

# Comparative analysis of inbred and hybrid maize at the diploid and tetraploid levels

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**Abstract** Heterosis often occurs in offspring derived from a cross between inbred or divergent parents and can be observed as the superior performance of these hybrids for a wide variety of characters. Heterosis was compared in maize lines at two ploidy levels, diploid and tetraploid, to gain a better understanding of the interaction of heterosis and ploidy level. Employing genetically identical diploid and tetraploid maize derived from four different inbred lines, we investigated heterosis for 11 morphological traits, including several plant height measures, as well as flowering time for both silks and anthers. We find that the heterotic response of a certain hybrid differs between diploid and tetraploid lines, and that the response at one ploidy cannot serve as a predictor for the other. Also, progressive heterosis was found for several of the characters in the tetraploid double-cross hybrid, which can have four different alleles at one locus, compared to the double-cross diploid hybrids, which can only possess two alleles per locus. Overall, the results indicate that the heterotic response of tetraploid maize lines differs significantly from that of the diploid.

## Introduction

Heterosis is the superior performance of hybrids over either parent for plant biomass, fertility and other characteristics. The genetic and molecular basis has remained unclear despite a great number of studies. A simple explanation has been that inbred lines are homozygous for a variety of slightly deleterious recessive mutations, which would be complemented in hybrids thus causing their superior phenotype (complementation model of heterosis). While this no doubt occurs, the behavior of polyploid hybrids is not easily reconciled with this hypothesis (Birchler et al. 2003).

Comparative studies of heterosis have been carried out mainly in alfalfa (*Medicago sativa*, diploid and tetraploid) and maize (*Zea mays*, diploid and tetraploid), from which autopolyploid lineages are known (Groose et al. 1989; Randolph 1935). In species for which autopolyploids exist, the comparison between identical diploid and tetraploid lines is particularly valuable due to the possibility for studying the effects of ploidy independent of heterozygosity and genetic distance. One common finding was that of progressive heterosis. Progressive heterosis refers to the observation that in polyploid hybrids, maximum performance is not reached in the F1 generation; rather, it increases further in double-cross hybrids in subsequent generations. For example, in tetraploids the heterotic response is often superior in a double-cross hybrid of the form ABCD than in single-cross hybrids (AABB, BBCC, CCDD, etc.). Population genetics theory predicts higher levels of heterozygosity which might serve as an explanation for the occurrence of progressive heterosis. Progressive heterosis has been observed for a variety of phenotypes such as forage yield and seed number per flower in alfalfa (Groose et al. 1989), tuber yield in potato (Mok and Pelouquin 1975), and for plant height, flowering time, and

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several yield characteristics in maize (Chase 1980; Levings et al. 1967; Sockness and Dudley 1989a).

Taking advantage of a method developed in our laboratory that allows for the generation of tetraploid versions of diploid lines of *Zea mays* (Kato and Birchler 2006), we undertook a series of experiments to investigate aspects of heterosis at two ploidy levels. Here, we report the results of a randomized field study comparing patterns of heterosis for 13 characters in diploid and tetraploid crosses for four inbred lines of *Zea mays*. As expected, we detect heterosis for the majority of characters both in diploid and tetraploid lines; however, the response of diploids and tetraploids to a specific cross differs in magnitude. In addition, a specific cross in the diploid lines was not a reliable predictor of the performance of the same lines at the tetraploid level despite being genetically identical. We also observe progressive heterosis for several of the characters measured. An analysis of variance indicates that the heterotic response in tetraploids is significantly different from that in diploid lines.

## Materials and methods

### Plant material

From diploid lines, tetraploid derivatives were generated using nitrous oxide gas as described previously (Kato and Birchler 2006). We used four diverse inbred lines: A188, B73, Oh43 and W22 (Liu et al. 2003; Senior et al. 1998). A complete list of the maize lines examined in this study is shown in Table 1. Hybrid F1 individuals were created by performing reciprocal crosses between individuals of the four lines, both at the diploid and tetraploid levels. By convention, the first individual noted in the cross is the female parent; the second genotype refers to the male parent. Quadruplex or double-cross hybrids of the tetraploid lines were generated by crossing F1 hybrids of different origin with each other (AABB × CCDD); the resulting offspring has the potential to carry four different alleles (ABCD). Double-cross hybrids of the diploids were generated by crossing F1 hybrids of different origins with each other (AB × CD); the double-cross hybrids thus contain a mosaic of genetic information from all four inbred lines, but carry at most two alleles per locus.

### Phenotypic measurements

In the summer of 2004, all lines listed in Table 1 were grown in three different sites (=blocks) near Columbia, MO, USA. We used a randomized complete block design for each of three blocks, where each line was represented once within each block in a random location by one row.

**Table 1** Maize lines included in the study

Inbred	Hybrid	Double-cross hybrid
A188 (2x, 4x)	A188/B73 (2x, 4x)	Oh43/A188/W22/B73 (4x)
W22 (2x, 4x)	A188/Oh43 (2x, 4x)	(A188/Oh43)/(B73/W22) (2x)
B73 (2x, 4x)	A188/W22 (2x, 4x)	(B73/W22)/(A188/Oh43) (2x)
Oh43 (2x, 4x)	B73/A188 (2x, 4x)	
	B73/Oh43 (2x, 4x)	
	B73/W22 (2x, 4x)	
	Oh43/A188 (2x, 4x)	
	Oh43/B73 (2x, 4x)	
	Oh43/W22 (2x, 4x)	
	W22/A188 (2x, 4x)	
	W22/B73 (2x, 4x)	
	W22/Oh43 (2x, 4x)	

Twenty seeds were planted per row. For each of the 35 lines, 10 individuals per block were used to collect the phenotypic data, and all plants were self-pollinated. In the analysis, the row was treated as the experimental unit, and individual measurements within the row represented subsamples.

In total, 13 phenotypic measures for each individual were collected. Two measures of flowering time were recorded: the number of days from planting to the emergence of silks and to the emergence of anthers. To investigate differences in growth rate and development, plant height was measured (from the soil to the top of the plant) at three time points: 4 and 6 weeks after planting as well as at maturity. It should be noted that tetraploid individuals of any type mature more slowly than diploid individuals; thus, the comparisons of plant height at 4 and 6 weeks after planting most likely involve different developmental stages for diploid and tetraploid lines. After flowering, when mature plant height is reached, the position/height of the primary ear in relation to the soil was measured (from the soil surface to the ear-carrying node), and the node number for this ear was recorded. The number of leaves and the number of tassel branches were counted at maturity. Using the third leaf from the top of the plant, leaf width (at the widest part of the leaf) and leaf length (from the base of the leaf to the leaf tip) measurements were obtained. In addition, the stem circumference in the second internode above the ear was recorded. Self-pollinated ears from all individuals were collected, and ear length was measured after harvest.

### Statistical analysis

All statistical analyses were performed using The SAS System for Windows Version 9.1.3. “Allele number”

refers to the hybridity of the line. All inbred lines have an allele number of 1, single-cross hybrids have an allele number of 2, and double-cross hybrids are assigned an allele number of 4, even though only in the tetraploid is there the potential for the presence of four alleles at each locus. “Family” refers to the exact genetic background of a line.

#### *Heterosis in diploid lines*

To quantify heterosis, mean values were calculated for each family and each measurement, and the values for the inbred parents were compared to that of the reciprocal hybrid offspring (Table 2). Using data from all diploid lines, an analysis of variance (ANOVA) was conducted. Because we considered the row of individuals as our experimental unit, all analyses used row means rather than the individual measurements. All results reported are from type III sums of squares due to the unbalanced nature of the data. The model included direct effects for “block” and “allele number,” as well as the nested effect of “family within allele number.” The two possible two-way interaction effects [“block with allele number” and “block with family nested within allele number”] were not included in the model. They all contain the “block” effect and thus represent part of the experimental error due to environmental variation and are more appropriately included in the overall error term of the model. The complete model is shown below:

$$y = \mu + \text{Block} + \text{Allele number} \\ + \text{Family}(\text{Allele number}) + \varepsilon$$

The factor of interest for our purpose of studying heterosis is the “allele number” effect. Comparisons between the individual allele number classes were carried out in order to determine which allele class comparison was responsible for significant allele number effects detected in the ANOVA. To do so, a comparison with adjusted class means was carried out (LSMEANS function in SAS).

