Genome Research on Peach and Pear

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Abstract A lot of SSRs (simple sequence repeats) in peach and pear from enriched genomic libraries and in peach from a cDNA library were developed. These SSRs were applied to other related species, giving phenograms of 52 Prunus and 60 pear accessions. Apple SSRs could also be successfully used in Pyrus spp. Thirteen morphological traits were characterized on the basis of the linkage map obtained from an F_2 population of peach. This map was compiled with those morphological markers and 83 DNA markers, including SSR markers used as anchor loci, to compare different peach maps. Molecular markers tightly linked to new root-knot nematode resistance genes were also found. A linkage map including disease-related genes, pear scab resistance and black spot susceptibility, in the Japanese pear Kinchaku were constructed using 118 RAPD markers. Another linkage map, of the European pear Bartlett, was also constructed with 226 markers, including 49 SSRs from pear, apple, peach and cherry. Maps of other Japanese pear cultivars, i.e., Kousui and Housui, were also constructed. These maps were the first results of pear species.

Key words: Genetic diversity, linkage map, morphological traits, pear scab, *Prunus persica*, *Pyrus pyriforia*, root-knot nematode, simple sequence repeats

Introduction

Linkage maps and molecular markers for agronomically important characters would be especially useful in traditional crossbreeding programs for long-lived crops such as fruit trees. Molecular markers are effectively utilized to select seedlings at the early stage of breeding before evaluating traits expressed only in mature plants such as fruit quality. Markers linked to disease and pest resistance genes (loci) can improve the efficiency of selection. Moreover, map-based cloning of interesting genes could help to improve fruit tree by genetic transformation. Various characters different from herb crops such as long juvenile period, dormancy and wood formation are important targets for fruit-tree genome analysis.

Peaches and pears are members of the family Rosaceae, which ranks as one of the most agronomically important plant families in temperate climate regions. It includes such economically important species as apple, apricot, plum, sweet and sour cherries, almond, strawberry and rose. Peach (*Prunus persica*) belonging to the sub-family Amygdaloideae, is the best genetically characterized species in the family; more than 30 of its morphological traits have been characterized. Peach is a self-pollinated diploid (2n=16), and has a small genome size of approximately 530 Mbp/diploid nucleus, about twice the size of the genome of *Arabidopsis thaliana*. Moreover, its juvenile stage is also shorter (about 3 years) than other perennial fruit trees. Therefore, peach is considered the ideal system for genome analysis in Rosaceae. Apple (*Malus domestica*) belonging to the sub-family Pomoideae, is also used in western countries as a material for genome research due to its economic importance as a tree-fruit. However, it has various faults such as a long germination time, a large chromosome number (2n=34) and self-incompatibility, as well as fewer morphological markers than peach. Pears, including a Japanese pear (*Pyrus pyrifolia*), Chinese pears (*P. bretschneideri, P. ussuriensis,* etc.) and a European pear (*P. communis*), of the same sub-family as apple, are also important tree-fruit in the temperate climate regions in the world, despite the fact they have disadvantages similar to apple. Thus we selected pears as materials for genome research.

Genome mapping of fruit trees has advanced rapidly in Europe and America in the past 10 years. Recently, peach research has been extensively carried out by the European *Prunus* Projects and Clemson University in the United States. DNA markers linked to

Table1. Segregation of 9 morphological characters.

Character	Symbol	Phenotypes of parents and F_1			Segregation in F_2	x_2-value
		Akame	Juseitou	F_1		
Flesh adhesion	*F/f*	clingstone	freesztone	freesztone	freestone:clingstone=98:24	1.85ns
Flesh color around stone	*Cs/cs*	white	red	red	red:white=96:26	0.89ns
Flower color	*Fc/fc*	pale pink	pink	pink	pink:pale pink=94:32	0.01ns
Fruit skin color	*Sc/sc*	red	green	red	red:green=97:29	0.26ns
Leaf color	*Gr/gr*	red	green	red	red:green=97:29	0.26ns
Plant height	*Dw/dw*	normal	brachytic dwarf	normal	normal:dwarf=98:28	0.52ns
Leaf shape	*Nl/nl*	wide	narrow	wide	wide:narrow=98:28	0.52ns
Resistance to *M. javanica*	*Mj/mj*	susceptible	resistant	resistant	resistant:susceptible=70:27	0.42ns
Resistance to *M. incognita*	*Mi/mi*	susceptible	resistant	resistant	resistant:susceptible=66:29	1.55ns

