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Two high-density AFLP® linkage maps of *Zea mays* L.: analysis of distribution of AFLP markers

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Abstract This study demonstrates the relative ease of generating high-density linkage maps using the AFLP® technology. Two high-density AFLP linkage maps of *Zea mays* L. were generated based on: (1) a B73 × Mo17 recombinant inbred population and (2) a D32 × D145 immortalized F_2 population. Although AFLP technology is in essence a mono-allelic marker system, markers can be scored quantitatively and used to deduce zygosity. AFLP markers were generated using the enzyme combinations *Eco*RI/*Mse*I and *Pst*I/*Mse*I. A total of 1539 and 1355 AFLP markers have been mapped in the two populations, respectively. Among the mapped *Pst*I/*Mse*I AFLP markers we have included fragments bounded by a methylated *Pst*I site (^{m}AFLP markers). Mapping these ^{m}AFLP markers shows that the presence of C-methylation segregates in perfect accordance with the primary target sequence, leading to Mendelian inheritance. Simultaneous mapping of *Pst*I/*Mse*I AFLP and *Pst*I/*Mse*I ^{m}AFLP markers allowed us to identify a number of epi-alleles, showing allelic variation in the CpNpG methylation only. However, their frequency in maize is low. Map comparison shows that, despite some rearrangements, most of the AFLP markers that are common in both populations, map at similar positions. This would indicate that AFLP markers are predominantly single-locus markers. Changes in map order occur mainly in marker-dense regions. These marker-dense regions, representing clusters of mainly *Eco*RI/*Mse*I AFLP and *Pst*I/*Mse*I ^{m}AFLP markers, co-localize well with the putative centromeric regions of the maize chromosomes. In contrast, *Pst*I/*Mse*I markers are more uniformly distributed over the genome.

Key words *Zea mays* L. · AFLP® · Methylation
AFLP® · Genetic map · DNA methylation

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Introduction

High-density genetic maps are becoming increasingly useful in fundamental and applied genetic research. They serve to (1) locate genes of interest, (2) facilitate marker-assisted breeding and map-based cloning and (3) provide the framework towards understanding the biological basis of complex traits. In genome projects, high-density genetic maps are central to localizing a large portion of the loci in the germplasm of interest and to top-down anchoring of physical maps.

Until recently, genetic maps of many plant species such as maize (*Zea mays* L.) were primarily based upon segregating restriction fragment length polymorphism

(RFLP) markers (Helentjaris et al. 1986, 1988; Burr et al. 1988; Beavis and Grant 1991; Shoemaker et al. 1992; Gardiner et al. 1993; Matz et al. 1994; Coe et al. 1995). The disadvantages of RFLPs include (1) large quantities of DNA are required, (2) analyses of large populations are costly and (3) the technique is difficult to automate. This has prompted the search for more efficient marker systems. Of these, the randomly amplified polymorphic DNA (RAPD) assay (Welsh and McClelland 1990; Williams et al. 1990) has been widely used in plant breeding and genetics (Waugh and Powell 1992). However, problems with the reproducibility of RAPD amplification have been reported (Demeke et al. 1997; Karp et al. 1997). Simple sequence repeat polymorphisms or microsatellites (SSR) (Tautz 1989), a marker system first made popular in mammalian genetics, has generated considerable interest among plant geneticists. SSR markers have been developed for many plant species, including maize (Senior et al. 1997). However, the high development and application costs may hinder their application in the large numbers needed to study, for example, a large germplasm collection.

Another efficient polymerase chain reaction (PCR)-based method, called AFLP®, has been developed (Vos et al. 1995), combining the restriction site recognition element of RFLP with the exponential amplification aspects of PCR-based DNA marker systems. The major advantages of AFLP are (1) a high multiplex ratio, (2) a limited set of generic primers is used, and (3) there is no need for sequence information. To date, the AFLP technique has been succesfully applied to identify markers linked to disease resistance loci (Meksem et al. 1995; Thomas et al. 1995; Cervera et al. 1996; Sharma et al. 1996; Brigneti et al. 1997; Simons et al. 1997; Vos et al. 1998), in germplasm analyses (Hill et al. 1996; Powell et al. 1996; Maughan et al. 1996; Milbourne et al.1997; Paul et al. 1997; Schut et al. 1997; Zhu et al. 1998; Cervera et al. 1998; Barrett and Kidwell 1998; Pejic et al. 1998) and in mapping barley (Becker et al. 1995; Qi et al. 1998; Castiglioni et al. 1998), eucalypts (Marques et al. 1998), potato (van Eck et al. 1995), rice (Maheswaran et al. 1997; Zhu et al. 1998), soybean (Keim et al. 1997) and sugar beet (Schondelmaier et al. 1996). The most recent AFLP publications on maize are those of Ajmone Marsan et al. (1998), Melchinger et al. (1998a) and Pejic et al. (1998). In the study presented here two high-density AFLP linkage maps of *Zea mays* L. were generated. The aim of this study was to (1) confirm the relative ease of generating high-density maps using the AFLP technology, (2) evaluate the efficiency of the AFLP technology in terms of the multiplex (*M*), effective multiplex (*EM*) and effective mapped multiplex (*EMM*) ratios for linkage analysis, (3) study the transmission of C-methylation from parent to offspring and (4) investigate the consistency of AFLP markers across populations. In addition, the data obtained enable a comparison of the genomic distribution of AFLP and ^{m}AFLP markers and of their position relative to the centromere.

Materials and methods

Plant material and DNA isolation

A recombinant inbred (RI) population (Senior et al. 1997) and an immortalized F_2 (IF_2) population (Xia et al. 1998) involving four different inbred lines of *Zea mays* L. were used for this study. The parents of the RI population were B73, a central corn belt line derived directly from Iowa Stiff Stalk Synthetic (BSSS), and Mo17, a central corn belt line derived from 'Lancaster' and 'Krug' germplasm. The RI population was set up as follows: an F_1 was produced from a cross between B73 and Mo17. Selfing for two generations produced a set of 264 F_3 lines. Two hundred and eight RI lines were developed from these F_3 plants by single-seed descent for three generations. DNA of the RIs was extracted by a modified CTAB procedure described by Saghai Maroof et al. (1984).

The parents of the IF_2 population were D32, a sugarcane mosaic virus (SCMV)- and maize dwarf mosaic virus (MDMV)-resistant European Dent line having an 'Iodent' and 'Illinois High Protein' (IHP) background, and D145, a SCMV- and MDMV-susceptible European Flint with 'Lancaster' background (Melchinger et al. 1998b). The IF_2 population was developed at the University of Hohenheim, Stuttgart, Germany and set up as follows: (1) an F_1 was produced from a cross between D32 and D145; (2) selfing for two generations produced a set of 220 F_3 families. Per F_3 family, a pool of 20 F_3 plants was chosen to generate the IF_2 lines: (1) random crosses were performed between 10 F_3 plants as female and the 10 F_3 plants as male;(2) seed obtained from the 10 females was subsequently pooled, representing an IF_2 line. Thus, 220 IF_2 lines were obtained. At the DNA level, each IF_2 line represented a pool of 60 sib-mated F_3 plants. DNA of the pooled sib-mated F_3 plants was extracted using a modified CTAB procedure described by Stewart et al. (1993).

AFLP and methylation AFLP analysis

The AFLP analysis was performed according to Vos et al. (1995), using the enzyme combinations (ECs) *Eco*RI/*Mse*I and *Pst*I/*Mse*I. Methylation AFLP® analysis was performed according to Vuylsteke et al. (submitted), using the EC *Pst*I/*Mse*I. The adaptor sequences specific for these enzymes were synthesized according to Zabeau and Vos (1993) and are as follows:

*Eco*RI-adapter:	5'-CTCGTAGACTGCGTACC-3'
	3'-CATCTGACGCATGGTTAA-5'
*Pst*I-adapter:	5'-CTCGTAGACTGCGTACATGCA-3'
	3'-CATCTGACGCATGT-5'
*Pst*I-adapter*:	5'-GCATCAGTGCATGCGTGCA-3'
	3'-GTAGTCACGTACGC-5'
*Mse*I-adapter:	5'-GACGATGAGTCCTGAG-3'
	3'-TACTCAGGACTCAT-5'
*Mse*I-adapter^{+}:	5'-CTCGTAGACTGCGTACC-3'
	3'-CTGACGCATGGAT-5'

The non-selective amplification of the restriction fragments during the methylation AFLP analysis is performed using both of the *Mse*I-primers shown below.

