Research Article

COMPARISON OF ECO-MSE AND PST-MSE PRIMER COMBINATIONS IN GENERATING AFLP MAP OF TOMATO

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ABSTRACT

An experiment was conducted at Wageningen University and Research Centre (WUR), Unifarm, Haarweg, Wageningen, The Netherlands in 2000 to compare Eco-Mse and Pst-Mse primer combinations to generate AFLP markers of tomato. Back cross first (BC_1) population of *Lycopersicon esculentum* cv money maker and *Lycopersicon pennellii* LA716 was used. Six primer combinations (three of Eco-Mse and three of Pst-Mse) were used. A total of 122 AFLP markers were found generating 76 markers by Eco-Mse primer combination and 46 markers by Pst-Mse primer combination. The average number of informative markers per primer combination was 20.33 ranging from 11 (P11M50) to 36 (E35M48). Similarly the average number of informative markers per chromosome was 10.16 ranging from 4 (chromosome number 8) to 16 (chromosome number 1). Within Eco-Mse primer combinations, E32M50 generated the least (18) and E35M48 generated the highest (36) AFLP markers. Similarly, within Pst-Mse primer combination, P14M50 generated the highest (20) and P11M50 generated the least (11) AFLP markers. The Eco-Mse primer combination generated the highest number of marker (12) in chromosome 9 and the lowest (2) in chromosome number 8. Similarly, the Pst-Mse primer combination generated the highest number of markers (9) in chromosome number 1 and the lowest (2) in chromosome number 3, 8, 11 and 12. The AFLP map spanned 843 cM. The longest AFLP map was found in chromosome 1 and spanned 98 cM and the shortest in chromosome 8 and spanned 46 cM.

Key words: Tomato, Back cross, Primer combination, AFLP map

INTRODUCTION

In Nepal, vegetable crops are grown in 181,000 ha of land and the production is achieved by 2038, 000 mt that contributes 5.73 % to the agricultural gross domestic products (MOAC, 2005). Tomato *(Lycopersicon esculentum)* is the most important and widely grown vegetable crop in the world. It is a day neutral crop and is found in both determinate and indeterminate types. The indeterminate types are grown in greenhouses whereas the determinate types are grown in field as well as greenhouse. Tomato is commercially important throughout the world in food processing industries. The indeterminate type is grown for fresh fruit market.

The progress in plant breeding is easy and quick for qualitative characters controlled by major genes whereas quantitative characters like yield, earliness, days to heading, stress tolerance, rate of development, quality, adaptation and height etc is still more complicated. These traits are controlled by several genes that have cumulative effects (Van Berloo, 2000). Quantitative Trait Loci (QTLs) harbours such genes and plant breeders are interested to find such genes that could efficiently help in crop improvement. DNA fingerprinting and QTL mapping are the powerful tools to unravel the genetic basis of polygenic traits. Creation of genetic variation and then selection of suitable genotypes is one of the common ways that can assist in crop improvement. It is becoming easier to enhance the exploitation of the germplasm of crop species with the advent of molecular markers like Amplified Fragment Length Polymorphism (AFLP), Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), Sequence Tagged Site (STS) and so on. These molecular or DNA markers have many applications in plant breeding such as estimation of genetic diversity, characterisation of germplasm resources, identification of crop varieties and marker assisted selection. The marker-assisted selection has been utilised in gene introgression, elimination of linkage drag and gene pyramiding. Successful pyramiding of genes Xa4, Xa5, Xa13 and Xa21 conferring resistance to 4 races of bacterial leaf blight pathogen has been achieved in rice (Huang et at, 1997) by using molecular markers, which was otherwise impossible through conventional methods.