An additional means comparison was conducted that only included the double-cross hybrid lines and the two single-cross lines from which the double-cross was derived (A188/Oh43, B73/W22, A188/Oh43/B73/W22 and B73/W22/A188/Oh43). In this case as before, adjusted means were used to compare the single-cross hybrids (allele number class 2) and the double-cross hybrids (allele number class 4).

In addition, a multivariate analysis of variance (MANOVA) was conducted using all 13 measurements. The model used was identical to the one employed in the

ANOVA described in the previous section. A contrast comparing the single-cross and double-cross hybrid classes was included in this analysis as before. Additionally, the same analysis was carried out on a subset of the diploid data which included only the double-cross hybrids and their two parents.

#### *Heterosis in tetraploid lines*

The statistical analysis of the data on tetraploid lines was carried out as described for the diploids. As outlined earlier, a means comparison between the four inbred lines and their hybrid derivatives was followed by ANOVA. The model used is the same as for the diploid lines:

$$y = \mu + \text{Block} + \text{Allele number} \\ + \text{Family}(\text{Allele number}) + \varepsilon$$

As described for the diploid lines, we considered the row of individuals as our experimental unit and used row means to carry out the analyses. Again, results reported are from type III sums of squares due to the unbalanced nature of the data. The initial comparisons between allele number classes were carried out using all tetraploid lines. An additional comparison of means was carried out using only the double-cross hybrid Oh43/A188/W22/B73 and the single-cross lines from which they were derived (Oh43/A188 and W22/B73). This analysis was conducted using the LS MEANS function in PROC GLM following the ANOVA.

Identical to the analysis carried out for the diploid lines, MANOVA was conducted using the data collected for all 13 characters in the tetraploid lines. The model used was the same as the one shown in the section describing the ANOVA. The analysis was carried out on two different subsets of data, once with all tetraploid lines, once with only the double-cross hybrid and its progenitor lines. These analyses contained a contrast between the single-cross and double-cross hybrid classes to determine if the two hybrid classes significantly differed from each other.

#### *Comparison of heterosis in diploid and tetraploid lines*

To compare the behavior of diploid and tetraploid lines, another ANOVA approach was used. The data were recorded so that individuals of the same genetic background but differing in ploidy (e.g. A188/Oh43 2× and A188/Oh43 4×) now are part of the same class. This classification was labeled “background” and included in the ANOVA model replacing the category of “family”. The new model is shown below:

**Table 2** Heterosis in diploid lines

	A188	A188/B73	B73/A188	B73
Days to anther emergence	60.38 ± 0.32	<b>59.70 ± 0.15</b>	<b>59.07 ± 0.20</b>	67.33 ± 0.40
Days to silk emergence	60.71 ± 0.34	<b>59.33 ± 0.18</b>	<b>59.07 ± 0.20</b>	66.54 ± 0.59
Tassel branch no.	15.17 ± 0.58	12.00 ± 0.36	13.42 ± 0.40	6.74 ± 0.26
Leaf number	10.00 ± 0.27	<b>12.57 ± 0.19</b>	<b>12.40 ± 0.18</b>	11.80 ± 0.26
Ear node number	3.92 ± 0.16	<b>5.80 ± 0.16</b>	<b>5.53 ± 0.13</b>	5.27 ± 0.22
Leaf length	56.13 ± 0.81	<b>69.04 ± 0.76</b>	<b>67.54 ± 0.86</b>	62.67 ± 1.07
Leaf width	9.88 ± 0.14	<b>10.71 ± 0.14</b>	<b>10.59 ± 0.20</b>	8.19 ± 0.22
Adult height	167.25 ± 3.68	<b>247.00 ± 3.80</b>	<b>255.88 ± 2.42</b>	209.38 ± 3.51
Ear height	52.71 ± 1.89	<b>93.72 ± 2.21</b>	<b>86.97 ± 1.36</b>	76.00 ± 2.38
Ear length	11.54 ± 0.29	<b>16.07 ± 0.19</b>	<b>16.17 ± 0.21</b>	12.50 ± 0.28
Circumference	5.81 ± 0.14	<b>6.77 ± 0.07</b>	<b>7.08 ± 0.09</b>	6.23 ± 0.13
Height at 4 weeks	22.75 ± 0.83	<b>35.25 ± 0.71</b>	<b>36.66 ± 0.75</b>	24.00 ± 0.64
Height at 6 weeks	54.82 ± 1.96	<b>92.05 ± 2.60</b>	<b>98.30 ± 2.51</b>	56.35 ± 2.32
	A188	A188/Oh43	Oh43/A188	Oh43
Days to anther emergence	60.38 ± 0.32	<b>58.17 ± 0.34</b>	<b>57.14 ± 0.17</b>	64.81 ± 0.95
Days to silk emergence	60.71 ± 0.34	<b>57.53 ± 0.20</b>	<b>56.45 ± 0.13</b>	65.00 ± 1.01
Tassel branch no.	15.17 ± 0.58	12.52 ± 0.43	13.45 ± 0.43	7.94 ± 0.44
Leaf number	10.00 ± 0.27	<b>11.00 ± 0.14</b>	<b>11.47 ± 0.15</b>	10.00 ± 0.35
Ear node number	3.92 ± 0.16	<b>4.87 ± 0.13</b>	<b>4.93 ± 0.14</b>	4.88 ± 0.20
Leaf length	56.13 ± 0.81	<b>68.07 ± 0.74</b>	<b>68.24 ± 0.76</b>	59.27 ± 1.31
Leaf width	9.88 ± 0.14	11.59 ± 0.21	11.55 ± 0.18	12.20 ± 0.28
Adult height	167.25 ± 3.68	<b>232.77 ± 2.2</b>	<b>240.41 ± 3.38</b>	187.87 ± 4.42
Ear height	52.71 ± 1.89	<b>76.17 ± 1.61</b>	<b>78.77 ± 2.45</b>	60.50 ± 2.28
Ear length	11.54 ± 0.29	<b>18.77 ± 0.40</b>	<b>18.61 ± 0.27</b>	17.88 ± 0.42
Circumference	5.81 ± 0.14	6.5 ± 0.10	6.93 ± 0.11	6.53 ± 0.14
Height at 4 weeks	22.75 ± 0.83	<b>37.42 ± 0.84</b>	<b>40.56 ± 0.57</b>	22.19 ± 1.51
Height at 6 weeks	54.82 ± 1.96	<b>95.84 ± 3.05</b>	<b>104.99 ± 2.01</b>	59.32 ± 3.61
	A188	A188/W22	W22/A188	W22
Days to anther emergence	60.38 ± 0.32	<b>59.47 ± 0.20</b>	<b>59.53 ± 0.19</b>	66.41 ± 0.51
Days to silk emergence	60.71 ± 0.34	59.73 ± 0.30	60.73 ± 0.34	68.69 ± 0.66
Tassel branch no.	15.17 ± 0.58	<b>17.47 ± 0.61</b>	<b>17.03 ± 0.45</b>	12.83 ± 0.53
Leaf number	10.00 ± 0.27	<b>12.13 ± 0.25</b>	<b>11.87 ± 0.18</b>	11.27 ± 0.20
Ear node number	3.92 ± 0.16	<b>5.10 ± 0.18</b>	<b>4.80 ± 0.13</b>	3.97 ± 0.19
Leaf length	56.13 ± 0.81	<b>65.71 ± 1.14</b>	<b>66.13 ± 0.81</b>	60.82 ± 0.81
Leaf width	9.88 ± 0.14	<b>11.18 ± 0.29</b>	<b>11.77 ± 0.10</b>	9.83 ± 0.20
Adult height	167.25 ± 3.68	<b>230.54 ± 3.22</b>	<b>233.59 ± 2.67</b>	176.25 ± 2.99
Ear height	52.71 ± 1.89	<b>80.90 ± 2.14</b>	<b>81.83 ± 1.85</b>	69.72 ± 2.27
Ear length	11.54 ± 0.29	<b>16.86 ± 0.37</b>	<b>17.34 ± 0.15</b>	12.86 ± 0.27
Circumference	5.81 ± 0.14	7.13 ± 0.10	6.77 ± 0.14	7.13 ± 0.12
Height at 4 weeks	22.75 ± 0.83	<b>34.33 ± 0.74</b>	<b>31.07 ± 0.58</b>	21.59 ± 0.81
Height at 6 weeks	54.82 ± 1.96	<b>89.45 ± 2.04</b>	<b>84.37 ± 3.23</b>	52.2 ± 1.82
	B73	B73/Oh43	Oh43/B73	Oh43
Days to anther emergence	67.33 ± 0.40	<b>61.73 ± 0.15</b>	<b>61.20 ± 0.11</b>	64.81 ± 0.95
Days to silk emergence	66.54 ± 0.59	<b>61.00 ± 0.13</b>	<b>60.47 ± 0.14</b>	65.00 ± 1.01
Tassel branch no.	6.74 ± 0.26	<b>9.97 ± 0.34</b>	<b>9.83 ± 0.34</b>	7.94 ± 0.44
Leaf number	11.80 ± 0.26	<b>13.03 ± 0.19</b>	<b>12.87 ± 0.17</b>	10.00 ± 0.35