A two-step amplification strategy was followed in the methylation AFLP as well as in the AFLP analysis: in a selective preamplification, the restriction fragments were amplified with AFLP primers both having a single selective nucleotide. In the second step, further selective amplification was carried out using primers having two (*Pst*I primer) or three (*Eco*RI and *Mse*I primer) selective nucleotides. The AFLP primers were designed based on the adapter sequence and restriction sites of *Eco*RI, *Pst*I and *Mse*I and have the following sequences:

*Pst*I-primer	5'-GACTGCGTACATGCAG...NN-3'
*Eco*RI -primer	5'-GACTGCGTACCAATTC...NNN-3'
*Mse*I-primer	5'-GATGAGTCCTGAGTAA...NNN-3'
*Mse*I-primer^{+}	5'-GTAGACTGCGTACCTAA-3'

Hereafter *Eco*RI/*Mse*I and *Pst*I/*Mse*I ECs will be referred to as E/M and P/M ECs and *Eco*RI+3/*Mse*I+3 and *Pst*I+2/*Mse*I+3 primer combinations (PCs) will be referred to as E/M PCs and P/M PCs. The P/M PCs used in the methylation AFLP analysis will be referred to as ^{m}P/M PCs.

AFLP marker nomenclature

Each polymorphic AFLP fragment was identified by: (1) the code referring to the corresponding PC (see Table 1), followed by (2) the estimated molecular size of the DNA fragment in nucleotides and (3) a code indicating the parental origin of the fragment (RI population: P1=B73; P2=Mo17; IF_2 population: P1=D32; P2=D145). SequaMark™ (Research Genetics, Huntsville, Ala., USA) was implemented as the size standard used to assign molecular weights to the AFLP fragments. Fragments and markers detected by E/M, P/M or ^{m}P/M PCs will be referred to as E/M, P/M or ^{m}P/M fragments and markers, respectively. Markers detected by ^{m}P/M PCs will also be referred to as AFLP markers, except when AFLP and ^{m}AFLP markers need to be distinguished.

Analysis of gel images

The AFLP technology predominantly produces mono-allelic markers: usually only one of the two alleles at a heterozygous locus is detected. However, since product concentration directly reflects initial template concentration, the expected difference between a heterozygous locus and a homozygous locus is approximately a two-fold difference in intensity of a band (= a reflection of the fragment quantity). This phenomenon is exploited to quantitatively analyze AFLP marker bands in order to deduce zygosity. Thus, AFLP markers can in principle be scored quantitatively as co-dominant markers, i.e. heterozygotes can be differentiated from both homozygous classes. For the analysis of complex AFLP fingerprint patterns, we have used proprietary software developed specifically for AFLP analysis at Keygene. This software allows the display and analysis of pixel images of X-Ray scans or phosphorimager scans. For the analysis of pixel images, the software has tools to navigate through the image and individual band signals and to size and quantify the AFLP bands with great precision. Each band of a specific marker is classified with respect to its intensity using a mixture model of normal distributions, as described by Jansen (1993). The basic idea behind quantifying band intensities is that the observed intensities of a marker are mixtures of two (RI lines) or three (F_2 plants) normal distributions. The estimated proportions, means and variances of the mixture components form the basis of band classification and of determination of genotypes. The algorithm can be set to identify either two classes (RI lines) or three classes (F_2 individuals) of intensities among the bands. Finally, genotypic data are exported to a file for each marker in each of the samples.

The absolute metrics multiplex ratio (*M*), effective multiplex ratio (*EM*) (Powell et al. 1996) and effective mapped multiplex ratio (*EMM*) define the number of fragments, polymorphic fragments and mapped polymorhic fragments, respectively, simultaneously analyzed in a single assay. For *M* to be calculated, the AFLP fragments in the two parental lanes had to meet the following criteria: (1) fragment size ranged from 50 bp to 500 bp; (2) the mean intensity of the two parental bands had to be higher than an intensity minimum (empirically defined by visual inspection). For *EM* to be calculated, polymorphic bands were discriminated from non-polymorphic bands by a two-fold difference (empirically determined) in intensity between the parental bands. The three metrics *M*, *EM* and *EMM* are suited to facilitate selection of an appropriate EC or appropriate PCs for a given application. Although these metrics are influenced by the number of selective nucleotides at the 3' ends of the PCR primers and can be manipulated by combining PCs in a multiplex reaction approach, it is useful to compare the *M*, *EM* and *EMM* afforded by the PCs in their standard implementation. *EMM* is especially suited for the selection of an appropriate EC or appropriate PCs for mapping.

Linkage analysis and segregation distortion tests

Linkage analyses and segregation distortion tests were performed with the software package JoinMap version 2 (JM) (Stam 1993; Stam and van Ooijen 1995) using the appropriate mapping population type; option RI6 for the RI population and option F_2 for the IF_2 population. Using normal LOD scores can result in spurious linkage of markers with segregation distortion. The LOD scores used by JM are based on the Chi-square test for independence of segregation. The rationale behind using a test of independence rather than the normal LOD score is that distortion of segregation affects normal LOD scores but does not affect the test of independence.

Linkage groups were assigned to the corresponding chromosomes of *Zea mays* L. by inclusion of segregation data of isozymes, RFLPs and SSRs obtained previously on the RI lines (Senior et al. 1997) and/or on the IF_2 lines (Xia et al. 1998). No order was forced during map construction, except for linkage group 1 of the RI population: a fixed order of 4 markers (*phi056*, *bnl5.62a*, *umc157(chn)* and *umc76*) belonging to bins 1.01 and 1.02 was forced in order to preserve their relative positions as given in Senior et al. (1997) and on the UMC 1998 map of maize (Davis et al. 1998). The recombination frequencies were converted to Kosambi centiMorgans (cM) (Kosambi 1944). Maps were drawn using proprietary software (see Fig. 1).

Distribution of AFLP markers over the genome

Equal representation of genomic regions in the map and genome coverage are a function of the distribution of markers over the linkage maps of chromosomes. In order to get information on the distribution of E/M, P/M and ^{m}P/M markers over linkage maps of chromosomes, their distribution has been determined statistically. The Kolmogorov assay was used to test the null-hypothesis:

H_0: $F(x) = F_0(x)$,

where F(x) represents the observed distribution function of the interval (expressed in cM) between 2 adjacent markers, either 2 E/M, 2 P/M or 2 ^{m}P/M-markers; $F_0(x)$ represents the corresponding distribution function under the null hypothesis (H_0); in this case we hypothesize that marker positions are independent and uniformly distributed over linkage maps of chromosomes. This implies an exponential distribution of inter-marker distances, i.e. $F_0(x) = 1\text{-}e^{-x/u}$, where u is the mean interval length.

Contingent on the rejection of H_0, the one-sided alternative hypothesis, H_1: $F(x) > F_0(x)$, for at least one value for x, is accepted. The test statistic D_n is defined as the largest difference between F(x) and $F_0(x)$ ($D_n = \max(F(x)\text{-}F_0(x))$).

The minimal size of the interval in which D_n is measured is determined by the resolution of the mapping population: 0.5 cM and 1.0 cM for the RI and the IF_2 population, respectively.

Since bi-allelic AFLP markers map to the same locus, they lead to an overestimation of the number of intervals of zero length and, hence, to erroneous rejection of the H_0. Therefore, bi-allelic AFLP markers are represented by only 1 marker in this analysis.

All calculations were carried out using the Genstat programme (Genstat-5-Committee 1993).

Co-localization of hypothetical centromeres and AFLP marker clusters

Map positions of putative centromeric regions were assigned using RFLP markers, segregating in one or both populations, which are known to map to the centromeres of the maize chromosomes, with the exception of chromosome 8 (Matz et al. 1994). Localization and visualization of clusters were assessed by scanning the linkage groups, using a 5-cM window, for the largest cluster of AFLP markers.

All calculations were carried out using the Genstat programme (Genstat-5-Committee 1993).

