Characterization of Morphological Traits Based on a Genetic Linkage Map in Peach

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Thirteen morphological characters including 9 simply inherited and 4 quantitative traits, were analyzed by using a genetic map constructed with F₂ progenies from a cross between the peach cultivars 'Akame' and 'Juseitou'. The map consisted of 92 markers (9 morphological and 83 DNA markers) covering approximately 1020 cM, in which 9 linkage groups were found. Eleven SSR (simple sequence repeat) markers were mapped. Segregation of 9 qualitative traits in the F₂ population fitted to the expected ratio, suggesting that these traits were controlled by single genes. Three characters relating to pigmentation showing a monogenic inheritance, i.e., flesh color around the stone, flower color (pink/pale pink) and fruit skin color, were newly identified. The fruit skin color cosegregated with the leaf color trait located on the linkage group 3. The flower color and the flesh color around the stone were located on the linkage group 4 with a recombination value of 0.13. Furthermore, several DNA markers tightly linked to flesh adhesion and 2 kinds of root-knot nematode resistance were obtained, which could be used as a reliable tool for trait selection. QTL analysis was conducted on 4 morphological traits, flowering time, maturation time, fruit dropping time, and fruit weight. Six, three, three and four QTLs were identified for these 4 traits, respectively. Among them, 12 QTLs tended to gather on the linkage groups 3 and 6, whereas no QTLs were detected on the groups 2, 4, 8 and 9.

Key Words: *Prunus persica*, quantitative trait loci, simple sequence repeat.

Introduction

Peach (*Prunus persica* (L.) Batsch) is one of the most important fruit species adapted to temperate and subtropical zones. More than 30 morphological traits of peach showing a simple inheritance have been revealed for tree, foliage, flower, and fruit habitats (Scorza and Sherman 1996). Analysis of several linkages among the morphological characters had been conducted. Bailey and French (1949) suggested

that 4 genes controlling flesh adhesion, melting flesh, soft melting flesh, and albino were located on the same chromosome. Chaparro *et al.* (1994) reported 2 linkages between pillar tree habit and double flower, and among white flower, pollen sterility and weeping growth. However, the number of characterized morphological traits is not sufficient for genetic studies as well as for breeding purposes and the information on their linkages is still very limited.

Recently, genetic linkage maps have been constructed in peach, in which some morphological traits were involved (Foolad *et al.* 1995, Rajapakse *et al.* 1995, Warburton *et al.* 1996, Dirlewanger *et al.* 1998, Lu *et al.* 1998). Rajapakse *et al.* (1995) could map 3 traits relating to the flesh color, double flower, and pillar tree habit, but did not determine the location of the red flower color, brachytic dwarf, and compact tree habit, on their RFLP- and RAPD-based linkage maps. The other traits, i.e., flesh adhesion, soft melting flesh, low malic acid, pubescent skin, pollen sterility, flat fruit shape, root-knot nematode resistance, have been mapped on different genetic maps of peach or interspecific populations resulting from crosses between peach and related species (Warburton *et al.* 1996, Dirlewanger *et al.* 1998, Lu *et al.* 1998). In addition, QTL analysis focusing on fruit quality, such as acid and sugar contents, pH, titratable acidity, soluble solid content and so on, was performed by using an F₂ population derived from an intraspecific cross in peach (Dirlewanger *et al.* 1999). Nevertheless, a large number of morphological trait loci have not been located on one genetic map in peach. Furthermore, linkage maps constructed by different groups have not been integrated yet. It is necessary to conduct analyses and mapping of a large number of trait loci, and to combine different genetic maps by using codominant DNA markers.

In our previous study, a linkage map was constructed by using 133 F₂ progenies from an intraspecific cross between a rootstock peach, 'Akame', and a flower-ornamental cultivar, 'Juseitou' (Shimada *et al.* 2000). Three morphological traits, leaf color, plant height and leaf shape, were found to be controlled by a single dominant gene and were mapped on the same linkage group.

In this study, the other 6 traits relating to fruit, flower and nematode resistance could be scored, since the F₂ progenies from 'Akame' × 'Juseitou' became mature and the trees bore fruits. Thus, we characterized 13 morphological traits including 9 simply inherited and 4 quantitative traits based on the genetic linkage map obtained from the same F₂ population derived from 'Akame' × 'Juseitou'. We also

Communicated by M. Hirai

Received March 21, 2001. Accepted June 25, 2001.

