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AFLP markers in a molecular linkage map of maize: codominant scoring and linkage group ditsribution

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Abstract We exploited the AFLP technique to saturate a RFLP linkage map derived from a maize mapping population. By using two restriction enzyme, *Eco*RI and *Pst*I, differing in methylation sensitivity, both in combination with *Mse*I, we detected 1568 bands of which 340 where polymorphic. These were added to the exitsing RFLP marker data to study the effects of incorporation of AFLPs produced by different restriction-enzyme combinations upon genetic maps. Addition of the AFLP data resulted in greater genome coverage, both through linking previously separate groups and the extension of other groups. The increase of the total map length was mainly caused by the addition of markers to telomeric regions, where RFLP markers were poorly represented. The percentage of informative loci was significantly different between the *Eco*RI and *Pst*I assays. There was also evidence that *Pst*I AFLP markers were more randomly distributed across chromosomes and chromosome regions, while *Eco*RI AFLP markers clustered mainly at centomeric regions. The more-random ditsribution of *Pst*I AFLP markers on the genetic map reported here may reflect a preferential localisation of the markers in the hypomethylated telomeric regions of the chromosomes.

Key words Genetic map · Linkage analysis · AFLP · Methylation sensitivity · Codominant markers · *Zea mays* L.

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Introduction

Extensive genome mapping based on DNA restriction fragment length polymorphism (RFLP) markers has been accomplished in many crop species (O'Brien 1993). These maps and their associated technology have been used successfully for a number of applications in genetic research and breeding, including gene tagging, evolutionary studies, marker-aided selection, and the analysis of quantitative trait loci (QTLs; for a review see Lee 1995, and references therein). However, RFLP analysis is an expensive and time-consuming technology and may not provide detailed coverage throughout the genome, which is a prerequisite for QTL analyses.

The development of the polymerase chain reaction (PCR; Saiki et al. 1988) has expanded the repertoire and efficiency of available DNA marker systems, which include the AFLP method (Vos et al. 1995). The advantage of the AFLP assay over other DNA marker techniques includes the detection of a larger number of polymorphisms from a single PCR reaction within a very short period of time, and the requirement for small amounts of DNA, thus reducing expenses and expediting the construction of high-density linkage maps. As described in many comparative studies AFLP is considered to be an efficient marker technology due to its high multiplex ratio (Powell et al. 1996; Pejic et al. 1998). The AFLP approach has recently been used to rapidly create linkage maps in a variety of plant species (Maheswaran et al. 1997; Alonso-Blanco et al. 1998; Castiglioni et al. 1998; Lu et al. 1998).

With the aim of exploiting AFLP markers in a maize genome-mapping program, we assayed the AFLP codominantly to test the distribution of these marker loci on the maize linkage groups, and to investigate enzyme combinations differing in sensitivity to DNA methylation.

Table 1 AFLP primer combinations generating polymorphic products after *Eco*RI/*Mse*I and *Pst*I/*Mse*I enzyme digestion, and distribution of AFLP markers

Primer combination	3′ Selective nucleotides		Visible bands (no.)	Polymorphic bands (no.)	Polymorphism (%)	Chromosomes covered (no.)
	*Eco*RI-*Pst*I	*Mse*I				
E32/M50	AAC	CAT	84	7	8.3	4
E33/M50	AAG	CAT	95	7	7.4	5
E33/M61	AAG	CTG	83	13	15.7	6
E35/M49	ACA	CAG	59	11	18.6	5
E35/M50	ACA	CAT	78	23	29.5	7
E38/M47	ACT	CAA	88	22	25.0	7
E38/M51	ACT	CCA	67	20	29.9	8
E32/M55	AAC	CGA	49	10	20.4	6
E32/M60	AAC	CTC	81	14	17.3	6
E33/M47	AAG	CAA	116	19	16.4	6
E33/M51	AAG	CCA	85	18	21.2	8
Average			80.5	14.9	18.5	
P12/M47	AC	CAA	74	19	25.7	7
P12/M48	AC	CAC	64	16	25.0	6
P12/M49	AC	CAG	75	26	34.7	9
P12/M50	AC	CAT	80	17	21.3	4
P12/M59	AC	CTA	70	20	28.6	8
P12/M61	AC	CTG	68	18	26.5	9
P12/M62	AC	CTT	78	18	23.1	5
P13/M50	AG	CAT	86	23	26.7	6
P13/M59	AG	CTA	88	19	21.6	5
Average			75.9	19.6	25.8	
Total			1568	340	21.9	

