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RESEARCH

New Insights into the Genetics of Haploid Male Fertility in Maize

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ABSTRACT

Doubled haploid (DH) lines have become widely used in maize (*Zea mays* L.) breeding. Haploid genome doubling is an important step in developing DH lines. The low rate of spontaneous genome doubling, which causes low haploid male fertility (HMF), seriously limits the largescale application of DH breeding without colchicine treatment. Our objective was to gain new insights into the genetics controlling HMF to improve the rate of HMF in DH breeding procedures. Haploid populations of 20 inbreds and their 31 single crosses derived from Chinese elite maize germplasm were screened for four traits related to HMF: anther emergence rate, pollen production rate, anther emergence score, and pollen production score. Haploid male fertility was compared between single crosses and their parents. Genotype effects were significant ($p < 0.01$) for all traits among Chinese elite maize lines and their single crosses, and interactions between genotype and environment were also significant ($p < 0.05$) for anther performance. Heritabilities ranged from 0.68 to 0.91 for these four traits. Haploid male fertility was controlled by additive effects with two or more genes. Anther emergence score proved to be the best trait for describing HMF and is the most practical trait for breeders. We propose that the potential use of HMF in breeding programs could reduce the need for toxic and costly artificial doubling treatments.

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Abbreviations: AER, anther emergence rate; AES, anther emergence score; DH, doubled haploid; FDR, first division restitution; HFF, haploid female fertility; HIR, haploid induction rate; HMF, haploid male fertility; PPR, pollen production rate; PPS, pollen production score; QTL, quantitative trait loci.

Doubled haploid (DH) technology is increasingly applied in commercial breeding and scientific research (Presterl et al., 2007; Wilde et al., 2010; Martin et al., 2011), while in vivo haploid induction (Röber et al., 2005) is the preferred method for maize DH line development (Gallais and Bordes, 2007). Compared with the conventional inbred line development process, DH technology only needs two generations to obtain homogeneous lines, which greatly speeds up the breeding process (De la Fuente et al., 2013). Doubled haploid lines are produced via haploid induction through cross pollination with a haploid inducer in the first generation, then haploid genome doubling through artificial or spontaneous genome doubling in the second generation. Doubled haploid breeding is considered to be an alternative breeding method compared with traditional successive self-pollination (Wedzony et al., 2009).

Despite the advantages of DH technology in maize breeding, not all steps involved in the process have been optimized. There are three subsequent steps required for acquiring a DH plant: (i) haploid induction, (ii) haploid identification, and (iii) genome doubling (Geiger and Gordillo, 2009; Kleiber et al., 2012). The first two steps have been improved substantially over decades. For example,

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haploid induction rate (HIR) has been increased from 0.6% (Chase, 1952) to >10% (Zhao et al., 2013). Several inducers, including WS14 (Lashermes and Beckert, 1988), ZMS (Chalyk, 1994), MHI (Eder and Chalyk, 2002), RWS (Röber et al., 2005), UH400 (Prigge et al., 2011), and CAU5 (Xu et al., 2013) have been developed and are available for maize breeding programs globally (Dwivedi et al., 2015). Two quantitative trait loci (QTL), *qhir1* and *qhir8*, with large effects on HIR have been fine mapped on chromosome 1 (Prigge et al., 2012; Dong et al., 2013) and chromosome 9 (Liu et al., 2015), respectively. Two QTL, *qmhir1* and *qmhir2*, which influence the maternal effect on induction rates, were detected on chromosomes 1 and 3, respectively (Wu et al., 2014b). Haploid identification is typically accomplished visually by using the "red color" marker *R1-nj*, but it is also performed using automated platforms based on near-infrared spectroscopy (Jones et al., 2012) or kernel oil markers in conjunction with nuclear magnetic resonance (Dong et al., 2014). Current DH technology relies on artificial genome doubling. A widely used artificial genome doubling method is based on the application of colchicine (Barnabás et al., 1999), a chemical agent affecting spindle tubes during mitosis and increasing genome doubling. Improved doubling methods may result in up to 27% of the treated haploid plants producing seed by selfing after colchicine treatment (Eder and Chalyk, 2002), compared with an average of 10% without colchicine treatment (Chase, 1949, 1952; Kleiber et al., 2012). Colchicine is a costly, highly toxic agent, and its application is labor intensive—in particular, it requires transplanting treated plants. Alternative chemical treatments or approaches such as trifluralin, amiprophos-methyl, and nitrous oxide gas have been suggested to improve doubling rates (Kato and Geiger, 2002; Klíma et al., 2008; Grzebelus and Adamus, 2004; Pintos et al., 2007; Kitamura et al., 2009; Häntzschel and Weber, 2010) but are not as effective as colchicine. Therefore, genome doubling remains a key bottleneck for improving the efficiency of DH technology.

Haploids may become fertile without particular treatment, i.e., by spontaneous haploid genome doubling. Spontaneous genome doubling is an alternative method to artificial genome doubling (Kleiber et al., 2012). Barnabás et al. (1999) reported that spontaneous haploid genome doubling rates ranged from 0 to 21.4% among maize germplasm. The spontaneous haploid genome doubling rates range from 10 to 40% in rape (*Brassica napus* L.) (Henry, 1998) and can reach 87% in *Hordeum vulgare* L., a variety of barley (Hoekstra et al., 1993).

After in vivo haploid induction and planting in the field, maize haploids show a high degree of haploid female fertility (HFF) (Chalyk, 1994; Geiger et al., 2006; Kleiber et al., 2012). More than 90% of haploid ears with kernels are obtained after crossing haploid plants with regular diploid maize pollen (Chalyk, 1994; Geiger et al., 2006). Compared with HFF, haploid male fertility (HMF) is strongly reduced (Chalyk, 1994; Kleiber et al., 2012), which limits the number of DH lines produced in a population without colchicine treatment. Thus, any methods to improve HMF are of interest to maize breeders. The choice of an appropriate environment is one option: HMF was found to be significantly higher for haploid maize cultivated in the greenhouse compared with the field (Kleiber et al., 2012). However, the use of greenhouses limits the number of DH lines that can be produced. Therefore, genetically improving HMF is a more realistic choice for large breeding programs.

