QTL mapping for haploid male fertility by a segregation distortion method and fine mapping of a key QTL *qhmf4* in maize

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Ren, Jiaojiao

Wu, Penghao

Tian, Xiaolong

Lübberstedt, Thomas §

Chen, Shaojiang

J. Ren and P. Wu contributed equally to this work.

J. Ren · X. Tian · S. Chen (✉)

College of Agriculture and Biotechnology, China Agricultural University,

No.2 Yuan Ming Yuan West Road, 100193 Beijing, China

e-mail: chen368@126.com

P. Wu

College of Agronomy, Xinjiang Agriculture University, 830052 Urumqi, China;

T. Lübberstedt

Department of Agronomy, Iowa State University, 50011 Ames, Iowa U.S.A.

Author contributions

S. Chen managed the project. J. Ren, P. Wu, and S. Chen designed the experiment. J. Ren, P. Wu, and X. Tian performed the experiment. J. Ren and P. Wu performed data analysis. J. Ren, P. Wu, and T. Lübberstedt wrote the paper.

Conflict of interest statement

We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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Key message

Four QTL related to haploid male fertility were detected by a segregation distortion method and the key QTL *qhmf4* was fine mapped to an interval of ~800 kb.

Keywords

Maize, haploid male fertility, QTL mapping, segregation distortion, progeny test

Abstract

Doubled haploid (DH) technology enables rapid development of homozygous lines in maize breeding programs. However, haploid genome doubling is a bottleneck for the commercialization of DH technology and is limited by haploid male fertility (HMF). This is the first study reporting the quantitative trait locus (QTL) analysis of HMF in maize. Four QTL, *qhmf1*, *qhmf2*, *qhmf3*, and *qhmf4*, controlling HMF have been identified by segregation distortion (SD) loci detection in the selected haploid population derived from 'Yu87-1/Zheng58'. Three loci, *qhmf1*, *qhmf2,* and *qhmf4*, were also detected in the selected haploid population derived from '4F1/Zheng58'. The QTL *qhmf4* showed the strongest SD in both haploid populations. Based on the sequence information of 'Yu87-1' and 'Zheng58', thirteen markers being polymorphic between the two lines were developed to saturate the *qhmf4* region. A total of 8168 H_1BC_2 (haploid backcross generation) plants produced from 'Yu87-1' and 'Zheng58' were screened for recombinants. All the 48 recombinants were backcrossed to 'Zheng58' to develop H_1BC_3 progeny. The heterozygous H_1BC_3 individuals were crossed with CAU5 to induce haploids. In each H_1BC_3 progeny, haploids were genotyped and evaluated for anther emergence score (AES). Significant (or no significant) difference ($P < 0.05$) between haploids with or without 'Yu87-1' donor segment indicated presence or absence of *qhmf4* in the donor segment. The analysis of the 48 recombinants narrowed the *qhmf4* locus down to an ~800 kb interval flanked by markers IND166 and IND1668.

Introduction

In vivo doubled haploid (DH) technology is recognized worldwide as an important means for enhancing breeding efficiency in maize breeding (Geiger and Gordillo 2009; Prasanna et al. 2012). Traditionally, it takes about 6-10 generations to produce inbred lines by recurrent selfing (Hallauer et al. 2010). DH technology reduces the time required for homozygous line development by more than half compared to traditional method and has been widely used in genetic research and maize breeding (Schmidt 2003; Seitz 2005; Smith et al. 2008; Chang and Coe 2009; Geiger 2009; Prigge et al. 2012). DH technology involves induction, identification, and genome doubling of haploids. In 1959, Stock 6 was reported to have a haploid induction rate (HIR) of 2.3% (Coe 1959). Based on inducer Stock 6, new inducers have been developed with a HIR of about 8% on average (Röber et al. 2005; Barret et al. 2008; Prigge et al. 2011, 2012). The genetic basis of in vivo haploid induction has been reported and discussed in several studies (Barret et al. 2008; Prigge et al. 2012; Xu et al. 2013; Dong et al. 2013; Liu et al. 2015; Kelliher et al. 2017). Eight QTL related to HIR have been detected by Prigge et al. (2012). The main effect QTL *qhir1* and *qhir8* have been fine mapped on chromosomes 1 and 9 (Dong et al. 2013; Liu et al. 2015), and MATRILINEAL (MTL), a pollen-specific phospholipase, controlling HIR has recently been isolated (Kelliher et al. 2017). Marker-assisted selection (MAS) can be used to develop new inducers (Dong et al. 2014). The dominant marker gene R1-*nj* (purple scutellum and a "purple crown" of the aleurone) is the most commonly used system for screening of haploids in maize (Nanda and Chase 1966; Rotarenco et al. 2010; Dwivedi et al. 2015). Haploid identification can also be automated using a high-throughput system based on a xenia effect for oil content and nuclear magnetic resonance (NMR) (Liu et al. 2012; Melchinger et al. 2013). The mean accuracy of the NMR screening system achieves 94% with an average speed of 4 seconds per kernel (Liu et al. 2012; Wang et al. 2016). Compared to haploid induction and identification, few studies have been reported on haploid genome doubling.

Artificial genome doubling is the most commonly used method for haploid genome duplication. Colchicine is a widely applied and highly effective genome doubling agent. Colchicine duplicates the chromosome set in single cells by binding to tubulin and inhibiting the formation of microtubules and the polar migration of chromosomes (Wan et al. 1989; Kleiber et al. 2012). Eder et al. (2002) reported that about 49% haploids produce fertile pollen and 27% produce seed by selfing after colchicine treatment in maize. However, colchicine is a costly and toxic agent and the related treatment is labor

intensive (Häntzschel et al. 2010). Nitrous oxide gas (Kitamura et al. 2009) is another genome doubling agent, but its effect on genome doubling is strongly influenced by genotype (Sugihara et al. 2013). Therefore, an alternative effective doubling method without artificial treatment is highly desirable for DH line production (Geiger and Schönleben 2011).