Materials and methods

Plant materials and DNA extraction

Two-hundred and twenty nine F_3 families, each tracing back to an individual F_2 plant, derived from a cross between the maize inbred lines B73 and A7, were used. This population has been described previously to construct an RFLP linkage map (Ajmone-Marsan et al. 1995). B73 and A7 are inbred lines, belonging to the ‘‘Stiff Stalk Synthetic’’ (BSSS) and ‘‘Lancaster Sure Crop’’ (LSC) heterotic groups, respectively. For DNA extraction, the seedlings of each F_3 family were grown in a growth chamber at 25°C with a 16-h photoperiod for 2 weeks. Genomic DNA was extracted from a pool of 15–20 shoots of each F_3 family using the CTAB method (Saghai-Maroof et al. 1984).

AFLP analysis

The protocol adopted for the generation of AFLP markers was essentially the same as that described by Vos et al. (1995). DNA was digested with an *Eco*RI/*Mse*I or *Pst*I/*Mse*I enzyme combination (EC). Two selective nucleotides for the *Pst*I primers were used instead of the three normally employed for *Eco*RI primers. Genomic DNA digested with *Pst*I/*Mse*I, where *Pst*I is a restriction enzyme sensitive to cytosine 5′-methylation in the sequences 5′-CNG-3′, results in a number of restriction fragments lower than in the *Eco*RI digestion. AFLP fingerprints were visualised using a Fuji BAS-2000 Phosphorimage analysis system.

Scoring AFLP markers

For the analysis of the complex AFLP fingerprint patterns, were used proprietary software developed specifically for AFLP analysis, at Keygene N.V. This software allows the identification and measurement of AFLP bands in a pixel image as produced by the Fuji BAS-2000. As a result, the presence/absence of a band can be scored. With refined quantification procedures, heterozygosity (corresponding to a 50% band intensity of homozygous bands) can also be identified. AFLP markers were codominantly scored (‘‘A’’ = homozygous band presence, ‘‘H’’ = heterozygous band presence, ‘‘B’’ = homozygous band absence) using code ‘‘C’’ (either ‘‘H’’ or ‘‘B’’) and ‘‘D’’ (either ‘‘A’’ or ‘‘H’’) for bands having intensities between heterozygous and homozygous. Markers not fitting the expected classes of intensities were excluded from further analysis. The bands were named with the capital letter of the six-cutter enzyme followed by the number referring to a certain nucleotide selectivity and the size of amplified product (i.e. P1262189 corresponds to a marker produced with the *Pst*12/*Mse*62 primer combination and a size of 189 bp). A7 and B73 AFLP pattern reporting the mapped bands is available on request.

Three-hundred and forty AFLP markers and 73 previous assayed RFLPs, were tested for their segregation according to the 1:2:1 expected Mendelian ratio using chi-square analysis. Markers showing distorted segregation ($P \leq 0.001$) were rejected; the remaining markers were used for map construction as well as to estimate the relative heterozygosity and the percentage of parental genome in each F_2 genotype. All calculations were performed with a PLABQTL software package (Utz and Melchinger 1996).

Map construction

Mapping was carried out using Mapmaker software (Lander et al. 1987; PC version/exp 3.0). Fifty six RFLP markers evenly spread over the genome and mapping in agreement with a maize reference map (Davis et al. 1998) were used as anchor probes. AFLP markers were assigned to chromosomes carrying the anchor RFLPs by two-point linkage analysis using a minimum LOD of 6.0 and a 50-cM maximum distance as significant thresholds. The main marker framework was built using a minimum LOD threshold of 3.0 to infer the most probable marker order along each chromosome. Remaining markers were placed on the map but did not contribute to the final map length. Finally, permutations among flanking markers were used to verify possible ambiguities.

