 cal BCE    | ND                   | Maya                   |
| PUXTABMEX005 | ND               | ND                   | ND                   | Maya                   |
| PUXTABMEX006 | 6270 ± 30 BP     | 5316–5211 cal BCE    | Tabular Oblique      | Maya                   |
| PUXTABMEX007 | 1290 ± 30 BP     | 660–770 cal CE       | Tabular Oblique      | Maya                   |
| PUXTABMEX008 | ND               | ND                   | Tabular Mimetic      | ND                     |
| PUXTABMEX009 | 3730 ± 30 BP     | 2206–2032 cal BCE    | Tabular Oblique      | Olmec                  |
| PUXTABMEX010 | 1280 ± 30 BP     | 662–774 cal CE       | Tabular Erect        | Maya                   |

Note: Possible ancestral lineage was determined based upon the calendar calibration age obtained by <sup>14</sup>C testing, ACD, and mtDNA haplotype network analyses.

Abbreviation: ND, not determined.

<sup>a</sup>Ethnicity: Possible ancestral origin determined in this study.

Individual PUXTABMEX006, from the Middle-Archaic Period, had an age at death of ~25–30 years, possessed tabular oblique ACD, and was assigned to mtDNA haplogroup A, because, this sequence does not have the transitions C16111T. Individual PUXTABMEX006 suffered from severe porotic hyperostosis, characterized by localized areas of spongy or porous bone tissue in the skull vault. This condition is often evidence of chronic or episodic malnutrition, common in ancient populations of the Americas (Brickley, 2018; El-Najjar & Robertson, 1976). Because the analyzed genetic sequences of individual PUXTABMEX006 were found to be closely related to contemporary Maya populations from Mexico and Guatemala, it can be inferred that this individual likely shared a common ancestor with members of these groups (Table 3). The most common mitochondrial subclade identified in Mesoamerican populations from the Classic Period is A2 (De Saint Pierre et al., 2012; Kumar et al., 2011). In contrast, haplogroup A is scarce among Indigenous Americans (ancient or contemporary), though was previously identified in a pre-Hispanic individual from Comalcalco, Tabasco (Muñoz-Moreno et al., 2021; Ochoa-Lugo & Muñoz, 2016).

The ACD and dental modification types of individual PUXTABMEX006 were historically common in the Maya cultures of Mexico and Guatemala. Particular ACD techniques have been reported to be associated with a given period of time and cultural affiliation (Bautista-Martínez, 2010; Monte de la Paz & Linares-Villanueva, 2015). ACD has been performed by human beings to differentiating from others. Different cultural affiliation has a specific ACD and can suggest the ancestral origins of the pre-Hispanic

remains for the specific ACD from the Puyil Cave skulls. For example, because the temporality of samples varies from the Archaic period to the Late-Classic period, we can follow a timeline of occupation in the cave and ACD modifications through the time, using unique configurations of contour and surface change (Lodder, 2008).

Jade figurines displaying persons with elongated heads have been previously attributed to Olmec culture (Childress & Foerster, 2012). Nevertheless, there is currently only one other study that we are aware of describing an ACD from Mesoamerica from the Archaic period (Tiesler, 2012) as PUXTABMEX006. Scant discoveries of this type in Mesoamerica may be due to the accelerated decomposition rates of human remains in tropical climates, where the Olmec, Zoque, and Maya largely resided.

Individual PUXTABMEX007, from the Late-Classic Period, was ~25–30 years old at death, and assigned to mtDNA haplogroup A. This individual exhibited a congenital fusion of the atlas to the base of the occiput, often referred to as “occipitalization of the atlas” (Gholve et al., 2007). This ACD type was common, specifically to Comalcalco, Tabasco and Palenque, Chiapas (Tiesler, 2012). According to network analysis, they appear on a branch where we observe the other ancient individuals PUXTABMEX003, PUXTABMEX005, PUXTABMEX006, and PUXTABMEX010 that show 2–3 different mutations among them and six mutations from the common haplotype H4 (18 individuals) observed among Maya individuals from Mexico (Figure 5\_Suppl). The tabular oblique type ACD identified has been observed in approximately 50% of Maya skulls from the Classic Period