Geiger and Schönleben (2011) studied European dent maize and used a scoring system with four distinct levels to distinguish different degrees of HMF. Their classification was from one, with very few extruding anthers on an unbranched tassel, to four, with several pollen-shedding anthers on all branches. By applying these scores, HMF varied significantly among the investigated European dent maize entries. They also found significant genetic variance for HMF within populations. Similar results have been found in tropical and temperate maize germplasm (Kleiber et al., 2012). So far, no studies are available for HMF in maize germplasm adapted to China. The main objectives of this study were: (i) to estimate means, genetic variances, and heritabilities for four traits—anther emergence rate (AER), pollen production rate (PPR), anther emergence score (AES), and pollen production score (PPS)—in describing HMF and determine, which would be the most practical trait for breeders; (ii) to investigate the impact of genotype, environment, and genotype × environment interactions affecting HMF; (iii) to classify the materials included in this study and identify germplasm with high HMF suitable for future breeding activities; and (iv) discuss genetic mechanisms underlying HMF.

MATERIALS AND METHODS

Germplasm and Experimental Design

Experiment 1: Haploids from Inbred Lines

Haploids from 20 elite Chinese maize inbred lines from the National Maize Improvement Center of China were produced in the summer of 2011 at Shangzhuang Experimental Station in Beijing and in the winter of 2012 at Nanbin Nursery Base in Hainan. These elite inbreds are members of five different heterotic groups (Xie et al., 2007): Reid (n = 8), Lancaster (n = 5), Lvdahonggu (n = 1), Tangsipingtou (n = 2), and Tropical germplasm (n = 4). Most agronomic traits differ significantly among these groups. The total number of inbred lines was 20, but there were minor differences between the 2 yr and two locations according to the available amount of haploid seed (Table 1). This source germplasm was crossed with the haploid inducer CAU5 (Xu et al., 2013). Haploids were identified with seed color marker *R1-nj* (Nanda and Chase, 1966), and haploids of all 20 lines were screened for HMF.

Evaluation of HMF for 20 haploid lines was conducted under field conditions during the summers of 2012 and 2013 at

Linze and Shangzhuang. The former site is located at the breeding station of Origin Seed Company in Linze, and it is located approximately 1100 km inland (38° N; 1500 m elevation). The latter site is located at the Shangzhuang experimental station of China Agricultural University in Beijing, and it is located less than 200 km away from the sea (39° N; elevation < 100 m). The climate characteristics of both sites differed in both years, especially in terms of temperature and humidity. At anthesis, both temperature and humidity in Linze (25°C on average, with large fluctuation per day and <50 mm precipitation) were lower than in Shangzhuang (32°C, with little fluctuation per day and >200 mm precipitation). The soils in Shangzhuang have a higher loam content than those in Linze. Two planting dates (half a month apart) were performed at the two sites: 10 May in Beijing and 25 April in Linze in 2012 and 12 May in Beijing and 27 April in Linze in 2013. Standard agronomic practices such as irrigation, fertilization, and weeding were applied during the entire growth period to ensure a uniform standard.

Table 1. Basic information of each inbred line: name, heterotic group membership, pedigree, planted year, and planted location as haploids.

Line name	Heterotic group	Pedigree	Year	Location†
Yu87–1	Tropical	Hybrid 78599	2012	B; L
			2013	B; L
Xu178	Tropical	Hybrid 78599	2012	B; L
			2013	B; L
Qi319	Tropical	Hybrid 78599	2012	B; L
			2013	B; L
1145	Tropical	Hybrid 78599	2012	B
			2013	B
4F1	Lancaster	Mo17	2012	B; L
			2013	B; L
By815	Lancaster	BHO population	2012	B; L
			2013	B; L
Mo17	Lancaster	Ex-pvp	\	\
			2013	B; L
Longkang11	Lancaster	Mo17 × Zi330	\	\
			2013	B; L
Gy923	Lancaster	BHO population	2012	B
			2013	B; L
EH759	Reid	Chang7–2 × zheng58	2012	B; L
			2013	B; L
Huang-C	Reid	(HX162×Zi330/O2) × Tuxpeno	\	\
			2013	B; L
K22	Reid	K11 × Ye478	2012	B; L
			2013	B; L
Kenzi167	Reid	C90–2 × Ye52106	2012	B; L
			2013	B; L
C8605	Reid	7922 × 5003	2012	B; L
			2013	B; L
B73	Reid	Ex-pvp	2012	B; L
			2013	B; L
Tie7922	Reid	Hybrid 3382	\	\
			2013	B; L
Zheng58	Reid	Ye478	2012	B; L
			2013	B; L
Dan598	Lvdahonggu	OH43 × Dan340	2012	B; L
			2013	B; L
Chang7–2	Tangsipingtou	(Wei95 × Huangzaosi) × S901	2012	B; L
			2013	B; L
EH766	Tangsipingtou	Chang7–2 × zheng58	2012	B; L
			2013	B; L

† B, Beijing; L, Linze.

A large sample size was used to evaluate the trait of HMF to increase accuracy. Considering limited haploid seed and maximizing the environmental divergence, haploid populations were grown in 10-row plots in a randomized block design with one replicate in each year for both locations (Table 1). In each row, 25 kernels were sown at a distance of 20 cm, and rows were spaced 50 cm apart. Haploids were not treated with any chemical reagent for genome doubling. Rogues (misclassified hybrids) were visually identified and removed at the six to seven leaf stage. The number of haploid plants for each inbred line was no less than 200 per plot.

Experiment 2: Haploids from Hybrids

Haploids from 31 elite single crosses were screened for HMF. These hybrids were chosen according to their parents' HMF, including high HMF × high HMF, high HMF × middle HMF, high HMF × low HMF, middle HMF × middle HMF, middle HMF × low HMF, and low HMF × low HMF (Supplemental Table S1). The source germplasm was crossed with the same inducer CAU5 used in experiment 1 in the winter of 2012–2013 at the Nanbin Nursery Base in Hainan. Parents of these 31 single crosses were all included as inbred lines in experiment 1.

In this experiment, the 31 haploid populations from elite single crosses were planted at both the Shangzhuang and Linze experimental stations in the summer of 2013 in a randomized complete block design with one replicate. In each site, 20-row plots containing 25 plants per row were grown with 50-cm spacing between rows and 20-cm spacing between plants within a row. Standard agronomic practices for growing maize were followed, as mentioned above.

