8. Pear Genomics

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1 Introduction

1.1 Origin, Speciation and Botanic Characteristics

Pear, like the other pip fruit species apple and quince, belongs to the sub-family *Maloideae* in the Rosaceae, sharing a basic chromosome number of x = 17 which indicates a polyploid origin. The genus *Pyrus* is believed to have arisen during the Tertiary period in the mountainous regions of western China. Dispersal and speciation is believed to have followed the mountain chains both east and west (Rubzov, 1944; Zeven and Zhukovsky, 1975). Wild pears can be found in the entire Eurasian zone. In Europe they are mostly *Pyrus communis* L. subsp. *pyraster* (L.) and in the Caucasus, *P. caucasica* (Fed.) Browicz. These pear trees produce small fruits of variable characteristics, which were probably picked and preserved dried by early humans. Domestication occurred from the better-fruited trees. As for apple, grafting played a key role in the diffusion of improved genotypes in Central Asia and in Eastern Mediterranean area. According to Hedrick et al. (1921), European pear culture was well established in Greece and cultivars with distinct names were propagated as early as 300 B.C. Oriental pears, which arose independently, were also grown in China for more than 2000 years (Kikuchi, 1946).

The taxonomy of the genus *Pyrus* is complex, due to synonymy between taxa, and to frequent interspecific crosses. A summary of the main recognized *Pyrus* species has been published by Bell et al. (1996) and is reproduced in Table 1. Economic usage of *Pyrus* species has been reviewed by Bell et al. (1996). *Pyrus communis* L. is the main edible pear species grown in Europe, North America, South America, Africa and Australia. The snow pear, *P. nivalis* Jacq., is also grown to a limited extent in Europe for making perry. In Asia, *P. pyrifolia* (Burm.) Nakai is the main cultivated species in southern and central China, Japan, Taiwan and countries

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Table 1 Primary species in the genus *Pyrus* (from Bell et al., 1996)

Species	Distribution
European :	
P. communis L.	West to SE Europe, Turkey, Eurasia
P. caucasica Fed.	SE Europe, Greece, Turkey
P. nivalis Jacq.	West, Central and Southern Europe
P. cordata Desv.	SW England, W France, Spain, Portugal
Circum-Mediterranean :	
P. amygdalyformis Vill.	Mediterranean Europe, Asia Minor
P. eleagrifolia Pall.	SE Europe, Russia, Turkey
P. syriaca Boiss.	Tunisia
P. longipes Coss & Dur.	Algeria
P. gharbiana Trab.	Morocco, W Algeria
P. mamorensis Trab.	Morocco
Mid-Asian	
P. glabra Boiss.	Iran
P. salicifilia Pall.	NW Iran, NE Turkey, South Russia
P. reqelii Redh.	South Central Asia (Afghanistan)
P. pashia D. Don.	Pakistan, India, Nepal
East Asian	
P. pyrifolia (Burm.) Nak.	China, Japan, Korea, Taiwan
P. pseudopashia Yu.	BNW China (Yunnan, Kweichow)
P. ussuriensis Maxim.	Siberia, Mandchuria, N China, Korea
P. calleryana Decne.	Central & S China, Vietnam
P. betulaefolia Bunge	Central & S China, S Mandchuria
P. fauriei Sceid.	Korea
P. hondoensis Kik. & Nak.	Japan
P. dimorphophylla Mak.	Japan
P. kawakamii Hayata	Taiwan, SE China

of Southeast Asia. Some cultivars of this species are also grown on a more limited basis in Europe, under their Japanese name, nashi. In northern China and Japan, *P. ussuriensis* Max. and *P.* × *bretschneideri* Rehd. are grown for edible pear production, as well as some selections of *P. pashia* P. Don. Various *Pyrus* species are also used as rootstocks, or as ornamentals.

Wild native trees are deciduous, generally medium sized trees, with few species being shrubs. The inflorescence is a loose corymb of 6–9 flowers. The fruit of pear is a pyriform (European) or round (Asian) pome. As in apple, the fleshy edible portion is derived from the receptacle and the base of the perianth. There are 5 central seed cavities, usually bearing 2 seeds each as in apple. The flesh contains grit cells which are thick-walled, lignified cells that give the characteristic European pear flesh texture.

