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RESEARCH ARTICLE

Wenying SUN, Yuxing ZHANG, Wenquan LE, Hai'e ZHANG

Construction of a genetic linkage map and QTL analysis for some leaf traits in pear (*Pyrus* L.)

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Abstract The major incompatibility barriers to specific inbred lines and the long generation duration in *Pyrus* L. may hinder the *Pyrus* breeding process. A genetic linkage map provides the foundation for quantitative trait loci (QTL) mapping and molecular marker-assisted breeding. In this study, we constructed a genetic map with 145 F_1 populations from a cross of two cultivars, Yali and Jingbaili, using AFLP and SSR markers. The map consisted of 18 linkage groups which included 402 genetic markers and covered 1395.9 cM, with an average genetic distance of 3.8 cM. The interval mapping was used to identify quantitative trait loci associated with four leaf agronomic traits in the F_1 population. The results indicated that four QTLs were associated with leaf length, two QTLs with leaf width, two with leaf length/leaf width, and three with petiole length. The eleven QTLs were associated with 9.9%–48.5% of the phenotypic variation in different traits. It is considered that the map covers almost the whole genome, and molecular markers will be greatly helpful to the related breeding.

Keywords *Pyrus* L., molecular linkage map, QTL analysis, leaf traits

1 Introduction

Pear (*Pyrus* L., AA, $2n = 34$) is one of the most important fruit crops in China, and the species includes many thousand varieties. To date, the pedigree relationships, the genetic diversity and the genetic breeding of pear have been studied. But the long generation duration severely hinders classical breeding and genetic studies. Furthermore, the most important traits are quantitative traits, which are complex traits and easily vary with the environment. Therefore, we need to divide single genes from multigenetic quantitative trait loci (QTL) for further analysis.

Constructing a genetic map represents a new tool to help traditional plant breeding methods through the identification of quantitative trait loci and their integration into marker-assisted selection programs. Today, many economic crops such as maize (Smith et al., 1997; Burr et al., 1988), rice (Harushima et al., 1998), tomato (Tanksley et al., 1992; Yu, 1998), wheat (Qi et al., 1998) and soybean (Cregan et al., 1999) have had their saturated maps constructed, which can not only map QTLs but also clone the genes by a map-based cloning method. The genetic researches in pear lag behind compared to these crops. Because there are no saturated genetic linkage maps to be used, the studies on genetic rules and evolution have been restricted. From a plant breeder's point of view, to study the genetic analysis and construct the genetic linkage map of pear and reveal the inheritance of a quantitative trait are fundamental for finding and cloning some major genes or major QTLs with large genetic effects.

Currently, some genetic linkage maps of pear based on the F_1 population have been developed independently in various labs for this species. But in most of the researches, the European pear and the Japan-Korea pear were used as materials (Yamamoto et al., 2002; Pierantoni et al., 2007), and the number of mapping populations was 63–82, the number of map markers was less than that of saturated maps, and the QTLs of the agronomic traits were not analyzed. Therefore, we selected an F_1 progeny derived from Yali and Jingbaili to construct their genetic linkage maps, respectively, and Yali and Jingbaili showed a similarity coefficient of 0.53. The genetic linkage map of pear was constructed in order to identify the potential

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Wenying SUN, Yuxing ZHANG (✉)
Key Laboratory of Fruit Setting Physiology and Molecular Biology, Agricultural University of Hebei, Baoding 071001, China,
E-mail: jonsonzhyx@yahoo.com.cn

Wenying SUN
Plant Science Department, Henan Vocational College of Agriculture, Zhengzhou 451450, China

Wenquan LE, Hai'e ZHANG
Hebei Changli Institute of Pomology, Changli 066600, China

molecular markers linked to leaf length, leaf width, leaf length/leaf width and petiole length.

2 Materials and methods

2.1 Plant materials

A total of 145 F_1 populations from a cross between Yali and Jingbaili were used to construct a genetic map. The self-incompatibility of Yali as the maternal line prevented the risk of contamination by self-pollination in the progeny. This population was generated by the Changli Institute of Pomology, Hebei, China. Plants were about six years old, and most of them were kept juvenile and did not flower.

