# Supplementary Information Appendix

# **The earliest maize from San Marcos Tehuacán**

# **is a partial domesticate with genomic evidence of inbreeding.**

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The authors declare no conflicts of interest.

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## **Materials and Methods.**

## **Archaeological Excavation, Sampling, and Dating.**

San Marcos cave is located in the limestone cliffs of the southeastern part of the so-called Tecorral Canyon, an arroyo depression that follows a northwest-southeast direction, ending about two kilometers West of the center of San Marcos Necoxtla. We surveyed 1-meter squares (quadrants) that were kept unexplored during the MacNeish expedition (*1*); these quadrants were either located East of the 7 to 9 meters long rectangle that was explored in 1962 (quadrants N1E2 and N1E3), in a small portion located close to the southwestern wall (W3), or below the large unremoved rock located at the center of the cave, within the surface that remained covered by its roof. Most of the area surveyed prove to be unaltered as the stratigraphy was well contextualized and respected the countour maps down to the rock floor, reaching surfaces of Zone D and E, as previously reported (*1*). All excavations were conducted under authorization and guidance of Consejo de Arqueologia, Instituto Nacional de Antropologia e Historia (INAH, México). Geographic and quadrant information followed coordinates reported in (*1*). Sampling was performed following all necessary procedures to avoid human-related or cross-sample contamination by wearing shoe covers for personnel working within the excavation quadrants, hazmat coverall suits, nitrile gloves, and surgical masks. Specimens were collected using previously bleached forceps, wrapped in aluminum foil, and placed in individual sealed bags that were kept at the ancient DNA facilities of Langebio CINVESTAV. For dating, 10 to 20 mg of each specimen was dated by Accelerator Mass Spectrometry (AMS) using the service provided by Beta Analytic (Miami, Florida); the Beta Analytic reference number is provided in Table S1 .

We recovered nine well-preserved macro-specimens of maize. All specimens except one (a carbonized cob corresponding to specimen SM4) were morphologically analyzed and sampled for accelerator mass spectrometry (AMS) dating. The most ancient specimen (SM10) was dated  $4240 \pm 30^{14}$ C years BP (5300 to 5040 2 $\sigma$  calibrated age years BP at 95% confidence). Three other specimens found in distinct sedimentary quadrants (SM3, SM5, and SM9) were dated 4220 to 4180 <sup>14</sup>C years BP (5300 to 4970  $2\sigma$  calibrated age years BP at 95% confidence). Three additional specimens (SM6, SM7, and SM8) were dated 3550 to 3500<sup> $14$ </sup>C years BP (about 4350 to 3990 calibrated years BP), and two (SM1 and SM2) were dated 1350 to 1310 <sup>14</sup>C years BP (about 1540 to 1400 calibrated years BP ; Table S1). In the case of the four most ancient specimens, SM9 and SM10 were two cobs morphologically reminiscent to those found in Zone E during the MacNeish expedition. These cobs had a fragile rachis and eight rows of kernels, as most of the cobs previously found in Zone E (*1*); their length was 34 and 30 mm, respectively. Their spikelet glumes were soft and long, confirming that the earliest maize found in San Marcos was tunicated; whereas SM10 was a complete cob devoided of kernels, the glumes of SM9 were missing in about one third of the apical portion, and folded back in the mid-region, suggesting manual removal of seeds. By contrast, SM3 was a well-preserved basal stalk fragment devoided of tillers with an estimated stem diameter of 20 mm. It included the first aerial internode and a basal portion containing the primary root system and close to 15 secondary roots. Finally, SM5 was an aerial leaf sheet containing part of the internode and a basal portion of a husk fragment that appeared to have been chewed. These results show that the new exploration of San Marcos cave yielded a small collection of non-manipulated specimens that are equivalent in age and state of preservation to some of the specimens originally collected during the MacNeish expedition, and currently preserved in several private or public collections.

