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Evolution

Evolution of maize inferred from sequence diversity of an *Adh2* gene segment from archaeological specimens

(*Zea*/molecular archaeology)

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ABSTRACT **A segment of the nuclear gene encoding alcohol dehydrogenase 2 (*Adh2*) was amplified and sequenced from extracts of archaeological maize specimens up to 4700 years old and from contemporary samples. Sequence diversity in ancient maize equals that of contemporary maize. Some ancient *Adh2* alleles are identical or closely related to contemporary alleles. The data suggest that the gene pool of maize is millions of years old and that domestic races of maize stem from several wild ancestral populations.**

The archaeological record indicates a sudden appearance, in Central America, of domesticated maize about 7000 years ago (1). Protein (2, 3), DNA (4–6), genetic, and cytogenetic (7–10) data imply that domestic maize (*Zea mays mays*) originated from a wild subspecies of teosinte, either *Zea mays mexicana* (7, 10) or *Zea mays parviglumis* (5, 6). One of the properties of maize is the spectacular diversity in morphology among races, which is seemingly paralleled by an extensive allelic variation as detected by molecular methods (2–4, 11–15). Although no correlation between morphological and genetic diversity has yet been shown in other organisms (14, 16, 17), the dramatic morphological and genetic diversity of maize has caused some geneticists to contemplate the idea that molecular evolution in maize has proceeded much faster since its domestication (3, 11, 14, 18). To investigate this question, we amplified a part of the nuclear gene alcohol dehydrogenase 2 (*Adh2*) from ancient maize remains, modern maize, and teosinte samples.

MATERIALS AND METHODS

Sources of DNA. Nucleic acids were extracted from archaeological and contemporary specimens of maize, teosinte, and *Tripsacum*. Table 1 describes the geographical affiliations of the samples and their estimated ages.

The specimen "Cabuza" comes from ≈10 noncharred kernels still attached to the same corn ear, from a burial site on the northern coast of Chile. The age of 1500 ± 50 years is based on cultural items.

The specimen "Charred" is a conglomerate of charred kernels and cobs from Junín, Peru. The elevation is 3700 m. The period is Wanka-II, dated 440 ± 30 years ago by ^{14}C.

The specimen named "Proto-Confite-Morocho" is a maize cob from the north coast of Peru, dated 4700 ± 500 years ago by thermoluminescence (19).

The *Adh2* sequence reported here for the inbred line named "Berkeley Fast" differs from the published sequence (20) by 3 nucleotides that are otherwise uncharacteristic of the *Zea* and *Tripsacum*. We used our sequence (the *BF* allele, Fig. 3) for the tree analysis.

Other sources and addresses (Table 1): USDA, U.S. Department of Agriculture; M. Freeling, Plant Biology Department, University of California, Berkeley; D. Bonavia and A. Grobman, Universidad Peruana, Cayetano Heredia, Lima, Peru; C. A. Hastorf, Department of Anthropology, University of Minnesota, Minneapolis; J. Tenney, Department of Pathology, Northern Inyo Hospital, Bishop, CA; NS/S, Native Seeds/Search, Tucson, AZ; J. Doebley, Department of Botany, University of Minnesota, St. Paul.

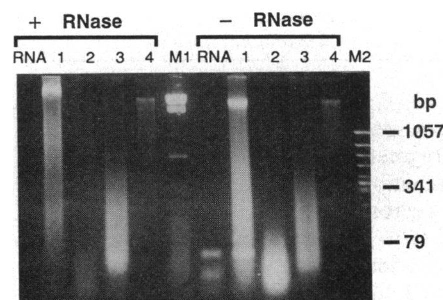

FIG. 1. Gel electrophoresis of nucleic acids extracted from modern and ancient maize specimens. A 15-μl sample of each DNA was loaded onto a 1.5% agarose gel and the gel was stained with ethidium bromide after electrophoresis. Lanes: RNA, 0.75 μg of RNA from yeast; 1, extract from modern Tabloncillo maize, Mexico; 2, 4700-year-old Proto-Confite-Morocho maize, Peru; 3, 1500-year-old Cabuza maize, Chile; 4, 440-year-old charred maize, Peru; M1, 0.2 μg of λ phage DNA cut with *Hin*dIII; M2, 0.1 μg of φX174 phage replicative form DNA cut with *Hin*cII. DNA extracts and RNA controls were treated for 30 min at 25°C with (+) or without (−) bovine RNase (10 μg/ml, Sigma type I-A).