2.2 Methods

2.2.1 DNA extraction

Harvested fresh leaves were freeze-dried and ground to powder. Total genomic DNA was extracted from fresh leaves of the parental lines and seedlings; DNA extraction was performed according to the CTAB method. DNA concentration was estimated by using a UV3802 spectrophotometer as well as by visual comparison to the known concentration of the λDNA marker.

2.2.2 AFLP analysis

AFLP procedure was as follows: An aliquot genomic DNA (450 ng) from each F_1 plant and the parent was digested with the restriction enzymes 3U *Eco*R I and 3U *Mse* I, and the digested fragments were ligated with *Eco*R I and a *Mse* I adapter. In a 20-μL volume, the digestions were carried out at 37°C for 5 h, and *Eco*R I and *Mse* I adapter were subsequently ligated to the digested DNA fragment by adding 5 μL of the adapter ligation solution. The ligation teations were incubated for 10 h at 37°C. Five microliters of ligation products from the 1∶10 dilution were used for the PCR pre-amplification step. Pre-amplification reactions were performed in a 20-μL volume. PCR cycles were performed at 95°C for 2 min, 95°C for 30 s, 56°C for 30 s and 72°C for 1 min for total 30 cycles, then at 72°C for 10 min on a 580BR0519 thermal cycler. Selective amplifications were performed using one hundred and fifty-four primer combinations of 16 *Eco*R I primers (E-acc, E-acg, E-agc, E-agg, E-act, E-atc, E-aaa, E-aat, E-aca, E-aag, E-aac, E-ag, E-tc, E-ac, E-tg, E-gc) and 18 *Mse* I primers (M-cag, M-cat, M-ctg, M-ctt, M-cca, M-cta, M-cac, M-caa, M-cag, M-cat, M-ctc, M-ctg, M-ctt, M-cca, M-cta, M-cac, M-caa). The following PCR reactions were done at 95°C for 2 min, 95°C for 50 s, 65°C (−0.7°C per cycle) for 40 s, 72°C for 1 min for 12 cycles, until reaching the optimal annealing temperature 56°C, thirty-one cycles were done at 95°C for 50 s, 56°C for 40 s, 72°C for 1 min, and then at 72°C for 10 min to complete the selective amplification.

2.2.3 SSR analysis

For SSR analysis, 18 pairs of primers were selected for amplification. The genomic DNA concentration was adjusted to 15 ng·μL^{-1}. Each 20-μL amplification reaction contained 30 ng of gDNA, 1.6 μL 10×PCR buffer, 1.6 μL 2.5 mmol·L^{-1} dNTP, 1.6 μL 25 mmol·L^{-1} Mg^{2+}, 0.8 μmol·L^{-1} forward primer and reverse primer, and 1 U of Taq DNA polymerase (TakaRa, Dalian, Liaoning, China). PCR amplifications were carried out under the following conditions: at 94°C for 2 min, followed by 35 cycles at 94°C for 1 min, 49°C–55°C for 1 min, 72°C for 2 min, and 72°C for 5 min.

