Proceedings of The National Conference
On Undergraduate Research (NCUR) 2014
University of Kentucky, Lexington
3-5 April 2014

Genetic Diversity in Totonacan-Speaking Populations from Veracruz and Puebla States of Mexico

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Abstract

The Totonac are an indigenous Mexican ethnic group living in the states of Puebla and Veracruz. To explore the linguistic, cultural, and geographical influences on the genetic composition and migration history of the Totonac, we PCR amplified, and sequenced the hypervariable 1 (HVSI) region of mitochondrial DNA (mtDNA) from 60 members of this group. Our results demonstrate that 97% of the mtDNA haplotypes were indigenous in origin, and belonged to one of three major indigenous haplogroups (lineages): A2, B2 and C1. The remaining 3% belonged to H, a common European haplogroup. This pattern suggests limited maternal admixture with non-native populations such as Spanish colonists and Africans introduced to Mexico as slaves in the last 500 years. When the Totonac mtDNA data were compared to those of other Native Mexican groups such as the Tepehua, Nahua and Otomi, we observed that linguistics rather than geography played a larger role in shaping the genetic composition of the Totonoc. Statistical tests were used to confirm this analysis and delineate the factors that shaped the observed pattern. The analysis of genetic diversity in the Totonac will further enable the reconstruction of prehistoric events and maternal patterns of migration within Central Mexico.

Keywords: Totonac, mtDNA, haplogroup

1. Introduction

1.1. Mitochondrial DNA Variation

In recent decades, studies of mitochondrial DNA (mtDNA) variation have come to the forefront of forensic and anthropological genetic research. Molecular anthropologists have used this 16,569 bp strand of DNA because of its rapid mutation rate, lack of recombination, and strict maternal transmittance[16]. These properties make it very useful for studying the genetic composition of various populations, and tracking their migrations over time from a biological standpoint. Therefore, mtDNA data can provide important information about previously unrecorded historical events while also verifying accounts of recorded ones, such as the movement of modern humans out of Africa[6] and the peopling of the Americas.[44,45]

More specifically, the process of studying intra- (within) and inter- (outside) population variation and interactions can be assessed through characterizing mutations, or single nucleotide polymorphisms (SNPs), in the mtDNAs of individuals from a particular geographic or linguistic group. Once the SNPs are determined, the mtDNAs can be assigned to a particular haplotype. Many SNPs that define haplotypes occur in the noncoding region of the mtDNA, which includes the hypervariable regions-I and -2 (HVSI and HVSII) of the mitochondrial genome[9]. Haplotypes sharing the same diagnostic SNPs belong to the same haplogroup, or maternal lineage. Haplogroups are assigned different letters, which denote that all individuals within a particular group share a common ancestral origin. In other words, an mtDNA haplogroup is an evolutionary branch of the human tree that can tell us about the divergence

of mtDNAs and their dispersal with migrating populations[10, 14].

Native American mtDNAs typically belong to one of four main haplogroups, namely, A2, B2, C1, and D1[21]. However, there have also been a few recorded instances of haplogroup X2a [13]. While the source of haplogroup X2a is debated,[42] all of the other haplogroups have their origins in Central and East Asia[42], but are thought to have migrated to the Americas across the Bering land bridge some time between 15,000 to 20,000 years ago[12, 24, 38]. The high prevalence of these four indigenous haplogroups in the Americas suggests that a limited number of founder haplogroups were brought to the Americas due to a founder effect[21]. This genetic bottleneck occurred as Asian ancestors migrated from Siberia to Mesoamerica by way of the Bering land bridge and Pacific coast [22,23]. Furthermore, through historical admixture, some Native American populations have acquired West Eurasian haplogroups H, I, J, K, T, U, and V [1], and African haplogroups L0, L1, L2, and L3[48].

