Sequence Diversity in the Tetraploid *Zea perennis* and the Closely Related Diploid *Z. diploperennis*: Insights From Four Nuclear Loci

Peter Tiffin and Brandon S. Gaut

Department of Ecology and Evolutionary Biology, University of California, Irvine, California 92697-2525

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ABSTRACT

Polyploidy has been an extremely common phenomenon in the evolutionary history of angiosperms. Despite this there are few data available to evaluate the effects of polyploidy on genetic diversity and to compare the relative effects of drift and selection in polyploids and related diploids. We investigated DNA sequence diversity at four nuclear loci (*adh1*, *glb1*, *c1*, and *waxy*) from the tetraploid *Zea perennis* and the closely related diploid *Z. diploperennis*. Contrary to expectations, we detected no strong evidence for greater genetic diversity in the tetraploid, or for consistent differences in the effects of either drift or selection between the tetraploid and the diploid. Our failure to find greater genetic diversity in *Z. perennis* may result from its relatively recent origin or demographic factors associated with its origin. In addition to comparing genetic diversity in the two species, we constructed genealogies to infer the evolutionary origin of *Z. perennis*. Although these genealogies are equivocal regarding the mode of origin, several aspects of these genealogies support an autotetraploid origin. Consistent with previous molecular data the genealogies do not, however, support the division of Zea into two sections, the section Zea and the section Luxuriantes.

THIRTY to 70% of angiosperm species have polyploid events in their evolutionary history (Stebbins 1950; Masterson 1994), and hence polyploid events are extremely common in the evolutionary history of flowering plants. Polyploidy is not, however, limited to plants. Yeast, insects, reptiles, mammals, and fish also have polyploid events in their evolutionary histories (Lewis 1980; Postlethwait *et al.* 1998; Seoighe and Wolfe 1998). Yet, despite the evolutionary importance of polyploidy, there have been few studies of its effect on genetic diversity at the DNA level.

Polyploids are often classified into allopolyploids, which result from interspecific hybridization, or autopolyploids, which form through intraspecific chromosomal duplications (Stebbins 1947). These modes of polyploid formation have different effects on the genome; allopolyploid results in twice the number of loci, whereas autopolyploid results in the same number of loci but twice the number of alleles segregating at each locus. However, both modes of formation are expected to result in polyploids having more genetic diversity than closely related diploids. Allopolyploids are expected to have greater genetic diversity because allopolyploids merge two independent evolutionary lineages (Clausen *et al.* 1945; Lewis 1980; Grant 1981).

In contrast, when autopolyploid species are formed they will have equal or less genetic diversity than the progenitor diploid species. However, because tetrasomic inheritance doubles the number of alleles segregating at each locus, autopolyploids have larger effective population sizes (N_e) than their diploid progenitors (all other things, like population size, being equal). Genetic drift is slowed with a larger N_e, and thus autopolyploids should maintain greater levels of neutral genetic variation than their diploid ancestors (Moody *et al.* 1993). The effect of selection on nonneutral variation is also expected to differ between autotetraploids and related diploids. For example, in a diploid population a recessive allele with a frequency of 0.1 is exposed to selection in 1% of individuals; whereas, a recessive allele in an autotetraploid population must reach a frequency of 0.3 before it will be exposed to selection in 1% of individuals. This difference in the efficacy of selection means that recessive alleles will be maintained in populations for much longer periods of time in autopolyploids than diploids (Haldane 1930; Wright 1938; Hill 1971; Otto and Whitton 2000). However, partially dominant alleles may respond to selection faster, and thus be maintained for shorter periods of time in autopolyploids than diploids (Otto and Whitton 2000).

Previous empirical investigations on the molecular diversity of tetraploids have relied primarily on isozyme surveys. The results from these studies are generally consistent with theoretical expectations of higher genetic diversity in tetraploids than their diploid progenitors (Soltis and Soltis 1993). However, isozymes capture only a fraction of allelic polymorphisms present in populations, and thus allozyme data are of limited use for assessing the relative effects of drift and selection. DNA sequence data provide both a more detailed mea-

Corresponding author: Peter Tiffin, Department of Ecology and Evolutionary Biology, 321 Steinhaus Hall, University of California, Irvine, CA 92697-2525. E-mail: ptiffin@uci.edu

sure of genetic diversity and a means to compare the relative effects of drift and selection between a polyploid and its diploid relatives. For example, interspecific differences in the frequency distributions of polymorphisms or in the ratio of nonsynonymous to synonymous variation may reflect differences in the relative strength of selection within the two species (Sawyer *et al.* 1987; McDonald and Kreitman 1991; Akashi 1999). Similarly, slowed drift in an autopolyploid may be reflected by higher diversity at synonymous sites. In short, comparisons of DNA sequence diversity between a polyploid and closely related diploids should provide insight into the relative strengths of the evolutionary forces acting on the different species.

Recent studies on gene expression and genomic divergence have begun to shed light on differences in the evolution of allopolyploids and diploids (Song *et al.* 1995; Cronn *et al.* 1999; Galitski *et al.* 1999; Small and Wendel 2000; Wendel 2000). In contrast, DNA sequence diversity in autotetraploids and closely related diploids has not been investigated. In this article we present data on DNA sequence diversity at four nuclear loci–*adh1*, *glb1*, *c1*, and *waxy*–in the tetraploid plant *Zea perennis* and its closest diploid relative *Z. diploperennis*. *Z. perennis* and *Z. diploperennis* are morphologically similar, primarily outcrossing species that are endemic to the Cerro de San Miquel, Sierra de Manantlan, in southwestern Jalisco, Mexico (Iltis *et al.* 1979; Benz *et al.* 1990). Both species are congeners of the domesticate *Z. mays* ssp. *mays* and are the only perennial species in the genus Zea.

Z. perennis is generally thought to be an autotetraploid that originated from a *Z. diploperennis*-like ancestor (Clausen *et al.* 1945). An autotetraploid origin is supported by morphological (Iltis *et al.* 1979), ribosomal internal transcribed spacer (ITS) sequence (Buckler and Holtsford 1996), chromosomal knob (Kato and Lopez 1990), and chloroplast restriction fragment length polymorphism (Doebley *et al.* 1987) similarities. Chromosome segregation patterns in *Z. perennis*, however, are equivocal with regard to origin. Randolph (1955) reported *Z. perennis* to form a prevalence of tetravalents at meiosis, as expected from an autotetraploid. However, Shaver (1962) found approximately equal frequencies of tetravalents and bivalents at meiosis. These data suggest that *Z. perennis* is either an autotetraploid that has partially diploidized or *Z. perennis* is actually an allotetraploid. If *Z. perennis* is an allotetraploid, the high numbers of tetravalents formed at meiosis may indicate that closely related species hybridized to form the polyploid or that the genetic contribution of the two progenitor species was not equal, perhaps due to more extensive introgression from one of the two progenitor species.