**FIGURE 6** Haplotype network of haplogroup C and D of mtDNA HVR-I region. (a) Haplogroup C network analysis from nucleotide positions 16,165–16,569 (404 bp) of individuals PUXTABMEX001 (H\_6), PUXTABMEX002 (H\_6), and PUXTABMEX004 (H\_112), and 242 sequences reported previously. Number of haplotypes, h: 112, Haplotype diversity, Hd: 0.9319. (b) Haplogroup D network analysis from nucleotide positions 16,064–16,569 (508 bp) of individual PUXTABMEX009 (H\_133), and 235 sequences reported previously. The delta symbol (Δ) prior to letters “CU,” “CH,” and “P” indicates the sequence of ancient samples from Cuba, Chile, and Peru, respectively.

and 70% from the Late-Classic Period (Nakbe, Uaxactun, Peten and Seiba) (Tiesler, 2012).

Individual PUXTABMEX008 had an age at death of ~25–30 years. The tabular mimetic type ACD that is possessed has been observed at a 77% frequency among populations surrounding the Usumacinta River in Petén and Copán, Guatemala (Tiesler, 2012). Since the ACD in individual PUXTABMEX008 was not symmetrical, and no sacrificial offerings were observable nearby, this individual may have been a companion of an individual with a high social or political status (Monte de la Paz & Linares-Villanueva, 2015).

Individual PUXTABMEX009, from the Late-Archaic Period, with an age at death of ~25–30 years was assigned to mtDNA subclade D4. The H\_133 node that this individual is placed at using a network analysis (Figure 6b) is proximal to the H\_119 and H\_2 nodes (five mutational steps), representative of a shared genetic lineage with contemporary populations from Brazil and East Asia (particularly, China). The ACD performed on this individual was tabular oblique and perfected. This specific type and refinement of ACD has been reported previously in the Late-Archaic Period in the central Andes and northern Patagonia (Alfonso-Durruty et al., 2015).

Individual PUXTABMEX010, from the Late-Classic Period, had an age at death of ~20–25 years, and was assigned to mtDNA haplogroup A. According to a network analysis, this individual (placed at the H\_109 node) clustered with individual PUXTABMEX007. Both of these individuals may belong to a high sociopolitical strata of Maya civilization, since both possessed perfected ACDs (Tiesler, 2012). Because these two individuals, however, exhibited different ACD types despite living at a similar historical period, it is plausible that they may have originated in different Maya regions of Mesoamerica (Table 3). The bodily remains of individuals PUXTABMEX002, PUXTABMEX007, and PUXTABMEX010 may have undergone the same funerary rituals, as they have similar radiocarbon ( $^{14}\text{C}$ ) dates (within the margin of error of being the same age) and share a resting site. Individual PUXTABMEX001 likely experienced a different funerary ritual, since radiocarbon ( $^{14}\text{C}$ ) dating placed the individual's lifespan before that of individuals PUXTABMEX002, PUXTABMEX007, and PUXTABMEX010.

Despite a low *n* value, the frequency of each mtDNA haplogroup across all of the studied specimens from Puyil Cave are as follows: A: 55.55%, C: 33.33%, and D: 11.11%. This proportion is very similar to the haplogroup frequencies found across other ancient Maya populations (A: 3%, A2v: 13%, A2: 45%, C: 3%, C1: 16%, C1b: 14%–16%, and D: 5%) (Ochoa-Lugo et al., 2016; Muñoz-Moreno et al., 2021), but notably, different from the Maya from Xcaret, Mexico (A: 84%, B: 4%, and C: 8%) (González-Oliver et al., 2001) and contemporary Maya populations, like those from Quintana Roo (A2: 64.9%, B2: 16.2%, C1: 14.9%, and D1: 4.1%), Yucatan (A2: 65%, B2: 12.5%, B4b1: 2.5%, C1: 12.5, and D1: 7.5%), Campeche (A2: 70.3%, B2: 5.4%, C1: 18.9%, and D1: 5.4%), and Chiapas (Tzotzil), Mexico (A2: 41.4%, B2: 24.1%, C1: 25.3%, and D4h3a: 9.2%) (González-Martín et al., 2015) and Guatemala (Tzotzil) (A2: 75%, B2: 14%, and C1: 10%) (Söchtig et al., 2015).

