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ORIGINAL PAPER

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Validation of *Dwarf8* polymorphisms associated with flowering time in elite European inbred lines of maize (*Zea mays* L.)

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Abstract The timing of transition from vegetative growth to flowering is important in nature as well as in agriculture. One of several pathways influencing this transition in plants is the gibberellin (GA) pathway. In maize (*Zea mays* L.), the *Dwarf8* (*D8*) gene has been identified as an orthologue of the gibberellic acid-insensitive (*GAI*) gene, a negative regulator of GA response in *Arabidopsis*. Nine intragenic polymorphisms in *D8* have been linked with variation in flowering time of maize. We tested the general applicability of these polymorphisms as functional markers in an independent set of inbred lines. Single nucleotide primer extension (SNuPe) and gel-based indel markers were developed, and a set of 71 elite European inbred lines were phenotyped for flowering time and plant height across four environments. To control for population structure, we genotyped the plant material with 55 simple sequence repeat markers evenly distributed across the genome. When population structure was ignored, six of the nine *D8* polymorphisms were significantly associated with flowering time and none with plant height. However, when population structure was taken into consideration, an association with flowering time was only detected in a single environment, whereas an association across environments was identified between a 2-bp indel in the promoter region and plant height. As the number of lines with different haplotypes within subpopulations was a limiting factor in the analysis, *D8* alleles would need to be compared in isogenic backgrounds for a reliable estimation of allelic effects.

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Introduction

In nature, the transition from vegetative to reproductive growth needs to be controlled in order to prevent flowering under unfavourable conditions. In crop plants, flowering time is an important trait that enables varieties to adapt to different latitudes and cropping seasons.

In *Arabidopsis*, the natural genetic variation for flowering time between accessions from different parts of the world has enabled a large number of flowering time genes to be identified and isolated (Mouradov et al. 2002; Putterill et al. 2004; Simpson and Dean 2002). Intragenic polymorphisms with an effect on flowering time have also been identified in *Arabidopsis* genes (Johanson et al. 2000; Gazzani et al. 2003; Le Corre et al. 2002; Olsen et al. 2004). Flowering time genes are integrated in several pathways, including the gibberellin (GA) pathway, and all act on relatively few floral pathway integrator genes that in turn regulate the expression of floral meristem identity genes.

Negative regulators of the GA response have been identified and cloned from several plant species. In *Arabidopsis*, the gibberellic acid-insensitive (*GAI*) gene was cloned and characterized as a transcription factor that negatively regulates gibberellin responses. A 51-bp deletion in the highly conserved, N-terminal DELLA domain (Silverstone et al. 1998) of *GAI* was identified as a dominant gain-of-function mutant (*gai*) with a dwarf phenotype caused by a reduced GA response (Peng et al. 1997). Several truncated *gai*-derivative alleles generated by γ-irradiation (Peng and Harberd 1993) were found to be recessive, loss-of-function mutations resulting in plants of normal height (Peng et al. 1997). The ''green

revolution" genes *Reduced height* (*Rht*) in wheat, *Dwarf8* (*D8*) in maize, *Slender1* (*SLN1*) in barley, and *Slender1* (*SLN1*) in rice are all functional orthologues of *Arabidopsis GAI* (Chandler et al. 2002; Ikeda et al. 2001; Peng et al. 1999). For all of these *GAI* orthologues, deletions in the DELLA domain result in dominant dwarf phenotypes.

Association analysis has revealed a relationship between maize *D8* polymorphisms and flowering time, but not plant height (Thornsberry et al. 2001). *D8* was sequenced for 92 diverse maize inbred lines, consisting of Stiff Stalk, non-Stiff Stalks and tropical and semi-tropical lines. While the DELLA domain was conserved across all inbreds, several polymorphisms were identified in the 5′-untranslated region (UTR) and the transcribed region. Association analysis including population structure (Pritchard et al. 2000a), identified nine of these polymorphisms to be significantly associated with flowering time. One of these polymorphisms, a 6-bp deletion near a SH2 domain (Koch et al. 1991; Peng et al. 1999) in the C-terminal region of the open reading frame (ORF), was suggested to affect the activity of the transcription factor. However, linkage disequilibrium prevented the additive effects of individual polymorphisms to be estimated (Thornsberry et al. 2001).

The polymorphisms identified by Thornsberry et al. (2001) can be converted into functional markers (FMs) for the control of flowering time (Andersen and Lübberstedt 2003). FMs are defined as being derived from intragenic polymorphisms causally affecting a trait of interest. Thus, the risk of recombination between marker and trait alleles is eliminated. This has several implications for marker-assisted selection (MAS) as well as for the screening of germplasm for specific alleles. In plant breeding, the use of FMs will enable markers to be used across populations without the risk of recombination events resolving the linkage between marker and quantitative trait locus (QTL) alleles (Lübberstedt et al. 1998).

In order to determine the general applicability of the previously identified *D8* polymorphisms affecting flowering time as FMs across plant populations, we evaluated the associations identified by Thornsberry et al. (2001) in a set of European maize inbred lines. The aim of our investigation was to (1) develop molecular marker assays for the nine *D8* polymorphisms reported by Thornsberry et al. (2001) and (2) employ association analysis to validate the associations between *D8* polymorphisms and flowering time or plant height in a population of 71 elite European maize inbred lines.

Materials and methods

Plant materials and phenotypic analysis

A total of 71 European inbred lines, developed by A.E. Melchinger and D. Klein at the University of Hohenheim, Stuttgart, Germany, were included in the analysis. These lines constituted four pools: Flint (F), Flint/Lancaster (L), Iodent and Iodent/Stiff Stalk (P), and Stiff Stalk (S). No public lines were included in the analysis.

Phenotypic data were recorded during the summer of 2003 at four climatically diverse locations in Germany: Hohenheim (HOH), Eckartsweier (EWE), Sünching (SÜN) and Pocking (POC) (Appendix 1). Days to male (DMF) and female flowering (DFF) were recorded from days after sowing, while plant height (PHT) was recorded after flowering when the maximum plant height was reached. The time of flowering was determined at half-way anthesis, i.e., the time at which anthers (DMF) or silks (DFF) were visible on 50% of all plants in a field plot. Compared to the long-term average, the growing season of 2003 was affected by high evaporation in combination with extreme and prolonged heat periods.

Broad-sense heritabilities (h^2) on an entry-mean basis (Fehr 1987) were estimated by the formula $h^2 = \sigma_g^2 \Big/ (\sigma^2/rt + \sigma_{ge}^2/t + \sigma_g^2)$ in which σ_g^2 is genetic variance, σ_{ge}^2 is genotype × environment interaction, σ^2 is experimental error, r is the number of replications and t is the number of test environments. Exact 95% confidence intervals of heritabilities were calculated according to (Knapp and Bridges 1987). The analysis was performed with PLABSTAT (Utz 2003).

