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An integrated high-density RFLP-AFLP map of tomato based on two *Lycopersicon esculentum* × *L. pennellii* F_2 populations

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Abstract Two independent F_2 populations of *Lycopersicon esculentum* × *L. pennellii* which have previously been investigated in RFLP mapping studies were used for construction of a highly saturated integrated AFLP map. This map spanned 1482 cM and contained 67 RFLP markers, 1078 AFLP markers obtained with 22 *Eco*RI + *Mse*I primer combinations and 97 AFLP markers obtained with five *Pst*I + *Mse*I primer combinations, 231 AFLP markers being common to both populations. The *Eco*RI + *Mse*I AFLP markers were not evenly distributed over the chromosomes. Around the centromeric region, 848 *Eco*RI + *Mse*I AFLP markers were clustered and covered a genetic distance of 199 cM, corresponding to one *Eco*RI + *Mse*I AFLP marker per 0.23 cM; on the distal parts 1283 cM were covered by 230 *Eco*RI + *Mse*I AFLP markers, corresponding to one marker per 5.6 cM. The *Pst*I/*Mse*I AFLP markers showed a more even distribution with 16 *Pst*I/*Mse*I AFLP markers covering a genetic distance of 199 cM around the centromeric regions and 81 *Pst*I/*Mse*I AFLP markers covering a genetic distance of 1283 cM on the more distal parts, corresponding to one marker per 12 and 16 cM respectively. In both populations a large number of loci showed a significant skewed segregation, but only chromosome 10 loci showed skewness that was similar for both populations. This ultra-dense molecular-marker map provides good perspectives for genetic and breeding purposes and map-based cloning.

Key words Molecular markers · Integrated linkage map · Tomato · *Lycopersicon* species · AFLP · RFLP

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Introduction

The development of molecular linkage maps for most cultivated crop species has made possible the application of a variety of new techniques in plant breeding; for example QTL mapping (reviewed by Young 1996), marker-assisted breeding (Tanksley et al. 1996) and map-based cloning (reviewed by Young 1995). Most of these linkage maps were generated using restriction fragment length polymorphisms (RFLPs) (Tanksley et al. 1992; Hauge et al. 1993; Kleinhofs et al. 1993). RFLP markers are locus-specific and co-dominant and, therefore, very informative. However, generating RFLP data is time- and labour-consuming and requires a relatively large amount of DNA. In addition, random amplified polymorphic DNA (RAPD) markers have also been used to construct genetic linkage maps (Giese et al. 1994; Uphoff and Wricke 1995). This PCR-based technique is fast, easy to perform and requires only small amounts of DNA. The disadvantages of

RAPDs are poor reproducibility (Penner et al. 1993), dominant inheritance and population specificity. Most other marker techniques (microsatellite, CAPS) make use of PCR with specific primers that are designed from known sequences. The advantage is the ease of this technique; however, sequence information is required to design the specific primers, which limits the usefulness of its application.

A relatively new technique, that does not require *a priori* sequence information, is the AFLP technique, which is very efficient and combines the advantages of PCR with high reproducibility and locus specificity among populations of RFLP markers (Zabeau and Vos 1993; Vos et al. 1995; Qi et al. 1998). AFLP maps have been constructed for potato (Van Eck et al. 1995), barley (Becker et al. 1995; Waugh et al. 1997; Qi et al. 1998), sugar beet (Schondelmaier et al. 1996), soybean (Keim et al. 1997), petunia (Gerats et al. 1995) and rice (Maheswaran et al. 1997), enabling the mapping of QTLs in these crops. For tomato (*Lycopersicon esculentum*), several inter- and intra-specific maps have been generated using RFLP markers (Paterson et al. 1991; Lindhout et al. 1994; Van Ooijen et al. 1994; Goldman et al. 1995; Maliepaard et al. 1995), which were originally mapped on an F_2 population of a cross between *L. esculentum* and *Lycopersicon pennellii* (Tanksley et al. 1992). The aim of the present study is the integration of AFLP markers in the RFLP map of tomato. To this end two populations were used. One population consisted of 67 F_2 plants from the interspecific cross *L. esculentum* cv VF36-*Tm2a* × *L. pennellii* LA716. This population has been used to construct a map containing 1030 RFLP markers (Tanksley et al. 1992). The resulting map has been employed for several mapping studies (Paterson et al. 1988; Mutschler et al. 1996) as well as for the construction of a backcross inbred line (BIL) population (Eshed and Zamir 1994; Paran et al. 1995). The second population was an F_2 of the same interspecific cross *L. esculentum* cv Allround × *L. pennellii* LA716 (Odinot et al. 1992) that has also previously been used in several mapping studies (Van der Beek et al. 1994; Arens et al. 1995; Van Tuinen et al. 1997). This map consists of 65 RFLP markers, which were selected by the criterion of even distribution over the tomato genome at an average distance of 25 cM, based on the first map (Tanksley et al. 1992).

Here, we describe the generation of an integrated genetic map containing 1175 AFLP markers by employing two independent F_2 populations of *L. esculentum* × *L. pennellii*.

Materials and methods

Plant material and DNA isolation

One population of 67 F_2 plants (the 'Cornell population') was derived from a cross between *L. esculentum* cv VF36-*Tm2a* and *L. pennellii* LA716 (Tanksley et al. 1992). The DNA isolation procedure has also been described by Tanksley et al. (1992). For the AFLP analysis, which was carried out at Keygene, DNA was available for 42 plants. Another F_2 population of 84 plants (the 'CPRO population') was derived from a cross between *L. esculentum* cv Allround and *L. pennellii* LA716 (Odinot et al. 1992). DNA was extracted from frozen leaves according to the method developed by the group of S.D. Tanksley with some minor modifications as described by Van der Beek et al. (1992). DNA of 80 plants was available for AFLP analysis, which was carried out at the Department of Plant Breeding of the Wageningen Agricultural University.

RFLP analysis and mapping

RFLP analysis and mapping on the Cornell population has been previously reported (Tanksley et al. 1992). RFLP analysis of the CPRO population was carried out as described by Van der Beek et al. (1992) using the RFLP probes developed and mapped by Tanksley et al. (1992).

Mapping of RFLP markers was performed using the computer program JoinMap (Stam 1993) and the resulting map has been described by Arens et al. (1995). The present map contains 13 more RFLP markers.

The AFLP protocol

The AFLP procedure as described by Vos et al. (1995) was applied to the Cornell population, except for the *Eco*RI adapter, which was used without the biotin label. Restriction enzymes, adapters and primers are listed in Table 1. The following primer combinations were employed: E32 + M47, E32 + M48, E32 + M49, E32 + M50, E32 + M59, E32 + M60, E32 + M61, E32 + M62, E33 + M47, E33 + M49, E35 + M47, E35 + M48, E35 + M49, E35 + M50, E35 + M59, E35 + M60, E35 + M61, E35 + M62, E38 + M61, E38 + M62 and E39 + M50.

For the AFLP analysis of the CPRO population, we used the protocol described by Qi and Lindhout (1997). The restriction enzymes, adapters and primers are listed in Table 1. The following primer combinations were employed: E32 + M47, E32 + M48, E32 + M49, E32 + M50, E32 + M61, E35 + M47, E35 + M48, E35 + M50, E35 + M58, E35 + M59, E35 + M62, E39 + M50, P11 + M50, P11 + M54, P14 + M49, P14 + M50, and P14 + M60. Primer combinations were selected as most informative from a previous study by Vos et al. (data not shown).

AFLP data analysis

Segregating AFLP markers in the mapping population were designated according to the primer combination employed, the parent species from which they were derived and the estimated fragment size (see Fig. 1).

Images of AFLP markers of the Cornell population were analysed and co-dominantly scored using the AFLP Image Analysis Software, which has been developed for internal use by Keygene N.V. For the CPRO population, markers were scored visually and co-dominantly. For both populations, bands occurring with an ambiguous intensity were scored as dominant. The frequency of dominant scored bands was higher in the CPRO population than in the Cornell population.

