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The maze of *Zea*: I. Chloroplast SSRs and evolution

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Abstract

The evolution of *Zea* and, especially, the domestication of maize have undergone multifaceted assessment for many decades. New analyses have often demonstrated inconsistencies that prompted new thinking. Reexamination of chloroplast microsatellite data from a broad sampling of *Zea* reveals clear phenetic patterns, notably: 1) The various teosinte taxa are related to each other as expected; 2) Chloroplasts of the tested maize landraces form two very different groups; 3) Mexican annual teosintes are closely linked to only one maize group. These and other results are supported by a wide range of older studies. The latter two patterns deserve careful consideration because they contradict the model that a Mexican annual teosinte was domesticated, then evolved to all present maizes. They do not contradict the model that a small, domesticated maize was greatly changed by introgression, probably by a teosinte in section *Luxuriantes* of *Zea*. The introgression probably also led to great evolutionary acceleration, several important bottlenecks and the enormous diversity of modern maize. These chloroplast microsatellite relationships have various implications and deserve careful attention in designing future studies (see discussion).

Keywords: maize, teosinte, phenetics, chloroplast SSRs, evolutionary acceleration

Introduction

A partial view of the family tree of *Zea* has come from an analysis of modern chloroplast DNA, one that provides information on relationships, timing of important events, and clues about evolutionary processes (Provan et al, 1999a). DNA sequences, both nuclear and chloroplast, are replete with microsatellites (simple sequence repeats: SSRs) that are composed of motifs repeated in tandem, each motif with one to many nucleotides (Powell et al, 1996). Because changes in SSRs seem usually to occur at a roughly predictable rate, the gains or losses of the motif units in the SSRs can be used to compute distance matrices and to draw phylograms, bases for quantitative comparisons to other studies.

SSRs of the chloroplast genome (cpSSRs) have been shown to change more slowly than nuclear SSRs and, being maternally inherited, are almost unaffected by sexual, outcrossing reproduction (Powell et al, 1995). Chloroplast DNA can provide long lineage histories independent of highly recombined, outcrossed *Zea* nuclear genomes (Doebley, 1990a; White and Doebley, 1999). When compared to phenotypic or nuclear DNA phylogenies, cpSSR relationships can help greatly in detecting introgression (Provan et al, 2001). CpSSR classifications of rice, pines, potatoes and *Phaseolus*, much of it done at the Scottish Crop Research Institute, are in close agreement with relationships determined using older, more tested methods (Provan et al, 1997, 2001; Echt et al, 1998; Angioi et al, 2009). Here, the results for *Zea* (Provan et al, 1999a: Table 1) are compared to prior data of several disciplines and to evolutionary hypotheses.

Materials and Methods

Accessions for the cpSSR study were chosen to represent much of the diversity of *Zea*: nine representative teosinte samples and 28 disparate Latin American maize races (Table 1; Bird, 1982; Provan et al, 1999a). DNA of one individual per race was subjected to polymerase chain reaction amplifications using 15 pairs of primers that define mononucleotide microsatellite loci in chloroplast DNA (Provan et al, 1999a). Long polyacrylamide gels revealed differences in amplicon lengths of one to nine nucleotide base-pairs (bp) per locus; a 43 bp shift, an anomalous, non-stepwise mutation, helped to distinguish the two *Zea* sections. The cpSSR diversity encountered was remarkably high, especially given the fairly limited sampling.

The data matrix of amplicon lengths per locus per accession was sorted and resorted resulting in clusters at various levels wherein variation was minimalized (Table 2); amplicon lengths unique in the clusters were made bold-face as were length shifts. Estimations of ancestral lengths (nodal values) for the major groupings were used to help draw a phylogram (Figure 1). Generally nodal values were the more common or average values within the cluster, sometimes adjusted by reference to values in closely related groups. The further left or more basal in the tree, the more conjectural is the graphing. The phylogram accessions follow the order in the data table, labeled to indicate the cpSSR shifts involved.

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Table 1 - Passport data for the 37 accessions used in the cpSSR study, in the order found in the phylogram. CIM No: accession number in the CIMMYT Maize Germplasm Bank.

Taxon / Landrace	Affiliation / Description	Country	Accession ID	CIM No.
Section *Luxuriantes*				
Z. nicaraguensis	diploid flood tolerant teosinte	Nic.	Ames 21893	11083
Z. diploperennis	diploid perennial teosinte	Mex.	Kato-Taba-87	9476
Z. perennis	tetraploid perennial teosinte	Mex.	Taba-79	9475
Section *Zea*				
Huehuetenango	*Z. mays* ssp. *huehuetenangensis*	Guat.	Iltis 76	9479
Dzit-Bacal	lowland white floury dent, narrow ear	Guat.	Guatemala 322	5253
Uchuquilla	Central Andean 8-rowed flint	Bol.	Bolivia 791	10222
Sabanero	North Andean Flints and Flours	Ecu.	Ecuador 496	8226
Chococeño	Tropical Northern Popcorns	Col.	Choco 340	3213
Huandango	North Andean Flints and Flours	Ecu.	Ecuador 510	8643
Coroico	Amazonian Interlocked Flours	Bol.	Bolivia 1063	6268
Cariaco	Tropical Lowland Flours	Col.	Cordoba 338	3130
Aragüito	Tropical Northern Popcorns	Ven.	Venezuela 568	9059
Huayleño	Central Andean Flours and Flints	Peru	Ancash 180	10381
Ladyfinger	Midwest U.S. yellow pearl popcorn	USA	(PI 217407[3])	Bird 11
Harinoso de Ocho	West Mexican narrow ear	Mex.	Nayarit 24	2250
Pira Naranja	Tropical Northern Popcorns	Col.	Nariño 363	3112
Zapalote Chico	Tropical Dents - Tuxpeño-Zapalote group	Mex.	Oaxaca 51	2270
Tuxpeño	Tropical Dents - Tuxpeño-Zapalote group	Mex.	Vera Cruz 225	467
Cónico	Mexican pyramidal ear - flinty dent	Mex.	Mexico 3	2232
Austria 14	small-grained yellow flint	Aus.	Austria 14	2571
Palomero Toluqueño	Mexican pyramidal ear - pointed white popcorn	Mex.	Mexico 5	2233
Pisankalla	Pointed White Popcorns - Andean group	Bol.	Cordoba 338	7861
Jala	Jala, a Mexican large-eared dent	Mex.	Nayarit 6	2246
Nal-Tel AmTiBa[1]	Caribbean yellow dent	Guat.	Guatemala 111	5183
Cateto Sulino	Cateto Sulinos, orange flints	Uru.	Uruguay 654	4276
Antigua 2	Caribbean small-grained orange flint	Ant.	Antigua 2	1241
Nal-Tel	Caribbean yellow dent	Mex.	Yucatan 7	815
Pollo	small North Andean pop-flint	Col.	Cundinamarca 366	3105
Confite Morocho	small highland Peruvian popcorn	Peru	Huancavelica 136	8381
Nobogame	*Z. mays* ssp. *mexicana*	Mex.	WST-85-2	11387
Chalco	*Z. mays* ssp. *mexicana*	Mex.	TKR-93-3	12823
Durango	*Z. mays* ssp. *mexicana*	Mex.	WST-92-1	11392
Central Guerrero - SE Balsas	*Z. mays* ssp. *parviglumis*	Mex.	K-67-5	8755
South Guanajuato	*Z. mays* ssp. *mexicana*	Mex.	WST-92-5	11396
East Michoacán	*Z. mays* ssp. *parviglumis*	Mex.	WS-92-16	11407
Chullpi	Central Andean Flours and Flints[24]	Peru	Ancash 391	10430
Canilla	Tropical Northern Popcorns[4]	Ven.	Venezuela 693	9063