Table 1 Overview of the 84 *Eco*RI/*Mse*I (E/M), 36 *Pst*I/*Mse*I (P/M) and 36 ^{m}*Pst*I/*Mse*I (^{m}P/M) primer combinations (PCs) that the two parental lines B73 and Mo17 of the RI population were screened with for polymorphism. The 41 E/M and 21 P/M PCs fulfilling the 'optimizing and minimizing effort' criteria are marked by ×; the 14 ^{m}P/M PCs are marked by ⊗ as a subfraction of P/M PCs

		M47 CAA	M48 CAC	M49 CAG	M50 CAT	M51 CCA	M54 CCT	M55 CGA	M58 CGT	M59 CTA	M60 CTC	M61 CTG	M62 CTT
E32	**AAC**		×	×	×			×			×		
E33	**AAG**	×			×	×	×		×		×	×	×
E35	**ACA**			×	×	×	×		×	×			
E38	**ACT**	×				×					×		
E39	**AGA**	×	×	×	×	×	×	×		×	×	×	×
E42	**AGT**	×			×						×	×	
E45	**ATG**		×	×							×	×	
P12	**AC**	⊗	⊗	⊗	⊗					⊗		⊗	⊗
P13	**AG**	×	×	⊗	×					×	×	×	×
P18	**CT**		⊗	⊗						⊗	⊗	⊗	⊗

Table 2 Overview of the total number of (1) AFLP fragments, (2) polymorphic AFLP fragments, (3) mapped polymorphism and (4) the corresponding multiplex ratio (*M*), effective multiplex ratio (*EM*) and effective mapped multiplex ratio (*EMM*) per enzyme combination [*Eco*RI/*Mse*I (E/M), *Pst*I/*Mse*I (P/M) and ^{m}*Pst*I/*Mse*I (^{m}P/M)] in the recombinant inbred (RI) and the immortalized F_2 (IF_2) mapping population

		E/M	P/M	^{m}P/M	Total
RI	*Total*	3297	1824	1083	6204
	M	92	87	77	87
	Polymorphic	1197(36.3%)	746 (40.9%)	483 (44.6%)	2425 (39.1%)
	EM	33	36	35	34
	Mapped	670 (20.3%)	565 (31.0%)	304 (28.1%)	1539 (24.8%)
	EMM	19	27	22	22
IF_2	*Total*	3182	1894	1023	6099
	M	88	90	73	86
	Polymorphic	1137(35.7%)	871 (46.0%)	491 (48.0%)	2499 (41.0%)
	EM	32	42	35	35
	Mapped	587 (18.4%)	550 (29.0%)	218 (21.3%)	1355 (22.2%)
	EMM	16	26	16	19

Results

Polymorphism rates among B73 and Mo17, and among D32 and D145

The first step required in the assembly of a linkage map is to screen the two parental lines for polymorphism. The two parental lines B73 and Mo17 of the RI population were screened for polymorphism with the 84 E/M, 36 P/M and 36 ^{m}P/M PCs listed in Table 1. This screening was designed to identify those PCs which fulfilled the 'optimizing and minimizing effort' criteria in that they: (1) gave patterns containing 50–100 fragments, (2) revealed a high polymorphism between the two parental lines and (3) did not amplify heavily repeated restriction fragments, visible as very intense bands. Forty one E/M, 21 P/M and 14 ^{m}P/M PCs fulfilled these criteria (see Table 1). Some E/M PCs were combined in a multiplex reaction approach, resulting in a total of 36 E/M PCs. For the parental lines D32 and D145, the same set of 71 PCs was used to ensure a high polymorhism rate and the maximum overlap of markers in the two maps. Table 2 gives an overview of the total number of AFLP fragments and polymorphic AFLP fragments counted for both pairs of inbred lines. The percentages of polymorphism reflect that the lines D32 and D145 are genetically slightly more divergent than the lines B73 and Mo17. ^{m}P/M PCs reveal the highest percentage of polymorphism, while E/M PCs revealled the lowest degree of polymorphism.

It is clear from Table 2 that E/M and P/M PCs yield on average the highest *M* and *EM*, respectively.

Construction of the B73 × Mo17 and D32 × D145 high-density AFLP linkage maps

Using the 36 E/M and 21 P/M PCs we recorded allelic segregation data for a first set of 90 RI lines; using the 14 ^{m}P/M PCs we recorded segregation data for a second set of 90 RI lines, that have 75 individuals in common with the first set of RI lines. This resulted in a total set of 105 RI lines genotyped either with E/M, P/M and ^{m}P/M PCs, or with ^{m}P/M PCs only.

Inclusion of segregation data of 184 isozymes, RFLPs and SSRs obtained previously on the set of 105 RI lines (Senior et al. 1997) totalized the dataset to 1723 markers. At a LOD=6, these 1723 markers were split into ten major and six minor linkage groups, each containing at least 1 anchor marker to assign to the ten maize chromosomes. The same set of 71 PCs was used to genotype a set of 88 IF_2 lines, yielding 1355 AFLP markers. Inclusion of segregation data of 47 RFLPs and SSRs obtained on the same set of 88 IF_2 individuals (Xia et al. 1998) to-

talized the dataset to 1402 markers. At a LOD threshold grouping value of 6.0, these 1402 markers were split into 11 linkage groups, ten major and one minor of 6 markers. The ten major linkage groups were assigned to the ten maize chromosomes, while the minor linkage group was assigned to chromosome 7 since it contained the core marker *asg8(myb)*.

The percentage of mapped polymorphic fragments was 24.8% and 22.2% for the RI and IF_2 linkage maps, respectively (Table 2). This means that 63.5% and 54.2% of the clearly visible polymorphic fragments of the RI and and IF_2 fingerprints, respectively, are of sufficient quality to allow quantitative scoring and reliable mapping.

It is clear from Table 2 that by choosing P/M PCs with a mean *EMM* of 27, the mapping efforts are minimized.

Map consistency

Although the number of mapped polymorphic AFLP fragments in the IF_2 map was lower than for the RI linkage map (Table 2), the IF_2 map spans 1376 cM, and the length of most of its linkage groups are systematically longer than in the RI map (1178 cM) (Table 3 and Fig. 1), and is more comparable with the lengths reported for other maps. For both maps, the position of RFLP, SSR and isozyme markers on this map were consistent with previously published *Zea mays* L. maps.

Distorted segregation ratios

Segregation ratios of the two homozygous classes at each marker mapped in the RI map were tested for the 1:1 expected proportion at the 5% level of significance. Chromosome 3 showed distorted segregation over almost its entire length with an excess of B73 alleles (Fig. 2), while on chromosomes 1, 2, 5 and 10 only minor regions showed distorted segregation. When the significance level was raised to 1%, chromosome 3 still showed distorted segregation over its total length. The largest distortions at chromosome 3 reached the 3:1 magnitude, while the remainder showed only a 2:1 ratio.

Segregation ratios of the three genotype classes of each marker mapped in the IF_2 map were tested for the 1:2:1 expected proportion at the 5% level and the 1% level of significance. Again chromosome 3 showed distorted segregation over a major part of its total length. The distortions for chromosome 3 ranged from magnitude 1:4:1 to 1:5:1 ($P<0.01$) for a smaller region (40–62 cM) and from magnitude 3:4:1 to 6:8:1 ($P<0.01$) (excess of D32 alleles) for a larger genomic region (79–117 cM), respectively. Minor regions showing distorted segregation ($P<0.05$) were found in only a few cases for other chromosomes (1, 2, 5 and 6) and were different from those on the identical chromosomes in the RI map.