Spontaneous genome doubling has been reported in maize for a long time (Chase 1952; Geiger et al. 2006; Geiger and Schönleben 2011; Kleiber et al. 2012; Sugihara et al. 2013; Wu et al. 2014). Exploiting the natural fertility of haploids in breeder's germplasm may enable to eliminate the use of artificial treatments and to increase the efficiency of DH line production (Kleiber et al. 2012). Kleiber et al. (2012) studied haploid fertility in temperate and tropical maize germplasm and found that the haploid fertility ranges from 0-20% under field conditions and from 0-70% under greenhouse conditions. Haploid fertility includes haploid male fertility (HMF) and female fertility (HFF). Genomes in both haploid male and female cell lines have to be doubled for successful gamete formation and subsequent DH line production. Female fertility is generally high in haploids without artificial treatment (Chase 1952; Chalyk 1994; Geiger et al. 2006; Wu et al. 2017). It is reported that more than 90% of haploid ears produce seed after pollination with normal pollen from diploid plants (Chalyk et al. 1994). Compared to HFF, HMF is strongly reduced and ranges from 2.8-46% (Liu and Song 2000; Wei and Chen 2006; Han et al. 2006). Therefore, haploid genome doubling and DH line production are mainly limited by HMF. Previous studies showed that HMF is highly genotype-specific (Vanous 2011) and can be slightly increased by choosing appropriate environments. Wu et al. (2017) screened 20 elite inbred lines adapted to China for HMF and found that the best HMF can be as high as 90%. Mutagenesis is another way to improve HMF. Sugihara et al. (2013) reported a sodium azide-induced maize mutation, first division restitution 1 (*fdr1),* which has a high spontaneous genome doubling rate in both tassel branches and ovules. The mechanism of spontaneous genome doubling is still unclear and may be due to somatic cell fusion, endoreduplication, endomitosis or other mechanisms (Jensen 1974; Testillano et al. 2004; Vanous 2011). To improve the degree of HMF to overcome the need for artificial genome doubling during the DH process, it is necessary to explore the genetic basis of HMF in germplasm with high HMF.

The statistical power of QTL detection largely depends on sample size and the amount of genetic variation for the target traits in the mapping population (Falconer and Mackay 1996; Lynch and Walsh 1998; Cui et al. 2015). QTL mapping is always conducted in large populations without selection (Zhan

and Xu 2011). Keeping undesirable individuals in the mapping population helps to estimate QTL effects, but causes additional costs for phenotyping of genotypes or families of no interest for breeding programs (Cui et al. 2015). QTL mapping can also be conducted in selected populations. In this case, only individuals with extreme phenotypic values are selected for QTL detection. If selection is effective, the frequencies of genes related to target traits will differ between selected and unselected populations (Hermisson and Wagner, 2004). Markers linked to QTL will show segregation distortion (SD), which refers to deviation of observed genotypic frequencies from expected Mendelian frequencies (Sandler et al. 1959). The level of SD is commonly estimated by a Chi-square (χ^2) test. Detection of SD loci is an alternative method for QTL mapping. Many factors may cause SD, such as gamete and zygote selection. Therefore, SD loci detected in multiple populations and environments are more dependable. QTL mapping by detecting SD loci has been done in various studies (Li et al. 2005; Venuprasad et al. 2009; Zhang et al. 2012, 2014; Cui et al. 2015). Venuprasad et al. (2009) detected QTL for drought tolerance in rice by selecting the 40 highest grain yield lines under stress and another 40 lines with highest grain yield under non-stress conditions.

In this study, we conducted a comparative QTL analysis for HMF in two haploid populations involving two inbred lines with high HMF and one inbred line with low HMF. SD loci detection was used to conduct QTL analysis. Our objectives were to (1) estimate the number and genomic positions of QTL associated with HMF, (2) fine map the *qhmf4* locus, a key QTL for HMF, and (3) identify markers closely linked to *qhmf4* for marker-assisted selection (MAS) in breeding programs to improve HMF in maize.

MATERIALS AND METHODS

Plant materials

To identify the appropriate mapping parents, field evaluation of maize HMF was conducted for several years in Hainan and Beijing, China. Maize inbred lines 'Yu87-1', '4F1' (both are donor parents with high HMF), and 'Zheng58' (recurrent parent with poor HMF) were selected to develop mapping populations, including two F_1 populations ('Yu87-1/Zheng58' and '4F1/Zheng58') and backcross populations of 'Yu87-1/Zheng58'. The two F_1 populations were pollinated with the haploid inducer CAU5, which has ~8% HIR (Xu et al. 2013). Haploids were identified with seed "red color" marker *R1-nj* (Nanda and Chase, 1966). Haploids with the best HMF were selected for initial QTL mapping.

Haploids with high HMF derived from the F_1 population of 'Yu87-1/Zheng58' were backcrossed to 'Zheng58' to produce H_1BC_1 (haploid backcross generation) population. H_1BC_1 individuals were subsequently backcrossed to 'Zheng58' to generate the H_1BC_2 population. Molecular markers within the *qhmf4* region were used to identify recombinants in the H_1BC_2 population and recombinants were backcrossed to 'Zheng58' to produce H_1BC_3 progeny. The H_1BC_3 progeny with heterozygous 'Yu87-1/Zheng58' genotype in the *qhmf4* region was pollinated by the haploid inducer CAU5 (Xu et al. 2013). Kernels with purple endosperm and colorless embryo were selected as haploids (Li et al. 2009, Dong et al. 2013). All haploids were grown in the field for the evaluation of HMF.

Scoring of haploids for HMF

HMF was evaluated by anther emergence score (AES). According to the fraction of anthers emerged on the tassel, anther emergence was rated using a rating scale of 0-5 (Table 1). A rating scale of 0 corresponds to sterility with no anthers emerged on the tassel. Scales 1-5, with less than 5%, 6-20%, 21-50%, 51-75%, and 76-100% anthers emerged on the tassel, respectively, correspond to different degrees of HMF (Wu et al. 2017). On a single haploid basis, AES was calculated by dividing the individual rating scale by 5. On a haploid population basis, AES was calculated by the following formula:

$$\boldsymbol{Y = \sum \mu_i \times n_j / n}$$

where Y is the average AES; μ_i is the individual AES; n_j is the number of haploids for each level of anther emergence; n is the total number of haploids per plot. The range of AES was zero to one (Wu et al. 2017).

Genotyping

At seeding stage, leaves were harvested separately in the field for DNA extraction according to the method of Murray and Thompson (1980). All primer sequences for markers were obtained from the Maize Genetics and Genomics Database (http://maizegdb.org/) and synthesized by TransGen Biotech in Beijing, China. PCR amplification was performed by the procedure described in Barret et al. (2004). PCR products were separated on either 3% agarose gel or 6% polyacrylamide gel.