Extraction of Modern and Ancient DNA. Between 300 and 700 mg of ancient and modern plant specimens was reduced to a thin powder in a DNA-free coffee grinder and then resuspended in 3 ml of sterile 10 mM Tris·HCl, pH 8.0/10 mM NaCl/10 mM dithiothreitol containing proteinase K (Boehringer Mannheim) at 0.4 mg/ml and 1% sodium dodecyl sulfate. After 15 hr of incubation at 37°C, the sample was extracted twice with phenol (pH 8.0; 1:1, vol/vol) and once with chloroform/isoamyl alcohol (24:1, vol/vol). The aqueous phase was filtered on a microconcentrator (Centricon-30, Amicon). The material on top of the membrane was washed four times with 3 ml of sterile water. The final volume of the samples was reduced to ≈300 μl.

DNA Primers. The target sequence is a fragment of the *Adh2* gene of variable size (315 ± 15 bp), spanning between positions 85 and 403 from the transcription start site of the gene. It includes the untranslated promoter region, exon 1, intron 1, and exon 2 (20). The restriction sites for *Eco*RI and *Hin*dIII were added to the sense primer 1 (5′-CTAAGAATTCTCGTGTTCTTGGAGTGGTC-3′) and to the antisense

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Table 1. Specimens used and alleles found

Plant	Specimen identity	Age, yr	Location	Source	Alleles found
Modern maize	Northern-Flint	Modern	Northeast USA	USDA 213760	*1A, 1B*
	Berkeley Fast	Modern	Corn belt USA	Freeling	*BF*
	Confite Morocho-4	Modern	Coast of Peru	Bonavia, Grobman	*3*
	Tabloncillo	Modern	Central Mexico	USDA 2835	*4*
	Kculli-47	Modern	Coast of Peru	Bonavia, Grobman	*5*
Ancient maize	Charred	440 ± 40	Peru highlands	Hastorf	*6*
	Cabuza	1500 ± 50	North Chile	Tenney	*7A, 7B, 7C*
	Proto-Confite-Morocho	4500 ± 500	Coast of Peru	Bonavia, Grobman	*8A, 8B*
Teosinte	*Zea mays mexicana*	Modern	Mexico highlands	NS/S Z121	*9A, 9B*
	Zea mays parviglumis	Modern	Mexico lowlands	Doebley GB-sn	*10A, 10B*
	Zea diploperennis	Modern	Jalisco, Mexico	NS/S Z120	*11A, 11B*
	Zea luxurians	Modern	Guatemala	Doebley HI G-5	*12A, 12B*
Tripsacum	*Tripsacum pilosum*	Modern	Jalisco, Mexico	Doebley JD467	*TP*

primer 2 (5′-CAGTAAGCTTCTGCGGCTAGAGATG-CAGC-3′), respectively, to facilitate subsequent cloning of the DNA fragments that had been amplified by the polymerase chain reaction (PCR). Amplified fragments were cloned in an M13 vector and sequenced.

PCR. *In vitro* DNA amplification was performed in the presence of primers 1 and 2, two units of *Taq* DNA polymerase, and 1 μl of various DNA extracts, in a final 25-μl reaction volume. Amplification was carried out in a Perkin–Elmer/Cetus thermal cycler for 40 cycles as follows: 40 sec at 93°C, 1 min at 55°C, and 1.5 min at 72°C. The reaction products were digested with the restriction enzymes *Eco*RI and *Hin*dIII and further purified by electrophoresis in a 4% low-melting agarose gel. DNA fragments were cloned in mp19 bacteriophage (21). Positive clones were sequenced with Sequenase (United States Biochemical).