Map construction

For both the Cornell population and the CPRO population, the computer program JoinMap 2.0 (Stam 1993; Stam and Van Ooijen

Table 1 List of primers and adapters

Primers/adapters		Sequences[a]
*Mse*I adapter		5′-GACGATGAGTCCTGAG-3′ 3′-TACTCAGGACTCAT-5′
M00 (universal primer)		GATGAGTCCTGAGTAA
*Mse*I + 1-primer	M02	M00 + C
*Mse*I + 3-primers	M47	M00 + CAA
	M48	M00 + CAC
	M49	M00 + CAG
	M50	M00 + CAT
	M54	M00 + CCT
	M58	M00 + CGT
	M59	M00 + CTA
	M60	M00 + CTC
	M61	M00 + CTG
	M62	M00 + CTT
*Eco*RI adapter		5′-CTCGTAGACTGCGTACC-3′ 3′-CTGACGCATGGTTAA-5′
E00 (universal primer)		GACTGCGTACCAATTC
*Eco*RI + 1-primer	E01	E00 + A
*Eco*RI + 3-primers	E32	E00 + AAC
	E33	E00 + AAG
	E35	E00 + ACA
	E38	E00 + ACT
	E39	E00 + AGA
*Pst*I adapter		5′-CTCGTAGACTGCGTACATGCA-3′ 3′-CATCTGACGCATGT-5′
P00 (universal primer)		GACTGCGTACATGCAG
*Pst*I + 2-primers	P11	P00 + AA
	P14	P00 + AT

[a] DNA sequences are always from a 5′ to 3′ orientation unless otherwise indicated

1996) was used to construct an RFLP-AFLP map. For the CPRO population, fixed-order files consisting of RFLP markers ordered according to the map described by Tanksley et al. (1992) were used. Fixed files consisting of AFLP markers common to both populations and at distances of about 15 cM were used to construct the integrated map of both populations. For calculating map distances, Kosambi's mapping function was employed (Kosambi 1944) together with a recombination threshold value of 0.49. The LOD threshold value for the CPRO map construction was 0.01 and for the Cornell map construction 0.1. For the integrated map the LOD threshold value for mapping was also set at 0.01.

Distorted segregation

The datasets of both populations were analysed for the occurrence of distorted segregation. Theoretically, in both F_2 populations the segregation of a marker into the three possible genotype classes *ee*, *ep* and *pp* (homozygous *L. esculentum*, heterozygous and homozygous *L. pennellii* respectively) should equal 1 : 2 : 1. The module JMSLA32 of JoinMap 2.0 (Stam 1993; Stam and Van Ooijen 1996) was used to test this hypothesis. This module calculates the probability that the observed ratio differs from the expected 1 : 2 : 1 ratio. Since dominant scoring results in a 3 : 1 segregation, which is less informative, only markers which were predominantly scored as co-dominant were employed for this analysis.

Results

AFLP markers in two *L. esculentum* × *L. pennellii* F_2 populations

In order to construct a reliable AFLP map, AFLP markers were evaluated in two independent *L. esculentum* × *L. pennellii* F_2 populations, which have previously been used in RFLP mapping studies. Tanksley et al. (1992) have mapped over 1000 RFLP markers in the Cornell population. By analysing 21 *Eco*RI + *Mse*I primer combinations in this population, 909 AFLP markers were scored, of which 433 were *L. esculentum*-specific and 476 *L. pennellii*-specific (Table 2). The average number of informative markers per primer combination was 43, ranging from 27 (E32 + M61) to 61 (E32 + M59).

As a skeleton map for the CPRO population, 65 RFLP markers were analysed at intervals of 25 cM, based on the map of Tanksley et al. (1992). By using 12 *Eco*RI + *Mse*I primer combinations and five *Pst*I + *Mse*I primer combinations, 642 AFLP markers were scored in the CPRO population, of which 303 were *L. esculentum*-specific and 339 *L. pennellii*-specific (Table 3). The actual number of polymorphic bands on a gel was higher; however, close migration of bands of nearly identical size prevented a reliable scoring of the bands. The average number of informative markers per *Eco*RI + *Mse*I primer combination was 42, ranging from 25 (E32 + M48) to 60 (E32 + M50), compared to 27 markers identified per *Pst*I + *Mse*I primer combination which ranged from 23 (P11 + M50) to 36 (P14 + M50).

The average total number of bands per *Eco*RI + *Mse*I primer combination was 111, compared to 93 per *Pst*I + *Mse*I primer combination. The *Eco*RI + *Mse*I primer combinations had an average polymorphism rate of 77% compared to 63% for the *Pst*I + *Mse*I primer combinations, which is significantly lower ($P < 0.01$).

Table 2 Number of amplification products and polymorphisms for ten of the 21 primer combinations used for the Cornell population

Primer combin.	Total # bands	# *L. esc.* specific	Polym. rate (%)	# Polym. scored
E32 + M47	141	45	67	36
E32 + M48	98	30	67	28
E32 + M49	90	28	70	36
E32 + M61	93	27	70	27
E35 + M47	173	53	66	35
E35 + M48	118	47	81	54
E35 + M50	131	42	63	36
E35 + M59	133	47	77	47
E35 + M62	136	50	77	45
E39 + M50	136	57	79	56
Average	125	43	71	40

Table 3 Number of amplification products and polymorphisms per primer combination used for the CPRO population

Primer combin.	Total # bands	# *L. esc.* specific	Polym. rate (%)	# Polym. scored
E32 + M47	130	42	68	49
E32 + M48	103	34	77	25
E32 + M49	83	27	76	33
E32 + M50	136	37	70	60
E32 + M61	78	31	77	44
E35 + M47	155	52	77	39
E35 + M48	100	36	82	40
E35 + M50	132	51	75	34
E35 + M58	86	33	79	33
E35 + M59	119	41	78	56
E35 + M62	106	42	84	39
E39 + M50	110	45	82	53
Average	112	39	77	42
P11 + M50	91	27	65	23
P11 + M54	95	25	65	27
P14 + M49	79	18	51	25
P14 + M50	110	37	72	36
P14 + M60	90	21	57	25
Average	93	26	63	27

Most AFLP markers were identical in both populations. However, because not all AFLP markers were evaluated in both populations, a total of 228 AFLP markers were common to both populations.

Map construction

After scoring the AFLP markers, separate maps of both populations were constructed. To assign markers to chromosomes, RFLP markers were used at an average distance of 25 cM, based on the RFLP map constructed by Tanksley et al. (1992). Markers were placed in one linkage group if they were at least linked to another marker in this group with a LOD value of 2.5–3.5. For map construction of the CPRO population, AFLP markers were selected with the following quality criterion: if a marker shows linkage to other markers with less than 5, 10 or 20% recombination the corresponding LOD values for linkage should be greater than 10, 5 and 1, respectively. If this criterion was not met for three or more times, such a marker was considered as not accurately scored and hence removed from the dataset. In addition, of marker groups that showed identical segregation, only one of these markers was taken as representative and the other markers were not used in further analysis. However, these markers were later positioned on the same locus as the representative marker.

The maps of both populations showed a high similarity (data not shown). Map positions of markers were generally similar and only three AFLP markers mapped on different chromosomes (indicated in Fig. 1). Consequently, an integrated map could be generated using a fixed order consisting of skeleton AFLP markers at strategic chromosome loci based on the most unambiguously mapped markers in both populations (Fig. 1). The resulting total map length was 1482 cM, with no gaps bigger than 21 cM.

Clustering of *Eco*RI + *Mse*I AFLP markers

Preferably, to obtain a genetic map with the smallest possible intervals between markers, these markers should be evenly distributed over the genome. However, often the distribution is not random and markers are clustered in certain regions on the chromosome (Tanksley et al. 1992). A very clear clustering of *Eco*RI + *Mse*I AFLP markers was observed on all chromosomes (Fig. 1). The vast majority of the *Eco*RI + *Mse*I markers (848) mapped in clusters (represented by closed bars on the chromosomes in Fig. 1) which together cover 199 cM, while 230 *Eco*RI + *Mse*I markers were distributed over the remaining 1283 cM. The positions of these clusters, as determined by using RFLP markers with known map position as landmarks, were in the centromere regions (Tanksley et al. 1992). A similar clustering was not observed for the *Pst*I + *Mse*I AFLP markers.

Distorted segregation

In F_2 populations, segregation of a single co-dominant locus should result in a 1 : 2 : 1 ratio of the three possible genotypes *ee* : *ep* : *pp* (homozygous *L. esculentum*, heterozygous and homozygous *L. pennellii* respectively). However, in both populations several chromosome regions showed distorted segregation, although the location of the distorted segregation could differ (Fig. 2 A, B). For the Cornell population skewness was observed in regions of chromosomes 2, 4, 7, 8, 10, 11 and 12, while the CPRO population showed skewness for regions of chromosomes 9, 10 and 12. This distorted segregation was observed for both RFLP and AFLP markers. For most of the markers that showed a skewed distribution, the *L. pennellii* allele was overrepresented. The most-skewed marker of the CPRO population was TG230 on chromosome 10. None of the plants was *ee* for this marker locus and the allele frequencies of *e* and *p* were 28% and 72%, respectively. This region on chromosome 10 was also the most distorted in the Cornell population with allele frequencies of 23% (*e*) and 77% (*p*) for TG230 (Fig. 2 B).