Verifying the method of SD mapping in haploid populations

QTL mapping by SD loci detection has not been conducted in haploid populations yet. Therefore, we used plant height to verify the method of SD loci detection in a haploid population. Plant height is a quantitative trait, for which more than 219 QTL have been reported across different mapping populations (Weng et al. 2011). One major QTL controlling plant height has been repeatedly detected between bins 5.05 and 5.06 when using the dwarf donor parent ‘Shen5003’. ‘Zheng58’ is an inbred line selected from ‘Shen5003’ and contains the SNP PZE-105115518 (Weng et al. 2011), which is significantly associated with plant height. In the summer of 2012 in Beijing, the 88 shortest haploids among 580 haploids induced from the F_1 population of ‘Zheng58/Yu87-1’ were used to detect the dwarf locus in bins 5.05-5.06. SD was examined within these 88 haploids using a χ^2 goodness of fit test compared to the expected segregation ratio of 1:1 of 'Yu87-1' or ‘4F1’ to 'Zheng58' alleles with Bonferroni correction for multiple testing. Significant deviation from a 1:1 ratio indicates the presence of a QTL for plant height near this marker. Fourteen polymorphic SSR markers across the maize chromosomes including five markers on chromosome 5 were chosen for our analysis.

Linkage map construction and QTL detection

The linkage map was constructed using MAPMAKER 3.0 (Lander et al. 1987). Linkage groups were constructed using the ‘Group’ command based on a logarithm of odds (LOD) score of 10.0 and a maximal recombination fraction of 0.30 (Zhou et al. 2015). For each linkage group, markers were ordered using the ‘order’ command at a minimum LOD score of 3.0, and the ‘Ripple’ command was used to get the best marker order. The order of the mapped markers in each linkage group was then confirmed by the IBM2 2008 Neighbors Map (http://www.maizegdb.org/) (Prigge et al. 2012). Recombination frequencies were converted into genetic distances (cM) using the Kosambi function (Kosambi 1944).

Analysis of QTL for HMF was performed by SD loci detection in haploid population. In the summer of 2012 in Beijing, more than 1000 haploids derived from 'Yu87-1/Zheng58' were screened for anther emergence score (AES). A total of 120 haploids with the most pronounced male fertility (AES of 1) were chosen to form the F_1 haploid mapping population for HMF. Segregation distortion of 130 markers was examined within the 120 haploids and five loci showed strong SD. The five loci were detected for SD in the F_1 selected haploid population of 'Yu87-1/Zheng58' in the winter of 2012 in Hainan (94 haploids with AES of 1 out of 765 haploids) and in the summer of 2013 in Beijing (94 haploids with AES of 1 out of 634 haploids). Only four loci showed strong SD across all the three environments. In the summer of 2013 in Beijing, the four loci were screened for SD in the F_1 selected haploid population of '4F1/Zheng58' (94 haploids with AES of 1 out of 934 haploids).

Development of molecular markers

To increase the resolution of the genetic analysis and for fine mapping of *qhmf4*, novel markers were designed in and near bin 6.07. The sequences of 'Yu87-1' and 'Zheng58' in this region were obtained from Dr. Lai (Jingsheng Lai's Lab, China Agricultural University, Beijing, China). Single-/low-copy sequences were detected by BLAST on NCBI (http://www.ncbi.nlm.nih.gov/) with the high throughput genomic sequence (HTGS) database. BLAST results were surveyed for at least 4 bp indels, recognized as 4 bp gaps in alignments of the two parents. For each indel with at least 150 bp flanking sequence on both sides, Primer 5.0 (http://www.primer-e.com/) was used for primer design based on the following settings: 20±2 nucleotides with 40-60% GC content, no secondary structure, and no consecutive tracts of a single nucleotide (Yang et al. 2010). Finally, 13 insertion/deletion (InDel) markers (Table S1) were developed and showed polymorphic for the two parental lines to cover this region.

Strategy for fine mapping of *qhmf4*

High-density molecular markers, a sufficient number of recombinants within the QTL region, and precise phenotype of each recombinant are indispensable for fine mapping. In this study, a robust progeny test strategy was used to accurately evaluate the HMF phenotype of all recombinants (Fig. 1). A large H_1BC_2 population (N = 8168 = 5000 + 3168) was developed through a haploid step and two backcross steps to identify a sufficient number of recombinants in the target region. All the 48 detected recombinants were backcrossed to 'Zheng58' to develop H_1BC_3 progeny and genotyped by the existing as well as newly designed markers to classify the recombinants to different types. In the H_1BC_3 generation, all individuals were genotyped by the appropriate flanking markers to identify individuals

with heterozygous ‘Yu87-1/Zheng58’ genotype in the *qhmf4* region. The selected H_1BC_3 individuals were crossed with CAU5 to induce haploids. Haploids identified by seed color were genotyped with appropriate markers and divided into two subgroups, class A with the ‘Yu87-1’ donor segment or class B without the ‘Yu87-1’ donor segment. For each group, at least 150 haploids were grown in the field and scored for HMF. A two-way ANOVA was used to test the difference of AES between the two subgroups. If there was a significant difference ($P > 0.05$) between the two subgroups for AES, the QTL-*qhmf4* was assumed to be present in the ‘Yu87-1’ donor segment. If there was no significant difference ($P > 0.05$) between the two subgroups, the QTL-*qhmf4* was assumed to be absent in the ‘Yu87-1’ donor segment.

RESULTS

Assessment of HMF

The inbred lines ‘Yu87-1’ and ‘4F1’ showed high HMF. More than 90% of ‘Yu87-1’ and ‘4F1’ haploid plants showed anther emergence, and the AES of ‘Yu87-1’ haploids was 0.96, which was significantly ($P<0.05$) higher than the AES of ‘4F1’ haploids (0.80) (Fig. 2a, b). In contrast, only ~40% of ‘Zheng58’ haploids showed anther emergence, and most of them exhibited less than 5% anthers emerged on a tassel (Fig. 2c). Haploids derived from the F_1 of ‘Yu87-1/Zheng58’ were also evaluated for their male fertility. About 50% haploids showed variable levels of HMF, ranging from high (rating of 5, 22.14%), intermediate (ratings of 3 and 4, 7.14%), and low (ratings of 1 and 2, 20.37%) (Fig. 2d).