1.2. Indigenous Mexicans

Over the course of many millennia, groups of migrants reached the geographic region that is known as present-day Mexico, possibly as early as 15,000 years ago[40]. Historians and archaeologists recognize Mexico as one of the largest and most significant cultural, economic, and political hubs of the ancient American world[17]. This is evidenced through archaeological remains of large empires, complex writing systems, and advanced agriculture present among central and coastal pre-Columbian civilizations like the Aztec, Maya, and Olmec[17].

In order to better contextualize this molecular anthropology study, we will describe indigenous Mexican history in some detail. Most researchers mark 5,000 BCE as a period when hunter-gatherers began to solidify into domesticated groups that focused on agriculture, particularly with crops like maize, beans, peppers, and potatoes[20]. During the Pre-Classic period from 1800 BCE to 300 AD, complex social structures, writing systems and new artistic forms emerged. With the rise of city-states during the Classic period from 300 AD to 1000 AD, indigenous peoples started to develop social, economic, and political systems that provided a degree of differentiation between themselves[20]. Furthermore, the implementation of social hierarchies and intricate government systems led to extended cultural differentiation into the Postclassic Period (1050 AD-1580s AD)[25]. Despite the rise of large empires during this period, such as the Aztecs, Maya, and Toltecs, smaller groups still maintained a large degree of cultural autonomy[25].

Essentially all indigenous groups had minor interaction with foreigners until the arrival of the Spanish conquistadors in the 16th century[2]. In February 1517, Francisco Hernandez de Cordoba left Cuba to explore the Mexican shoreline. While venturing along the coast, de Cordoba stumbled across a Mayan village, which he later described as his first sighting of advanced civilization in the New World[2]. Two years later, in 1519, Cortes landed on the Mexican shoreline at Veracruz destined to colonize Mexico for Spain. After conquering the Aztecs and Mayas, two of the greatest empires in Mesoamerica, he established Mexico as New Spain, which would later become one of the largest and most significant colonies of the Spanish empire[35].

Although the Spanish government introduced social and political reforms, some of the most drastic changes came in the economic sector. Officials implemented forced labor through the *economienda* system, which pressed indigenous communities for quotas of resources and services; despite this systemic exploitation, native peoples were not considered slaves[53]. As the local Mexican population began to decline during the early colonial period (1590-1630) from war and disease, the Spanish turned to alternate sources of forced labor. Accordingly, they brought enslaved Africans from the Caribbean to Mexico to assist with ore mining[51].

A majority of the members of these indigenous communities, many of whom had ancestors who interacted extensively with European and African peoples, typically belong to large groups like the Nahua and Maya. However, there were many other smaller ethnic groups that lived in proximity to these vast empires, including the Totonac, which also have an intricate ancestry and numerous descendants[3]. Although most research today has been conducted on the aforementioned larger indigenous populations, the mtDNAs of smaller ethnolinguistic groups also need to be analyzed in order to understand their genetic history. This kind of work will help to place them within the larger framework of indigenous migration history and population interactions in Mesoamerica.

1.3. The Totonac

This project focuses on the Totonac, an indigenous group living in the Central Valley and Gulf coast regions of Mexico. According to tradition, "Totonaco" is derived from two words: tuta (three) and naku (heart)[25]. People belonging to this group typically reside in the Puebla, Veracruz, and Hidalgo states (**Figure 1**). These regions

contained a stable agricultural base that allowed this group to thrive for hundreds of years. Although still currently debated among archaeologists, the earliest historical literature on Mexican indigenous groups suggested that Teotihuacan was the first major Totonacan city, which was located approximately 40 km from current day Mexico City[37]. However, this city declined soon after its creation due to issues related to population overburden, famine, drought, and civil unrest[3]. Thus, early Totonacan peoples migrated to Totonacapan to establish several large hubs in Veracruz, including Cempoala and Tajin[25] (**Figure 1 and 2**).