[1]Nal-Tel Amarillo de Tierra Baja

[2]Chulpi-Paru in Sánchez et al 2006

[3]Ladyfinger available from the USDA maize germplasm collection as accession PI 217407

[4]Unexpected cpSSR affiliation

Table 2 - Chloroplast SSR amplification product lengths, sorted by relationship. Bold-face: shift from antecedent nodal/root value. Locus o shows only an indel. Source: Provan et al, 1999a.

					cpSSR Locus = position (bp) in sequence GenBank X86563														omit
					65013	12968	17192	18704	8372	3764	7430	20824	59488	16073	58414	36547	56136	6359	20597
Branch	Taxon/Landrace	Ctry.	No.	Acc. No.	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o
	Overall Root Estimate		1-37		129	145	97	103	125	142	80	119	171	124	101	137	172	180	---
L	Sect. Luxuriantes - node L		1-3		**131**	145	97	**102**	**123**	**141**	80	119	171	**123**	**103**	137	**171**	180	**153**
	nicaraguensis	Nic.	1	11083	**135**	**142**	97	102	123	141	80	119	171	**122**	103	**138**	171	180	153
	diploperennis	Mex.	2	9476	**125**	145	97	102	123	141	80	119	171	123	**104**	137	171	180	153
	perennis	Mex.	3	9475	**125**	**146**	**96**	102	123	141	80	119	171	123	**104**	137	171	180	153
Z	Sect. Zea - node Z		4-37		129	**147**	97	**106**	125	142	80	119	**172**	**126**	101	**136**	172	180	**196**
ZA	Huehuetenango - ZA	Guat.	4	9479	129	147	**96**	**104**	**124**	142	80	119	172	126	101	136	172	180	196
ZB	node		5-18		**128**	**150**	97	**107**	125	142	80	**120**	**173**	126	101	136	172	180	196
	ZB-1 node		5-12		**127**	150	97	**108**	125	142	80	120	173	126	101	136	172	180	196
	Dzit-Bacal	Guat.	5	5253	127	**145**	97	108	125	142	80	120	173	126	101	136	172	180	196
	Uchuquilla	Bol.	6	10222	127	**149**	**96**	**106**	125	142	80	120	173	126	101	136	172	180	196
	Sabanero	Ecu.	7	8226	**129**	150	97	108	125	142	80	120	173	126	101	136	172	180	196
	Chococeño	Col.	8	3213	**128**	150	97	108	125	142	80	120	173	126	101	136	172	180	196
	Huandango	Ecu.	9	8643	**128**	150	97	108	125	142	80	120	173	126	101	136	172	180	196
	Coroico	Bol.	10	6268	127	150	97	108	125	142	80	120	173	126	101	136	172	180	196
	Cariaco	Col.	11	3130	127	**151**	**98**	108	125	142	80	120	173	126	101	136	172	180	196
	Aragüito	Ven.	12	9059	127	150	**96**	108	125	142	80	120	173	126	101	**135**	172	180	196
	Huayleño	Peru	13	10381	128	**147**	97	107	125	142	80	120	173	126	101	136	172	180	196
	Ladyfinger	USA	14	Bird 11	128	**147**	97	107	125	142	80	120	173	126	101	136	172	180	196
	Harinoso de Ocho	Mex.	15	2250	**126**	150	**98**	107	125	142	80	120	173	126	101	136	172	180	196
	Pira Naranja	Col.	16	3112	128	150	97	107	125	142	80	120	173	126	101	136	172	180	196
	Zapalote Chico	Mex.	17	2270	128	150	97	107	125	142	80	120	173	126	101	136	172	180	196
	Tuxpeño	Mex.	18	467	128	**148**	97	107	125	**144**	80	120	173	126	101	136	172	180	196
ZC-ZD	node		20-36		129	**148**	97	106	125	**143**	**83**	119	172	126	101	136	172	180	196
ZC	node		20-30		129	148	97	**107**	125	**144**	**84**	119	172	126	101	136	172	180	196
	Cónico	Mex.	20	2232	129	148	97	107	125	144	84	119	172	126	101	136	172	180	196
	Austria 14	Aus.	21	2571	129	148	97	107	125	144	84	119	172	126	101	136	172	180	196
	Palomero Toluqueño	Mex.	22	2233	129	148	**98**	107	125	144	84	119	172	126	101	136	172	180	196
	Pisankalla	Bol.	23	7861	129	148	97	**108**	125	144	84	**120**	172	126	101	136	172	180	196
	Jala	Mex.	24	2246	**130**	148	97	107	125	144	84	119	172	126	101	136	172	180	196
	Nal-Tel Am. Ti. Baja*	Guat.	25	5183	**130**	148	97	**108**	125	144	84	119	172	126	101	136	172	180	196
	Cateto Sulino	Uru.	26	4276	**130**	148	97	**108**	125	144	84	119	172	126	101	136	172	180	196
	Antigua 2	Ant.	27	1241	129	**147**	97	107	125	144	84	119	172	126	**100**	136	172	180	196
	Nal-Tel	Mex.	28	815	**130**	**147**	**96**	107	125	144	**87**	119	172	126	**100**	136	172	180	196
	Pollo	Col.	29	3105	**128**	148	**98**	107	**126**	**143**	84	119	172	126	101	136	172	**179**	196
	Confite Morocho	Peru	30	8381	**130**	148	**98**	107	**126**	**143**	**83**	119	172	126	101	136	172	180	196
ZD	node		31-36		129	**145**	97	106	125	143	83	119	172	126	101	136	172	180	196
	Nobogame	Mex.	31	11387	**127**	145	97	106	**126**	143	83	119	172	126	101	136	172	180	196
	Chalco	Mex.	32	12823	**127**	145	**98**	106	**126**	143	83	119	**173**	126	101	136	172	180	196
	Durango	Mex.	33	11392	**130**	145	97	106	**126**	**144**	83	119	172	126	101	136	172	180	196
	South Guanajuato	Mex.	34	11396	**131**	**144**	**96**	106	**126**	143	83	119	172	126	101	136	172	180	196
	Central Guerrero	Mex.	35	8755	**130**	145	**96**	**107**	**126**	143	83	119	172	126	101	136	172	180	196
	East Michoacán	Mex.	36	11407	**123**	**147**	**95**	106	125	143	83	119	172	126	101	136	172	180	196
Affiliations unsettled																			
	Canilla (maize)	Ven.	19	9063	125	150	96	108	126	143	86	119	173	125	101	136	172	180	196
	Chullpi (maize)	Peru	37	10430	129	145	98	106	126	143	83	119	172	126	101	136	172	180	196