Discussion

Map construction

Using a limited number of only 27 primer combinations, we were able to construct highly saturated AFLP

maps of two interspecific populations. Both individual maps were very similar in marker order as well as in map distances, facilitating the integration of both maps. Clearly, this indicates that AFLP markers are reliable, reproducible and locus-specific. Waugh et al. (1997) showed that 78 out of 81 co-migrating AFLP markers segregating in more than one population, mapped to similar loci in three different barley populations, while

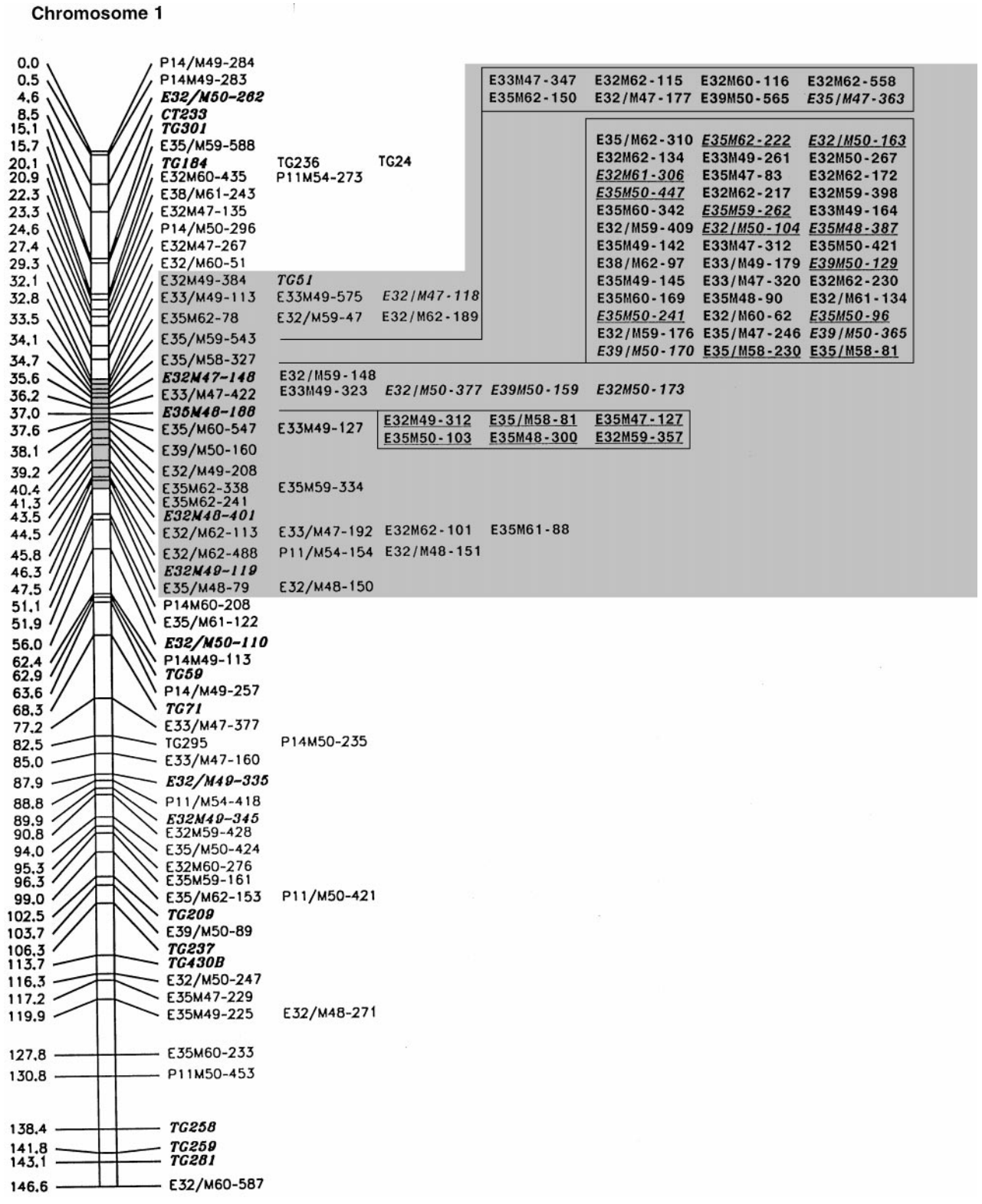
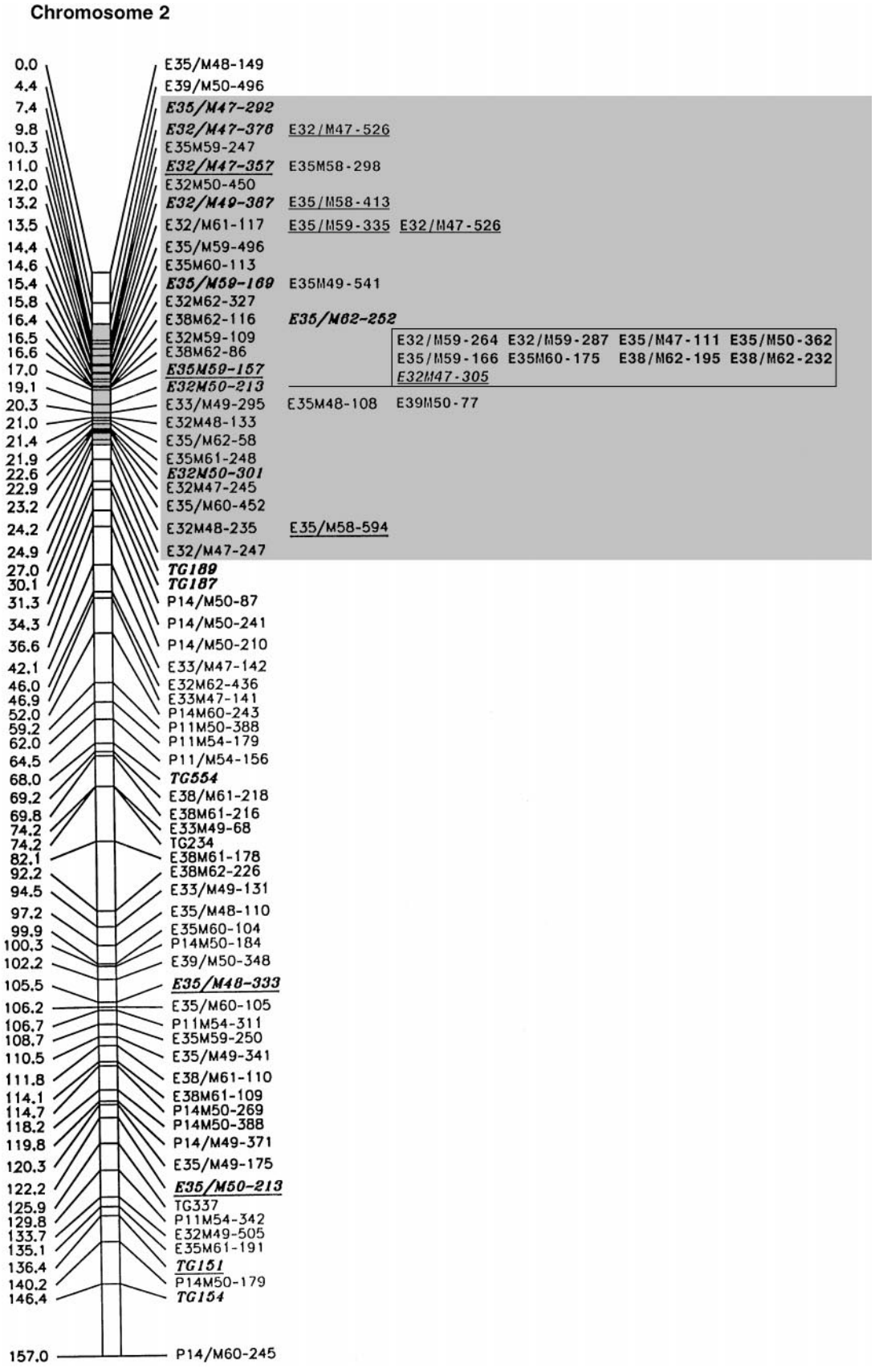