Relatively little is known about the history of this ethnic group until the arrival of Spanish settlers in 1519. As Hernan Cortes moved into the interior of Mexico from the coast, he stopped in Cempoala and convinced the Totonac to help wage war against the Aztecs[3]. After the conquest of the Aztecs, the Spanish decided to move further inland from this region due to the regions humid climate and uneven terrain. This was important from an indigenous standpoint because Totonac peoples were able to maintain a cultural autonomy for many centuries due to limited foreign influence on their land[37]. Despite this independence, many Totonac were still peacefully coerced into the Spanish army, which contributed significantly to the conquest of the Aztecs and other indigenous groups[11].

The Totonac faced numerous problems following these Spanish expeditions. In the late 16th century, the Totonac were ravaged by several mysterious diseases, which were estimated to have destroyed 2,000,000 of their people[36]. Furthermore, Totonac groups coexisted with other native groups and settlers for many years until political turmoil ensued. The Mexican revolution in the 19th century caused mestizos and other groups to invade Totonac villages[25]. This social unrest soon led to economic hardship because conflicts over land ownership arose between native and mestizo groups [25]. As a result of this turmoil, along with the various diseases brought through trans-Atlantic movements over the years, only 200,000 people belonging to this indigenous group today[29]. Despite their complicated history, current-day Totonac peacefully coexist with other surrounding groups and have done much to preserve their customs and traditional farming lifestyles[36].

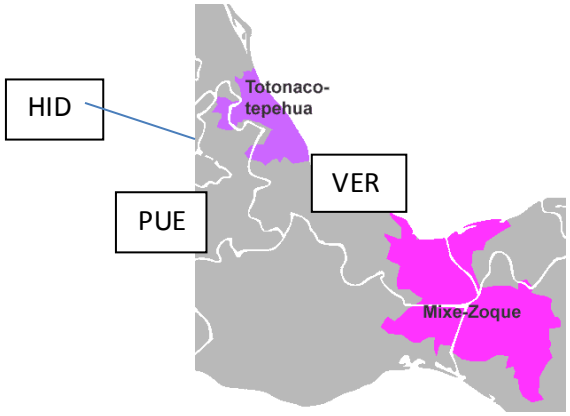

Figure 1. Map of Totozoquean language family

Figure 1. Source: Davius of "Totozoquean" article in *Wikimedia Commons*, May 2011

Figure 1. Map of Totozoquean language family, consisting of two language isolates: the Totonaco-tepehua and the Mixe-Zoque. The Totonaco-tepehua is found in Veracruz (Ver), Puebla (Pue), and Hidalgo (HID) states of Mexico.

In this context, language is also an important variable to understand in order to reconstruct the prehistorical and historical contexts of these and other indigenous Mexican groups (**Figure 2**). The Totonac and Tepehua speak languages that are part of the Totonacan language family[41]. However, this linguistic family, which has been recently hypothesized to be part of the Totonacan and Mixe-Zoque language groups, form their own language family because they both have no genealogical relationship to other languages in Mexico[12]. Despite the lack of linguistic relationship with other groups, researchers have hypothesized considerable borrowing from the Huastec and Aztec languages[36]. In a recent survey, the Totonacan language group was currently spoken by ~290,000 people in the Mexican states of Veracruz, Puebla and Hidalgo[30].

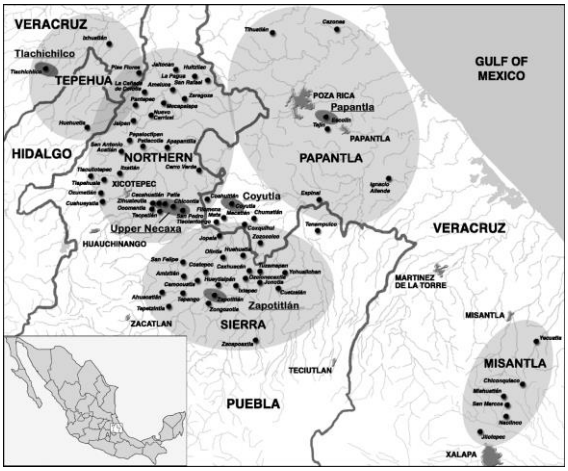

Figure 2. Map of Totonacan language groups (Totonacan and Tepehuan) in Veracruz (VER), Puebla (PUE), and Hidalgo (HID) [29].