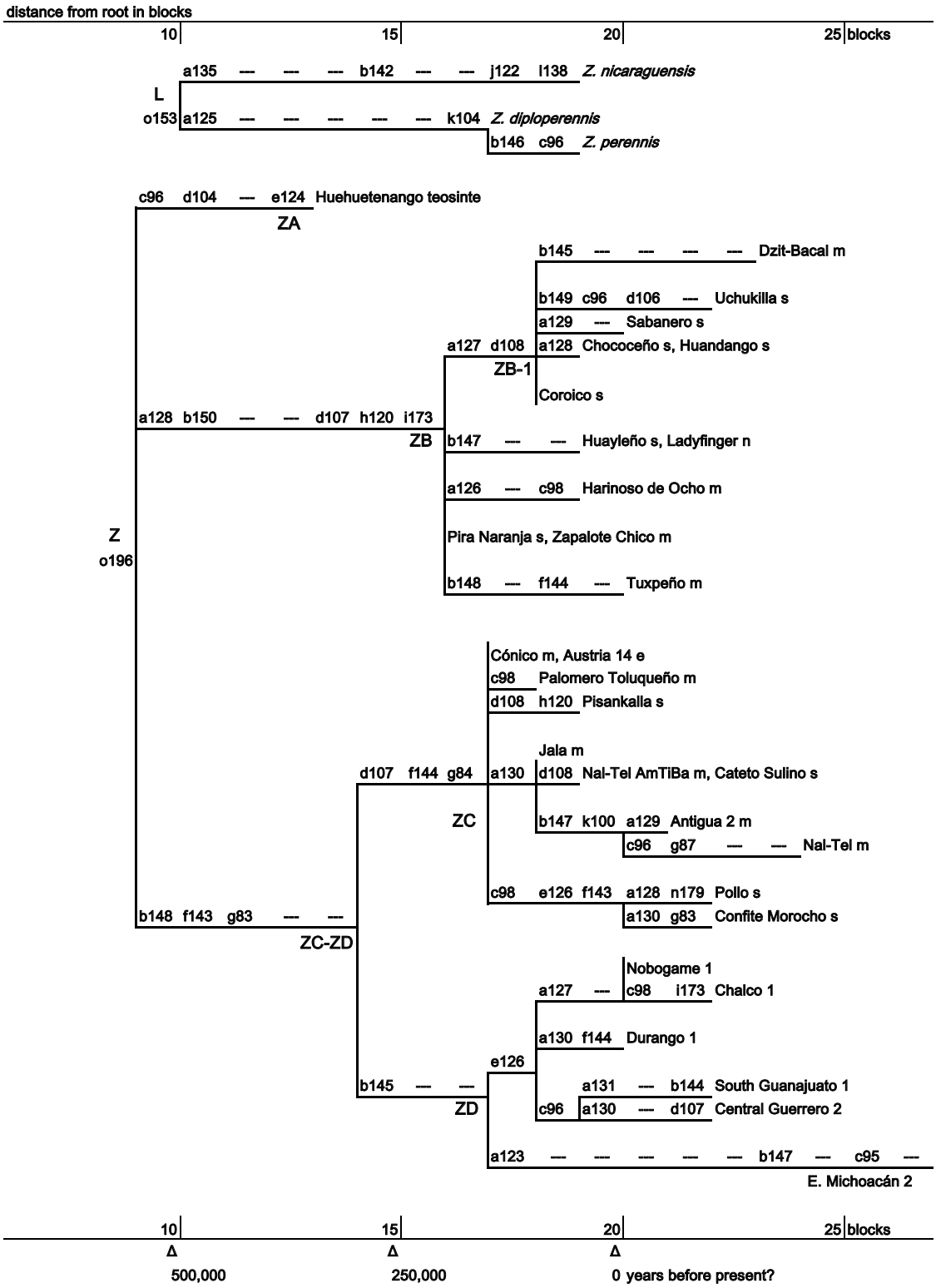

Figure 1 - Phylogram based on mononucleotide cpSSR amplification product lengths (Table 2). The Z and L branches contain accessions of sect *Zea* and sect *Luxuriantes*, respectively. Branch ZA equals *Z. m. huehuetenangensis*, ZB and ZC hold the maizes, and ZD includes all Mexican annual teosintes. Letters indicate cpSSR loci, and numbers provide product lengths (e.g., e123); the amount of change from an estimated earlier state is shown by numbers of blocks. Scales show the amount of change since the provisional L-Z root, in cpSSR blocks, and the estimated number of years since divergence of the sections, here 1 x 10^6 years. Branch tips are fairly even because root and internode values have been balanced (estimates in Table 2).
e: European; m: Middle American-Caribbean; n: North American; s: South American; 1: *Z. m. mexicana*; 2: *Z. m. parviglumis*.

Results

Seven major features of the cpSSR phylogram and data base are striking, usually supported by results of earlier studies of *Zea* morphology, ecology, cytology, isozymes and nuclear DNA thus increasing confidence in the observed relationships. The following discussion is organized around these features: great separation of the *Zea* sections, relationships of some unusual teosintes, early divergence of two large maize groups, the proximity of Mexican annual teosintes (MATs) to just one maize group, notable subdivisions of the maize and MAT branches, prominent early bottlenecks, and great, late acceleration in the rate of cpSSR change. For each feature, the cpSSR results are compared to a range of evidence.

Deep sectional division of Zea

The cpSSR data of Provan et al (1999) demonstrate a profound separation between two groups of *Zea* accessions – L and Z – equivalent to the two generic sections, *Luxuriantes* and *Zea* (Figure 1). The L and Z nodes are separated by 19 SSR blocks involving ten SSRs (the number varies somewhat according to nodal estimates; Table 2); the distances from the hypothetical ancestral *Zea* root to the L and Z nodes are unsettled. The sect *Luxuriantes* accessions are 7-9 blocks from the L node, the sect *Zea* accessions 7-15 from the Z node.

The morphological definition of two sections in *Zea* (Doebley and Iltis 1980; Doebley, 1983) culminated a long period of changing views of teosinte relationships (Wilkes, 1967; Bird, 1978). The form of the male lower glumes and phyllotaxy of the tassel central spike distinguish sect *Luxuriantes* (with Z. *luxurians*, *Z. nicaraguensis*, *Z. perennis*, and *Z. diploperennis*) from sect *Zea* (with the four subspecies of *Z. mays*). Except for Huehuetenango teosinte ("Huehue"), cytological results clearly separate the sections: knobs terminate most chromosome arms in sect *Luxuriantes*, while the great majority of knob positions in sect *Zea* are internal (intercalary) between centromere and terminus (Kato, 1976; Kato and Lopez, 1990; Ellneskog-Staam, 2007). Isozyme frequencies, especially of *Got1*, *Mdh2*, *Pgm1*, and *Acp1*, differentiate teosintes of the two sections, clearly demonstrated in principal component graphs (Smith et al, 1984; Doebley, 1990b).