The absence of mtDNA haplogroup B from the human remains of Puyil Cave may be explained by its late independent arrival to

this geographic region (Lalueza-Fox et al., 2003), when it subsequently mixed with populations carrying mtDNA haplogroups A and C (Ochoa-Lugo & Muñoz, 2016). Alternatively, the absence of mitochondrial DNA haplogroup B may be due to the effects of genetic drift and/or selective pressure in small populations (Muñoz-Moreno et al., 2021; Ochoa-Lugo & Muñoz, 2016). The Asian mtDNA haplogroup, B1, is the most closely related haplogroup to the Indigenous American branch of haplogroup B2 and can be traced to the Altai-Sayan region that forms the borderland between present-day China, Mongolia, Kazakhstan, and Russia (Starikovskaya et al., 2005). Both subclades share identical genetic motifs between mitochondrial control region positions 827–4820 and 13,590–15,535.

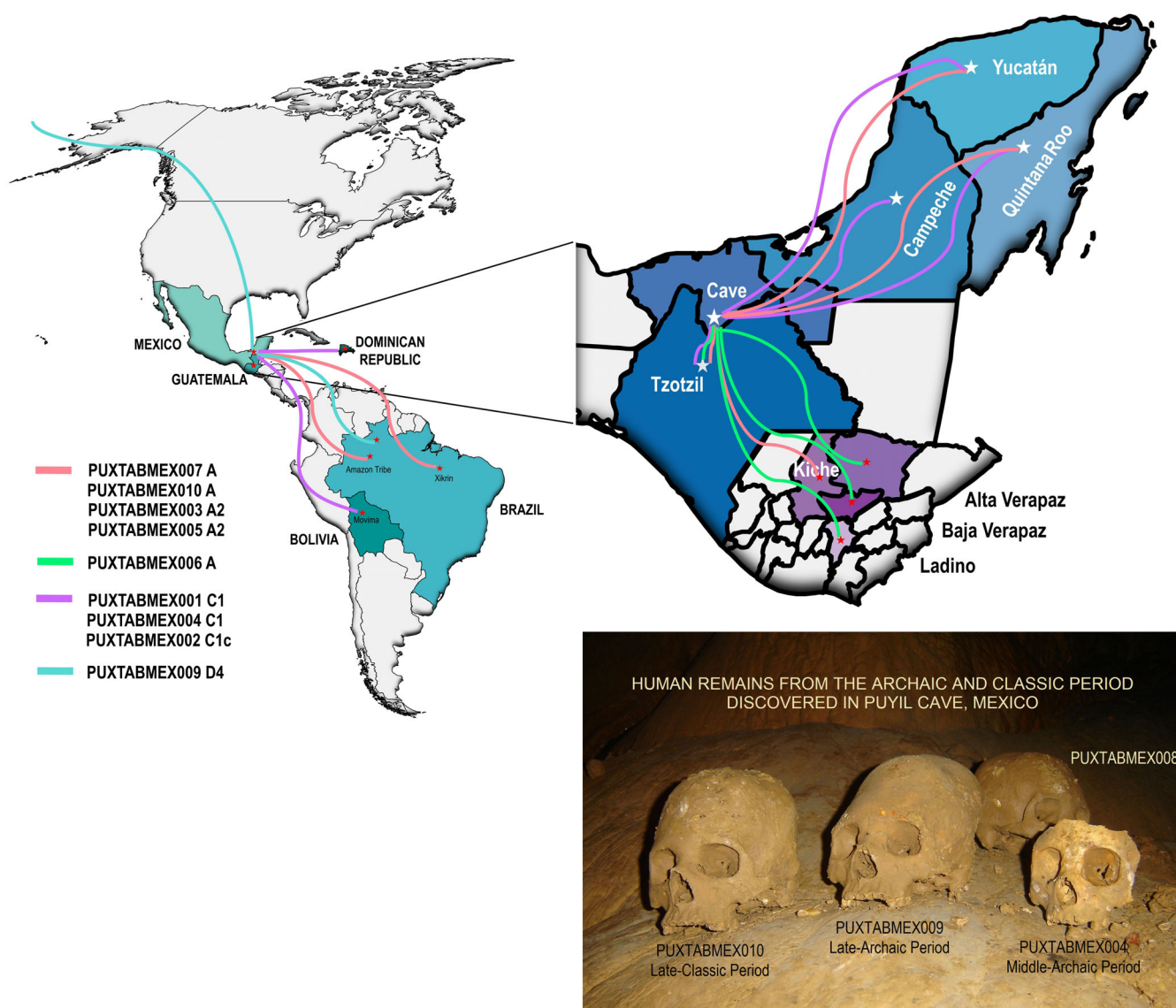
Haplogroup frequency in contemporary Indigenous populations of Mexico are very variable. Most of these populations either do not contain mtDNA haplogroup D, or the frequency is very low (0.0 to 16.2%) (Mizuno et al., 2014). MtDNA haplogroup A is in the range of 25.7% to 98.8%, mtDNA haplogroup B from 0.0% to 58.1%, and mtDNA haplogroup C from 0.0% to 25.3% (Mizuno et al., 2014). No contemporary populations of Mexican Mesoamerica display mitochondrial haplogroup frequencies similar to those from Puyil Cave (A: 55.55%, C: 33.33%, and D: 11.11%). Nevertheless, the reported frequency of mtDNA haplogroup A was similar to that of Nahua Ixhuatlancillo, Nahua Necoxtla, Purepecha, Otomi, and Tzeltal populations. Frequencies of mtDNA haplogroup C were similar to Tarahumara, Ticuna, and Zenu populations. Frequencies of mtDNA haplogroup D were similar to Mixe, Nahua Chilachapa, and Tzeltal populations (Mizuno et al., 2014).