(Yamamoto et al. 2001b. Reproduced with permission from the Japanese Society of Breeding)

several kinds of morphological loci have been detected. Linkage maps of apple have been mainly constructed by the European Apple Genome Project, which is particularly interested in the map-based markers linked to economically important genes such as blackspot disease resistance. To the best of our knowledge, no one has conducted studies on pear except for our group.

Our genome projects for peach and pear started in 1997 and 1995, respectively. This paper introduces our genome research on peach and pear, including the development of DNA markers and linkage map constructions.

Development of Molecular Markers and Genetic Diversity

Random amplified polymorphic DNA (RAPD) has been widely used on genetic studies. RAPD markers are generated by PCR amplification of random genomic DNA segments with single arbitrary primers (usually 10 nucleotides long). Amplified fragment length polymorphism (AFLP) is based on selective amplification of restriction enzyme-digested DNA fragments. A large number of fragments (50 to 100) are generated in each amplification reaction that originates from random sequences in genomes. Most maps of early stage peach and apple have been constructed by RAPD and AFLP markers because they are powerful and cost-effective methods to identify DNA polymorphism. Since these markers show generally dominant inheritance, they must be converted to sequence tagged sites (STSs) in order to apply them to comparative mapping studies and use them in marker-assisted selection.

SSRs (simple sequence repeats, also called microsatellites) are excellent sources of polymorphisms in eukaryotic genomes. Microsatellites are comprised of tandem arrays of 2- to 5-bp repeat units. Polymorphisms appear to be generated from variation in the number of tandem repeats in a given repeat motif. Designing primers from the sequenced regions flanking the repeat motifs are amplified to induce such polymorphisms. SSRs are currently the best marker systems because they have high reliability and codominant inheritance, in spite of the cost and time required to develop them. In plants, SSR markers have been used widely for cultivar identification and genetic mapping.

The technical development of SSRs from genomic libraries has not yet been completed. We succeeded in developing SSRs from peach and pear enriched genomic libraries. We also found SSR sequences from a peach cDNA library, which helped us to create an alternative method for mapping expressed genes.

SSRs from Peach

A genomic library enriched with (AG)/TC) sequences was constructed from the peach variety Akatsuki using the magnet beads method. We obtained 60 independent sequences containing 8 to 36 microsatellite repeats, 21 repeats on the average. The average insert size of the clones obtained was 465 bp, with a range of 110 to 1300 bp. Twenty-two SSR clones showed polymorphisms in 12 peach accessions with 3-9 alleles per locus.

cDNA clones containing SSRs of peach were also found. cDNA libraries were obtained from fruits of Akatsuki, harvested 25 and 110 days after flowering. Each clone was sequenced and searched for homology of nucleotide sequences. Twelve cDNA clones containing microsatellite repeats were collected from about 800 expressed sequence tag (EST) sequences, in which about 700 clones were from young peach fruits of 25 days after flowering and about 100 clones were from mature fruits of 110 days after flowering.