DNA extraction

Total genomic DNA was extracted for simple sequence repeat (SSR) analysis by employing a modified CTAB procedure from a bulk of five individual plants of each inbred line (Saghai-Maroof et al. 1984). For the single nucleotide primer extension (SNuPe) assay, DNA was extracted from 20 mg freeze-dried leaf materials using the DNeasy kit (Qiagen, Hilden) according to manufacturer's protocol.

Oligonucleotides

The SSR analysis was performed with 55 publicly available SSR markers (Appendix 2) that provided an even coverage of the maize genome. Primer sequences were obtained from MaizeGDB (http://www.maizegdb.org). The primers for the SSR analysis were synthesized by Sigma-Aldrich (St. Louis, Mo.); one primer of each pair was labelled with Cy5.

Primers for detecting polymorphisms in the *D8* gene associated with flowering time were designed on the basis of the alignment published by Thornsberry et al. (2001). All single nucleotide polymorphisms (SNPs) (positions 677, 1,663, 3,490 and 3,570) and the 2-, 3- and 6-bp indels (positions 1,044, 1,964 and 3,471, respectively) were analysed as SNPs. SNuPe primers were designed so that the 3′ end terminating 1 bp before the polymorphic site. Furthermore, primers were designed

flanking both the putative miniature transposable element (MITE) and the 18-bp deletion in the promoter region. All *D8* primers were synthesized by MWG Biotech (Ebersberg, Germany). Table 1 lists the primer sequences.

SSR analysis

The PCR reactions for SSR analysis were performed in a total volume of 9.75 µl containing 60 ng of template DNA, 154 µ*M* of each dNTP, 256 n *M* of each primer, 2.56 m *M* $MgCl_2$, 1× PCR Buffer (Mg^{2+}-free), and 0.5 U *Taq* polymerase (Invitrogen, Karlsruhe). Thermocycling consisted of an initial denaturation of the template DNA at 94°C for 2.5 min, followed by 33 cycles of 93°C for 45 s, 52–60°C (depending on the primer set) for 45 s and 72°C for 45 s, with a final extension of 10 min at 72°C. For some primer sets, a touchdown step from between 58°C and 64°C down to 55°C was included in the protocol.

The amplified DNA samples were analysed on polyacrylamide gels (Ultra Pure SequaGel-XR; National Diagnostics, Atlanta) on an ALF DNA sequencer (Amersham Biosciences, UK) equipped with ALFWIN v2.00.15 software. The DNA fragments were sized automatically and assigned to specific alleles based on a modified stepwise mutation model (Kimura and Ohta 1978) adjusted to two inbred line controls, Mo17 and B73.

Amplification of SNuPe templates and indel markers

The PCR amplifications of SNuPe templates and indel marker fragments were carried out using the primer combinations listed in Table 1. The PCR reactions occurred in a total of 10 µl containing 50 ng total genomic DNA, 1× HotMaster *Taq* PCR buffer (with Mg^{2+}), 1 µ*M* of forward and reverse primers, 300 µ*M* of each dNTP and 0.25 U of HotMaster *Taq* (Eppendorf, Hamburg). Following an initial denaturation at 96°C for 5 min, a touchdown PCR cycle profile was applied: 12 cycles of 96°C for 20 s, 62–50°C (72–60°C for D8_3for + rev) for 20 s (decreasing with 1°C per cycle) and 65°C for 1 min, followed by 25 cycles of 96°C for 20 s, 50°C (60°C for D8_3for + rev) for 20 s and 65°C for 1 min, with a final extension step at 65°C for 5 min. All PCR reactions were carried out in a MJ Research (Waltham, Mass.) PTC-225 thermocycler. Indel markers were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. The MITE polymorphism was scored on the basis of size differences, while the 18-bp deletion was scored on the basis of the presence/absence of amplified fragments.

SNuPe reactions

Following amplification of the SNuPe templates, 20 U of Exonuclease I (Fermentas, Hanover, Md.) and 4 U of shrimp alkaline phosphatase (SAP) (Promega, Mannheim, Germany) were added to the 10-µl PCR reactions in order to remove primers and dNTPs. The reactions were incubated at 37°C for 15 min followed by enzyme inactivation at 80°C for 15 min. The purified reactions were diluted tenfold with milliQ water, and then 2-µl aliquots of this dilution were used as the templates for the SNuPe reaction. The SNuPe reactions were set up in a total volume of 10 µl and included 4 µl of SNuPe premix (Amersham Biosciences), 200 n *M* of SNP pri-

Table 1 Names, sequences and combinations of primers used for detection of *Dwarf8* polymorphisms

Polymorphism[a,b]	Indel marker and SNuPe template fragments		SNuPe primer	
	Name	Sequence (5′ → 3′)	Name	Sequence (5′ → 3′)
MITE (185)	D8_1for	ACA CTA TCA CCG CTC TAT TG		
	D8_1rev	ACT CTT TCC CTG ACT TCA TT		
C/G SNP (677)	D8_1for		D8_SNP5	AAG GAT TGA TGT TGG TGG CT
	D8_1rev			
18-bp deletion (702)	D8_2for	CCA CCA ACA TCA ATC CTT CT		
	D8_2rev	ATC CAG CCT TCC CTT TAC TC		
2-bp indel (1,044)(C/T SNP)	D8_4for	TTG CAT TGA TCT TGG CTT GG	D8_SNP1	CCT CCC CAC TCG CGA CAT
	D8_4rev	GGG GAA GGA AAG GGT AGG G		
C/T SNP (1,663)	D8_4for		D8_SNP2	GGG AGG GAC CTG CCA GG
	D8_4rev			
3-bp indel (1,964)(C/T SNP)	D8_SNP2		D8_SNP6	CAA GAC GCC GGC GGG AG
	D8_5rev	GAC GAA CGC ACC TTG TAC C		
6-bp indel (3,472)(G/T SNP)	D8_3f(3234)	CGA TGA CAC GGA TGA CGA	D8_SNP7	TCT CGA GGG CGC CGG C
	D8_3r(3679)	AGG CAT TGG AGC CCA GGT		
G/A SNP (3,490)	D8_3f(3234)		D8_SNP3	GCC GGG GAG GCG TCG G
	D8_3r(3679)			
C/G SNP (3,570)	D8_3f(3234)		D8_SNP4	CCG GCA GAT CTG CAA CGT
	D8_3r(3679)			

[a]Numbers in parenthesis denote location of the polymorphisms in the alignment produced by Thornsberry et al. (2001)
[b]Primary PCR fragments were either used directly as indel markers (MITE and 18-bp deletion) or as templates for SNP detection by the SNuPe assay (SNP primers)

mer and 2 µl of purified, diluted DNA template. Individual polymorphisms according to primer name are described in Table 1.