Table 3 Comparison of the genetic length and numbers of *Eco*RI/*Mse*I (E/M), *Pst*I/*Mse*I (P/M), ^{m}*Pst*I/*Mse*I (^{m}P/M) and non-AFLP markers (RFLPs, isozymes and SSRs) mapped per linkage group, of the recombinant inbred (RI) and the immortalized F_2 (IF_2) mapping population

RI map

Chromosome	*cM*	*E/M*	*P/M*	*^{m}P/M*	*non-AFLP*	*Number of markers*
1	129.2	104	87	57	25	273
2	123.4	67	66	32	13	178
3	105.3	77	64	38	13	192
4	152.4	71	55	33	24	183
5	121.6	57	64	31	29	181
6	99.3	67	54	34	17	172
7	122.1	50	43	21	12	126
8	127.0	58	47	22	17	144
9	124.2	72	41	15	17	145
10	73.6	47	44	21	17	129
Total	**1178.1**	**670**	**565**	**304**	**184**	**1723**

IF_2 map

Chromosome	*cM*	*E/M*	*P/M*	*^{m}P/M*	*non-AFLP*	*Number of markers*
1	184.8	65	68	31	6	170
2	151.0	76	70	27	9	182
3	144.1	74	64	24	6	168
4	133.2	75	59	33	1	168
5	149.0	55	75	18	8	156
6	109.6	44	50	15	6	115
7	143.3	61	34	11	4	117
8	142.8	55	57	16	2	130
9	126.2	38	33	13	3	87
10	91.6	44	40	23	2	109
Total	**1375.6**	**587**	**550**	**218**	**47**	**1402**

Allelism of AFLP fragments and epi-alleles

Due to occasional length polymorphism, some of the AFLP markers could be used as codominant markers. These allelic AFLP fragments have to meet the following two criteria: (1) they originate from a different parent and amplify with the same PC; (2) they map to the same locus (complementary segregation). Putative allelic AFLP fragments were observed in both AFLP linkage maps. The majority of the bi-allelic markers differed in size by only a few bp (1–20 bp), probably the result of a small insertion/deletion, while a few pairs of markers were of a large size difference (100–487 bp), reflecting neighboring restriction sites. In terms of bi-allelic pairs of markers, the P/M EC predominated: 48 (17.1%) and 32 (11.7%) pairs of mapped P/M markers in contrast with only 36 (10.9%) and 23 (7.8%) pairs of mapped E/M markers and 11 (7.3%) and 10 (9.2%) pairs of mapped ^{m}P/M markers for the RI and IF_2 maps, respectively.

Allele-specific methylation (methylation polymorphism) results in ^{asm}AFLP markers. Since DNA methylation is the only source of allelic difference between epi-alleles, some of the scored P/M and ^{m}P/M fragments might be epi-allelic. To identify a AFLP and a ^{m}AFLP

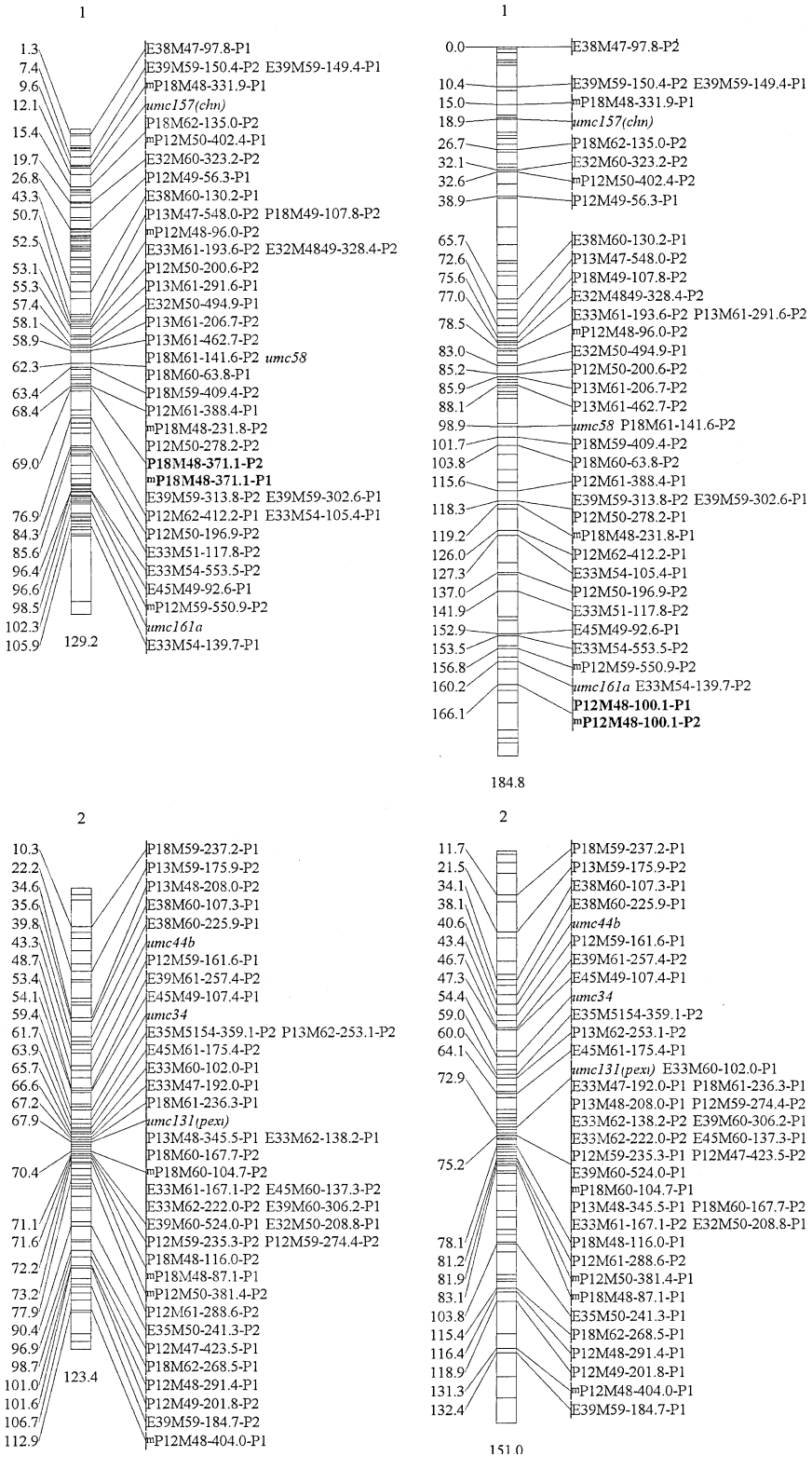
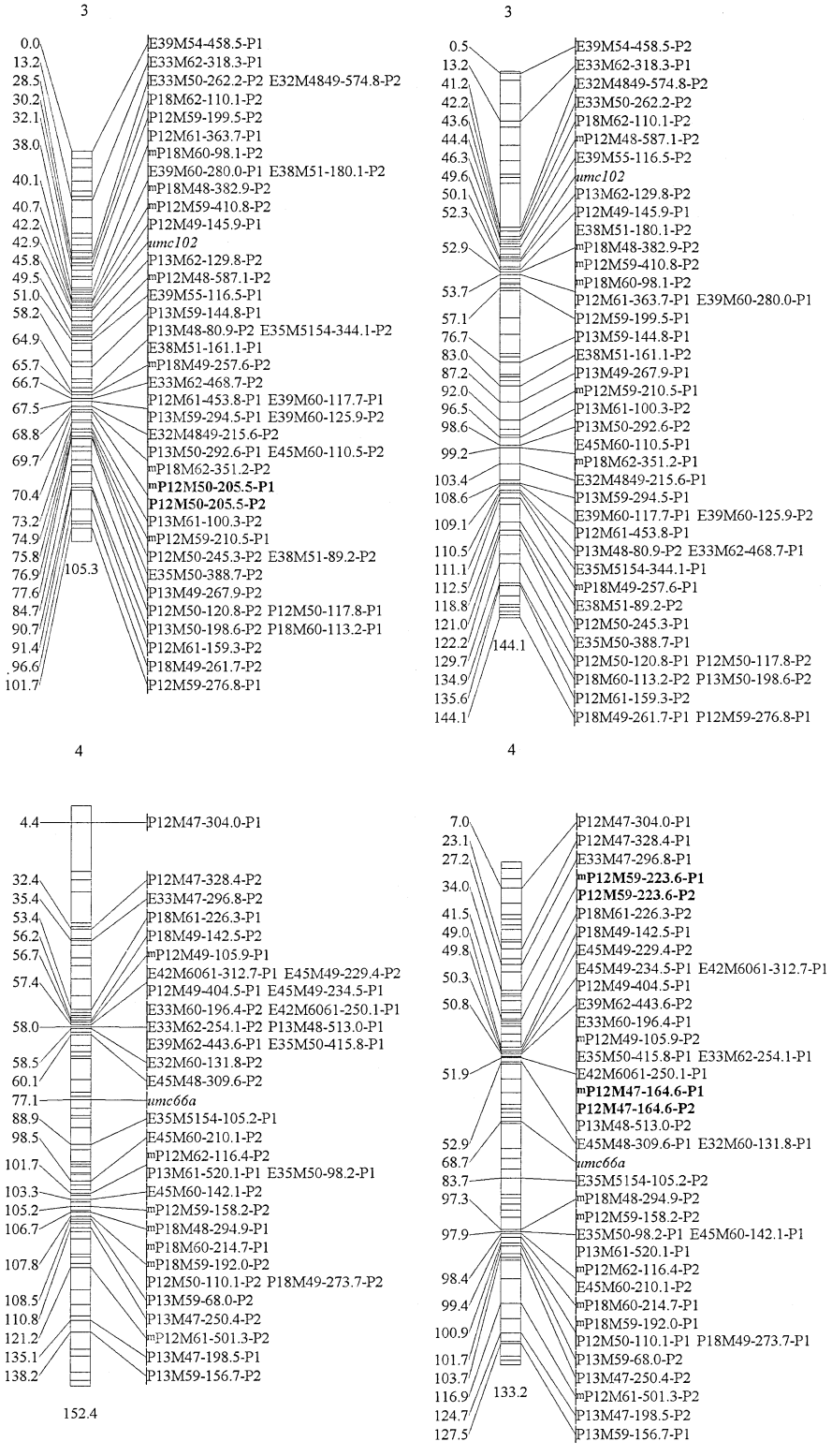