Verifying the method of SD mapping in haploid population

In the 88 shortest haploids, of the 14 markers tested, 4 markers showed strong SD ($P<0.01$) based on χ^2 tests with Bonferroni correction (Table S2). All the four SD markers are around chromosomal bins 5.05/5.06, and umc2164 showed the strongest SD (Table 2). In contrast, the other ten markers segregated indistinguishable from a 1:1 ratio.

Construction of the linkage map

In this study, a total of 563 SSR markers were screened for polymorphisms between ‘Zheng58’ and ‘Yu87-1’. In total, 132 polymorphic markers covering the entire maize genome were chosen for this analysis. Two markers, umc1223 and umc2039, could not be placed on any chromosome, were excluded from the linkage mapping. The remaining 130 of the polymorphic markers were used to develop the genetic linkage map using MAPMAKER 3.0 (Lander et al. 1987). The whole length of the genetic linkage map was 1484.5 cM with an average distance of 11.4 cM between adjacent markers (Fig. S1).

Mapping of QTL for HMF

Genotyping of the 120 haploids selected for high HMF in Beijing 2012 with the 130 SSR markers identified five loci with strong SD, located on chromosomes 1, 3, 4, 6 and 10 (Table 2, S3). SD can be caused by different factors, including sampling. To overcome false positives, the F_1 haploid population of ‘Yu87-1/Zheng58’ was screened for AES in two additional environments in Hainan (2012 winter season) and Beijing (2013 summer season). Four loci showed significant SD across all three environments (Fig. 3; Table 2, S3-S5). For one QTL located in bin 1.11 (*qhmf1*), the HMF increasing allele came from inbred line ‘Zheng58’. For the other three QTL, located in bins 3.06, 4.02/03, and

6.07 (*qhmf2, qhmf3, and qhmf4*), the HMF increasing alleles were provided by 'Yu87-1'. All four QTL were also screened in the second F_1 haploid population, '4F1/Zheng58', and three QTL, *qhmf1*, *qhmf2*, and *qhmf4* showed strong deviation (Fig. 3; Table S6). SD analysis defined an interval of 61.2 cM (from umc1306 to umc1064) for the *qhmf1* locus, an interval of 42 cM (from bnlg1035 to umc1528) for *qhmf2*, an interval of 7.3 cM (from umc1294 to umc2082) for *qhmf3*, and an interval of 31.0 cM (from umc2170 to IND169) for *qhmf4*.

Fine mapping of *qhmf4*

According to the initial mapping of HMF, we decided to conduct the fine mapping of QTL *qhmf4*, which was detected and showed the strongest SD in the two populations. To narrow down the region of *qhmf4*, 5000 H_1BC_2 plants grown during the winter of 2013 in Hainan and genotyped with flanking markers umc2170 and IND169. A total of 39 recombinants were found and further genotyped with nine newly designed markers (IND161, IND162, IND163, IND164, IND165, IND1658, IND166, IND167, and IND168) (Table S1), which were divided into ten (R1-R10) types of recombinant due to their genotypes (Fig. 4). All 39 H_1BC_2 recombinants were backcrossed to 'Zheng58' to develop the H_1BC_3 progeny. In the summer of 2014, individuals with heterozygous 'Yu87-1/Zheng58' genotype in the *qhmf4* region from the 39 H_1BC_3 progenies were crossed with inducer CAU5. Haploids selected by seed color marker *R1-nj* (Nanda and Chase, 1966) were divided into two subgroups, class A or class B (with or without the 'Yu87-1' donor segment) by appropriate flanking markers, and screened for AES in the winter of 2014. A progeny test was conducted by a two-way ANOVA ($P<0.05$) to infer the phenotypes of the 39 H_1BC_2 recombinants. Type R6 recombinants showed a significant different ($P<0.05$) AES between the two subgroups, which indicated that the 'Yu87-1' donor segments contained *qhmf4*. Both types R9 and R10 recombinants showed no significantly different ($P<0.05$) AES between the two subgroups, which suggested that the 'Yu87-1' donor segments did not harbor *qhmf4*. Types R6 and R10 indicated that *qhmf4* was upstream of IND167, and type R9 supported that *qhmf4* was downstream of IND1658. The analysis of the 39 recombinants narrowed down *qhmf4* locus between markers IND11658 and IND167 with a distance of about 1.1 Mb based on the maize genome sequence (http://www.maizesequence.org). By analyzing type R1 to R6 recombinants, AES of haploids in genotype class A (with the HMF genotype from 'Yu87-1' at *qhmf4*), which was 0.25, was significantly ($P<0.01$) higher than AES of haploids in genotype class B (lacking the HMF genotype from 'Yu87-1' at

qhmf4), which was 0.08 (Fig. 5). The standard deviation of HMF in genotype class A is also higher than that in genotype class B.

In the summer of 2015, another 3168 H_1BC_2 plants were genotyped with the flanking markers IND1658 and IND167, and nine new recombinants were identified. These nine H_1BC_2 recombinants were further classified into two recombinant types (R11-R12) by genotyping with four markers (IND166, IND1662, IND1666, and IND1668). Each H_1BC_2 recombinant was backcrossed to 'Zheng58' to produce the H_1BC_3 mapping population. In the winter of 2015, individuals with heterozygous 'Yu87-1/Zheng58' genotype in the *qhmf4* region from the H_1BC_3 mapping population were induced by inducer CAU5 to produce haploids. The same progeny test strategy was used to infer the phenotypes of the nine H_1BC_2 recombinants. Type R11 recombinants indicated that *qhmf4* was located upstream of the marker IND1668 and type R12 recombinants showed that *qhmf4* was downstream of IND166 (Fig. 4). Thus, *qhmf4* was mapped between markers IND166 and IND1668 with a physical distance of ~800 kb.

DISCUSSION

Strategies for initial mapping of HMF

QTL mapping is generally conducted in unselected populations, but it can also be done in selected populations. Genotyping of selected individuals can be used to detect QTL and linked markers via detecting SD (Darvasi and Soller, 1992). QTL mapping by SD loci detection can be used in breeding populations. In a breeding program for a target trait such as drought tolerance, breeders may only have individuals selected for the target trait. Cui et al. (2015) detected QTL for grain yield by SD loci detection in two rice breeding populations selected for high grain yield. Detecting SD loci in selected populations focusing on the individuals with desired phenotype is thus a low-cost and efficient method for QTL mapping and can be better integrated into plant breeding programs (Navabi et al. 2009).