Seven, two, two, and one cDNA clones contained microsatellite repeats in the putative 5′-untranscribed region (UTR), 3′-UTR, transcribed region (TR) and in an unknown region, respectively. Out of 12 sequences, 8 had complete or interrupted motifs of AG or TC repeats, whereas others contained microsatellite repeats of

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Housui                  1: TTCCAATTCACATGAGGCTGATAATCTGCTGCTCCAACACTAAAAACAAACAATCCCATG :60
Kousui                  1: TTCCAATTCACATGAGGCTGATAATCTGCTGCTCCAACACTAAAAACAAACAATCCCATG :60
Cox's Orange Pippin     1: TTCCAATTCACATGAGGCTGATGATCTGCTGCTCCAACACTAAAAACAAACAATCCCATT :60
Golden Delicious        1: TTCCAATTCACATGAGGCTGATGATCTGCTGCTCCAACACTAAAAACAAACAATCCCGTT :60
                                  **  ********************************** *

Housui                 61: GATAACAAGAGCATTGTAATCCTCCATTTCGCCATCTTCGATGTCTCCACTTCTCGCTCA :120
Kousui                 61: GATAACAAGAGCATTGTAATCCTCCATTTCGCCATCTTCGATGTCTCCACTTCTCGCTCA :120
Cox's Orange Pippin    61: ACTAACAAGAGCAATATACTCCTCCTTTTCCCCATCATCGATCTCTCCGCTTCTCACTCA :120
Golden Delicious       61: ACTAACAAGAGCAATAAACTCCTCCTTTTCCCCATCATCGATCTCTCCGCTTCTCACTCA :120
                             *********** *  * ****** **** ***** ***** ***** ****** ****

Housui                121: CAATCTTGCAGAGGGGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG---- :176
Kousui                121: CAATCTTGCAGAGGGGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG :180
Cox's Orange Pippin   121: CAATCTTGCACAC---AGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG-------- :169
Golden Delicious      121: CAATCTTGCACAC---AGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG-------- :169
                           ********** *

Housui                177: GAATGTGTCTGGTTTGAGAGGACGAAGATAAGAATGCCTTGATTATATACAGTGCGTGCA :237
Kousui                181: GAATGTGTCTGGTTTGAGAGGACGAAGATAAGAATGCCTTGATTATATACAGTGCGTGCA :240
Cox's Orange Pippin   170: GAATGTGTCTGGTTTGAGAGGAGGAAGATAAGAATGTCTTGATTATATACCGTGCGTGCA :229
Golden Delicious      170: GAATGTGTCTGGTTTGAGAGGAGGAAGATAAGAATGTCTTGATTATATACCGTGCGTGCA :229
                           ********************** ************ ************ *********

Housui                238: TGCCCTCATGGCTTCAGTTT :256
Kousui                241: TGCCCTCATGGCTTCAGTTT :260
Cox's Orange Pippin   230: TGCCCTCATGGCTTCAGTTT :249
Golden Delicious      230: TGCCCTCATGGCTTCAGTTT :249
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3-6 bp units. Seven sequences showed significant homology to the registered genes in a database. They were cytochrome c reductase, hypersensitive-induced response protein, antimicrobial protein, 60S ribosomal protein L27a, water-stress inducible protein, embryo-abundant protein like and CS-IAA2. Seven clones showed polymorphisms in peach with 2-7 alleles per locus. All 12 microsatellite clones developed from peach cDNAs can be transferred to other *Prunus* species, such as almond, apricot, and mume.

Twelve SSR markers, 8 from cDNA and 4 from the genomic library, genetically identified 52 *Prunus* species including 31 peaches, 10 species related to peach (almond, *P. mira, P. davidiana, P. kansuensis,* etc.), 3 apricot, 4 plum, 3 Japanese apricot and 1 cherry. All accessions could be successfully differentiated. A phenogram based on the SSR genotypes was obtained, showing 6 major groups corresponding to the above *Prunus* species. Almost all SSR markers from cDNA produced amplified fragments in all accessions, perhaps because the primer sequences from cDNA are stably conserved among accessions. SSR markers were highly polymorphic and could be utilized as a reliable tool for cultivar identification in *Prunus*.