Evidences have been obtained that wild species of *Lycopersicon* have alleles associated with desirable traits

(Bernacchi *et al.*, 1998; Vicente and Tanksley, 1993; McNally and Mutschler, 1997; Fulton *et al.,* 1997; Fulton *et al.,* 2000). Various researches were carried out to find valuable QTLs for different characters in crop plants. In wild relatives of tomato, QTLs were found for valuable traits (Paterson et al., 1988; Lefort et al., 1990; Alpert *et al.*, 1995; Doganlar *et al.*, 2000; Thoquet *et al.*, 1996; Carlos, 1998; Foolad *et al.*, 1998; Fulton *et al.*, 1997; McNally and Mutschler, 1997).

AFLP technique is one of the most efficient and realistic techniques used in generating linkage map because it combines the advantages of RFLPs and RAPDs with high reproducibility and locus specific. *Lycopersicon esculentum* cv Money Maker is a high yielding cultivar whereas *Lycopersicon pennellii* is a wild species that has genes for increase in yield (Vicente and Tanksley, 1993) and insect resistance (McNally and Mutschler, 1997).

The Eco-Mse and Pst-Mse primer combinations are used in generating AFLP map. These primer combinations produce more AFLP markers than other combinations. Pst-Mse AFLP markers are found in the distal part of the chromosomes. The majority of the Eco-Mse AFLP markers mapped in clusters are found in the central part of the chromosomes. If the markers are distributed evenly in the entire chromosomes, a large genome can be found. In past most of the researches were mainly carried out with regard to varietal adaptation, characterisation of germplasm and study of different traits (Upreti and Adhikary, 1984; Upreti *et al.*, 1985; Shakya *et al.*, 1993; Ojha, 2004). But some researches were directed towards this approach and satisfactory results were found (Ojha, 2001; Ojha, 2003; Haanstra, 2000; and Vos *et al.*, 1995). To give continuity in this area and to verify the previous findings, this study was conducted to compare Eco-Mse and Pst-Mse primer combinations in generating AFLP map of tomato that helps in finding QTLs important for crop improvement.

MATERIALS AND METHODS

AFLP map of tomato was developed from 44 BC_1 populations. To obtain this map, DNA was extracted from leaves and AFLP data was analysed. Then with the help of JoinMap 2.0 program an AFLP map was constructed. The detail procedure in generating AFLP map is explained below.

Plant material

Forty four plants in the Backcross first (BC_1) population of the interspecific cross *Lycopersicon esculentum* cv Money Maker X *Lycopersicon pennellii* LA716 were genetically analysed with the help of AFLP markers.

Experimental design

The research was carried out in the Unifarm, Haarweg, Wageningen, The Netherlands. Plants were grown under the conditions that are similar environments to those in commercial greenhouse tomato cultivation. The BC_1 population seeds were sown in trays filled with a commercial potting soil mixture. After three weeks, the seedlings were transferred to rockwool and placed on benches. The plants were grown by giving 100 cm row to row distance and 50 cm plant to plant distance. After every two BC_1 plants, one recurrent parent Money Maker was planted to facilitate the crossing.

Isolation of DNA from leaves

The protocol for the isolation of DNA was used according to the standard tomato DNA isolation protocol developed in the Laboratory of Plant Breeding, Wageningen University and Research Centre (WUR). Young leaf of 2-3 cm^2 of each BC_1 plant was collected. The sample of leaf was crushed with the help of grinder. Four hundred µl extraction buffer, 500 µl nuclei lysis buffer and 50 µl sarkosyl were added. Then the samples were shaked and vortexed and incubated in a water bath at 65^0C for one hour. The tubes were taken out and 800 µl chloroform and isoamylalcohol in 24:1 ratio was added to each sample. Centrifuging was done and supernatant was pipetted. Eight hundred µl isopropanol was added to each sample. Pellet was seen after centrifuging and rinsed for 20 min in 250 µl 76 % ethylalcohol with 10 mM ammonium acetate (NH4Ac). The pellets were dried and dissolved in 100 µl tris EDTA (TE) buffer (+ 1µg Rnase).