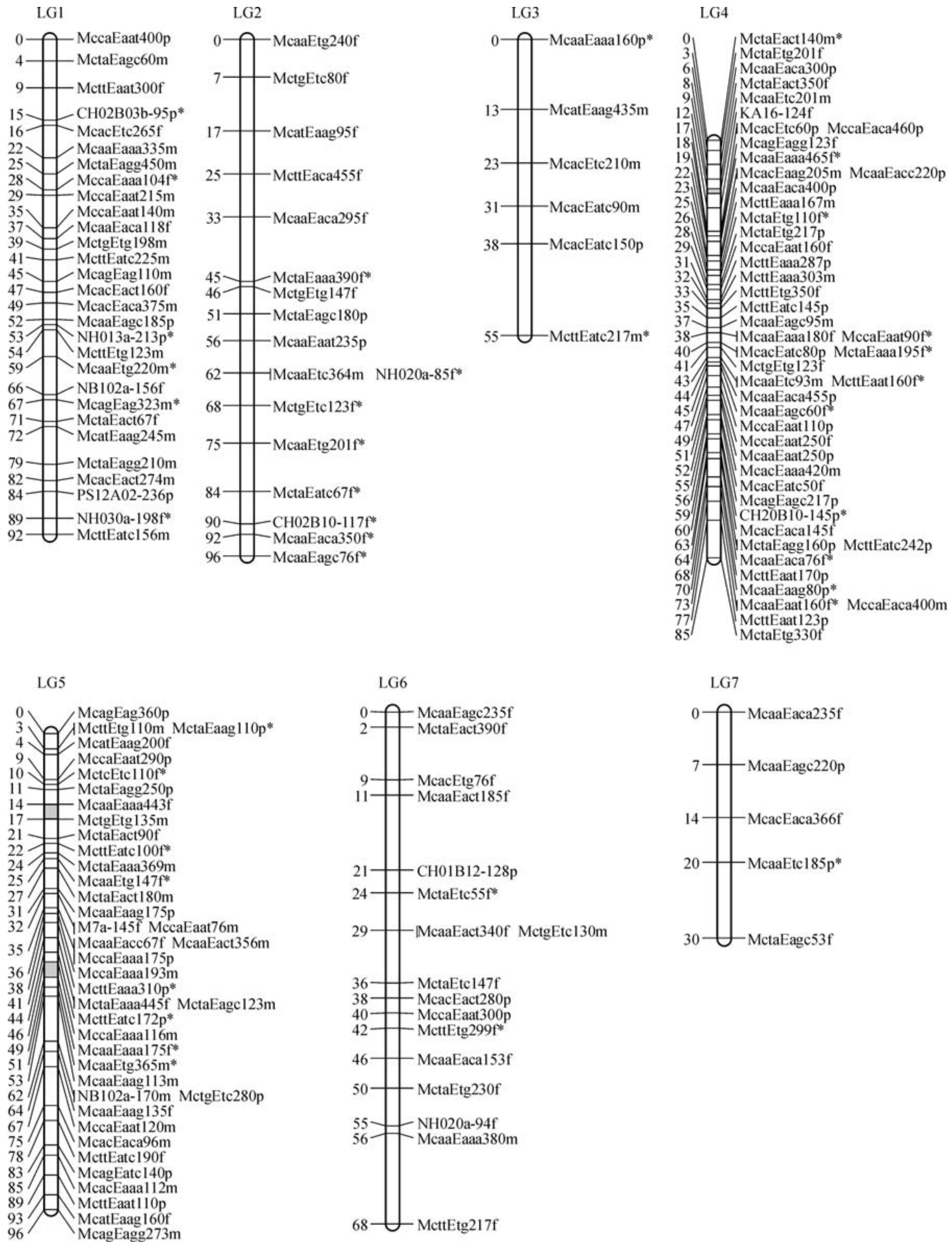
2.2.4 Band and data analysis

Data were scored on the basis of the presence or absence amplified fragments (1 = presence, 0 = absence). Markers obtained with the AFLP and SSR procedures were separated into three genotypes: (1) those heterozygously segregated for the female parent, which were signed as "m" in the map; (2) those heterozygously segregated in the progeny for the male parent, which were signed as "f" in the map; (3) those polymorphically segregated in the progeny for both parents, which were signed as "p" in the map. Only markers (1) and (2) were segregated in 1∶1 (present and absent) ratio, markers (3) were segregated in 3∶1 ratio. Segregation distortion from the expected 3∶1 and 1∶1 Mendelian ratio was evaluated by applying the Chi-square tests of goodness-of-fit at 1% and 0.1% levels of probability. All of the markers were used for mapping. Segregation distortion markers were marked by "*" in the map. The map was constructed using Jionmap version 3.0. Kosambi function was used to convert the recombination frequency into genetic distance in centimorgans (cM), and the map was constructed at a LOD threshold value 3.0–8.0.

3 Results

3.1 Linkage analysis

Linkage analysis was done using Jionmap version 3.0 software at a LOD score of 4.0–7.0. Four hundred and fifty-four polymorphic AFLP markers and 41 SSR markers were used to generate the map of pear based on the segregation data obtained from 145 F_1 plants; eighteen linkage groups were obtained, and a total of 402 loci were made, which constituted the data set (Fig. 1). These 402 markers covered 1395.9 cM. The average spacing between markers for each group ranged from 2.0 to 11.0 cM. The