1.2 Production and Utilization

Pears rank second to apples in the amount of worldwide production of deciduous tree fruit species The world production of pear has reached 19 MT in 2005

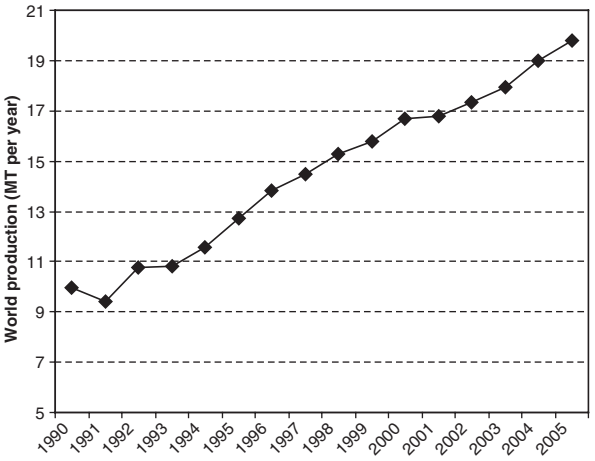

Fig. 1 World pear production (FAO, 2007)

(FAOSTAT, 2007). This total production has doubled over the past 15 years (Fig. 1). It consists mostly of Asian pears (70%), China being the main producer, with more than 11 MT. European pears are produced mainly by Italy, United States, Argentina and Spain, each country producing between 0.5 and 1 MT. Most of the crop is utilized as fresh fruit, but significant amounts are processed and marketed as canned pears, puree, juice, or other products.

1.3 Traditional Breeding

Pear breeding already has a very long history which deals with improvement of European as well as Asian pears. Breeding objectives are complex, but most pear breeders share a number of common purposes such as fruit quality, storage ability, consistent production, disease and pest resistance for cultivar improvement, and dwarfing ability, graft compatibility, iron chlorosis tolerance, disease resistance, cold hardiness for rootstock improvement (Bell et al., 1996). Information on objectives and recent introductions can be found in review by Bellini (1995).

Fruit tree breeding methods generally involve three major steps: creation of genetic variation, selection of elite material and extended experimentation of promising pre-selection before market release (Schmidt and van de Weg, 2005). Crossing is still by far the more largely used method of creation of genetic variability for pear. The genetic pool available for the pear breeder is wide. Most species of *Pyrus* are diploid ($2n = 34$; $x = 17$) and no significant interspecific cross-incompatibility is known to exist. Crossing programs rely on the availability of appropriate genetic resources, which can comprise recent breeding selections, old and new cultivars and various species of the genus *Pyrus*. Most pear varieties are diploid (2n=2x=34), with a few triploids (2n=3x=51) or tetraploids (2n=4x=68).

Most varieties are self-sterile, due to a strong gametophytic self-incompatibility controlled by a single S locus. This self-sterility leads to a high degree of heterozygosity.

Many new European pear scion cultivars have been developed from hybridization programs and released within the last 20 years with major improvements in the quality, storage and shelf life of early season pears, as well as in late season pears. Important progress has also been made in the field of disease resistance, particularly concerning fire blight. Countries with the highest number of pear scion releases since 1990 have been USA, Germany, France, Russia and Italy. However, very few of these novel varieties have the potential to replace the classical pear cultivars whose agronomical and commercial limits are already well known and which have gained a solid market (Bellini and Nin, 2002). Rootstock breeding programs are considerably longer than scion breeding programs, because no reliable laboratory technique is available so far to preselect for vigor and compatibility before testing rootstock/scion combinations in the orchard. Despite hybridization programs in several countries and release of several new rootstocks, there is still a lack of compatible dwarfing pear rootstocks of *Pyrus* type, combining a good propagation ability with disease resistance and an adaptation to difficult environmental conditions (cold, drought or lime-induced chlorosis) (Wertheim, 2002).

Mutation induction, although efficient for some characteristics such as skin color, compact growth or tetraploidy, never took a large place in pear breeding. The occurrence of chimeras, which are often unstable, is one of the major obstacles to the use of spontaneous or induced mutations for pear breeding (Chevreau et al., 1989). Recently, in vitro systems for pear mutation breeding have been developed to decrease the risk of obtaining chimeric plants, either by using adventitious regeneration or by applying rapid cycles of micropropagation to separate mutated from non-mutated sectors (Predieri and Zimmerman, 2001). So far, successes of induced mutagenesis for pear improvement are still limited. Three mutants of Asian pear ('Gold Nijisseiki', 'Kotobuki Shinsui' and 'Osa Gold') have been released. They were obtained after chronic γ-irradiation of plants and were selected for increased resistance to black spot disease, caused by *Alternaria alternata* (Yoshioka et al., 1998).