Buckler and Holtsford (1996) studied nuclear single nucleotide polymorphisms (nrSNPs) in cloned sequences of ribosomal internal transcribed spacer alleles in 24 *Zea* accessions (66 clones) and four species of Tripsacum (12 clones). Their phylogram showed 1) that the Tripsacum and *Z. luxurians* clones formed two monophyletic clades; 2) that clones of *Z. luxurians* (including the Nicaraguan taxon) and most clones from perennial teosintes were closer to each other than to *Z. mays* (*sensu latu*); and 3) that the perennials were further than *Z. luxurians* from the maizes and MATs with three exceptions (relative distances were estimated using the phylogram). Section *Zea* differed more from the Tripsacum species than did sect *Luxuriantes*. They also found evidence of intersectional introgression: three of eleven allelic clones from perennials closely resembled sect *Zea* alleles.

Nuclear SNPs of the *Adh2* locus (Goloubinoff et al, 1993) and of *c1* (Hanson et al, 1996) did not clearly separate the sections, perhaps due to introgression. The complexity of the results, the apparent recombining of haplotype blocks, the meagerness of the sampling and the brevity of discussion show that further study and interpretations of nrSNPs are needed.

Doebley (1990a, b) used 49 mutations at endonuclease restriction sites of cpDNA to classify 151 *Zea* accessions (9 from sect *Luxuriantes*). The two sections were separated by 17-20 mutations, roughly equally distributed, rooted by two Tripsacum species.

Unique teosintes

The relationships among the sect *Luxuriantes* teosintes (cpSSR branch L) are fairly consistent: two similar perennial Mexican taxa are well separated from two Central American species that share many traits. The perennials differ from *Z. nicaraguensis* by ≥16 cpSSR blocks and from each other by 2 blocks, less than the differences among the maizes (0-9 blocks for branch ZB; 0-12 for ZC; Figure 1). Roughly the same is often seen in other data. The perennials are similar in form, with thin, tall culms and rather small tassels, although the diploid's culms and leaves are notably more robust and it has distinctive rhizomes (Iltis et al, 1979: Figure 2; Sánchez-G et al, 1998). The two species share 17 terminal chromosome knob positions with small, inconspicuous or absent knobs in the tetraploid (*Z. perennis*, 2n = 40) versus large to small or absent knobs in the diploid (*Z. diploperennis*; 2n = 20), both of Jalisco, Mexico (Kato and Lopez, 1990). Seven allozymes distinguish *Z. luxurians* from the perennials and ten separate *Z. diploperennis* from *Z. perennis* (Smith et al, 1984; data of Sánchez-G, Goodman and Stuber). Doebley (1990b), using cpDNA restriction fragments, reported that the then known species of sect *Luxuriantes* were clearly differentiated, although in his graphs the two perennials were no more separated than the MAT subspecies of *Z. mays*. Buckler and Holtsford (1996: Figure 2) found 1) that three ribosomal DNA clades holding *Z. luxurians* were well separated from the perennial clades; 2) that in the latter, seven diploid and tetraploid sequences formed two mixed clades; and 3) that three perennial sequences were more like those in two *Z. mays* (mostly MAT) clades. Grebenshchikov (1986) referred to the diploid as a subspecies of *Z. perennis*, without a careful rationale but defensible. The relationships between *Z. luxurians*, *Z. nicaraguensis* and the three new sect *Luxuriantes* populations (Sánchez-G et al, 2011) need considerable study in all disciplines.