The amplified fragment length polymorphism (AFLP) protocol

The AFLP procedure as described by Vos et al (1995) was applied to perform the experiment. The adapters and primers used for this experiment are listed in Table 1. The primer combinations: E32M49, E32M50, E35M48, P11M50, P14M49 and P14M50 were used. Most informative primer combinations were selected from a previous study done by Haanstra, 2000 and Vos *et al.*; 1995.

Table 1. List of primers and adapters

Primers/Adapters	Sequences
Mse 1 Adapter	5'-GACGATGAGTCCTGAG-3'
	3'-TACTCAGGACTCAT-5'
MOO (Universal primer)	GATGAGTCCTGAGTAA
Mse1 + 3 primers	
M48	MOO+CAC
M49	MOO+CAG
M50	MOO+CAT
EcoR1 Adapter	5'-CTCGTAGACTGCGTACC-3'
	3'-CTGACGCATGGTTAA-5'
EOO (Universal primer)	GACTGCGTACCAATTC
EcoR1 + 3 primers	
E32	EOO+AAC
E35	EOO+ACA
Pst1 Adapter	5'-CTCGTAGACTGCGTACATGCA-3'
	3'-CATCTGACGCATGT-5'
POO (Universal primer)	GACTGCGTACATGCAG
Pst + 2 primers	
P11	POO+AA
P14	POO+AT

AFLP data analysis and Chi-square of homogeneity test

Each sample containing 0.6 µl was loaded in the licor gel with the help of micro pipette. The samples of *Lycopersicon esculentum* cv Money maker and *Lycopersicon pennellii* were kept as control. After running licor gel, the AFLP picture of forty four BC_1 plants was taken out. AFLP markers were scored dominantly and visually with the help of developed picture. The scoring was tabulated giving symbols H for heterozygousness of *L. pennellii* specific, A for homozygousness of Money Maker specific and U for doubtful bands. Chi square of homogeneity test between two groups as well as within group of primer combinations was performed.

Map construction

The computer programme JoinMap 2.0 (Stam and Van Ooijen 1996) was used to construct an AFLP map. The modules JMGRP 32, JMTREE 32, JMSPL 32, JMSLA 32, JMREC 32 and JMMAP 32 were used. For calculating mapping distances, Kosambi's maping function was used. Likewise 0.1, 0.49, 5, 3 and 7 values were used as LOD threshold, recombination threshold, jump value, ripple value and triplet threshold respectively. The Map Chart software programme developed by Voorrips 1999, was used for drawing the map of the chromosomes. The data was scored visually with the help of developed pictures of 44 BC_1 plants (Table 2). These data were used in constructing AFLP map.

RESULTS AND DISCUSSION

Analysis of AFLP markers

Based on Haanstra, 2000 findings, 6 informative primer combinations (3 Eco-Mse and 3 Pst-Mse) were used for this research. AFLP markers were evaluated in back cross 1 population of *Lycopersicon esculentum* X *Lycopersicon pennellii*. The AFLP map was constructed based on pennellii specific markers.

A total of 122 markers were found in this experiment. Seventy six markers was found in Eco-Mse primer combination and 46 markers in Pst-Mse primer combination. The number of AFLP markers generated by different Eco-Mse and Pst-Mse primer combinations in different chromosomes is presented in Table 2.

Table 2. The number of AFLP markers generated by different Eco-Mse and Pst-Mse primer combinations in different chromosomes

Primer combinations	Chromosomes												Total markers
	1	2	3	4	5	6	7	8	9	10	11	12	
E32M49	5	1	3	1	0	1	1	2	5	0	1	2	22
E32M50	0	2	1	1	1	2	1	0	2	2	4	2	18
E35M48	2	1	4	5	6	0	5	0	5	5	1	2	36
EcoMseTotal	7	4	8	7	7	3	7	2	12	7	6	6	76
P11M50	1	1	0	1	2	0	2	0	1	1	2	0	11
P14M49	5	0	1	2	1	0	1	1	0	3	0	1	15
P14M50	3	4	1	0	0	3	5	1	2	0	0	1	20
Pst-MseTotal	9	5	2	3	3	3	8	2	3	4	2	2	46
Total Markers	16	9	10	10	10	6	15	4	15	11	8	8	122