Fig. 1 See page 269 for legend



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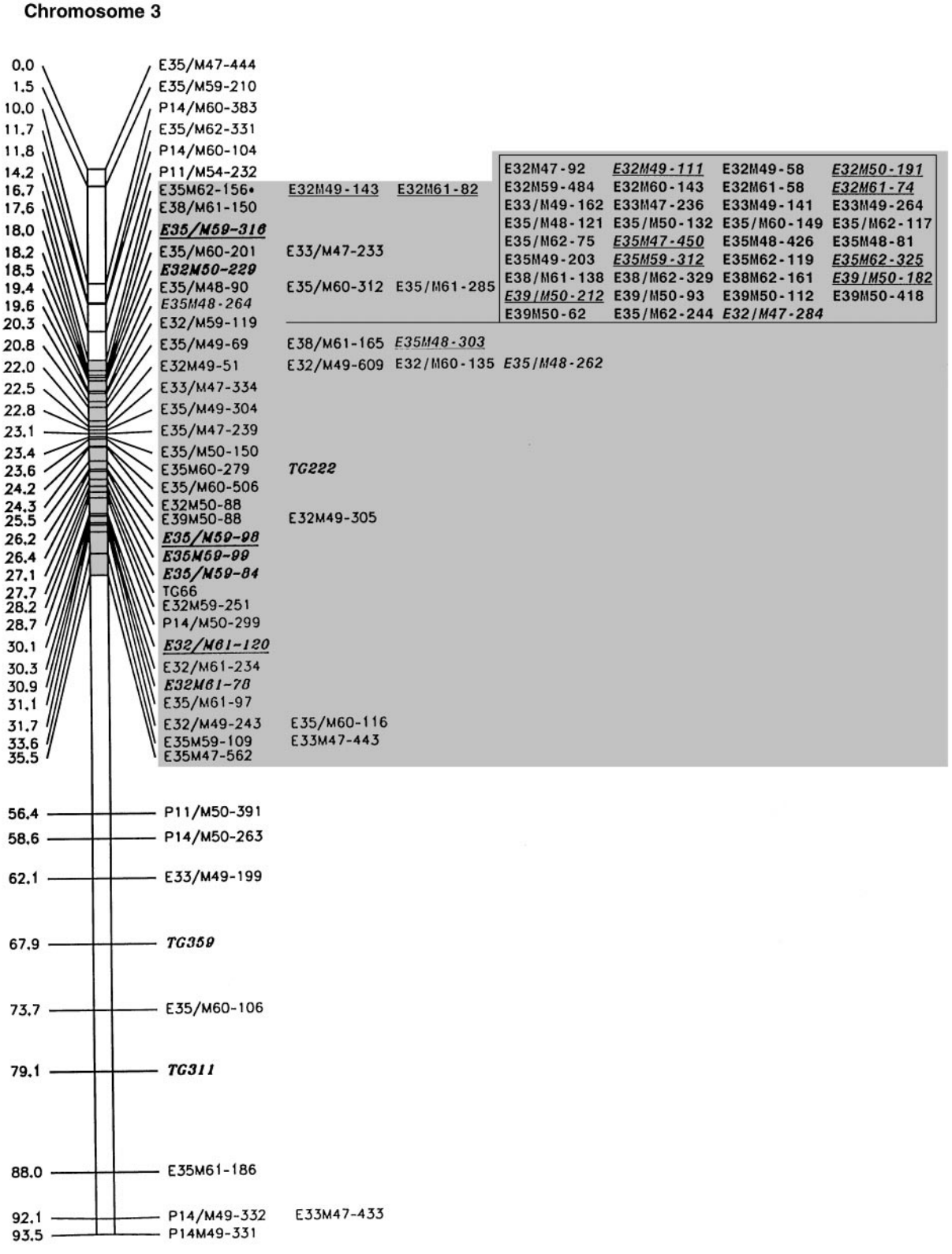
Qi et al. (1998) found that all 38 co-migrating AFLP markers, mapped to the same position on two barley maps.

The integrated map presented in this paper was 1482 cM in length, which is considerably longer than the map of 1276 cM presented by Tanksley et al. (1992).



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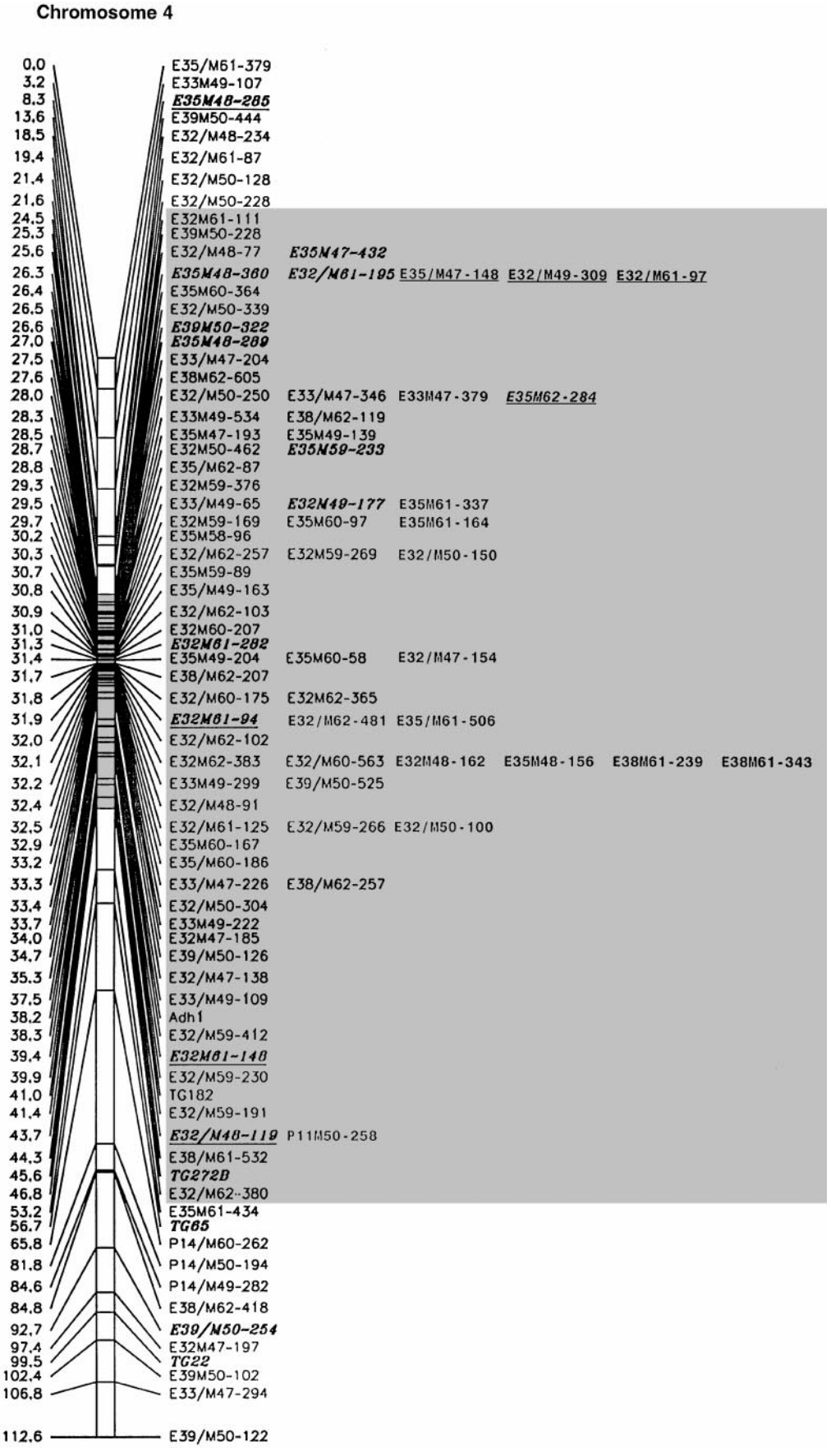

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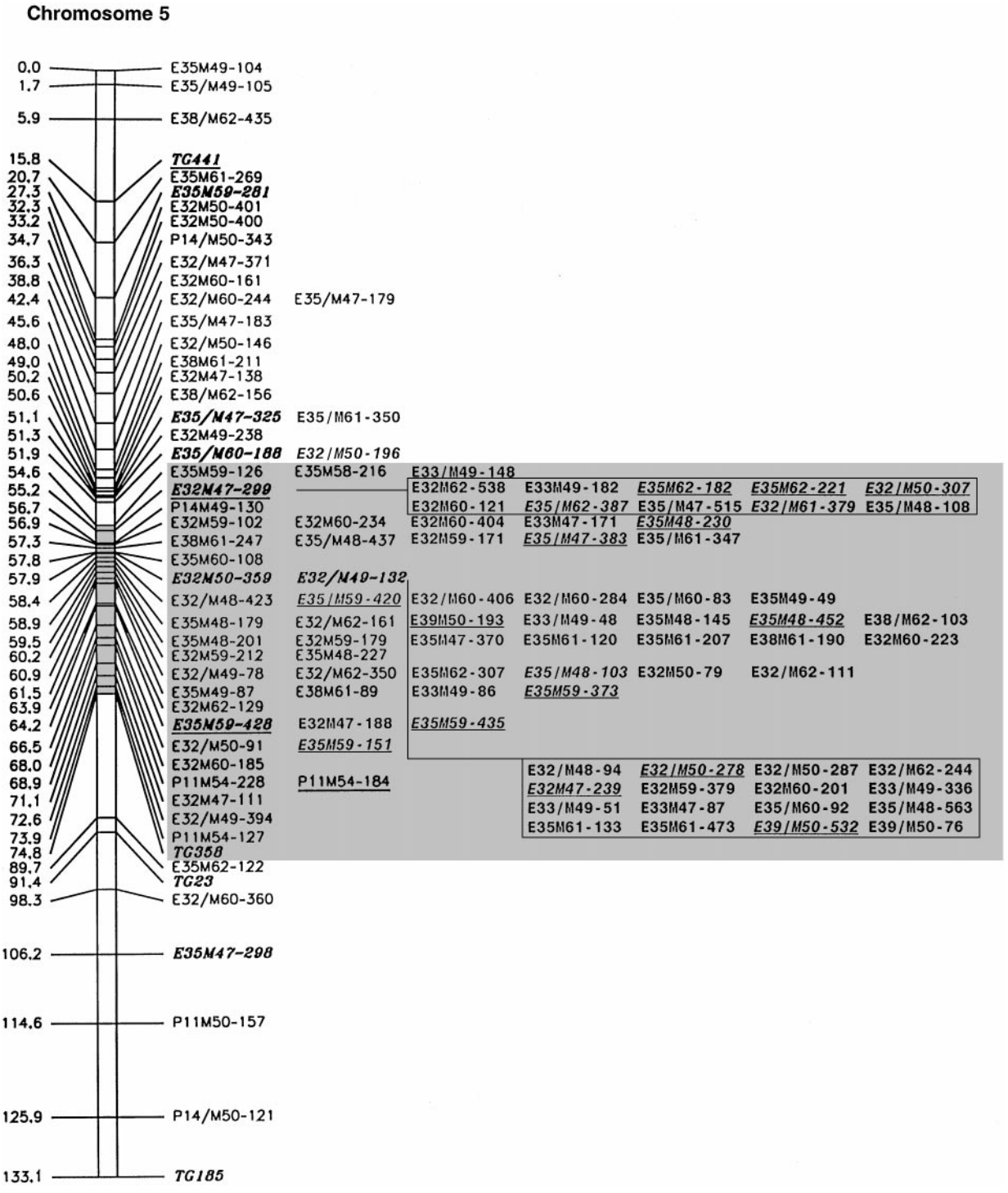
A major reason for this increase is due to more distal markers, adding approximately 110 cM to the total map length. Furthermore, chromosomes 5 and 10 were each 30 cM longer at the most distal RFLP markers on both chromosome ends, compared to the map presented by Tanksley et al. (1992). Part of the increase in map length may be due to errors in the scoring of markers. Lincoln and Lander (1992) described that an error rate of 1% increases the map length by a factor of two for markers with low average spacing (1 cM). Since the