LG1
0 MccaEaat400p
4 MctaEagc60m
9 McttEaat300f
15 CH02B03b-95p*
16 McacEtc265f
22 MccaEaaa335m
25 MctaEagg450m
28 MccaEaaa104f*
29 MccaEaat215m
35 MccaEaat140m
37 MccaEaca118f
39 MctgEtg198m
41 McttEatc225m
45 McagEag110m
47 McacEact160f
49 McacEaca375m
52 McaaEagc185p
53 NH013a-213p*
54 McttEtg123m
59 McaaEtg220m*
66 NB102a-156f
67 McagEag323m*
71 MctaEact67f
72 McatEaag245m
79 MctaEagg210m
82 McacEact274m
84 PS12A02-236p
89 NH030a-198f*
92 McttEatc156m
LG2
0 McaaEtg240f
7 MctgEtc80f
17 McatEaag95f
25 McttEaca455f
33 McaaEaca295f
45 MctaEaaa390f*
46 MctgEtg147f
51 MctaEagc180p
56 McaaEaat235p
62 McaaEtc364m NH020a-85f*
68 MctgEtc123f*
75 McaaEtg201f*
84 MctaEatc67f*
90 CH02B10-117f*
92 McaaEaca350f*
96 McaaEagc76f*
LG3
0 McaaEaaa160p*
13 McatEaag435m
23 McacEtc210m
31 McacEatc90m
38 McacEatc150p
55 McttEatc217m*
LG4
0 MctaEact140m*
3 MctaEtg201f
6 McaaEaca300p
8 MctaEact350f
9 McaaEtc201m
12 KA16-124f
17 McacEtc60p MccaEaca460p
18 McagEagg123f
19 McaaEaaa465f*
22 McacEaag205m McaaEacc220p
23 McaaEaca400p
25 McttEaaa167m
26 MctaEtg110f*
28 MctaEtg217p
29 MccaEaat160f
31 McttEaaa287p
32 McttEaaa303m
33 McttEtg350f
35 McttEatc145p
37 McaaEagc95m
38 McaaEaaa180f MccaEaat90f*
40 McacEatc80p MctaEaaa195f*
41 MctgEtg123f
43 McaaEtc93m McttEaat160f*