## **Sequencing of Ancient Samples.**

Three SOLiD (for SM3 and SM10) and four indexed Illumina (SM3, SM5, and SM10) DNA libraries were built for subsequent shotgun sequencing. In summary, fragment length distributions in the aDNA extracts were determined with a High Sensitivity DNA Assay Chip Kit (Agilent, Waldborn Germany) on a Bioanalyzer 2100. Size-based selection targeting 100 bp DNA fragments (size select) was performed using Agencourt® AMPure® XP (Beckman & Coulter Inc, California USA) according to manufacturer's instructions. Genomic libraries were constructed using Illumina TruSeq Nano DNA LT library prep kit (cat no. FC-121- 4001) and SOLiD library preparation 5500 series kit (SOLiD, Part Number. 4460960 Rev. A), with 9 cycles of PCR for each library amplification. Fragmentation steps were avoided. DNA library concentration was quantified by qPCR and using a Bioanalyzer assay. Detailed results are shown in Supplementary Table S2. SOLiD libraries were sequenced at the Genomic Core Facility of PennState University, and Illumina libraries were sequenced at Langebio CINVESTAV or the Core Services at the University of California at Davis. SOLiD color space libraries were filtered to remove all reads with missing bases and converted to standard base space fastq-format files using SoliD2std.pl.

## **Read Processing, Mapping, and Genotyping.**

Index sequences of 16 nucleotides were used to tag libraries described above. Only reads with the correct index were used in downstream analysis. All libraries were filtered to remove adaptors and low quality reads using Cutadapt (*2*) and keeping reads longer than 50 bp with a quality above 10 Phred score. Repetitive adenines (A) and thymines (T) were invariably removed from read ends. Filtered reads were mapped using the BWA MEM algorithm with default conditions (*3)*. *Zea mays* B73 RefGen\_v3 (*4*) was used as the reference sequence after masking repetitive genomic regions with RepeatMasker (*5*). Reads with multiple hits were removed using *SAMtools* map quality filters. As a clonal removal strategy, sequence duplication in reads was filtered with the *rmdup* function of *SAMtools* (*6*), and sequences were locally re-aligned around insertion/deletions (indels) using GATK IndelRealigner (*7*). A map quality filter of a minimum value of 20 Phred score was applied in order to eliminate reads with low certainty assignments. This resulted in an average Phred score of 51 for SM10 and 50 for SM3, and a mapping efficiency of 15.19% for SM10 and 2.18% for SM3. SNP and genotype calling was performed as previously described *(8-10*). Variation information was extracted and called using the *mpileup* and *bcftools* functions of *SAMtools* (*6*), generating a VCF file containing genotypes based on a majority rule and requiring a minimal depth-ofcoverage of 2.

### **Metagenomic Analysis and Postmortem Damage.**

Cytosine deamination rates and fragmentation patterns were estimated using mapDamage2.1 (*11*) based on all reads mapping to the B73 reference genome, revealing expected patterns of postmortem damage in the form of C>T substitutions at the 5' termini, and G>T substitutions at the 3' termini. The excess of purines observed near read-termini furthermore supports fragmentation driven by depurination (Figure S1). All Indels and sites behaving as molecular damage (CG->TA) (*12,13*) were excluded. A metagenomic filter was applied in order to discard reads that aligned to sequences in the GenBank NCBI database of all bacterial and fungal genomes using default mapping quality parameters of BWA (*3*).