Fig. 1 Two high-density linkage maps of maize based on 105 recombinant inbred (RI) lines from a B73 × Mo17 cross (*left map*) and 88 immortalized F_2 (IF_2) lines from a D32 × D145 cross (*right map*). Distances are given in Kosambi centiMorgans. For simplicity, only common markers, including those that map to different chromosomes in both maps, and pairs of allele specific methylation AFLP markers ([asm]AFLP markers) are shown. The AFLP markers are named with (1) the code referring to the corresponding primer combination (see Table 1), followed by (2) the estimated molecular size of the DNA fragment in nucleotides and (3) a code indicating the parental origin of the fragment (RI population: *P1*=B73; *P2*=Mo17. IF_2 population: *P1*=D32; *P2*=D145). The pairs of [asm]AFLP markers, located on chromosome 1, 3 and 6 and on 1 and 4 in the RI and IF_2 map, respectively, are indicated in ***bold***

marker as a possible [asm]AFLP marker pair, we applied the following criteria: (1) both AFLP fragments have exactly the same size, (2) both AFLP fragments are derived from different parents, with the same complementary PCs and (3) both AFLP fragments map to the same locus (complementary segregation). Only three pairs of epialleles were identified in both the RI and IF_2 map (Fig. 1). This means that only 1% of a total of 673 and 595 mapped [(m)]P/M markers (obtained with the 14 complementary P/M PCs), respectively, showed allelic variaton in CpNpG methylation.