Trait Assessment

Haploid male fertility was divided into two traits: anther emergence and pollen production. Maize haploids were scored for the two traits in reference to maize cytoplasmic male sterile classification (Duvick, 1965). For scoring anther emergence, haploids were classified on the basis of a five-point scale according to the density of anthers on the tassel (including main and side branches): (1) <5% (Fig. 1a), (2) 6 to 20% (Fig. 1b), (3) 21 to 50% (Fig. 1c), (4) 51 to 75% (Fig. 1d), and (5) >75% of anthers emerged on the tassel (Fig. 1e). For scoring pollen production, haploids were scored on a three-point scale according to the quantity of pollen grains and the percentage of fertile pollen grains as determined by 3% iodine-potassium iodide solution: (1) difficult release of pollen—little pollen was obtained only by pressing anthers, <5% of pollen grains showed viability using the iodine test, and few kernels were harvested after cross pollination with regular diploid maize ears (Fig. 1f); (2) a moderate amount of pollen was obtained by shaking a tassel, about 6 to 30% of pollen grains were viable based on iodine test evaluation, and a few kernels were obtained after cross pollination with regular diploid maize ears (Fig. 1g), but the overall seed set was poor (10–30%); (3) ample pollen was released on touching the tassel, >30% fertile pollen grains and a good

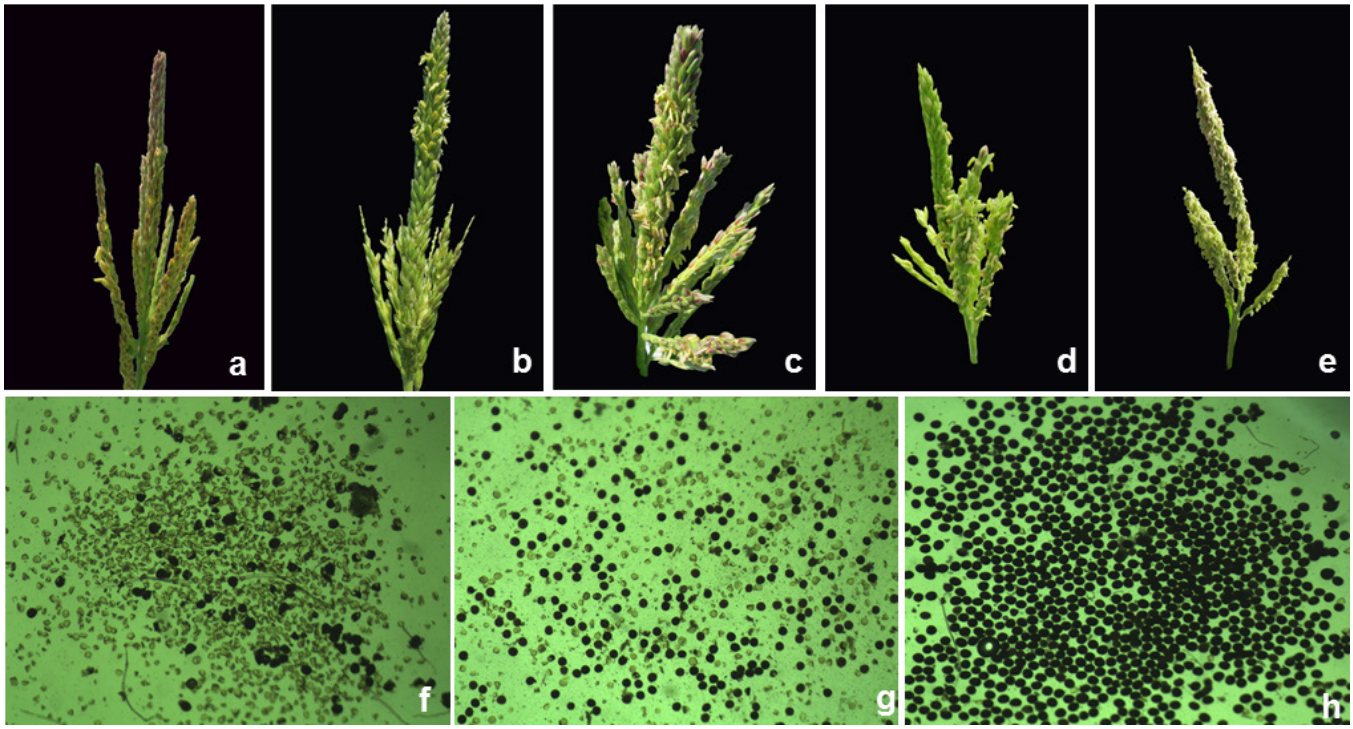

Fig. 1. Different levels of anther emergence (a–e) and pollen production (f–h) in a haploid population. Percentage of anthers on the tassel: (a) level 1, 0–5%; (b) level 2, 6–20%; (c) level 3, 21–50%; (d) level 4, 51–75%; (e) level 5, >75%. The quantity of fertile pollen grains by iodine test: (f) level I, few pollen grains showed fertility; (g) level II, some parts of the pollen grains showed fertility but other parts were infertile; (h) level III, almost all of the pollen showed fertility.

seed set (Fig. 1h). The total length of the flowering period was 35 to 40 d, from the end of June to the beginning of August. Flowering haploid plants were scored daily for male fertility from the beginning to the end of pollen shed. Only the results from the pollination peak period were used for analysis. After flowering, AER and AES were used to describe anther emergence, and PPR and PPS were used to describe pollen production. Anther emergence rate was calculated by dividing the total number of haploid plants with emerged anthers by the total number of haploid plants per plot; PPR was calculated by dividing the total number of pollen-producing haploid plants by the total number of haploid plants per plot. Anther emergence score and PPS, as the new methods for HMF evaluation of a haploid population, were calculated as follows:

$$Y_{AS} = \sum \mu_{Ai} \times n_{Aj}/(nh_{Amax}) \qquad [1]$$

where Y_{AS} is the average AES, μ_{Ai} is the individual AES and the individual score equal to the evaluated scale for each haploid, n_{Aj} is the number of haploids for each level of anther emergence, n is the total number of haploids per plot, and h_{Amax} is the highest level of anther emergence, which is 5.

$$Y_{PS} = \sum \mu_{Pi} \times n_{Pj}/(nh_{Pmax}) \qquad [2]$$

where Y_{PS} is the average PPS, μ_{Pi} is the individual PPS and the individual score equal to the evaluated scale for each haploid, n_{Pj} is the number of haploids for each level of pollen production, n is the total number of haploids per plot, and h_{Pmax} is the highest level of pollen production, which is 3. The range of the four traits was zero to one.