**Table 2** continued

	B73	B73/Oh43	Oh43/B73	Oh43
Ear node number	5.27 ± 0.22	<b>6.53 ± 0.16</b>	<b>6.10 ± 0.14</b>	4.88 ± 0.20
Leaf length	62.67 ± 1.07	<b>67.62 ± 0.85</b>	<b>66.79 ± 1.09</b>	59.27 ± 1.31
Leaf width	8.19 ± 0.22	10.68 ± 0.20	10.31 ± 0.19	12.20 ± 0.28
Adult height	209.38 ± 3.51	<b>277.92 ± 3.04</b>	<b>269.21 ± 3.34</b>	187.87 ± 4.42
Ear height	76.00 ± 2.38	<b>109.93 ± 2.10</b>	<b>106.81 ± 2.64</b>	60.50 ± 2.28
Ear length	12.50 ± 0.28	<b>17.90 ± 0.39</b>	<b>18.36 ± 0.29</b>	17.88 ± 0.42
Circumference	6.23 ± 0.13	<b>6.72 ± 0.08</b>	<b>6.67 ± 0.09</b>	6.53 ± 0.14
Height at 4 weeks	24.00 ± 0.64	<b>39.84 ± 1.06</b>	<b>40.26 ± 0.65</b>	22.19 ± 1.51
Height at 6 weeks	56.35 ± 2.32	<b>99.65 ± 1.65</b>	<b>101.18 ± 2.53</b>	59.32 ± 3.61
	B73	B73/W22	W22/B73	W22
Days to anther emergence	67.33 ± 0.40	<b>62.83 ± 0.13</b>	<b>62.31 ± 0.19</b>	66.41 ± 0.51
Days to silk emergence	66.54 ± 0.59	<b>62.80 ± 0.15</b>	<b>62.45 ± 0.24</b>	68.69 ± 0.66
Tassel branch no.	6.74 ± 0.26	10.80 ± 0.37	12.52 ± 0.34	12.83 ± 0.53
Leaf number	11.80 ± 0.26	<b>14.10 ± 0.14</b>	<b>13.79 ± 0.15</b>	11.27 ± 0.20
Ear node number	5.27 ± 0.22	<b>6.50 ± 0.14</b>	<b>6.45 ± 0.13</b>	3.97 ± 0.19
Leaf length	62.67 ± 1.07	<b>66.61 ± 0.75</b>	<b>67.93 ± 0.93</b>	60.82 ± 0.81
Leaf width	8.19 ± 0.22	9.32 ± 0.21	9.79 ± 0.16	9.83 ± 0.20
Adult height	209.38 ± 3.51	<b>283.89 ± 1.89</b>	<b>269.29 ± 3.27</b>	176.25 ± 2.99
Ear height	76.00 ± 2.38	<b>121.03 ± 1.92</b>	<b>112.72 ± 2.05</b>	69.72 ± 2.27
Ear length	12.50 ± 0.28	<b>16.9 ± 0.18</b>	<b>17.36 ± 0.22</b>	12.86 ± 0.27
Circumference	6.23 ± 0.13	<b>7.23 ± 0.11</b>	<b>7.33 ± 0.08</b>	7.13 ± 0.12
Height at 4 weeks	24.00 ± 0.64	<b>35.77 ± 0.46</b>	<b>37.47 ± 1.22</b>	21.59 ± 0.81
Height at 6 weeks	56.35 ± 2.32	<b>97.87 ± 1.40</b>	<b>94.37 ± 1.42</b>	52.20 ± 1.82
	Oh43	Oh43/W22	W22/Oh43	W22
Days to anther emergence	64.81 ± 0.95	<b>61.63 ± 0.18</b>	<b>61.50 ± 0.20</b>	66.41 ± 0.51
Days to silk emergence	65.00 ± 1.01	<b>61.41 ± 0.18</b>	<b>61.10 ± 0.23</b>	68.69 ± 0.66
Tassel branch no.	7.94 ± 0.44	12.28 ± 0.44	12.97 ± 0.46	12.83 ± 0.53
Leaf number	10.00 ± 0.35	<b>12.53 ± 0.16</b>	<b>12.13 ± 0.18</b>	11.27 ± 0.20
Ear node number	4.88 ± 0.20	<b>5.77 ± 0.16</b>	<b>5.50 ± 0.16</b>	3.97 ± 0.19
Leaf length	59.27 ± 1.31	<b>64.45 ± 0.92</b>	<b>66.76 ± 1.08</b>	60.82 ± 0.81
Leaf width	12.20 ± 0.28	11.68 ± 0.21	11.87 ± 0.20	9.83 ± 0.20
Adult height	187.87 ± 4.42	<b>254.55 ± 2.84</b>	<b>249.76 ± 2.72</b>	176.25 ± 2.99
Ear height	60.50 ± 2.28	<b>95.44 ± 2.12</b>	<b>89.07 ± 1.73</b>	69.72 ± 2.27
Ear length	17.88 ± 0.42	<b>17.97 ± 0.48</b>	<b>19.31 ± 0.41</b>	12.86 ± 0.27
Circumference	6.53 ± 0.14	6.98 ± 0.09	7.23 ± 0.12	7.13 ± 0.12
Height at 4 weeks	22.19 ± 1.51	<b>37.51 ± 0.66</b>	<b>36.07 ± 0.83</b>	21.59 ± 0.81
Height at 6 weeks	59.32 ± 3.61	<b>94.06 ± 2.10</b>	<b>90.30 ± 1.47</b>	52.20 ± 1.82

Mean character measures and standard errors of the mean are shown for each of the reciprocal crosses included in the study. Measures that are marked in bold exhibit heterosis, as they are more extreme in the hybrids than in either inbred parent. Measurements are in centimeter unless otherwise noted

$$\begin{aligned}
 y = & \mu + \text{Block} + \text{Allele number} + \text{Ploidy} \\
 & + \text{Background}(\text{Allele number}) \\
 & + \text{Allele number} * \text{Ploidy} \\
 & + \text{Background} * \text{Ploidy}(\text{Allele number}) + \varepsilon
 \end{aligned}$$

Only interactions not involving the block factor were included in the model for the same reasons as specified under the original ANOVA model. This full model was used in the initial analysis of each character but was simplified in subsequent analyses by removing interaction

effects that did not significantly contribute to the model. To examine the possibility of a differential response to allelic diversity in diploid and tetraploid lines of maize, the interaction effect between allele number and ploidy was investigated in detail. As before, type III sum of squares were used due to the unbalanced nature of the data.