Figure 2.The Totonac language family is divided into four primary branches: Northern Totonac, Papantla Totonac, Misantla Totonac, and Sierra Totonac. All four are found within distinct places of Hidalgo, Puebla, and Veracruz.

Although many of these Totonac groups have remained genetically independent from Europeans since their arrival in the 16th century, some have become culturally intertwined through language and other cultural practices [52]. By the 19th century, the Spanish wanted to eliminate all remnants of Totonac customs and traditions in order to retain dominance over this group. Thus, they eliminated traditional religious practices and mandated the teaching of Spanish to the entire populace, particularly young schoolchildren [36]. As a result, the number of speakers of Totonacan languages has dropped by more than half since that time [37].

In this project, we focused on analyzing the genetic diversity of the Totonac in Veracruz and Puebla regions of Mexico, and comparing the resulting data to the genetic diversity of the Nahua, Tepehua, Otomi, Chichimeca and other groups from the neighboring state of Hidalgo. By analyzing the mtDNA data from Totonac individuals, several aspects of their genetic diversity were elucidated. First, we examined the intra-population diversity of the Totonac and related it to historical and linguistic evidence. We then compared the maternal genetic diversity in Totonac with that of surrounding groups (interpopulation diversity). Computational and statistical methods were employed to help elucidate the forces influencing the pattern of diversity of these movements [31,43]. The resulting data provided a detailed biological perspective of the origin of the Totonac, their interaction with other populations, and the influence of language and geography on patterns of genetic diversity in Central Mexico.

2. Materials and Methods

2.1. Research Permissions And Sample Collection

In 2012, researchers from the University of Pennsylvania and Centro de Investigación y de Estudios del Instituto Politéchnico Nacional (CINVESTAV-IPN) travelled to the Mexican states of Veracruz and Puebla in order to enroll Native Mexican participants in the Genographic Project. Prior to taking DNA samples, written consent was obtained from all research participants that were involved, and oral interviews of participant genealogy was obtained, using translators when necessary. After obtaining informed consent, blood and/or mouthwash samples were collected from approximately 60 individuals. Researchers from CINVESTAV-IPN extracted the DNA with the Qiagen Puregene® Blood Core Kit B, following the manufacturer's main protocol. Approval for this study was obtained from the University of Pennsylvania IRB #8 under protocol 803115, CINVESTAV-IPN and La Comisión Nacional para el Desarrollo de los Pueblos Indígenas (CDI) [National Commission for the Rights of Indigenous

Peoples of the United Mexican States].

2.2. Molecular Genetic Analysis

Maternal genetic ancestry was determined through the analysis of mtDNA variation in 60 male and female participants. For all individuals, the HVS1 and HVS2 of the mtDNA control region was directly sequenced. For this analysis, a ~860 base pair (bp) segment of the HVS1 was amplified by polymerase chain reaction (PCR) using 0.25 ul of primers 15997FOR and 269REV (10 pmol dilutions). These primers were combined with a PCR mix containing 1.25 ul 10x Taq Buffer, 0.25 ul dNTPs, 0.05 ul Taq polymerase, 0.75 ul $MgCl_2$, and 7.7 ul H_2O per sample. A 639 base pair (bp) segment of the HVSII region was also amplified using the same method with primers 1FOR and 639REV. Single-stranded DNA was eliminated from the PCR products using 0.1 ul of Exonuclease I, 0.1 ul of tSAP (Thermosensitive Shrimp Alkaline Phosphatase), and 1.9 ul of ddH_2O per sample. A 861 bp segment was primed for sequencing using 0.5 of primers 15977F and 269R (3 pmol dilution), and a mixture of 0.5 ul of BigDye Terminator Pre-Mix v. 3.1, 2 ul Big Dye buffer, and 3 ul H_20 per sample (**Table 1**). After the sequencing products were generated, they were then purified of unincorporated ddNTPs using a mixed solution consisting of 10 ul X-Terminator and 45 ul SAM per sample (ABI).