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conducted mapping of SSR markers used as anchor loci to compare different peach maps.

Materials and Methods

Plant materials and trait evaluation

The 126 F₂ plants were obtained by selfing a single F₁ plant derived from a cross between the peach (*Prunus persica* (L.) Batsch) cultivars 'Akame' (accession No. NIFTS6111027-0367) and 'Juseitou' (accession No. NIFTS6111027-0378). Nine morphological traits showing a simple segregation were investigated for each F₂ individual, parents, and their F₁ (5 individuals) as follows: leaf color (red/green), plant height (normal/brachytic dwarf), leaf shape (wide/narrow), flower color (pink/pale pink), fruit skin color (red/green), flesh adhesion (freestone/clingstone), pigmentation around the stone (red/none), resistance to the nematode *Meloidogyne incognita* (resistant/susceptible), and resistance to the nematode *M. javanica* (resistant/susceptible). The former 3 traits had already been characterized in our previous report (Shimada *et al.* 2000). The fruit skin color was evaluated by using young fruits (early July), while the flesh adhesion and pigmentation around the stone were evaluated in mature fruits. Resistance to the root knot nematodes *M. incognita* and *M. javanica* was bioassayed by using 5 to 15 rooted cutting clones of each F₂ individual (Yoshida 1981). The strains of the nematodes (preserved at the National Institute of Fruit Tree Science, Ibaraki, Japan) were grown with garden balsam plants (*Impatiens balsamina* L.) in potted soils. The rooted F₂ clones were transplanted to the soils for inoculation and the presence or absence of galls and egg masses was investigated for the determination of the resistance or susceptibility at 3 months after inoculation.

Four quantitative characters, i.e., flowering time, maturation time, fruit dropping time, and fruit weight, were also investigated. The flowering time corresponded to the time when more than 20% of the inflorescences were open. The maturation time corresponded to the time when flesh softening was observed for more than half of the fruits. The fruit dropping time corresponded to the time when more than 30% of the fruits spontaneously dropped. The fruit weight was measured for 10 fruits of each tree harvested at maturation time.

DNA analyses

Total genomic DNA was isolated from fresh young leaves of each individual according to the CTAB-based method. RAPD, AFLP, ISSR, PCR-RFLP markers were characterized and described (Shimada *et al.* 2000). The designation of AFLP is based on the primer combination and the sizes indicated as bp. The designations of RAPD and ISSR are based on the primer and the sizes expressed as kbp. For example, EAAC/MCTC-315, OPI6/0.4 and ISSR811/0.6 indicate the 315 bp AFLP fragment from the combination of E-AAC and M-CTC primers, the 0.4 kbp band from the RAPD primer OPI6 and the 0.6 kbp band

from the ISSR primer No. 811, respectively. One PCR-RFLP marker is indicated by the clone name pc48.

Eleven SSRs (simple sequence repeats), i.e., UDP96-001, UDP96-003, UDP96-005, UDP96-015, UDP96-019, UDP98-406, UDP98-408, DP98-409 (Cipriani *et al.* 1999), SSR-M2b, SSR-M3b, and SSR-M6a were used. The latter 3 SSRs were developed from cDNA clones obtained from young fruit tissues of the peach cultivar 'Akatsuki'. Three sets of oligonucleotide sequences were used as primers for the amplification of these SSRs: SSR-M2b (5'-GCCCTCCTCCTCTCAGCCA-3', 5'-GCATCCTTTTTTTGGACATAACCAC-3'), SSR-M3b (5'-CGAGAACTCTGCACAGAGA-3', 5'-GTAGCCGATTCAAAGCCTCC-3'), SSR-M6a (5'-AGAAGGGCAAGCCCAAGTGC-3', 5'-TGCAAAGCCA GAGCCCACAA-3'). PCR amplification was performed in a 20 µl solution of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, 0.2 mM each of dNTPs, 10 pmoles of each forward primer labelled with fluorescent chemical (FAM or TET or HEX) and unlabelled reverse primer, 10 ng of genomic DNA, and 0.5 unit of Taq polymerase (Life Technologies, USA). Amplification was performed for 35 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 2 min, for denaturation, annealing, and primer extension, respectively. The PCR products were separated and detected using a PRISM 377 DNA sequencer (PE Applied Biosystems, USA). The size of the amplified bands was calculated based on an internal standard DNA (GeneScan-350TAMRA, PE Applied Biosystems, USA) with GeneScan software (PE Applied Biosystems, USA).