An allotetraploid origin is also suggested by an investigation of restriction sites that identified a chloroplast haplotype from the Piedra Ancha population of *Z. perennis* (accession per6 in this study) that is considerably different from chloroplast haplotypes isolated from *Z. diploperennis* or other *Z. perennis* individuals (Doebley 1989). This atypical chloroplast haplotype, although not found in any other Zea species, is more closely related to *Z. mays* than any other Zea species and thus seems to support the possibility of an allotetraploid origin of *Z. perennis* (Doebley 1989).

The main objective of this study is to investigate the effects of polyploidy on genetic diversity by comparing DNA sequence diversity in a presumed autotetraploid to its most closely related diploid relative. In addition, because there is some uncertainty as to the origin of *Z. perennis,* we ask whether genealogical data support an intraspecific or interspecific origin of this species. Sequence data for the four genes we studied are also available from all other diploid species within the genus Zea, providing opportunities both to estimate the relatedness of *Z. perennis* alleles to those of other possible progenitors and to evaluate phylogenetic relationships among Zea species.

MATERIALS AND METHODS

Sampling DNA sequences: We PCR amplified the following sections of DNA: a 1400-bp portion of *adh1* (alcohol dehydrogenase-1), a 1200-bp portion of *glb1* (globulin-1, a nonessential seed storage protein), a 750-bp portion of *c1* (a bHLH transcriptional regulator of enzymatic genes in the anthocyanin biosynthetic pathway), and a 1400-bp portion of *waxy* (granule bound starch synthase; Mason-Gamer *et al.* 1998). *adh1* and *glb1* are located within 12 cM of one another on chromosome 1, and *c1* and *waxy* are located within 30 cM of one another on chromosome 9. *waxy* was amplified using PCR with 35 cycles of 1 min at 94°, 2 min at 62°, and 2 min at 72° (forward primer, 5′ tgcgagctagacaacatcatgcgcc 3′; reverse primer, 5′ agggcgcggccacgttctcc 3′); and *c1* was amplified using PCR with 35 cycles of 1 min at 94°, 2 min at 60°, and 2 min at 72° (forward primer, 5′ cactggggatccttagttactggcatg 3′; reverse primer, 5′ cataggtaccagcgtgctgttccagtagt 3′). Details of the PCR amplification conditions and primers for *adh1* and *glb1* are reported in Eyre-Walker *et al.* (1998; *adh1*) and Hilton and Gaut (1998; *glb1*). In most cases, sequences were amplified from a single individual from each of eight accessions of *Z. diploperennis* and six accessions of *Z. perennis* (Table 1). However, we were unable to obtain full-length sequences for *adh1* from *Z. diploperennis* accession 2 or *glb1* from *Z. perennis* accession 2. DNA was extracted from leaves using QIAGEN (Chatsworth, CA) DNeasy DNA extraction kits.

We sampled multiple alleles at each locus from all *Z. perennis* and several *Z. diploperennis* individuals. For each of the diploids we performed a single PCR reaction. PCR products were cloned into pGEM-T vectors (Stratagene, La Jolla, CA) and plasmid DNA was isolated from three clones using QIAGEN minipreps. Each of these clones was partially sequenced, using one of the amplification primers. Nonidentical clones from a single individual were then sequenced in their entirety using the PCR primers as well as internal primers (except for *c1* for which only the two PCR primers were used), Perkin Elmer (Norwalk, CT) Big-Dye sequencing chemistry, and an ABI 377 automated sequencer.

Because we were unsure of the origin of *Z. perennis* and we wanted to ensure sufficient sample to detect evidence for a

TABLE 1

***Zea perennis* and *Z. diploperennis* accessions, geographic source, sample numbers used in this study, and GenBank accession numbers**

Accession	Geographic source	Allele	GenBank nos.			
			adh1	*glb1*	*c1*	*waxy*
	Z. diploperennis					
M005	Zarza, Jalisco, Mexico	1a	329404	329790	329762	329764
		1b	—	329791	329761	—
9476	Las Joyas, Jalisco, Mexico	2a	—	329792	329763	329736
		2b	—	329793	329764	329735
10003	San Miguel, Jalisco, Mexico	3a	329405	329794	329765	329737
		3b	329406	—	329766	—
Ames 2317	Jalisco, Mexico	4a	329408	329795	329767	329738
		4b	329407	—	—	329739
PI 441932	Jalisco, Mexico	5a	329409	—	329768	329743
PI 462368	Jalisco, Mexico	6a	329410	329796	329770	329740
		6b	—	—	329769	—
Ames 21884	Jalisco, Mexico	7a	329411	329797	329771	329741
		7b	—	—	329772	—
PI 441931	Jalisco, Mexico	8a	329412	329798	329773	329742
		8b	—	—	329774	—
	Z. perennis					
Ames 21869	Teosjar, Jalisco, Mexico	1a	329413	329801	329776	329745
		1b	329414	329799	329775	329744
		1c	329415	329800	—	329746
		1d	—	329801	—	—
Ames 21870	Teosjar, Jalisco, Mexico	2a	329416	—	329777	329748
		2b	329417	—	329778	329747
		2c	—	—	—	329749
Ames 21873	Teosjar, Jalisco, Mexico	3a	329418	329803	329779	329750
		3b	329419	329805	329780	329751
		3c	329420	329804	—	329752
Ames 21874	Teosjar, Jalisco, Mexico	4a	329421	329806	329781	329753
		4b	329422	329807	329782	329754
		4c	329423	329808	—	—
Mo10	Ciudad Guzman, Jalisco, Mexico	5a	329427	329811	329787	329757
		5b	329428	329813	329789	329758
		5c	—	329812	329788	329760
		5d	—	—	—	329759
Jal-88	Piedra Ancha 9475, Jalisco, Mexico	6a	329424	329809	329784	329755
		6b	329425	329810	329783	329756
		6c	329426	—	329785	—
		6d	—	—	329786	—

The GenBank accession numbers listed are all preceded by AF. For example, the GenBank accession number for the *glb1* sequence from *Zea diploperennis* accession 1, allele *a*, is AF329790.

possible allotetraploid origin, we sampled *Z. perennis* much more extensively than *Z. diploperennis*. Our primary motivation for this extensive sampling was that if loci within *Z. perennis* were disomic (*i.e.*, the loci were duplicated), we wanted to be certain that we sampled alleles from both loci. For each *Z. perennis* individual we performed two PCR reactions. The products from each of these reactions were cloned and a total of 12 clones were chosen for each *Z. perennis* individual, six colonies from each of the two original PCR reactions. Each of these 12 clones was partially sequenced using one end primer to identify alleles that differed within individuals. Nonidentical clones from a single individual were sequenced in their entirety as described above. This sampling scheme resulted in full sequences of multiple alleles from each individual.