In the present study, SD loci detection was used for mapping of HMF QTL in selected haploid populations. Only 120 out of more than 1000 haploids derived from 'Yu87-1/Zheng58' were genotyped with 130 SSR markers. SD loci were detected in two additional environments to reduce the number of false positive loci. Four QTL related to HMF were identified on chromosomes 1, 3, 4, and 6. The loci *qhmf1*, *qhmf2* and *qhmf4* were also detected in the haploid population of '4F1/Zheng58'. Except for *qhmf1*, the source of HMF alleles was 'Yu87-1'. This indicates that both parents contain beneficial alleles for HMF, but the parent with high HMF has more beneficial alleles compared to the parent with low HMF. Stacking of the four loci would result in an improvement of HMF.

Strategies for fine mapping of *qhmf4*

There are numerous studies about QTL fine mapping in maize. How to acquire the accurate phenotypic value is one of the most important issues in QTL mapping (Zhang et al. 2012). Lander et al. (1989) used a large number of progenies to evaluate the phenotype more accurately. The mean value of the progenies reduces the statistic error and the effect of genetic background (Yang et al. 2010; Dong et al. 2013). Fine mapping by progeny test has been reported in several studies (Yang et al. 2010; Zhang et al. 2012; Dong et al. 2013; Liu et al. 2015). Yang et al. (2010) used a progeny test to obtain the accurate evaluation of resistance to *Gibberella* stalk rot for fine mapping of the major QTL *qR*fg*1* in maize. Zhang et al. (2012) conducted the fine mapping of the minor QTL *qR*fg*2* by a progeny test using a larger progeny population. The increased progeny size reduces the statistical error and reveals the genetic effect of *qRfg2*.

In this study, a progeny test was used to get accurate phenotypes for all recombinants. By planting haploids derived from H_1BC_3 progeny with heterozygous 'Yu87-1/Zheng58' genotype from these recombinants and by genotyping and phenotyping the haploids, we accurately estimated the AES for the two subgroups with or without 'Yu87-1' donor segment in the *qhmf4* region for fine mapping. The *qhmf4* has been narrowed down to ~800 kb by the analysis of 48 recombinants using the progeny test. Another highlight for fine mapping is the use of a haploid backcross population. To develop the fine mapping population, haploids with high HMF of 'Yu87-1/Zheng58' were backcrossed to 'Zheng58', which confirms that the backcross individuals contain the QTL of HMF. This shows that HMF can be efficiently increased by phenotypic in a backcross procedure. Marker-assisted backcrossing is preferable for efficient fore- and background selection (Francia et al. 2005).

Putative candidate genes for HMF

Identification of candidate genes for HMF is essential for understanding the underlying genetic mechanisms. Previous studies showed that the leaves of male fertile haploids are still haploids, which indicates that the process of somatic and sexual cell doubling is not synchronized (Kleiber et al. 2012; Wu et al. 2014). Genes related to meiosis may be involved in haploid genome doubling. The maize mutant *fdr1* showed high HMF due to first division restitution (Sugihara et al. 2013). *fdr1* only affects male fertility in haploids but not in diploids. In Arabidopsis, haploids can be fertile and generate homogeneous lines by combining three mutations: *spo11-1,* which abolishes recombination, *rec8,* which with *spo11-1* leads to sister chromatids segregation into two balanced groups *at anaphase I*, and *osd1,* which leads to exit from meiosis before meiosis II (Cifuentes et al. 2013). This genotype called MiMe results in a meiosis replaced by a mitotic-like division.

In this study, the HMF QTL *qhmf4* was narrowed down to an ~800 kb region and the corresponding B73 sequence was retrieved from Gramene (http://www.gramene.org/). Gene prediction by FEGNESH 2.6 has revealed ~22 putative genes in the *qhmf4* region. The gene absence of first division1 (*afd1*), a maize *rec8* homolog, affecting sister chromatid cohesion (SSC) is the most likely candidate gene. AFD1 protein is necessary for axial element elongation and controls homologous pairing (Golubovskaya et al. 2006). In the mutant of *afd1*, meiotic first (reduction) division is replaced by a single mitotic (equational) division. While *afd1* is a good candidate for *qhmf4,* further experiments such as genetic mapping by screening new recombinants within the *qhmf4* locus or gene expression and transgenic testing studies must be performed to restrict the *qhmf4* locus to a single gene.

Implications for breeding of HMF development

Haploid male fertility is a quantitative trait controlled by several loci and *qhmf4* is a key QTL for HMF. In the H_1BC_2 population, haploids with the *qhmf4* genotype from 'Yu87-1' had a large variance for HMF, which can be explained by the interaction between *qhmf4* and other QTL related to HMF. HMF can be improved by targeted parental recombination and selection. To reduce the effort for phenotypic selection of HMF, which is laborious and time-consuming, marker-based methods such as MAS could be used to select individuals with favorable alleles for HMF to increase HMF in breeding materials (Francia et al. 2005). Markers closely linked to HMF QTL can be used for MAS to improve HMF. In the present study, the QTL *qhmf4* has narrowed down to an ~800 kb region and a number of markers have been designed around the *qhmf4* region. The *qhmf4* region can be quickly fixed by MAS, followed phenotypic selection within this genotype class for high HMF.

Kleiber et al. (2012) reported that artificial genome doubling is not necessary for DH line production from germplasm with high HMF. For efficient DH line production, haploids with high HMF identified by MAS will be duplicated by spontaneous genome doubling. Haploids with poor HMF identified by MAS will be doubled by artificial genome doubling. In conclusion, our study provides a starting point for understanding the genetics of HMF. More recombinants are required for the fine mapping of *qhmf4*. Understanding the biological mechanisms of HMF will benefit the development of high HMF breeding materials and increase the efficiency of DH technology.

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version.