## **Evolutionary Analysis and SNP Genotype Comparisons.**

Patterns of divergence were analyzed by generating maximum likelihood (ML) trees using Treemix (*14*) and the intersection of SNPs passing quality filters for the ancient specimens and 44 selected individuals of the publically available database HapMap3 without imputation (*15*). The list of selected individuals is presented in Table S3. The topologies were generated with each ancient sample individually or including both samples together. In each case, no less than 10,000 bootstrap pseudoreplicas were generated with a parallelized version of a public script (*16*), which uses the *sumtree* function in *DendroPy* (*17*) to obtain a consensus ML bootstrapped tree. The same SNP alignments were also used to assign the identity of each ancient SNP genotype to shared or exclusive SNP genotypes of the selected HapMap3 individuals. According to their SNP identity, the ancient genotypes were assigned exclusively to one of six categories: B73 genotypes, maize landraces genotypes, *Zea mays* ssp. *parviglumis*, *Zea mays* ssp. *mexicana*, *Tripsacum dactyloides*, or those not present in the dataset. Neighbor-joining topologies were generated on the basis of 100,540 shared SNPs between SM10, SM3 and HapMap3 that were concatenated for each genotype and imported into MEGA 7 software (*18*). Analysis was performed by applying the p-distance method and uniform rates at all transition and transversion sites, applying a test based on 1000 replicates of the bootstrap method. A pairwise deletion method was applied on missing data, and resulting trees were visualized using figtree software.

## **Nucleotide Variability and Frequency of Segregating Sites at Domestication Loci.**

The genomic coordinates of selected loci previously reported as affected by domestication were obtained from B73 RefGen\_V3 from MaizeGDB (*4,19*). All SNPs represented in HapMap3 (15) from more than 1,180 extant maize and 18 Balsas teosinte accessions were identified and compared to quality mapped sequences obtained for SM10. *Zea mays ssp. mexicana* was excluded to avoid any overestimation of nucleotide diversity in the teosintes. For each HapMap3 mapping to a 20 Kb region spanning a selected gene known to be affected by domestication, the corresponding nucleotide variant from SM10 was identified and compared to all nucleotide variants present in the Balsas teosinte or extant maize accessions. The relative frequency of each allele present in at each independent site was plotted, assigning the same color to identical nucleotide variants present in extant maize, Balsas teosinte and SM10. Color assignment is independent at each site. Additionally, the average value of the genetic diversity index θ was calculated for each class (Balsas teosinte and extant maize), and compared to the θ value for SM10 and *Palomero toluqueño*. For estimation of the frequency of segregating sites per individual (FSSI) at each locus, the number of independent sites showing at least two nucleotide variants was divided by the total number of sites covered, and subsequently divided by the total number of accessions in each group for either Balsas teosinte or extant maize. In the case of Hufford et al. regions previously identified as having a general tendency to be selected during domestication (*20*), filtered reads mapping to these regions defined segments of SM10 coverage that were used to calculate the genetic diversity index θ for maize landraces (23 accessions) and Balsas teosinte (15 accessions). θ values were compared using the Kolmorov-Smirnov non-parametric test (KS test); a false discovery rate (FDR) was applied to p values obtained for the KS test with a 0.05 cut-off value. Only segments having a FDR<0.05 were considered for  $\theta$  comparison between landraces and SM10, by correcting significance values for null hypothesis using an R library described as p.adjust. Since no formal tests of likelihood can be calculated without a distribution for ancient maize (SM10 represents a single sample), we compared the value of  $\theta$  in SM10 to the one sigma θ value interval for the landraces as a test for significance.

## **Estimation of identity by descent (IBD).**

Identity by descent (IBD) was calculated using *plink* V1.9 (*21*) using either all single nucleotide variants common ancient samples (in pairwise comparisons) or all heterozygous SNPs shared between ancient maize samples and B73. To avoid any bias caused by low-depth coverage, only bi-allelic sites with at least 10X depth coverage were used for IBD calculation in all pairwise comparisons between SM3, SM5, and SM10.