Linkage and QTL analysis

A total of 102 markers including 37 AFLPs, 38 RAPDs, 11 SSRs, 5 ISSRs, 2 PCR-RFLPs and 9 phenotypic traits, were analyzed for the construction of a genetic linkage map using Mapmaker/EXP v3.0 (Lander *et al.* 1987, Lincoln *et al.* 1993b). The Kosambi Function was used to convert the recombination units into genetic distances. The mapping analysis was conducted using a minimum LOD score of 4.0 and recombination frequency of 0.3. QTL analysis was performed for 4 quantitative characters using Mapmaker/QTL 1.1 (Lincoln *et al.* 1993a). A threshold LOD score value of 2.5 was selected for the presence of a QTL.

Results and Discussion

Evaluation of qualitative traits

Nine morphological characters showing a simple segregation were investigated for the F₂ plants, their parents and F₁ plants. Segregation of 3 traits, i.e., flesh adhesion (*F/f*), resistance to the nematode *Meloidogyne javanica* (*Mj/mj*), and resistance to *M. incognita* (*Mi/mi*), observed in an F₂ population fitted to the ratio of 3:1, based on the Chi-square test (Table 1). No segregation of phenotypes was detected in 5 F₁ individuals. Furthermore, the uniformity of these qualitative traits was observed in our rootstock breeding population consisting of 14 F₁ hybrids derived from

Table 1. Segregation of 9 morphological characters

Character	Symbol	Phenotypes of parents and F ₁			Segregation in F ₂	χ^2 -value
		Akame	Juseitou	F ₁		
Flesh adhesion	<i>F/f</i>	clingstone	freestone	freestone	freestone : clingstone = 98 : 24	1.85ns
Flesh color around stone	<i>Cs/cs</i>	white	red	red	red : white = 96 : 26	0.89ns
Flower color	<i>Fc/fc</i>	pale pink	pink	pink	pink : pale pink = 94 : 32	0.01ns
Fruit skin color	<i>Sc/sc</i>	red	green	red	red : green = 97 : 29	0.26ns
Leaf color ¹⁾	<i>Gr/gr</i>	red	green	red	red : green = 97 : 29	0.26ns
Plant height ¹⁾	<i>Dw/dw</i>	normal	brachytic dwarf	normal	normal : dwarf = 98 : 28	0.52ns
Leaf shape ¹⁾	<i>Nl/nl</i>	wide	narrow	wide	wide : narrow = 98 : 28	0.52ns
Resistance to <i>M. javanica</i>	<i>Mj/mj</i>	susceptible	resistant	resistant	resistant : susceptible = 70 : 27	0.42ns
Resistance to <i>M. incognita</i>	<i>Mi/mi</i>	susceptible	resistant	resistant	resistant : susceptible = 66 : 29	1.55ns

¹⁾ described in Shimada *et al.* (2000)

‘Akame’ and ‘Juseitou’ (Yoshida and Seike 1981). It was previously reported that these 3 traits were controlled by single genes (Bailey and French 1949, Blake 1937, Lammerts 1945, Scorza and Sherman 1996, Weinberger *et al.* 1943, Yoshida and Seike 1981). The results obtained were in good agreement with previous reports, reconfirming that the 3 morphological characters were controlled by single genes.

We also evaluated 3 characters relating to pigmentation, i.e., flesh color around the stone (red/white), flower color (pink/pale pink), and fruit skin color (red/green), which had not been thoroughly identified up to now. The results are shown in Table 1. The segregation for color of the flesh around the stone fitted to the ratio of 3 : 1 in the F₂ individuals, which suggested that the red color flesh around the stone was controlled by a single dominant gene designated as *Cs/cs*, since no gene symbol had been adopted. Although Blake and Connors (1936) stated that the red color around the stone was dominant over its absence, no data were presented. It was possible to score the pink or pale pink flower color. The segregation of the F₂ individuals with the flower color fitted to the ratio of 3 : 1 based on the Chi-square test. The gene controlling the flower color (pink/pale pink) appeared to be a single gene and was designated as *Fc/fc*. Lammerts (1945) reported that the flower color was controlled by 3 pairs of genes, i.e., colored/white (*W/w*), pink/red (*R/r*), and dark pink/light pink (*P/p*). We considered that *P/p* (dark pink/light pink) might be different from *Fc/fc* (pink/pale pink), because it was reported that peach with ‘dark pink flowers’ produced progenies with red flowers (Lammerts 1945). It will be necessary to conduct allelism tests to determine whether *Fc/fc* is controlled by the same locus as *P/p*. Another trait relating to pigmentation, fruit skin color (*Sc/sc* as gene symbol), was also investigated. The segregation of red or green fruit skin color fitted to the ratio of 3 : 1 in the F₂ population and showed an identical pattern with that of the leaf color.