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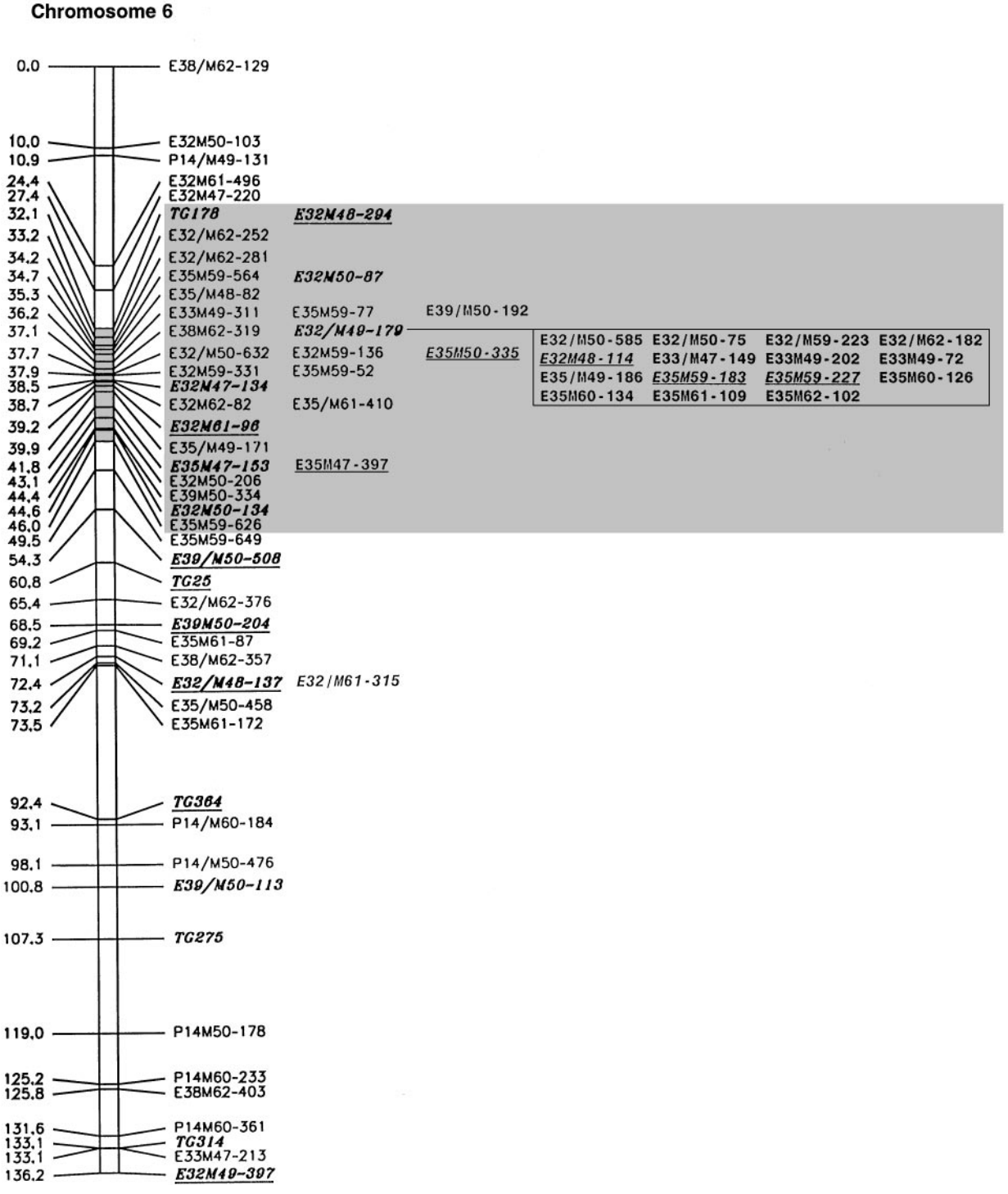
majority of the markers in the present map are clustered with an average spacing of less than 1 cM, it is remarkable that the total map length did not increase by several hundred cM. This suggests that most markers were accurately positioned.

Clustering of markers

By constructing an AFLP map, it was found that the vast majority of *Eco*RI + *Mse*I markers occurred in clusters. The positions of the clusters in the present map



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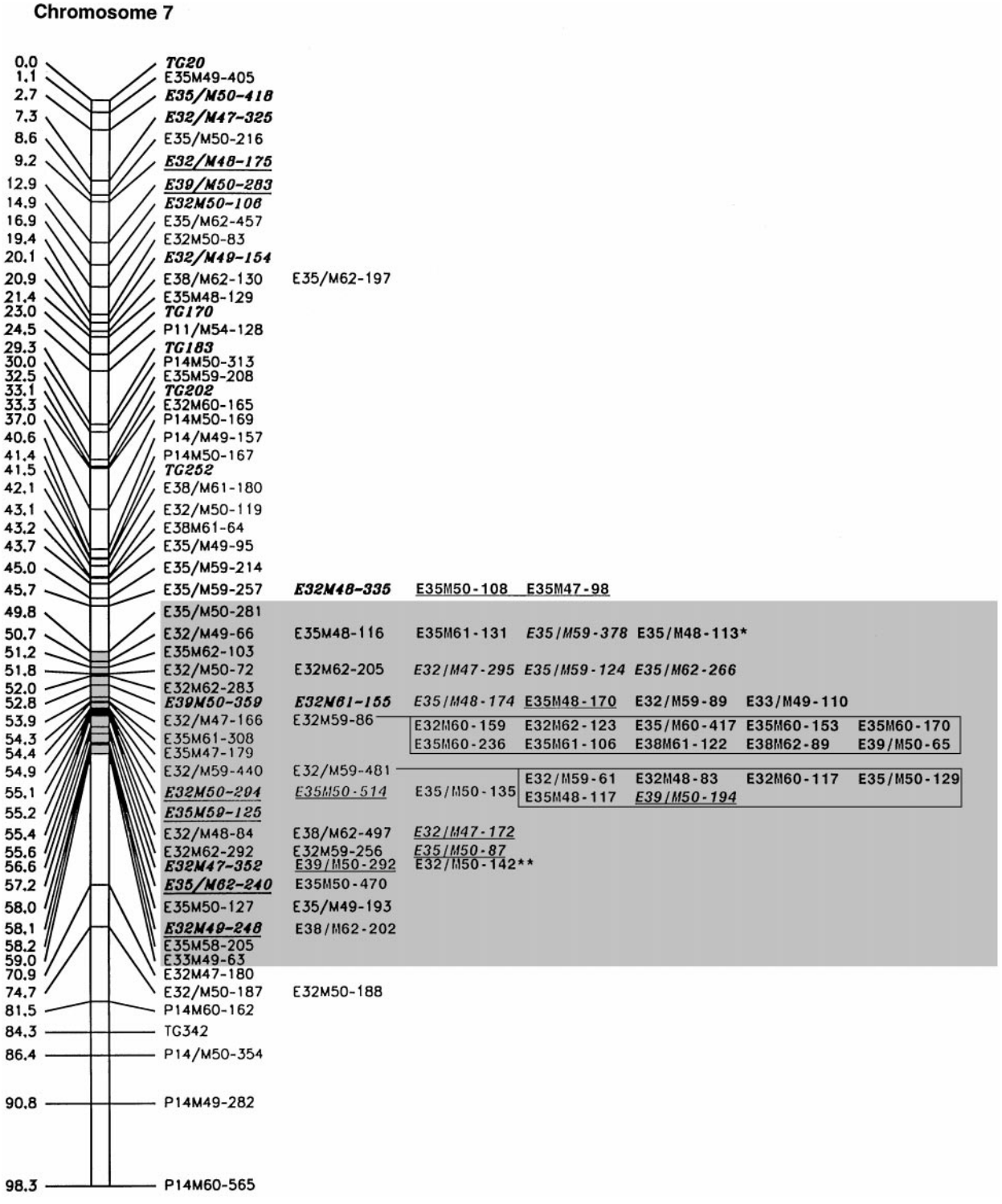
are presumably the heterochromatic regions around the centromeres (Tanksley et al. 1992). A similar strong clustering of *Eco*RI + *Mse*I markers around the putative centromeres has also been reported in barley (Qi et al. 1998) and maize (Vuylsteke et al. 1997). In addition, in soybean, sugar beet and potato, a clustering of *Eco*RI + *Mse*I AFLP markers has also been observed, although it was not known whether the clusters were in the centromeric regions (Keim et al. 1997; Schondelmaier et al. 1996; Van Eck et al. 1995). By determining