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Fig. 1 Strategy for fine mapping of the *qhmf4* locus

[a] HMF: haploid male fertility

[b] SD: segregation distortion

[c] AES: anther emergence score

[d] class A: with the 'Yu87-1' donor segment

class B: without the 'Yu87-1' donor segment

Fig. 2 Haploid male fertility (HMF) for the three parental lines and F_1 plants. a High HMF parental line 'Yu87-1'; b High HMF parental line '4F1'; c Low HMF parental line 'Zheng58'; d The 'Yu87-1/Zheng58' F_1 hybrids

Fig. 3 The position, direction, and segregation distortion (SD) level of QTL for haploid male fertility (HMF) in the two selected haploid populations

Fig. 4 Fine mapping of *qhmf4* using a progeny test strategy. The left side of the figure is the genetic structure of each recombinant. The progeny with heterozygous 'Yu87-1/Zheng58' alleles in the *qhmf4* region for each type of recombinant was divided into two subgroups: class A with 'Yu87-1' allele and class B without 'Yu87-1' allele. A two-way ANOVA is used to compare the difference of AES between the two subgroups. Significant differences ($P > 0.05$) between the two subgroups indicate that *qhmf4* is present in the donor segment. No significant differences ($P > 0.05$) between the two subgroups indicate that the *qhmf4* is absent in the donor segment

Fig. 5 Effect of the *qhmf4* region on haploid male fertility (HMF) in maize by the analysis of type R1 to R6 recombinants. Class A with the HMF genotype from 'Yu87-1' at *qhmf4;* class B without the HMF genotype from 'Yu87-1' at *qhmf4*

Table 1 Description of different levels of anther emergence for haploid male fertility (HMF)

Rating scale	Anther emergence score (AES)	Description
0	0	No anthers emerged on a tassel
1	0.2	Less than 5% anthers emerged on a tassel
2	0.4	About 6-20% anthers emerged on a tassel
3	0.6	About 21-50% anthers emerged on a tassel
4	0.8	About 51-75% anthers emerged on a tassel
5	1	More than 75% anthers emerged on tassel

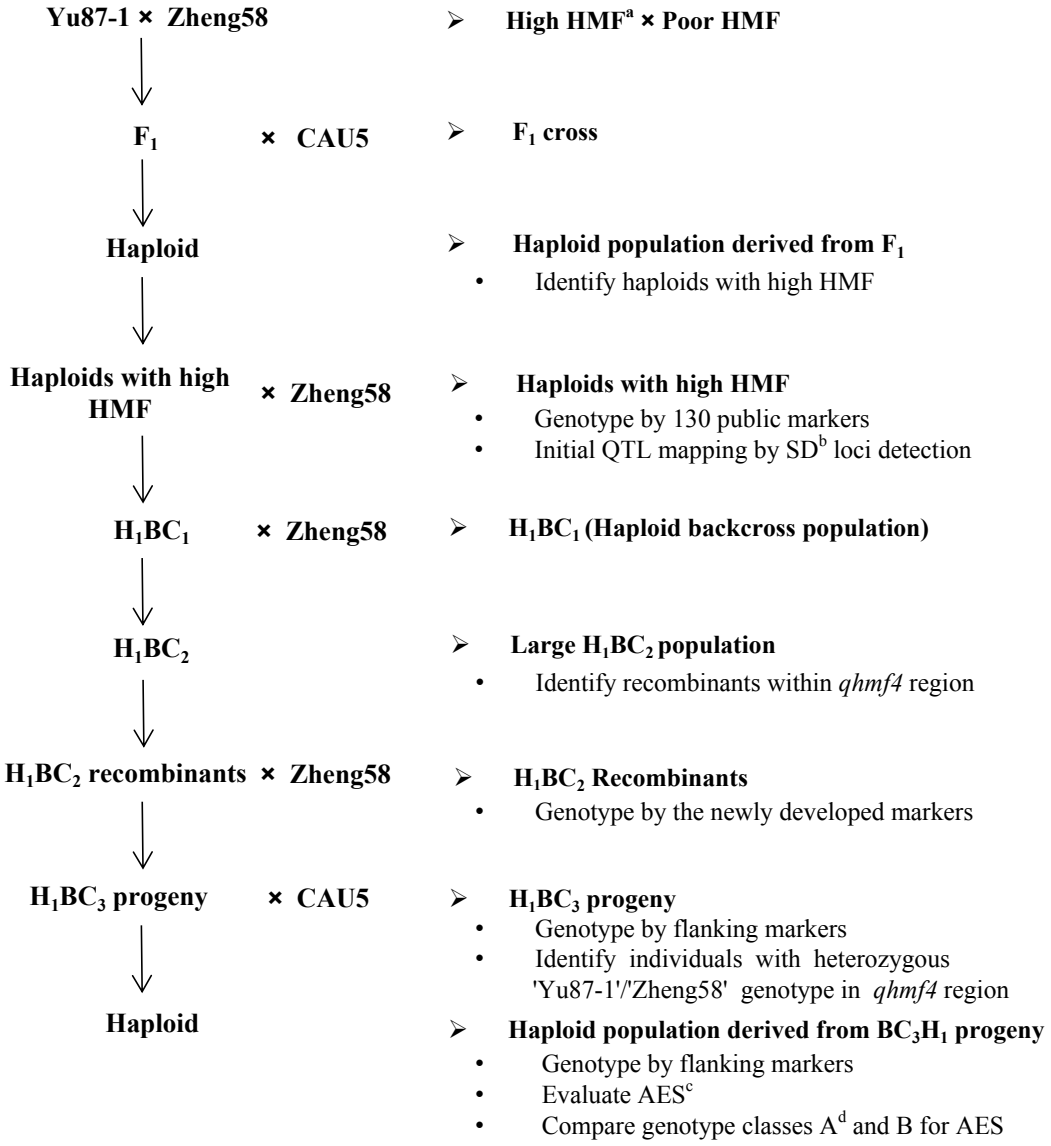
Table 2 Analysis of the five segregation distortion (SD) loci in the selected haploid population of 'Yu87-1/Zheng58' detected for haploid male fertility (summer of 2012 in Beijing)