44 McaaEaca455p
45 McaaEagc60f*
47 MccaEaat110p
49 MccaEaat250f
51 McaaEaat250p
52 McacEaaa420m
55 McacEatc50f
56 McagEagc217p
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60 McacEaca145f
63 MctaEagg160p McttEatc242p
64 McaaEaca76f*
68 McttEaat170p
70 McaaEaag80p*
73 McaaEaat160f* MccaEaca400m
77 McttEaat123p
85 MctaEtg330f
LG5
0 3 4 9 10 11 14 17 21 22 24 25 27 31 32 35 36 38 41 44 46 49 51 53 62 64 67 75 78 83 85 89 93 96
McagEag360p
McttEtg110m MctaEaag110p*
McatEaag200f
MccaEaat290p
MctcEtc110f*
MctaEagg250p
McaaEaaa443f
MctgEtg135m
MctaEact90f
McttEatc100f*
MctaEaaa369m
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MctaEact180m
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McaaEaag113m
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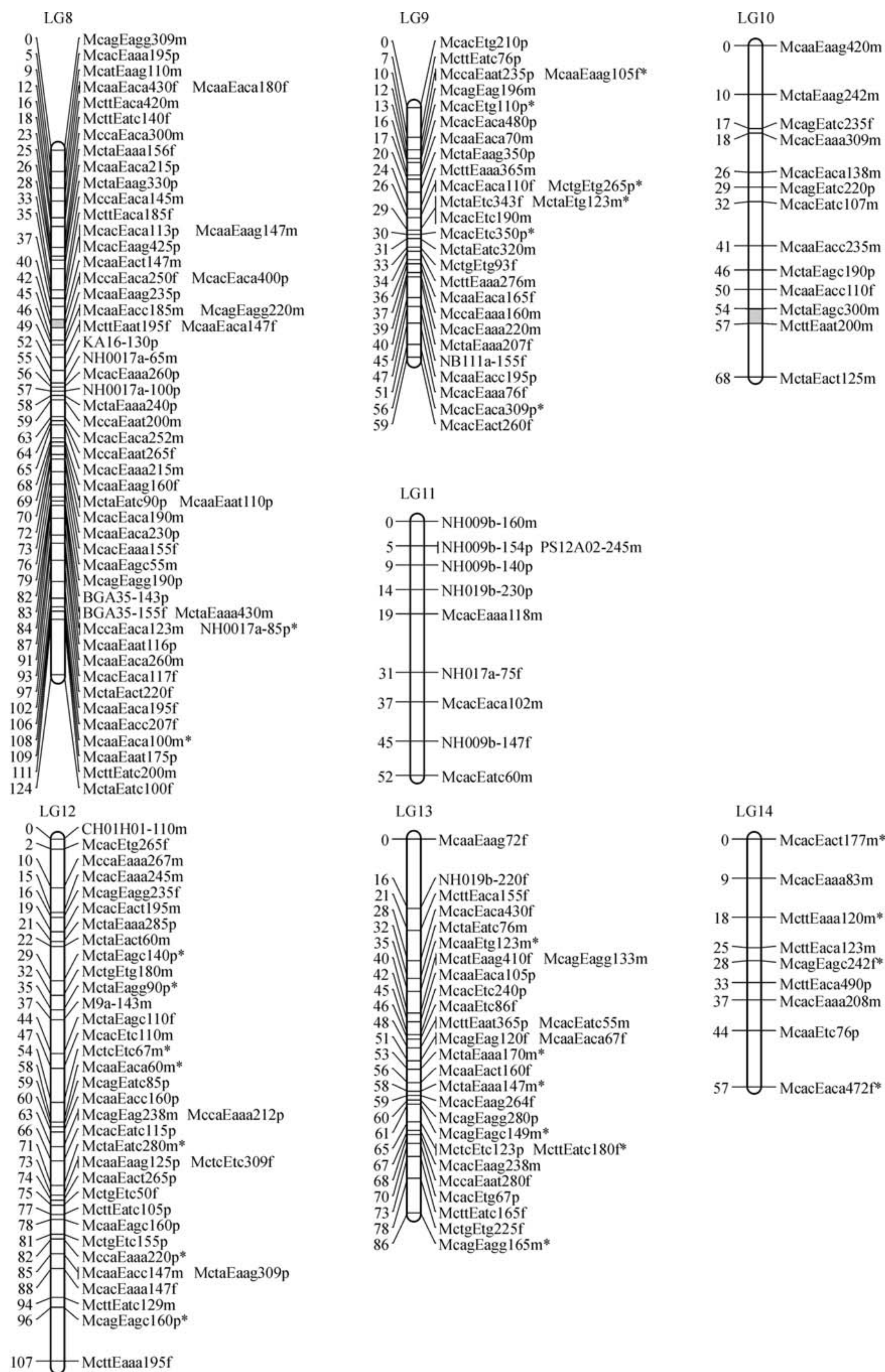
McacEaca96m
McttEatc190f
McagEatc140p
McacEaaa112m
McttEaat110p
McatEaag160f
McagEagg273m
LG6
0 McaaEagc235f
2 MctaEact390f
9 McacEtg76f
11 McaaEact185f
21 CH01B12-128p
24 MctaEtc55f*
29 McaaEact340f MctgEtc130m
36 MctaEtc147f
38 McacEact280p
40 MccaEaat300p
42 McttEtg299f*
46 McaaEaca153f
50 MctaEtg230f
55 NH020a-94f
56 McaaEaaa380m
68 McttEtg217f
LG7
0 McaaEaca235f
7 McaaEagc220p
14 McacEaca366f
20 McaaEtc185p*
30 MctaEagc53f