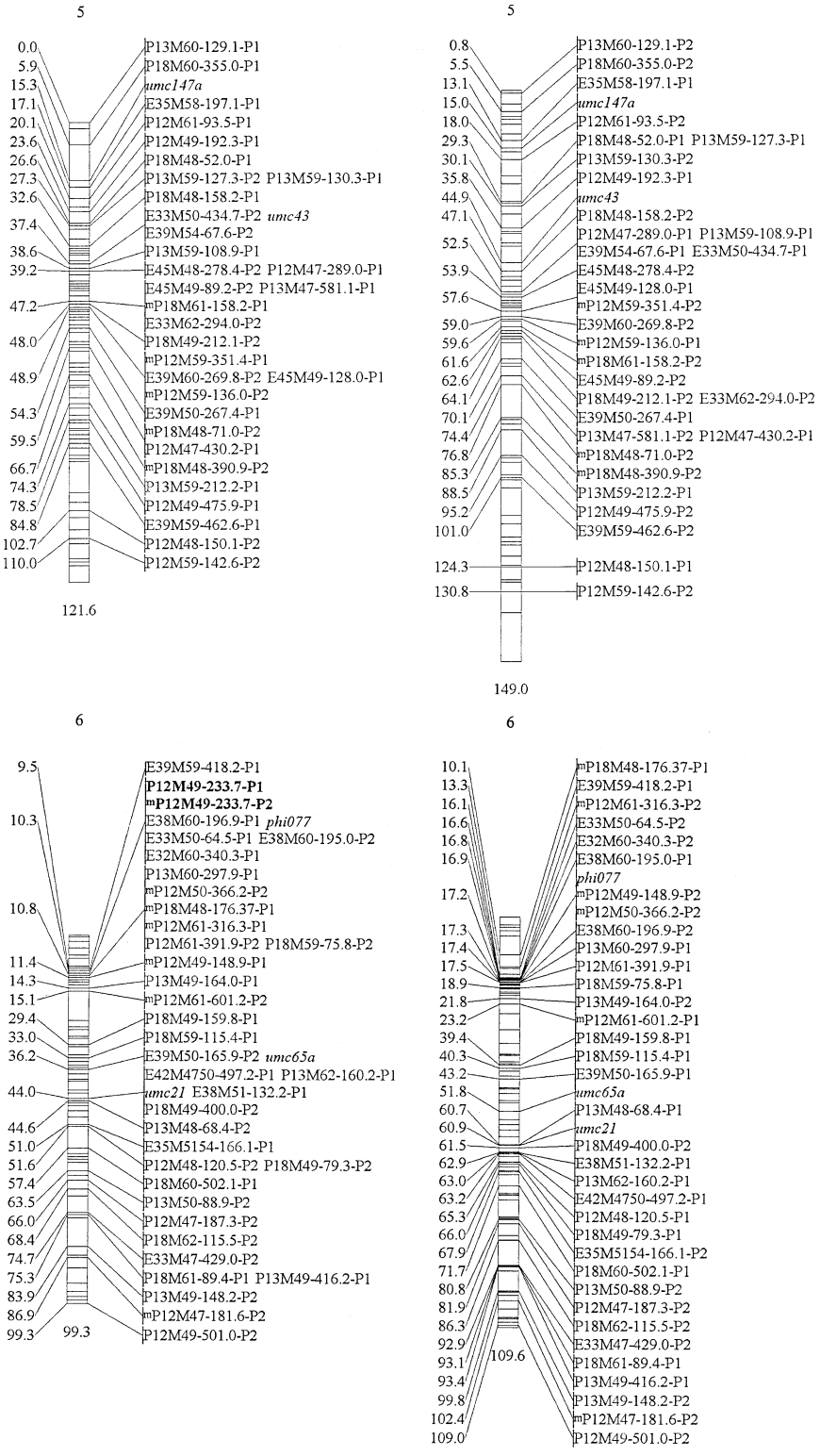
Fig. 1



Colinearity between the RI and the IF_2 high-density AFLP linkage maps

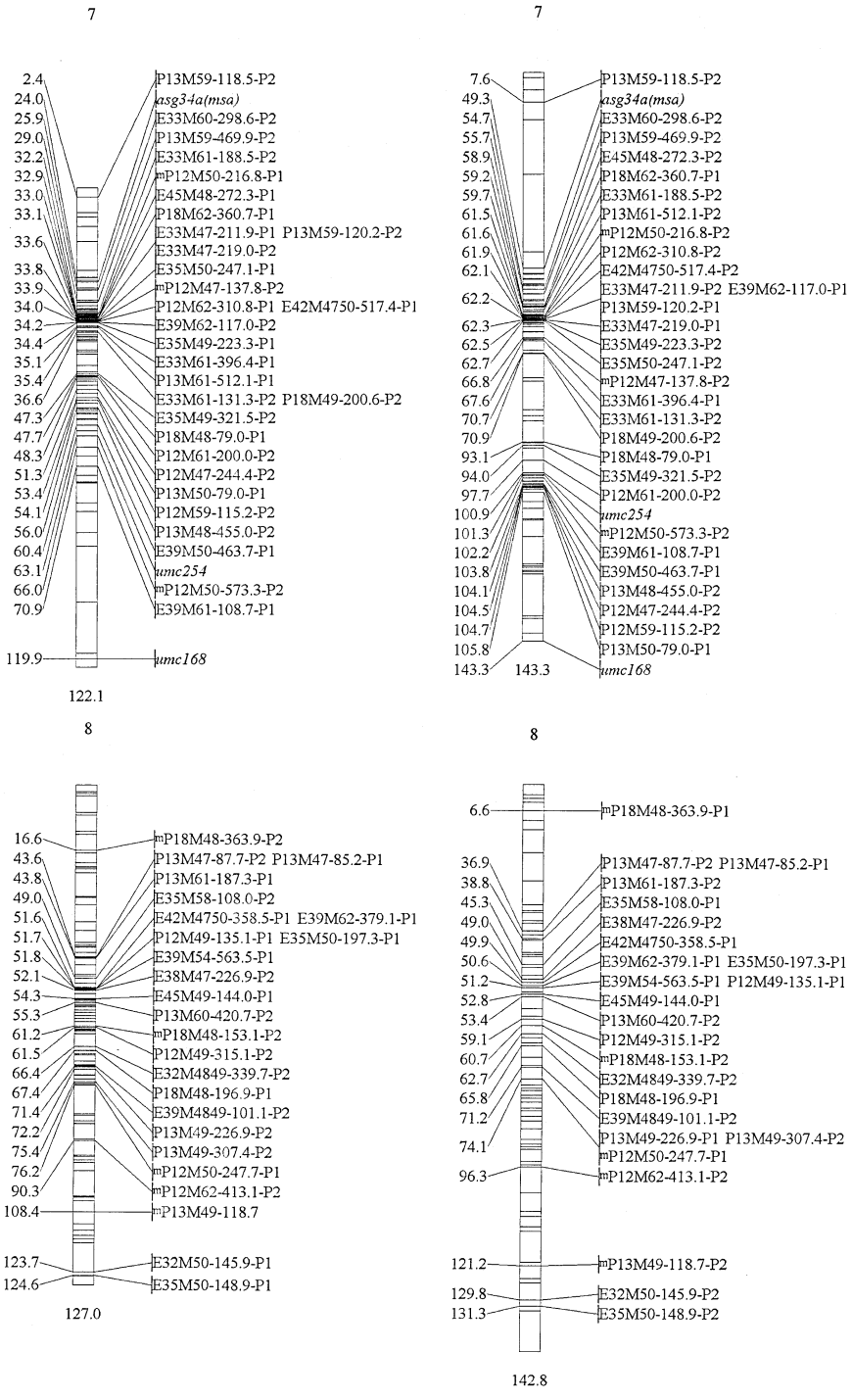
The two maps had 353 AFLP markers of identical size and amplified by the same PC, representing 23% and 26% of the AFLP markers mapped in RI and the IF_2 linkage map, respectively. For 327 out of these 353 AFLP markers, linkage maps of chromosomes were moderately (chromosomes 3, 4, 5, 6, 7 and 10) to highly (chromosomes 1, 2, 8, and 9) colinear (Fig. 1). Rearrangements occurred in the map order of some markers, predominantly those residing in or flanking marker-dense regions.

Fig. 1



With respect to the RI map in Fig. 1, major rearrangements occurred in chromosome 3 (38.0–51.0 cM; 64.9–77.6 cM), chromosome 4 (98.5–106.7 cM), chromosome 5 (47.2–48.9 cM), chromosome 6 (9.5–10.8 cM) and chromosome 7 (33.6–35.4 cM; 51.3–70.9 cM). Alternative marker orders, involving the inversion of 2 adjacent markers or segments of more than 2 markers, without any increase in total chromosomal map length, were evaluated. The most likely map location of a marker was given as a mean rank order and its variance. Variance in the rank order of markers residing in marker-dense regions was among the highest (data not shown), indicating

Fig. 1



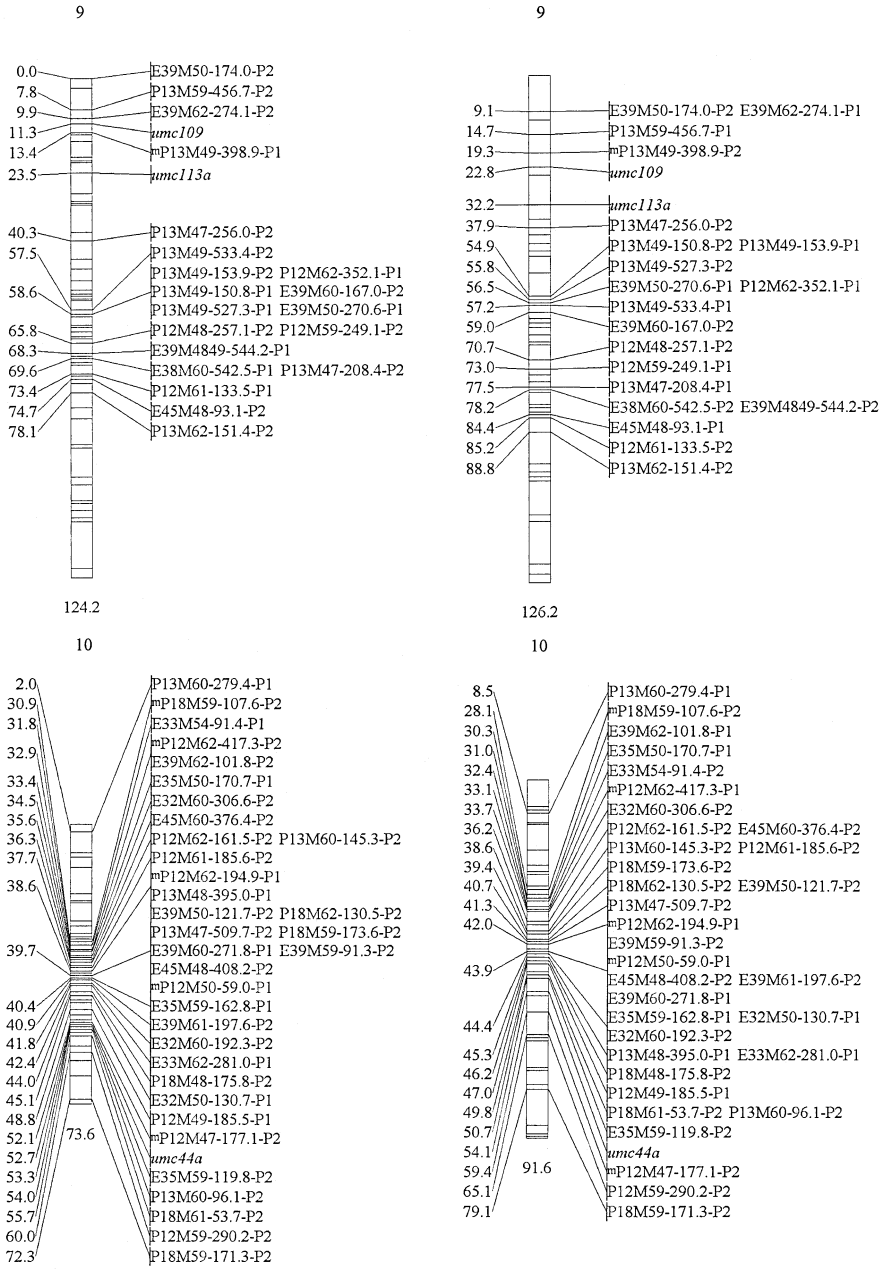
that different map orders in these specific chromosomal regions can be produced with the same dataset.

A total of 26 (7.4%) of the 'common' AFLP markers mapped to two different chromosomes. To elucidate the nature of this behavior, we chose 13 pairs of these 'common' markers from the two populations (estimated molecular size >100 bases) for comparison. For 3 pairs of markers, the SeqmanII™ module of Lasergene (DNASTAR, Madison, Wis., USA) could assemble a contig of each set, indicative for sequence identity. Although the distal sequences (restriction sites + selective nucleotides) were similar for the remaining 10 pairs of markers, they could not be build in contigs by SeqmanII™, suggesting a low homology between the pairs of sequences.

Distribution of the AFLP markers over the maize genome

Distribution of the E/M, P/M and ^{m}P/M markers was determined using the Kolmogorov-assay (Table 4). E/M markers are not randomly distributed on any of the ten

Fig. 1



linkage groups (P<0.05) in either map. In contrast, P/M markers are not randomly distributed on only four out of the ten linkage groups of the RI map (P<0.05) and on only two out of the ten linkage groups of the IF_2 map (P<0.05). The distribution of ^{m}P/M markers is intermediate, with a tendency to non-uniform distribution: ^{m}P/M markers are not randomly distributed (P<0.05) on nine and six of the ten linkage groups of the RI map and the IF_2 map, respectively.

Scanning for the largest cluster of AFLP markers revealed that for some linkage groups of the RI linkage map the largest cluster co-localized well with RFLP markers residing on the hypothetical centromeric region. The IF_2 map did not contain sufficient centromeric RFLPs to perform an identical analysis as described above. Nevertheless, clustering of AFLP markers on the IF_2 and RI map co-localized well. Taken together the results suggest that indeed large clusters of AFLP markers occur in the regions of the hypothetical centromeres of the maize chromosomes.