More than half of the alleles identified after the initial data collection contained one or more unique single base pair changes (or "singletons") relative to the remainder of the sequences. Because singletons in individual cloned products can represent either true sequence variation or polymerase error, we reamplified all DNAs containing singletons to determine which singletons were true variants and which were the results of PCR error. We checked singletons in *Z. diploperennis* by reamplifying and resequencing the appropriate allele from each individual. The task was more burdensome in *Z. perennis* because we did not know the number of different alleles present within each tetraploid individual. It was therefore not always possible to determine if a clone obtained upon reamplification represented the allele without the singleton (thus indicating that the initial singleton was due to PCR error) or an allele that was not sampled previously. Because of this

TABLE 2

Summary of variation, tests of neutral evolution, and estimates of recombination at the *adh1*, *c1*, *glb1*, and *waxy* loci in the diploid *Zea diploperennis* and tetraploid *Zea perennis*

Gene	Species	N	H	θ_S	π_S	θ_N	π_N	D	D^*	*MK*	C
adh1	*Z. diploperennis*	9	8	0.018	0.017	0.0018	0.001	0.15	−0.24	NS	5.6
	Z. perennis	16	10	0.016	0.017	0.0038	0.003	0.25	0.16	$P < 0.05$	12.9
glb1	*Z. diploperennis*	9	6	0.017	0.012	0.0065	0.006	−1.20	−1.47	NS	0.6
	Z. perennis	15	10	0.018	0.022	0.008	0.008	0.49	0.24	NS	13.8
c1	*Z. diploperennis*	14	11	0.009	0.010	0.006	0.0045	0.019	−0.38	NS	62.1
	Z. perennis	15	10	0.008	0.007	0.0028	0.0036	−0.26	0.098	NS	28.5
waxy	*Z. diploperennis*	10	9	0.005	0.006	0.003	0.003	−0.33	0.015	NS	14.7
	Z. perennis	20	12	0.010	0.010	0.0016	0.0016	0.13	0.20	NS	25.2

NS, nonsignificant at $P > 0.05$. N, number of alleles; H, number of haplotypes; θ_S and π_S (per site) were calculated using only silent sites; θ_N and π_N (per site) were calculated using only nonsynonymous sites; D, Tajima's D; D^*, Fu and Li's D; MK, McDonald-Kreitman test; C, recombination parameter per gene. The numbers of silent sites used to calculate θ and π were 832 for *adh*, 408 for *c1*, 582 (*Z. diploperennis*), and 563 (*Z. perennis*) for *glb*, and 687 (*Z. diploperennis*) and 628 (*Z. perennis*) for *waxy*. The numbers of sites in the two species differ because of indels.

uncertainty, we employed an extensive sampling strategy to confirm the presence of singletons. For each tetraploid DNA from which at least one allele contained a singleton, we performed two additional PCR reactions, cloned the products from these reactions, and isolated and partially sequenced 6 isolates from each reaction (a total of 12 isolates from each tetraploid DNA). Assuming that each tetraploid plant contained four distinct alleles, and that there was no amplification bias in the PCR reactions, sampling 12 isolates results in a >95% probability of resampling the allele that initially contained the singleton. Approximately one-half of the singletons in the initial data set were confirmed by this strategy, while the other half were assumed to have resulted from polymerase error and excluded from the data set. Our estimated rate of polymerase error was ~1 in 1200 bp, which is similar to previously reported polymerase error for Zea DNAs (Eyre-Walker *et al.* 1998).

Corrected sequences were aligned manually along with previously published sequences from other Zea species. Sequences have been submitted to GenBank (Table 1).

Sequence analyses: For each of the four loci, genealogies were constructed using the neighbor-joining method (Saitou and Nei 1987) on the basis of Kimura's two-parameter genetic distance, using a sequence from *Tripsacum dactyloides* as an outgroup. Data were resampled 1000 times for bootstrap analyses. When constructing genealogies, we included sequences from other members of the genus Zea. Genealogies were constructed with PAUP* version 4.0b (Swofford 1990). The data included sequences from *adh1* (GenBank nos. L08587–L0591, AF044289–AF044307, AF045548, and AF293887–AF293894; Eyre-Walker *et al.* 1998), *c1* (GenBank nos. AF292540–AF292553; Hanson *et al.* 1996; L. Zhang, A. Peek, D. Dunams and B. S. Gaut, unpublished results), *glb1* (GenBank nos. U28017, X59084, and AF064212–AF064233; Hilton and Gaut 1998), and *waxy* (GenBank nos. AF292500–AF292539; L. Zhang, A. Peek, D. Dunams and B. S. Gaut, unpublished results).

Estimates of genetic diversity, π (Tajima 1983) and θ (Watterson 1975), were calculated separately on the basis of silent (synonymous and intron sites) and nonsynonymous sites. The recombination rate at each locus C ($= 4Nc$) was estimated using the method of Hudson (1987). Evidence for nonneutral evolution was investigated using the tests of Hudson *et al.* (HKA; 1987), Tajima (1989), McDonald and Kreitman (MK; 1991), and Fu and Li (1993). HKA tests were performed with pairs of genes, resulting in six HKA tests for both *Z. perennis* and *Z. diploperennis*. For all HKA and MK tests, *T. dactyloides* sequences were used as the reference species. Ratios of the number of synonymous to nonsynonymous segregating sites and the frequency of singleton and nonsingleton segregating sites in *Z. diploperennis* and *Z. perennis* were compared using 2×2 contingency tests (Sawyer *et al.* 1987; Akashi 1999). Sites that were polymorphic in one species were included in the analyses, regardless if that site was fixed or polymorphic in the second species. Polymorphism metrics and tests of selection were calculated with DnaSP version 3.0 (Rozas and Rozas 1999). All nucleotide sites (synonymous, nonsynonymous, and intron sites) were used for phylogenetic reconstruction and indels were excluded from all analyses.

RESULTS

Patterns of DNA sequence diversity within and among species: Estimates of DNA sequence diversity, θ and π, made separately on silent sites and nonsynonymous sites provided no clear evidence for more genetic diversity in tetraploid *Z. perennis* than diploid *Z. diploperennis* (Table 2). Similarly, estimates of C, the number of recombination events per gene (Hudson *et al.* 1987), were also not consistently different across genes between species.