To complement visual scoring of ploidy levels, the flag leaves of three representative plants of each anther level and each pollen level, harvested during flowering, were examined by flow cytometry as described by Palomino et al. (2008) using a CAII flow cytometer (Partec GmbH, Münster, Germany). Additionally, 10 normal diploids and 10 completely male-sterile haploids were sampled as controls.

Statistical Analyses

The means and variance components were estimated for logit-transformed data $\{\gamma_{trans} = \log [(\gamma + 0.005)/(1 - \gamma + 0.005)]\}$ using SAS PROC MIXED (Kleiber et al., 2012). Data from experiment 1 were analyzed using the model:

$$Y_{ijk} = \mu + T_i + L_j: G_k + TG_{ik} + LG_{jk} + \varepsilon_{ijk,} \qquad [3]$$

where μ is the overall mean, T_i is the effect of the ith year, L_j is the effect of the jth location, G_k is the effect of the kth haploid genotype, TG_{ik} is the effect of the kth genotype within the ith year, LG_{jk} is the effect of the kth genotype within the jth location, and ε_{ijk} is the effect of the experimental error. The terms on the left of the colon refer to fixed effects and the rest of the terms refer to random effects.

Data from experiment 2 were processed using the following model:

$$Y = \mu + G + L + \varepsilon \qquad [4]$$

where μ is the overall mean, G is the effect of haploid genotype, L is the effect of the location, and ε is the effect of the experimental error. All of the factors were treated as random effects.

Heritability was estimated on an entry-mean basis (Hallauer et al., 2010). All of the factors were treated as random effects when calculating heritability. The median values of AER, AES, PPR, and PPS correspond to retransformed results using the formula:

$$z = \exp^r + 0.005(\exp^r - 1)/(1 + \exp^r) \qquad [5]$$

where r is the result value and z is the median (Connolly and Wachendorf, 2001; Kleiber et al., 2012).

Since a wide range of materials were used in the two experiments and four traits were evaluated, a cluster analysis (average linkage method) was conducted to combine all traits, AER, PPR, AES, and PPS, in a single analysis. Each two of these four traits were analyzed by a Spearman's rank correlation test. Cluster and correlation analysis were all performed using statistical analysis software (SAS 9.2; SAS Institute, 2008).

Single-cross derived haploid populations were compared with parent performance. Five classes were differentiated (Fig. 2): (A) below the low parent performance (underdominance), (B) low parent performance (low parent dominance), (C) mid-parent performance (additivity), (D) high parent performance (high parent dominance), (E) above the high parent performance (overdominance) (Swanson-Wagner et al., 2006).

RESULTS

Characteristics of Traits Associated with Haploid Male Fertility

Ploidy level determination via flow cytometry confirmed that the randomly selected representative plants in the experiments were indeed haploid. Inbred lines had a mean AER of 47.2%, a mean PPR of 36.6%, a mean AES of 0.25, and a mean PPS of 0.17, respectively (Table 2). Compared with inbred lines, the four parameters in single-cross offspring had no significantly ($p < 0.05$) different mean values at 55.3%, 47.0%, 0.29, and 0.21, respectively (Table 2). The four traits showed slightly wider ranges of values for inbred lines than for single cross offspring. Anther emergence rate ranged from 9.8 to 89.8% for inbred lines and from 27.5 to 85.5% for single crosses. Pollen production rate ranged from 4.8 to 85.5% for inbred lines and from 20.2 to 81.0% for single crosses. Anther emergence score ranged from 0.02 to 0.83 for inbred lines and from 0.10 to 0.73 for single crosses. Pollen production score ranged from 0.02 to 0.69 for inbred lines and from 0.07 to 0.47 for single crosses (Table 2).

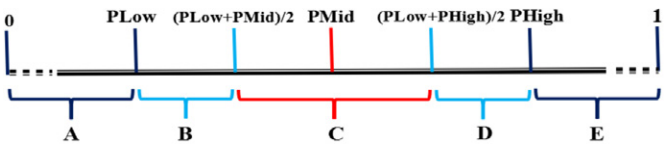

Fig. 2. Classification of the five categories when comparing the performance of single crosses with their parents: (A) below the low parent performance, (B) low parent performance, (C) mid-parent performance, (D) high parent performance, (E) above the high parent performance.

Haploid male fertility-related traits (AER, PPR, AES, and PPS) were significantly and closely correlated in both experiments ($p < 0.01$) (Spearman's rank correlation) (Table 3). For inbred lines, pairwise correlation coefficients ranged from 0.83 to 0.95. For hybrids, correlation coefficients ranged from 0.76 to 0.96 (Table 3). The correlation coefficients between AES and PPS were the lowest and the correlation coefficients between AER and PPR were the closest, both in inbred lines and single-cross offspring (Table 3).

Mixed Model Analysis of Haploid Male Fertility

Combined analysis of the haploid populations of inbred lines cultivated in two different environments over a period of 2 yr revealed significant ($p < 0.01$) genetic variances for each trait with respect to HMF (Table 4). Genotype × environment interaction variance in inbred lines was significant ($p < 0.05$) for AER and AES, but not for other parameters. The inbred lines had heritabilities for AER of 0.68, for PPR of 0.85, for AES of 0.84, and for PPS of 0.89, respectively. Analysis of variance of these haploid populations of single crosses also revealed significant genetic variance for each trait (Table 4). Single crosses had heritabilities for AER of 0.78, for PPR of 0.80, for AES of 0.91, and for PPS of 0.85, respectively.

No significant differences for AER, PPR, and AES were observed between both locations and years (Table 5). For PPS, no significant difference was observed between years, but a significant ($p < 0.05$) difference was found between locations (Table 5). The median PPS in Beijing was 0.12, which was significantly higher than 0.08 in Linze.

Table 3. Spearman's rank correlation coefficients (*r*) among anther emergence rate (AER), pollen production rate (PPR), anther emergence score (AES), and pollen production score (PPS) for the haploid population from inbred lines and hybrids.

Germplasm	Trait	AER	PPR	AES	PPS
Inbred	AER	1.00			
	PPR	0.95**	1.00		
	AES	0.95**	0.95**	1.00	
	PPS	0.83**	0.90**	0.83**	1.00
Hybrid	AER	1.00			
	PPR	0.96**	1.00		
	AES	0.92**	0.92**	1.00	
	PPS	0.80**	0.84**	0.76**	1.00

** Significant at the 0.01 probability level.

Table 2. Mean, SE, and range of anther emergence rate (AER), pollen production rate (PPR), anther emergence score (AES), and pollen production score (PPS) for inbred lines and hybrids.