Table 1. A list of primers and their functions and PCR amplicon lengths

mtDNA Region	Primer Set	Function	Amplicon (bp)
HVSI	15997F/269R	Amplification	861
HVSI	15997F/269R	Sequencing	861
HVSII	1F/639R	Amplification	639
HVSII	1F/639R	Sequencing	639

All samples were also surveyed for SNP variation to confirm their haplogroup identity. SNP genotyping was conducting using Custom TaqMan assays. These assays screened samples for phylogenetically informative single nucleotide polymorphisms (SNPs) that define major branches of the human mtDNA phylogeny (**Table 2**). Thus, multiple confirmatory techniques were used in order to ensure the accuracy of the haplogroup identifications.

Table 2. A list of custom TaqMan assays

Marker	Macrohaplogroup	Ancestral Allele	Derived Allele
mt7256	L3	T	C
mt9540	N	C	T
mt12705	R	T	C
mt14783	M	T	C

2.3. mtDNA Sequence Analysis

Each mtDNA sequence was read on an ABI 3130xl Gene Analyzer and aligned for reading to the Cambridge Reference Sequence[4,5] (rCRS) using the SEQUENCHER 4.8 software tool. Mutations were manually determined through comparison with the rCRS and were confirmed for each sample through the analysis of forward and reverse DNA strands. Samples were assigned haplogroups and haplotypes based on the reference sequence (rCRS) and PhyloTree mtDNA tree, Build 15[49].

2.4. Phylogenetic Analysis

Median-joining (MJ) networks were generated using the mtDNA HVS1 sequences with Network 4.500[7]. To resolve some of the reticulations in the networks, the C16111T mutation was down-weighted to two, the T16311C mutation

was down-weighted to one, and the G16129A mutation was down-weighted to two. All other polymorphisms were set at a default weight of ten. Moreover, mutations at T16182C and T16183C were not used in the phylogenetic analysis due to their different mutational basis (insertion or deletion)[8]. Likewise, the T16519C mutation was not used because of being a hypervariable site and appearing in multiple difference haplogroups. Coalescence time estimations were performed with Network using a mutation rate of 1 mutation per 16,677 years, as described by Soares[46]

2.5. Statistical Analysis

Comparisons that involved pairwise F_{ST} genetic distances within and between populations were calculated from the frequency of HVS1 haplotypes in population, using Arlequin 3.5.1.3[19]. These calculations were based of 100 permutations at a significance level of 0.05. The F_{ST} estimates were then used by Dr. Miguel Vilar to create a multidimensional (MDS) scaling plot in SPSS v. 17.0, to visualize the relative genetic distances among various indigenous Mexican groups. Furthermore, respective p-values for all the pairwise comparisons were estimated in order to provide better insight into the statistical significance of the F_{ST} test[19].

3. Results

Out of 60 individuals sampled, 97% belonged to Amerindian haplogroups and 3% to European haplogroups. All of the Totonacan samples belonged to four haplogroups: A2 (65%; 39 individuals), B2 (26.6%; 16 individuals), C1 (5%; 3 individuals) and H (3.3%; 2 individual), with none belonging to the other Amerindian haplogroups X2a or D1. A total of 31 distinct haplotypes were observed amongst the 54 HVS1 sequences.