To determine whether the selective histories differed between the two species and among genes, we applied four tests of neutrality to the sequence data. These tests—Tajima's D (Table 2, all $P > 0.2$), Fu and Li's D (Table 2, all $P > 0.10$), Fu and Li's F (all $P > 0.10$, data not shown), and HKA (all $P > 0.3$, data not shown)—revealed no evidence of deviation from the neutral model. MK tests also were nonsignificant for *c1*, *glb1*, and *waxy*. In contrast, MK tests of *adh1* data indicated significant departure from neutral evolution in *Z. perennis* ($P < 0.05$). However, this result is not significant after Bonferroni correction for multiple tests, and it seems unlikely that *adh1* is under strong selection, given that HKA tests, intraspecific tests of selection, and a

TABLE 3

Numbers of synonymous, nonsynonymous, singleton ($r = 1$), and nonsingleton ($r > 1$) sites in *Z. perennis* and *Z. diploperennis*

					Coding					
		All sites			Synonymous			Nonsynonymous		
Gene	Species	Total	$r = 1$	$r > 1$	Total	$r = 1$	$r > 1$	Total	$r = 1$	$r > 1$
adh1	*Z. diploperennis*	38	18	20	5	1	4	2	2	0
	Z. perennis	51	16	35	7	3	4	5	3	2
glb1	*Z. diploperennis*	21	14	7	4	2	2	7	4	3
	Z. perennis	43	11	32	10	1	9	14	6	8
c1	*Z. diploperennis*	16	7	9	3	2	1	5	3	2
	Z. perennis	13	4	9	3	1	2	2	0	2
waxy	*Z. diploperennis*	16	6	10	4	1	3	5	2	3
	Z. perennis	29	8	21	9	2	7	3	1	2
Total	*Z. diploperennis*	91	45	46	16	6	10	19	11	8
	Z. perennis	136	39	97	29	7	22	24	10	14

2 x 2 contingency tests were conducted to compare the distribution of (i) singleton *vs.* nonsingleton polymorphisms within each species and between the two species at all, synonymous, and nonsynonymous sites; and (ii) total synonymous to total nonsynonymous polymorphisms within each species. Tests were conducted for each gene separately and for all of the data together. Fisher's exact tests comparing the distribution of singleton to nonsingleton polymorphisms at all sites in *glb1* and all genes together were both significant at $P < 0.01$ (underlined data). All other tests were not significant at $P > 0.10$.

Data from the putatively introgressed *glb1* allele (*Zea diploperennis* 1a) are not included.

previous study of *adh1* in Zea (Eyre-Walker *et al.* 1998) have failed to reject neutral evolution for this gene.

A primary motivation for this study was to compare the distribution and pattern of polymorphism between a tetraploid and closely related diploid species. Interspecific differences in the frequency distributions of nonsynonymous to synonymous polymorphisms and rare to common polymorphisms can reflect differences in the relative strengths of selection and drift operating within each species (Sawyer *et al.* 1987; Akashi 1999). We therefore compared the ratio of nonsynonymous to synonymous polymorphic sites with *Z. perennis vs. Z. diploperennis*. The ratio did not differ significantly between the two species for any of the loci (Fisher's exact test, all $P > 0.20$) or when data from all genes were analyzed together (Fisher's exact test, $P > 0.5$; Table 3).

We also compared the distribution of singleton *vs.* nonsingleton polymorphisms at synonymous, nonsynonymous, and all sites. For all genes and at all classes of sites the ratio of singleton to nonsingleton polymorphisms was lower in *Z. perennis* than *Z. diploperennis*. For three of the four genes these differences were not significant (*G*-test, all comparisons $P > 0.10$; Table 3), whereas at *glb1* the distribution of singleton to nonsingleton polymorphisms at all sites, including introns, differed significantly between the two species ($P < 0.01$). Likewise, a contingency test comparing the distribution of singleton to nonsingleton polymorphic sites over all genes was significant ($P < 0.01$), although the significance of this comparison was largely due to the *glb1* data. Removing the *glb1* data resulted in a difference that was only marginally significant ($P = 0.062$). Overall, the tetraploid appears to have a lower relative frequency of singletons, although the significance of this result is due largely to one of the four genes, *glb1*.

Genealogical analysis: Several aspects of the genealogies (Figures 1–4) support an autotetraploid origin of *Z. perennis* from *Z. diploperennis*. First, with one exception we found no well-supported clades (*i.e.*, bootstrap support >50%) that contained alleles from either *Z. perennis* or *Z. diploperennis* and any other Zea species. Second, several well-supported clades contained alleles isolated from both *Z. perennis* and *Z. diploperennis*. Third, the majority of clades that contained *Z. perennis* alleles also contained *Z. diploperennis* alleles or were sister to clades containing *Z. diploperennis* alleles. The only strong evidence for an autotetraploid origin, however, comes from the *c1* locus; all *c1* alleles isolated from these two species formed a single monophyletic clade.

The genealogies are not, however, entirely consistent with an autotetraploid origin. The strongest evidence against an autotetraploid origin comes from the *adh1* locus where one clade of *Z. perennis* alleles (per7a, per4b, per1a, and per2b) is sister to a clade of *Z. mays* ssp. *mays* alleles (Figure 1). However, the node joining these two clades and other internal nodes within the *adh1* genealogy are poorly supported, and thus any inferences regarding the genealogical placement of these alleles should be viewed with caution. It is also possible that these *adh1* alleles are the result of introgression of