The SNuPe reactions were thermocycled at 96°C for 10 s, 50°C for 5 s and 60°C for 10 s for 25 cycles in a MJ Research PTC-225 thermocycler. The excess ddNTPs were subsequently removed by incubating the reactions with 0.5 U of SAP (Promega) at 37°C for 30 min followed by enzyme inactivation at 80°C for 15 min.

SNP detection by the MegaBACE1000

Five-microlitre aliquots of individual SNuPe reaction samples were added to 4.975 µl loading buffer and 0.025 µl injection marker. Samples were loaded onto the MegaBACE1000 capillary sequencer applying default parameters. Peaks were subsequently detected and scored by the SNP PROFILER software (Amersham Biosciences).

Data analysis

Differences in mean phenotypic values between *D8* haplotypes were determined by single factor ANOVA. Cluster analysis was based on modified Rogers distance (MRD) values (Gower 1966), and the MRD value between two individuals was calculated based on SSR data according to Wright (1978) and Goodman and Stuber (1983). The analyses were carried out with the PLABSOFT software (Maurer et al. 2004), which is implemented as an extension to the statistical software R(Ihaka and Gentleman 1996).

Population structure was inferred from SSR data by using the STRUCTURE ver. 2.0 software (Falush et al. 2003; Pritchard et al. 2000b). This software applies a Bayesian clustering approach to identify subpopulations, each modelled by a characteristic set of allele frequencies which, in this case, were based on genotyping data from 55 microsatellites. The procedure assigns individuals to these populations while simultaneously estimating the population allele frequencies. STRUCTURE produces a Q-matrix that lists the estimated membership coefficients for each individual in each cluster. The ADMIXTURE model was applied, as several individuals in our plant material were derived from crosses between pools; i.e., were of mixed ancestry. A burn-in length of 50,000 followed by 50,000 iterations was used (for further discussion, see the STRUCTURE 2.0 documentation: http://pritch.bsd.uchicago.edu/). The correlation between population structure and phenotypic values as well as between population structure and *D8* polymorphisms were determined by regression—the least-squares method (Searle 1987).

The estimated Q-matrices were used in the subsequent association analysis carried out by logistic regression in the TASSEL ver. 1.0 software (Thornsberry et al. 2001; http://www.maizegenetics.net/bioinformatics/tasselindex.htm). This software applies a logistic regression ratio test to calculate the likelihood of either (1) the candidate gene distribution (in this case, *D8* polymorphisms) being associated with population structure and phenotypic variation or (2) the candidate gene distribution being associated with population structure only. The test statistic (Λ)—the ratio between these two likelihoods—indicates associations between individual polymorphisms and traits (in this case, flowering time and plant height). Furthermore, the general linear model (GLM) analysis in TASSEL, based on the least-squares method (Searle 1987), was employed to identify associations between polymorphisms and markers not taking population structure into account. The *P* value for individual polymorphisms was estimated based on 1,000 permutations of the dataset.

Results

Field data of inbred lines

Days to male flowering were not recorded in POC, while plant height was not recorded in SÜN and POC. In general, plants in EWE were the shortest (148.9 cm) and flowered the earliest (DFF = 74.8 days), while plants in HOH were the tallest (182.7 cm) and flowered the latest (DFF = 81.3 days). Flowering time in POC and SÜN were in general intermediate (DFF = 78.3 and 79.0 days, respectively) relative to EWE and HOH (Table 2). Overall averages for DMF, DFF and PHT across environments were 77.0 days, 78.4 days, and 165.8 cm, respectively.

On average, Flint lines were shorter than other lines, and Flint lines and Flint/Lancaster lines flowered earlier than Stiff Stalk, Iodent and Iodent/Stiff Stalk lines (Ta-

Table 2 Summary of phenotypic data[a] at four locations[b] for 71 European inbred maize lines

	EWE			HOH			SÜN		POC
	DMF	DFF	PHT	DMF	DFF	PHT	DMF	DFF	DFF
Mean	74.4 days	74.8 days	148.9 cm	79.3 days	81.3 days	182.7 cm	77.2 days	79.0 days	78.3 days
Standard deviation	4.12	4.50	16.22	3.87	3.95	12.76	3.45	3.29	3.93

[a]Days to male flowering (DMF) and days to female flowering (DFF) were the period between sowing and flowering, while plant height (PHT) was recorded after flowering

[b]HOH, Hohenheim; EWE, Eckartsweier; SÜN, Sünching; POC, Pocking

Table 3 Mean and minimum/maximum phenotypic values for individual traits within pools, across environments

	DMF	DFF	PHT
Mean values (min/max)			
Flint	73.8 days (68.8/83.1)	75.9 days (69.9/84.8)	162.1 cm (142.0/193.9)
Flint/Lancaster	74.9 days (70.7/78.9)	76.2 days (72.0/80.3)	166.0 cm (151.5/181.8)
Iodent and Iodent/Stiff Stalk	79.0 days (72.6/87.2)	80.3 days (74.7/87.6)	166.0 cm (143.1/189.4)
Stiff Stalk	78.4 days (73.8/82.0)	79.4 days (74.3/82.5)	168.4 cm (144.8/201.7)
Variance components[a]			
Environment	6.01**	7.16**	569.63**
Genotype	13.03**	13.46**	136.23**
Genotype by environment	0.90**	1.44**	52.65**
Error	1.35	1.22	48.35
Heritability[a]	0.961	0.963	0.780
95% Confidence interval	0.943; 0.973	0.947; 0.974	0.657; 0.859

**Significant at $P \leq 0.01$

[a]Variance components and heritability estimates are given for days to male flowering (DMF), days to female flowering (DFF) and plant height (PHT)

ble 3). Heritabilities for DMF, DFF and PHT were 0.96, 0.96 and 0.78, respectively. Variance components for DMF, DFF, and PHT were significant ($P < 0.01$) for genotype, environment and genotype-by-environment effects.

Cluster analysis and population structure

Of the total number of genotype-SSR marker combinations (71 × 55), 93.4% were homozygous, 3.5% heterozygous and 3.1% were missing.