Germplasm	AER		PPR		AES		PPS	
	Mean ± SE	Range	Mean ± SE	Range	Mean ± SE	Range	Mean ± SE	Range
	%							
Inbred	47.2 ± 6a†	9.8–89.8	36.6 ± 6a	4.8–85.5	0.25 ± 0.06a	0.02–0.83	0.17 ± 0.04a	0.02–0.69
Hybrid	55.3 ± 2a	27.5–85.5	47.0 ± 2a	20.2–81.0	0.29 ± 0.02a	0.10–0.73	0.21 ± 0.01a	0.07–0.47

† Values within each trait followed by a common letter are not significantly different at $p < 0.05$.

Table 4. Estimates of genetic (σ^2_g), genotype × environment interaction ($\sigma^2_{g \times e}$), and error (σ^2_ε) variance components and heritability (h^2) for anther emergence rate (AER), pollen production rate (PPR), anther emergence score (AES), and pollen production score (PPS) in inbred lines and hybrids. Analyses were based on logit-transformed data.

		Trait			
Germplasm	Parameter	AER	PPR	AES	PPS
Inbred	σ^2_g	1.52**	2.13**	2.40**	1.48**
	$\sigma^2_{g \times e}$	0.59*	0.02	0.37*	<0.01
	σ^2_ε	0.48	1.41	0.39	0.75
	h^2	0.68	0.85	0.84	0.89
Hybrid	σ^2_g	0.38**	0.43**	0.56**	0.29**
	σ^2_ε	0.22	0.22	0.11	0.11
	h^2	0.78	0.80	0.91	0.85

* Significant at the 0.05 probability level.

** Significant at the 0.01 probability level.

Table 5. Median of anther emergence rate (AER), pollen production rate (PPR), anther emergence score (AES), and pollen production score (PPS) at two locations and 2 yr. Analyses were based on logit-transformed data.

		Trait medians†			
Environment		AER	PPR	AES	PPS
		——— % ———			
Location	Beijing	52.02a‡	34.95a	0.18a	0.12a
	Linze	40.14a	25.50a	0.14a	0.08b
Year	2012	49.70a	32.78a	0.19a	0.10a
	2013	42.39a	27.39a	0.13a	0.10a

† Retransformed value.

‡ Values within one experiment–trait combination followed by a common letter are not significantly different at $p < 0.05$.

Cluster Analysis to Evaluate Haploid Male Fertility in Different Germplasm

The result of cluster analysis revealed that the 20 inbred lines could be classified into three major groups (Fig. 3). Cluster 1 included inbred lines Yu87–1, 4F1, EH759, and Huang-C, which exhibited superior HMF, and their AER, PPR, and AES values were >83%, >71%, and >0.68, respectively (Table 6). Cluster 3 included inbred lines Longkang11, Tie7922, Gy923, Zheng58, 1145, EH766, and Dan598, with a lower HMF than the lines in cluster 2, which included the remaining lines. In cluster 3, the AER, PPR, AES, and PPS values were <24%, <15%, <0.06, and <0.06, respectively (Table 6). Haploid male fertility of lines in cluster 2 was intermediate between clusters 1 and 3. The haploid populations derived from single crosses were also divided into three major groups based on cluster analysis (Fig. 4). Cluster 1 was comprised of three single crosses, while clusters 2 and 3 were comprised of 8 and 20 single crosses, respectively. For all the single-cross offspring in cluster 1, AER, PPR, and AES values were >82%, >77%, and >0.54, respectively (Table 6). For single-cross offspring in cluster 3, AER and PPR values were <63% and <53%, respectively (Table 6). The performance of the single-cross offspring in cluster 2 was between those in clusters 1 and 3.

Considering the limited number of inbred lines in this study, Tropical germplasm showed the best HMF among all these heterotic groups, while Tangsipingtou and Lvdahonggu groups showed the lowest HMF (Table 7). Crosses of Tropical × Tropical germplasm showed the best HMF among all these heterotic patterns. Three crosses, Lancaster × Lancaster, Reid × Tangsipingtou, and Reid × Lvdahonggu, showed a lower HMF than the remaining seven crosses.

With regard to relative performance of single-cross offspring compared with their two parental lines for each trait (AER, PPR, AES, and PPS), almost half of the single crosses showed mid-parent performance (C), about a quarter of the single crosses were similar to one of the parents (B and D), and about a quarter of the single crosses performed lower than the inferior (A) or higher than the better parent (E) (Fig. 5).

DISCUSSION

Assessment of Traits Associated with Haploid Male Fertility

Male fertility depends on both anther emergence and pollen production. Theoretically, there are four types of male fertile haploids with the following properties: (i) abundant pollen in many anthers, (ii) limited pollen in few anthers, (iii) limited pollen in many anthers, and (iv) abundant pollen in few anthers. In previous studies, the percentage of male fertile haploids in a haploid population was used as a phenotypic description to evaluate HMF. A few former studies classified HMF into four or five groups, considering the portion of anthers that emerged on the main and side branch (Geiger and Schönleben, 2011; Kleiber et al., 2012). Currently, no uniform standard exists for the evaluation of HMF.

In our study, first, AER and PPR were calculated according to previous studies, focusing on the percent of haploid plants with anther emergence or pollen production in a haploid population. Geiger and Schönleben (2011) studied European dent maize and claimed that the proportion of male fertile haploids reached 51.5% in the field and 66.0% in the greenhouse. We measured haploid AER and PPR values only under field conditions. The average AER was found to be 47.2% in haploids derived from inbred lines and 55.3% in haploids derived from hybrids. The average PPR was 36.6% in inbred lines and 47.0% in hybrids.

