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## Kernel number as a positive target trait for prediction of hybrid performance under low-nitrogen stress as revealed by diallel analysis under contrasting nitrogen conditions

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Environmental sustainability concerns make improving yield under lower N input a desirable breeding goal. To evaluate genetic variation and heterosis for low-N tolerance breeding, 28 F1 hybrids from a diallel scheme, along with their eight parental lines, were tested for agronomic traits including kernel number per ear (KNE) and grain yield per plant (GY), in replicated plots over two years under low-nitrogen (LN, without nitrogen application) and normal-nitrogen (NN, 220 kg N ha<sup>-1</sup>) conditions. Taken together the heritability in this and our previous studies, the correlation with grain yield, and the sensitivity to the stress for target trait selection, KNE was a good secondary target trait for LN selection in maize breeding. KNE also showed much higher mid-parent heterosis than hundred-kernel weight under both nitrogen levels, particularly under LN, indicating that KNE contributed the majority of GY heterosis, particularly under LN. Therefore, KNE can be used as a positive target trait for hybrid performance prediction in LN tolerance breeding. Our results also suggest that breeding hybrids for LN tolerance largely relies on phenotypic evaluation of hybrids under LN condition and yield under LN might be improved more by selection for KNE than by direct selection for GY *per se*.

**Key Words:** maize, heterosis, genetic variation, low-nitrogen tolerance, nitrogen-use efficiency.

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### Introduction

Global food demands pose huge challenges for sustainable production of cereal crops including maize (*Zea mays* L.) (Tilman *et al.* 2002). Two major innovations to increase grain yield (GY) to meet these demands are the use of hybrid maize and application of nitrogen fertilizers (Hirel *et al.* 2007).

N fertilization has made great contributions to increased GY in maize (Hirel *et al.* 2001). However, some serious environmental problems have resulted from application of high-level N in light of the relatively low N-use efficiency of maize (Guo *et al.* 2010, Ju *et al.* 2009). Developing maize varieties with improved N-use efficiency (NUE) or low-N (LN) tolerance will be essential for reducing N fertilization costs and the negative impact of excessive N on the environment (Wu *et al.* 2011). Furthermore, heterosis has been successfully used to increase maize GY (Shull 1946). One would also expect that hybrids might better tolerate stress and undergo developmental transitions better than their inbred parents (Schnable and Springer 2013). There-

fore, fundamental genetic and agronomic knowledge will improve understanding of how to combine maize hybrid vigor with N stress tolerance and provide new strategies to address nitrogen-related issues.

When breeding hybrids for low-N stress tolerance, assessing genetic variation including heterosis, general combining ability (GCA), and specific combining ability (SCA) (Le Gouis *et al.* 2002) under both LN and normal N (NN) conditions is key to understanding and enhancing maize NUE traits. The heredity of and genetic variation in N nutrition has been of interest for over 7 decades (Abdel-Ghani *et al.* 2013, Coque and Gallais 2007, Harvey 1939, Presterl *et al.* 2003, Worku *et al.* 2012, Wu *et al.* 2011). However, reports on genetic variation in the response to N (Azeez *et al.* 2006, Bänziger and Cooper 2001, Bänziger *et al.* 2002, Bolaños and Edmeades 1996, Lafitte and Edmeades 1994a, 1994b, Worku *et al.* 2012) have focused on tropical germplasm because inadequate inorganic N supply limits maize production in tropical regions (Lafitte and Edmeades 1994a, 1994b). Recent N-associated heterosis analyses have also been centered mainly on tropical germplasm (Amiruzzaman *et al.* 2013, El-Badawy 2013). However, heterosis has been frequently demonstrated and widely used in temperate maize. The objectives of this study were to (i) evaluate the genetic variation of temperate hybrids and their parental

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lines in response to nitrogen input levels, (ii) estimate associated quantitative genetic parameters, and (iii) analyze heterosis for low-N tolerance. These research findings would help us to design and optimize breeding programs for improving low-N tolerance and NUE in maize.

## Materials and Methods

### Maize germplasm

Eight parental inbred lines, 81865, Qi209, Zheng30, Qi318, Jinhuang96B, CA156, Qi205, and 89-1 (Table 1) were used to produce 28 crosses from an  $8 \times 8$  partial diallel that did not include reciprocals. The elite hybrid, Zhengdan958, and its two parental lines, Chang7-2 and Zheng58, were also planted together as checks for hybrids and their parental inbred lines, respectively. All of these lines are temperate-adapted germplasm and were selected to represent the different heterotic groups that have been developed during the past decades in Chinese maize breeding programs (Xie *et al.* 2007). The control hybrid, Zhengdan958, was the one of most successful temperate hybrids and still the leading hybrid now in China with a total of growing area of over 33 Mha (<http://baike.baidu.com/view/264391.htm> Internet news in Chinese, accessed on June 9, 2013).

### Field preparation and nutrient management

The experiment was conducted at two locations in China: at Shunyi, Beijing (40.2°N 116.5°E, 44 MASL elevation) from May to October in 2009 and 2010; and at Tonghua, Jilin (42.5°N 125.6°E, 320 MASL) from May to October in

2010 and 2011 (Table 2). Field soil samples from each of the two locations were collected from soil layers at 0 to 30 cm depth prior to fertilization and planting, and then mixed, air dried, and sieved to remove undecomposed organic materials. Soil nutrients were tested to determine organic matter (Walkley 1947), total N (Bremner 1996), total P (HClO<sub>4</sub>-H<sub>2</sub>SO<sub>4</sub> digestion), total K, Olsen-P (Olsen *et al.* 1954), NH<sub>4</sub> OAc-K (Van Reeuwijk 1992), and pH (pHS-3C pH meter, Nanjing) The water: soil ratio was 2.5 : 1). The data on nutrients along with geographic information for each location are both provided in Table 2. Pairwise t-test revealed no significant differences in soil nutrients or pH prior to establishment of LN and NN conditions at either location.