To investigate the distribution of AFLP markers over tha maize genome, we have compared the number of markers present on each

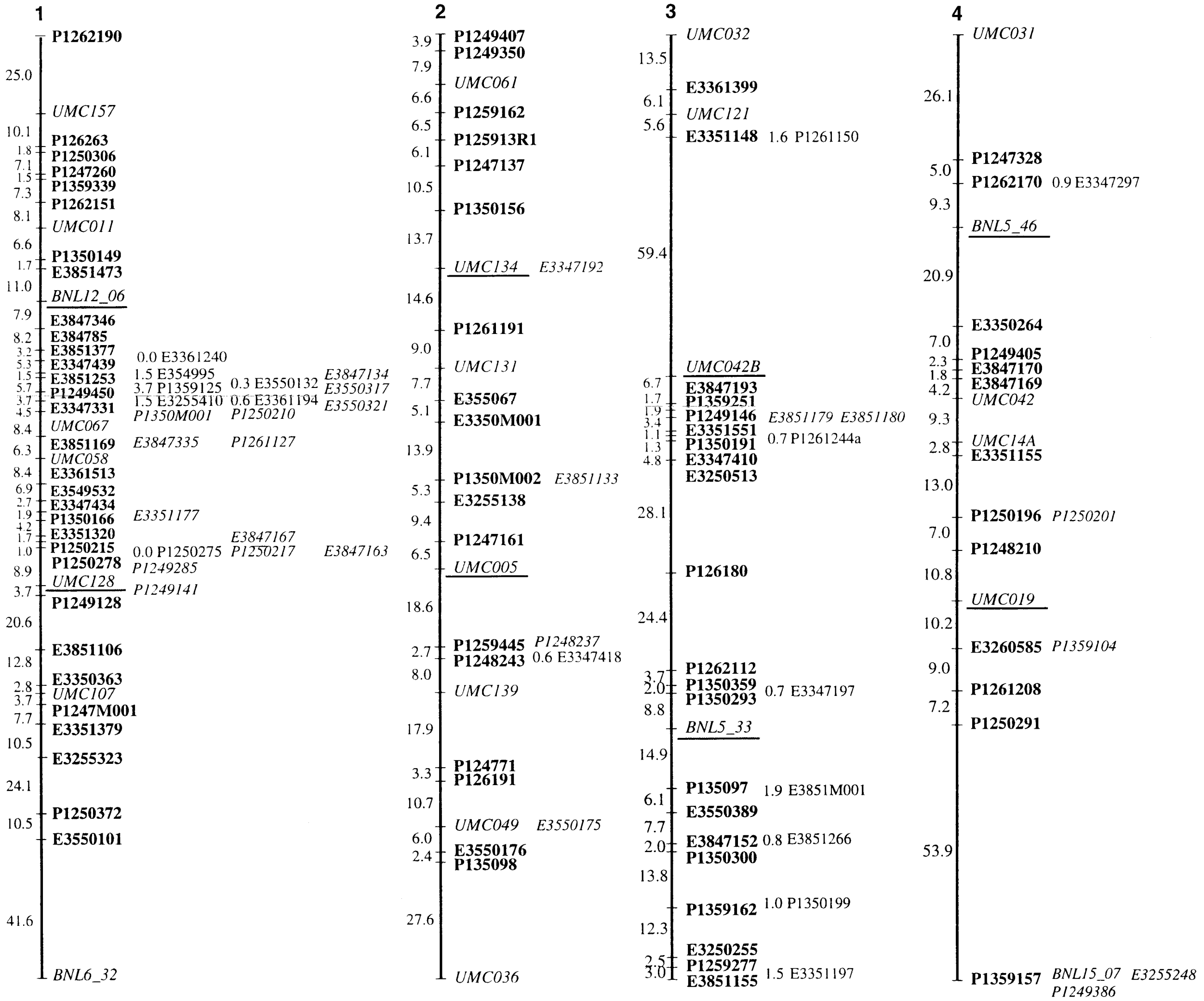


Fig. 1 Linkage map of the ten maize chromosomes based on the F_2 mapping population derived from the cross of inbred lines B73 and A7. To define chromosome regions, RFLP probes which localise centromeric regions on the reference map by Davis et al. (1998), and in common with our map, have been selected (*underlined markers*). Map distances, on the left side of the bars are in centimorgans (cM) calculated using the Haldane function

chromosome as well as on centromeric and non-centromeric (herein named "telomeric") chromosome regions, defined as reported in Fig. 1, to the expected number following a random distribution of AFLP markers over the genome. The expected values were calculated as: (1) relative chromosome length, in percentage of the genome, multiplied by the total number of mapped markers; (2) within each specific chromosome, relative length of the telomeric and centromeric regions, expressed in percentage of the chromosome length, multiplied by the number of markers present in that chromosome. To avoid bias, the selection of RFLP markers defining chromosome regions and all calculations of the relative lengths were based on the reference map reported by Davis et al. (1998).