Second, to evaluate HMF more objectively in different germplasm, we introduced AES and PPS. For haploid populations, the ratio calculation accounts for the number of male fertile plants but ignores differences between each male fertile haploid. Some germplasm had similar AER or PPR, but the degree of expression of HMF in each individual was significantly different. For example, there were no significant differences between EH759 and Huang-C based on the evaluation of proportion (about 80%), but based on the values of AES and PPS, Huang-C had significantly higher scores (0.83 and 0.69, respectively) than

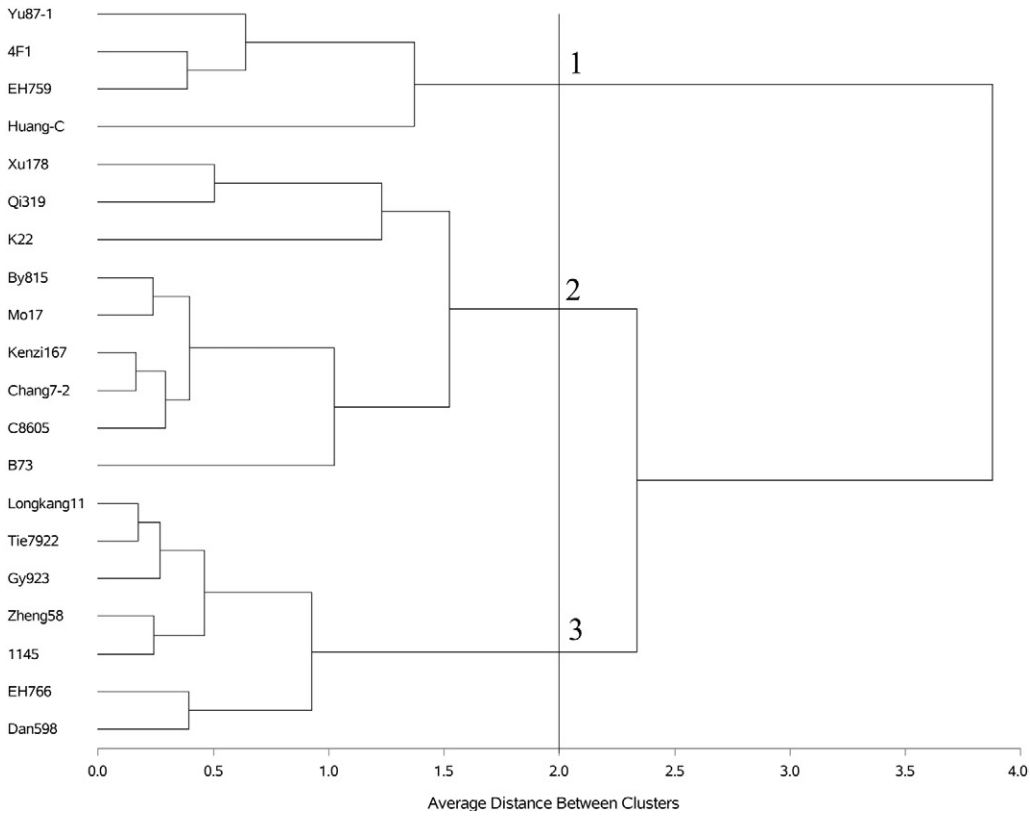

Fig. 3. Cluster analysis of the inbred lines according to the four haploid male fertility (HMF)-related traits.

Table 6. Median and range for the cluster group of anther emergence rate (AER), pollen production rate (PPR), anther emergence score (AES), and pollen production score (PPS) within the groups of inbred lines or single crosses. Results are based on 20 inbred lines evaluated at two sites in 2 yr and 31 single crosses in 1 yr.

	Cluster group	Number of lines	AER		PPR		AES		PPS	
			Median†	Range	Median	Range	Median	Range	Median	Range
			%							
Inbred	1	4	89.6	83.4–89.8	83.5	71.3–85.5	0.79	0.69–0.83	0.39	0.25–0.69
	2	9	51.0	40.1–68.9	34.3	20.5–57.0	0.15	0.10–0.35	0.13	0.08–0.48
	3	7	13.8	9.8–23.2	6.9	4.8–14.0	0.03	0.02–0.05	0.02	0.02–0.05
Hybrid	1	3	84.0	82.1–85.5	80.2	78.0–81.0	0.63	0.55–0.73	0.36	0.31–0.47
	2	8	68.7	64.3–71.7	60.2	55.6–64.3	0.39	0.26–0.52	0.29	0.21–0.46
	3	20	45.6	27.5–62.5	36.1	20.2–52.9	0.19	0.10–0.37	0.15	0.07–0.22

† Retransformed value.

EH759 (0.69 and 0.29, respectively). Moreover, heritability of AES, which was 0.84 and 0.91 in inbreds and hybrids, respectively, was relatively higher than that of AER, with 0.68 and 0.78 in inbreds and hybrids, respectively. Heritability of PPS, which was 0.89 and 0.85 in inbreds and hybrids, respectively, was slightly higher than that of PPR, with 0.85 and 0.80 in inbreds and hybrids, respectively. Heritability can be affected by many factors, but here we are only comparing differences between two estimators, holding all other factors constant. Taken together, AES and PPS could be more reliably scored and were more informative than the other HMF traits.

Hybrid female fertility, HMF, anthesis–silking interval, and many other factors contribute to seed production of fertile haploids, which is the ultimate goal of DH breeding. In this study, we focused on HMF. For practical breeding, it is preferable to select only one or as few as possible traits to maximize genetic gain. Significant and close correlations between all four traits related to HMF suggest that each single trait may be representative of all four traits. From a breeder's perspective, both the number of anthers or pollen grains per D_0 plant and the number of D_0 plants with anthers or pollen grains are important. The number of anthers or pollen grains per D_0 plant determines the kernel number after self-pollination. The number of D_0 plants with anthers or pollen grains determines the number of DH lines produced in a haploid population. That is why we introduce AES and PPS, in addition to AER and PPR, to describe HMF. Taking all above into account, both AES and PPS are the better choices to describe HMF. Compared with PPS, which involves laboratory assays, AES is preferable because it is easier to evaluate and is more stable under varying environments.

Factors Affecting Haploid Male Fertility

There was a significant ($p < 0.01$) genetic variance for HMF among pure lines and single crosses. For example, AER values ranged from 9.8 to 89.8% for inbred lines and from 27.5 to 85.5% for single crosses. The best AER value achieved was almost nine times higher than the lowest AER value achieved. Based on the results of cluster analysis, the germplasm was divided into three groups with different HMF. Our results demonstrated that different materials have divergent degrees of HMF under natural conditions and corroborated the results of Chase (1969). The average heritability estimated for Chinese maize lines (0.83) was similar to the estimate of Kleiber et al. (2012) for elite US Corn Belt lines (0.79). These