RESULTS

Quality of Ancient DNA. The extract from modern maize presents long RNase-resistant molecules and shorter RNase-sensitive molecules, of the size expected for rRNA and tRNA molecules (Fig. 1, lane 1). Extract from the 4700-year-old specimen yields mainly short RNase-sensitive molecules and traces of longer (500- to 2000-bp) RNase-resistant molecules (Fig. 1, lane 2). Remarkably, despite its charred appearance, the 440-year-old specimen contains traces of long RNase-resistant molecules (Fig. 1, lane 4), whereas most of the molecules from the macroscopically well-preserved 1500-year-old Cabuza sample are short (20–250 bp), though mostly RNase-resistant (Fig. 1, lane 3). This confirms reports (22) of both RNase-resistant and RNase-sensitive nucleic acid molecules in extracts of ancient maize remains. Interpretation calls for caution, since DNA contamination from bacteria and fungi is possible. Furthermore, lesions in ancient DNA, such as baseless sites, are sensitive targets to RNase (23–25). At the moment, we conclude that the successful amplification of nuclear sequences from these samples (see below) demonstrates that these extracts contain some genuine ancient DNA.

Reconstruction of Ancient Alleles. In Fig. 2, the sequences of 12 clones from the 4700-year-old Proto-Confite-Morocho maize cob are shown as an example of how ancient alleles were reconstructed from clones of PCR products. Two length mutations and nine base substitutions (Fig. 2, arrows),