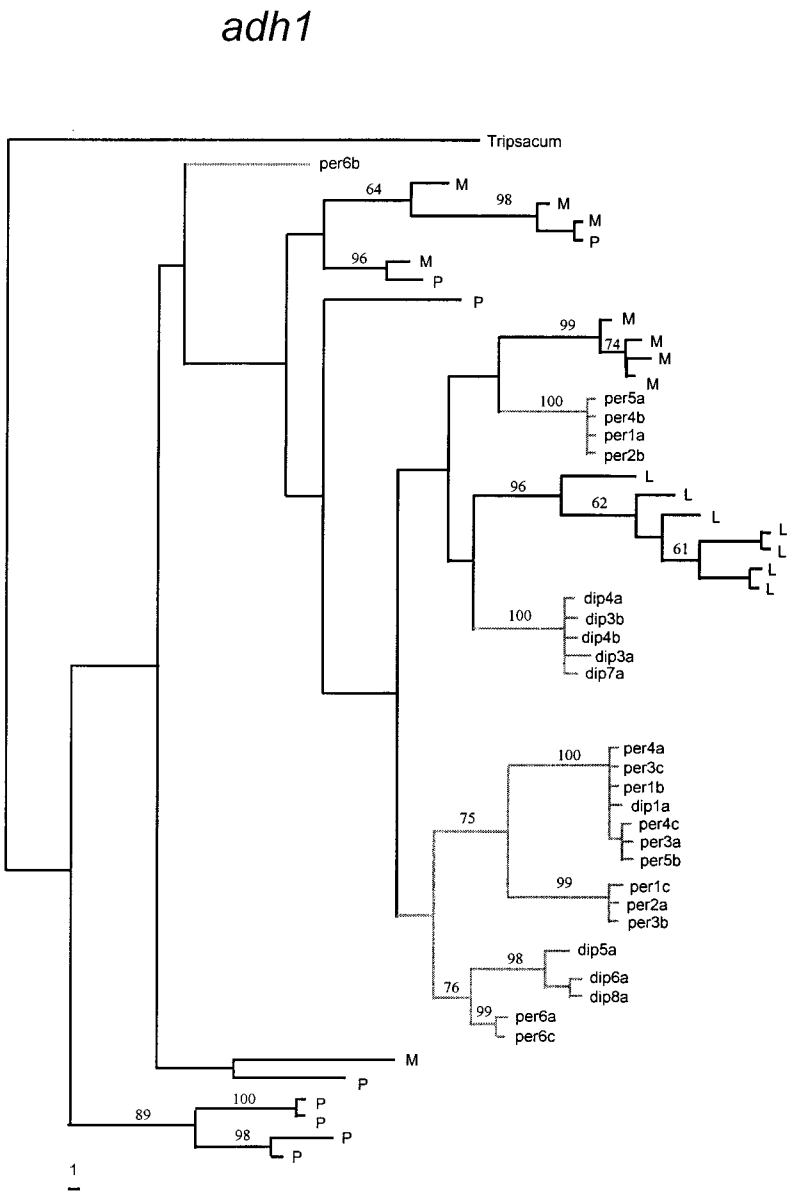

FIGURE 1.—Neighbor-joining reconstructions of the genealogical relationships among *adh1* alleles. Bootstrap values for nodes supported in >50% of 1000 bootstrap replicates are given above the branches leading to those nodes. *Zea perennis* and *Z. diploperennis* alleles are named as indicated in Table 1. *Z. mays* ssp. *mays* (M), *Z. mays* ssp. *parviglumis* (P), *Z. mays* ssp. *mexicana* (MX), and *Z. luxurians* (L) sequences were published previously (see MATERIALS AND METHODS for references).

Z. mays ssp. alleles into *Z. perennis*. Nevertheless, these alleles could be interpreted as evidence of an allotetraploid origin for *Z. perennis*.

In addition to the genealogical data, the ratio of shared polymorphisms and fixed differences between *Z. perennis* and three of the diploid species of the genus Zea (*Z. diploperennis*, *Z. luxurians*, and *Z. mays* ssp. *parviglumis*; Table 4) is not entirely consistent with an autotetraploid origin. If *Z. perennis* were an autotetraploid we expect the ratio of shared polymorphisms to fixed differences to be much higher between *Z. perennis* and *Z. diploperennis* than between *Z. perennis* and any of the other species. The ratio of shared polymorphisms to fixed differences between *Z. perennis* and *Z. diploperennis* is, in fact, higher than between other species pairs but is not significantly higher than the ratio between *Z. perennis* and *Z. mays* ssp. *parviglumis*.

DISCUSSION

Genetic diversity: The primary purpose of this study was to compare genetic diversity between a tetraploid, *Z. perennis*, and a closely related diploid *Z. diploperennis*. In theory, allotetraploids should have higher genetic diversity than diploids because they are formed through hybridization of two divergent genomes. Likewise, autopolypoidy can slow the effects of both drift and selection on genetic diversity, potentially resulting in both greater diversity and different patterns of molecular diversity in autotetraploids than diploids (HALDANE 1930; WRIGHT 1938; HILL 1971; MOODY *et al.* 1993). Contrary to these expectations, we detected little evidence for differential patterns of drift and selection in tetraploid *Z. perennis* relative to its closest diploid relative *Z. diploperennis*.

There are at least two possible explanations for the absence of substantial differences in the pattern and

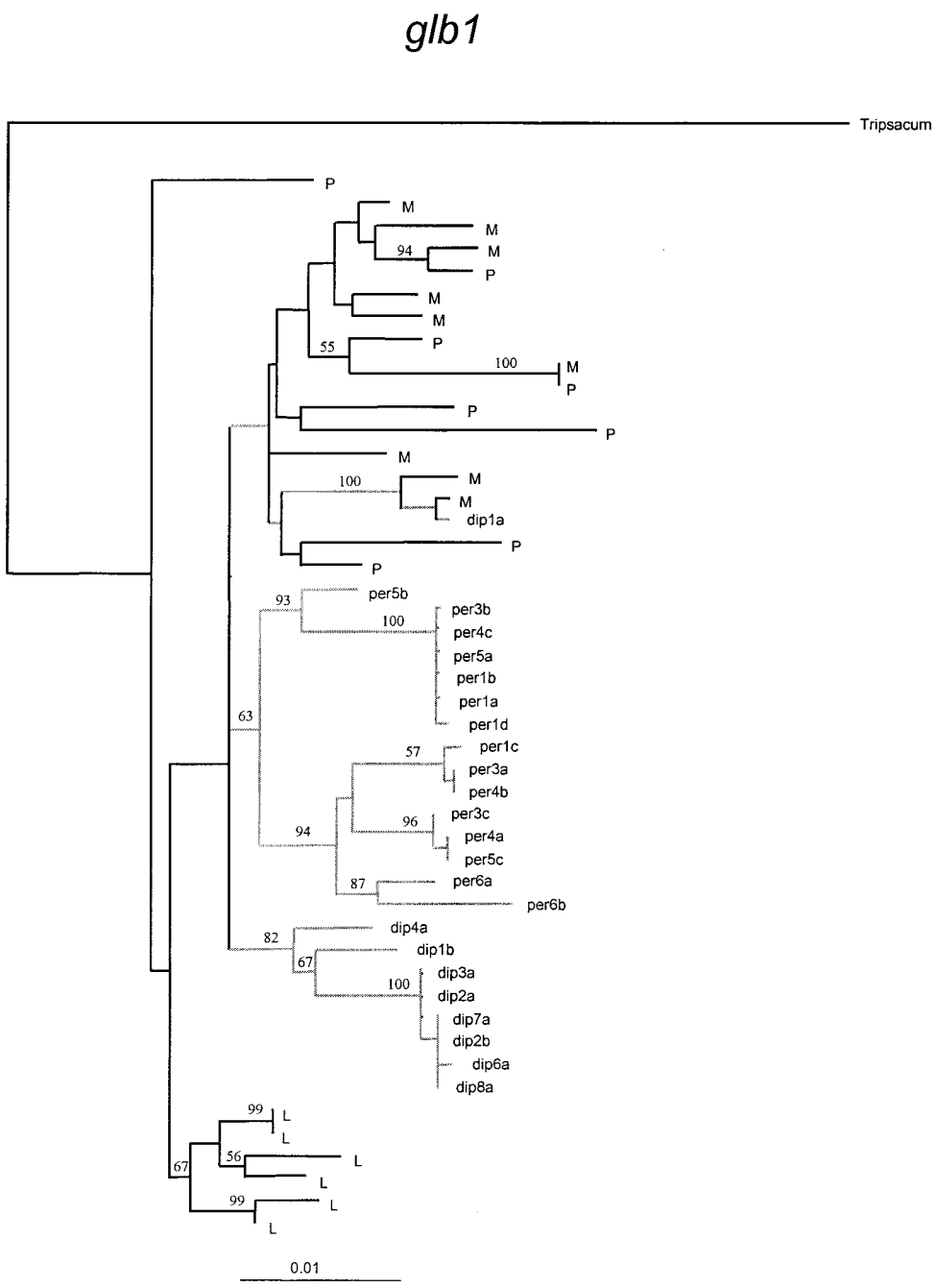

FIGURE 2.—Neighbor-joining reconstructions of the genealogical relationships among *glb1* alleles. Allele designations are described in Figure 1.