The F1 hybrids and their parental inbreds were evaluated separately under NN and LN conditions. The NN experiment was managed under well-fertilized conditions, so as to provide an ideal maize growing environment. For the NN experiment, 80.5 kg nitrogen (N) ha<sup>-1</sup> in the form of urea, 51 kg phosphorous ha<sup>-1</sup> in the form of calcium superphosphate, and 90 kg potassium ha<sup>-1</sup> as muriate of potash were applied as a basal dressing before planting, and a second application of 161 kg N ha<sup>-1</sup> and 90 kg potassium ha<sup>-1</sup> was made as a top dressing at V8 stage. For the LN experiment, no nitrogen was applied as either a basal dressing or top-dressing, but phosphorous and potassium were applied in the same manner as for the NN experiment.

All hybrids and their parental lines along with hybrid and parental line controls were planted in the field using a randomized block design with two replications. The hybrids

**Table 1.** Parental inbred lines and their pedigree information, in addition to parental line and hybrid checks used in this study

	No.	Name	Pedigree	Note
Inbred lines	1	81865	Tropical germplasm, Mexico	
	2	Qi209	Qi205 × Ye107	
	3	Zheng30	Zheng20 × Ye478	
	4	Qi318	American hybrid 78599	
	5	Jinhuang96B	Zhongzong3 C2 (synthetic)	
	6	CA156	pool 33 temperated adapted	
	7	Qi205	Wei ai 141 × Zhongxi017	
	8	89-1	American hybrid 78599	
Hybrid	CK1	Chang7-2	(Huangzao4 × Wei95) × S901	parental line check
	CK2	Zheng58	improved Ye478	parental line check
Hybrid	CK	Zhengdan958	Zheng58 × Chang7-2	hybrids check

**Table 2.** Mean with standard deviation (mean ± SD) and pairwise t-tests for background level of soil nutrients between LN and NN at two locations

Locations	Latitude, longitude and elevation	N treatments	pH	Soil nutrients				
				Organic matter (g/kg)	Total N (g/kg)	Olsen-P (mg/kg)	Total P (g/kg)	Total K (g/kg)
Tonghua, Jilin	42.5°N 125.6°E, 320 MASL	NN	8.30 ± 0.10	24.69 ± 3.57	0.83 ± 0.03	39.78 ± 3.78	1.50 ± 0.08	31.37 ± 0.44
		LN	8.35 ± 0.03	29.40 ± 1.12	0.89 ± 0.02	36.38 ± 3.23	1.61 ± 0.09	29.80 ± 14.96
		Pairwise t-test	0.28	0.11	0.08	0.01	0.01	0.43
Shunyi, Beijing	40.2°N 116.5°E, 44 MASL	NN	8.36 ± 0.10	20.48 ± 2.04	0.87 ± 0.05	17.23 ± 12.01	0.87 ± 0.09	41.20 ± 3.41
		LN	8.19 ± 0.21	17.72 ± 3.57	0.88 ± 0.03	20.08 ± 0.15	0.92 ± 0.03	43.24 ± 1.35
		Pairwise t-test	0.21	0.14	0.36	0.36	0.19	0.27

LN, low nitrogen; NN, normal nitrogen; MASL, meters above sea level.

and the inbred lines were tested in different blocks to avoid plant height interactions between hybrids and inbred lines. Each entry was planted in two rows, each 4.0 m long, with 0.20 m space between plants and 0.5 m between rows. The borders of both the LN and NN field trials were protected by two rows of the CK hybrids (Table 1) for removing the border effect.

#### Measurement of agronomic traits

The number of leaves was scored by counting the number of nodes on the stalks of six plants. Senesced leaves were also counted by marking young leaves during early development. Plant height (PH) was recorded on ten plants per block after silking, as the distance between the ground surface and the first tassel branch of the same plant.

After harvest, shoot biomass (SB), grain yield per plant (GY), kernel number per ear (KNE) and hundred-kernel weight (HKW) were recorded trait by trait for 10 plants per block. SB for each entry was recorded at time of harvest on a whole plant dry weight basis as the average of three plants selected at random per block. Fallen leaves were not included in the shoot biomass measurement. And the same time, GY was recorded on a 100% dry matter basis. Harvest index (HI) was then calculated as:  $HI = \frac{GY}{SB}$ . KNE was obtained by multiplying the number of rows in the middle ear and the average kernel number per row together, measured on ten similar dry ears per block and averaged. HKW was recorded by weighing 100 kernels for three replications and averaging per block.

#### Statistical analysis

Differences among parental lines or hybrids were tested by analysis of variance (ANOVA) conducted for each measured trait using PROC GLM (SAS 1999) with the following model (1):

$$Y_{ijkl} = \mu + E_k + B(E)_{l(k)} + G_{ij} + G_{ij} \times E_k + \varepsilon_{ijkl} \quad (1),$$

where  $Y_{ijkl}$  is the observed value of a parental line or hybrid,  $\mu$  is the population mean,  $E_k$  is the main effect of the environment,  $B(E)_{l(k)}$  is the main effect of block within environment,  $G_{ij}$  is the genotypic effect (hybrids or parental inbred lines),  $G_{ij} \times E_k$  is the genotype-by-environment interaction ( $G \times E$ ), and  $\varepsilon_{ijkl}$  is the random residual effect. Environments and genotypes were considered fixed effects.