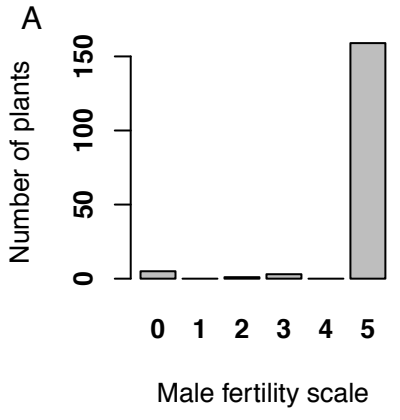
Position		Marker	Genotype[a]		χ^2	*P* value	Adjusted *P* value[b]
Chromosome	Bins		A	B			
1	1.07	umc1128	42	78	10.80	0.0010	0.1320
1	1.09	umc1306	40	80	13.33	0.0003	0.0339
1	1.11	umc1553	38	82	16.13	0.0001	0.0077
1	1.11	umc1064	46	74	6.53	0.0106	1.0000
1	1.12	umc1797	49	71	4.03	0.0446	1.0000
3	3.05	bnlg1035	78	42	10.80	0.0010	0.1320
3	3.06/7	umc1949	82	38	16.13	0.0001	0.0077
3	3.07	umc2050	82	38	16.13	0.0001	0.0077
3	3.07	umc1528	80	40	13.33	0.0003	0.0339
3	3.08	phi046	79	41	12.03	0.0005	0.0679
4	4.01	phi072	63	57	0.30	0.5839	1.0000
4	4.02	umc1294	80	40	13.33	0.0003	0.0339
4	4.03	umc2082	80	40	13.33	0.0003	0.0339
4	4.05	phi079	74	46	6.53	0.0106	1.0000
4	4.05	bnlg1217	73	45	6.64	0.0099	1.0000
6	6.06	umc2170	80	40	13.33	0.0003	0.0339
6	6.07	bnlg1740	80	40	13.33	0.0003	0.0339
6	6.07/8	bnlg1521	86	34	22.53	<0.0001	0.0003
6	6.08	IND169	82	38	16.13	0.0001	0.0077
10	10.01	umc1291	71	49	4.03	0.0446	1.0000
10	10.02	bnlg1451	80	39	14.13	0.0002	0.0222
10	10.02	umc1432	83	37	17.63	<0.0001	0.0035
10	10.02	phi059	75	45	7.50	0.0062	0.8021
10	10.02	umc1576	68	52	2.13	0.1441	1.0000

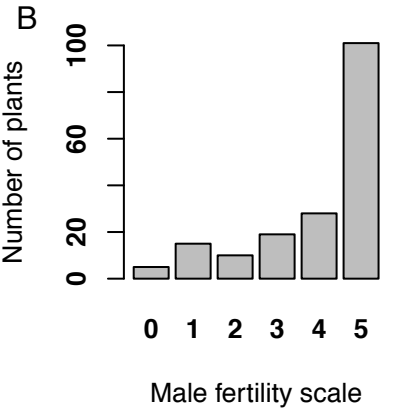
[a] A: 'Yu87-1' allele

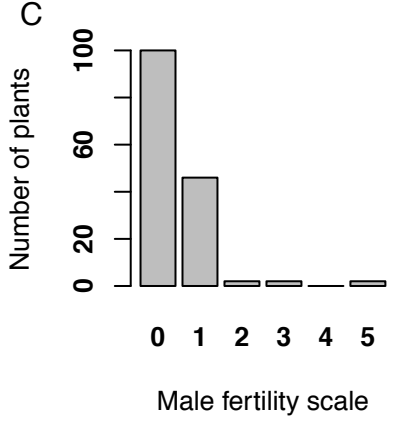
B: 'Zheng58' allele

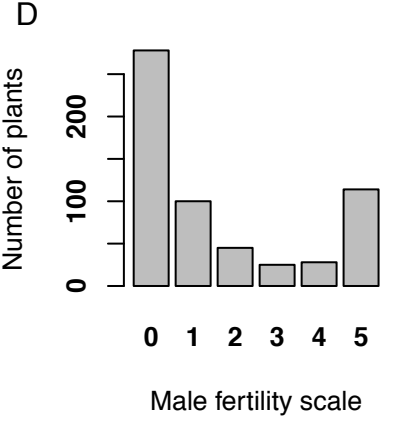
[b] Adjusted *P* value was obtained by Bonferroni correction for multiple testing

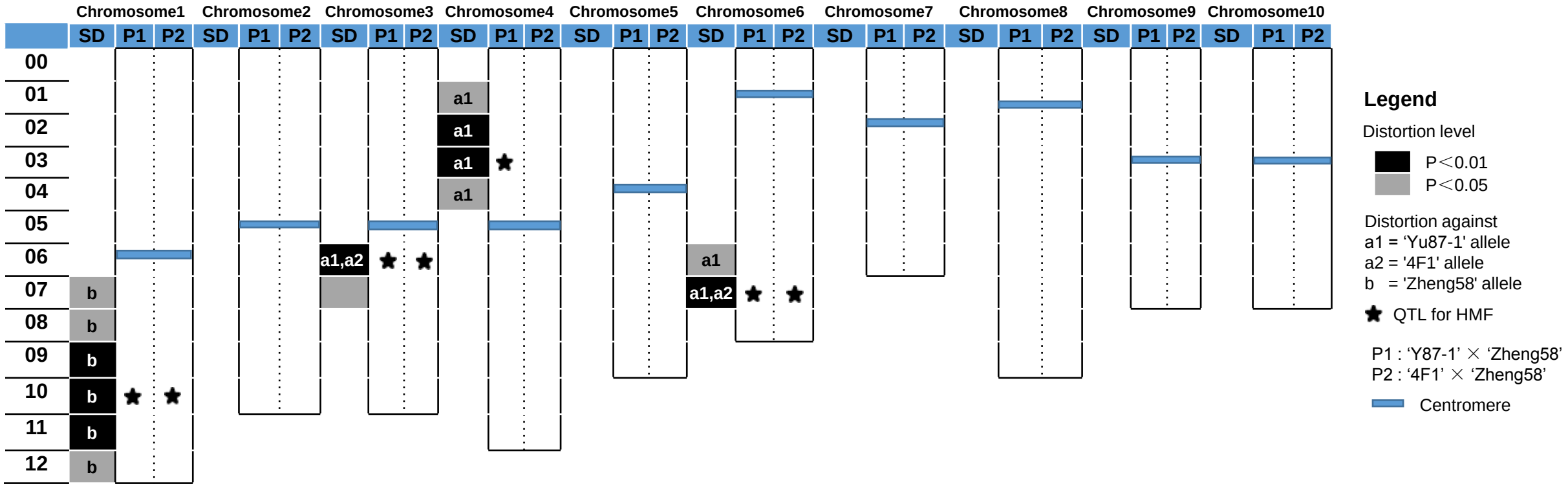

Yu87-1 × Zheng58
High HMF[a] × Poor HMF
F1 × CAU5
F1 cross
Haploid
Haploid population derived from F1
Identify haploids with high HMF
Haploids with high HMF × Zheng58
Haploids with high HMF
Genotype by 130 public markers
Initial QTL mapping by SD[b] loci detection
H1BC1 × Zheng58
H1BC1 (Haploid backcross population)
H1BC2
Large H1BC2 population
Identify recombinants within qhmf4 region
H1BC2 recombinants × Zheng58
H1BC2 Recombinants
Genotype by the newly developed markers
H1BC3 progeny × CAU5
H1BC3 progeny
Genotype by flanking markers
Identify individuals with heterozygous 'Yu87-1'/'Zheng58' genotype in qhmf4 region
Haploid
Haploid population derived from BC3H1 progeny
Genotype by flanking markers
Evaluate AES[c]
Compare genotype classes A[d] and B for AES