The primer combination E35M48 generated the highest number of markers (36). On the other hand, P11M50 generated the least number of markers (11). The AFLP markers were found the highest number (16) in chromosome 1 whereas the least (4) in chromosome 8. In chromosome 9, the Eco-Mse primer combination produced the highest number of marker (12). In constrast, this primer combination produced the least marker (2) in chromosome 8. Similarly, the Pst-Mse primer combination generated the highest number of markers (9) in chromosome 1 and the least (2) in chromosomes 3, 8, 11 and12.

The Eco-Mse primer combination produced in equal markers (7) in chromosomes 1, 4, 5, 7 and 10, and 6 markers in each chromosome 11 and 12. Likewise, the Pst-Mse primer combination produced equal markers (3) in chromosomes 4, 5, 6 and 9, and 2 markers in each chromosome 3, 8, 11 and 12. The Eco-Mse and Pst-Mse combination generated equal number of markers (3 and 2) in chromosome 6 and 8 respectively.

Equal number of markers (10) were found in chromosome 3, 4 and 5; 8 markers in chromosome 11 and 12; and 15 markers in chromosome 7 and 9. E32M49 produced no markers in chromosome 5 and 10 and produced equal markers in chromosomes 2, 4, 6, 7 and 11. No markers in chromosome 1 and 8 were produced by E32M50 and generated equal marker (1) in chromosomes 3, 4, 5 and 7 and 2 markers in chromosomes 2, 6, 9, 10 and 12. Similarly, E35M48 produced no markers in chromosomes 6 and 8 but generated equal markers (5) in chromosomes 4, 7, 9 and 10; 2 markers in chromosomes 1 and 12; 1 marker in chromosome 2 and 11. Likewise, the primer combination P11M50 produced no markers in chromosomes 3, 6, 8, 12 but generated equal markers (1) in chromosomes 1, 2, 4, 9 and 10 and 2 markers in chromosomes 5, 7 and 11. Similarly, the primer combination P14M49 produced no markers in chromosomes 2, 6, 9, and 11 but generated equal markers (1) in chromosomes 3, 5, 7, 8 and 12. Likewise, the primer combination P14M50 also produced no markers in chromosomes 4, 5, 10 and 11 but generated equal markers (3) in chromosomes 1 and 6; 1 marker in chromosomes 3, 8 and 12. All primer combinations produced markers in chromosome 7. P14M49 and P14M50 generated no markers in chromosome 11 but generated equal markers in chromosomes 3, 8 and 12. All Eco-Mse primer combinations produced same number of marker (2) in chromosome 12. Two primer combinations E35M48 and P11M50 produced no markers in chromosomes 6 and 8. Similarly, two primer combinations namely E32M49 and P14M50 produced no markers in chromosomes 5 and 10. The average number of markers per Eco-Mse primer combination was 25.3, and per Eco-Mse primer combination per chromosome was 6.3. Similarly, the average number of markers per Pst-Mse primer combination was 15.3 whereas per Pst-Mse primer combination per chromosome was 3.8. In chromosome number 2, 6, 8, 11 and 12, the Eco-Mse primer combination generated less AFLP markers than average (6.3). Similarly, in chromosomes 3, 4, 5, 6, 8, 9, 11 and 12 the Pst-Mse primer combination generated less AFLP markers than average (3.8). Both primer combinations Eco-Mse and Pst-Mse generated more markers in chromosomes 1, 7 and 10 than their respective averages.

As a whole, the Eco-Mse primer combination produced more AFLP markers than the Pst-Mse primer combination. This findings was in agreement with the findings of Haanstra, 2000; Carlos, 1998; Vos *et al.*, 1995 and Ojha, 2001.