Genetic linkage map

In our previous genetic linkage map, 10 linkage groups included 86 markers with 3 trait loci, encompassing 960 cM (Shimada *et al.* 2000). In this study, we reconstructed a link-

age map including newly scored 6 morphological trait loci and 11 SSRs. The map consisted of 9 morphological markers and 83 DNA markers (35 AFLPs, 31 RAPDs, 11 SSRs, 5 ISSRs, 1 PCR-RFLP). Distorted dominant DNA markers were excluded by preliminary linkage analysis because it was rather difficult to determine whether the distortion was due to problems associated with band detection (ex. the same fragments from more than 2 loci) or from the hybrid nature (ex. dysfunctional or lethal factors). Nine linkage groups were identified that covered approximately 1020 cM with an average distance of 12 cM between each pair of loci (Fig. 1). The linkage groups 1 to 4 were basically the same as the groups previously identified by Shimada *et al.* (2000). Group 5 consisted of the previous groups 7 and 9, and group 6 consisted of the previous groups 5 and 6. The size of the linkage groups ranged from 205 cM (Group 1) to 20 cM (Group 9). Six RAPD, 3 AFLP, and 1 PCR-RFLP markers could not be assigned to any linkage groups. All the 11 SSR markers could be mapped, among which 2, 5, 2, 1 and 1 SSRs were assigned to the linkage groups 1, 3, 5, 6 and 7, respectively. Only one marker UDP98-406 showed a distorted segregation. Mapping of additional markers (especially codominant markers such as SSRs) should be performed to develop a saturated genetic map to cover the whole genome, since the number of linkage groups does not correspond to the basic chromosome number (*n* = 8).

Interestingly, the loci controlling 3 characters relating to color were mapped at different positions. The *Gr* locus (cosegregating with *Sc*) was mapped on the linkage group 3 and did not show any linkage with the flower color (*Fc*) and the flesh color around the stone (*Cs*). The *Fc* and *Cs* were located on the same linkage group 4, with a recombination value of 0.13. There are no reports, to our knowledge, dealing with the linkage among the characters relating to color. In this study, we revealed that *Gr* cosegregated with *Sc* and the relationship of the 3 loci relating to color was demonstrated for the first time. The female parent ‘Akame’ was characterized by the presence of a red leaf, pale pink flower, white flesh around the stone, compared to the male parent ‘Juseitou’ which showed a green leaf, pink flower, and red flesh around the stone. When the composition of antho-

Fig. 1. Genetic linkage map obtained in the 'Akame' × 'Juseitou' F_2 progeny. Loci and genetic distances are listed on the right and the left sides, respectively. Nine morphological markers are shown as gene symbols. The designation of the AFLP is based on the primer combination and the sizes expressed as bp (Eco primer/Mse primer-size). RAPD markers are indicated as the primer name and its fragment size (kbp). ISSR markers are based on the primer name and fragment size as kbp indicated by ISSR-. SSR markers are denoted by UDP- or SSR-.

cyanins of the red leaf, pink flower and red flesh around the stone was analyzed and compared by HPLC, there were no differences in the main components. These results suggested that the expression of color was regulated by several different factors (genes), controlling specific pigmentation of different tissues.