Using chloroplast SSRs, Guatemalan "Huehue" teosinte (*Z. mays ssp huehuetenangensis*) is the sole member of the ZA branch, 15-20 blocks from MAT accessions in branch ZD (Figure 1). This supports Doe-

bley's moving it from the MATs to a new subspecies of *Z. mays* (1990b). It is peculiar in many ways. Morphologically, Huehue resembles *Z. mays ssp parviglumis*, a MAT (Wilkes, 1967; Iltis and Doebley, 1980; Doebley, 1983; Sánchez-G et al, 2011), but it has a chromosome knob pattern much like that of *Z. luxurians* – 18 often large, terminal knobs – but five more than *Z. luxurians* (Longley, 1937; Kato, 1976). Its large 9S knob links it only to sect *Zea*. An internal knob was once reported (Longley, 1937). High frequencies of allozymes Got1-6 and Pgd1-2 link Huehue to sect *Luxuriantes* and highland Andean maize races; Idh2-4 and Mdh3-18 tie it to sect *Zea*; and Acp1-3.8 and Mdh1-2.8 help to distinguish it from other *Zea* taxa (Doebley et al, 1984; data of Sánchez-G, Goodman and Stuber). The first four principal components of isozyme allele frequencies show Huehue with no close linkage to any taxon, even equaling *Z. perennis* in distance to the three other subspecies of *Z. mays* (Smith et al, 1984; Buckler et al, 2006). Huehue, *Z. luxurians* and *Z. nicaraguensis* when flooded develop adventitious roots above the soil surface (Mano and Omori, 2007; Mano et al, 2009).

Doebley (1990b) found Huehue was distinguished from maize and the MATs by 1-2 cpDNA mutations and by 18-19 from sect *Luxuriantes*. However, Buckler and Holtsford (1996) sequenced five ribosomal DNA (rDNA) clones from two Huehue accessions and found them to be intermediate between the sections although closer to a minority of *Z. mays* than to sect *Luxuriantes*. Doebley (1990b: 147) said, "[It] may be of hybrid origin [or] . . . may have experienced some form of genetic revolution . . . in a very short period of time," well founded and prophetic statements. Kato and López (1990) suggested moving it to a new, third section, but its probable origin (below) and many relationships would argue for its subspecific status in *Z. mays*.

Two distinct maize groups

Using cpSSRs, maize accessions group into two large, well separated branches: ZB holds most highland South American accessions, while most maize accessions from Middle America and around the Caribbean are in ZC. The distances between the tested materials of the two maize branches range 15-26 (29) blocks. This cpSSR separation is mirrored in various older results as are many subdivisions of the two branches, although four Middle American-Caribbean (MA-C) races are in ZB (below).

McClintock (1959, 1961) showed that almost all highland maize races in Andean countries south of Colombia are clearly separated cytologically from most lowland races. Many lowland eastern South American dent and flint accessions have large to medium knobs at positions 2L to 5L, 7L1 and 8L1, much like the pattern of the more northerly lowland MA-C races which generally have larger and more frequent knobs (McClintock et al, 1981; Bird, 1980b). Lowland maizes from Oaxaca to Colombia to Trinidad also have a large terminal 9S knob, as do most teosintes of sect *Zea*. Frequencies and sizes at these positions are notably reduced in high altitude races of Mexico and Guatemala (*loc cit*; Bretting and Goodman, 1989).

In sharp contrast, highland Central Andean races almost always lack knobs at all positions except 7L1 (always found) and 6L3 (often) (*loc cit*). The pattern is much the same in the Amazonian Interlocked Flours (AIF), but these can have occasional small knobs at eight other positions. However, small infrequent knobs at up to 11 positions differentiate the Andean pointed white popcorns from other races of the region; these and other traits link them to Mexican Pyramidal races (below).

Using isozyme alleles, Goodman and Stuber (1983) found that again Bolivian lowland races, except the AIF, were clearly separated from nearby highland races. For instance, frequencies of the Got1-6 and Glu1-7 allozymes were usually much higher in the highland races than in lowland groups related to MA-C races. Sánchez-G and associates analyzed allozyme frequencies and found much the same in seven Andean countries (2006) and in eight southern and eastern South American countries (2007); the MA-C and other lowland races were united by high frequencies of Got1-4 and Glu1-2. The Central Andean pointed white popcorns differed from other highland races by allele frequencies at over six isozyme loci, although, using isozymes, they were not close to the Mexican Pyramidal races. When Sánchez-G and associates combined three types of data, ecogeographic, morphological and isozyme, the contrast between South American lowland and highland races was even clearer.

Races of the Central Andean Cluster are grown from 3840 masl to 2000 masl (rarely lower), plants are shorter at higher altitudes, ears are usually somewhat rotund and 6-17 cm long, and kernels are often long or elongate (Grobman et al, 1961 and other reports on races; Sánchez-G et al, 2006, 2007). Lowland races near the Bolivian Andes, including the AIF, and tropical groups further east generally have more cylindrical ears (15-25 cm long), tassels with more branches, and plants and leaves that are larger (*loc cit*). They are seldom grown above 1900 masl. Many South American lowland races morphologically and cytologically overlap lowland MA-C races, especially dents and flints – they are thought to have migrated south from various locales over many centuries (Paterniani and Goodman, 1977; Sánchez-G et al, 2006, 2007).