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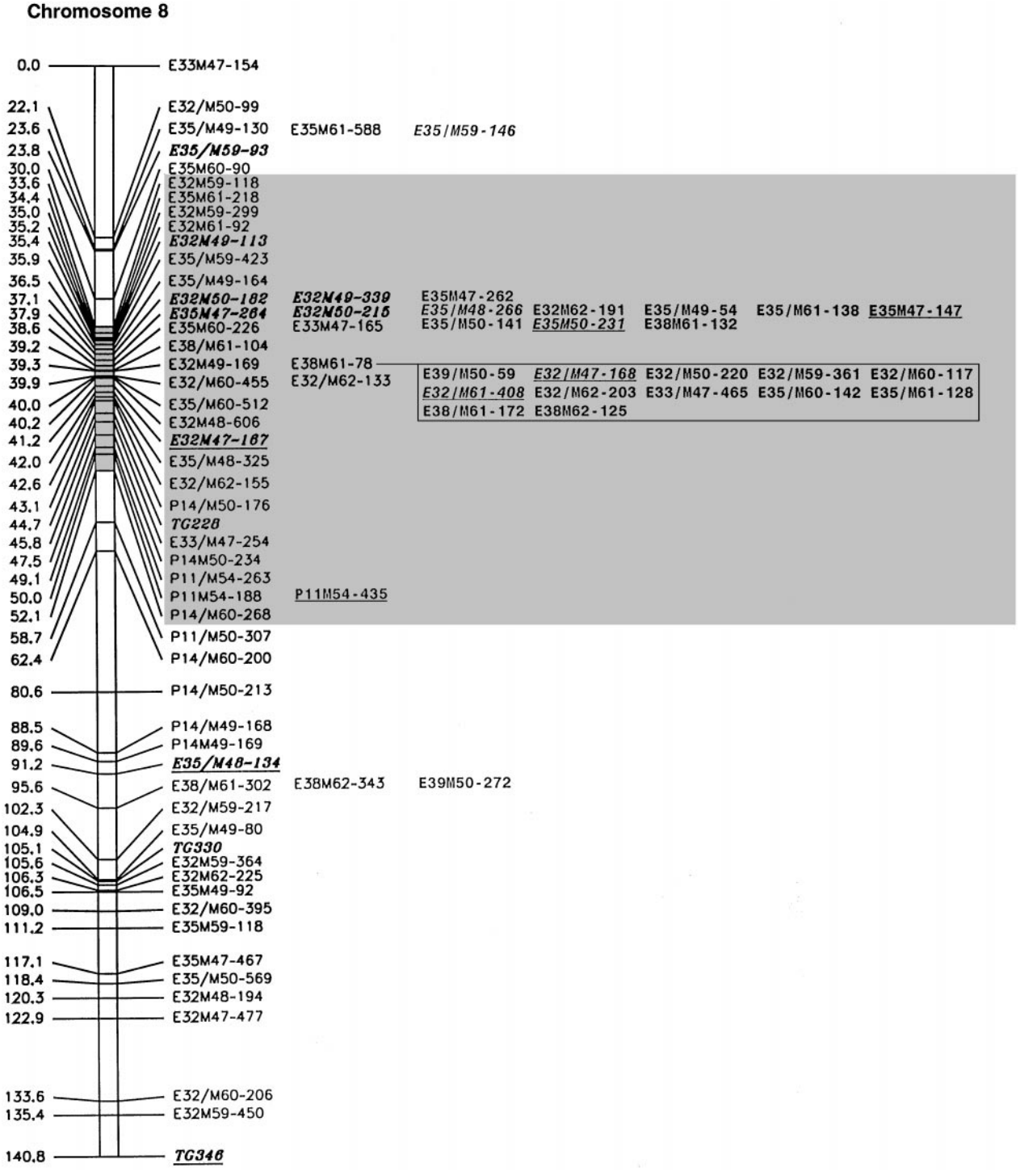
the frequency and distribution of recombination nodules on tomato synaptonemal complexes, Sherman and Stack (1995) observed a much lower frequency of recombination nodules in heterochromatin compared to euchromatin. We found 848 of the 1078 *Eco*RI + *Mse*I AFLP markers in clusters, which equals 78.7% of the mapped markers. This corresponds remarkably well with the percentage of DNA present in the heterochromatic regions, which is 77% according to Peterson et al. (1996) who studied the pachytene chromosomes



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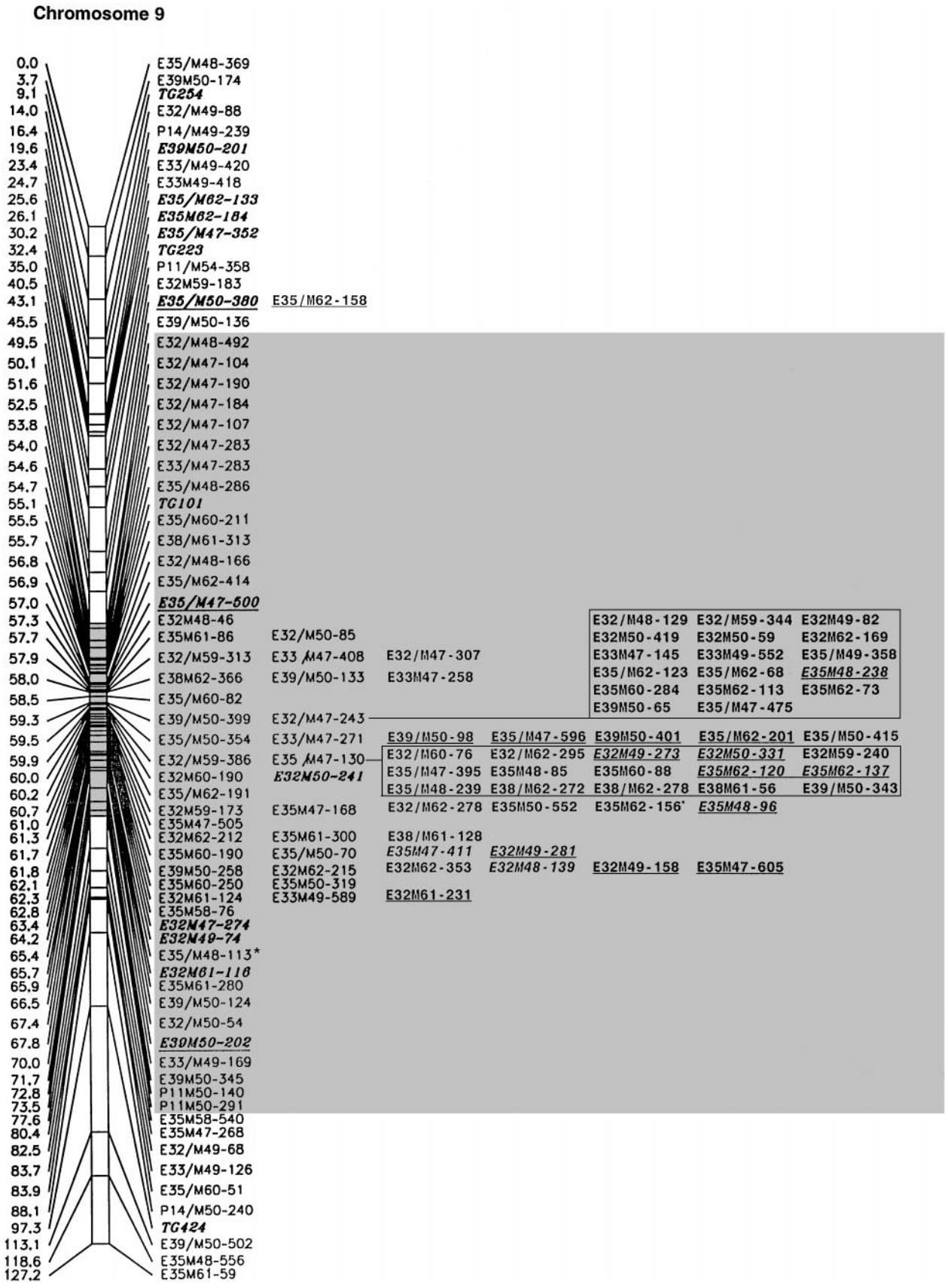