Broad-sense heritability ( $h_B^2$ ) was estimated on a block basis (Harvey 1939) as Eq. (1):

$$h_B^2 = \frac{\sigma_G^2}{\sigma_G^2 + \sigma_{G \times E}^2 + \sigma_\varepsilon^2} \quad (1),$$

where  $\sigma_G^2$  is genotypic variance,  $\sigma_{G \times E}^2$  is the variance due to the  $G \times E$  interaction, and  $\sigma_\varepsilon^2$  is the error variance. The narrow-sense heritability ( $h_N^2$ ) of hybrids was calculated based on variance in combining ability, which is derived by

Eq. (2) from the variances of general combining ability (GCA) and special combining ability (SCA), according to Griffing's method 4 (Griffing 1956):

$$h_N^2 = \frac{2\sigma_{GCA}^2}{2\sigma_{GCA}^2 + \sigma_{SCA}^2 + 2\sigma_{GCA \times E}^2 + \sigma_{SCA \times E}^2 + \sigma_\varepsilon^2} \quad (2),$$

where  $\sigma_{GCA}^2$ ,  $\sigma_{SCA}^2$ ,  $\sigma_{GCA \times E}^2$ , and  $\sigma_{SCA \times E}^2$  were estimated from the subsequent diallel analysis.

When genotypic effects for traits in hybrids are significant according to ANOVA, diallel analysis of combining ability is then conducted according to Griffing's method 4 (Griffing 1956) using a special SAS program for diallel analysis with missing crosses developed by Wu and Matheson (2000) that considers environment and genotype as fixed effects. The response  $Y_{ijkl}$  of the cross between parents  $i$  and  $j$  was modeled as model (2):

$$Y_{ijkl} = \mu + E_k + B(E)_{l(k)} + GCA_i + GCA_j + SCA_{ij} + GCA_i \times E_k + GCA_j \times E_k + SCA_{ij} \times E_k + \varepsilon_{ijkl} \quad (2),$$

where  $\mu$ ,  $E_k$ ,  $B(E)_{l(k)}$  and  $\varepsilon_{ijkl}$  are the same as in model (1);  $GCA_i$  and  $GCA_j$  are the GCA of parents  $i$  and  $j$ ;  $SCA_{ij}$  is the SCA of hybrid  $ij$ ;  $GCA_i \times E_k$ ,  $GCA_j \times E_k$ , and  $SCA_{ij} \times E_k$  are interactions between GCA and SCA and the environment.

The relative importance of GCA and SCA was estimated as the ratio:  $\frac{2K_{GCA}^2}{2K_{GCA}^2 + K_{SCA}^2}$ , modified from Baker (1978), where  $K_{GCA}^2$  is the quadratic form (analogous to a variance component, but referring to a fixed effect) derived from the mean square of the GCA effect and  $K_{SCA}^2$  is the quadratic form of the SCA effects. As this ratio approaches unity, GCA becomes more important, and the predictability of the performance of a specific hybrid could be based on GCA alone.

The variance of the SCA effect of parent  $i$  ( $S_{si}^2$ ) indicates the differences in SCA among a series of hybrids involving one line, estimated as Eq. (3) (Griffing 1956):

$$S_{si}^2 = \frac{1}{P-2} \sum_{i \neq j} SCA_{ij}^2 - \frac{P-3}{P-2} \sigma_\varepsilon^2 \quad (3)$$

The low-N tolerance index (*LNTI*) was estimated by Eq. (4) to evaluate the ability of parental lines and hybrids to tolerate low nitrogen in terms of the traits measured.

$$LNTI = \frac{LN}{NN} \times 100\% \quad (4)$$

A higher *LNTI* indicates a stronger ability to adapt to low nitrogen.

Heterosis (%) was calculated over the high-parent and mid-parent values. High-parent heterosis (*HPH*) was calculated using Eq. (6) (Hill *et al.* 1987). Similarly, mid-parent heterosis (*MPH*) was estimated using Eq. (7).

$$HPH(\%) = \frac{F_1 - HP}{HP} \times 100 \tag{6}$$

$$MPH(\%) = \frac{F_1 - MP}{MP} \times 100 \tag{7}$$

$F_1$  is the performance of each hybrid; HP indicates the performance of the best parent and MP represents the mean performance of two parental inbred lines. For each hybrid, the difference between the hybrid and the mean of its two parents as well as the best parent was tested by a t-test to determine whether MPH or HPH was significant (Le Gouis *et al.* 2002).

The relationship between the LNTI of parental inbred lines and that of hybrids was measured using PROC REG in SAS (SAS 1999). Similarly, correlations between SCA, heterosis, and least square mean values of hybrids, as well as correlations between performance of hybrids and the mean performance of two parents for all measured traits were calculated using PROC CORR in SAS (SAS 1999).

The correlation between inbred line and hybrid performance was estimated by linear regression analysis of the GCA values on inbred performance.

**Results**

*The effect of low nitrogen on agronomic performance of the hybrids and their parental inbreds*

Significant differences were found between LN and NN trials for most of the measured traits except of leaf number (LEAF) and harvest index (HI) for both parental lines and their half-diallel cross hybrids (Table 3). Among these traits, GY and SB showed the largest differences between LN and NN treatments. For example, average GY of the parental inbred lines and hybrids under LN was only 74.1% and 64.0% of those under NN, with an average reduction of 25.9% and 36.0%, respectively. Additionally, the differences between the inbred lines and hybrids were also very significant due to heterotic effect. The hybrids showed significantly higher phenotypic performance than their parental lines under both NN and LN conditions, indicating significant heterosis. For