Vigouroux and associates (2008) analyzed 964 plants of ca 310 races and found that nuclear SSRs (n = 96) resolved many major maize groupings, especially Tropical Lowland (MA-C races), Highland Mexican (West, Central and Northwest), Andean (including AIF), Southwestern US and Northern US The Andean and Northern US clusters were further from *Z. m. parviglumis*, which rooted the calculations, than were

the others. van Heerwaarden and others (2011) used nrSNPs to confirm most of this pattern for a slightly larger set of accessions.

MATs and branch ZC: sister groups

The MATs in cpSSR branch ZD are closer to maize branch ZC than to ZB by at least nine blocks (Figure 1; Table 1). Branches ZC and ZD share an internode five blocks long before they separate, and both have significant internodes (apparent bottlenecks) before sub-branches diverge. This important relationship parallels the sharing of high frequencies of seven large chromosome knobs (including 9S) by lowland MA-C maizes, largely in branch ZC, and the MATs of ZD (Kato, 1976; McClintock et al, 1981). However, MATs are distinct from all maizes by having infrequent knobs at up to 13 positions, usually small or medium. All but two of the 20 knobs are absent from the highland Central Andean Cluster, typically in branch ZB. MATs also share low frequencies of allozymes Acp1-2, Glu1-7 and Got1-6 with tropical lowland dent maizes, in contrast to high frequencies of these in the Central Andean Cluster (*loc cit*).

Morphologically, the MATs differ greatly from all maizes, especially in their ear spikes and branching patterns, although there is some sharing of traits by Chalco teosinte and the sympatric Mexican Pyramidal maizes: leaf sheaths hairy, swollen and pigmented, tassel branches fewer (Anderson, 1946; Wilkes, 1967; Sánchez-G et al, 1998). No known morphological traits uniquely link the MATs to the tropical lowland MA-C.

Doebley (1990b), using cpDNA restriction sites, demonstrated no consistent distinctions between the 80 maizes and the 60 MATs analyzed. Of the five shallow clades he defined for sect *Zea*, four were mixes of MATs and maizes: *Z. mays* ssp *parviglumis* was in four and *Z. m. mexicana* was in three, essentially no taxonomic resolution. The ribosomal allele phylogram of Buckler and Holtsford (1996: Figure 2) shows that of nine *Zea mays* clades, three were mixes of maize and MAT alleles. Six of the nine clades separated at about the same level and diverged internally about equally, whether they included mostly maize or mostly MAT alleles. A mixed maize-MAT-*Z. perennis* clade was basal to the other eight. Both these studies provide support for a late and incomplete separation of MATs from Middle American-Caribbean maizes (Central Andean and upper Amazonian races seem not to have been included). These studies illustrate the problems of interpretation and sampling so often found with DNA analyses. A recent broad and inclusive nrSNP analysis of 1131 accessions showed both separation of MATs from maizes and much sharing of SNPs by Mexican highland maizes and *Z. m. mexicana* (van Heerwaarden et al, 2011).

Grouping within sect Zea branches

Within cpSSR branch ZB there are a large group, ZB-1, and four small ones (Figure 1). ZB-1 includes two races from the North Andes (Sabanero, Huandango), three from lower altitudes nearby (Chococeño, Cariaco, Aragüito), two from the highland Central Andes (Uchukilla, Huayleño) and one from the proximal upper Amazon (Coroico). Many of these are floury, and at least Coroico and Cariaco have multiple aleurone layers (Wolf et al, 1972; author's observation). Two of the small ZB sub-branches include accessions that are not closely related, geographically or systematically (e.g., Huayleño - Peru and Ladyfinger - USA). Four Middle American lowland accessions, marked "m" in Figure 1, are unexpectedly included in four ZB sub-branches although by chromosome knobs and other features they are typical MA-C races (McClintock et al, 1981; Doebley, 1994a). Perhaps there were introgressions between branches ZB and ZC, in Central America or the North Andes where 2300-1700 years ago there were broad intercontinental cultural exchanges (Willey, 1971; Bird, 1980a, 1984a; Sánchez-G, 1994).

Branch ZC has three moderately sized sub-branches, mostly reasonable groupings. The white highland popcorns with pointed kernels from Mexico (Palomero Toluqueño) and Bolivia (Pisankalla) have very similar cpSSRs, reflecting cytological and morphological descriptions of the unique Mexican Pyramidal complex (Wellhausen et al, 1952; Ramírez et al, 1960; McClintock et al, 1981). The second group, two Nal-Tel and other MA-C accessions, is also well defined. The round-kernelled, yellow Andean popcorns, Pollo and Confite Morocho, have similar ears, but their chromosome knob patterns are very different (Roberts et al, 1957; Grobman et al, 1961; McClintock et al, 1981). It is remarkable that such widespread and distinctive races as Cónico (Mexico), Cateto Sulino (Uruguay) and Confite Morocho (highland Peru) share the three cpSSR blocks found below node ZC. Oddly, Cónico shows no further change above that node, while Confite Morocho has five more.

Separation of the MAT subspecies is only partial using cpSSRs – of the two *Z. m. parviglumis* accessions, the East Michoacán sample lies to one side and that from Central Guerrero is close to the several members of *Z. m. mexicana*. Similar lack of resolution is seen in other studies where indications of introgression cloud the picture (Doebley, 1989, 1990b; Buckler and Holtsford, 1996; *loc cit*). Better sampling of the MAT races and use of more cpSSR traits may improve resolution of the subspecies and their populations as otherwise defined (Sánchez-G et al, 1998; Buckler et al, 2006).

Judging by cpSSRs, the sampled MATs have diverged internally only slightly more than the two major maize groups (Figure 1), paralleling the ribosomal allele study of Buckler and Holtsford (1996). Distances from the ZD node to ZD branch tips range 3-5 (10) blocks, while the equivalent distances for ZC and ZB range 0-7 (averages: 5.2, 2.7, and 3.3, respectively). The 6-block shift of a123 in East Michoacán is extreme.

The pattern of maize evolution expected in the Domestication of Teosinte (DoT) Model (Goodman 1988; Wilkes, 2004) is one of differentiation of the MATs followed by evolution of all maizes from one MAT branch, but here MAT cpSSRs were apparently not the source of ZB cpSSRs and probably not of ZC cpSSRs. In the Intersectional Introgression (II) Model (Bird, 1979; Wilkes, 1979), MAT-maize divergence would have occurred after the initial intersectional hybridization and after divergence of Central Andean maizes but before Mexican highland and lowland maizes separated.