Results

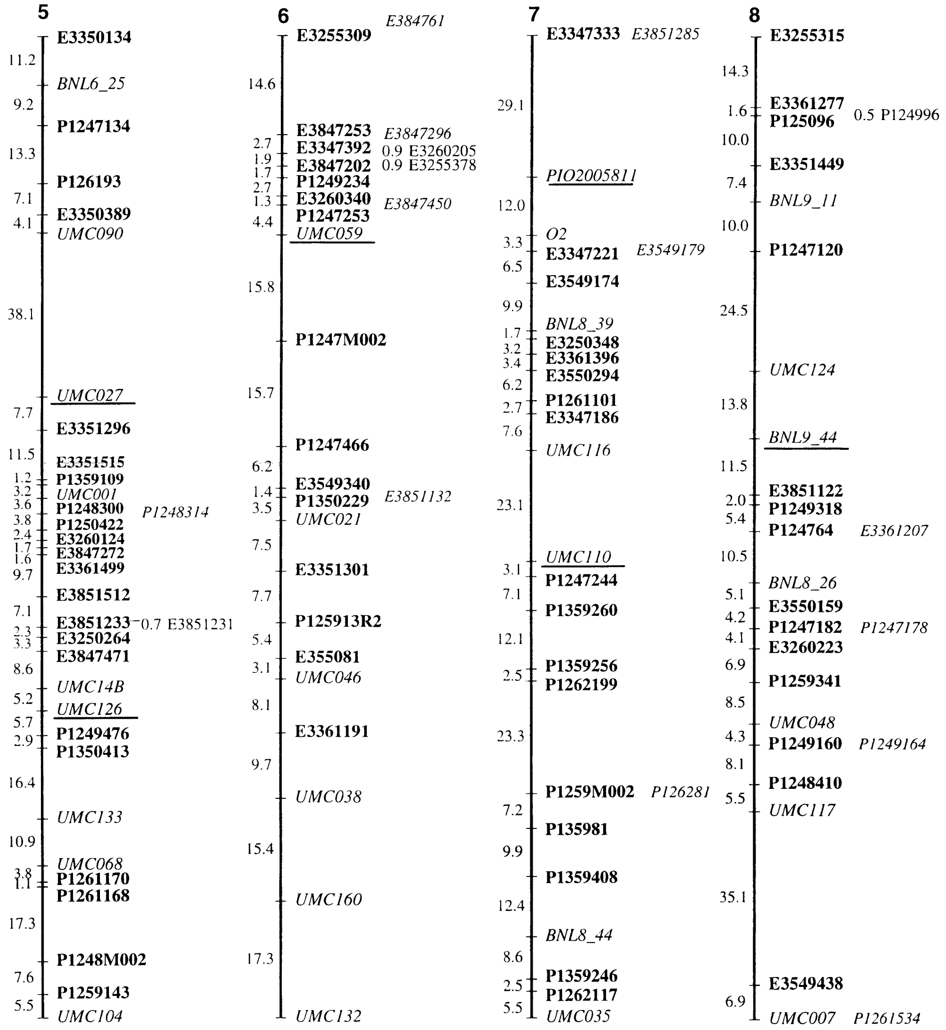
AFLP polymorphism

A survey of different primer combinations (PCs), the number of visible bands, polymorphisms, and the distribution of AFLP markers across chromosomes is shown in Table 1. The F_2 mapping population was assayed with a total of 79 RFLP probe-enzyme combinations and 20 AFLP PCs. A total of 1568 AFLP bands were amplified by the 20 PCs (885 by *Eco*RI/*Mse*I and 683 by *Pst*I/*Mse*I PCs). A total of 340 out of 1568 bands were polymorphic (21.7%) ranging from 7.4% to 34.7% for individual primer combinations. The percentage of informative loci was significantly different between the *Eco*RI and *Pst*I assays; in fact, *Eco*RI/*Mse*I and *Pst*I/*Mse*I produced averages of 14.9 and 19.6 polymorphic fragments per PC, respectively. In addition, the profiles generated by *Pst*I/*Mse*I PCs were clearer and easier to score due to a lower number of bands per gel and a reduced background.