**Table 3.** Differences between parental lines and diallel cross F1s under low- (LN) or normal-nitrogen (NN) conditions

		LN							NN						
		LEAF	PH	HKW	KNE	GY	SB	HI	LEAF	PH	HKW	KNE	GY	SB	HI
		(number)	(cm)	(g)	(number)	(g)	(g)	(%)	(number)	(cm)	(g)	(number)	(g)	(g)	(%)
Parental lines	Avg.	18.2	193.9	26.6	155.6	41.1	108.0	32.7	18.9	210.8	30.5	181.4	55.5	156.8	34.9
	Max.	20.8	239.5	36.5	251.6	72.2	134.4	51.4	22.3	277.7	36.8	271.8	83.8	192.0	52.6
	Min.	16.0	170.9	18.6	87.5	22.5	87.2	13.8	17.0	191.8	23.5	81.2	23.8	106.3	14.0
	CV (%)	7.7	10.9	21.0	33.2	48.5	10.2	35.0	10.4	12.1	13.9	23.3	38.3	19.4	31.2
	Avg. LN/NN (%)	96.4	92.0	87.2	85.8	74.1	68.8	93.9							
F1s	Avg.	19.3	280.5	29.9	366.3	109.3	217.5	46.7	20.1	298.8	35.2	483.7	170.7	335.8	51.6
	Max.	21.3	339.4	38.3	445.8	149.9	323.0	54.1	23.8	356.4	42.5	578.6	208.7	428.6	61.1
	Min.	17.3	223.3	24.2	182.8	53.3	154.0	30.3	18.0	242.5	29.3	351.6	114.7	278.5	38.9
	CV (%)	6.0	9.5	13.5	15.3	17.7	18.7	8.4	6.1	9.6	11.3	13.6	14.5	11.2	10.2
	Avg. LN/ NN (%)	95.8	93.9	85.0	75.7	64.0	64.8	90.5							
Parental Lines/F1s (%)		94.4	69.1	89.1	42.5	37.6	49.6	70.1	93.8	70.6	86.7	37.5	32.5	46.7	67.5

LN, low nitrogen; NN, normal nitrogen; LEAF, the final number of leaves; PH, plant height; HKW, hundred-kernel weight; KNE, kernel number per ear; GY, grain yield; SB, shoot biomass; HI, harvest index; Avg., average; Max., maximum; Min., minimum.

**Table 4.** Variance analysis of parental lines and their diallel cross F1 hybrids under two nitrogen levels in two locations across two years

Types	Source	LN							NN						
		LEAF	PH	HKW	KNE	GY	SB	HI	LEAF	PH	HKW	KNE	GY	SB	HI
F1 hybrids	Genotype	5.20*	2863.77**	65.12**	21682.29**	2133.59**	6284.09**	0.012**	6.63**	2807.15**	62.89**	24999.81**	2613.65**	6057.49**	0.015**
	Env.	0.81	7941.65**	994.01**	2580.03	21633.11**	65712.76**	0.290**	0.81	391.73	39.6	509.63	3747.83*	5495.26	0.093**
	Rep.	1.45*	4331.74**	45.03	7539.64*	229.31	4220.85	0.001	1.13	287.58	1.77	1266.36	655.08	637.60	0.002
	Genotype*Env.	0.06	170.88	26.29*	3660.65*	784.85	3175.65**	0.006*	0.25	219.93	23.17	2616.01	927.38	1717.58	0.003
	Error	0.43	150.42	14.84	1638.87	739.36	1385.59	0.00	0.57	125.27	14.47	1785.43	646.05	2283.60	0.002
	$h_B^2$	0.822	0.833	0.365	0.678	0.335	0.261	0.265	0.791	0.828	0.326	0.759	0.379	0.332	0.579
	$h_N^2$	0.683	0.639	0.315	0.436	0.101	0.047	0.011	0.476	0.745	0.245	0.547	0.133	0.077	0.413
Parental lines	Genotype	8.93**	1642.61**	129.12**	17921.77**	1219.49**	767.46*	0.064**	14.42**	3810.04**	86.35**	16462.04**	1727.41**	3625.80**	0.049**
	Env.	0.50	3130.40**	124.39**	79693.63**	794.36*	117.05	0.024*	0.28	390.60*	4.54	16066.28*	1287.42*	5067.48*	0.008
	Rep.	0.63	14.60	17.81*	23.64	117.83	333.07	0.009	0.16	305.33	6.72	42.37	437.07	687.12	0.014
	Genotype*Env.	0.07	137.16*	30.50**	9991.51*	86.08	595.27	0.005	0.07	107.95	45.99**	1946.11	456.10	730.90	0.008
	Error	0.20	46.32	4.37	1515.44	121.26	274.60	0.005	0.58	83.94	9.76	2490.93	174.47	613.85	0.005
	$h_B^2$	0.917	0.813	0.641	0.416	0.694	0.221	0.753	0.855	0.907	0.407	0.584	0.552	0.528	0.640

LN, low nitrogen; NN, normal nitrogen; LEAF, the final number of leaves; PH, plant height; HKW, hundred-kernel weight; KNE, kernel number per ear; GY, grain yield; SB, shoot biomass; HI, harvest index; Geno., genotype; Env., environment; Rep., replication;  $h_B^2$ : broad-sense heritability;  $h_N^2$ : narrow-sense heritability. \*,\*\* indicates significance at 5% and 1% levels of probability, respectively.

instance, the inbred lines had only 37.6% of the average GY of hybrids, indicating that the heterosis effect was very strong for grain yield under LN stress.

#### Genetic variation in response to LN stress revealed by analysis of variance

Significant differences were observed among both the eight parental lines and their hybrids for all measured traits under both nitrogen levels (Table 4). Furthermore, both environmental variance and the  $G \times E$  interaction for most of the measured traits were more frequent and significant for both hybrids and inbred lines under LN than NN. This suggests that LN treatment in this study at each location across years increased phenotypic variance because of the increase in effects due to environment and  $G \times E$  interaction. This is also an indication of differential responses to LN stress among tested hybrids and their parental inbred lines.

Broad-sense heritability for KNE was relatively high for both parental lines and hybrids under both LN and NN conditions. Narrow-sense heritability of KNE for hybrids under both LN and high NN conditions was higher than that of GY and its component trait HKW. These data indicate that KNE could respond better to selection than GY for a breeding program directed at maintaining or improving grain yield under LN stress.