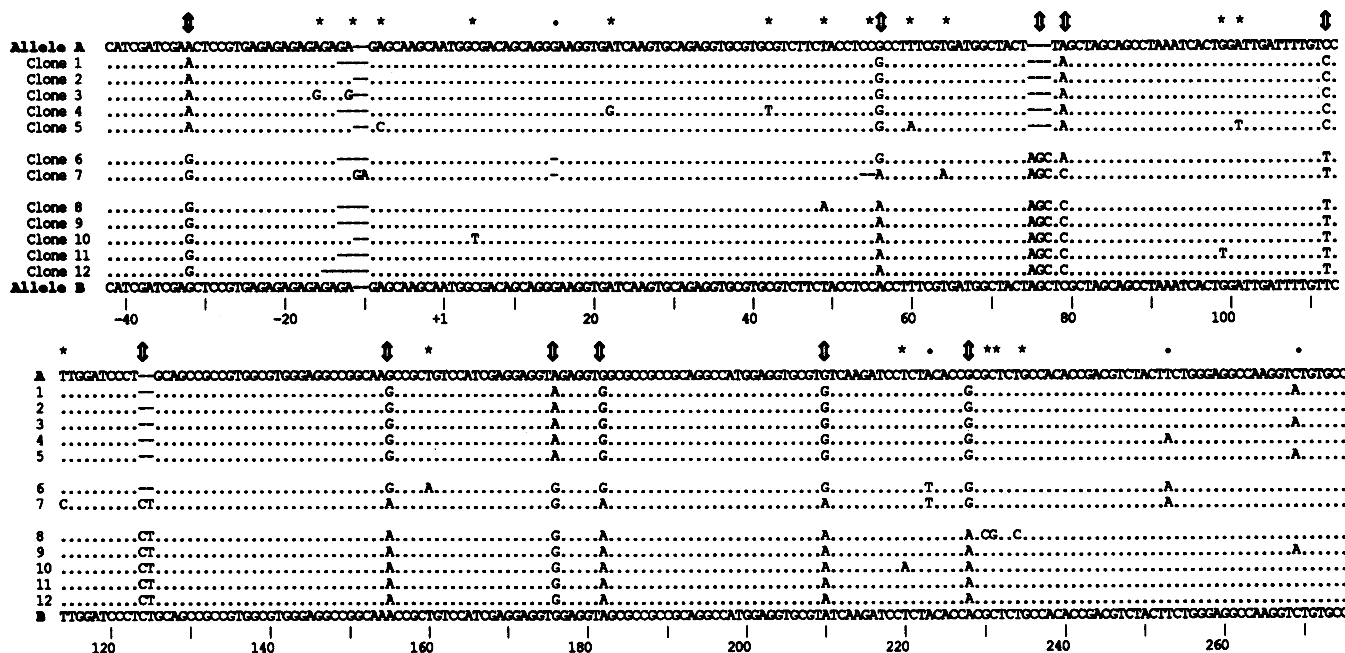

FIG. 2. *Adh2* sequences for 12 M13 clones from a 4700-year-old maize specimen. Dots mark bases shared by all clones. Dashes mark missing bases. The sequences from clones 1–5 and 8–12 fall into two categories, *A* and *B*, according to 11 cosegregating characters (arrows). Asterisks mark the 18 positions that vary in only 1 of the 12 clones, and dots above the sequence mark those that may vary in 2–4 clones (see text for discussion). Clones 6 and 7 are chimeras, displaying composite characters from both *A* and *B* sequences. The reconstructed consensus sequences for allele *A* and allele *B* are labeled with bold characters. The translation start site is numbered +1.