Segregation of AFLP markers

The majority (90.3%) of markers showed a 1:2:1 segregation ratio for the two parental alleles ($P < 0.05$), as expected in an F_2 population. Among the three RFLP and 37 AFLP markers with distorted segregation, 22 were skewed towards B73 alleles and 17 towards A7 alleles, while a single AFLP marker showed an excess of heterozygosity. Those markers displaying anomalous results

Fig. 1 (Continued)



were eliminated from further analysis. Considering the information collected from the remaining 373 loci, the percentage of the B73 genome in the F_2-derived progenies was on average 49.6%, ranging from 24.9 to 68.3%. Mean heterozygosity in the experimental progenies was 49.4%, ranging from 26.1 to 86.6%. For each marker system, the averages of parental genome contribution and heterozygosity were in agreement with expectation (49.6% for RFLP vs 49.6% for AFLP markers of the B73 genome, and 51.7% vs 48.7% of the average heterozygosity).

Map construction

The 373 markers (71 RFLPs and 302 AFLPs) used for mapping produced a data set of 85 417 potentially informative data points. Two-point linkage analysis revealed that all the 317 non-anchor markers were linked to one of the ten chromosomes defined by the 56 RFLP anchor probes. Sixty two markers (1 RFLP and 61 AFLP markers) were not consistently ordered along the respective chromosomes by multi-point analysis and, therefore, were not included in the map.

Table 2 Number of observed and expected (in brackets) AFLP markers across chromosomes

Chromosome	Total	Chromsome region	
		Centromeres	Telomeres
1	51 (*34.9*)*	34 (*19.6*)*	17 (*31.4*)*
2	23 (*28.5*)	8 (*8.9*)	15 (*14.2*)
3	30 (*23.5*)	15 (*11.6*)	15 (*18.4*)
4	18 (*24.2*)	8 (*6.5*)	10 (*11.5*)
5	24 (*24.9*)	14 (*8.9*)*	10 (*15.2*)*
6	21 (*24.0*)	12 (*4.3*)*	9 (*16.7*)*
7	20 (*21.0*)	8 (*8.2*)	12 (*11.8*)
8	20 (*23.9*)	6 (*6.9*)	14 (*13.1*)
9	19 (*21.4*)	6 (*6.7*)	13 (*12.3*)
10	20 (*19.7*)	12 (*7.4*)*	8 (*12.6*)*
Total	246	123 (*88.8*)*	123 (*157.2*)*

* Significantly different at $P \leq 0.05$

The final map contained 312 markers (66 RFLPx and 246 AFLP markers) covering a distance of 2057 cM, corresponding to approximately 6.6 cM per marker (Fig. 1). Chromosome 1 had the largest number of markers with the longest genetic distance; chromosome 9 dis-