Chi-square test

The AFLP markers produced by Eco-Mse and Pst-Mse primer combinations was observed in 76 and 46 numbers respectively. It was assumed that the two groups of primer combinations would produce the same

proportion of markers. On the basis of chi-square test, Eco-Mse primer combination appeared significantly different from Pst-Mse primer combination at 5 % and 1 % level of significance for 1 df. It was shown that both groups of primer combinations did not generate the same proportion of AFLP markers (Table 3). Within group of Eco-Mse primer combination, equal proportion of AFLP markers were not generated by Eco-Mse primers whereas within group of Pst-Mse primer combination, equal proportion of AFLP markers were generated by Pst-Mse primers on the basis of chi square test (Table 3). This findings is seemed similar to the findings of many authors (Haanstra, 2000; Carlos, 1998; Paterson *et al.*, 1998).

Table 3. Chi-square of homogeneity test within and between groups of primer combinations

SN	Particulars	AFLP Markers		χ^2 'Cal'	χ^2	χ^2
1	Both groups	Expected	Observed	value	'tab' at 5 %	'tab' at 1 %
	Eco-Mse	61	76	3.69		
	Pst-Mse	61	46	3.69		
	Sum of χ^2			**7.3****	**3.84**	**6.63**
2	Eco-Mse group					
	E32M49	25.33	22	0.44		
	E32M50	25.33	18	2.12		
	E35M48	25.33	36	4.49		
	Sum of χ^2			**7.05***	**5.99**	**9.2**
3	Pst-Mse group					
	P11M50	15.33	11	1.22		
	P14M49	15.33	15	0.01		
	P14M50	15.33	20	1.42		
	Sum of χ^2			**2.65[ns]**	**5.99**	**9.2**

Map construction

The computer programme JoinMap 2.0 (Stam and Van Ooijen 1996) was used to construct an AFLP map. The Map Chart software programme developed by Voorrips 1999, was used for drawing the map of the chromosomes. The longest AFLP map was found in chromosome 1 and spanned 98 cM whereas the shortest was found in chromosome 8 and spanned 46 cM.