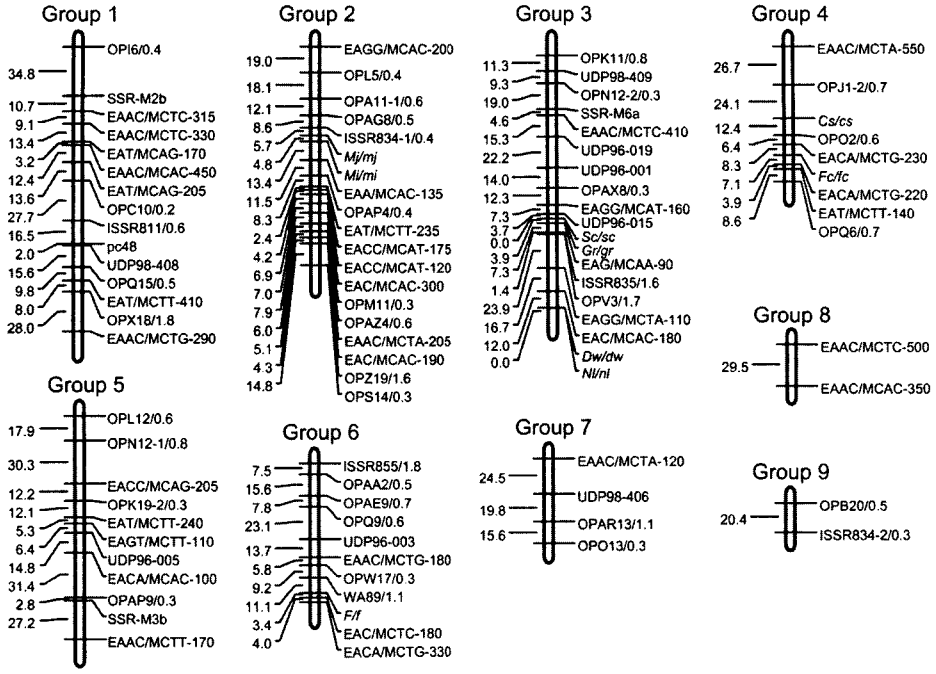
SSRs from Pear

A genomic library enriched with (AG)/TC) sequences was constructed from the Japanese pear variety Housui using the magnet beads method (Yamamoto et al. 2002a, 2002b). We obtained 85 independent sequences containing 8-36 microsatellite repeats, 20 repeats on average. The average insert size of the clones obtained was 360 bp, and the range was from 136 to 880 bp. Out of the 85 sequences, 59 contained complete (AG)/(TC) repeats and the others had interrupted repeats or combined motifs of (AG)/(TC) and other units. Thirteen primer pairs could successfully amplify the target fragments, and showed a high degree of polymorphism in the Japanese pear with 3-6 alleles per locus. Ten microsatellites successfully amplified loci in the European pear, and were found to be highly polymorphic as well.

Nine SSR markers with a total of 133 putative alleles genetically identified 58 Asian pear accessions from 6 *Pyrus* species (Kimura et al. 2002). All the SSR markers produced 1 or 2 discrete amplified fragments for all the diploid accessions, whereas a triploid variety showed 3 fragments with some SSRs. The number of putative alleles ranged from 7 to 20, with an average value of 14.8. A phenogram based on the SSR genotypes was obtained, showing 3 major groups corresponding to the Japanese, Chinese and European groups. The SSR markers were highly polymorphic and could be utilized as a reliable tool for cultivar identification in Asian pears.

Application of Apple SSRs to Pear

Nine apple SSRs were intergenetically applied to the characterization of 36 pear accessions, including Japanese pears, Chinese pears, European pears, wild relatives and hybrids (Yamamoto et al. 2001a). All of the tested SSR primers derived from apple produced discrete amplified fragments in all pear species and accessions. The differences in fragment size among pears or between pears and apples were, in many cases, due to the



differences in repeat number. Interestingly, the DNA sequence of franking regions in apple was highly conserved in pear (Figure.1). A total of 79 alleles were detected from seven SSR loci in pear, and all pear varieties except for the mutants could be differentiated.