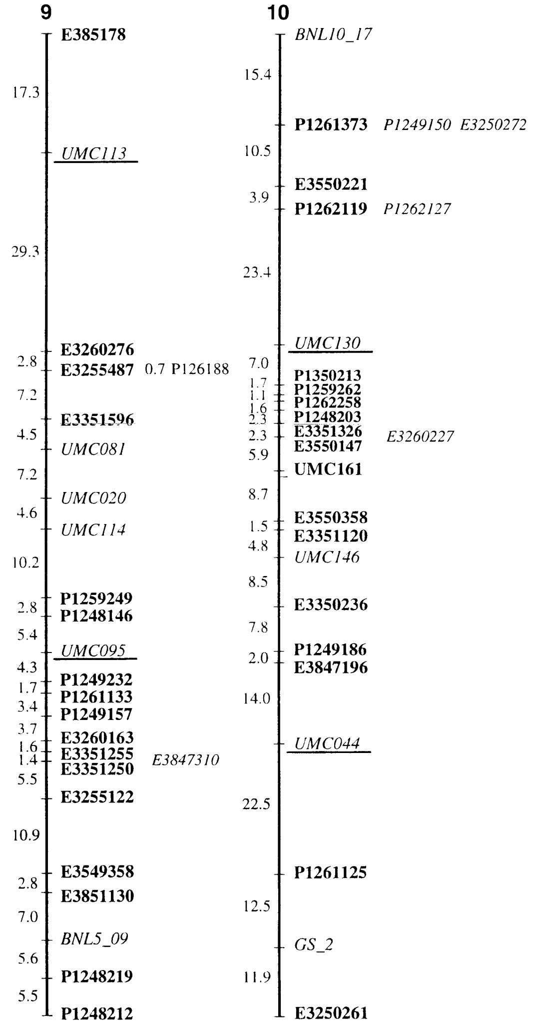


Fig. 1 (Continued)

played the shortest genetic length, while chromosome 4 had the lowest number of markers. Despite the relatively small average distance between markers, five gaps larger than 30 cM, located on chromosomes 1, 3, 4, 5 and 8, were still present on the map.

The AFLP-enriched map was 440-cM longer than the previous RFLP map (Ajmone-Marsan et al. 1995). Map expansion was largely caused by the addition of telomeric AFLP markers located in genomic regions previously uncovered by RFLPs (342 cM added), while distances between anchor RFLP markers remained almost unchanged (98 cM added). A high correlation (r = 0.86) between chromosome length and number of markers per chromosome was found. On chromosomes 1 and 3, the AFLP markers were useful to link two RFLP probes (bnl 6.32 and bnl 5.33, respectively) that were not previously associated with any linkage group.

Marker distribution

The ability of AFLP markers to uniformly cover the maize genome has been investigated analysing the expected and observed marker distribution across chromosomes by chi-square test. In Table 2 the observed and expected number of AFLP markers for each chromosome are reported. The distribution of these markers across chromosomes was random, with the exception of chromosome 1; within chromosomes, AFLP markers were significantly more frequent than expected in the centromeric regions of chromosomes 1, 5, 6 and 10.

*Eco*RI/*Mse*I AFLP markers appeared well distributed over the genome, with the exception of chromosome 1, were a clustering of markers was found (Table 3). Interestingly, the distribution of markers along chromosomes showed that the *Eco*RI-generated AFLP markers localise preferentially in centromeric regions. A similar inspection indicated a significantly different distribution of *Eco*RI markers than expected for chromosomes 1, 5, 6, 7 and 10, with a notable concentration of markers in

Table 3 Number of observed and expected (in brackets) *Eco*RI- and *Pst*I-based AFLP markers along maize chromosome

Chromosome	*Eco*RI markers			*Pst*I markers		
	Total	Chromosome region		Total	Chromosome rerion	
		Centromeres	Telomeres		Centromeres	Telomeres
1	29 (*17.7*)*	23 (*11.1*)*	6 (*17.9*)*	22 (*17.2*)	11 (*8.4*)	11 (*13.6*)
2	8 (*14.5*)	5 (*3.1*)	3 (*4.9*)	15 (*14.0*‘)	3 (*5.8*)	12 (*9.2*)
3	16 (*11.9*)	7 (*6.2*)	9 (*9.8*)	14 (*11.5*)	8 (*5.4*)	6 (*8.6*)
4	7 (*12.3*)	4 (*2.5*)	3 (*4.5*)	11 (*11.9*)	4 (4.0)	7 (*7.0*)
5	12 (*12.6*)	10 (*4.4*)*	2 (*7.6*)*	12 (*12.2*)	4 (*4.4*)	8 (*7.6*)
6	15 (*12.2*)	10 (*3.1*)*	5 (*11.9*)*	6 (*11.8*)	2 (*1.2*)	4 (*4.8*)
7	9 (*10.7*)	7 (*3.7*)*	2 (*5.4*)*	11 (*10.3*)	1 (*4.5*)*	10 (*6.5*)*
8	8 (*12.1*)	3 (*2.8*)	5 (*5.2*)	12 (*11.7*)	3 (*4.2*)	9 (*7.8*)
9	11 (*10.9*)	3 (*3.9*)	8 (*7.2*)	8 (*10.5*)	3 (*2.8*)	5 (*5.2*)
10	10 (*10.0*)	7 (*3.7*)*	3 (*6.3*)*	10 (*9.7*)	5 (*3.7*)	5 (*6.3*)
Total	125	79 (*44.5*)*	46 (*80.5*)*	121	44 (*44.4*)	77 (*76.6*)

* Significantly different at $P \leq 0.05$

the centromeric regions. *Pst*I/*Mse*I-generated markers were randomly distributed among and along chromosomes with the exception of chromosome 7, where an excess of AFLP markers was observed in the telomeric regions.