# **Table S1. Radiocarbon and calibrated dates of new maize specimens from San Marcos cave.**

a Following coordinates described in (*1*)



**Table S2. Paleogenomic characterization of two ancient maize samples from San Marcos cave.**



**Table S3. Distribution, coverage and depth of total mapped reads from SM3 and SM10 ancient maize samples across the 10 chromosomes of the B73 maize reference genome.**

Depth	SM <sub>3</sub>	<b>SM10</b>
$\mathbf{1}$	41,420,620	88,078,128
$\boldsymbol{2}$	16,083,544	46,494,648
3	3,946,553	22,845,457
$\overline{\mathbf{4}}$	2,026,559	11,431,780
5	690,923	5,819,978
6	404,726	3,098,243
$\overline{7}$	207,294	1,787,255
8	139,704	1,110,243
9	94,134	14,444
10	72,221	16,277
>10	488,693	597,616

**Table S4. Total number of unique genomic sites covered at variable depths in SM3 and SM10 ancient maize samples** 



# **Table S5. Description of all HapMap3 and ancient maize genotypes included in this study.**



**Table S6. Gene identity, number of sites with SM10 coverage, and average number of modern maize and Balsas teosinte accessions included per each domestication locus used in this study.**

<sup>a</sup> INDELs were discarded from the analysis

## **Table S7. Comparison of** θ **at each selected locus affected by domestication.**



<sup>a</sup>In the cases of extant maize and Balsas teosinte, the estimate represents the average of the genetic diversity index θ per individual for more than 1,180 maize accessions at 15 Balsas teosinte individuals, respectively. Values in blue represent a deviation from the 1 sigma range of extant maize.

# **Table S8. Frequency of segregating sites per individual.**



<sup>a</sup>For extant maize and Balsas teosinte, the average takes in consideration all pairwise comparisons between each individual and B73, across a region of 25Kb spanning each selected gene.











Regions in green correspond to a θ difference between SM10 and extant landraces, i.e. beyond the 1 sigma value of for extant landraces within the shared segment (FDR Teosintes vs Landraces <0.05).

Regions in blue do not show a θ for SM10 beyond the 1 sigma value of for extant landraces within the shared segment (FDR Teosintes vs Landraces <0.05).

Regions in yellow show FDR Teosintes vs Landraces >=0.05 within the segment covered by SM10.

**ID**: Region number following reference (*20*).

**Region Coordinates**: chromosome number and B73 genomic coordinates for each region.

**Mean** θ **Region**: mean value of the θ index for maize landraces calculated for each region.

**St. Deviation** θ **Region**: standard deviation of the θ index for maize landraces calculated for each region.

**Mean** θ **Segment**: mean value of the θ index for maize landraces calculated for the segment covered by SM10 sequence.

**St. Deviation** θ **Segment**: standard deviation of the θ index for maize landraces calculated for the segment covered by SM10 sequence.

θ **for SM10:** value of θ for SM10 sequence within the segment.

**FDR Teosintes vs Landraces:** false discovery rate corresponding to a non-significant difference in genetic diversity between Balsas teosinte and extant landraces within the segment of SM10 coverage.

**Coverage SM10**: total length of the segment covered by SM10 sequence (in nucleotides).

## **Table S10. Comparison of single nucleotide polymorphic variants among ancient samples and extant maize.**



<sup>a</sup>Comparison of three ancient samples from San Marcos cave.<br><sup>b</sup>Comparison of three randomly selected *Cacahuacintle* individuals from an open-pollinated population of 4,500 plants.

#### **Identity by Descent (IBD)**



<sup>ap</sup>robability that at any given SNV 0 alleles are identical by descent

Probability that at any given SNV 0 alteres are identical by descent<br>
<sup>b</sup>Probability that at any given SNV 1 allele is identical by descent<br>  $P$ robability that at any given SNV 2 alleles are identical by descent<br>  $P$ ropor

**Table S11. Genetic comparison of single nucleotide polymorphic variants among three ancient maize samples.**



aPolymorphic sites correspond to those having at least one identical variant with the B73 reference genome



# **Table S12. Estimation of heterozygosity in SM10, selected genotypes of HapMap3, and a maize open-pollinated landrace individual.**

<sup>a</sup>Based on 12,999,553 shared SNPs between the SM10 genome and selected members of the HapMap3 panel of diversity.