## Results

### Heterosis in diploid lines

In a randomized field study we quantified heterosis in hybrids derived from a set of four diverse inbred maize lines. Eleven morphological traits and flowering time for both silk and anther emergence were recorded for the four inbred diploid lines as well as all possible reciprocal hybrids. Representative individuals for the cross between the inbred lines W22 and B73 are shown in the top panel of Fig. 1. In Table 2, the mean measurements of all 13 characters for the inbred parent lines as well as their F1 hybrid offspring are presented. Based on the deviation from the mid-parent expectation, we find heterosis in all crosses and for all phenotypes (data not shown). Using the better parent heterosis criterion, approximately 85% of measures show heterosis, as the mean values for the hybrid lines surpass the superior parent (marked in bold in Table 2). From a total of 78 hybrid measurements, only 12 do not show heterosis. Interestingly, these 12 cases are not randomly distributed among the various characters. Non-heterotic behavior is mostly seen in the traits tassel branch number, leaf width and stem circumference, which account for 11 of the 12 cases.

The strength of heterosis observed in the hybrids included in our study ranges from a low value of 1% (Oh43/B73 hybrid for the character leaf width) to an 84% increase in plant height at 4 weeks observed for the Oh43/A188 hybrid. An example of the range of heterosis values observed for a single trait is illustrated in Fig. 2, which shows the percentage increases observed for one of the plant height measurements. The heterosis values differ by a factor of two for this character, whereas for other characters the differences in heterosis between the various crosses can be up to 20-fold. Thus, as expected, the impact of heterosis differs in strength depending on the exact cross and also on the particular trait in question.

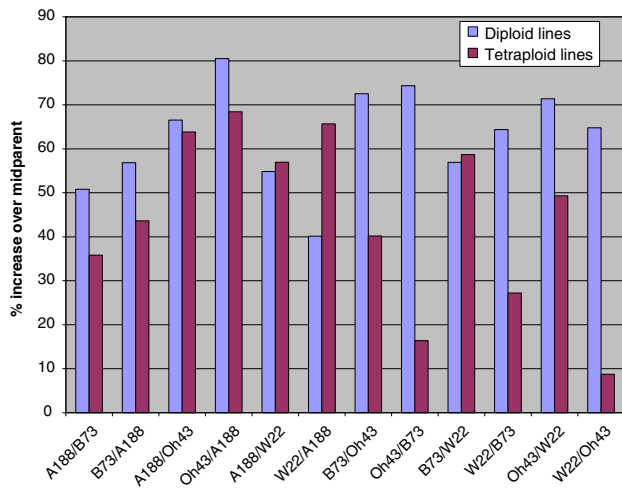
Using a number of different analysis of variance approaches, we investigated the heterotic behavior and the influence of allelic diversity present within diploid maize lines on the morphological measurements (for details as to which analysis was used for each character, see [Materials and methods](#)). The ANOVA model included the direct effects of block to account for environmental variation



**Fig. 1** Heterosis in diploid and tetraploid maize. B73 is shown on the left, W22 on the right. In the middle are the two reciprocal hybrids, B73  $\times$  W22 and W22  $\times$  B73. Top panel diploid lines, bottom panel tetraploid lines. A meter stick is shown as a size marker

between the three blocks, and allele number, to account for the effects of hybrid vigor/heterozygosity, as well as the nested effect of family within allele number, which accounts for the effect of the genetic background from which a certain line is derived. For 8 of the 13 characters, the factor “block” had a significant impact on the measurements. All but 3 of the 13 characters were strongly influenced by the genetic background of the line (family nested within allele number).

To investigate the difference between inbred and hybrid lines of diploid maize, we examined the factor allele number in our ANOVA model. This factor differentiates between the inbred, single-cross and double-cross hybrids and is expected to be significant if heterosis affects the phenotypes included in this study. We find that the inbred or hybrid state has a strong influence on all characters, as expected. This finding is illustrated in the statistical analyses, where the factor “allele number,” a measure of heterozygosity, consistently exhibits highly significant



**Fig. 2** Strength of heterosis. Percent increase over the mid-parent value for the character “plant height at 4 weeks after planting” is plotted for the various hybrid lines for diploids and tetraploids

*P*-values (see Table 3). The class means for hybrid individuals of either class are always higher than those for the inbred individuals with allele number = 1. Examples of this behavior can be seen in Fig. 1, which shows a cross between W22 and B73, and also in Fig. 3, which graphs flowering time for all diploid lines.

Having documented the magnitude of heterosis in the phenotypes investigated, we compared the single- and double-cross hybrids in more detail. The diploid double-cross was included in this study specifically to serve as control and to allow for a comparison to the tetraploid double-cross, described below. In tetraploids a double-cross can increase allelic diversity by potentially having

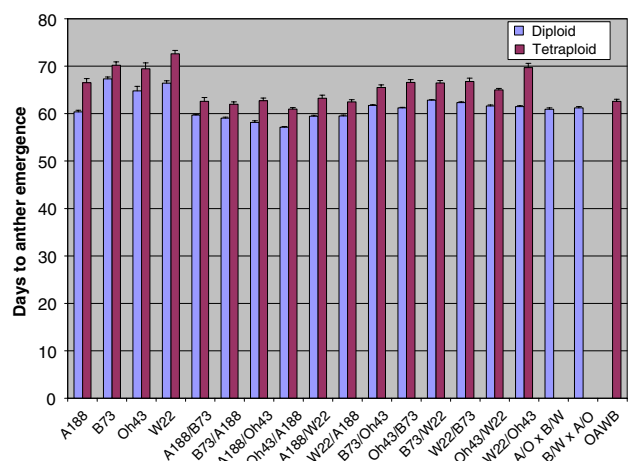
four different alleles per locus; in the diploid, an increase in allelic diversity is not possible. When means between the double-cross hybrids and all single-cross hybrids are compared in diploids, they are statistically not different for any of the 13 measurements. If compared to the average single-cross line, characters often exhibit a trend towards more extreme phenotypes in the double-cross hybrid; this observation will be explored in more detail below and in the multivariate portion of the analysis.

In the above comparison between single-cross and double-cross hybrids, we used pooled data from all single-cross lines, not just from the two lines from which the double-cross hybrids were derived. This approach was taken to account for the fact that the double-cross hybrid will carry a complex mixture of genetic information from all four inbred lines. The individual double-cross hybrids will have different combinations of alleles compared to the uniform combinations in each of the single-cross hybrids. However, as heterosis is defined specifically as the superior performance of a hybrid offspring, we carried out a second analysis, which examined the exact parent lines (A188/Oh43 and B73/W22) and compared their performance to the double-cross hybrids. The results of this analysis are illustrated in Table 4, which displays the mean phenotypic measures for the double-cross hybrids and their single-cross parent lines. Only for the tassel branch count, does the reciprocal double-cross hybrids exceed the single-cross lines; however, this difference is not statistically significant. For one of the flowering time measures, time to silk emergence, the difference between double- and single-cross hybrids is significant, but the double-cross hybrids do not exceed both parental lines. The second measure to show a significant difference in this analysis is plant height 6 weeks after planting, but this measure is inferior in the double-cross hybrids. Thus, as expected, the majority of

**Table 3** ANOVA results for diploids

Character	DF	SS	<i>F</i>	<i>P</i> -value
Days to flowering: anthers	2	194.10	98.14	<0.0001
Days to flowering: silks	2	256.80	123.63	<0.0001
Tassel branch number	2	59.37	18.16	<0.0001
Leaf number	2	32.91	38.00	<0.0001
Ear node number	2	12.99	39.81	<0.0001
Leaf length	2	476.92	47.37	<0.0001
Leaf width	2	7.83	19.44	<0.0001
Adult height	2	46,304.48	274.39	<0.0001
Ear height	2	8,802.36	125.64	<0.0001
Ear length	2	138.15	135.30	<0.0001
Stem circumference	2	2.88	27.39	<0.0001
Height at 4 weeks	2	1,863.60	89.82	<0.0001
Height at 6 weeks	2	14,858.84	184.06	<0.0001