3.1 Phylogenetic Analysis Of mtDNA Data

Three MJ networks were created from the Totonac HVS1 sequences. One of them included haplotypes from all three haplogroups, and two others used just A2 or B2 haplotypes. The network containing all of the indigenous haplotypes (**Figure 3**) shows the relative diversity of haplotypes in A2, B2, and C1. Haplogroup C1 showed no diversity because only one haplotype was found in the Puebla population. As seen in **Figure 4a**, haplogroup A2 contains the highest amount of diversity as illustrated by the 11 branches originating from the large central ancestral node (n=14). In addition, most of the derived lineages have individuals with secondary branches. With few exceptions, there were no missing internodes, which indicated that there has been a significant loss of diversity over time in the Totonac.

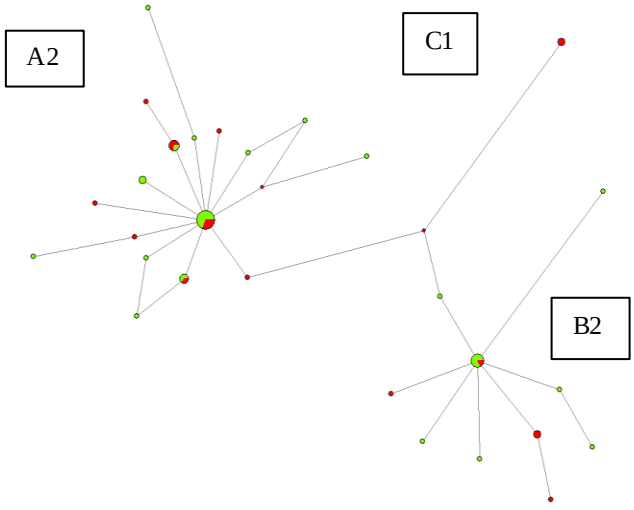

Figure 3. HVSI network for all Totonacan HVS1 sequences.
Figuere 3. The green color represents samples from Veracruz and red color represent those from Puebla.

Figure 3. Each node (circle) corresponds to a distinct single nucleotide polymorphism. Clusters of SNPs denote certain haplotypes, which are indicative of the diversity of entire haplogroups.

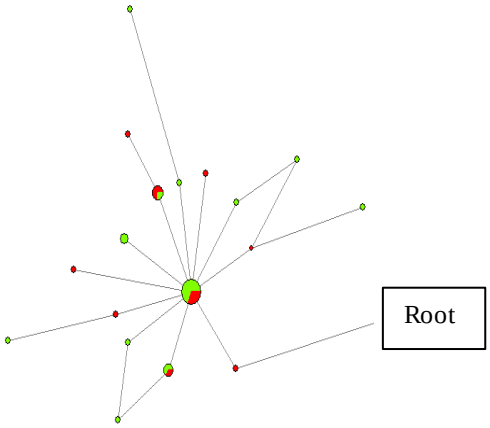

Figure 4a. HVSI network of A2 haplotypes in the Totonac. The central node represents the ancestral haplotype containing mutations C16111T, C16223T, C16290T, 16319A, T16362C.
Figure 4a. Each branch corresponds to a mutational event that leading to a derived haplotype.

Figure 4a. Each node (circle) corresponds to a distinct single nucleotide polymorphism. Combinations of SNPs denote certain haplotypes indicated by the branching. All of these haplotypes belong to haplogroup A.

The MJ network for haplogroup B (Figure 4b) also demonstrated some diversity from the ancestral node (n=5), albeit not as large. It contains seven secondary derived lineages surrounding the ancestral node along with some secondary branching. In this haplogroup, however, there appears to be no sharing of derived lineages between the Veracruz and Puebla Totonac populations.