Although both pear and apple are classified into the Rosaceae family and the Pomoideae sub-family, their genetic relationship remains unclear. This study demonstrated that apple SSRs could be successfully used in *Pyrus* spp. Our results confirm that *Pyrus* spp. has a close genetic relationship with Malus spp.

Map Construction of Peach

Recently, genetic linkage maps have been constructed for 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; 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 study, we characterized 13 morphological traits including 9 simply inherited and 4 quantitative traits based on the genetic linkage map obtained from an F_2 population of peach. We also mapped SSR markers used as anchor loci to compare different peach maps.

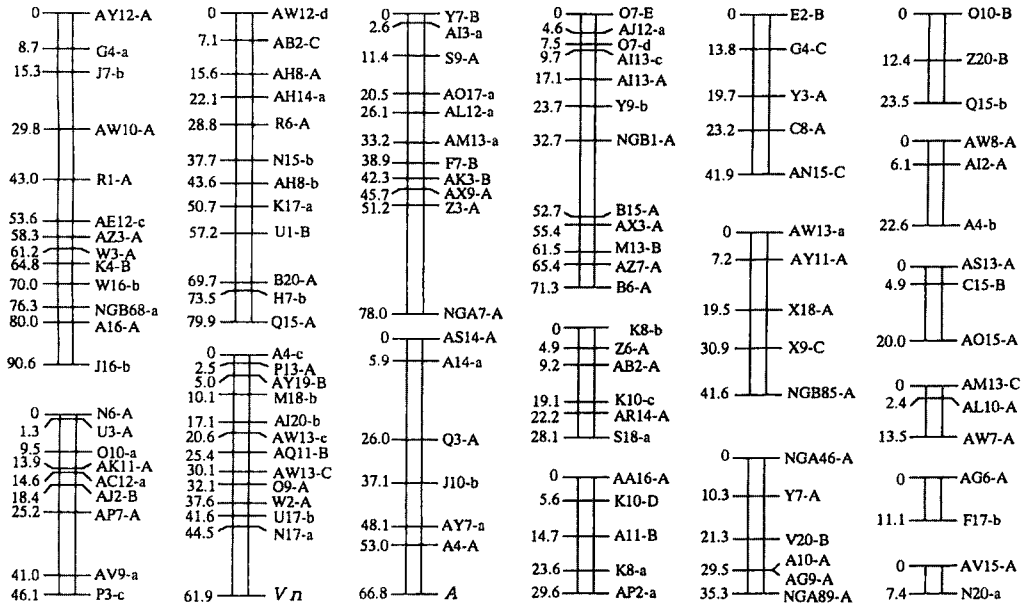
Evaluation of qualitative traits

One hundred and twenty-six F_2 progenies from an intraspecific cross between a rootstock peach, Akame, and a flower-ornamental cultivar, Juseitou, were used for analyses of morphological characters. Nine characters showing a simple segregation were investigated for the F_2 plants, their parents and the F_1 plants. The results are shown in the Table (Shimada et al. 2000; Yamamoto et al. 2001b).

Each of brachytic dwarf (Dw/dw), red leaf (Gr/gr), and narrow leaf (Nl/nl) fitted to the segregation ratio of 3:1, confirming that these traits were controlled by a single dominant gene. Dwarf growth habit and red leaf phenotype are linked to each other. Narrow leaf phenotype (nl) co-segregated with the dw locus is controlled by a recessive gene. We believe that the dw gene has a pleiotropic effect to control the leaf shape of the peach.

The segregation of 3 traits, i.e., flesh adhesion (F/f), and resistance to the root-knot nematode *Meloidogyme javanica* (Mj/mj) and *M. incognita* (Mi/mi), suggested that each of them was controlled by a single dominant gene.

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. The segregation test for color of the flesh around the stone suggested that this was controlled by a single dominant gene designated as Cs/cs. The gene controlling the flower color (pink/pale pink) appeared to be a single gene and was designated as Fc/fc. Another trait relating to pigmentation, fruit skin



color (Sc/sc), showed an identical pattern with that of the leaf color.