Cluster analysis alloted the lines from the four pools, Flint (F), Flint/Lancaster (L), Iodent and Iodent/Stiff Stalk (P), and Stiff Stalk (S), two distinct clusters, one consisting of the F and L lines and the second consisting of the P and S lines (Fig. 1). Within the P/S cluster, two distinct subgroups were identified, one subgroup included only P lines and the other included all of the S lines and three P lines. No clear distinction of subgroups was found in the F/L cluster.

This clustering was supported by the population structure analysis carried out with STRUCTURE, which determined that the optimal number of subpopulations was three ($K = 3$; Figs. 3, 4). Specifying two subpopulations ($K = 2$) led to a distinct decrease in the likelihood value, indicating that three subpopulations were more likely than two, while specifying a K greater than three led to only minimal increases in the likelihood value, indicating the absence of additional subpopulations. Population structure explained 32%, 24% and 3% of the variation in DMF, DFF, and PHT, respectively, as well as up to 17% of the frequency distribution of individual *D8* polymorphisms. While the MITE and ORF polymorphisms contributed most to the correlation between population structure and *D8* allele frequency distribution in subpopulation 1 (Flint and Flint/Lancaster), the remaining promoter polymorphisms contributed most to the correlation between population structure and *D8* allele frequency distribution in subpopulation 2 (Iodent and Iodent/Stiff Stalk).

Analysis of *Dwarf8* polymorphisms

Eight haplotypes were revealed (Fig. 2, Appendix 3) following the genotyping of the 71 inbred lines for nine polymorphisms in the *D8* gene sequence that had previously been shown to be associated with flowering time (Thornsberry et al. 2001). One haplotype (haplotype 7) was most prominent and present in 42 lines across all four pools. In contrast, haplotypes 3 and 5 were represented by only one line each. A larger than expected (1.5 kb) MITE fragment was observed in four lines (haplotype 8).

At least five recombination events have to have occurred to explain the eight haplotypes. The difference in recombination events between the promoter and open reading frame (ORF) region is significant ($P < 0.05$) as determined by a χ^2-test. ANOVA, not including haplotypes 3 and 5 consisting of only one genotype each, indicated significant differences in flowering time ($P < 0.01$), but not in plant height, between *D8* haplotypes.

Association analysis: general linear model

The GLM analysis without accounting for population structure was performed between *D8* polymorphisms and phenotypic values across environments. The MITE (position 185), the 18-bp deletion (position 702) and the polymorphisms in the ORF (positions 1,964, 3,472, 3,490 and 3,570) were all significantly associated with flowering time (DMF and DFF; $P < 0.001$) (Table 4). An association between the SNP at position 1,663 and DMF ($P < 0.05$) was non-significant after the permutation test. None of the nine polymorphisms were significantly associated with plant height. Based on the GLM analysis and considering the eight haplotypes (Fig. 2) as markers, the haplotypes were associated with flowering time ($P < 0.001$) and plant height ($P < 0.05$). Overall, the *D8* haplotypes explained 42% of the variation in flowering time.

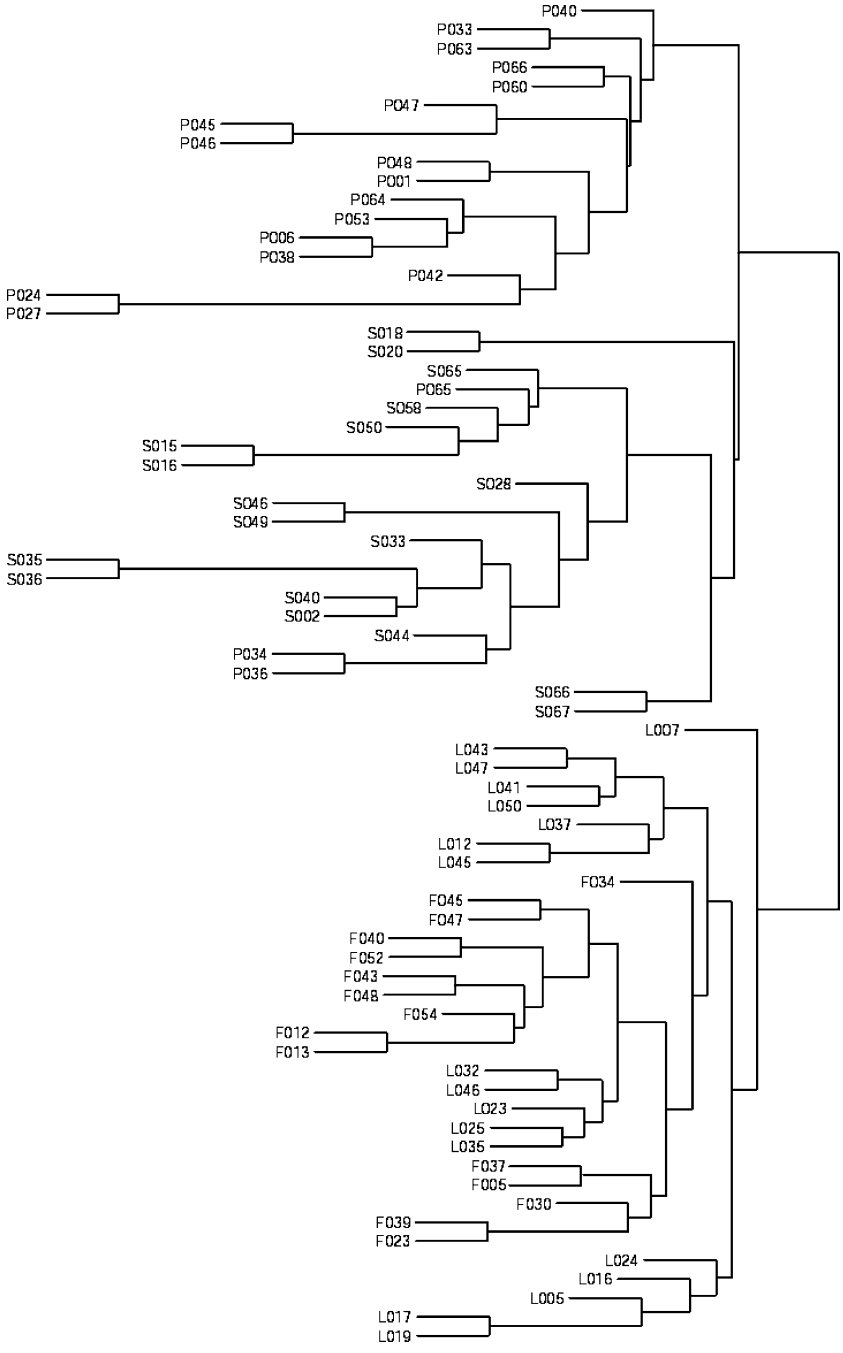


Fig. 1 Cluster analysis based on modified Rogers distance values of 71 genotypes of four inbred pools: *F* Flint, *L* Flint/Lancaster, *P* Iodent and Iodent/Stiff Stalk, *S* Stiff Stalk

Structured association analysis: logistic regression

Logistic regression was carried out taking into consideration the Q-matrix produced by STRUCTURE for three subpopulations. This analysis revealed that no polymorphisms were significantly associated with flowering time. However, a SNP in the promoter region (position 1,044), representing a 2-bp indel, was significantly ($P < 0.05$) associated with plant height across the two environments in which this trait was recorded.