#### Diallel analysis

GCA and SCA were also analyzed in this study. GCA

and SCA variation across these lines were significant for all measured traits under both LN and NN conditions (Table 5).  $GCA \times Env$  interactions and  $SCA \times Env$  interactions were not significant for most traits. These results indicate that genetic effects were the main contributors to combining ability variance. Under both LN and NN conditions, PH, KNE, GY, SB, and HI showed high HPH and MPH, indicating that they would be good traits to target for exploiting heterosis (Table 6). GY can be dissected into its components KNE and HKW, and average HPH and MPH for KNE were much higher than those for HKW under both conditions, and were even larger under LN than under NN. Therefore, the KNE component contributes the majority of heterosis for GY under both LN and NN conditions, but particularly under LN. GY exhibited the largest range of heterosis among measured traits, ranging from 38.7% to 501.1% and from 71.9% to 436.2% for HPH under LN and NN, respectively, and from 70.7% to 517.2% and from 101.4% to 502.6% for MPH under LN and NN, respectively. The LEAF trait exhibited the lowest range of heterosis; from -8.0% to 9.9% and -9.0% to 23.2% for HPH under LN and NN, respectively, and from -0.7% to 15.6% and from -1.4% to 24.1% for MPH under LN and NN, respectively. The ranges of values for heterosis for all traits except LEAF increased concomitantly with the lower nitrogen levels under the LN treatment, indicating that the hybrids showed much larger differential responses to LN stress compared to their parental lines.

The GCA effect, which determines the average

**Table 5.** Mean squares for general combining ability (GCA) and specific combining ability (SCA) under low-nitrogen and normal-nitrogen conditions

		LEAF	PH	HKW	KNE	GY	SB	HI
LN	GCA	14.7**	7287.4**	159.5**	46553.5**	3876.3**	7809.4**	0.014**
	SCA	1.3**	882.7**	26.3	11441.2**	1416.0*	5656.0**	0.012**
	$GCA \times Env.$	0.009	231.2	25.7	3120.1	821.1	3330.1*	0.009*
	$SCA \times Env.$	0.08	150.2	26.5	3883.2**	769.9	3112.1*	0.005
	Ratio	0.66	0.53	0.60	0.34	0.35	0.15	0.13
NN	GCA	14.8**	8588.1**	158.5**	61286.9*	1881.1*	7795.69**	0.037**
	SCA	3.3**	480.6**	23.5	10058.1**	2915.3**	5341.8*	0.006**
	$GCA \times Env.$	0.2	340.5*	56.5**	3622.5	1610.0*	1377.8	0.005*
	$SCA \times Env.$	0.3	169.6	9.4	2201.6	646.3	1857.5	0.002
	Ratio	0.38	0.74	0.65	0.46	0.06	0.17	0.48

LEAF: the final number of leaves; PH, plant height; HKW, hundred-kernel weight; KNE, kernel number per ear; GY, grain yield; SB, shoot biomass; HI, harvest index; Env., environment; \*, \*\* Significant at 5% and 1% levels of probability, respectively; Ratio:  $2K_{GCA}^2 / (2K_{GCA}^2 + K_{SCA}^2)$ .

**Table 6.** Heterosis for measured traits under two nitrogen levels

		LN							NN						
		LEAF	PH	HKW	KNE	GY	SB	HI	LEAF	PH	HKW	KNE	GY	SB	HI
HPH	Aver.	1.5	33.9	-0.9	114.3	159.8	89.4	39.3	1.5	28.2	4.2	99.5	192.7	97.1	43.7
	Max.	9.9	69.4	40.6	239.7	501.1	176.4	170.7	23.2	47.4	38.2	133.3	436.2	163.5	111.7
	Min.	-8.0	10.9	-27.7	39.7	38.7	33.3	-9.4	-9.0	15.3	-12.5	56.6	71.9	46.2	-13.0
MPH	Aver.	6.5	42.7	11.3	147.2	206.3	100.9	68.3	7.1	37.8	14.0	118.8	257.4	120.1	69.3
	Max.	15.6	72.2	51.9	267.3	517.2	193.6	195.6	24.1	53.0	46.8	150.8	502.6	193.0	135.0
	Min.	-0.7	21.0	-12.7	59.5	70.7	49.9	12.6	-1.4	25.5	-4.7	83.1	101.4	53.8	19.9

LEAF, the final number of leaves; PH, plant height; HKW, hundred-kernel weight; KNE, kernel number per ear; GY, grain yield; SB, shoot biomass; HI, harvest index; Aver., average; Max., maximum; Min., minimum; LN, low nitrogen; NN, normal nitrogen; HPH, high-parent heterosis; MPH, mid-parent heterosis.

**Table 7.** General combining ability effects (GCA) and variance of specific combining ability (SCA)( $S_{Si}^2$ ) of parent *i* for all measured traits under two N levels at two sites

GCA $S_{Si}^2$	LN							NN						
	LEAF	PH	HKW	KNE	GY	SB	HI	LEAF	PH	HKW	KNE	GY	SB	HI
1	1.43**	38.38**	3.80**	7.61	21.03**	32.22**	-0.0017	1.65**	37.90**	2.24**	-8.49	6.79	37.47**	-0.0328**
	0.02	180.47	6.99	1433.15	12.62	837.89	0.0015	0.45	21.23	6.81	2796.70	345.96	1162.62	0.0011
2	-0.24	-0.38	3.66**	-82.56**	-15.58**	-27.46**	-0.0353**	-0.22	11.35**	3.60**	-73.46**	-1.03	4.05	0.0071
	0.17	101.26	1.42	5173.06	263.78	542.77	0.0024	0.80	64.39	2.16	2418.59	388.23	530.87	0.0001
3	-0.58**	-10.20**	1.32	17.97*	10.70*	21.07**	0.0232*	-0.54**	-16.24**	2.97**	-8.18	6.15	1.67	0.0255**
	0.10	216.37	0.06	597.10	50.77	1469.72	0.0005	0.95	105.51	2.50	584.33	558.59	839.53	0.0003
4	0.02	4.59	-0.65	1.91	4.22	-2.88	0.0209	0.11	-3.53	0.01	-12.15	-1.53	0.96	-0.0128
	0.40	142.23	0.21	27.70	139.90	801.82	0.0006	0.47	123.31	0.35	88.31	458.91	1374.88	0.0001
5	-0.48**	-10.02**	-2.62**	84.54**	10.22*	2.12	0.0435*	-0.64**	-12.49**	-2.63**	83.33**	6.95	-12.34	0.0514**
	0.06	23.66	1.13	5662.20	162.89	687.07	0.0002	0.00	18.91	3.10	2272.00	203.76	131.00	0.0003
6	-0.21	-16.34**	-0.75	2.96	-17.09**	-19.18*	-0.0141	-0.67**	-19.47**	-1.31	24.09**	-8.33	-30.76**	0.0028
	0.04	237.92	4.72	596.05	92.06	443.95	0.0028	0.01	125.19	6.29	1759.83	533.21	622.28	0.0010
7	-0.90**	-19.77**	-2.69	42.72**	-3.26	2.80	0.0003	-0.43**	-17.88**	-2.15**	66.64**	10.03	-1.12	0.0365**
	0.44	68.42	2.66	833.07	212.55	932.65	0.0013	1.28	64.42	1.41	1631.57	164.87	233.81	0.0001
8	0.97**	13.75**	-2.07*	-39.21**	-10.23	-8.68	-0.0368**	0.74**	20.37**	-2.73**	-71.78**	-19.03**	0.07	-0.0777**
	0.28	304.19	4.13	1902.60	22.09	1160.75	0.0015	0.43	208.93	1.86	2043.33	217.27	479.14	0.0011