It was observed that vast majority of Eco-Mse markers occurred in clusters. In chromosomes 1, 3, 4, 5 and 7, majority of the Eco-Mse AFLP markers were clustered in distances between 25-28 cM, 15-21 cM, 26-39 cM, 24-36 cM and 14-30 cM respectively. Similarly, in chromosomes 9, 10, 11 and 12 these markers were clustered in distances between 18-19 cM, 20-24 cM, 47-49 cM and 14-16 cM respectively. On the other hand, Pst-Mse markers were not occurred in clusters. Eco-Mse markers were found in the central part of the chromosomes but the Pst-Mse markers were found in the distal part of the chromosomes. This result supported the previous findings of many author (Ojha, 2001; Haanstra, 2000; Paterson, 1998; Carlos, 1998). The map of the chromosomes is given in Figure 1.
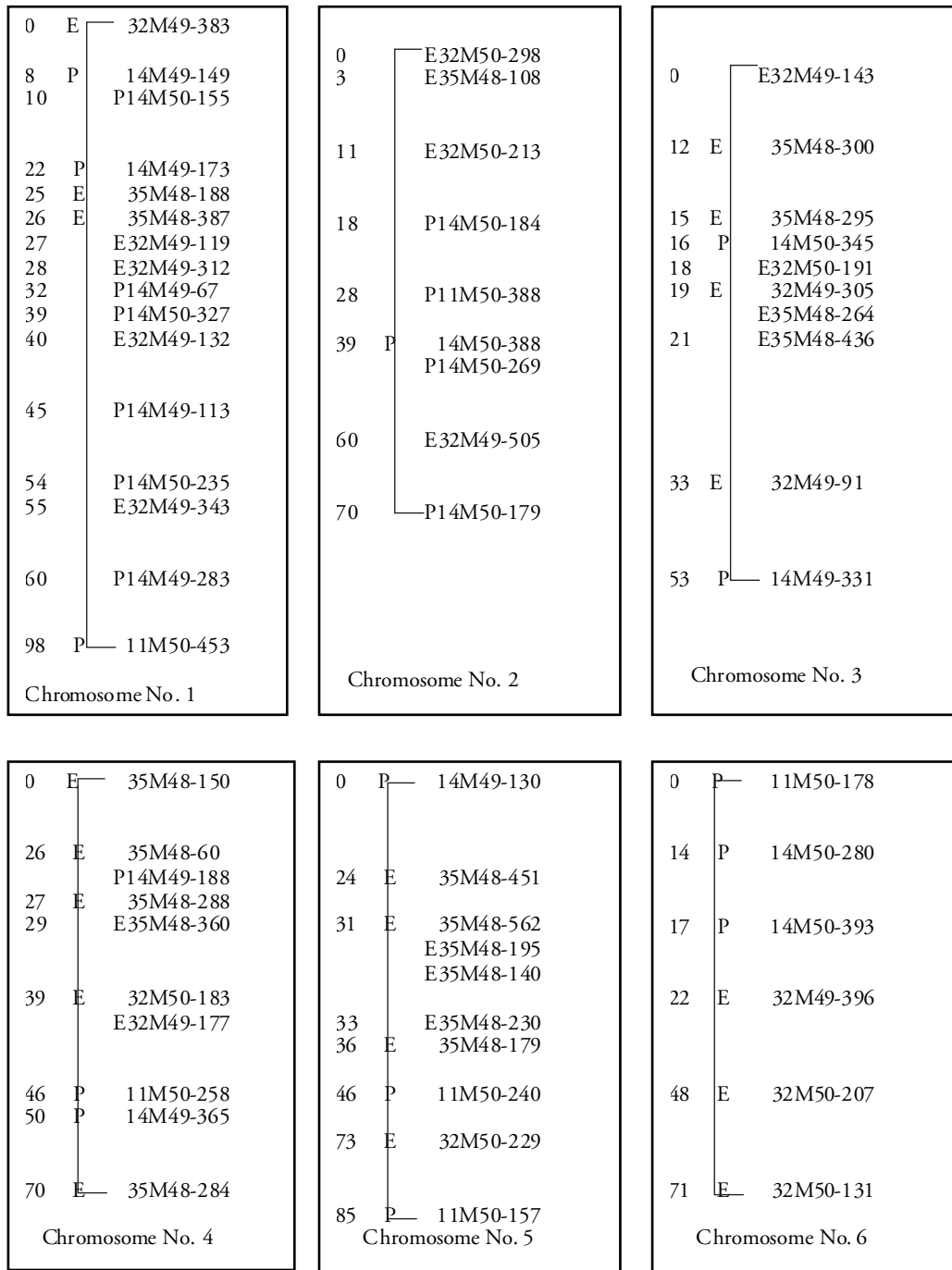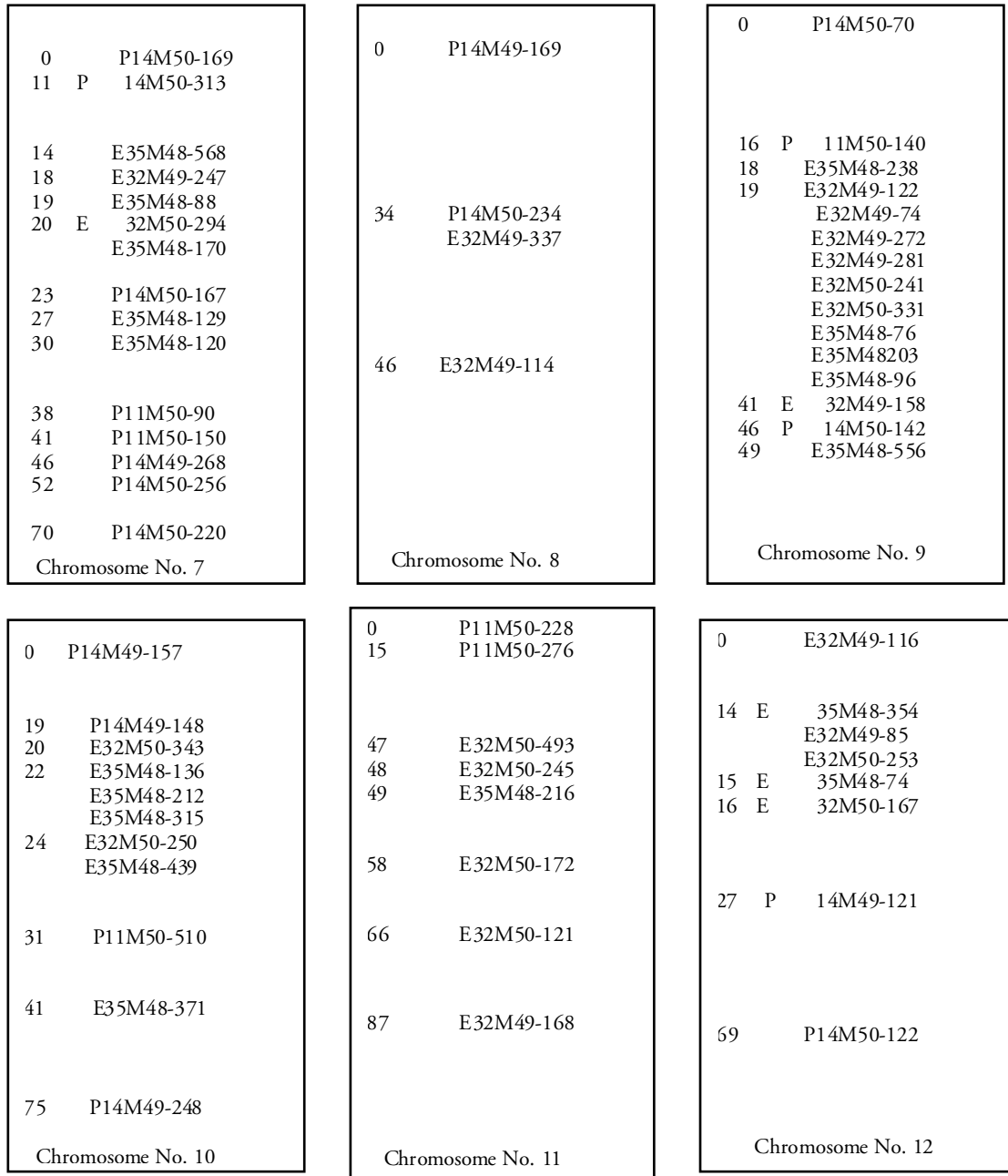
Chromosome No. 1