level of genetic diversity between the tetraploid and the diploid. One possibility is that *Z. perennis* is an autotetraploid that originated too recently to allow for the accumulation of mutations that differentiate the two species. We estimated the time of *Z. diploperennis*-*Z. perennis* divergence using a method of NEI (1987, p. 277), which is based on the average number of nucleotide substitutions within and between populations, and estimated substitution rates of 5×10^{-9}–30×10^{-9} substitutions per site per year (WOLFE *et al.* 1987; GAUT and CLEGG 1991). The estimated divergence times based on the high and low substitution rates, respectively, were 63,500–381,000 years (*adh1*), 194,700–1,168,000 years (*glb1*), 41,800–251,000 years (*c1*), and 24,000–142,000 years (*waxy*). Thus the preponderance of evidence supports divergence within the past few hundred thousand years.

A recent divergence between these species is also supported by the large number of polymorphisms that are shared between *Z. perennis* and *Z. diploperennis* and several strongly supported clades that are composed predominantly of *Z. perennis* or *Z. diploperennis* alleles but also contain alleles from the other species. Interestingly, the *glb1* gene, which shows no evidence of lineage sorting, is also the only gene with a ratio of common to rare polymorphisms that is significantly higher in *Z.*

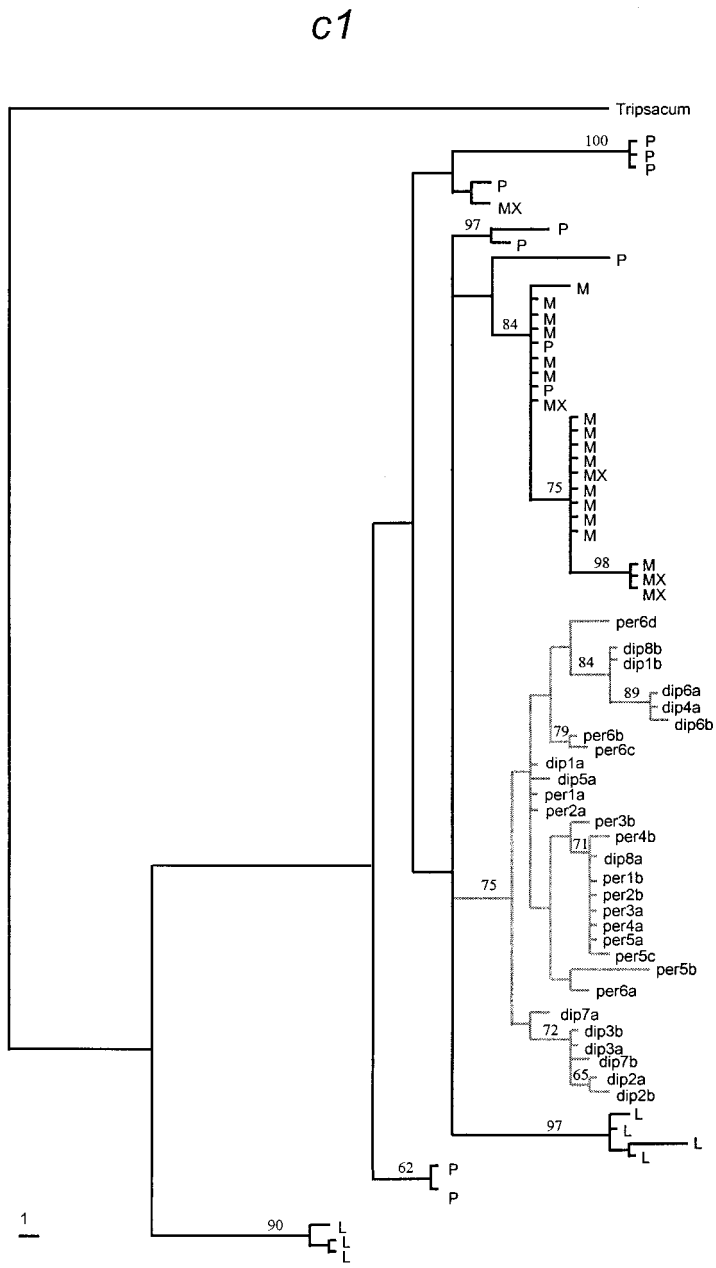

FIGURE 3.—Neighbor-joining reconstructions of the genealogical relationships among *c1* alleles. Allele designations are described in Figure 1.

perennis than *Z. diploperennis*. This, together with higher ratios of common to rare polymorphisms in all four genes, as would be expected if drift were slowed in the tetraploid (MOODY *et al.* 1993), could be indicative of increasing diversity in *Z. perennis*.

A second possible explanation for finding approximately equal amounts of genetic diversity in the tetraploid and diploid is that demographic forces associated with polyploid formation offset the forces that lead to greater diversity. Theoretical arguments for greater genetic diversity in autotetraploids are based on the assumption that increasing the number of chromosomes per individual increases effective population size. This implicitly assumes that the tetraploid and diploid species have had approximately equal population sizes. However, autotetraploid formation may involve a diversity-reducing population bottleneck (STEBBINS 1980). Nevertheless, our data provide no evidence for a severe bottleneck in the formation of *Z. perennis*. If *Z. perennis* has experienced a severe bottleneck, then the Tajima's *D*'s should be both negative and lower than Tajima's *D*'s calculated from *Z. diploperennis*; but this was not the case (Table 2). It is possible that *Z. perennis* has had multiple origins and this is the reason for finding little evidence for a bottleneck. Distinguishing multiple origins from gene flow and segregation of ancient polymorphisms is not possible from our data; however, the wide distribution of *Z. perennis* alleles within the genealogies suggests that multiple origin events are a possibility. The primary point of this discussion is that the demographic