Discussion

In this study we were able to detect 1568 visible bands and map 246 AFLP markers covering 2057 cM. Our data are in good agreement with previous studies (Dudley et al. 1991; Smith et al. 1997; Ajmone-Marsan et al. 1998, and references therein), which reported that the degree of polymorphism in maize detectable by DNA markers is very high. The efficiency of generating AFLP markers appears substantially higher relative to RFLP mapping in the same population (Ajmone-Marsan et al. 1995), and the speed at which they can be generated shows a great potential for application in marker-assisted breeding. The appropriate selection of primer combinations that generates a high level of polymorphism with markers well-distributed over the genome plays a crucial role. We have observed that some primer combinations produced as many as 19 polymorphic markers distributed over as many as nine chromosomes.

The majority of AFLP markers (89.1%) followed Mendelian segregation. They showed allelic frequencies in agreement with expectation, and were unambiguously placed on linkage groups (72.4%). The addition of a large number of AFLP markers to the map did not disturb the original order or the relative distances of the previously mapped RFLP markers (Ajmone-Marsan et al. 1995). In contrast, substantial expansios of linkage maps were found in similar mapping studies in other crops (Becker et al. 1995; Cho et al. 1998). In the experiment reported here, the assay of a relatively large mapping population, the high level of informativeness of codominant scored AFLP markers and the rejection of markers with unexpected behaviour, have probably minimised the map inflation; typing errors have been credited to be in part responsible for map inflation (Lincoln and Lander 1992).

By adding AFLP markers, we generated a map which is 440-cM longer than the map generated with RFLP markers alone (Ajmone-Marsan et al. 1995). The increase of the total map length was mainly caused by the addition of markers to telomeric regions, where RFLP markers were poorly represented. The current study indicated that *Pst*I/*Mse*I PCs were more efficient in detecting polymorphism than *Eco*RI/*Mse*I primers. In addition, *Pst*I/*Mse*I AFLP markers are more randomly distributed across chromosomes and chromosome regions, while *Eco*RI/*Mse*I AFLP markers clustered mainly on centromeric regions and on chromosome 1. Specific regions were observed, in which only markers produced with either *Pse*I/*Mse*I or *Eco*RI/*Mse*I restriction-enzyme combinations were located (i.e. 1S, 2S, 5L, 7S and 7L). A high degree of clustering of AFLP markers around the centromeres was a notable feature also in wheat (Hart 1994), barley (Castiglioni et al. 1998), rice (Nandi et al. 1997), and potato (VanEck et al. 1995); this may be attributed to suppressed recombination, which stems from a direct inhibitory effect on recombination of the centromere itself and/or adjacent centromeric heterochromatin. These findings are in agreement with previous studies suggesting that centromeric regions are embedded in repetitive sequences (Peacock et al. 1981). As the amplification products generated by the *Eco*RI/*Mse*I AFLP technique map contain repetitive sequences, there is a higher probability of identifying *Eco*RI/*Mse*I AFLP markers than *Pst*I/*Mse*I AFLP markers and RFLPs in highly repetitive regions near the centromeres.

The more random distribution of *Pst*I/*Mse*I-based AFLP markers on the genetic map reported here may reflect a preferential localisation of the markers in the hypomethylated non-centromeric regions of the chromosomes. There is considerable evidence that hypomethylated regions of the maize genome are associated with genes (Bennetzen et al. 1994, and references therein) and that recombination occurs primarily within genes, or perhaps unique sequences, and rarely in intergenic regions (Dooner and Martinez-Perez 1997, and references therein; Okagaki and Weil 1997). These observations fit previous findings concerning the presence of large amount of repetitive sequences in the maize genome and their preferential chromosomal distribution in centromeric regions, while genes seem to concentrate in non-centromeric regions (Carels et al. 1995, and references therein).

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