Partial results for the factor “allele number” from all characters investigated. Reported are type III sum of squares and associated *P*-values unless otherwise noted



**Fig. 3** Flowering time measured by the days to anther emergence in diploid and tetraploid lines of *Zea mays*

**Table 4** Comparison between diploid double-cross hybrids and their single-cross parents

	A188/Oh43	A188/Oh43 × B73/W222	B73/W22 × A188/Oh43	B73/W22
Days to anther emergence	58.17 ± 0.34	60.90 ± 0.30	61.20 ± 0.26	62.83 ± 0.13
Days to silk emergence*	57.53 ± 0.20	60.83 ± 0.29	60.97 ± 0.25	62.80 ± 0.15
Tassel branch number	12.52 ± 0.43	<b>14.04 ± 0.64</b>	<b>13.48 ± 0.53</b>	10.80 ± 0.37
Leaf number	11.00 ± 0.14	12.87 ± 0.21	12.79 ± 0.22	14.10 ± 0.14
Ear node number	4.87 ± 0.13	5.87 ± 0.18	5.73 ± 0.14	6.50 ± 0.14
Leaf length	68.07 ± 0.74	66.81 ± 1.43	66.50 ± 1.20	66.61 ± 0.75
Leaf width	11.59 ± 0.21	10.61 ± 0.23	11.10 ± 0.24	9.32 ± 0.21
Adult height	232.77 ± 2.2	265.46 ± 3.56	256.29 ± 4.23	283.89 ± 1.89
Ear height	76.17 ± 1.61	98.04 ± 3.08	95.12 ± 2.88	121.03 ± 1.92
Ear length	18.77 ± 0.40	17.50 ± 0.31	18.41 ± 0.32	16.9 ± 0.18
Stem circumference	6.5 ± 0.10	7.10 ± 0.14	6.97 ± 0.10	7.23 ± 0.11
Height at 4 weeks	37.42 ± 0.84	36.53 ± 1.16	35.52 ± 1.03	35.77 ± 0.46
Height at 6 weeks*	95.84 ± 3.05	90.64 ± 1.98	88.43 ± 1.69	97.87 ± 1.40

When the character means for the single-cross and double-cross hybrids are compared, the difference is statistically significant for these characters; mean character measures and standard errors of the mean are shown; characters that exhibit superior performance in double-cross hybrid are marked in bold. Measurements are in centimeter unless otherwise noted

phenotypes investigated, 11 of 13 measures, show no clear difference between the double-cross and single-cross hybrids in these diploid lines, which is very similar to what was observed when the average single-cross values were used for analysis.

Two multivariate analyses were carried out to determine if there is an effect of allele number on the overall plant phenotype. While heterosis is usually studied on the level of a single trait, natural selection operates on the entire organism, and thus benefits conveyed by heterosis to the individual might not be evident by studying particular traits. Thus, we explored the effects of single- and double-crosses with multivariate analyses as well. Using the same model as for the single character ANOVA and including all diploid lines, we find that allele number significantly impacts the multivariate phenotypes ( $P < 0.0001$ ). Again, we find that the comparison between the two types of hybrids is not significant. In an analysis that includes only the two parent single-cross lines and their double-cross offspring, the contrast of means is not estimable. These results indicate that overall, single- and double-cross hybrids are not different from each other, as might be expected based on the analysis of individual phenotypes. In addition, the results do not indicate a trend towards greater heterosis in one configuration versus the other.

#### Heterosis in tetraploid lines

Tetraploid lines identical to the diploid lines allow for a comparison of heterosis between different ploidy levels. The tetraploid inbred lines were derived from the four diploid inbred lines and differ only in genome dosage. For

the four inbred lines as well as all possible single-cross hybrids and one double-cross hybrid, the same 11 morphological traits and flowering time for both silk and anther emergence were recorded. In Table 5, the mean measurements for the inbred parent lines as well as their F1 hybrid offspring are presented. For each of the six reciprocal crosses, all F1 means exceed the midparent values, and 88% of the measures show better-parent heterosis, as demonstrated by the fact that the mean values for the hybrid lines exceed that of both parents (marked in bold in Table 5). This behavior is also evident in the bar graphs of Figs. 3 and 4, which show the mean measurements for days to anther emergence and plant height at 4 weeks after planting in the diploid as well as in the tetraploid lines. From a total of 78 hybrid measurements, nine do not show heterosis. Non-heterotic behavior is mostly seen in tassel branch number, stem circumference (similar to what is observed in the diploids), and leaf number; these three characters account for seven of the nine cases.

The heterotic strength in the tetraploid hybrids is similar to that observed in the diploid lines, ranging from 1% (Oh43/B73 hybrid for flowering time for silks) to 102% (B73/Oh43 hybrid for tassel branch number). An example of the range of heterosis values observed for a single phenotype is illustrated in Fig. 2, which shows the percentage increases observed for one of the plant height measurements. The heterosis values differ by a factor of seven for this character, whereas for other characters the differences in heterosis between the various crosses can be up to 12-fold. Thus, as expected, the impact of heterosis differs in strength depending on the exact cross and also on the particular phenotype in question. While the overall midparent heterosis levels between diploids and tetraploids