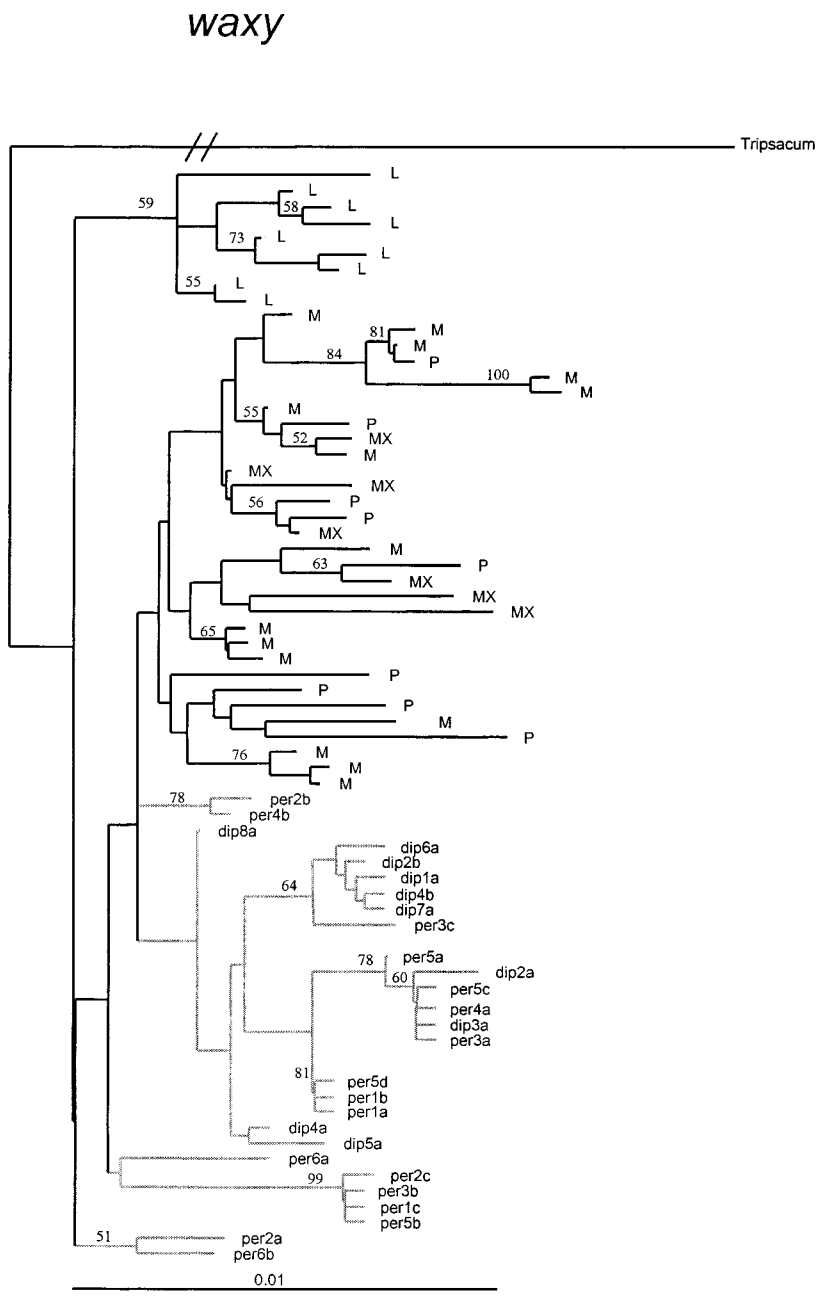

FIGURE 4.—Neighbor-joining reconstructions of the genealogical relationships among *waxy* alleles. Allele designations are described in Figure 1.

forces associated with tetraploid formation may offset the evolutionary forces contributing to greater genetic diversity in tetraploids.

If *Z. perennis* is in fact an autotetraploid, then our finding of approximately equal DNA sequence diversity in *Z. perennis* and its closest diploid relative appears inconsistent with the general finding that autotetraploids have a higher number of allozyme alleles than their diploid progenitors (reviewed in SOLTIS and SOLTIS 1993). However, allozyme studies that find greater genetic diversity in autotetraploids than diploids may be biased by unequal sampling. SOLTIS and SOLTIS (1993) reviewed data from six studies comparing autotetraploids to their diploid progenitors and found a greater number of allelic types in the tetraploid than the diploid species in five of six of these comparisons. However, at least five of these studies sampled considerably more alleles from the tetraploid (1.5 to nearly 4 times more alleles were sampled from the tetraploids; sampling data from the sixth study were not available). Because greater sampling effort is likely to result in the detection of a greater number of allelic types, the results from these allozyme studies must be interpreted with caution. In contrast, estimates of θ and π correct for sample size and thus these measures of diversity should be robust to deviations in sampling intensity.

Because genetic diversity has been measured for other diploid species of Zea, we can compare sequence diver-

TABLE 4

Distribution of fixed and shared polymorphisms between *Zea mays* ssp. *parviglumis*, *Z. luxurians*, *Z. diploperennis*, and *Z. perennis*

Species pairs	Fixed	Shared
diploperennis-parviglumis	6	25
diploperennis-luxurians	6	9
perennis-parviglumis	4	34
perennis-luxurians	7	12
diploperennis-perennis	2	45

Data from the putatively introgressed *glb1* allele (*Zea diploperennis* 1a) are not included.

sity among several other members of the genus (Table 5). At the four loci we studied, both *Z. perennis* and *Z. diploperennis* harbor lower levels of genetic diversity than *Z. mays* ssp. *parviglumis* and in general have lower diversity than the domesticate *Z. mays* ssp. *mays*. In contrast, *Z. perennis* has higher diversity than *Z. luxurians*, whereas *Z. diploperennis* and *Z. luxurians* have approximately equal amounts of diversity. The exception to these general patterns is the *c1* locus. At *c1*, *Z. mays* ssp. *mays* has lowest diversity and *Z. perennis* has lower diversity than either *Z. luxurians* or *Z. diploperennis*. *c1* has previously been hypothesized to evolve in response to selection during domestication, which could explain the low level of diversity in *Z. mays* ssp. *mays*. However, it is not clear why *Z. perennis* should also have relatively low diversity at this locus, especially since we found no evidence of selection having acted on this locus in this taxon. Altogether, these sequence data are in broad agreement with earlier isozyme studies of Zea species. Both sequence and isozyme data show that among all Zea species, *Z. mays* ssp. *parviglumis* has the greatest amount of genetic diversity and *Z. luxurians* has the least amount. However, sequence and isozyme data do not agree with regard to the relative levels of diversity within *Z. perennis* and *Z. diploperennis*. The sequence data revealed generally higher diversity in *Z. perennis* than *Z. diploperennis* or Z. *luxurians*, whereas isozyme data revealed higher diversity in *Z. diploperennis* than in *Z. perennis* or *Z. luxurians* (Doebley *et al.* 1984).

Origin of *Z. perennis* and phylogenetic relationships among Zea species: Genealogical data may be useful for inferring the evolutionary origin of tetraploid species (Okane *et al.* 1996; Small *et al.* 1998; Barrier *et al.* 1999; Small and Wendel 2000; Wendel 2000). Alleles sampled from an allopolyploid are expected to form two clades, one of which is either interspersed with or sister to clades formed by alleles from each of the parental taxa. In contrast, alleles sampled from an autopolyploid are expected to group phylogenetically with alleles from the species from which the polyploid formed. If the autopolyploid evolved recently, the alleles from the polyploid should be interspersed with alleles from the diploid progenitor; whereas, alleles from older autopolyploids should form clades that are monophyletic sister clades to the diploid progenitors.