To test the effect of altered population structure in the analysis, we performed the structured association analysis with the Q-matrix for two subpopulations ($K = 2$) containing F/L and P/S lines, respectively. The *P* values were determined as described above and did not indicate significant associations between markers and traits across environments. However, within one environment (EWE), a deletion in the promoter (position 702) was significantly ($P < 0.05$) associated with flowering time (DFF).

We also tested an alternative approach (Olsen et al. 2004) in which association analysis was applied for each of the individual subpopulations identified by STRUCTURE. In subpopulation 1, GLM revealed that all polymorphisms except for the three SNPs in the promoter (positions 677, 1,044 and 1,663) were significantly

				Promoter					ORF				
			Position	185	677	702	1044	1663	1964	3472	3490	3570	
Haplotype	DMF	DFF	PHT	MITE	C/G SNP	INDEL	C/T SNP	C/T SNP	C/T SNP	G/T SNP	G/A SNP	C/G SNP	F:L:P:S
1	72.3(*63.6*)	73.5(*65.2*)	159.4(*124.7*)	+	G	DEL	T	T	C	T	G	C	5:2:0:0
2	78.5(*67.6*)	79.7(*68.1*)	164.5(*153.5*)	-	G	DEL	T	T	T	G	A	G	0:0:2:8
3	77.4	79,1	189.4	-	G	INS	T	T	T	G	A	G	0:0:1:0
4	72.5	73.3	154.4	-	C	DEL	C	C	T	G	A	G	1:1:0:0
5	75.5	77.4	164.4	ND	ND	DEL	T	T	T	G	A	G	0:1:0:0
6	76.9(*73.3*)	78.6(*75.3*)	176.4(*156.3*)	-	C	INS	T	C	T	G	A	G	0:4:0:0
7	77.1(*77.1*)	78.6(*78.8*)	164.0(*162.8*)	-	C	INS	C	C	T	G	A	G	9:10:16:7
8	77.8	79.1	158.3	++	C	INS	C	C	T	G	A	G	0:0:1:3

Fig. 2 Haplotypes based on the nine *Dwarf8* polymorphisms. The average phenotypic value for days to male flowering (*DMF*), days to female flowering (*DFF*) and plant height (*PHT*) are shown for individual haplotypes. *Numbers in parenthesis* are phenotypic values for the same haplotypes in the study of Thornsberry et al. (2001) (phenotypic data available through the TASSEL software). *Position* refers to the basepair position in the alignment produced by Thornsberry et al. (2001), and the *overlying bar* denotes the promoter region and open reading frame (*ORF*). The number of haplotypes by heterotic pools is shown (*F* Flint, *L* Flint/Lancaster derivates, *P* Iodent and Iodent/Stiff Stalk derivates, *S* Stiff Stalk). The exact correspondence between genotypes and haplotypes can be seen in Appendix 3. + denotes the presence and – denotes the absence of the MITE; + + denotes a larger than expected *MITE* (see text). *ND* Not determined

associated with flowering time (DFF and/or DMF). In subpopulation 2, the MITE (position 185) and two SNPs in the promoter region (positions 677 and 1,663) were significantly associated with DFF but not with DMF. In subpopulation 3, there were no identifiable significant associations between markers and phenotypes. No significant associations were identified between markers and plant height in any of the individual subpopulations. To test for associations between SSR markers and traits, we identified 16 SSR markers with PIC values similar to those of the *D8* polymorphisms, with a range of 0.18 to 0.44 (data not shown). Logistic regression taking population structure into account ($K=3$) indicated that one of these SSRs (phi032, Appendix 2) was significantly ($P<0.01$) associated with flowering time across environments.

Discussion

The SSR markers were used to determine population structure. The cluster analysis (Fig. 1) and estimation of population structure by STRUCTURE (Fig. 3) revealed two major clusters consisting of F/L inbred lines and P/S inbred lines, respectively. This was not a surprising result as the P lines include Iodent/Stiff Stalk, some of which could be expected to group together with pure Stiff Stalks, and the L lines consist of Flint/Lancaster and thus could be expected to group together with pure Flint lines.

Cluster analysis revealed the presence of two subgroups in the P/S cluster, while in the F/L cluster, no further subgrouping was apparent. This result is supported by STRUCTURE; thus, three subpopulations are the most likely case. The F and L lines grouped together in subpopulation 1, while the P and S lines were assigned to subpopulations 2 and 3, respectively (Fig. 3), suggesting a greater diversity within the P/S cluster than within the F/L cluster, which is also indicated by the genetic composition of each of the three subpopulations estimated by STRUCTURE (Fig. 4).

Although three subpopulations seemed the most likely possibility, two subpopulations (fusion of the P and S lines) were also an acceptable alternative based on the cluster analysis and STRUCTURE (Figs. 1, 3). We, therefore, determined population structure estimates for both two and three subpopulations for the association analysis.

Table 4 General linear model (GLM) analysis between individual *Dwarf8* polymorphisms and phenotypic values across environments, not considering population structure (*ns* not significant)

Locus[a]	Trait	$P(X>F)$[b]	Trait	$P(X>F)$[b]	Trait	$P(X>F)$[b]
Haplotype	DMF	<0.0001***	DFF	<0.0001***	PHT	0.0674 ns
MITE (185)	DMF	<0.0001***	DFF	<0.0001***	PHT	0.1406 ns
SNP (677)	DMF	0.8704 ns	DFF	0.6666ns	PHT	0.7023 ns
DEL (702)	DMF	0.0107*	DFF	0.0013**	PHT	0.1553 ns
SNP (1,044)	DMF	0.3776 ns	DFF	0.2526 ns	PHT	0.0779 ns
SNP (1,663)	DMF	0.0417*	DFF	0.0581 ns	PHT	0.5931 ns
SNP (1,964)	DMF	<0.0001***	DFF	<0.0001***	PHT	0.3774 ns
SNP (3,472)	DMF	<0.0001***	DFF	<0.0001***	PHT	0.1575 ns
SNP (3,490)	DMF	<0.0001***	DFF	<0.0001***	PHT	0.1838 ns
SNP (3,570)	DMF	<0.0001***	DFF	<0.0001***	PHT	0.1953 ns