<sup>b</sup>Based on 45,281 shared polymorphic sites between SM10 and CCH1, as compared to the B73 reference genome



**Figure S1. Post-mortem DNA damage and fragmentation patterns of ancient maize samples SM3 and SM10.** DNA composition around read-termini (top four plots), and DNA mis-incorporation errors relative to the 5' and 3' read (bottom plot) ; the two distributions for *post-mortem* damage signatures (C>T and G>A) are shown in red and blue respectively, while other types of substitutions are shown in gray.



**Figure S2. Total number of covered sites of the unique genome for SM3 and SM10 ancient maize samples.**



**Figure S3.** Venn diagram illustrating the distribution of SNPs shared by SM3, SM10, and the HapMap3 group of 15 Balsas teosinte and 22 extant maize landraces accessions (*15*).



**relatives.** Maximum likelihood tree from an alignment of 100,540 genome-wide SNPs covering nonrepetitive regions of the reference maize genome. SM3 and SM10 represent two maize samples dating 5300-4970 calibrated years BP; SNPs obtained from 77,960,582 mapped reads of the *Palomero Toluqueño* landrace (PT2233) were also included in the analysis. The teosinte and landrace accessions follow the previously reported nomenclature (*20*).



**Figure S5. Evolutionary relationships between SM3 ancient maize and its wild or cultivated relatives.** Maximum likelihood reconstruction from an alignment of 201,450 genome-wide SNPs covering non-repetitive regions of the reference maize genome. SM3 represents a maize sample dating 5280-4970 cal. years BP; the teosinte and landrace accessions follow the nomenclature reported in (*20*), and described in Table S5.



**Figure S6. Evolutionary relationships between SM10 ancient maize and its wild or cultivated relatives** Maximum likelihood reconstruction from an alignment of 892,033 genome-wide SNPs covering non-repetitive regions of the reference maize genome. SM10 is a maize sample dating 5040- 4970 years BP; the teosinte and landrace accessions follow the nomenclature reported in (*20*), and described in Table S5.

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**Figure S7. Evolutionary relationships between both SM3 and SM10 ancient maize samples and its wild or cultivated relatives.** Maximum likelihood reconstruction from an alignment of 13,079 genome-wide heterozygous SNPs showing at least 10X coverage and corresponding to non-repetitive regions of the reference maize genome. SM3 and SM10 represent two maize samples dating 5300- 4970 years BP; the teosinte and landrace accessions follow the nomenclature reported in (*20*), and described in Table S5.



**Figure S8.** Maximum likelihood topology based on 1,665,533 SNPs of OAX70 obtained from the same sequence dataset as the one used in HapMap3 (OAX70\_2), but independently called with the pipeline used in this study. Although the intra-lineage topology is modified on the basis of the nature of the SNP dataset, both OAX70 samples group adjacently within the landrace lineage; accessions follow the nomenclature reported in (*20*), and described in Table S5.



Figure S9. Neighbor-joining tree of ancient (blue) and domesticated maize (red), as well as their wild teosinte relatives (green). A total of 100,540 SNPs shared between SM3, SM10, and selected HapMap3 accessions were concatenated for each genotype. SM3 and SM10 represent two maize samples dating 5300-4970 years BP; the teosinte and landrace accessions follow the nomenclature reported in (*20*), and described in Table S5.



**Figure S10. Distribution and density maps of single nucleotide variants having at least 10X coverage for the three ancient maize genotypes SM3, SM5 and SM10**. (A) Total number SNVs called in each genotype; it includes both homozygous and heterozygous variants. (B) Only SNVs corresponding to heterozygous variants.



**Figure S11. Comparison of single nucleotide polymorphic variants (SNVs) among ancient samples and extant maize.** Pairwise comparisons of three ancient samples from San Marcos cave (SM3, SM5, and SM10) as compared to pairwise comparisons of three randomly selected *Cacahuacintle* landrace individuals from an open-pollinated population of 4,500 plants (CCH1, CCH2, and CCH3). All values are given as a percentage of total.

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