LG8
LG9
LG10
LG11
LG12
LG13
LG14

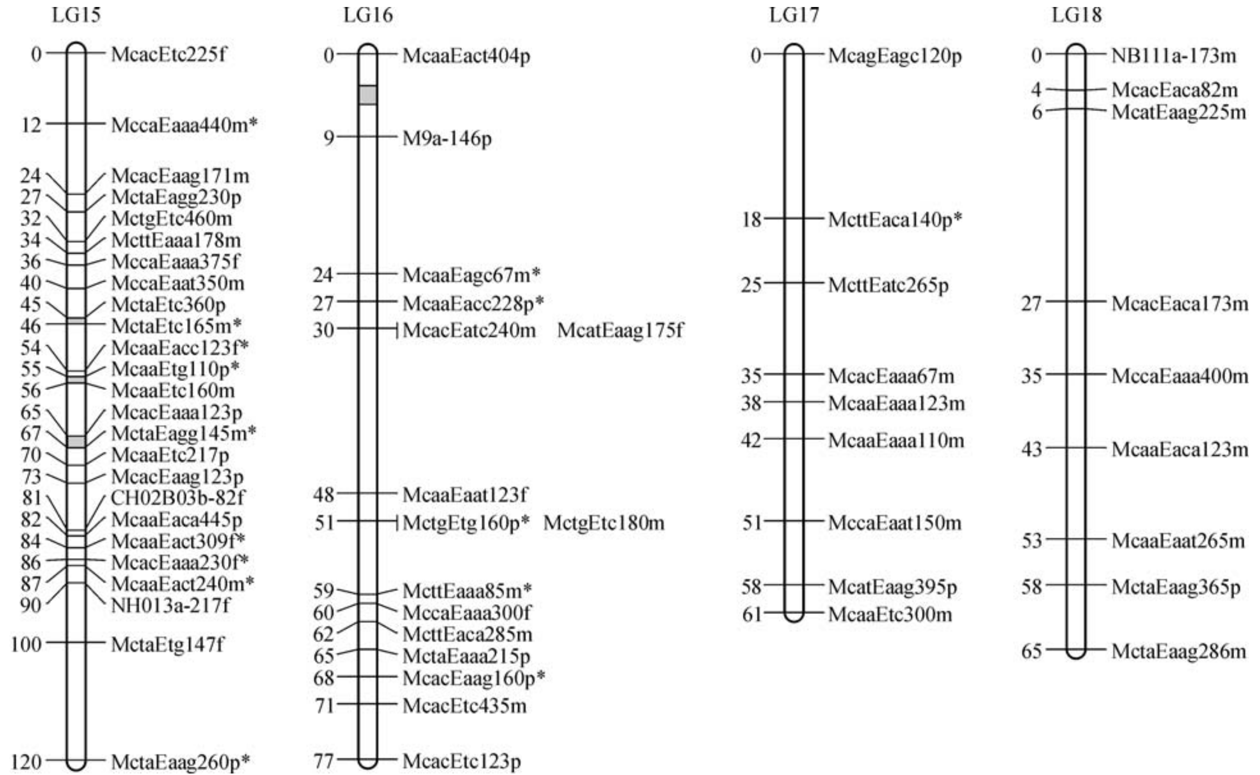


Fig. 1 Molecular genetic maps in pear based on AFLP and SSR markers

average length of each linkage group was 77.6 cM, with the lowest value of 29.6 cM in LG7 and the highest of 123.7 cM in LG8. The number of loci per linkage group was from 5 to 56, and there were eight linkage groups whose numbers of loci were more than 20, with 56 in LG8, 50 in LG4, 42 in LG5, 36 in LG12, 29 in LG13, 29 in LG1, 28 in LG9 and 25 in LG15. We observed only four intervals longer than 15 cM, which were distributed in four of the eighteen linkage groups. Eighty markers in the map deviated from the expected segregation at a threshold of 1% and 0.1%, with 19.7% deviated percentage.

3.2 Correlation analysis of some leaf traits

We analyzed the main genetic parameters and the correlation of leaf length, leaf width, leaf length/width and petiole length of the 145 F_1 populations of Yali × Jingbaili. The results showed that different relationships existed among the four leaf traits, and the kurtosis and skewness were less than two. The frequency distribution further showed that the four traits were of single peak histogram, which belonged to a normal distribution (Fig. 2). However, the kurtosis of leaf length/width was 1.88, and the skewness was 0.95. So we inferred that the trait was controlled by the unequal effect of genes or major genes.

The correlation analysis showed that the four traits were correlated to different degrees. For example, there was a very significant positive correlation between leaf length and leaf width and leaf length/width (R = 0.550** and R = 0.370**), and a positive correlation with petiole length (R = 0.177*). There was a very significant negative correlation between leaf width and leaf length/width (R = −0.556**), and a very significant positive correlation with petiole length (R = 0.238**). The correlation traits may be mapped at the adjacent or recent position in the same linkage group.

3.3 QTL analysis

The data on total leaf length, leaf width, leaf length/width and petiole length from the 145 F_1 populations derived from Yali × Jingbaili were analyzed for mapping QTL by using the MapQTL version 4.0 software based on the genetic linkage map of pear. A LOD value of 2.5 was used as the threshold value for the existence of a QTL. If the LOD value was more than 3.5, then we could infer that there would be major genes, by analyzing eleven QTLs associated with the four leaf traits, which were detected and mapped on the linkage groups of LG4, LG5, LG8, LG10, LG15 and LG16 (Fig. 1, Table 1).