1.4 Biotechnical Approaches to Pear Improvement

The contribution of in vitro methods to create novel genetic variability in pear has advanced considerably during the two last decades. Haploidization via in situ parthenogenesis induced by irradiated pollen and in vitro rescue of the haploid plantlets has been successfully developed for pear (Bouvier et al., 1993). Techniques of adventitious bud regeneration from in vitro leaves have been developed for several genotypes of European and Asian pear and for quince. So far, applications of these techniques for the induction of somaclonal variation have been very limited. The occurrence of somaclonal variation has been demonstrated for fire blight resistance and iron-chlorosis tolerance, albeit at low

frequency (Chevreau and Bell, 2005). Protoplast technology has also been applied to pear since 1986 by Ochatt and Caso. However, somatic hybridization between *Pyrus* and an incompatible genus, *Prunus*, has been reported only once (Ochatt et al., 1989).

Marketing of pears both within and among nations is characterized by limitations on the number of cultivars, because both producers and consumers prefer the old traditional cultivars, which are unique and clearly recognized. Therefore, acceptance of new hybrid cultivars is very slow. In this context, gene transfer offers pear breeders new tools to directly improve existing elite cultivars without changing their main recognizable characteristics. Transformation of pear is based on the co-culture of in vitro leaves with a disarmed *A. tumefaciens* strain carrying the gene(s) of interest in a binary vector. Since the first report of pear transformation on three European pear cultivars (Mourgues et al., 1996), several other genotypes have been transformed by various groups in Europe, United States and Asia.

Genetic engineering has already been applied to pear with the aim to modify important agronomical traits by several groups since 1999. These reports, mostly on European pears, are summarized in Table 2. Increased resistance to fire blight is the main objective of the pear genetic engineering program in France (Reynoird

Table 2 Reports of introduction of transgenes in pear with the aim of modifying agronomical traits

Country	Pear variety	Gene : trait of interest	Reference
USA	Beurre Bosc	Rol C / plant architecture	Bell et al. (1999)
France	Passe Crassane	Attacin / fire blight resistance	Reynoird et al. (1999)
France	Passe Crassane	Lysozyme / fire blight resistance	Malnoy et al. (2000)
USA	Bartlett	Lytic peptide DRC1 / fire blight resistance	Puterka et al. (2002)
Russia	Burakovka	Thaumatin II : taste improvement	Lebedev et al. (2002a)
Russia	Burakovka	Defensins Rs-AFP2 / resistance to fungal pathogens	Lebedev et al. (2002b)
Sweden	BP10030	rolB / rooting efficiency	Zhu et al. (2003)
France	Passe Crassane	Lactoferrin / fire blight resistance	Malnoy et al. (2003a)
China	Fertility	Defensins Rs-AFP2 / resistance to fungal pathogens	Zhao et al. (2004)
Israel	Spadona	Stilbene synthase / health promoting	Flaishman et al. (2005)
France	Passe Crassane	Depolymerase / fire blight resistance	Malnoy et al. (2005a)
France	Passe Crassane	Harpin N / fire blight resistance	Malnoy et al. (2005b)
Japan	La France, Ballade	CiFT / flowering	Matsuda et al. (2006)
Japan	La France	ACC oxidase / fruit ripening	Gao et al. (2007)
China	Xueqing	Cry1Ac / insect resistance	Tang et al. (2007)
Japan	Ballade	Spermidine synthase / abiotic stress tolerance	Wen et al. (2008)
Israel	Spadona	TFL1 / flowering	Flaishman et al. (2007)

et al., 1999, Malnoy et al., 2000, 2003a, 2005a, b). Promising strategies include the establishment of a competition for iron with the bacterial siderophores and the induction of plant defenses by expression of a bacterial effector. Transfer of exogenous transgenes for fungal and insect resistance has also been reported (Lebedev et al., 2002b, Zhao et al., 2004, Tang et al., 2007). Modification of polyamine levels has been attempted recently to confer abiotic stress tolerance (Wen et al., 2008). The *rol* B and C genes from *Agrobacterium rhizogenes* have been introduced into pear for modification of scion development (Bell et al., 1999) or for improvement of rootstock rooting ability (Zhu et al., 2003). A few reports indicate the use of transgenes related to fruit quality (Lebedev et al., 2002a, Flaishman et al., 2005, Gao et al., 2007). Finally, the development of juvenile-free pear has been accomplished by *CiFT* over-expression (Matsuda et al., 2006) and by a *TFL1*-RNAi strategy (Flaishman et al., 2007).