**Table 5** Heterosis in tetraploid lines

	A188 4x	A188/B73	B73/A188	B73 4x
Days to anther emergence	66.54 ± 0.84	<b>62.61 ± 0.80</b>	<b>61.97 ± 0.51</b>	70.20 ± 0.72
Days to silk emergence	67.75 ± 0.90	<b>63.74 ± 0.92</b>	<b>62.93 ± 0.71</b>	71.65 ± 0.49
Tassel branch no.	14.48 ± 0.97	11.26 ± 0.54	12.03 ± 0.71	4.03 ± 0.19
Leaf number	9.43 ± 0.35	<b>10.95 ± 0.32</b>	<b>11.47 ± 0.15</b>	10.87 ± 0.20
Ear node number	4.58 ± 0.18	<b>5.47 ± 0.22</b>	<b>5.10 ± 0.15</b>	4.81 ± 0.25
Leaf length	53.78 ± 1.15	<b>67.47 ± 1.42</b>	<b>66.50 ± 1.01</b>	62.71 ± 1.13
Leaf width	7.83 ± 0.35	<b>10.63 ± 0.26</b>	<b>9.37 ± 0.26</b>	7.75 ± 0.22
Adult height	123.63 ± 4.28	<b>218.39 ± 3.98</b>	<b>221.66 ± 4.62</b>	180.25 ± 3.99
Ear height	41.96 ± 2.12	<b>79.21 ± 1.95</b>	<b>86.79 ± 2.47</b>	73.27 ± 2.21
Ear length	8.26 ± 0.53	<b>14.39 ± 0.45</b>	<b>12.93 ± 0.44</b>	8.38 ± 0.27
Circumference	5.98 ± 0.15	8.21 ± 0.16	7.35 ± 0.17	7.78 ± 0.21
Height at 4 weeks	16.09 ± 0.77	<b>27.26 ± 1.23</b>	<b>28.83 ± 0.64</b>	24.05 ± 0.89
Height at 6 weeks	32.67 ± 1.68	<b>66.29 ± 3.71</b>	<b>76.62 ± 2.95</b>	59.01 ± 2.29
	A188 4x	A188/Oh43	Oh43/A188	Oh43 4x
Days to anther emergence	66.54 ± 0.84	<b>62.75 ± 0.55</b>	<b>60.97 ± 0.32</b>	69.47 ± 1.24
Days to silk emergence	67.75 ± 0.90	<b>63.42 ± 0.80</b>	<b>61.53 ± 0.42</b>	72.24 ± 1.17
Tassel branch no.	14.48 ± 0.97	13.00 ± 0.60	12.17 ± 0.53	4.00 ± 0.34
Leaf number	9.43 ± 0.35	<b>11.13 ± 0.21</b>	<b>10.83 ± 0.22</b>	9.11 ± 0.17
Ear node number	4.58 ± 0.18	<b>5.58 ± 0.16</b>	<b>4.47 ± 0.16</b>	4.22 ± 0.19
Leaf length	53.78 ± 1.15	<b>64.21 ± 1.58</b>	<b>61.89 ± 1.31</b>	52.79 ± 1.59
Leaf width	7.83 ± 0.35	<b>11.63 ± 0.32</b>	<b>10.59 ± 0.29</b>	10.05 ± 0.39
Adult height	123.63 ± 4.28	<b>204.74 ± 2.91</b>	<b>182.03 ± 4.20</b>	148.11 ± 6.94
Ear height	41.96 ± 2.12	<b>78.30 ± 2.97</b>	<b>60.57 ± 2.32</b>	46.38 ± 2.02
Ear length	8.26 ± 0.53	<b>17.27 ± 0.52</b>	<b>16.17 ± 0.51</b>	12.81 ± 0.71
Circumference	5.98 ± 0.15	<b>7.54 ± 0.19</b>	<b>7.23 ± 0.15</b>	6.55 ± 0.22
Height at 4 weeks	16.09 ± 0.77	<b>30.43 ± 0.96</b>	<b>31.28 ± 0.62</b>	21.06 ± 1.40
Height at 6 weeks	32.67 ± 1.68	<b>69.43 ± 1.96</b>	<b>68.41 ± 1.66</b>	43.38 ± 2.45
	A188 4x	A188/W22	W22/A188	W22 4x
Days to anther emergence	66.54 ± 0.84	<b>63.23 ± 0.68</b>	<b>62.5 ± 0.46</b>	72.63 ± 0.71
Days to silk emergence	67.75 ± 0.90	<b>64.93 ± 0.84</b>	<b>64.71 ± 0.54</b>	74.72 ± 0.83
Tassel branch no.	14.48 ± 0.97	<b>16.47 ± 0.86</b>	<b>15.96 ± 0.65</b>	7.47 ± 0.76
Leaf number	9.43 ± 0.35	11.40 ± 0.17	11.36 ± 0.19	11.68 ± 0.38
Ear node number	4.58 ± 0.18	<b>5.00 ± 0.17</b>	<b>5.25 ± 0.14</b>	4.50 ± 0.20
Leaf length	53.78 ± 1.15	<b>60.34 ± 1.31</b>	<b>60.25 ± 0.96</b>	50.47 ± 2.02
Leaf width	7.83 ± 0.35	<b>10.07 ± 0.35</b>	<b>10.39 ± 0.25</b>	8.26 ± 0.30
Adult height	123.63 ± 4.28	<b>199.33 ± 4.33</b>	<b>217.57 ± 4.32</b>	154.78 ± 5.54
Ear height	41.96 ± 2.12	<b>73.70 ± 2.16</b>	<b>80.39 ± 2.93</b>	59.00 ± 2.97
Ear length	8.26 ± 0.53	<b>14.59 ± 0.51</b>	<b>14.65 ± 0.62</b>	7.65 ± 0.37
Circumference	5.98 ± 0.15	<b>7.60 ± 0.11</b>	<b>7.41 ± 0.15</b>	7.39 ± 0.18
Height at 4 weeks	16.09 ± 0.77	<b>25.74 ± 1.37</b>	<b>27.17 ± 0.85</b>	16.71 ± 0.97
Height at 6 weeks	32.67 ± 1.68	<b>63.54 ± 2.82</b>	<b>65.41 ± 2.28</b>	39.90 ± 1.36
	B73 4x	B73/Oh43	Oh43/B73	Oh43 4x
Days to anther emergence	70.20 ± 0.72	<b>65.53 ± 0.53</b>	<b>66.56 ± 0.62</b>	69.47 ± 1.24
Days to silk emergence	71.65 ± 0.49	<b>66.75 ± 0.81</b>	<b>71.27 ± 1.28</b>	72.24 ± 1.17
Tassel branch no.	4.03 ± 0.19	<b>8.13 ± 0.39</b>	<b>7.04 ± 0.39</b>	4.00 ± 0.34
Leaf number	10.87 ± 0.20	<b>12.70 ± 0.19</b>	<b>11.52 ± 0.22</b>	9.11 ± 0.17

**Table 5** continued

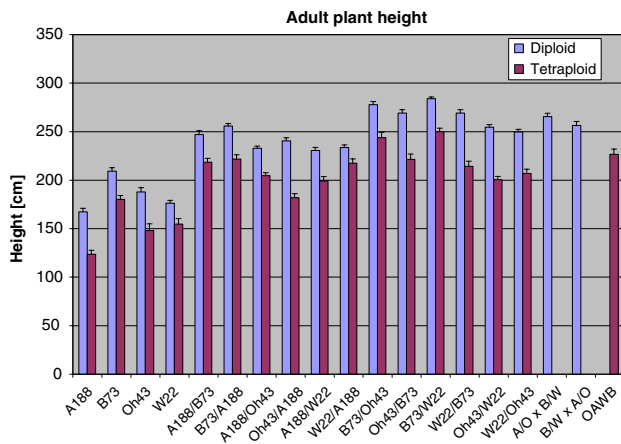
	B73 4x	B73/Oh43	Oh43/B73	Oh43 4x
Ear node number	4.81 ± 0.25	<b>5.90 ± 0.18</b>	<b>5.19 ± 0.19</b>	4.22 ± 0.19
Leaf length	62.71 ± 1.13	<b>64.52 ± 1.85</b>	<b>63.00 ± 2.43</b>	52.79 ± 1.59
Leaf width	7.75 ± 0.22	9.86 ± 0.23	9.59 ± 0.22	10.05 ± 0.39
Adult height	180.25 ± 3.99	<b>243.79 ± 5.72</b>	<b>221.38 ± 5.47</b>	148.11 ± 6.94
Ear height	73.27 ± 2.21	<b>96.83 ± 3.20</b>	<b>88.24 ± 4.13</b>	46.38 ± 2.02
Ear length	8.38 ± 0.27	<b>16.41 ± 0.60</b>	<b>15.77 ± 0.79</b>	12.81 ± 0.71
Circumference	7.78 ± 0.21	7.66 ± 0.13	7.67 ± 0.17	6.55 ± 0.22
Height at 4 weeks	24.05 ± 0.89	<b>31.62 ± 1.24</b>	<b>26.25 ± 1.58</b>	21.06 ± 1.40
Height at 6 weeks	59.01 ± 2.29	<b>76.50 ± 3.22</b>	<b>64.58 ± 3.99</b>	43.38 ± 2.45
	B73 4x	B73/W22	W22/B73	W22 4x
Days to anther emergence	70.20 ± 0.72	<b>66.48 ± 0.50</b>	<b>66.76 ± 0.73</b>	72.63 ± 0.71
Days to silk emergence	71.65 ± 0.49	<b>68.38 ± 0.65</b>	<b>67.93 ± 0.59</b>	74.72 ± 0.83
Tassel branch no.	4.03 ± 0.19	<b>8.64 ± 0.42</b>	<b>8.59 ± 0.43</b>	7.47 ± 0.76
Leaf number	10.87 ± 0.20	<b>13.59 ± 0.20</b>	<b>12.62 ± 0.22</b>	11.68 ± 0.38
Ear node number	4.81 ± 0.25	<b>6.41 ± 0.20</b>	<b>5.46 ± 0.19</b>	4.50 ± 0.20
Leaf length	62.71 ± 1.13	<b>64.89 ± 1.38</b>	<b>66.24 ± 1.05</b>	50.47 ± 2.02
Leaf width	7.75 ± 0.22	<b>8.62 ± 0.26</b>	<b>8.86 ± 0.20</b>	8.26 ± 0.30
Adult height	180.25 ± 3.99	<b>249.86 ± 3.82</b>	<b>214.21 ± 5.37</b>	154.78 ± 5.54
Ear height	73.27 ± 2.21	<b>111.97 ± 3.43</b>	<b>89.73 ± 3.37</b>	59.00 ± 2.97
Ear length	8.38 ± 0.27	<b>15.13 ± 0.44</b>	<b>14.96 ± 0.45</b>	7.65 ± 0.37
Circumference	7.78 ± 0.21	<b>8.09 ± 0.22</b>	<b>9.13 ± 0.19</b>	7.39 ± 0.18
Height at 4 weeks	24.05 ± 0.89	<b>32.34 ± 0.84</b>	<b>25.93 ± 1.39</b>	16.71 ± 0.97
Height at 6 weeks	59.01 ± 2.29	<b>75.63 ± 2.59</b>	<b>63.59 ± 2.81</b>	39.90 ± 1.36
	Oh43 4x	Oh43/W22	W22/Oh43	W22 4x
Days to anther emergence	69.47 ± 1.24	65.03 ± 0.30	69.68 ± 0.91	72.63 ± 0.71
Days to silk emergence	72.24 ± 1.17	<b>67.48 ± 0.51</b>	<b>71.25 ± 0.73</b>	74.72 ± 0.83
Tassel branch no.	4.00 ± 0.34	10.57 ± 0.49	6.64 ± 0.64	7.47 ± 0.76
Leaf number	9.11 ± 0.17	11.86 ± 0.25	11.45 ± 0.25	11.68 ± 0.38
Ear node number	4.22 ± 0.19	<b>5.17 ± 0.18</b>	<b>4.59 ± 0.18</b>	4.50 ± 0.20
Leaf length	52.79 ± 1.59	<b>59.89 ± 0.96</b>	<b>56.45 ± 1.87</b>	50.47 ± 2.02
Leaf width	10.05 ± 0.39	<b>10.26 ± 0.26</b>	<b>10.18 ± 0.24</b>	8.26 ± 0.30
Adult height	148.11 ± 6.94	<b>200.39 ± 3.61</b>	<b>206.95 ± 4.39</b>	154.78 ± 5.54
Ear height	46.38 ± 2.02	<b>73.11 ± 2.40</b>	<b>67.24 ± 2.46</b>	59 ± 2.97
Ear length	12.81 ± 0.71	<b>15.76 ± 0.60</b>	<b>15.29 ± 0.68</b>	7.65 ± 0.37
Circumference	6.55 ± 0.22	<b>7.91 ± 0.15</b>	<b>7.61 ± 0.25</b>	7.39 ± 0.18
Height at 4 weeks	21.06 ± 1.40	28.20 ± 0.76	20.54 ± 1.55	16.71 ± 0.97
Height at 6 weeks	43.38 ± 2.45	<b>60.82 ± 1.88</b>	<b>48.59 ± 2.87</b>	39.9 ± 1.36