Important early bottlenecks

Chloroplast SSRs indicate there were several ancient bottlenecks with small population subsamples eventually giving rise to numerous derivatives. These are shown by the unbranched internodes, each with many SSR blocks, subtending lower-level nodes: ≥9 blocks below the Z node, 7 blocks below the ZB node, and 5 blocks below the ZC/ZD node which is followed by 3-block internodes in the ZC and ZD branches (Figure 1; Table 2). There is much branching above these nodes. Doebley (1990b) demonstrated similarly long cpDNA internodes for the sect *Luxuriantes* and sect *Zea* clades.

The low isozyme and knob diversity of the highland Central Andean races is probably due to founder effect, a bottleneck associated with the ancient introduction to that region of a limited, ancestral seed sample (McClintock et al, 1981; Sánchez-G et al, 2006, 2007). That branch's great morphological diversity probably resulted later from many cultural and natural selective forces. The unique eco-morphological and cytological characteristics of the Mexican Pyramidal complex and of Northern Flints (US) were probably shaped by the same factors (Wellhausen et al, 1952; McClintock et al, 1981; Doebley et al, 1986). The lowland MA-C races that share so much cytologically possibly had a more complex history of early divergence.

Chronometrics and acceleration

Frequencies of nuclear SNPs at *Zea* loci have provided various estimates of evolutionary rates and divergence dates, calibrated by using the fossil record – panicoid grasses (e.g., maize and sorghum) seem to have diverged from subfamily *Ehrhartoideae* (rice) and the *Pooideae* (wheat, barley) 50-60, even 80, million years ago (Stebbins, 1981; Gaut et al, 1991; Doust, 2007). These molecular clocks are based on counts of polymorphic synonymous base-pairs (those exon nucleotides not involved in protein determination, generally at third codon positions). For over five *Zea* genes, these have provided synonymous rate estimates of 4.7 to 7.9 substitutions per year per billion bases (reviewed by White and Doebley, 1999). They are complicated by indications of introgression (Goloubinoff et al, 1993; White and Doebley, 1999). Based on these rates and pertinent counts, the sections of *Zea* have been diverging for one or more million years (Gaut and Clegg, 1993).

Accessions in the cpSSR phylogram are about 20 shift-blocks from an unsettled common root, on average. This and a low estimate of 1 Myr of L-Z divergence allow an initial estimate of divergence rate of one block every 50,000 years (2×10^{-5}, below the 3-8 x 10^{-5} cpSSR rate of Provan et al, 1999b). The tips (accessions) on maíze branches ZB and ZC average about 11 and 12 blocks from node Z where the ZA, ZB and ZC/ZD branches diverge, so one might calculate that the two major maize cpSSR branches separated ca. 550,000-600,000 years ago. By 50,000 b.p. at least a dozen sub-branches might seem ancestral to present maize, quite unlikely. Rather, the divergence rate accelerated sometime post-domestication. If domestication and initial maize divergence occurred ~6,000 b.p. (uncalibrated ^{14}C years before present), then cpSSR evolution would have been ca. 92-100 times faster after domestication than before it, with one block change about each 500 years (Benz, 2001; Sluyter and Dominguez, 2006). If ZB-ZC divergence started with intersectional hybridization ca. 4,000 b.p., acceleration would have been 138-150 times faster.

Acceleration has also been proposed or can be deduced in studies of isozymes and nrSNPs, at least in maize and perhaps throughout sect *Zea*. Especially interesting is an attempted chronology by Hanson et al, (1996: Figure 5C) based on available isozyme allele frequencies of most teosinte races and a few modern Mesoamerican maizes (Doebley et al, 1984). They used a moderate rate of isozyme mutation – ca. one mutation per 10^6 years – to provide two dates for divergence of maize from *Z. m. parviglumis*: ca. 12,700 b.p. and ca. 18,500 b.p., both being too early for the two *Zea* evolution models. If the 12,700 b.p. estimate is adjusted to ~6,000 b.p., the rate would be about 2.1 x 10^{-6}. They used the first rate to estimate that sectional divergence was ca. 134,800 b.p. – more recent by a factor of 7.4 than the minimal estimate based on grass fossils and nuclear SNPs. Maize isozyme estimates would be more parsimonious if the calculated natural rate of *Zea* isozyme mutation were ca. 0.135 x 10^{-6} and if, after the proposed intersectional hybridization (ca. 4,000 b.p.), the rate increased to ca. 3.18 x 10^{-6} for sect *Zea*, 23.5 times the natural rate.

The available chronologies involving sect *Zea* often, even usually, seem affected by accelerations of mutation rates (Hake and Walbot, 1980; Werr et al, 1985; White and Doebley, 1999; Lukens and Doebley, 2001; Clark et al, 2005). This acceleration might be due to intersectional introgression, which provided great recombination potential and transposon activation, and to the "capturing" of curious new recombinants and mutants by diverse peoples cultivating early maize in various habitats and regions. Could cpSSRs have been affected by these factors?

There are abundant morphological data for mod-

Table 3 - Chloroplast SSR states per locus per major branch. Commas indicate two states per locus; hyphens over two.

	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o
	65013	12968	17192	18704	8372	3764	7430	20824	59488	16073	58414	36547	56136	6359	20597
L	125,135	142-146	96,97	102	123	141	80	119	171	122,123	103,104	137,138	171	180	153
ZA	129	147	96	104	124	142	80	119	172	126	101	136	172	180	196
ZB	126-129	145-151	96-98	106-108	125	142,144	80	120	173	126	101	135,136	172	180	196
ZC	128-130	147,148	96-98	107,108	125,126	143,144	83,84	119,120	172	126	100,101	136	172	179,180	196
ZD	123-131	144-147	96-98	106,107	125,126	143,144	83	119	172,173	126	101	136	172	180	196

ern maizes and teosintes, but only a few chronological estimates can be based on these. However, when archaeobotanical evidence and cultural histories are factored in, some estimates are possible. For instance, maize that arrived on the coast of Peru at about 2,500 b.p. had evolved considerably from the early domesticate of 1,500 years earlier; this Confite Iqueño started a lineage leading to the Central Andean Rotund Flours (Grobman et al, 1961; Benz et al, 2006; Sánchez-G et al, 2006; Blake et al, 2010).