On the RI map, 44% of the E/M and 43% of the ^{m}P/M markers reside within the 5-cM windows co-localizing with the putative centromeres, whilst only 21% of the P/M markers reside on these clusters. Figure 3 shows the distribution of E/M, P/M and ^{m}P/M markers over the total length of linkage group 10 of the RI map and illustrates the strong clustering of E/M and ^{m}P/M markers in the centromeric region. The same pattern of distribution of E/M, P/M and ^{m}P/M markers was found for the nine other chromosomes of the RI map, although to a lesser extent.

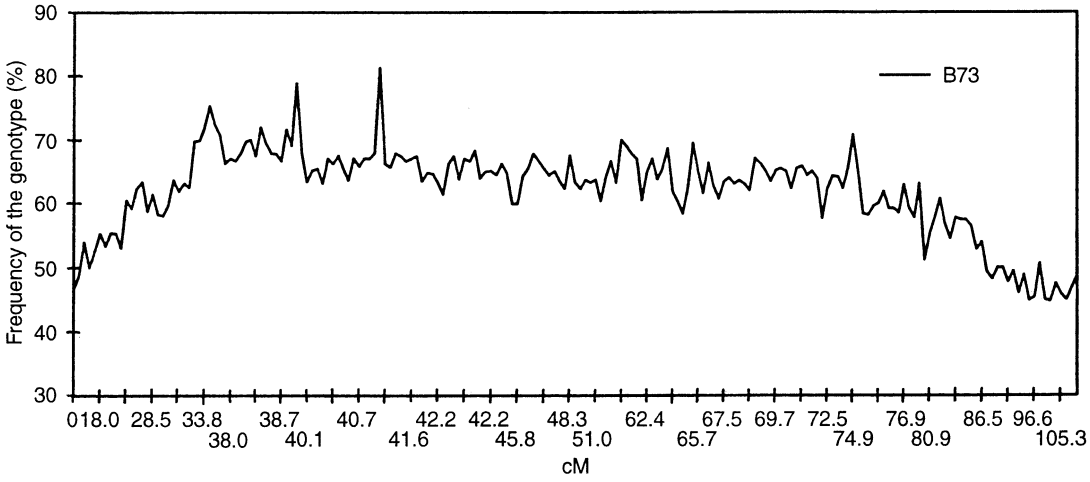


Fig. 2 Distorted segregation on the recombinant inbred (RI) chromosome 3 linkage map

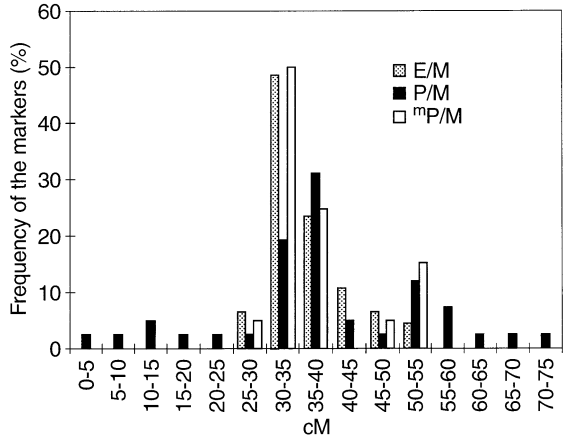


Fig. 3 Distribution of *Eco*RI/*Mse*I (*E/M*), *Pst*I/*Mse*I (*P/M*) and m*Pst*I/*Mse*I (*mP/M*) markers over the total length [expressed in centiMorgans (cM)] of the recombinant inbred (RI) chromosome 10 linkage map; clustering of *E/M* and *mP/M* markers in the centromeric region

Discussion

Construction of the RI and the IF_2 high-density AFLP maps of maize

The high multiplex ratio of the AFLP technique, combined with the high level of polymorphism of maize, was exploited to generate a large number of markers with relative ease. It is clear from the *EMM* that P/M PCs, rather than mP/M and E/M PCs, are to be chosen to minimize the mapping efforts while maximizing the number of markers to be mapped. Ideally, *EMM* equals *EM*. However, bands identified as polymorphic and taken up in the calculation of *EM* can be too close to allow reliable quantification and mapping. This causes a drop in *EMM* relative to *EM*. Furthermore, it should be emphasized that the technical skills that the performer displays in slabgel electrophoresis can highly influence the metric *EMM*. Any deviation from good laboratory practice may cause a drop in the *EMM*.

There are two additional explanations for the lower than expected *EMM* of mP/M markers, based on their high *EM*: (1) although mP/M PCs were screened and selected against highly abundant restriction fragments, numerous repetitive restriction fragments remain, leading to a poorer quality of the mP/M fingerprints; (2) an additional amplification step makes the methylation AFLP technique more complex than the AFLP technique. This affects the linear relation between band intensity and initial template concentration, thereby broadening the variances of the mixture components, and finally leads to inaccurate band classification and rejection of the marker.

Differences in genetic map length

Despite the larger number of markers, the total genome coverage and the length of the individual linkage groups in both maps are systematically shorter than the ones already published (Helentjaris et al. 1986, 1988; Burr et al. 1988; Beavis and Grant 1991; Shoemaker et al. 1992; Gardiner et al. 1993; Matz et al. 1994; Causse et al. 1995; Coe et al. 1995; Senior et al. 1997). This observation does not support the assumption that the complete coverage of the maize genome is approached asymptotically as the number of mapped markers increases. On the other hand, it does not show evidence that the larger the number of mapped markers, the more false recombinants are induced, resulting in inflation of the total genetic map length.

A difference in the map function used can hardly be the only explanation for the observed discrepancy in map length. A difference in mapping algorithm is a more plausible explanation. The maps reported above, with exception of that of Causse et al. (1995) were constructed with MAPMAKER (MM) (Lander et al. 1987), whereas in this study the JM package was used. To estimate the distance between a pair of adjacent markers, MM only uses the information of that pair of markers, whereas JM uses all of the pairwise recombination estimates in a da-

Table 4 Statistical determination of the distribution of the markers obtained from *Eco*RI/*Mse*I (E/M), *Pst*I/*Mse*I (P/M) and ^{m}*Pst*I/*Mse*I (^{m}P/M) enzyme combinations (EC) over the recombinant inbred (RI) and the Immortalized F_2 (IF_2) linkage maps of chromosomes. The Kolmogorov assay was used to test whether marker positions are independent and uniformly distributed over linkage maps of chromosomes. The test statistic D_n is defined as the largest difference between F(x) and $F_0(x)$ (D_n = max(F(x)-$F_0(x)$)), where (1) F(x) represents the observed distribution function of the interval (expressed incentiMorgans) between 2 adjacent markers, either 2 E/M, 2 P/M or 2 ^{m}P/M markers; and (2) $F_0(x)$ represents the corresponding distribution function under the null hypothesis (H_0); in this case we hypothesize that marker positions are independent and uniformly distributed over linkage maps of chromosomes

Chromosome	*EC*	**RI map** *Number of intervals*	*D_n*	**IF_2 map** *Number of intervals*	*D_n*
1	E/M	102	0.210**	61	0.189*
	P/M	80	0.194*	65	0.089
	^{m}P/M	57	0.291**	32	0.185
2	E/M	67	0.286**	73	0.252**
	P/M	61	0.084	68	0.195**
	^{m}P/M	31	0.373**	26	0.334**
3	E/M	76	0.197**	71	0.215**
	P/M	54	0.056	58	0.307**
	^{m}P/M	39	0.256**	25	0.481**
4	E/M	64	0.404**	71	0.245**
	P/M	52	0.184*	58	0.139
	^{m}P/M	32	0.342**	31	0.385**
5	E/M	54	0.226**	55	0.347**
	P/M	58	0.103	66	0.135
	^{m}P/M	31	0.231*	18	0.372**
6	E/M	64	0.365**	44	0.285**
	P/M	52	0.174	48	0.150
	^{m}P/M	34	0.314**	16	0.238
7	E/M	47	0.348**	60	0.311**
	P/M	38	0.154	33	0.192
	^{m}P/M	19	0.340*	17	0.484**
8	E/M	51	0.210*	52	0.181*
	P/M	42	0.204*	51	0.136
	^{m}P/M	22	0.207	16	0.077
9	E/M	66	0.346*	38	0.237*
	P/M	40	0.229*	30	0.153
	^{m}P/M	16	0.356*	13	0.267
10	E/M	48	0.326**	42	0.401**
	P/M	43	0.072	37	0.188
	^{m}P/M	21	0.391**	23	0.324**

**, * Significant at the 0.01 and 0.05 probability level, respectively

taset simultaneously. The other difference is the way mapping functions are applied. In the primary estimation procedure MM assumes absence of interference, and only afterwards adjacent recombination frequencies are translated to map distances with a given mapping function. In JM, on the contrary, all calculations are based on map distances that are obtained by applying a mapping function to recombination estimates, thus accounting for a given level of interference. Due to these differences in estimation procedure, JM will produce shorter maps than MM, whenever the assumed level of interference by Kosambi is less than the true degree of interference (P. Stam, personal results). Castiglioni et al. (1998) noticed also a drastic contraction in map length using JM compared to MM: analyzing segregating data obtained from 113 doubled haploid barley lines using MM with the ERROR DETECTION resulted in a map spanning 1597 cM; when JM was used, the total map length resulted in 1264 cM.