Four quantitative characters, flowering time, maturation time, fruit developing time, and fruit weight, were subjected to quantitative trait loci (QTL) analysis. Six, three, three and four QTLs were identified for these 4 traits, respectively.

Genetic linkage map of Peach

We constructed a linkage map composed of 9 morphological markers and 83 DNA markers (35 AFLPs, 31 RAPDs, 11 SSRs, 5 ISSRs, 1 PCR-RFLP) (Yamamoto et al. 2001b). This linkage map is shown in Figure. 2. We identified 9 linkage groups that covered approximately 1020 cM with an average distance of 12 cM between each pair of loci. The size of the linkage groups ranged from 205 cM (Group 1) to 20 cM (Group 9). 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. Additional markers (especially codominant markers such as SSRs) should be mapped 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. Analyses of the composition of anthocyanins of the red leaf, pink flower and red flesh around the stone by HPLC showed no differences in the main components. These results suggest 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 linkage group 3. 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.

Flesh adhesion (F/f), one of the most important characters of fruits, was mapped on linkage group 6. No linkages were observed for the other characters. We were able to obtain 2 tightly linked AFLP markers to the flesh adhesion. These will be used as selection markers.

New Root-knot Nematode Resistance Genes

Root-knot nematodes are ones of the most serious parasites in peach as well as the other *Prunus* species and the family Rosaceae, since it is impossible to remove infected nematodes from the roots of perennial fruit trees. In peach, the existence of two genes, Mi/mi and Mij/mij, showing resistance to *M. incognita* and to both *M. incognita* and *M. javanica*, respectively, has been indicated by analysis with DNA markers (Lu et al. 1999). It was reported that an STS (sequence tagged site) marker (EAA/MCAT10) from an AFLP marker showed a linkage with the Mij gene in an F_2 population of 'Nemared' × 'Lovell'. One SSR marker (pchgms1) was also found to be located on the same linkage group as the nematode resistance loci Mi and Mij in the same population.

These two DNA markers EAA/MCAT10 (STS) and pchgms1

(SSR) were analyzed and mapped in our F_2 population derived from a cross between resistant Juseitou and susceptible Akame. These 2 markers, which showed a significant linkage in our mapping population, did not show any linkages with the resistance loci to *M. incognita* and *M. javanica* of Juseitou. These results indicate that the nematode resistance character of Juseitou is different from that of Nemared and is controlled by new genes. Five STS markers, tightly linked to the resistance genes of Juseitou, were successfully developed. One of these markers cosegregated with the resistance gene to *M. javanica*. The STS markers obtained will be utilized for the introgression of new root-knot nematode resistance genes from Juseitou into peach rootstocks.

Map Construction of Pear

No linkage map for pears has been reported yet, despite the many papers on apple mapping. Because apple and pear are considered to be in very low differentiation in a sub-family Pomoideae, we constructed maps of pear species for comparative mapping with apple.

The preparation of F_2 or backcross populations is generally required for mapping. In the case of fruit tree, F_2 is used for species with short generation and a self-pollination nature such as peach, but is difficult to apply it to long generation species such as apple and pear. Here, we applied the pseudo-testcross method (Grattapaglia and Sederoff 1994), in which two maps of their parents are constructed by analysis of F_1 population.

Mapping of Disease-Related Genes in Japanese Pear

Black spot caused by *Alternaria altemata* and pear scab caused by *Venturia nashicola* are the most severe diseases of Japanese pear. The delicious Nijisseiki is particularly vulnerable to black spot. Housui and Kousui produced by crossbreeding and Gold Nijisseiki produced by mutation with gamma-irradiation are successful resistant cultivars derived from Nijisseiki. On the other hand, damage from pear scab is still serious because almost all commercially important cultivars are susceptible. A survey of *Pyrus* germplasm has identified Kinchaku as the only Japanese pear cultivar having resistance, although there are some Chinese pear and European pear cultivars that are also resistant.