Mean character measures and standard errors of the mean are shown for each of the reciprocal crosses included in the study. Measures that are marked in bold exhibit heterosis, as they are more extreme in the hybrids than in either inbred parent. Measurements are in centimeter unless otherwise noted

are similar, when compared in more detail, the midparent heterosis averages are larger for 8 of the 13 characters in the tetraploid hybrids.

Employing the same strategy described in the previous section for the diploid maize lines, we investigated the 13

characters using a variety of ANOVA approaches to examine the influence of allelic diversity on the various characters and the strength of heterosis. As with the diploid lines, we find a “block” effect for some of the characters (5 out of 13 characters), and there is a clear effect of the genetic



**Fig. 4** Adult plant height in diploid and tetraploid hybrids

background, as measured by the significant effect “family (allele number)” detected for all characters. The influence of allelic diversity on the 13 characters was assessed by the factor “allele number” included in the ANOVA model. This factor is highly significant for all characters examined, as illustrated in Table 6, demonstrating that allelic diversity has a strong effect on morphological characters.

By comparing the character means of the three classes of tetraploid maize lines—inbred, single-cross hybrid and double-cross hybrid—we wanted to determine if the observed relationship between character measurements and allelic diversity is due to extreme measurements observed in just one class, or if all classes are significantly different from each other. For all but one character, the comparisons between inbred lines and both classes of hybrid lines are highly significant. One exception is ear node number, where

**Table 6** ANOVA results for tetraploids

Character	DF	SS	F	P-value
Days to flowering: anthers	2	232.47	66.06	<0.0001
Days to flowering: silks	2	265.84	81.13	<0.0001
Tassel branch number	2	110.41	50.94	<0.0001
Leaf number	2	18.01	31.28	<0.0001
Ear node number	2	4.91	12.70	<0.0001
Leaf length	2	594.97	41.39	<0.0001
Leaf width	2	18.34	33.08	<0.0001
Adult height	2	34,450.32	148.37	<0.0001
Ear height#	2	5,954.97	47.25	<0.0001
Ear length	2	292.19	144.08	<0.0001
Stem circumference	2	7.14	20.67	<0.0001
Height at 4 weeks#	2	579.49	40.00	<0.0001
Height at 6 weeks	2	4,415.62	44.21	<0.0001

Partial results for the factor “allele number” from all characters investigated. Reported are type III sum of squares and associated P-values unless otherwise noted

the comparison between inbred and double-cross hybrid individuals is not significant. When comparing the class means from single- and double-cross hybrids, 2 of the 13 characters show a significant difference between the two types of hybrids (ear length, and the time to silk emergence), one is a borderline case (height at 6 weeks after planting with a P-value of  $P = 0.0536$ ), and the other measures do not show a significant difference (Table 7). These data indicate that with the specific inbred lines included in this study, progressive heterosis can be observed.

The comparison between single-cross and double-cross hybrids described above includes all tetraploid single-cross lines, and thus compares the double-cross hybrid to the average single-cross hybrid. In order to investigate the difference between single- and double-cross hybrids, it is also appropriate to compare the double-cross hybrid only to the two lines from which it was derived. If the analysis is carried out with only Oh43/A188 and W22/B73 contributing to the single-cross class, the results are slightly different. Again, most character measures do not exhibit a statistically significant difference between the single- and double-cross hybrids. However, the characters’ adult height and ear length significantly differ between single- and double-cross hybrids in this analysis ( $P < 0.05$ ; data not shown) with the double-cross hybrids being larger.

As in the analysis for the diploid lines, a multivariate analysis was carried out to determine if overall, there is a significant difference between single- and double-cross hybrids in the tetraploid lines. In this analysis, allele number significantly influences the multivariate phenotypes, which confirms the finding of the ANOVA results ( $P < 0.0001$ , Wilks’ lambda). If the single- and double-cross hybrid classes are contrasted, we find that this comparison is not significant ( $P = 0.49$ ). These findings indicate that while in single-character comparisons there are differences between the single- and double-cross hybrids in the tetraploid lines, there is not a comprehensive trend for either the single- or double-cross hybrids.