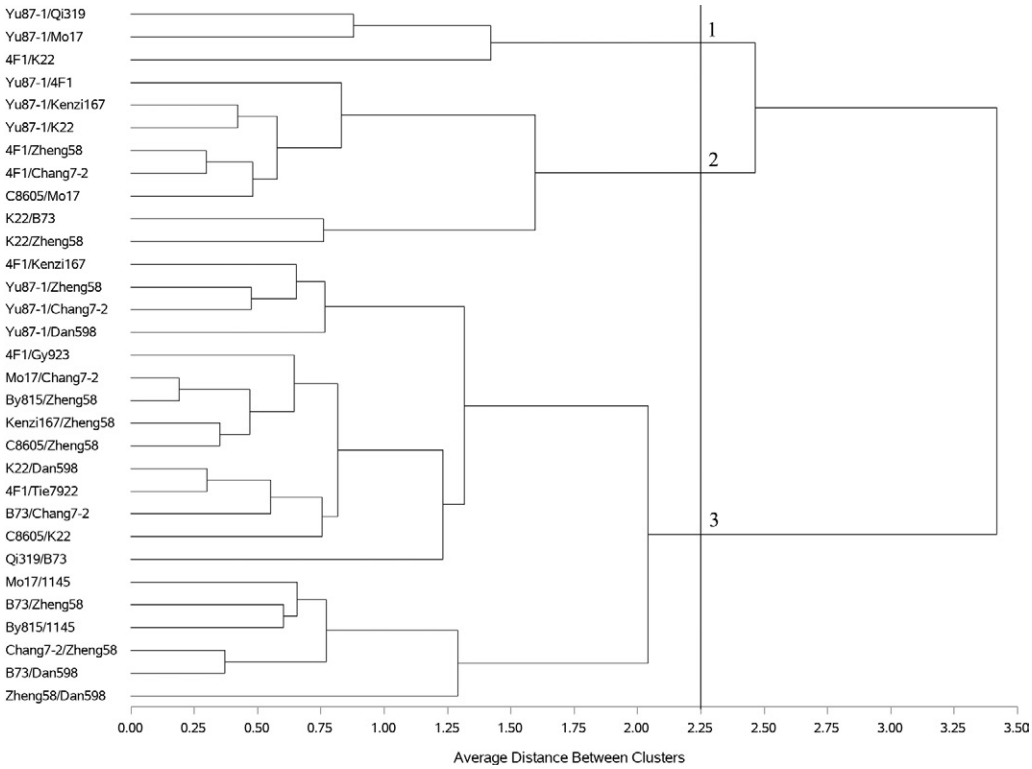

Fig. 4. Cluster analysis of single-cross offspring according to the four haploid male fertility (HMF)-related traits.

Table 7. Median and range for the cluster group of anther emergence rate (AER), pollen production rate (PPR), anther emergence score (AES), and pollen production score (PPS) within the groups of inbred lines or single crosses according to the heterotic group or heterotic pattern they belong to. Results are based on 20 inbred lines evaluated at two sites in 2 yr and 31 single crosses in 1 yr.

	Cluster group	Heterotic group pattern†	Number of lines	AER		PPR		AES		PPS	
				Median‡	Range	Median	Range	Median	Range	Median	Range
				%							
Inbred	1	P	4	67.3	13.8–89.2	59.2	6.8–87.9	0.37	0.03–0.84	0.14	0.02–0.25
	2	R	8	51.7	17.9–92.6	34.6	6.9–84.3	0.18	0.04–0.85	0.14	0.02–0.69
		La	5	51.8	17.7–91.0	29.3	7.6–82.4	0.16	0.04–0.74	0.10	0.03–0.37
	3	T	2	20.9	8.9–41.4	9.6	4.9–18.7	0.05	0.02–0.11	0.04	0.02–0.08
		Lv	1	5.3	/	4.1	/	0.02	/	0.02	/
Hybrid	1	P/P	1	83.5	/	81.5	/	0.73	/	0.31	/
	2	R/La	6	65.3	44.7–82.1	53.7	37.3–77.9	0.33	0.17–0.55	0.23	0.17–0.47
		T/P	1	58.1	/	52.9	/	0.32	/	0.20	/
		R/P	4	59.8	48.0–69.4	53.3	41.4–63.5	0.31	0.13–0.46	0.21	0.11–0.29
		T/La	2	61.2	53.1–68.7	47.2	38.3–56.3	0.29	0.22–0.37	0.20	0.18–0.24
		La/P	4	59.4	34.5–86.1	50.5	24.9–80.9	0.30	0.16–0.59	0.17	0.10–0.31
		Lv/P	1	49.6	/	47.2	/	0.27	/	0.17	/
		R/R	6	53.7	33.2–66.4	42.8	21.9–62.2	0.23	0.14–0.34	0.21	0.08–0.46
	3	La/La	1	44.5	/	33.0	/	0.22	/	0.15	/
		R/T	2	40.1	37.7–42.7	31.2	29.9–32.6	0.14	0.13–0.15	0.17	0.13–0.21
		R/Lv	3	34.8	27.3–31.9	29.0	20.1–39.2	0.13	0.09–0.20	0.12	0.06–0.18

† P, Tropical germplasm; R, Reid; La, Lancaster; T, Tangsipingtou; Lv, Lvdahonggu.

‡ Retransformed value.