According to the APHIS Field Test Releases Database (updated October 9, 2007), two groups have already released transgenic pears for field trials in USA. The USDA-ARS in West Virginia has released transgenic pears carrying a cecropin gene for fire blight resistance, and the *rolC* gene for dwarfing. Exelixis in Oregon and Washington, has released transgenic pears carrying the *sam-k* gene for delayed fruit ripening. So far in Europe, only one field trial has been conducted by the Swedish University of Agriculture Sciences, with transgenic pear rootstocks BP10030 containing the *rolB* gene.

Practical applications of gene transfer for pear breeding are facing many obstacles, in particular the reluctance of the public in Europe to accept genetically modified fresh products such as fruits and the limited economical value of this fruit crop compared to the cost of intellectual property associated with the development of a GMO variety. Research projects to develop targeted expression of transgenes (Malnoy et al., 2003b) and marker-free transformation systems (Djennane et al., 2007) should contribute to minimize public concern. Even though the direct use of gene transfer for pear breeding seems a long distance target, the various possibilities offered by gene transfer to over-express or silence a precise gene constitutes a unique tool for the progress of genetic knowledge of this species. With the increased speed of gene discovery in fruit species, gene transfer will become a necessary tool to demonstrate the function of these genes.

2 Structural Genomics

2.1 Genetic Diversity

2.1.1 Genetic Diversity in Asian Pears, European Pears, and Other Pyrus

The genus *Pyrus* contains at least 22 widely recognized primary species, all indigenous to Asia, Europe, and the mountainous area of North America. Presently, some pear species are cultivated commercially in the temperate regions of more than 50 countries around the world (Bell, 1990; Bell et al., 1996). It is considered that

there are at least 10 naturally and artificially occurring interspecific hybrid species. The major edible species *P. communis* L. is used for cultivation in Europe, North America, South America, Africa and Australia. The other major edible species, *P. bretschneideri* Rehd., *P. ussuriensis* Maxim. and *P. pyrifolia* (Burm.) Nakai, are cultivated in East Asian countries. All the species of *Pyrus* are intercrossable and there are no major incompatibility barriers to interspecific hybridization in *Pyrus*, in spite of the wide geographic distribution of the genus (Westwood and Bjornstad, 1971). Although interspecific hybrids among *Pyrus* spp. have been tested to improve disease and pest resistances, fruit quality and adaptability were generally low (Bell et al., 1996). During the past decade, a lot of attempts have been tried to evaluate genetic diversity in Asian pears, European pears, and other *Pyrus*, using several types of DNA markers, i.e., RAPD, AFLP, SSRs (microsatellites) and ISSR.

Ten or more primary species are naturally distributed in East Asia, including *Pyrus pyrifolia*, *P. pashia*, *P. hondoensis*, *P. ussuriensis*, *P. kawakamii*, *P. calleryana*, *P. koehnei*, *P. fauriei*, *P. dimorphophylla* and *P. betulaefolia* (Bell, 1990, Bell et al., 1996). It is considered that some species such as *P. bretschneideri* and *P. phaeocarpa* are naturally occurring interspecific hybrids. Genetic diversity and genetic relatedness within species as well as between species in East Asian pears have been examined by DNA marker systems. Nineteen Japanese pear (*P. pyrifolia*) cultivars were successfully discriminated by 82 RAPD primers and 6 SCAR (sequence characterized amplified regions) markers converted from RAPD fragments (Kim et al., 2000a, b). Kim and Ko (2004) analyzed 33 Asian pears from 12 *Pyrus* species by 60 RAPD primers. Four groups were obtained based on a cladogram. A total of 118 *Pyrus* spp. and cultivars native mainly to east Asia were analyzed by 20 RAPD primers to evaluate genetic variation and relationships among the accessions (Teng et al., 2001, 2002). According to their reports, RAPD markers specific to species were identified, and the grouping of the species and cultivars by RAPD largely agrees with morphological taxonomy. Cultivars of *P. sinkiangensis* were suspected to be of hybrid origin involving *P. communis* and *P. bretschneideri* (Teng et al., 2001).