*$P \le 0.05$, **$P \le 0.01$, ***$P \le 0.001$

[a]Numbers in parenthesis correspond to the location of individual polymorphisms according to the alignment by Thornsberry et al. (2001)

[b]*P* values (based on the *F*-distribution) for associations between the entire haplotype and individual polymorphisms and days to male flowering (DMF), days to female flowering (DMF), and plant height (PHT) are shown

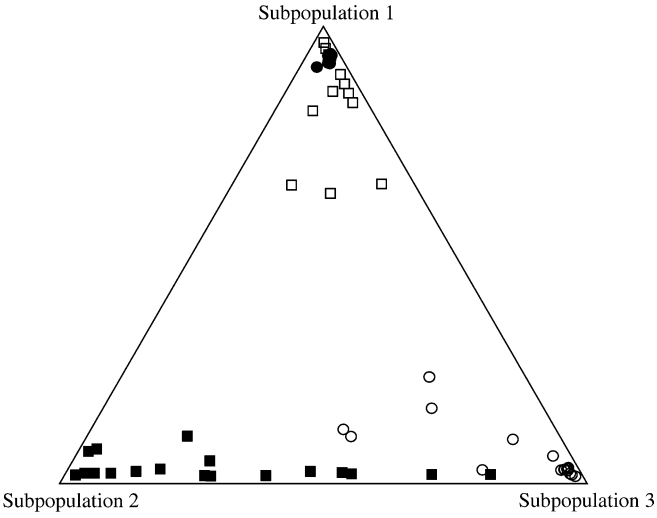


Fig. 3 Triangle plot of the Q-matrix for three subpopulations ($K = 3$) estimated by STRUCTURE. Each inbred line is represented by a *symbol*: *closed circles*, *open squares*, *open circles* and *closed squares* represent Flint, Flint/Lancaster derivates, Iodent and Iodent/Stiff Stalk derivates and Stiff Stalk lines, respectively. The contribution to each inbred line from each of the three subpopulations is given as the distance from the point to each of the three edges of the *triangle*

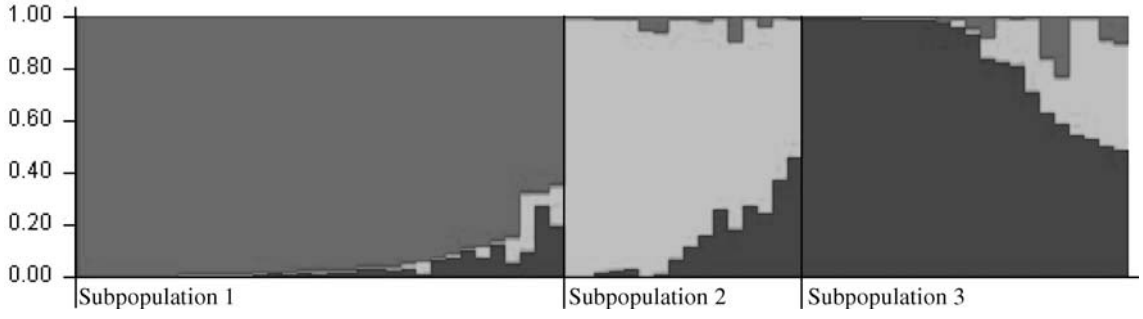
We genotyped our plant materials for nine *D8* polymorphisms previously shown to be associated with flowering time (Thornsberry et al. 2001) to reveal eight haplotypes within the 71 inbred lines included in this study (Fig. 2). Only haplotype 7 was present in all four inbred pools, while the other seven were restricted to either the F/L group or P/S groups. The occurrence of recombination events between *D8* polymorphisms indicates less selective constraints in the promoter region than in the ORF region. Furthermore, no recombination was observed between the MITE (position 185) and the ORF polymorphisms—i.e. these polymorphisms are in complete linkage disequilibrium in this plant material. This supports the findings of Thornsberry et al. (2001), in which only one of 92 individuals showed recombination between the coding region polymorphisms and the MITE.

Four of the *D8* haplotypes identified in the present study (1, 2, 6 and 7; Fig. 2) were also identified in the study by Thornsberry et al. (2001), which enables us to directly compare haplotype phenotypes. In general, the ranking of early- and late-flowering haplotypes was in agreement between the studies (Fig. 2), with only a few exceptions. Haplotype 2 flowered the latest in our study, while in the study of Thornsberry et al. (2001), it flowered earlier than haplotypes 6 and 7. For plant height, haplotype 1 was the shortest in both studies. However, while haplotype 6 was the tallest in our study, haplotype 7 was tallest in the study by Thornsberry et al. (2001). This difference could be due to a relatively low number of individuals for haplotype 6 in both studies and/or different genetic backgrounds (affecting plant height) of the individuals in the two studies. Furthermore, the relative difference between haplotype 1 and haplotypes 2, 6 and 7 was considerably greater in the study of Thornsberry et al. (2001). While the effects of different genetic backgrounds cannot be excluded, these quantitative differences are most likely due to different environments. Based on these haplotype data, it could be suggested that two "functional" haplotypes exist: haplotype 1 (early flowering and short plants) and haplotype 7 (late flowering and tall plants). With respect to these two haplotypes, both the ANOVA and GLM analyses indicated a significant effect of haplotypes on flowering time but not on plant height (data not shown). However, haplotype 1 is confounded with population structure (Fig. 2), and the associations disappeared when population structure was taken into account (data not shown).

Not considering population structure, the MITE (position 185), the 18-bp deletion (position 702), and the ORF polymorphisms were associated with flowering time (Table 4). No significant associations between polymorphisms and plant height were identified by this method. This indicates that these six polymorphisms and/or the haplotype as a whole explain a significant proportion of the phenotypic variation for flowering time in our set of inbred lines. However, on the basis of this method, we cannot exclude the possibility of population stratification resulting in false positive associations.

The above-mentioned significant associations disappeared when the population structure of the three sub-

Fig. 4 Bar plot of the genetic composition of individual lines based on 55 SSR markers. Each *column* represents an individual genotype and is partitioned into three segments, the *length* of which represents the estimated genetic fraction in each individual of each of the three inferred subpopulations



populations was taken into account (Fig. 3) in the association analysis. If we consider the confounding of *D8* haplotypes with population structure (Fig. 2), the absence of flowering time associations is not surprising. In particular, the fixation of the early-flowering haplotype 1 in the F/L subpopulation would affect the analysis. Interestingly, an association was detected between a 2-bp indel in the promoter region (position 1,044) and plant height when population structure was included in the analysis. This result could indicate that population structure, if not included in the analysis, can possibly mask genuine associations.