LEAF, the final number of leaves; PH, plant height; HKW, hundred-kernel weight; KNE, kernel number per ear; GY, grain yield; SB, shoot biomass; HI, harvest index; LN, low nitrogen; NN, normal nitrogen; GCA, general combining ability; SCA, specific combining ability.

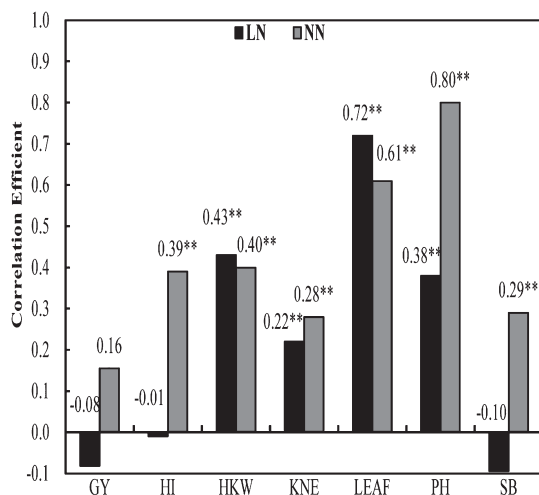
performance of a parent in a series of crosses, was calculated for each parental line for all traits along with their corresponding SCA variances (Table 7). Combined data analysis provides average performance values and reveals the outstanding hybrids with high SCA variance for a given line. Jinhuang96B has the highest GCA along with high SCA variance for KNE, which makes it the best line to use for LN tolerance as defined by this component of yield. Conversely, the parental line 2 Qi209 had the lowest GCA but high SCA, which means it could be useful for selection of elite hybrids based on high SCA estimates alone although with less confidence.

#### Correlation between inbred line *per se* and hybrid performance

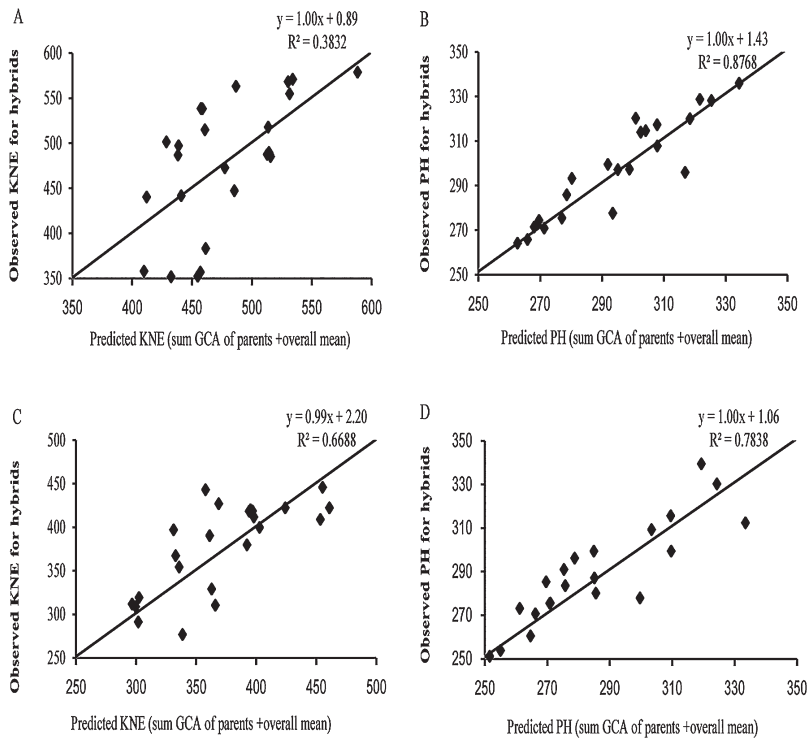
the correlation between hybrids and mid-parents was positive and significant for all traits under NN (Fig. 1). However, for the GY related traits, only KNE and HKW were significantly positively correlated under LN. Under LN, KNE and PH both fit a linear regression with high correlation coefficients ( $R^2$ ) of 0.6688 and 0.7838 (Fig. 2) between *per se* and hybrid performance, respectively. However, under NN,  $R^2$  for KNE decreased to 0.3832. In summary, KNE may be a positive trait with predictive value for the relationship between performances of an inbred line *per se* and in hybrids for a maize low-N tolerance breeding program. However, inbred lines and hybrids should be evaluated under LN conditions to identify inbreds with good breeding value and outstanding hybrids for low-N tolerance.