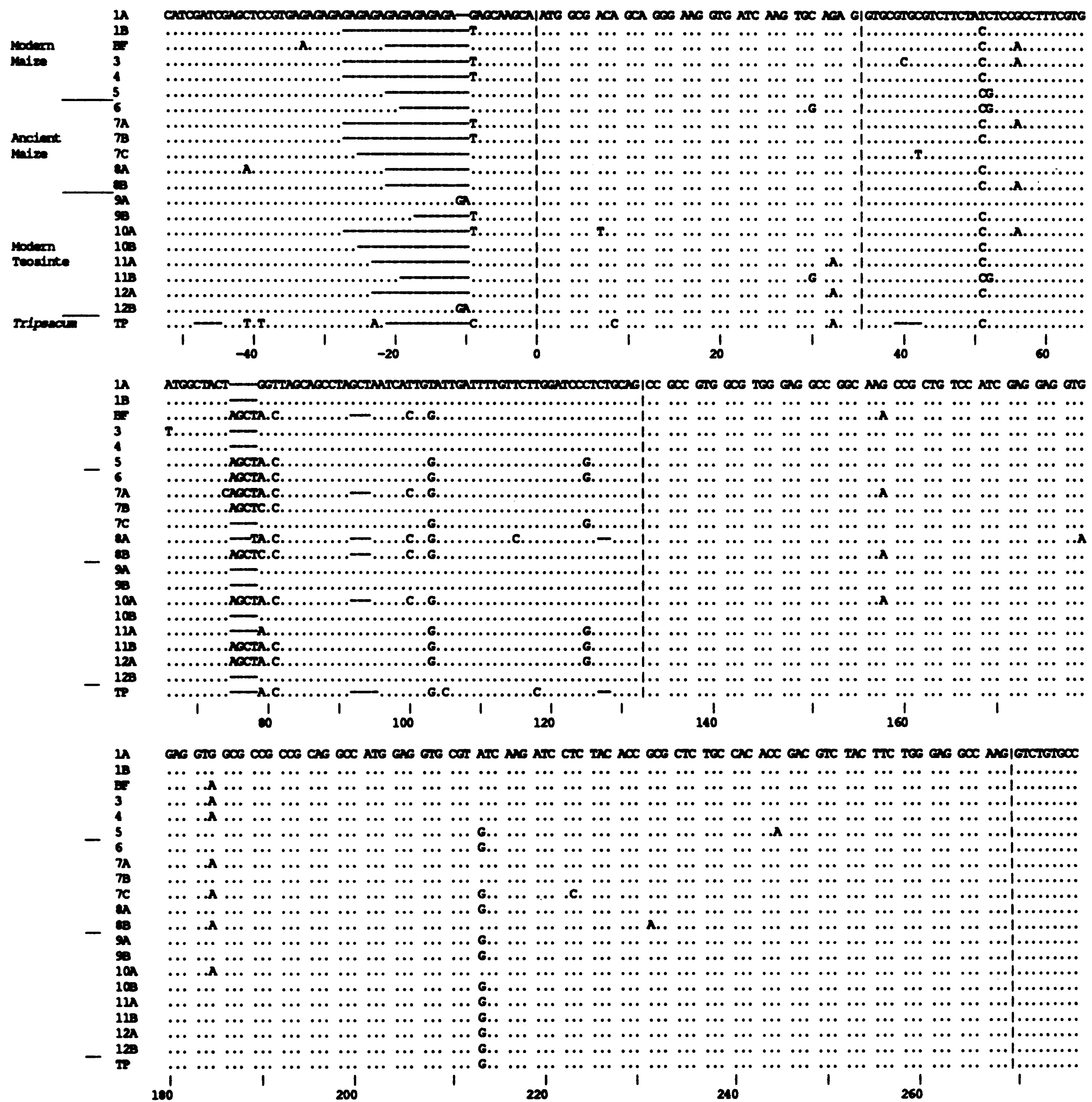

FIG. 3. Nucleotide sequences for *Adh2* alleles from maize, teosinte, and *Tripsacum pilosum*. Dots mark the bases shared with allele *1A*. Vertical lines mark intron–exon boundaries. The affiliations of the alleles are listed in Table 1. The numbering refers to the translation start site.

equally divide 10 clones among two consensus sequences, *A* and *B* (clones 1–5 and 8–12, respectively). After exclusion of artifacts (see below), we interpret the *A* and *B* sequences as two genuine ancient alleles.

Artifacts. Clones 6 and 7 share mixed *A* and *B* characters and are probably mosaic sequences, as expected from PCR with damaged DNA (24–26). Seventeen base substitutions and one deletion appear only once in 12 clones (Fig. 2, asterisks). They are interpreted as PCR artifacts because of their dispersion and their uncharacteristic transition-to-transversion ratio (7:10), which contrasts with the 8:1 ratio of the nine base substitutions between the *A* and *B* alleles.

There are four variable positions (dots above sequence in Fig. 2), which occur more than once among the 12 clones. Three of these are found in the mosaic clones 6 and 7 and would either destroy the reading frame (a deletion at position +15) or cause amino acid replacements (positions 223 and 253). We interpret these changes as artifacts related to the assembly of amplification products from short and damaged templates. Position 269 is ambiguous: in three clones of the *A* type and one clone of the *B*, an adenine replaces a cytosine that is observed in all other maize, teosinte, and *Tripsacum* alleles (see Fig. 3); therefore, we have not counted this ambiguous site in the further analysis.

Finally, a region of GA repeats around positions −15 to −10 displays a high incidence of length mutations. These do not cosegregate with the nine base substitutions defining the two ancient alleles. Dinucleotide repeats have been observed to cause slippage artifacts during enzymatic amplification (27), and GA tracts can arrest DNA synthesis by *Taq* polymerase (28). Therefore, this region was excluded from the evolutionary analyses.

Additional *Adh2* Alleles from Ancient and Modern Specimens. When the approach discussed above was used for reconstruction of *Adh2* alleles, a 440-year-old charred maize specimen from Peru was inferred to be homozygous (Fig. 3, allele *6*), and 1500-year-old Cabuza kernels from Chile were in turn inferred to contain three alleles (Fig. 3, alleles *7A–7C*),

Table 2. Genetic distances among *Adh2* alleles from maize, teosinte, and *Tripsacum*

			Number of differences																			
Specimen	Allele	bp	*1A*	*1B*	*BF*	*3*	*4*	*5*	*6*	*7A*	*7B*	*7C*	*8A*	*8B*	*9A*	*9B*	*10A*	*10B*	*11A*	*11B*	*12A*	*12B*
Modern maize	*1A*	324			Mean = 2.2% ± 1.1%																	
	1B	308	2																			
	BF	315	9	9																		
	3	308	5	3	8																	
	4	308	3	1	8	2																
	5	318	8	8	9	11	9															
Ancient maize	*6*	320	8	8	9	11	9	2		Mean = 2.8% ± 0.6%												
	7A	309	10	8	3	7	7	10	10													
	7B	312	4	2	8	5	3	7	7	7												
	7C	310	5	7	10	8	6	7	7	11	9											
	8A	310	9	9	8	12	10	7	7	9	8	10										
	8B	315	9	9	3	8	8	10	10	4	7	10	9									
Teosinte	*9A*	326	1	3	10	6	4	7	7	11	5	4	8	10		Mean = 1.9% ± 1.1%						
	9B	318	3	1	10	4	2	7	7	9	3	6	8	10	2							
	10A	309	10	8	3	7	7	10	10	2	7	11	9	4	11	9						
	10B	310	2	2	9	5	3	6	6	10	4	5	7	9	1	1	10					
	11A	312	6	6	9	9	7	4	4	10	7	5	7	10	5	5	10	4				
	11B	320	8	8	9	11	9	2	0	10	7	7	7	10	7	7	10	6	4			
	12A	316	7	7	8	10	8	3	3	9	6	6	6	9	6	6	9	5	1	3		
	12B	326	1	3	10	6	4	7	7	11	5	4	8	10	0	2	11	1	5	7	6	
Tripsacum	*TP*	300	12	11	13	14	12	10	10	13	10	13	10	14	11	10	13	10	8	10	7	11