Two kinds of nematode resistance loci *Mi* and *Mj* were closely located at a distance of 4.8 cM on the linkage group 3. Five recombinants were detected for resistance to *M. incognita* and *M. javanica*. Four F_2 individuals showed a resistance to *M. javanica* and susceptibility to *M. incognita*, compared to 1 F_2 plantlet which showed the reverse phenotype. We were able to confirm that the resistance to the nematodes *M. incognita* and *M. javanica* was not controlled by the same gene but by different genes with a very close location. A large number of DNA markers linked to the resistance loci were found in this study, for use as selection markers. For example, the ISSR marker ISSR834-1/0.4 and the AFLP markers EAA/MCAC-135, were obtained. These markers are currently being investigated for conversion into STS markers. It was reported that two resistance loci, *Mi* and *Mj* derived from the rootstock cultivar 'Nemared',

were linked together on an AFLP-based map and that the DNA markers obtained had been evaluated in several rootstock cultivars (Lu *et al.* 1998, Lu *et al.* 1999). It was also described that *Mj* showed a resistance to both nematodes *M. incognita* and *M. javanica*. Since the origin of the resistance gene of 'Nemared' is unclear, it will be necessary to determine whether the resistance alleles *Mi*, *Mj* derived from 'Juseitou' and *Mi*, *Mj* from 'Nemared' are identical, and whether the strains (races) of nematodes are identical.

Flesh adhesion (*F/f*), one of the most important characters of fruits, was mapped on the linkage group 6. No linkages were observed for the other characters. Warburton *et al.* (1996) mapped this trait on their map derived from an interspecific cross. In this study, we were able to obtain 2 tightly linked AFLP markers to the flesh adhesion, EAC/MCTC-180 and EACA/MCTG-330. These AFLP markers will be used as selection markers.

QTL analysis

Four quantitative characters, flowering time, maturation time, fruit dropping time, and fruit weight, were subjected to QTL analysis. Frequency distributions in the F_2

individuals and the values of the parents, F_1 for the 4 traits are shown in Fig. 2. The flowering time in each F_2 individual was distributed in the range of 12 days, compared to 'Akame', 'Juseitou' and F_1 which bloomed on the 9th day, the 5th day and the 7th day after the earliest day of F_2 , respectively. The maturation time and fruit dropping time of the F_2 individuals were widely distributed over a range of 24 days. The values of the parents and F_1 were observed at intermediate positions between the F_2 distribution. In contrast, more than 80% of the F_2 individuals showed a larger fruit weight than the parents. The fruit weight of each F_2 individual ranged from 55g to 131g. The F_1 plants also exhibited a larger weight than the parents, suggesting that the heterosis effect might be reflected on the fruit weight.

Six QTL loci for the flowering time were found at the LOD value of 2.5-5.6 (Fig. 3, Table 2). Among them, 3 loci were observed in group 3, which showed similar effects to those of Juseitou's alleles for the acceleration of the flowering time. The other 3 loci, which produced opposite effects to those of the alleles of 'Akame', were detected on groups 1, 5 and 6. Three QTLs controlling the maturation time were observed at the LOD values of 2.78, 3.30 and 2.71 in groups 3, 6 and 7, respectively. Two loci in groups 3 and 6 showed

similar effects to those of Akame's alleles for the retardation of the maturation time, compared to the locus of group 7 which showed an opposite effect. For the fruit dropping time, 3 QTLs were found in regions which almost overlapped those for the maturation time in the same groups 3, 6 and 7. All the loci showed similar effects to those of the alleles of 'Akame'. These results indicated that the genes controlling the maturation time and fruit dropping time were closely related, even if the effects of the loci in group 7 were different between the two traits. There were 4 loci (3 loci in group 3 and 1 locus in group 6) corresponding to the fruit weight. All the loci produced the same effects as the Akame's alleles.

We identified a total of 16 QTL loci controlling 4 traits, flowering time, maturation time, fruit dropping time and fruit weight. Eight and 4 QTL loci were located on groups 3 and 6, respectively. In contrast, no QTL loci were observed on groups 2, 4, 8 and 9. Dirlewanger *et al.* (1999) reported that there were 1 locus for the blooming date, 2 loci for the maturation date, and 1 locus for the fruit fresh weight in their linkage map constructed from a cross between the non-acid peach cultivar 'Ferjalou Jalousia' and the acid nectarine cultivar 'Fantasia'. For these quantitative traits, the fre-

Fig. 2. Frequency distribution of 4 characters in the F_2 progeny. a: flowering time (zero corresponding to the earliest day, March 26th), b: maturation time (zero corresponding to the earliest day, September 7th), c: fruit dropping time (zero corresponding to the earliest day, September 7th), and d: fresh fruit weight. The values of the female parent, male parent and their F_1 , are denoted by A, J and F_1 , respectively.