#### Discussion

Development of hybrid varieties and application of N fertilizers are two of the most important proven approaches to meeting increased future demands for maize. Breeding for improved maize low-N tolerance can be expected to also effect positive genetic gains under high N because approximately two-thirds of genetic gains for grain yield at high N can be explained by improvements in grain yield at low N (Haeghele *et al.* 2013). Moreover, GY of modern maize hybrids depends largely upon sowing density, as GY has been shown to be a function of plant population density and high planting-density tolerance, rather than intrinsic yield potential (Duvick 2005, Tollenaar and Lee 2002). At higher plant populations, low-N stress should be more severe due to competition for resources including N. Therefore, LN stress tolerance results from an important intersection of grain



**Fig. 1.** Correlations between midparent and hybrid performance for each trait under LN and NN. LEAF: the final number of leaves; PH, plant height; HKW, hundred-kernel weight; KNE, kernel number per ear; GY, grain yield; SB, shoot biomass; HI, harvest index.



**Fig. 2.** Observed and predicted KNE and PH across 28 F1 diallel crosses at NN (A, B) and LN (C, D) conditions calculated for each cross from the GCA effect of two parents and over-all means. KNE: kernel number per ear; PH: plant height.

yield, NUE, and heterosis in maize. However, the success of maize breeding for high NUE or low-N tolerance largely relies on acquiring relevant knowledge and understanding the genetic control of these traits at the population level. For example, the responses of hybrids and their parental lines to nitrogen input levels in terms of genetic variation and the magnitude of heterotic effects are important parameters for improving NUE and low N tolerance. Understanding the relevant kinds of genetic variation might direct breeders to the appropriate traits to target during line development or test crossing and reveal to what extent hybrid performance can be predicted based on parental lines and their testcrosses.

#### *Genetic variability reveals KNE as a breeding target for LN tolerance*

ANOVA revealed significant differences among these eight parental lines and their hybrids for all traits under two nitrogen levels (Table 4). However, the significances of the variances among traits differed between N levels. The heritability of each of these traits can explain their genetic contribution to the differences among variances. Under both N levels, genetic variability in the hybrids is higher than that in the parental lines for all traits except LEAF and HI (Table 4). Under LN conditions, the magnitude of the genetic variability decreased in the parental lines for all traits except HKW, but increased in the hybrids for all traits except LEAF. Compared with parental lines, hybrids showed lower heritability for all traits except KNE under NN, indicating that the performance of parental lines is more stable genetically than that of their hybrids under NN. LEAF showed the highest heritability, while KNE showed highest heritability

among all measured yield traits in both parental-line and hybrid-based analyses except for parental line under the LN condition (Table 4). However, our previous report using a larger panel of inbred lines ( $n = 189$ ), which included all parental lines in this study, tested under both LN and HN conditions revealed that KNE had higher heritability than any other yield component traits under LN (Wu *et al.* 2011). The genetic parameters characterized among those border genetic basis should be more credibly to justify that KNE was a good selecting trait which respond to LN selection at maize breeding. In addition to heritability, the sensitivity of the secondary trait other than GY *per se* was very important for improving the precision and demonstrating the degree of stress to crop to low nitrogen stress (Bänziger 2002). In this study, KNE was found to have a larger coefficient variation (CV) than HKW under LN and NN conditions in both parental lines and hybrids (Table 3), indicating that KNE shows a larger range of variation in both parental lines and hybrids under two nitrogen conditions. KNE, as well as GY, was more sensitive to nitrogen than HKW in both parental lines and hybrids (Table 3). For example, average KNE of the parental inbred lines and hybrids under LN was only 85.8% and 75.7% of those under NN, while HKW was 87.2% and 85.0%, respectively. In addition, KNE showed a more significant correlation with grain yield than HKW in both parental lines and hybrids under LN stress. For example, the correlation coefficients ( $R^2$ ) between GY and KNE are 0.68 and 0.60 for parental lines and hybrids respectively under the LN condition, while HKW are only 0.48 and 0.35 for parental lines and hybrids, respectively (data not shown). Therefore, concerning the heritability in this and

previous studies, the correlation with grain yield, and the sensitivity to the stress, for target trait selection, KNE was better than HKW as a secondary target trait for LN selection in maize breeding. A primary goal of NUE breeding and production is to maintain high crop productivity under lower N application and to determine whether it is possible to select good genotypes that are adapted to LN, NN, or both nitrogen conditions (Hirel *et al.* 2007). As a result, we could achieve ideal GY under LN stress. Thus, in terms of the agronomic performance of inbred lines under LN, KNE would be the best target trait for selection. Evaluation of hybrid performance also revealed that KNE had higher heritability than other GY component traits under both LN and HN conditions, predicting good response to selection for KNE as a target trait for improvement of N stress tolerance in future hybrid breeding programs.

#### Genotype by environment interaction

Furthermore, parental lines showed more significant  $G \times E$  interaction than did hybrids. The  $G \times E$  was significant for HKW, KNE, GY, and HI under both N levels. For hybrids, the environment effect was significant only for GY and HI, while  $G \times E$  was not significant for most of the measured traits under NN. These results indicate that inbred lines were more susceptible to environmental factors, particularly under LN, and that the performance of the hybrids should be relatively more stable. Therefore, hybrid evaluation under LN should be more reliable than the evaluation of parental lines in breeding programs for LN tolerance.

#### Heterosis for LN tolerance and its breeding implications

Maize hybrid vigor for grain yield is determined by the heterosis associated with plant biomass, kernel number, and harvest index (Munaro *et al.* 2011). Heterosis revealed in this study for HPH and MPH for PH and KNE tended to be relatively greater under LN conditions but lower under NN conditions (Table 6), consistent with a previous report (Zaidi *et al.* 2003). In this study, heterosis levels for HKW, GY, SB, and HI were greater under NN conditions. In particular, heterosis results for GY agreed with the conclusions from a previous study on a tropical maize population (Gama *et al.* 2002). Further, low heterosis was found for LEAF under both LN and NN conditions in this study, as also found for leaves above the ear in a previous report (Mehta and Sarkar

1992). Our findings suggest that leaf number is a developmental trait with large genotypic differences but low heterosis effects. Under NN, environmental effects in parental lines were significant for PH, KNE, GY, and SB; however, under LN, environmental effects were significant for PH, HKW, KNE, GY, and HI. MPH for KNE was much higher than that for HKW at both N levels, but particularly under LN. Therefore, KNE contributed the majority of GY heterosis under both LN and NN conditions, but especially under LN.