Four QTLs for the leaf length trait were detected (*pyc-1*,

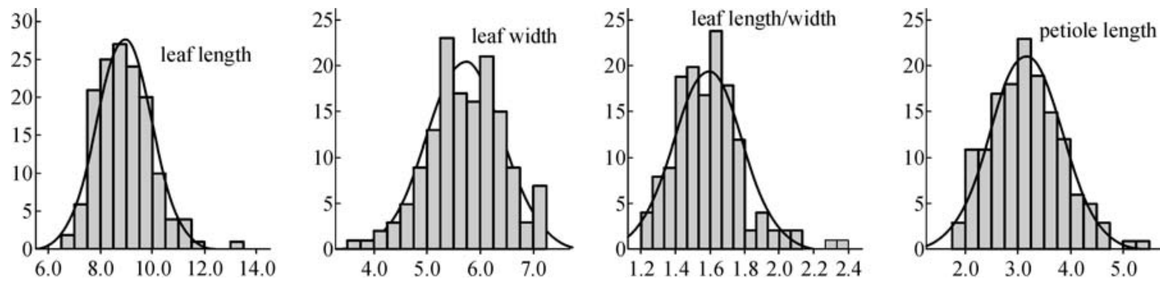


Fig. 2 The frequency distribution of four leaf traits in pear mapping population

pyc-2, *pyc-3* and *pyc-4*), among which was *pyc-2*, with a LOD value greater than 3.5. The distance between the four QTLs and the nearest molecular markers was 0.2–4.0 cM, the minimum distance between *pyc-1* located on LG8 and the McaaEact147 m AFLP marker was 0.2 cM, and the maximum distance between *pyc-3* located on LG 16 and the M9a-146p SSR marker was 4.0 cM. The four QTLs explained the variance of 10.7%, 9.9%, 10.6% and 10.1%.

Two QTLs for the leaf width trait were detected (*pyk-1* and *pyk-2*), with *pyk-1* and *pyk-2* located on LG10 and LG15, respectively. The LOD values of the two QTLs were less than 3.5, with the distance between the two QTLs and the nearest molecular markers being 0.2–0.4 cM. The two QTLs explained the variance of 48.5% and 47.8%.

Another two QTLs for the leaf length/width trait were detected (*ycw-1* and *ycw-2*), and both *ycw-1* and *ycw-2* were located on LG5, with the LOD value being less than 3.5, the distance between the two QTLs and the nearest molecular markers being 0.1–0.5 cM and the variance being 7.9% and 9.8%.

The other three QTLs for the petiole length trait were detected (*ybc-1*, *ybc-2* and *ybc-3*), and *ybc-1* was located on LG4, *ybc-2* and *ybc-3* were located on LG15, and among them *ybc-1* and *ybc-3* had LOD values more than 3.5. The distance between the three QTLs and the nearest molecular markers was 0.1–3.8 cM. The maximum distance between *ybc-3* and the McacEaaa123p AFLP marker was 3.8 cM. The three QTLs explained the variance of 39.2%, 40.6% and 39.4% of the traits.

4 Discussion

4.1 Map construction

Most fruit crops are self-incompatible and have a long breeding period, so it is difficult to create an F_1 population and the recombinant inbred strain population for mapping. These characteristics restrict the development of the study for constructing linkage maps in fruit crops. Hemmat et al. (1994) put forward the feasibility of constructing a molecular genetic map using a "double pseudo-testcross" strategy through establishing an F_1 population. It was suggested that if any one of the mapping parents would have recessive genes, then polymorphic marker segregation in the F_1 hybrid progeny were in a 1∶1 (present and absent) ratio, and the 1∶1 segregation type was based on the source of heterozygosis in order to construct the female and male linkage map. The idea was widely used in constructing linkage maps in fruit crops.