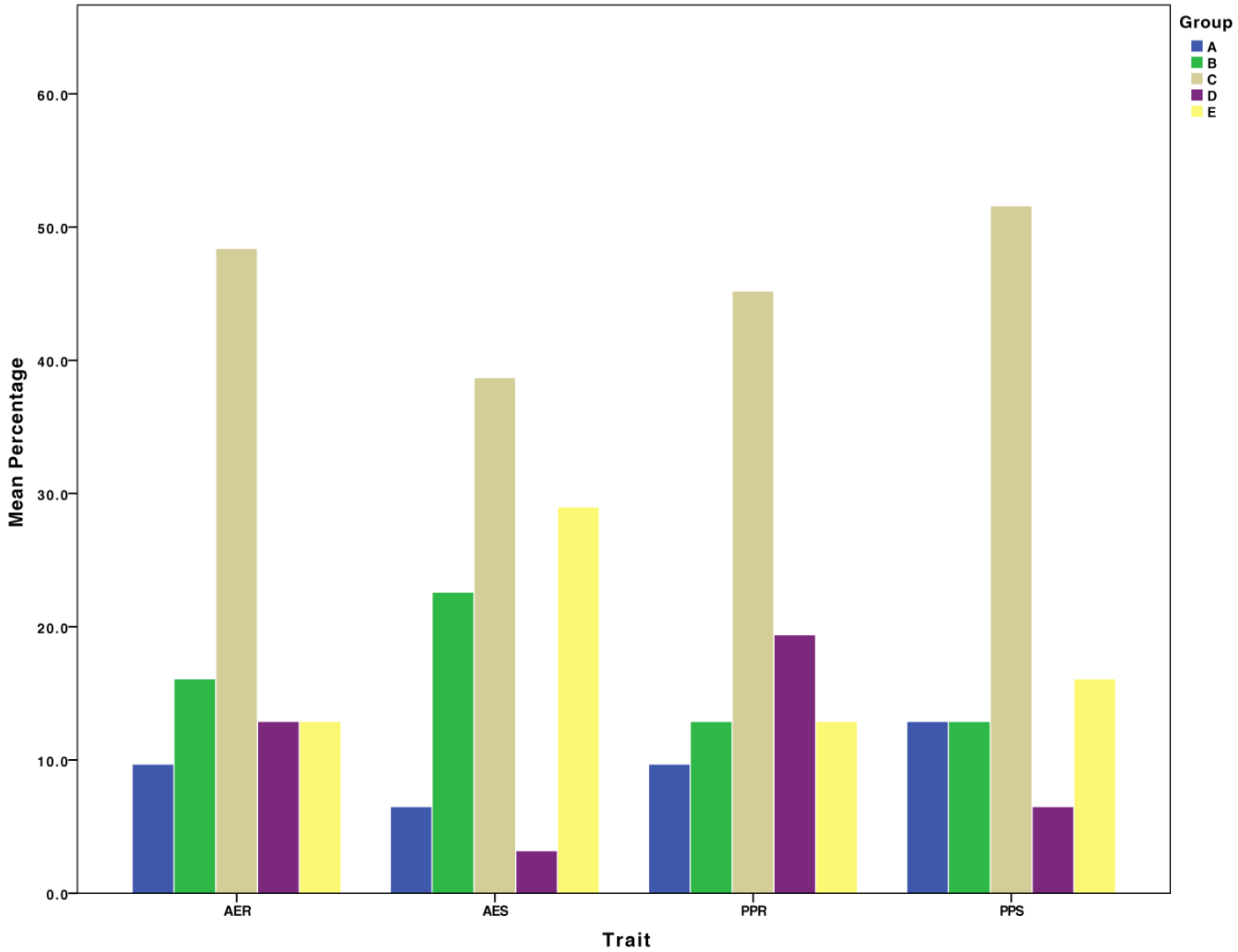

Fig. 5. Summary of the classification of the five categories: AER, anther emergence rate; PPR, pollen production rate; AES, anther emergence score; PPS, pollen production score; (A) below the low parent performance; (B) low parent performance; (C) mid-parent performance; (D) high parent performance; (E) above the high parent performance; NS, no significant difference in comparison of the value between two parents.

results confirm their findings that recurrent selection to increase HMF is promising. Therefore, identifying genes controlling HMF would be an effective way to improve HMF. Increased HMF has also been observed in particular mutants, such as the sodium azide-induced mutant *fdr1* (first division restitution) in maize (Sugihara et al., 2013). Furthermore, the present study suggests that germplasm with substantial HMF exists in breeding materials. Thus, elite lines with high HMF performance can be used as parents for QTL mapping of HMF or as donor parents to introgress high HMF into breeding programs by recurrent selection. We believe that efficient largescale DH production via this approach may reduce the need for toxic and costly artificial doubling treatments in the DH breeding process (Geiger and Gordillo, 2009; Prigge et al., 2011).

In this study, the environments used seemed to have a small effect on HMF, yet a wider range of environments might have revealed larger environmental effects. Only PPS was found to be significantly different across two locations. Generally, favorable environmental factors, such as stable day temperatures of about 30°C without large changes and precipitation during anthesis, may improve pollen production of fertile haploid plants. This effect was reflected in pure lines, but not in single-cross offspring. The other three traits did not show significant differences across locations or years. This finding indicates that the different locations or years of sowing may contribute little to anther emergence and pollen production in haploid populations. This result differed slightly from the findings of Kleiber et al. (2012), who found that HMF can be increased through improved environmental conditions under greenhouse and field conditions.

Interaction between germplasm and environment should also be a consideration. Particularly with respect to anther emergence in pure lines, there was a significant ($p < 0.05$) difference in genotype × environment

interaction variance for AER and AES. This is the first study to report this finding. Some haploid populations performed better in Shangzhuang, some performed better in Linze, and some performed similarly at both sites. For example, Qi319 had better anther emergence in Beijing than in Linze in both years. In contrast, Kenzi167 had better anther emergence in Linze than in Beijing (data not shown). This means that maize breeders might be able to increase the efficiency of DH line production by choosing the best-matching nursery locations for their germplasm.

Possible Genetic Mechanisms of Haploid Male Fertility

Two inbred lines, EH759 and EH766, were selected from an F_2 population derived from a cross of Zheng58 by Chang7–2. Haploid male fertility of EH759 and EH766 were significantly different from each other (Supplemental Table S2). The four values of EH759 were significantly higher than the values of both parents, Zheng58 and Chang7–2. EH766 showed lower HMF than its parents. Because of significant transgression, two or more genes control HMF instead of a single gene. By comparing the four traits between single-cross offspring and their parents, most of the single crosses expressed mid-parent inheritance. This indicates that genes controlling HMF showed mostly additive effects. In conclusion, HMF is controlled by two or more genes mostly showing additive gene action.

In our study, germplasm with high levels of HMF are available to identify loci controlling HMF by high-density genotyping and as donor materials for introgression into current breeding programs to overcome the need of artificial genome doubling in future. These genotypes exhibit HMF at a level that might be sufficient for the development of new breeding strategies, where selection can be conducted at the haploid level, which can improve the per se and hybrid performance of DH lines (Kleiber et al., 2012). To make genomic selection at the haploid level cost effective, a success rate of >17% for genome doubling without colchicine would be needed (Wu et al., 2014a).

CONCLUSION

We used four different traits to describe HMF in maize and found a close correlation between each two of the four traits. This study is the first to use AES and PPS to describe HMF. Anther emergence score was found to be the most appropriate and most practical trait for breeders to evaluate HMF. Haploid male fertility showed significant genetic variation among inbred lines and their single crosses. Inheritance of HMF was predominantly additive. Germplasm with the highest HMF identified in this study exceeded the performance of lines reported earlier, and these lines could be used to improve HMF in future breeding programs by recurrent selection. Increased HMF would facilitate genomic selection at the haploid level.

Conflict of Interest

The authors declare there to be no conflict of interest.

Supplemental Material Available

Supplemental material for this article is available online.

Acknowledgments

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