When our material was grouped into two subpopulations, the plant height association disappeared. However, an association was identified between the 18-bp deletion in the promoter (position 702) and flowering time within one environment. This shows that population structure estimates as well as environmental effects are crucial for the identification of associations.

An alternative approach to structured association analysis has been published for *Arabidopsis* (Olsen et al. 2004). In this approach, association analysis is carried out within subpopulations, assuming that within subpopulations the genetic background is uniform and will not influence phenotypic trait expression. When we applied this method, several of the polymorphisms were significantly associated with flowering time in subpopulation 1 (F/L genotypes) and, to a smaller degree, in subpopulation 2 (P genotypes); no significant associations with plant height were identified. However, subpopulations 2 and 3 harboured relatively few individuals (16 and 22, respectively), thus limiting the power of the analysis. The limitation of this method, with regard to population structure, is that even within the three subpopulations included in this study there was considerable variation for genetic background markers (Fig. 4). This within-subpopulation variation is not taken into account in this approach, whereas genetic background is considered throughout the analysis by logistic regression (Thornsberry et al. 2001). When logistic regression was performed within subpopulation 1 taking population structure estimates into account, the only significant association identified was between the 18-bp indel in the promoter (position 702) and all traits (data not shown). Thus, depending on the level of the genetic background variation within subpopulations, logistic regression that takes population structure into account will be more restrictive than the method employed by Olsen et al. (2004) in identifying significant associations.

The fact that we did not identify significant associations between *D8* polymorphisms and flowering time when population structure was considered in the analysis could reflect the choice of plant materials rather than actual functional effects of the *D8* polymorphisms. This situation illustrates a dilemma of association analysis; on the one hand, it is important to avoid false positives caused by population structure, but on the other hand, taking population structure into account in the analysis might create false negative results; i.e., "true" functional polymorphisms will prove to be non-significant if they are confounded with population structure, as illustrated by haplotype 1 and the F/L subpopulation in this study.

The GLM analysis excluding population structure (Table 4) as well as the GLM analysis in the F/L lines exclusively associated six of the nine polymorphisms with flowering time. These polymorphisms include the MITE and 18-bp deletion in the promoter, which possibly affect expression level of the transcript, as well as the polymorphisms in the ORF. The deletion of two amino acids in the C-terminus region (position 3,472) of the ORF has been proposed to be a causative factor with respect to flowering time (Thornsberry et al. 2001) as it resides near a SH2-like binding domain important for the functionality of this class of transcription factors (Koch et al. 1991; Peng et al. 1999). However, no recombination was observed between the MITE and ORF polymorphisms, which prevented the estimation of additive effects of individual polymorphisms. The association analysis, as well as the similar phenotypes of haplotypes 2 and 7 (Fig. 2, supported by ANOVA; data not shown), indicate that the promoter polymorphisms at positions 677, 1,044 and 1,663 are not causative for flowering time. The significant association we observed between the promoter indel (position 1,044) and plant height is based on the phenotype of four individuals (haplotype 6) only, as the association disappears if these individuals are excluded from the analysis (data not shown). Deletions in the DELLA domain at the N-terminus of *D8* have previously been shown to result in dwarf phenotypes (Peng et al. 1999). It can be speculated that the promoter indel itself affects expression of the transcript, or that it is linked to DELLA polymorphisms in the ORF, which in turn affects functionality of the transcription factor and, consequently, plant height. However, Thornsberry et al. (2001) did not identify associations between plant height and polymorphisms in the DELLA region, as this region was completely conserved across lines.

Confirmation of associations in very large populations has been proposed (Cardon and Palmer 2003; http://www.ukbiobank.ac.uk/) for association studies in humans in order to minimize the risk of false positive associations. However, even in large populations, haplotypes might be confounded with population structure. Thus, for the systematic development of FMs in plants, it might be more efficient to test candidate polymorphisms in an isogenic background than to validate associations in large populations. This can be achieved by marker-assisted introgression or, alternatively, by screening TILLING populations, which have recently become available as a public resource (http://genome.purdue.edu/maizetilling/) for maize.

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Appendix 1

The 71 inbred lines included in the analysis with recorded phenotypic data[a] at the four locations[b]. DMF, DFF and PHT were recorded in EWE and HOH; DMF and DFF were recorded in SÜN, while DFF was recorded in POC