Fig. 3. Location of QTLs controlling flowering time (Ft), maturation time (Mt), fruit dropping time (Dt), and fruit weight (Fw). The confidence intervals of QTLs are denoted by vertical blocks and the peak position is indicated by horizontal lines. The length of the horizontal lines corresponds to the LOD values. Mark 'A' indicates the alleles of 'Akame' that increase the value of each trait. Mark 'J' indicates the alleles of 'Juseitou' that increase the value of each trait.

quency distribution in the F_2 plantlets and the values of the parents and F_1 in our population were markedly different from those of the Dirlwanger's group. These differences were presumably due to differences in the gene(s) controlling QTL traits. Unfortunately, no anchor markers are available to compare both maps. Therefore, it will be necessary to combine different maps and to compare QTL loci.

Comparison of linkage maps

Several groups of researchers have constructed linkage maps in peach (Dirlwanger *et al.* 1998, Rajapakse *et al.*

1995, Warburton *et al.* 1996) and interspecific crosses between peach and almond (Foolad *et al.* 1995, Joobeur *et al.* 1998). Although these maps included some morphological traits, they have not been sufficiently integrated. Thus, linkages and relationships among phenotypic traits are still unclear. It is very important that codominant DNA markers such as SSRs and CAPS (cleaved amplified polymorphic sequence) are developed and mapped on the different peach maps used as anchor loci for integration.

Eight out of 17 SSR markers developed by Cipriani *et al.* (1999) were mapped on our linkage map. Some of the

Table 2. Detected QTLs and their locations, LOD scores, and effects and modes of gene action

Characters	Location	Nearest marker	LOD score	Additive effect ¹⁾	Dominance effect
Flowering time	group 1	EAAC/MCTG-290	3.25	-0.98	2.34
	group 3	UDP96-019	3.06	0.93	-0.62
	group 3	UDP96-015	2.52	0.84	-0.61
	group 3	Dw	2.53	0.11	2.08
	group 5	EACA/MCAC-100	5.59	-1.47	1.35
	group 6	OPQ9/0.6	3.06	-1.24	-0.40
Maturation time	group 3	OPAX8/0.3	2.78	-1.10	-2.21
	group 6	UDP96-003	3.30	-2.39	0.66
	group 7	UDP98-406	2.71	0.25	3.23
Fruit dropping time	group 3	UDP96-001	5.25	-2.79	0.15
	group 6	EAAC/MCTG-180	3.58	-2.26	-0.44
	group 7	EAAC/MCTA-120	3.37	-0.21	4.88
Fruit weight	group 3	EAAC/MCTC-410	4.41	-9.17	2.95
	group 3	OPV3/1.7	6.30	-11.39	7.51
	group 3	Dw	10.75	-13.61	5.70
	group 6	OPQ9/0.6	2.57	-7.44	2.21

¹⁾ -, + values indicate the direction of the additive effect on the phenotype for 'Akame', 'Juseitou', respectively.

SSR markers developed by Cipriani *et al.* (1999) were assigned to a BC₁ interspecific cross population from *P. persica* × (*P. persica* × *P. ferganensis*) (Quarta *et al.* 1998) and to an interspecific cross between peach and almond (Joobeur *et al.* 1998) (Dr. I. Verde, personal communication). Therefore, our linkage map is partially anchored to these maps, in which linkages between SSRs are in good agreement.

In this study, two-fifths of the SSRs examined showed polymorphisms between the parents in our mapping population, compared to only 37 AFLPs and 38 RAPDs which could be scored and screened from 72 combinations of AFLP primers and 1080 RAPD primers, respectively. It was reported that the level of polymorphism of the RAPD and AFLP markers in intraspecific crosses in peach was low (Chaparro *et al.* 1994, Lu *et al.* 1998). These results indicated that SSR markers could be much more powerful tools than AFLP and RAPD for genome mapping of closely related populations.

Acknowledgements

The authors thank Drs. I. Kajiura, M. Yoshida, H. Iketani for their discussions and valuable suggestions. Contribution No. 1221 of the National Institute of Fruit Tree Science. This work was supported in part by a Grant-in-Aid (Development of DNA Marker-aided Selection Technology of Plants) from the Ministry of Agriculture, Forestry and Fisheries.

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