#### Comparison of heterosis in diploid and tetraploid lines

We also compared the behavior of inbred, single-cross and double-cross hybrids between diploid and tetraploid lines. Figures 3 and 4 show a comparison between the behavior of the diploid and the tetraploid lines for the character days to anther emergence and plant height at 4 weeks after planting, respectively. Days to anther emergence exhibit a similar pattern in the diploid and tetraploid lines, whereas for the plant height measure, there are several marked differences between them. When comparing the percent increase over the midparent value, the tetraploid hybrids show a greater level of heterosis than the diploid hybrids in

**Table 7** Comparison between tetraploid double-cross hybrids and the parental lines from which they were derived

	Oh43/A188 4x	Oh43/A188 × W22/B73	W22/B73 4x
Days to anther emergence	60.97 ± 0.32	62.59 ± 0.44	66.76 ± 0.73
Days to silk emergence	61.53 ± 0.42	64.19 ± 0.55	67.93 ± 0.59
Tassel branch number	12.17 ± 0.53	11.52 ± 0.70	8.59 ± 0.43
Leaf number	10.83 ± 0.22	11.44 ± 0.22	12.62 ± 0.22
Ear node number	4.47 ± 0.16	4.89 ± 0.15	5.46 ± 0.19
Leaf length	61.89 ± 1.31	66.04 ± 1.19	66.24 ± 1.05
Leaf width	10.59 ± 0.29	10.12 ± 0.32	8.86 ± 0.20
Adult height <sup>a</sup>	182.03 ± 4.20	<b>226.63 ± 5.49</b>	214.21 ± 5.37
Ear height	60.57 ± 2.32	85.54 ± 3.42	89.73 ± 3.37
Ear length <sup>a</sup>	16.17 ± 0.51	<b>16.96 ± 0.59</b>	14.96 ± 0.45
Stem circumference	7.23 ± 0.15	8.27 ± 0.20	9.13 ± 0.19
Height at 4 weeks	31.28 ± 0.62	30.48 ± 1.45	25.93 ± 1.39
Height at 6 weeks <sup>a</sup>	68.41 ± 1.66	<b>76.48 ± 3.64</b>	63.59 ± 2.81

Mean character measures and standard errors are shown and characters that exhibit superior performance in double-cross hybrid are marked in bold. Measurements are in centimeter unless otherwise noted

<sup>a</sup> Measurements for which the comparisons between double-cross and single-cross hybrid are significant in both cases

more than 60% of cases (48 of 78 cases, significantly different from 50%,  $P < 0.05$ ), indicating that based on midparent levels, heterosis is more pronounced at the tetraploid level. When heterosis levels are averaged over all crosses, eight traits show higher levels of heterosis in the tetraploids, while five traits show larger increases in the diploid hybrids. The tetraploid inbreds, however, show lesser stature and robustness, so even though the increase over the parental lines is greater at the tetraploid level, in the absolute comparisons, tetraploids do not exceed the diploids. Also, by comparing Tables 2 and 5, it is clear that for only 6 of the 13 characters, the best performing F1 combination is the same in the diploid and tetraploid crosses.

As our field study included identical lines at the diploid and tetraploid level, a comparison can be carried out employing an ANOVA model that included an effect for genotype representing the specific genetic background independent of genome dosage and an effect for ploidy. Additional main effects included effects for block and ploidy as well as allele number nested within genetic background. We were mainly interested in the interaction effect between ploidy and allele number, as significance would indicate a difference in response to allele number or heterozygosity between diploid and tetraploid lines (the ANOVA tables for this analysis can be found in the supplemental material). For 7 of the 13 characters, we detect a significant ploidy–allele number interaction effect in the analysis of variance, while 6 do not show such an effect. The characters that do not exhibit a significant interaction effect between allele number and ploidy are ear height, adult height, leaf length and width, leaf number and tassel branch number. As the majority of characters demonstrate

a significant interaction effect, these data indicate a different phenotypic response to allelic diversity in diploid versus tetraploid lines of maize.

## Discussion

The goal of this field study was to compare the heterotic response of crosses of diverse maize inbred lines at the diploid and tetraploid level. We employed previously generated diploid and tetraploid lines that were identical in all aspects but ploidy level (Kato and Birchler 2006). This approach allowed us to delineate effects of ploidy on the heterosis response without having confounding effects of potentially altered genotypes.

For the diploid crosses, we find that heterosis occurs for most of the characters and crosses identified, indicating that the maize lines we chose for our study are well suited. These results are consistent with previous reports that find similar levels of heterosis for these characters in other crosses (for example, see Auger et al. 2005). The analysis of variance, as well as visual inspection of the data, clearly demonstrates that there is a strong effect of the specific line utilized in the cross. Crosses with W22, for example, often resulted in the largest percentage increase over the midparent value in the F1 hybrids. As has been shown in previous studies, heterosis levels very strongly depend on the inbred parent lines used to create the F1 hybrids, and the suitability of any line combination has to be evaluated by a test cross (Dias et al. 2004). Our data are consistent with these findings, and also indicate, that different crosses have maximum heterosis for different characters, such that the hybrids between Oh43

and A188 show the greatest heterosis for the early height measures at 4 and 6 weeks, but that adult height heterosis is strongest in B73/W22 hybrids.

The existence of heterosis for most characters in the diploid lines is also demonstrated by the ANOVA results showing a significant effect of the factor “allele number”. These data show that the performance of inbred individuals is inferior to the single- and double-cross hybrids in all cases. When the difference between single- and double-cross hybrids is examined via the pooled data from all diploid crosses and inbred lines, none of the characters show a significant difference between the two hybrid classes. Also when the direct parents of the double-cross hybrid are used for this comparison, only the character time to silk emergence passes the statistical significance level of  $P < 0.05$ . However, the differences detected between the two hybrid classes are small, and this analysis mainly serves as control for comparison to the tetraploids.

Evaluation of the tetraploid lines demonstrated that most F1 hybrid lines show heterosis for the majority of characters. The ANOVA demonstrates a strong influence of the genetic background on the F1 phenotypes and on the level of heterosis they exhibit. The factor of allele number was highly significant for all characters, and the parental inbred lines are inferior in 12 of 13 measurements. In addition, if data from all tetraploid lines are used in the analysis, a total of four characters exhibit a significant difference between the single- and double-cross hybrids in the various analyses, indicating that these characteristics potentially exhibit progressive heterosis.

The comparison between the heterotic patterns between diploids and tetraploids revealed a number of interesting observations. Generally, the response of diploid and tetraploid lines of maize to the hybrid state is different for most characters (9 of 13), demonstrated by the significant ploidy by allele number interaction detected. This point is illustrated by the fact that of the 13 measurements, only 5 show the highest level of heterosis in the same hybrid cross in the diploid and tetraploid lines. These are the two flowering time measures, tassel branch number, ear node number and plant height at 4 weeks after planting. In addition, for most characters, the correlation coefficients between the midparent heterosis levels observed in diploid and tetraploid lines are low. Only for ear length and leaf width there is a correlation significantly different from 0 ( $P < 0.05$ , data not shown). The difference observed between the heterotic effects in the diploid and tetraploid lines implies that heterosis measured in diploid strains cannot be used to predict the behavior of tetraploid strains. Similar results have been obtained in alfalfa (Groose et al. 1989) and maize (Sockness and Dudley 1989b).

In addition, the comparison between the behavior of diploid and tetraploid maize lines revealed that heterosis

measured as a percent improvement over the expected midparent value is stronger in tetraploid than in diploid lines. In particular, the average level of heterosis calculated over all 13 traits investigated in this study was 28.3% in tetraploid hybrids, whereas the average level of heterosis recorded in diploid hybrids was 24.8%. Also, we observed four measurements that showed progressive heterosis in the tetraploids included in this study in both analyses. These characters were plant height at 6 weeks after planting, adult height, ear length and the time to silk emergence. These results are consistent with previous findings in maize (Chase 1980; Levings et al. 1967; Sockness and Dudley 1989a) that indicate double-cross tetraploid hybrids exhibit greater plant height and yield than either single-cross hybrid used to produce them. Although different combinations of lines appear to produce progressive heterosis for different plant characteristics beyond both single-cross parents, the double-cross genotype was not found to be inferior to either single-cross hybrid parent for any measured characteristic in the various comparisons. Thus, the double-cross hybrid exhibits superior performance for some characteristics, but is otherwise similar to the single-cross tetraploid hybrids. In alfalfa, progressive heterosis is used to produce tetraploid strains which in general outperform the diploid strains (Bingham et al. 1994; Groose et al. 1989). Collectively, the results suggest that maximizing allelic diversity in tetraploids will maximize the heterotic response.

Our results indicate that in maize increasing ploidy of an inbred has a detrimental effect on plant stature and overall morphology (for a more detailed discussion of the morphological changes associated with ploidy change, see Riddle et al. 2006). Interestingly, the magnitude of heterosis at the tetraploid level is greater than for the diploids that are otherwise genetically identical. Also, maximizing allelic diversity in tetraploids also appears to maximize the heterotic response. These observations will need to be considered in formulating a genetic model of heterosis.

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