Although the genealogical relationships among the sequences we sampled are not well enough resolved to provide conclusive evidence, our data largely support an autopolyploid origin of *Z. perennis* from a *Z. diploperennis*-like ancestor. This support comes from four observations. First, with one exception there were no well-supported clades that contained alleles from *Z. perennis* and any other Zea species. Second, several well-supported clades contained alleles isolated from both *Z. perennis* and *Z. diploperennis*. Third, the majority of clades that contained *Z. perennis* alleles also contained *Z. diploperennis* alleles or were sister to clades containing *Z. diploperennis* alleles. The strongest evidence for an autotetraploid origin, however, comes from the *c1* locus; all *c1* alleles isolated from these two species formed a single monophyletic clade. These genealogical inferences are consistent with the preponderance of evidence from morphological, isoenzyme, and meiotic pairing data

TABLE 5

π values and their standard errors (in parentheses) of *adh1*, *glb1*, *c1*, and *waxy* from five taxa of Zea

	ssp. *mays*	ssp. *parviglumis*	*luxurians*	*diploperennis*	*perennis*
adh1	22.0	27.0	12.0	18.5	17.4
	(12.2)	(15.2)	(7.2)	(10.4)	(9.2)
glb1	26.2	36.7	16.1	12.1	22.5
	(14.7)	(20.7)	(10.0)	(7.1)	(12.1)
c1	4.0	25.6	19.6	9.9	6.7
	(2.8)	(14.4)	(11.4)	(5.9)	(4.2)
waxy	12.4	15.6	9.0	5.9	10.2
	(6.9)	(8.9)	(5.4)	(3.6)	(5.6)

All values are per site and multiplied by 1000. Standard errors were calculated using formula 2.10 in Hartl and Clark (1997). All values were calculated on silent sites. Data for *Z mays* ssp. *mays* and *Z. mays* ssp. *parviglumis* are from Eyre-Walker *et al.* (1998; *adh1*); Hilton and Gaut (1998; *glb1*); Hanson *et al.* (1996); L. Zhang, A. Peek, D. Dunams and B. S. Gaut, (unpublished results; *c1* and *waxy*).

that also support an intraspecific origin of *Z. perennis* (Clausen *et al.* 1945; Shaver 1962; Kato and Lopez 1990).

Two aspects of the data, however, appear to conflict with an autotetraploid origin but are consistent with *Z. perennis* forming through interspecific hybridization between ancestors of *Z. diploperennis* and *Z mays* ssp. *parviglumis* or a now-extinct Zea species (Doebley 1989). First, one clade of *Z. perennis adh1* sequences clusters with *Z. mays* ssp. *mays* sequences, although the branches connecting these clades have weak phylogenetic support. The second apparent conflict with an autotetraploid origin is that the ratio of fixed to shared polymorphisms between *Z. perennis* to *Z. mays* ssp. *parviglumis* is approximately equal to the ratio between *Z. perennis* and *Z. diploperennis* (Table 4). However, this too is mitigated by the high ratio of fixed to shared polymorphisms between *Z. diploperennis* and *Z. mays* ssp. *parviglumis*, suggesting that the high ratio of shared polymorphisms may be due to lineage sorting and not reflective of an interspecific origin. Although evidence for an interspecific origin is certainly no stronger than evidence for an autopolyploid origin, we cannot exclude the possibility that *Z. perennis* was formed through interspecific hybridization. If *Z. perennis* was formed through interspecific hybridization it may be most similar to *Z. diploperennis* today because of a high rate of *Z. diploperennis* introgression, or because *Z. diploperennis* genes were preferentially maintained as the *Z. perennis* genome evolved.

The genus Zea has been separated into two sections, section Luxuriantes, composed of *Z. luxurians, Z. diploperennis,* and *Z. perennis*; and section Zea, composed of *Z. mays* ssp. *mays, Z. mays* ssp. *mexicana, Z. mays* ssp. *Parviglumis*, and *Z. mays* ssp. *huehuetenagensis* (Doebley 1990). This division is supported by chloroplast restriction site polymorphism data (Doebley *et al.* 1987) and weakly supported by morphological (Doebley 1983) and isoenzyme data (Doebley *et al.* 1984). In contrast, none of the four loci we studied formed a monophyletic group that included *Z. perennis*, *Z. diploperennis*, and *Z. luxurians*. Moreover, the distribution of shared polymorphisms and fixed differences between *Z. perennis*, *Z. diploperennis*, and *Z. luxurians* suggests that both *Z. diploperennis* and *Z. perennis* are more distantly related to *Z. luxurians* than to *Z. mays* ssp. *parviglumis* (Table 4). As such, our data do not support the section Luxuriantes as a natural grouping, but this conclusion is subject to the caveat that *Z. mays* ssp. *parviglumis* is known to segregate ancient alleles, thus complicating phylogenetic conclusions (Gaut and Clegg 1993). However, previous molecular sequence data, including ITS data (Buckler and Holtsford 1996), limited restriction site data from the IGS (ribosomal intergeneric spacer) and 5SDNA (Zimmer *et al.* 1988), and Magellan retrotransposon sequence (Purugganan and Wessler 1994) also do not support section Luxuriantes as a natural grouping.

Our data also provide some evidence that the phylogenetic relationships among the genus Zea may be complicated by introgression between taxa. In particular, a *glb1* allele from *Z. diploperennis* (dip1a, Figure 3) was very similar to and formed a well-supported clade with two *Z. mays* ssp. *mays* alleles. In contrast, all other *Z. perennis* and *Z. diploperennis glb1* alleles formed well-supported clades that contained no alleles from other Zea species. Previous investigations of nuclear genes in the genus Zea also provide evidence of possible introgression between *Z. mays* ssp. *mays* and *Z. diploperennis*. Isozyme surveys of Zea found a *Z. diploperennis* individual with two alleles that were otherwise unknown in *Z. diploperennis* but common in *Z. mays* ssp. *mays* (Doebley *et al.* 1984). In addition, several *Z. mays* ssp. *mays*-like ITS sequences were isolated from *Z. diploperennis* and *Z. perennis* individuals (Buckler and Holtsford 1996). Introgression between *Z. mays* ssp. *mays* and *Z. diploperennis* may not be surprising given that hybrids between these species are reported to be viable and fertile (Paspupuleti and Galinat 1982; Benz *et al.* 1990). Moreover, farmers in the Sierra de Manantlan region of Mexico, where *Z. perennis* and *Z. diploperennis* grow, reportedly hybridize *Z. diploperennis* and *Z. mays* ssp. *mays* in an attempt to improve productivity and impart pest resistance to traditional maize races (Benz *et al.* 1990). These hybrids are often left to grow in the maize field for 3–5 years. Because populations of *Z. diploperennis* often surround maize fields in this area, the hybrids may provide a bridge for introgression of *Z. mays* ssp. *mays* alleles into the *Z. diploperennis* gene pool.

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