#### Prediction of hybrid performance from parental lines

Using inbred line information to predict hybrid performance could reduce the need for hybrid evaluation. An early study reported that the contribution of mid-parent performance for GY was comparatively greater than mid-parent heterosis under both low and normal nitrogen conditions (Zaidi *et al.* 2003), which encouraged us to explore the correlation between hybrid and mid-parent performance for all measured traits. A similar result for PH was obtained in an earlier study, with a lower correlation observed under severe low nitrogen stress than under optimal conditions (Zaidi *et al.* 2003) and stronger correlations observed for HKW under low-N stress (Bolaños and Edmeades 1996). However, correlations between performances of parent and hybrid have often been reported to increase under extreme environmental conditions (Good *et al.* 2004). The extent of hybrid performance that we could predict will determine whether this approach will be useful in breeding programs. Our results suggest that both KNE and PH can fit the linear regression with a high correlation coefficient under LN conditions (Fig. 2). Actually, most of the traits studied here, except HI, show positive correlations under N stress (data not shown). Linear regression revealed a correlation between parental lines and hybrids for low-N tolerance (Table 8). Among all the traits measured here, the regression coefficients between hybrids and parents estimated for the low-N tolerance index (LNTI) were only significant for HKW ( $R^2 = 0.2334$ ) and SB ( $R^2 = 0.2770$ ). This indicates that the nitrogen sensitivity of HKW and SB in hybrids is more related to that of their parents than are the nitrogen sensitivities of other traits we studied. However, our experiment revealed that for most of the other traits we studied, low-N tolerance in hybrids had no certain correlation with that of their parental lines. Based on the definitions of GCA and SCA (Griffing 1956), the

**Table 8.** Parent-offspring correlations for the low-N tolerance index (LNTI) between the parental lines and their diallel cross F1 hybrids

Traits	F1, MP		F1, HP		F1, LP		F1, (HP LP)		
	Reg.	R-square	Reg.	R-square	Reg.	R-square	Reg. (HP)	Reg. (LP)	R-square
LEAF	0.135	0.0031	0.231	0.0117	-0.019	0.0001	0.282	-0.119	0.0149
PH	0.054	0.0075	0.213	0.0185	0.023	0.0044	0.207	0.003	0.0186
HKW	0.073	0.0039	-0.185	0.0387	0.329	0.1039	-0.370	0.492*	0.2334
KNE	-0.030	0.0035	-0.059	0.0243	0.043	0.0075	-0.097	0.105	0.0584
GY	-0.089	0.0181	-0.187	0.1106	0.057	0.0104	-0.264*	0.172	0.1878
SB	0.403	0.1215	0.127	0.0204	0.588**	0.2600	-0.135	0.675**	0.2770
HI	0.097	0.0432	0.071	0.0387	0.079	0.0278	0.054	0.040	0.0435

\*, \*\* Significant at 5% and 1% levels of probability, respectively; Reg., Regression coefficient; MP, mid-parent; HP, high parent; LP, low parent.



**Table 9.** Correlations among F1 and MPH, HPH, and SCA for traits measured under two nitrogen levels

Traits	LN					NN				
	r(F1,MPH)	r(F1,HPH)	r(SCA,MPH)	r(SCA,HPH)	r(F1,SCA)	r(F1,MPH)	r(F1,HPH)	r(SCA,MPH)	r(SCA,HPH)	r(F1,SCA)
LEAF	0.47*	0.16	0.79**	0.61**	0.43*	0.29	0.05	0.82**	0.74**	0.56**
PH	0.66**	0.46*	0.53**	0.61**	0.46*	0.37	-0.40*	0.95**	0.42*	0.35
HKW	0.55**	0.50*	0.47*	0.44*	0.53**	0.62**	0.64**	0.51**	0.52**	0.51**
KNE	0.36	0.17	0.40*	0.25	0.59**	0.57**	0.38	0.77**	0.64**	0.50**
GY	0.55**	0.42*	0.44*	0.40*	0.63**	0.28	0.31	0.49*	0.45*	0.89**
SB	0.91**	0.84**	0.79**	0.75**	0.80**	0.52**	0.53**	0.66**	0.47**	0.79**
HI	0.09	0.08	0.46*	0.44*	0.81**	-0.09	0.11	0.41*	0.35	0.55**

LEAF: the final number of leaves; PH, plant height; HKW, hundred-kernel weight; KNE, kernel number per ear; GY, grain yield; SB, shoot biomass; HI, harvest index; LN, low nitrogen; NN, normal nitrogen.

correlations between MPH or HPH and F1 performance and between SCA and F1 performance for many traits might also be used for parent-hybrid performance prediction. A large number of positive correlations were found between these combinations of parameters in this study (Table 9), so it does seem that both GCA and SCA play important roles in F1 performance. Finally, these results provide further evidence that prediction of hybrid performance based on that of parental lines, although a major challenge in low-N tolerance breeding, may be amenable to the type of analysis presented here.

## Conclusions

From a breeder's point of view, selection of the appropriate maize hybrids to be cultivated under LN input will be facilitated by identification of high GCA and SCA effects. Improving low-N tolerance in maize can be expected to also result in genetic gain for yield under high N. Genetic variation and heterosis analysis showed that genetic improvement of hybrids for LN tolerance is an effective approach to enhancing maize production under lower N input settings. Selection for KNE, rather than for GY itself, may be more effective for genetic improvement of yield under LN, or LN tolerance. Therefore, evaluation of hybrids under LN is essential for breeding hybrid maize for LN tolerance.

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