Many studies of linkage maps in pear at present use the "double pseudo-testcross" strategy. That is, female and male maps can be constructed respectively by using the BC_1 model of the Mapmaker.exe software, but only the segregation 1∶1 markers can be used to construct a female or male map. Because the abundance of 3∶1 markers can not determine the source of alleles, we could not analyze the individual genotypes. To some degree, this may increase the difficulty in constructing the linkage map. Fang et al. (2003) analyzed the 3∶1 markers in mango

Table 1 The QTLs distribution of 4 leaf traits in pear genetic linkage map

trait	linkage group	QTL	LOD	the nearest marker	position/cM	distance/cM	Expl/%
leaf length	LG8	*pyc-1*	2.47	McaaEact147m	40.3	0.2	10.7
		pyc-2	3.57	McaaEaag253p	45.0	0.1	9.9
	LG15	*pyc-3*	2.92	MctaEagg145m*	67.4	0.4	10.6
	LG16	*pyc-4*	2.92	M9a-146p	5.0	4.0	10.1
leaf width	LG10	*pyk-1*	2.50	McttEaat200m	56.8	0.2	48.5
	LG15	*pyk-2*	2.56	MctaEtc360p	44.6	0.4	47.8
leaf length /width	LG5	*ycw-1*	2.57	McaaEaaa443f	14.5	0.5	7.9
		ycw-2	2.52	McaaEaag113m	48.9	0.1	9.8
petiole length	LG4	*ybc-1*	3.66	MctaEact350f	8.2	0.2	39.2
	LG15	*ybc-2*	3.38	McaaEtc160m	55.9	0.1	40.6
		ybc-3	3.73	McacEaaa123p	61.2	3.8	39.4

interspecific hybridization and constructed a map of fourteen linkage groups and a total of 39 loci based on the binomial distribution principle. However, because the "F_2 map" and "BC_1 map" did not have the same probe, then the maps could not be integrated with each other.

Stam (1995), who created the "CP" mapping strategy in the Jionmap version 2.0 software, solved the question. The "CP" strategy could be produced in two heterozygous genotypes or one heterozygous genotype and one homozygous genotype for the diploid hybridization parents. The idea was suitable for outcrossing fruit crops or F_1 population of many trees to construct a high-density genetic linkage map. Shen (2005) analyzed 419 polymorphic AFLP markers using Jionmap 3.0 based on the segregation data obtained from 150 F_1 jujuba plants, and fourteen linkage groups were obtained successively, making a total of 333 loci. We analyzed 454 polymorphic AFLP markers and 41 SSR markers also using Jionmap 3.0, and eighteen linkage groups were obtained, making a total of 402 loci. These 402 markers covered 1395.9 cM. The average spacing between markers was 3.8 cM. AFLP markers mapping efficiency was 81.9% and SSR markers mapping efficiency was 82.9%.

The frame of a linkage map needed 20-cM spacing between markers for each group, the objective was for the major gene location, and the average spacing was 10–20 cM or smaller (Fang et al., 2001). In the study, the linage map of the pear was constructed based on AFLP and SSR markers, and it could primarily locate the major gene and map QTL. However, the saturation of the map was not enough, and there was still more than 10 cM spacing between the two nearest markers in some regions. So in the future, we need to increase the map density.

4.2 QTL mapping and their relationship

Some QTLs with correlation traits were easy to aggregate on an adjacent region of the same linkage group. The aggregation phenomenon for the function genes with similarities was ubiquitous. We thought that these genes could express and adjust the genetic information in harmony. Meanwhile, some research results were reported about QTLs with correlations located on specific regions or QTLs tightly linking with other QTLs in tomato (Parniske and Jones, 1999), potato (van der Vossen et al., 2000), lettuce (Meyers et al., 1998) and so on.

Studies on the four traits showing some QTLs with very significant or significant correlation were located on the same linkage groups, and these QTLs were co-segregated or tightly linked. For instance, four QTLs associated with leaf length trait, leaf width trait and petiole length trait were located on linkage group 15, and their distribution intervals were 44.6–67.4 cM.

QTLs for co-segregation or tightly linking could be the genetic bases for phenotypic correlation traits, and the traits and QTLs were not always relative. In this study, we failed in detecting QTLs for co-segregation or tight linking, although these traits had a very significant or significant correlation. For instance, the leaf length trait was correlated to leaf width trait (R = 0.370**), while we failed to detect the co-segregation or tightly linking QTLs with the two traits.

The QTLs for co-segregation or tight linking were beneficial to marker-assisted selection, however, we should not only choose the genes through the two sides' markers or a linkage marker, but we also should consider the effects of other QTLs. Therefore, it was difficult to choose objective traits to analyze these genes. We will further study the above results, find the map objective region and test the relationship of the markers and traits.

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