0	E	32M49-383
8	P	14M49-149
10		P14M50-155
22	P	14M49-173
25	E	35M48-188
26	E	35M48-387
27		E32M49-119
28		E32M49-312
32		P14M49-67
39		P14M50-327
40		E32M49-132
45		P14M49-113
54		P14M50-235
55		E32M49-343
60		P14M49-283
98	P	11M50-453

Chromosome No. 2

0		E32M50-298
3		E35M48-108
11		E32M50-213
18		P14M50-184
28		P11M50-388
39	P	14M50-388
		P14M50-269
60		E32M49-505
70		P14M50-179

Chromosome No. 3

0		E32M49-143
12	E	35M48-300
15	E	35M48-295
16	P	14M50-345
18		E32M50-191
19	E	32M49-305
		E35M48-264
21		E35M48-436
33	E	32M49-91
53	P	14M49-331

Chromosome No. 4

0	E	35M48-150
26	E	35M48-60
		P14M49-188
27	E	35M48-288
29		E35M48-360
39	E	32M50-183
		E32M49-177
46	P	11M50-258
50	P	14M49-365
70	E	35M48-284

Chromosome No. 5

0	P	14M49-130
24	E	35M48-451
31	E	35M48-562
		E35M48-195
		E35M48-140
33		E35M48-230
36	E	35M48-179
46	P	11M50-240
73	E	32M50-229
85	P	11M50-157

Chromosome No. 6

0	P	11M50-178
14	P	14M50-280
17	P	14M50-393
22	E	32M49-396
48	E	32M50-207
71	E	32M50-131

Figure 1. Map of chromosomes

Figure 1. Contd......