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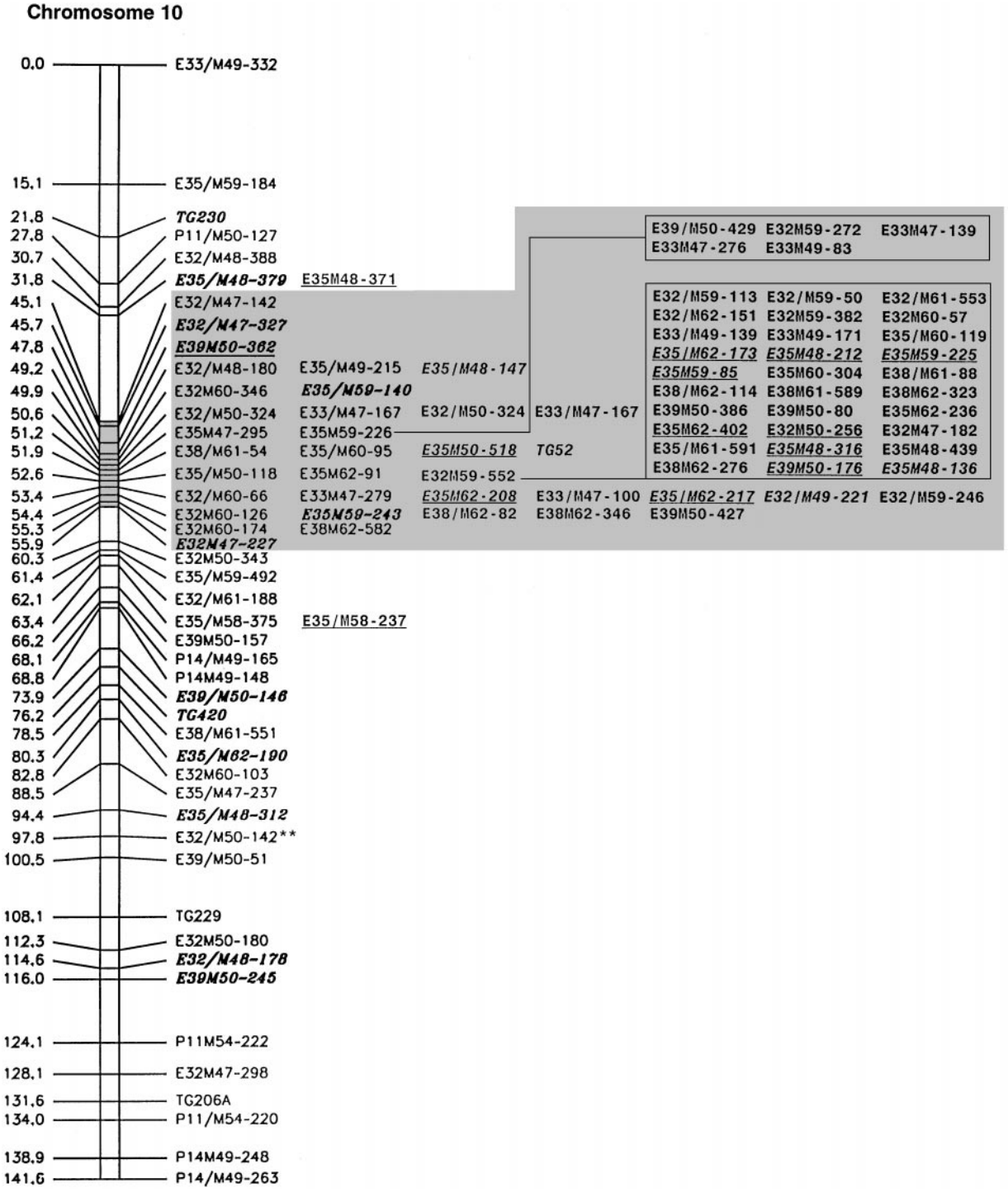
of *L. esculentum*. This suggests that the clustering of *Eco*RI + *Mse*I markers is due to a suppression of recombination in the heterochromatic regions near the centromeres rather than to a non-random distribution of markers on the chromosomes.

It was expected that the methylation-sensitive restriction enzyme *Pst*I would recognise restriction sites in non-methylated euchromatin but not in the methylated heterochromatin (Gruenbaum et al. 1981). Consequently, fewer *Pst*I + *Mse*I markers should be obtained, but these markers should be located in the more distal parts of the chromosomes. To avoid the reduction in the number of markers, only two selective bases were used at the 3′ end of the *Pst*I primer. Indeed,



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*Pst*I + *Mse*I markers were not clustered like *Eco*RI + *Mse*I markers; only 16 *Pst*I + *Mse*I AFLP markers mapped in the clusters, which span 199 cM, while 81 *Pst*I + *Mse*I AFLP markers mapped to the remaining 1283 cM. In agreement, using only *Pst*I + *Mse*I AFLP markers in a doubled-haploid rice population, Maheswaran et al. (1997) showed a random distribution of AFLP markers on the genetic map. These differences between *Eco*RI + *Mse*I and *Pst*I + *Mse*I AFLP markers have implications for the choice of restriction enzymes in different studies. In most genetic studies, especially QTL mapping, it is desirable to have markers evenly distributed over the genetic map. Consequently, *Pst*I + *Mse*I AFLP markers are more