An F_1 population composed of 82 individuals between Kinchaku and Kousui was used to construct a linkage map (Iketani et al. 2001). The linkage map for Kinchaku is shown in Figure 3. Eighteen linkage groups, consisting of 120 loci, including 118 RAPD and 2 morphological markers, with a total length of 768 cM were constructed for Kinchaku. The resistance allele of pear scab (Vn) and the susceptibility allele of black spot (A) in Kinchaku were mapped in different linkage groups. Furthermore, twenty-two linkage groups, consisting of 78 loci, with a total length of 508 cM, were constructed for Kousui.

Comparison of scab resistance genes in pear and apple will be important to identify the pear gene because the two pathogens may be differentiated into two species from one genus.

Mapping of European pear

All species of pear, Japanese pear, European pear and Chinese pears are intercrossable and there are no major incompatibility barriers to interspecific hybridization. Thus 63 F_1 individuals obtained from an interspecific cross between the European pear cultivar, Bartlett, and the Japanese pear cultivar, Housui, were used to construct a linkage map of Bartlett. This map consisted of 226 markers, including 175 AFLPs, 32 pear SSR loci, 12 apple SSRs, 3 peach SSRs, 2 cherry SSRs, 1 isozyme and 1 self-incompatibility (S) locus. Eighteen linkage groups were identified that covered 949 cM, with an average distance of 4.2 cM between each pair of loci. The size of the linkage groups ranged from 10.7 cM to 90.3 cM.

Another linkage map of Housui was also constructed. It consisted of 154 loci, including 105 AFLPs, 42 SSRs (29, 7, 4 and 2 were derived from pear, apple, peach and cherry), 3 isozymes, 1 phenotypic trait leaf color (Lc) and the S locus. This map contained 17 linkage groups ranging from 91.3 cM to 14.5 cM and encompassed 926 cM.

Some linkage groups of Bartlett and Housui could be connected together by using 19 SSR loci and the S locus as anchors, resulting in partial identification of the relationship between the two groups. The position of 14 SSR loci originating from apple could be successfully determined in the pear maps. Eleven SSR markers from the *Prunus* (peach, cherry) could also produce segregating genotypes in the F_1 progenies obtained from a cross between the European and Japanese pears. SSR markers could be useful for comparisons of the apple, pear and *Prunus* genome organization and for comparative mapping among those species.

Conclusion

Linkage maps and molecular markers for agronomically important characters could be especially useful in traditional crossbreeding programs for fruit trees. Furthermore, map-based

cloning of interesting genes could help to improve fruit trees through genetic transformation. SSRs (simple sequence repeats) are the best marker systems of choice because they have high reliability and codominant inheritance. We developed a lot of SSRs in peach and pear, and applied these SSRs to other related species, giving phenograms of many *Prunus* and *Pyrus* accessions. The SSR markers were highly polymorphic and could be utilized as a reliable tool for cultivar identification in *Prunus* and *Pyrus*. Apple SSRs could be successfully used in *Pyrus* spp., suggesting that pears display a close genetic relationship to apples. We characterized many morphological traits based on the linkage map obtained from an F_2 population derived from an intraspecific cross of peach. This map was compiled with those morphological markers and DNA markers, including SSR markers used as anchor loci, to compare different peach maps. Molecular markers tightly linked to new root-knot nematode resistance genes were also found, and will be utilized for selecting resistance varieties among peach rootstocks. A linkage map including disease-related genes, pear scab resistance and black spot susceptibility, in the Japanese pear were constructed. Other linkage maps of a European pear and Japanese pears were also constructed with DNA markers, including SSRs from pear, apple, peach and cherry. These maps were the first results of pear species, and are being developed for comparative mapping with apple and peach. We are now stepping up efforts to develop more SSRs and to construct fine linkage maps in order to obtain selecting markers and interesting genes.

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