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   0        P14M50-169
  11    P    14M50-313


  14         E35M48-568
  18         E32M49-247
  19         E35M48-88
  20    E    32M50-294
             E35M48-170

  23         P14M50-167
  27         E35M48-129
  30         E35M48-120


  38         P11M50-90
  41         P11M50-150
  46         P14M49-268
  52         P14M50-256

  70         P14M50-220
        Chromosome No. 7
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   0        P14M49-169




  34        P14M50-234
            E32M49-337



  46        E32M49-114




        Chromosome No. 8
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   0        P14M50-70



  16    P    11M50-140
  18         E35M48-238
  19         E32M49-122
             E32M49-74
             E32M49-272
             E32M49-281
             E32M50-241
             E32M50-331
             E35M48-76
             E35M48203
             E35M48-96
  41    E    32M49-158
  46    P    14M50-142
  49         E35M48-556


        Chromosome No. 9
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   0    P14M49-157


  19        P14M49-148
  20        E32M50-343
  22        E35M48-136
            E35M48-212
            E35M48-315
  24        E32M50-250
            E35M48-439


  31        P11M50-510


  41        E35M48-371



  75    P14M49-248
        Chromosome No. 10
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   0        P11M50-228
  15        P11M50-276


  47        E32M50-493
  48        E32M50-245
  49        E35M48-216


  58        E32M50-172


  66        E32M50-121


  87        E32M49-168



        Chromosome No. 11
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   0        E32M49-116


  14    E    35M48-354
             E32M49-85
             E32M50-253
  15    E    35M48-74
  16    E    32M50-167


  27    P    14M49-121



  69         P14M50-122


        Chromosome No. 12
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CONCLUSIONS

The progress in plant breeding is easy and quick for qualitative characters controlled by major genes with easily identifiable effect on the phenotype. The identification of desirable genotypes for the quantitative characters is still more complicated. DNA fingerprinting and QTL mapping are the powerful tools to unravel the genetic basis of quantitative traits. Making distant crosses of complementary genotypes creates the new genetic variation. Molecular markers AFLP, RFLP, RAPDs and STS have many applications in plant breeding. The marker-assisted selection has been utilised in gene introgression, elimination of linkage drag and gene pyramiding. Most of the wild species of tomato have desirable characters, like insect resistance, disease resistance, firmness and earliness that can be introduced to the elite cultivated species. So these wild species are widely used in crossing programme because of the certain agriculturally important genes. These genes are easily and quickly trapped with the help of molecular markers.

A total of 122 AFLP markers were found generating 76 markers by Eco-Mse primer combination and 46 markers by Pst-Mse primer combination. The Eco-Mse primer combinations produced more markers than Pst-Mse combination. The average number of informative markers per primer combination was 20.33 ranging from 11 (P11M50) to 36 (E35M48). The average number of informative markers per chromosome was 10.16. The Eco-Mse primer combination generated the highest number of marker (12) in chromosome nine and Pst-Mse primer combination generated the highest number of markers (9) in chromosome number one. The majority of Eco-Mse markers occurred in clusters but not the Pst-Mse markers. Eco-Mse markers were found in the central part of the chromosomes but the Pst-Mse markers were found in the distal part of the chromosomes. More number of AFLP markers are obtained using Eco-Mse and Pst-Mse primer combinations and then a large genome can be found. When there is large genome, the chance of obtaining many QTLs is high. QTLs harvor such genes that govern agriculturally important traits and plant breeders are interested to grap these genes. A precise genetic map is obtained if the distribution of markers is evenly over the entire genome.

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