For each pair of sequences (Fig. 3), the number of base differences is given below the diagonal. Above the diagonal mean pairwise differences (±SD) appear in percent for intragroup comparisons.

a possible condition since each kernel results from an independent pollination event. In addition, the sequences were determined for six *Adh2* alleles from five different contemporary races of maize, for eight alleles from four teosinte taxa, and for one allele from *Tripsacum pilosum* (see Fig. 3). The observed sequence differences are computed in Table 2.

DISCUSSION

Sequence Variation in Ancient and Modern Maize. The mean pairwise difference due to base substitutions among ancient maize alleles is 2.8% ± 0.6% (maximal difference: 3.7%) and among modern maize alleles it is 2.2% ± 1.1% (maximal difference: 3.7%) (Table 2). Thus, the extent of sequence variation among the ancient alleles is similar to that among contemporary alleles. Even the two 4700-year-old alleles (*8A* and *8B* in Fig. 3) are more related to some of the modern alleles (1%) than to each other (3.1%). The extent of sequence difference remains constant, rather than diminishing, from the present to a time that is about midway in the domestication era. If maize had originated from one domestication event and subsequently evolved at an accelerated pace, we would predict less diversity among ancient alleles than among modern alleles.

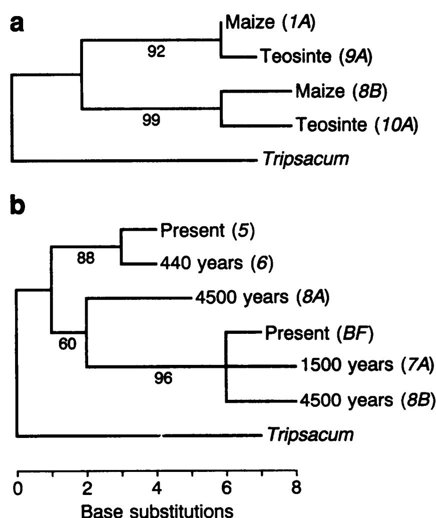

FIG. 4. Parsimony trees relating *Adh2* alleles from teosinte to alleles from ancient and modern maize. The sequence of the *Tripsacum pilosum* allele was used as the outgroup for both trees. The numbers under the internal branches are bootstrap values in percent of 200 replications (30). (*a*) Most parsimonious tree for two maize and two teosinte alleles. (*b*) Most parsimonious tree relating six alleles from ancient and modern maize.

The rate of sequence divergence in the grass family has been calculated, from comparisons of alcohol dehydrogenase 1 (*Adh1*) sequences (15), to be about 1.6% per million years for synonymous sites and 0.05% for nonsynonymous sites. These calibration values are within the ranges of those estimated for other plants and even for animals (29). Among the *Adh2* alleles of maize, the pairwise sequence difference averages 2.5% ± 0.9% (4.0% for untranslated regions and 1.3% for translated regions). Accordingly, the gene pool of maize must be at least several million years old and must vastly predate the domestication era. One possible explanation for the existence of a deep gene pool in maize is that a constant flow of teosinte alleles into domesticated maize has occurred by cross-pollination. However, teosinte does not grow naturally in the Andean area, from where the ancient samples stem. Thus, introgression of teosinte alleles must have occurred before introduction of these maize races to South America, in the case of the Peruvian sample, before 4700 years ago. Therefore, most of the significant teosinte contributions must have taken place early in maize history.