	Line name	DMF, EWE	DFF, EWE	PHT, EWE	DMF, HOH	DFF, HOH	PHT, HOH	DMF, SÜN	DFF, SÜN	DFF, POC
Flint	F005	71.6	71.9	174.9	74.4	78	187.6	72.9	77.5	74
	F012	65	63.9	127.9	73.1	73	178.4	71	71.5	74
	F013	63.9	63	132.8	72.4	73	182.4	70	71	73
	F023	74.4	75.4	134.3	77.6	81.5	199	73.1	78.5	76.5
	F030	71.7	72.1	129.6	75.9	80	181.2	74	76	74
	F034	71.7	71.5	130.1	74.1	77.5	178.3	73.1	77.5	76
	F037	69.3	68.9	148.7	73.1	74	200.2	72	73.5	74.5
	F039	74.5	76.9	127.8	74.5	81.5	197.3	74	80	76.5
	F040	64.1	63.8	119	72	71.5	165	71	71.5	73
	F043	71.9	71.9	125.8	74	74.5	169.7	73.7	75	74.5
	F045	66.7	66.5	131.3	73	75	179.2	74.5	76	75.5
	F047	70.4	72.9	153.8	74.5	75	177.4	73	75	73
	F048	71.6	71.9	142	75	76.5	189.6	72.6	75.5	73
	F052	67.6	68.6	141.3	74.3	77	184.1	74	76.5	76.5
	F054	69.9	69.3	146.7	74.8	75	190.6	72	73	73.5
Flint/Lancaster	L005	78.3	78.9	127.5	77	84.5	160.2	81.5	82	82
	L007	79.9	80.8	163.3	86.5	89.5	192.2	83	82.5	85.5
	L012	78.3	81.3	191.8	82.3	87	196	80	84	87
	L016	73.1	74.6	145.1	78	81	184.6	77.5	80	77.5
	L017	71.8	71.7	132	75	78.5	171	76.4	76.5	77
	L019	73.6	74.1	145.9	78.4	80.5	180.9	79	79	76
	L023	73.6	74.5	161.7	79	80.5	201.9	77.7	77.9	75
	L024	73.9	74.7	150.5	78.2	80	182.9	75.5	78	77
	L025	72.4	72.8	166.9	78.5	80	187.6	77	77	76.5
	L032	75.1	74.9	148.7	80	82.5	181.3	77.5	79.5	77
	L035	71	70.2	144.8	76	79	184.8	75	76	76.5
	L037	73.6	73.1	151.4	77	80	177.4	75.9	79	77
	L041	67	67.1	137.2	74.5	79	175.8	72.9	75	74.5
	L043	73.6	74	145	78	80.5	179.2	77.5	79	75
	L045	73.7	76.7	159.6	81	84.5	196.1	78	79.5	80.5
	L046	69.4	69.4	138	75	79	183.7	73.5	77	73.5
	L047	66.8	65.4	146.1	73.9	75	172.4	71.5	73	74.5
	L050	74.3	74	168.4	82.4	83	180.1	79.9	79.5	77
Iodent and Iodent/Stiff stalk	P001	79.3	79.8	158	83.4	85.5	185.1	80.9	85	86.5
	P006	73.9	75.3	157	79.4	79.5	193.6	75	78.5	78.5
	P024	78.8	79.9	167.3	82.1	85	187	79.5	82	80.5
	P027	77.9	78.7	154.5	82.2	82.5	188.3	79.6	80.5	78
	P033	77.1	76.8	149.3	80.5	82	163.9	79	79.5	78
	P034	76.7	76.7	163.3	78.5	80	215.5	77	80.5	77.5
	P036	74.7	72.9	158	79	78.5	192	78.5	76.5	76
	P038	73.9	75.5	121.6	80.1	83	164.6	75.1	79	77
	P040	77.8	78.4	148.7	82.1	86.5	182.7	78.9	80.5	80.5
	P042	77.3	76.7	160.6	80.9	81	193.7	80.5	80	76.5
	P045	70.7	70.2	138.4	77.4	79	179	73	76	75.5
	P046	69.4	68.6	139	75.5	78	173.7	73	77	75
	P047	73.5	72.7	140.5	78.6	78.5	177.3	74.9	75.5	76.5
	P048	75.1	75.3	143.1	80.5	81.5	168.2	76.5	78	81.5
	P053	75.3	77.5	144.4	80.2	83.5	166.4	76.5	80	80.5
	P060	77.4	77.3	132	82.5	86	187.3	79	81.5	84
	P063	78.2	80.9	149	83	86.5	183.7	81.4	83	82
	P064	79.7	80.7	160.4	83.5	86.5	190.8	79.3	83.9	86
	P065	81.1	81	167.9	84.7	85	204.5	80.9	82.5	86
	P066	74.7	75.7	144.2	80	81	189.3	79	79.5	77.5
Stiff stalk	S002	74.6	74.8	142.9	81	83	163.7	77	78.5	76.5
	S015	80	80.3	148.4	84	86	184.8	81.5	82.5	83.5
	S016	75.1	76.3	132.5	82.5	83.5	169.6	80	81	82
	S018	82.8	84	155.1	89.5	90	187.2	86	86.5	86.5
	S020	85	85.8	168.2	90.5	90.5	187.9	86	86.5	87.5
	S028	76.5	76.5	161.1	82.5	84	180.7	79.9	81	80
	S033	79.7	78.9	160.3	82.9	86.5	183.1	81.5	84.5	82.5
	S035	73.9	74.3	136.5	79	81	169.9	77	80	77
	S036	73.3	75.3	144.3	80.5	83.5	166.6	78.5	80	76.5

Appendix 1 (Contd.)

Line name	DMF, EWE	DFF, EWE	PHT, EWE	DMF, HOH	DFF, HOH	PHT, HOH	DMF, SÜN	DFF, SÜN	DFF, POC
S040	73.4	71.8	144.4	77.5	78.5	185.9	74.5	74	73
S044	79.4	79.1	197.4	82.9	83.5	206	81.1	81.5	82
S046	75	74.6	138	81	81.5	161.4	79.4	80	78.5
S049	76	75.1	149.6	81.5	81.5	175	79.9	80	80.8
S050	78.3	78.9	166.6	81.5	83.5	180.2	75.5	80.5	78
S058	75.1	74.7	113.3	80	80.5	151.8	76.5	78	74.5
S065	72.5	72.7	132.2	77	78.5	163.8	77.1	77.5	77
S066	73	73.2	155.4	75.4	79	163.7	73	77	76
S067	73.7	73.3	127.1	79	80	162.5	79	79.5	76.5

[a]DMF, Days to male flowering; DFF, days to female flowering; PHT, plant height
[b]EWE, Eckartsweier; HOH, Hohenheim; SÜN, Sünching; POC, Pocking

Appendix 2

The SSR analysis was performed with 55 publicly available SSR markers providing an even coverage of the maize genome

Chromosome	Bin	Marker name
1	1	phi427913
	3	phi109275
	4	umc1169
	6	umc1122
	9	phi011
	11	phi064
2	1	phi96100
	3	umc1555
	4	phi083
	8	phi127
	10	phi101049
3	1	phi104127
	2	phi374118
	5	phi053
	6	phi102228
	7	umc1489
	9	umc1641
4	1	phi072
	1	phi213984
	4	phi308090
	5	phi079
	8	phi093
	10	umc1180
5	2	phi396160
	4	phi331888
	5	phi333597
	7	phi128
	9	umc1153
6	0	umc1143
	1	phi423796
	3	umc1887
	4	phi031
	7	phi123
	8	phi089
7	0	umc1545
	3	phi114
	4	phi328175
	5	phi069
	6	phi116
8	0	phi420701
	2	umc1304
	3	phi121
	3	phi100175
	8	phi015
	9	phi233376
9	0	umc1279
	3	phi065
	4	phi032
	5	phi108411
	7	umc1675
10	0	phi041
	1	umc1152
	3	phi050
	4	phi084
	6	umc1061

Appendix 3

Correspondence between *D8* haplotypes (Fig. 2) and the 71 maize lines included in the analysis

Haplotype	Lines
1	F012, F013, F034, F045, F048, L016, L035
2	P036, P060, S002, S015, S028, S033, S035, S036, S040, S050
3	P034
4	F040, L024
5	L037
6	L012, L045, L047, L050
7	F005, F023, F030, F037, F039, F043, F047, F052, F054, L005, L007, L017, L019, L023, L025, L032, L041, L043, L046, P001, P006, P024, P027, P033, P038, P040, P042, P045, P046, P047, P048, P053, P064, P065, P066, S016, S018, S020, S044, S046, S058, S065
8	P063, S049, S066, S067

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