Relative utility of the chloroplast microsatellites

Individual cpSSR loci differ in their utility in distinguishing *Zea* branches (Table 3). Readings of cpSSRs labeled a to d vary notably within groups, and for these four, separate branches often share a cpSSR state (product length), while cpSSRs g to i vary less and distinguish better (Tables 2 and 3). Chloroplast SSRs j to m contribute much to distinguishing the two sections but are less useful within branches. In combination, all help to determine relationships in the genus. Subsets of cpSSRs could be used for studies of different groups – shifts in cpSSRs a to i are key to separating the sub-groups of branch Z, but several cpSSRs are almost invariant within one or another sub-group (e.g., h and i in branch ZC). Chloroplast SSR 20597 (o) is 153 nucleotides long in the three sect *Luxuriantes* accessions and 196 nucleotides long in all sect *Zea* accessions, a non-stepwise shift, perhaps an indel. Some shifts seem to be convergent (e.g., a129 in node Z shifts to a130 in three separate sub-branches of the ZC-ZD branch). Overall results would be similar if cpSSRs a, b, l, m, n and o were omitted.

Discussion

cpSSR features, problems and prospects

Rather impressively, cpSSR phylogenetic features fit many prior phenetic results at all levels, from the sections and major branches to the racial complexes near the branch tips.

The great cpSSR contrast between sect. Luxuriantes and sect. *Zea* is supported by data from morphological, cytological and isozyme studies, which also attest to the separation of highland Central Andean races (plus several lowland Amazonian races) from lowland maize races of Middle America, the Caribbean and eastern South America (MA-C races). Even cpSSR results for the pointed white popcorns parallel a wide range of studies. Given these correlations, we can have confidence in other cpSSR results: the apparent acceleration in evolutionary rate in maize, the various bottlenecks, and the pattern of close relationships between branches ZC (largely MA-C maizes) and ZD (Mexican annual teosintes). In the two major maize branches, the distances of accessions from an estimated node Z and the distances within the branches indicate that their chloroplast genomes have experienced rapid and extensive change – acceleration – after domestication, a feature also seen in nrSNP and isozyme results. The lengths and consistencies of the internodes subtending the ZB and ZC-ZD nodes indicate major bottlenecks, apparently related to early maize history. Such acceleration, the bottlenecks and the phenetic relationships demonstrate the need for reconsidering prior evolutionary interpretations.

The cpSSR observations, in combination with other molecular results and archaeobotanical evidence, help to judge the parsimony of models of maize evolution. The Intersectional Introgression Model - domestication of wild maize with subsequent introgression between the sections - can explain seeming inconsistencies found when considering the Domestication of Teosinte Model:

- The MA-C maize lineage is closer to the MATs than either is to Central Andean races.
- Maize shows both exceptionally high diversity and evidence of great evolutionary acceleration.
- Archaeobotanical teosinte and teosintoid traits in maize cobs appeared about or after 3300 b.p., after two millennia of dominance by small Tehuacán Early Domesticate.

These observations can be incorporated into the II Model more efficiently than into the DoT Model.

The cpSSR study generates many questions. Why are most of the Central Andean maize races that are united by cpSSRs, chromosome knobs and allozymes so consistently different from races found in Middle America and the Caribbean? Why, if maize evolved from a Mexican annual teosinte, do the two maize cpSSR branches, ZB and ZC, not share a bottleneck linked to one of the ZD (MAT) sub-branches, instead of branches ZC and ZD sharing a bottleneck well separated from branch ZB? Did evolutionary acceleration affect all sect *Zea* branches roughly equally? How much of the acceleration was due to ongoing recombination of two very different genomes, possibly after 4000 b.p.? Is the unique cpSSR status of the east Michoacán teosinte accession found in other accessions from there and nearby western Mexico state (possibly this accession is more parsimoniously linked to the ZC/ZD node than to ZD)? Is a shift of three in amplicon length as important in phenetic description as single shifts at three loci? Importantly, is an increase in cpDNA mutation rate seen in artificial wide-cross hybrid derivatives?

Although analysis of cpSSRs of teosintes and maizes from South America, Middle America and the Caribbean was fairly balanced, material selection by this author was interrupted and incomplete. Teosinte taxa in this and many other studies need further attention, especially *Z. luxurians* and *Z. m. parviglumis* from México and Jalisco states. Three teosinte populations were recently found in Mexico and have been partially studied; all are in sect *Luxuriantes*, possibly new taxa (Sánchez-G et al, 2011). Some maize groups with distinctive form, cytology and/or isozymes need cpSSR characterization, especially races from northern and southwestern US and northwest Mexico, highland Guatemalan flints, South American Morotís, Guaraní Popcorns and various small-eared, small-grained races (e.g., McClintock et al, 1981; Bird, 1982; Doebley et al, 1986; Sánchez-G et al, 2007). Analysis of these would test the present results and probably add important new dimensions. Corroborations of the anomalous Canilla and Chullpi readings are needed (Table 2). Accessions that gave unusual results in other studies might prove particularly interesting (e.g., Iltis et al, #1050, a *Z. perennis* accession from Piedra Ancha, Jalisco, that yielded unusual cpDNA restriction fragments, Doebley, 1989). One or more species of both *Tripsacum* and *Coelorachis* should be included to root Zea relationships. Wherever possible, knowing the morphology, knobs and allozymes of the chosen accessions would allow more careful comparisons. Broadened sampling within each selected race could prove very useful. Concomitant sequence analysis of certain nrDNA loci could reveal tentative introgression events. Also, the set of 15 cpSSRs might be expanded by including di- and tri-nucleotide SSRs or reduced by omitting cpSSR o and cpSSRs involved only in defining the sections.

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