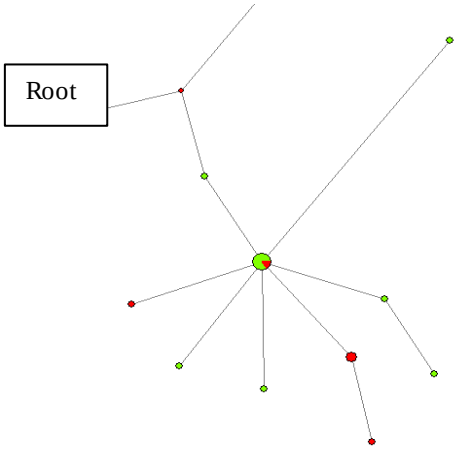

Figure 4b. The central node represents the ancestral haplotype containing mutations 16189C and 16217C.
Figure 4b. Each branch corresponds to a mutational event that leading to a derived haplotype.

Figure 4a. Each node (circle) corresponds to a distinct single nucleotide polymorphism. Combinations of SNPs denote certain haplotypes indicated by the branching. All of these haplotypes belong to haplogroup B.

3.2 Statistical Analysis Of Mtdna Data

There were two primary clusters in the MDS plot of F_{ST} estimates based on HVS1 sequence data (**Figure 5**). The two Totonac populations (one from Veracruz and one from Puebla) were relatively close to one another and formed the first cluster. The Totonac from Puebla were also somewhat closer to the central population cluster including the Otomi, Tepehua, and Nahua. The Otomi, Tepehua, and Nahua populations all reside in the state of Hidalgo, and form the second population cluster. The Chichimeca from Guanajato were not part of either population cluster and remained isolated from them, as seen in the top left corner of the plot.

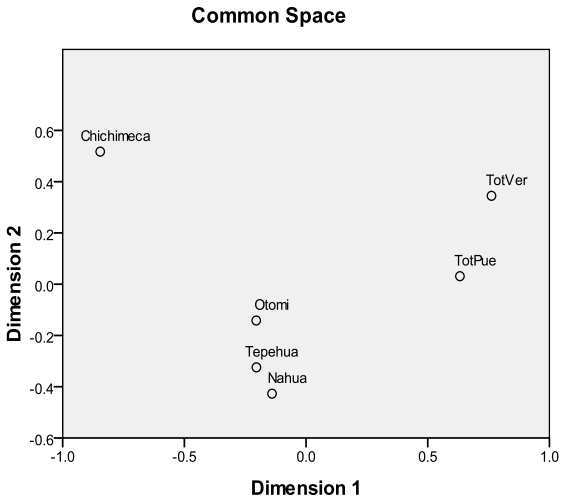

Figure 5. A MDS plot of F_{ST} estimates based on HVS1 sequence data for six Native Mexican populations. Figure 5. Source: Schurr et al., unpublished data

Figure 5. An MDS plot tracks the genetic distances between various populations. As illustrated by the points, the Otomi, Tepehua, and Nahua are clustered together. In addition, the Totonac from Veracruz and Puebla are also close to one another. This indicates that these two clusters share genetic similarity between each other.

4. Discussion

The distribution of mtDNA haplogroup frequencies in the Totonac was relatively consistent with that for other Mesoamerican groups in the surrounding area. In recent studies, Mesoamerica is often characterized by high frequencies of haplogroup A2, lower frequencies of two other two major haplogroups B2 and C1, and, in most cases, very low frequencies of haplogroups D1 or D4h3[28,34,38,43].

Haplogroup frequencies in the Totonac were also similar to data from Totonacan groups obtained by Watkins et al.[51]. For example, 66% of all samples in the Watkins et al. data set[51] were A2 compared to 67% in our Totonac samples. Haplogroups B and C also showed similar frequencies, although our data set also had two H mtDNAs. The presence of H mtDNAs suggests non-native admixture in our Totonac samples, which wasn't evident in the Watkins et al. study[51]. Such data could indicate a more interrelated and dynamic history of indigenous Mexicans than originally proposed by Mesoamerican historians and anthropologists alike[1,21,22,24,48].