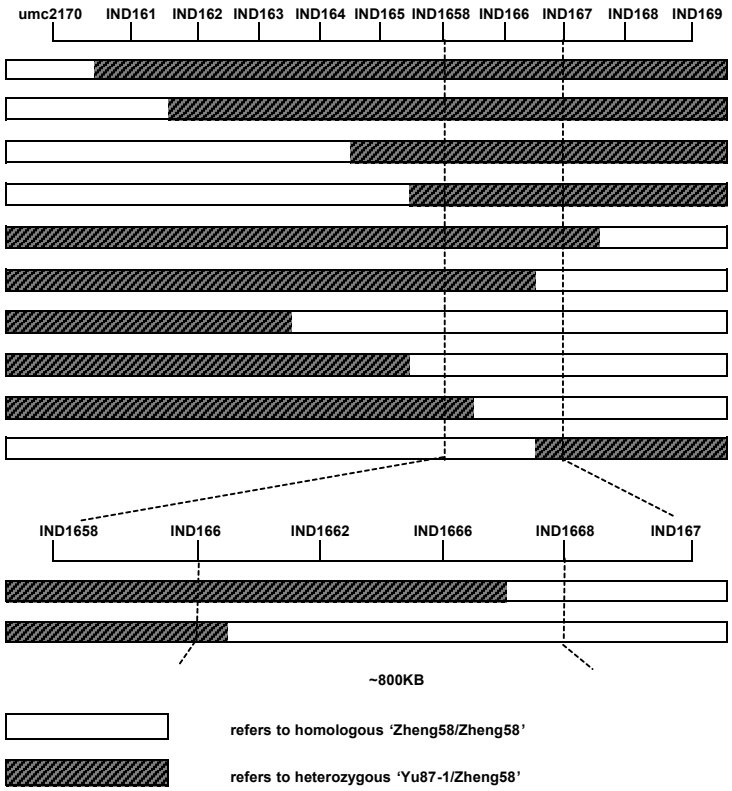

A
Number of plants
0
50
100
150
0 1 2 3 4 5
Male fertility scale


B
Number of plants
0
20
60
100
0 1 2 3 4 5
Male fertility scale


C
Number of plants
0
20
60
100
0 1 2 3 4 5
Male fertility scale


D
Number of plants
0
100
200
0 1 2 3 4 5
Male fertility scale


Chromosome1
Chromosome2
Chromosome3
Chromosome4
Chromosome5
Chromosome6
Chromosome7
Chromosome8
Chromosome9
Chromosome10
SD
P1
P2
00
01
02
03
04
05
06
07
08
09
10
11
12
a1
a1
a1
a1
a1,a2
a1
a1,a2
b
b
b
b
b
b
Legend
Distortion level
P＜0.01
P＜0.05
Distortion against
a1 = 'Yu87-1' allele
a2 = '4F1' allele
b = 'Zheng58' allele
QTL for HMF
P1 : 'Y87-1' × 'Zheng58'
P2 : '4F1' × 'Zheng58'
Centromere



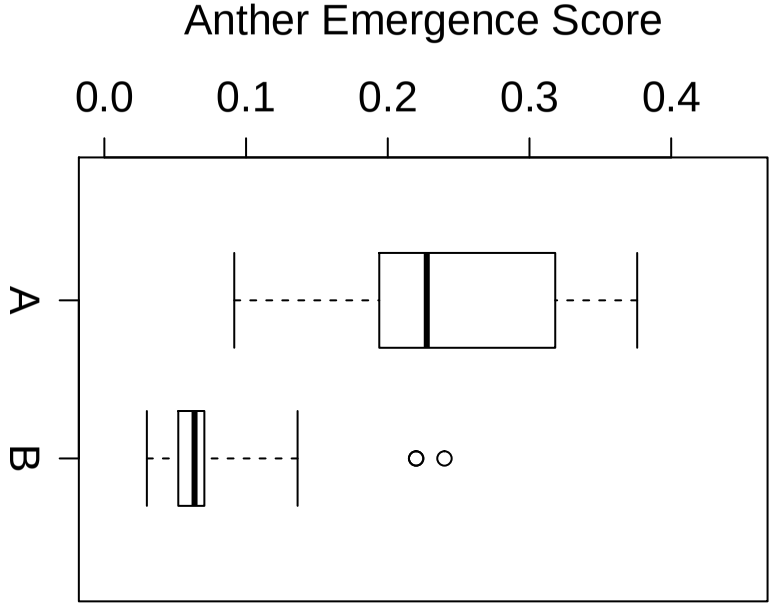
Type of recombinant	No. of recombinants	No. of progeny[a]	AES[b] of BC_3H_1 haploid		P-Value	Inference[d]
			A[c]	B		
R1	4	1362	0.24	0.06	0.003**	Y
R2	5	1713	0.24	0.13	0.009**	Y
R3	2	704	0.26	0.07	0.040*	Y
R4	5	1733	0.24	0.11	0.007**	Y
R5	4	1328	0.20	0.05	0.003**	Y
R6	5	1741	0.25	0.06	0.013*	Y
R7	3	1105	0.08	0.10	0.303	N
R8	5	1782	0.06	0.10	0.105	N
R9	3	1071	0.06	0.05	0.672	N
R10	3	1038	0.04	0.07	0.107	N
R11	5	1927	0.20	0.07	0.007**	Y
R12	4	1596	0.04	0.04	0.574	N

[a] Total number of progeny derived from the same recombinant type

[b] AES: anther emergence score

[c] A: haploids with 'Yu87-1' allele
B: haploids with 'Zheng58' allele

[d] Y: the 'Yu87-1' donor segments harbors the QTL *qhmf4*
N: the 'Yu87-1' donor segments does not harbor the QTL *qhmf4*


Anther Emergence Score
0.0
0.1
0.2
0.3
0.4
A
B