Fig. 4*a* shows a representative tree for two maize and two teosinte alleles. Neither the maize nor the teosinte alleles form monophyletic groups. Rather, many maize alleles are more closely related to teosinte alleles than to the other maize alleles and vice versa. This applies not only to alleles from teosinte lines known to be related to maize, such as *Z. mays parviglumis* and *Z. mays mexicana* (alleles *9A–10B* in Table 2) but also to the more distant taxa *Z. luxurians* and *Z. diploperennis* (alleles *11A–12B* in Table 2) which do not commonly cross-pollinate with maize (6, 31). Hence, a phy-

logenetic analysis yields no evidence in support of the notion that modern races of maize emerged from a single common ancestor, such as a specific line of *Z. mays parviglumis* or *Z. mays mexicana* (5, 7) or a hypothetical line of "wild maize" (32). Rather, despite its spectacular display of morphological variability, domestic maize remains, from the standpoint of the *Adh2* gene, genetically indistinguishable from the morphologically more uniform teosinte species.

Fig. 4*a* also shows that the rates of evolution of maize and teosinte alleles are similar in relation to an outgroup such as *Tripsacum*. Fig. 4*b* similarly illustrates that modern maize alleles have not evolved more extensively than ancient maize alleles compared with *Tripsacum*. Thus, relative rate tests (33) give no indication of a particular acceleration of evolution in maize. In Fig. 4*b* it can be seen that some ancient alleles are closely related to modern alleles. For example, a 440-year-old maize allele (allele *6*, Table 2) differs by only two base substitutions from a modern maize allele (allele *5*) and is identical to a modern *Z. diploperennis* allele (*11B*). Similarly, only two base substitutions distinguish a 1500-year-old Chilean maize allele (*7B*) from a modern maize allele (*1B*). Such close associations between ancient and modern alleles are incompatible with the notion that there has been an acceleration of the base substitution rate in maize, since even an acceleration as low as 10-fold would be expected to produce differences detectable over the periods tested.

Implications for the Process of Maize Domestication. The demonstration that no acceleration of DNA sequence evolution has taken place in maize and that the maize gene pool predates domestication leads to three, not mutually exclusive, scenarios for maize domestication: (*i*) Maize was domesticated from a single wild ancestor that was subsequently introgressed by wild teosinte prior to its exportation to South America. (*ii*) Maize was domesticated from a population of wild ancestors that initially contained, and later perpetuated, a high degree of allelic polymorphism. (*iii*) Maize was domesticated independently from several distinct wild ancestors that have been subsequently interbred among themselves and with wild teosinte. A more extensive survey of ancient and modern maize, teosinte, and *Tripsacum* is needed to determine the contribution of each process to the domestication and early evolution of maize.

All the above scenarios invoke multiple events, such as active selection of mutants and their crossing with wild teosintes as well as other domesticated strains. The idea of multiple origins for maize was first proposed by Randolph (34) and was further developed on the basis of morphological, cytogenetic, and physiological considerations (8, 9, 35–37). This model was supported by studies of the distribution of chromosome knobs (33, 38) and has been proposed also on archaeological grounds (1, 19). To support this idea, one does not have to invoke the notion that all of the four or five loci that are responsible for the unique morphological characters of domestic maize (39–41) were acquired several times independently. The acquisition of any one trait could have provided early breeders with a substantial agricultural advantage. Afterwards, these traits would have been gradually pooled during exchanges of maize between Indian tribes (1) in an effort to improve crop yields, to prevent inbreeding depression, and to obtain hybrid vigor. These antique breeding efforts may also have included intentional and unintentional crosses with teosinte, thus contributing further to the highly divergent gene pool. These practices could also have triggered the activation of transposable elements (42), which have been speculated to be involved in DNA sequence diversity (43) and in the generation of the morphological and allelic diversity of maize (14, 44). However, our data suggest that the depth of the maize gene pool is such that no additional genetic mechanism needs to be invoked to explain the overall genetic variability in maize.

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