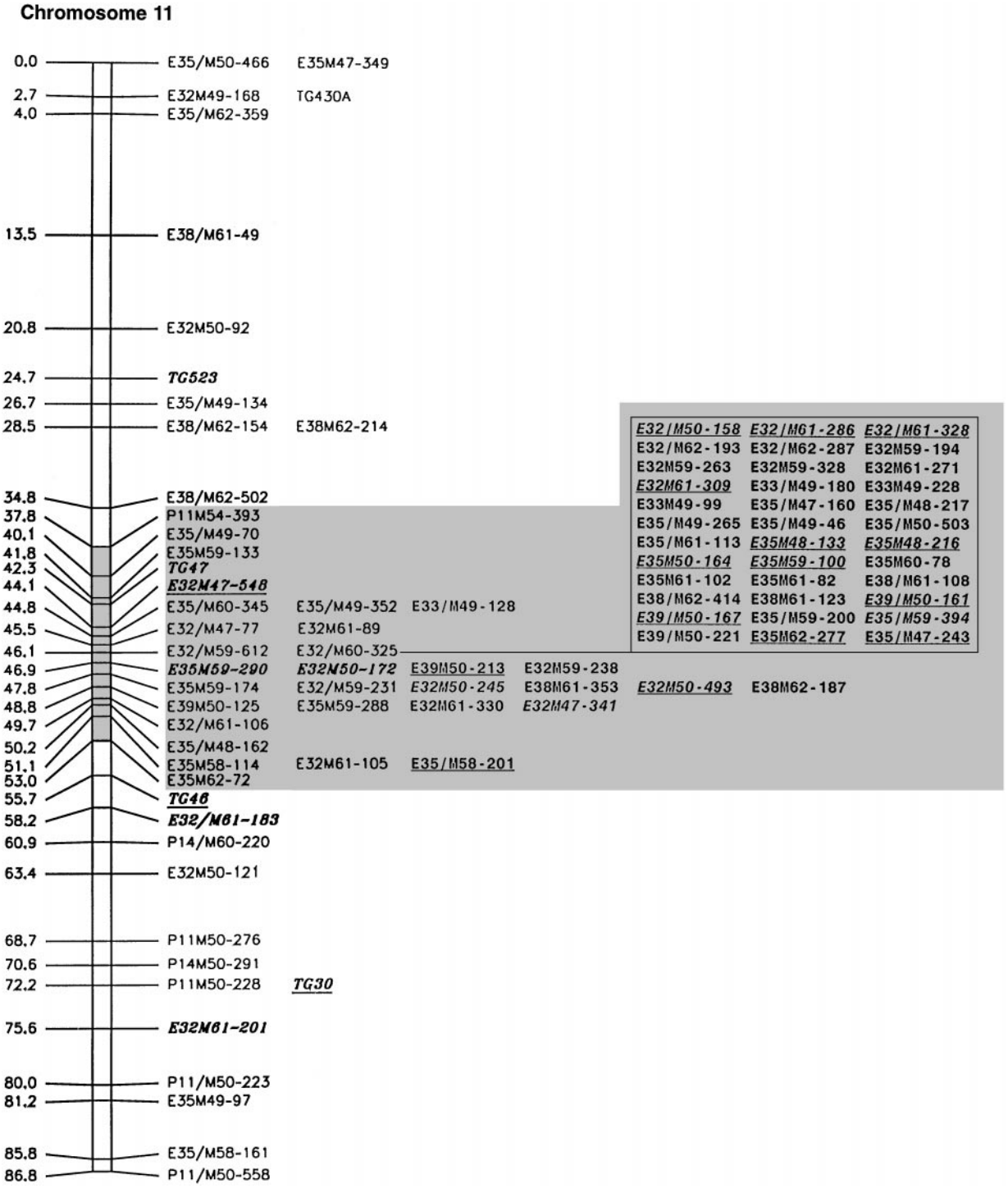

Fig. 1 See page 269 for legend

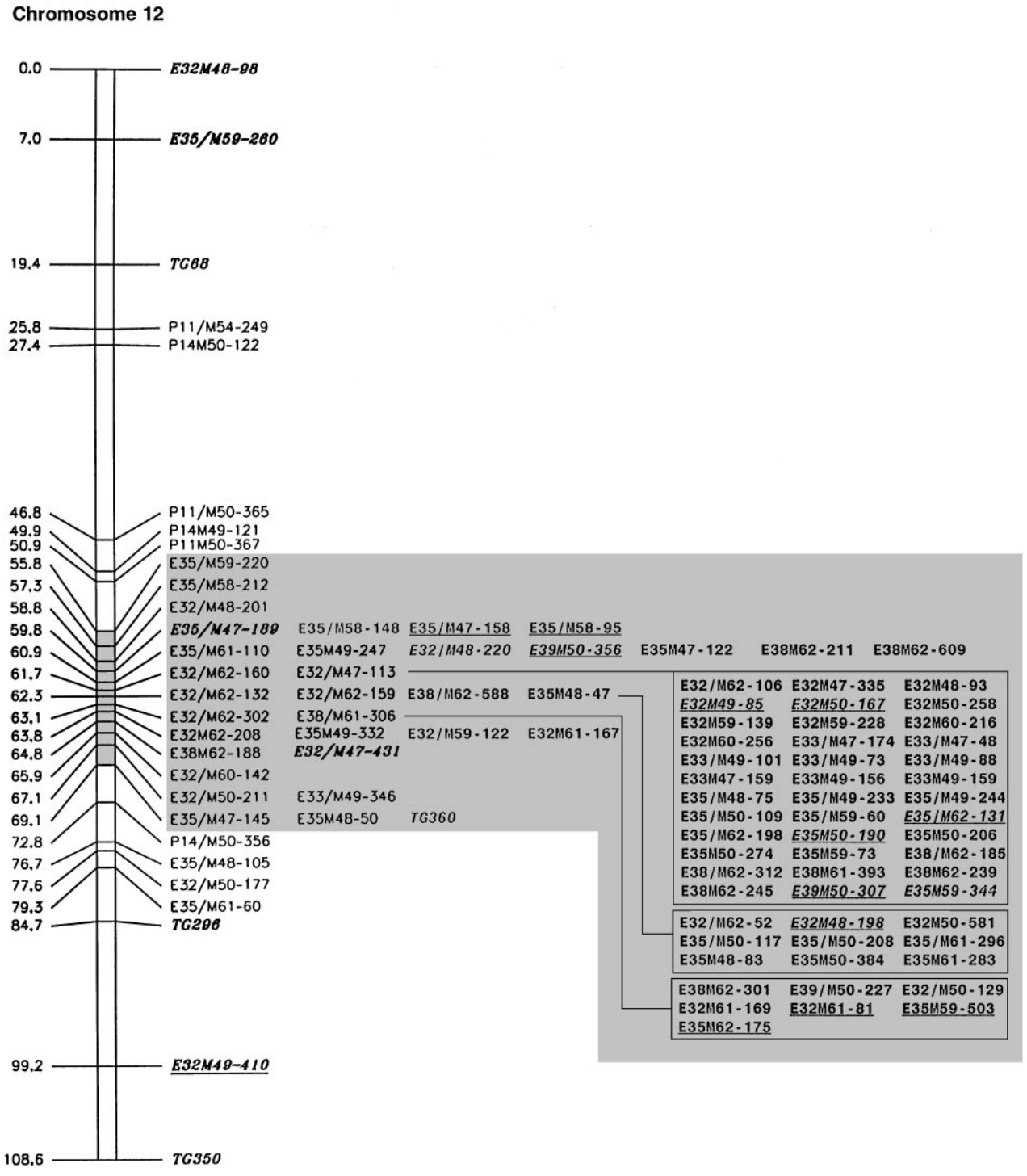

Fig. 1 An integrated map based on two interspecific *L. esculentum* × *L. pennellii* F_2 populations. Markers that were scored in both populations are printed *boldface in italics. Underlined* markers represent markers of the CPRO population with no recombination with other markers of the CPRO population. Markers that were scored but not mapped in the CPRO population and were mapped in the Cornell population are printed *boldface in italics and underlined*. Three markers were scored in both populations but were mapped in different linkage groups. These markers are indicated with ·, * and **. The *solid bars* within the chromosomes represent the clusters. When six or more markers mapped to identical loci, these loci where chosen as part of a cluster. If marker loci were separated by 2.5 cM or more, the cluster ceased

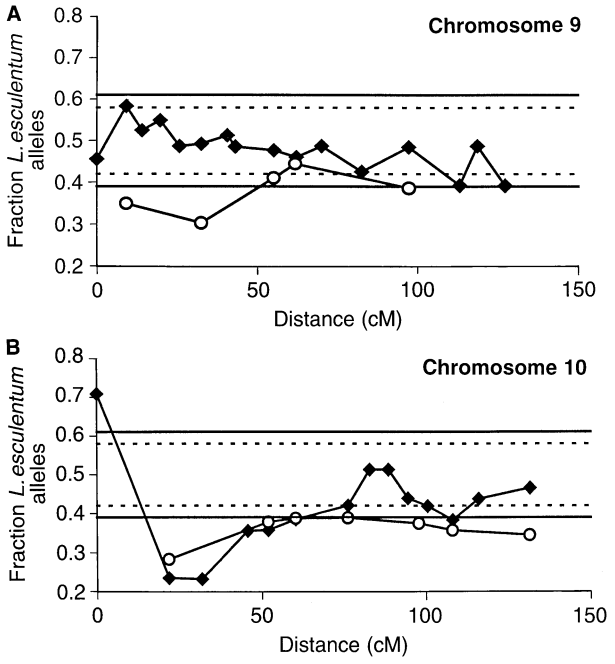

Fig. 2A, B The frequency of alleles originating from *L. esculentum* per marker locus of the Cornell population (–◆–), and the CPRO population (–○–). Panel **A** refers to chromosome 9 and panel **B** to chromosome 10 respectively. For the Cornell population, values between 0.39 and 0.61 (as indicated by the *horizontal lines*) do not differ significantly from 0.5 ($P < 0.05$). For the CPRO population, values between 0.42 and 0.58 (as indicated by the *dashed horizontal lines*) do not differ significantly from 0.5 ($P < 0.05$)

suitable for these purposes. However, it has to be stated that methylation varies between genotypes and during plant development, and this can influence marker analysis and the predictive value of such markers within the germplasm. For map-based cloning, the choice of AFLP markers is dependent upon the position of the gene of interest. For example, the tomato gene *Cf-9*, conferring resistance to *Cladosporium fulvum*, is located in the euchromatin and consequently *Pst*I + *Mse*I AFLP markers were preferred to fine-position this gene (Thomas et al. 1995). On the other hand, the tomato *Mi* gene, conferring resistance to *Meloidogyne incognita*, is located in the heterochromatin and *Eco*RI + *Mse*I markers were preferred to clone this gene (Kaloshian et al. 1998).

Distorted segregation

Distorted segregation has been observed in both populations. However, it is remarkable that not all regions that were skewed showed this distorted segregation in both populations, since both are derived from similar crosses. For most of the skewed markers, prevalence for the *L. pennellii* allele was found, although some regions with minor distortion towards the *L. esculentum* allele were also found. A similar distortion in favour of *L. pennellii* alleles has been observed before by DeVicente and Tanksley (1993). The most skewed region in both populations was around the RFLP marker TG230 on chromosome 10. There are two likely hypotheses to explain this skewness. The first hypothesis is selection against zygotes of the *ee* genotype, resulting in an expected 0:2:1 segregation of *ee*:*ep*:*pp*. The second model is selection against pollen with an *e* allele, which would result in a 0:1:1 segregation of *ee*:*ep*:*pp*. The *ee*:*ep*:*pp* ratio for the most extreme marker TG230 of 2:25:35 for the Cornell population and 0:44:34 for the CPRO population better resembles a 0:1:1 rather than a 0:2:1 segregation, which indicates that pollen selection is more likely than zygote abortion of the homozygous *L. esculentum* genotype.

In conclusion, AFLP provides a rapid way to construct reliable integrated genetic linkage maps. Especially when special considerations with respect to the choice of restriction enzymes are made, this technique enables both researchers and breeders to use AFLP markers for a variety of purposes, such as gene and QTL mapping, map-based cloning and marker-assisted breeding.

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