Despite the fact that both maps are produced with JM, the total genome coverage and the length of most linkage groups in the RI map are systematically shorter than in the IF_2 map. In addition to differences in true recombination rates and environmental conditions, differences in reliability of the data may affect the observed recombination rates. It is well known that even small error rates in genotyping leads to map inflation, especially in high-density maps (Lincoln and Lander 1992). The AFLP fingerprint patterns of the IF_2 population are more complex by nature (three zygosity classes), making genotyping more prone to misclassification. In the RI population, there is a more clear-cut difference in band intensities. This might explain the difference in total length between the IF_2 and RI map. So, population types with two instead of three genotype classes and a higher level of recombination are preferred to generate a high-density AFLP linkage map.

Distorted segregation ratios

It is not unusual to find distorted Mendelian segregation ratios in populations where a moderate numbers of markers were analyzed, but the observed conformity in segregation distortion across major genomic regions in chromosome 3 in both high-density AFLP linkage maps is striking and can hardly be explained by sampling bias.

The excess of D32 alleles at the major genomic region in chromosome 3 in the IF_2 map is in good agreement with the excess of B73 alleles at chromosome 3 in the RI map: D32 is partially composed of BSSS genetic material, while B73 is a BSSS inbred line. Also Lübberstedt et al. (1998), analyzing a KW1265 × D145 and a D145 × KW1292 F_4 population, found underrepresentation of the D145 allele in a region of chromosome 3 comprising *umc10* and *bnl6.06*. In contrast, neither Senior et al. (1997), genotyping 192 RI lines of the same B73 × Mo17 RI population, nor Beavis and Grant (1991), analyzing a B73 × Mo17 F_2 population, found evidence for segregation distortion on chromosome 3. Only Gardiner et al. (1993) found distorted segregation towards a heterozygote excess on chromosome 3 between markers *umc92* and *bnl5.37*.

Colinearity between the RI and the IF_2 linkage maps

Although the two populations have no parent in common, and the four parental lines are not highly related lines, sufficient common AFLP markers were generated to align the two maps. The comparison between the two linkage maps, based on the 92.6% of common markers, shows that linkage maps of chromosomes were, despite

some rearrangements, moderately to highly colinear (Fig. 1). Rearrangements in the map order of some markers, predominantly those residing in or flanking marker-dense regions, were also observed by Castiglioni et al. (1998). Mapping using JoinMap results in the most likely marker order according to the parameter settings. However, especially when the number of informative recombination events in a particular region is low, for example, the centromeric region, alternative marker orders with an equivalent goodness of fit are possible.

For the 7.4% 'common' AFLP markers that map to different chromosomes in both maps, there are two plausible explanations: (1) coincidental co-migration of two non-related AFLP fragments or (2) areas of genomic duplications in the maize genome. Helentjaris et al. (1988) observed that 29% of their cloned maize sequences hybridized to at least two different genomic regions. These duplicate loci suggested that the maize genome either contains a partial duplication or is tetraploid in origin. Recent analyses confirmed the tetraploid nature of the maize genome, possibly being derived from the hybridization of two parents with different arrangements of rice linkage segments constituting their chromosomes (Moore 1995). However, for two unrelated maize crosses, AFLP markers of equal size and generated by the same PC map in more than 90% of the cases to the same map location, making the transfer of AFLP markers of one population to the other feasible with minor risk. The expectations are that for two more related maize crosses, or for two maize crosses with one common parent, the frequency of these 'ambiguous' common markers will decrease.

Distribution of the AFLP markers over the maize genome

Considering that the underlying basis for AFLP polymorphisms (point-mutation or insertion/deletion) is evenly distributed over the DNA and that rare-cutter sites are also randomly distributed, one could assume an even sampling of the physical genome. However, the observed tendency is that genetically there is an overrepresentation of the centromeres. So, clustering of AFLP markers around the centromere raises the question as to whether recombination is predominantly confined to the distal regions, with the centromeric regions being recombination 'cold spots'. Investigation of the genetic location of AFLP markers obtained with different restriction enzymes and with different levels of methylation revealed that clusters which co-localize with the putative centromeric regions of maize are enriched especially by E/M and ^{m}P/M markers, while the P/M markers are shown to be more randomly spread over the genome.

The clustering of E/M markers in specific chromosomal regions also appears in other plant AFLP linkage maps, such as potato (van Eck et al. 1995), barley (Becker et al. 1995; Powell et al. 1997; Qi et al. 1998), soybean (Keim et al. 1997) and *Arabidopsis* (Alonso-Blanco et al. 1998). In *Arabidopsis* it was shown that pericentromeric heterochromatin fluoresces brightly when stained with the fluorochrome DAPI (Ross et al. 1996), which is known to show preference for AT-rich DNA. This is a plausible explanation of the enrichment of E/M AFLP markers in the *Arabidopsis* centromeres (Alonso-Blanco et al. 1998) and possibly in other plant genome centromeres, since the restriction enzymes *Eco*RI and *Mse*I have AT-rich target sequences (*Mse*I recognizes 5'-TTAA-3', while *Eco*RI recognizes 5'-GAATTC-3').

In maize the heterochromatin, enriched with methyl groups, is concentrated in the centromeric regions, the nucleolus organizer region, telomeres and knobs, mainly consisting of particular regions that are not transcribed. Consequently, the lower frequency of P/M markers and the clustering of ^{m}P/M markers in the centromeric regions is consistent with the enrichment of methyl groups in heterochromatin.

The good agreement between the prevalence of P/M markers in the distal genomic regions, which harbor the gene spaces, and the prevalence of P/M markers among the bi-allelic markers can be explained as follows: maize transposable elements, which can easily induce allelism by integration and deletion, show a preference for gene-rich transcriptionally active regions. Thus, P/M PCs give not only a better genome coverage with fewer markers but also plausible landmarks for genes.

Conclusion

The high multiplex ratio of the AFLP technique, combined with the high polymorphism rate of maize, was exploited to generate a large number of markers. The highly effective mapped multiplex ratio of AFLP in maize has the potential to improve the efficiency of genetic map construction of maize and to generate high-density maps around loci that control commercially important traits.

To our knowledge this is the first detailed report of mapping C-methylation and epi-alleles. Although the rules governing the transmission of methylation from one generation to the next are still unclear, we were able to show that C-methylation can be inherited in a Mendelian way. ^{m}AFLP markers are also of practical use in genome research. Like AFLP markers, most ^{m}AFLP markers correspond to unique positions in the genome, and, hence, can be exploited as landmarks in and as bridging tools between, genetic and physical maps. Native methylated sites are present on cloned DNA segments, for example, yeast artificial chromosomes (YACs) and bacterial artifical chromosomes (BACs), as unmethylated sites. Hence, native methylated sites can not be distinguished from native unmethylated sites on a physical map. However, lining up the physical map with a genetic map containing ^{m}AFLP markers may help to identify native methylated sites on the physical map.

Beside top-down anchoring of physical maps and mapping commercially important traits like grain yield, these linkage maps can be used (1) for map-based AFLP

fingerprinting of maize inbred lines in order to determine the levels of genetic diversity in different regions of the maize genome and (2) as main framework in a unified AFLP linkage map for maize. Although the relative map position of markers in an integrated linkage map is less reliable due to statistical errors associated with the recombination estimates and to differences in recombination frequency among crosses, an integrated AFLP linkage map for maize is an inexhaustible resource of markers, encouraging the use of the AFLP technique in maize breeding.

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