The haplogroup A2 network showed a star-like pattern with many individual branches emerging from the ancestral node (C16111T, C16223T, C16290T, G16319A, T16362C). Haplogroup B2 had a similar pattern with a few short branches from the ancestral node (T16189C, T16217C). A star-like pattern is commonly seen in A2 networks from other indigenous Mesoamerican studies and suggests a demographic expansion since populations colonized the Americas [24,43]. The limited number of haplotypes in and reduced diversity of the haplogroups and the coalescence time of 16,203.4 ± 4069.43 for A2 and 15,689 ± 475.2 years for B2 suggests these two lineages arose relatively early in Mexican prehistory.

The MDS plot of the F_{ST} genetic distances suggested that linguistic affiliation rather than geography has been more influential in shaping the genetic composition of the Totonac (**Figure 7**). The two Totonac groups (Veracruz and Puebla) speak the same language and are positioned close to each other on the MDS plot. However, geography does play some role, albeit minor, in shaping genetic variation, as the Totonacs from Veracruz and Puebla were more genetically similar to the Tepehua, Nahua, and Otomi from Hidalgo than the Chichimeca from Guanjato.

The only C1 haplotype in the Totonac is rare. It contains a C16327T back mutation, which is not present in other C1 haplotypes in Mexican populations like the Otomi, Nahua and Tepehua. The emergence of this SNP probably resulted from a back mutation[18]. Stochastic factors, such as non-random mating and small sample sizes in the parental population, could have reduced the frequency of this haplotype, perhaps through genetic drift. Although selection is another important genetic pressure, it cannot be factored into this situation because HVSI and HVSII mutations are located in non-functional region of the mtDNA. Thus, these haplotypes are not likely to be under selection[16]. However, it is difficult to infer the exact origin of this haplotype, and further analysis of the HVSII region and the mtDNA coding region sequence for this sample must be conduct to learn more about its history.

5. Conclusions

The purpose of this study was to use molecular genetic techniques to characterize genetic variation in the Totonac and determine the historical, cultural and linguistic factors influencing it. Previous studies have typically included only theoretical information about this group from archaeological studies. There has been little archaeological analysis done on sites related to this ethnic group[15,47]. However, in an earlier study, Watkins et al.[51] noted that the Totonac belonged to haplogroups A2, B2, C1, and D1, consistent with its pre-Columbian New World maternal ancestry. Similar to the Watkins et al. study[51], we noted that A2 was the dominant haplogroup in our Totonac populations, whereas they lacked haplogroup D mtDNAs all together.

In the future, additional steps will help to enhance the existing Totonac mtDNA data analysis. For one, it will be necessary to complete the sequencing of the HVSI and HVSII regions for all these samples in order to verify the haplogroups (and fully define their CR haplotypes). Most of this work is already in progress, and will be finished within the next month. If possible, it would also be informative to sequence the entire mtDNA genomes to check for other sub-haplogroup defining SNPs that could verify the diversity seen with control region sequences. In addition, mitogenome data is also important for resolving phylogenetic relationships among mtDNAs and provide insights into Native Mexican phylogeography[39]. Furthermore, mitogenome analysis could be important to study functional regions of mtDNA, which could be important for analyzing selective factors and their roles on a population's genetic diversity[33].

In addition, it will be important to conduct Y-chromosome analysis in the Totonac to compare the paternal genetic diversity with the maternal genetic diversity. This comparison will be beneficial because it can provide a paternal perspective on population narratives and genetic structure[43]. Together, these data sets will help to delineate the role of the Totonac in the larger historical and cultural context of indigenous Mexicans.

6. Acknowledgments

I would like to thank Drs. Theodore Schurr and Miguel Vilar for their numerous hours of contribution towards this research project. In addition, I would like to thank CURF for providing me funding through Pincus-Magaziner Award. The Genographic Project, in which this study is a smaller analysis, is supported by the National Geographic Society, IBM, and the Waitt Family Foundation, and the University of Pennsylvania.

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