The primary objective of this study was to elucidate the cultural affiliation and ancestral origins of pre-Hispanic remains discovered in Puyil Cave, Mexico. Taking into account ACD type, carbon ( $^{14}\text{C}$ ) dating, mitochondrial DNA haplogroup, and a biodemographic context, it is likely that individuals PUXTABMEX006 and PUXTABMEX004 share a common ancestor with pre-Hispanic Maya, individuals PUXTABMEX001, PUXTABMEX002, PUXTABMEX003, PUXTABMEX005, PUXTABMEX007, and PUXTABMEX0010 with various contemporary Maya subpopulations, and PUXTABMEX009 probably with Olmec peoples because the skull deformation, and possibly Indigenous South Americans. Future studies will look forward to study Olmec pre-Hispanic samples to know more about the relationship of this individual with the Olmecs.

## 5 | CONCLUSIONS

The analysis of mitochondrial DNA from pre-Hispanic human remains from Puyil Cave, Mexico aids in the wider elucidation of Indigenous American populations. Our findings support the affinity between the Maya and Puyil Cave individuals. Network analysis illustrates the relationship between ancient populations from Puyil Cave and other Indigenous American populations, as well as those from East Asia (Figure 7). Results also suggest that ACD and dental filing practices began in the Mesoamerican Archaic Period.





**FIGURE 7** Geographic distribution of shared mtDNA haplotypes between. This map shows the geographic distribution of shared mtDNA haplotypes between the human remains of Puyil Cave and those reported previously from ancient and contemporary people. Additionally, in the bottom right corner are shown the skulls on the surface of Chamber 4, discovered in this cave from Tabasco.

#### AUTHOR CONTRIBUTIONS

**María Teresa Navarro-Romero:** Conceptualization (equal); data curation (lead); formal analysis (equal); investigation (equal); methodology (lead); software (equal); validation (equal); writing – original draft (equal); writing – review and editing (equal). **María de Lourdes Muñoz:** Conceptualization (lead); data curation (equal); formal analysis (equal); investigation (lead); methodology (supporting); project administration (lead); resources (lead); software (supporting); supervision (lead); validation (lead); visualization (lead); writing – original draft (equal); writing – review and editing (equal). **Ben Krause-Kyora:** Data curation (equal); methodology (equal); resources (equal); supervision (supporting); writing – original draft (supporting). **Javiera Cervini-Silva:** Data curation (equal); formal analysis (equal); funding acquisition (equal); methodology (equal); validation (equal); writing – review and editing

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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

## DATA AVAILABILITY STATEMENT

The sequences of all samples discussed in this study are available in the NCBI GenBank nucleotide database with the follow accession numbers: PUXTABMEX001, MK483648; PUXTABMEX002, MK483649; PUXTABMEX003, MK483657; PUXTABMEX004, MK483650; PUXTABMEX005, MK483651; PUXTABMEX006, MK483652; PUXTABMEX007, MK483653; PUXTABMEX009, MK483655; PUXTABMEX010, MK483656.

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