Kimura et al. (2002) identified 58 Asian pear accessions from 6 *Pyrus* species using 9 SSR markers with a total of 133 putative alleles. They obtained a phenogram based on the SSR genotypes, showing 3 major groups corresponding to the Japanese, Chinese and European groups. Bao et al. (2007) evaluated 98 pear cultivars native mainly to East Asia by 6 SSR markers. Chinese sand pear (*P. pyrifolia*) and Chinese white pear (*P. bretschneideri*) presented a large genetic diversity. Occidental pears generally had low affinities to Asian pears.

Edible European pears (*P. communis*) are derived from wild relatives native to the Caucasus Mountain region and eastern Europe. Thirteen SSR loci were used to determine the relationships among 145 wild and cultivated individuals of *P. communis* maintained in the National Plant Germplasm System (NPGS, USA) (Volk et al., 2006). Twelve clusters were obtained based on individual SSR genotypes by Bayesian clustering method. *Pyrus communis* ssp. *caucasica* which is native to the Caucasus Mountains can be genetically differentiated from *P. communis*

ssp. *pyraster* native to eastern European countries. The domesticated pears cluster closely together and are most closely related to a group of genotypes that are intermediate to the *P. communis* ssp. *pyraster* and the *P. communis* ssp. *caucasica* groups. Dolatowski et al. (2004) studied the variability and genetic relationship of wild and semi-wild pears (*P. pyraster*) in Poland using AFLP markers.

Oliveira et al. (1999) investigated molecular characterization and phenetic similarities between several cultivars of *P. communis* and *P. pyrifolia* and several wild species by RAPD markers. Monte-Corvo et al. (2000) investigated the genetic relationships among 39 cultivars including 35 *P. communis* and 4 *P. pyrifolia* cultivars using AFLP and RAPD markers. They confirmed that AFLP markers were five times more efficient in detecting polymorphism per reaction. Although some differences can be noticed between the dendrograms resulting from AFLP and RAPD analyses, both techniques produced similar results. Lee et al. (2004) reported that RAPD, SCAR and the conserved 18S rDNA could be used to classify and identify cultivars of *P. pyrifolia* and *P. communis*.

2.1.2 Taxonomical Relationships in Pyrus Assessed by DNA Markers

Taxonomy in *Pyrus* is conducted mainly by morphology and geographic distribution. Classification of species of pears is very problematic and is often confused due to following reasons: (1) lack of wild populations, especially in cultivated species; (2) poor morphological diversity and lack of distinguishing characters among species; (3) widespread crossability and consequent interspecific hybridization and introgression among species. Recently, many efforts using DNA markers from the nuclear genome as well as chloroplast DNAs have been conducted, and reveal taxonomical relationships and the course of evolution in pears.

SSR markers are efficient tools to assess taxonomical relationships in pear, because of their advantages over other markers, i.e., co-dominant and typically neutral inheritance, large number of alleles per locus, abundance in genomes, and suitability for automation. Fifty-eight Asian pear accessions from 6 *Pyrus* species, 98 pear cultivars native mainly to East Asia, and 145 wild and cultivated individuals of *P. communis*, were analyzed by 9, 6 and 13 SSR loci, respectively (Kimura et al., 2002, Bao et al., 2007, Volk et al., 2006). SSR markers could reveal genetic and taxonomical relationships in pears and showed distinctive groups generally corresponding to species in taxonomy. It appears that exact identification of species may be difficult, for closely related varieties, and hybrid and introgressed germplasms.

Chloroplast DNA (cpDNA) usually shows maternal inheritance in angiosperms. Sequence conservation within cpDNA allows us to compare phylogenetic relationships at various taxonomic levels (Palmer et al., 1985). In spite of the conservation within the chloroplast genome, structural alterations such as insertions, deletions, inversions and translocations in cpDNA have been found in related plants by comparing the structure of cpDNAs. Tracing the mutational events in cpDNA provides useful tools to trace the course of evolution by reconstructing the plant phylogeny (Downie and Palmer, 1992). A physical map of cpDNA of pear was constructed

using 5 restriction enzymes (Katayama and Uematsu, 2003). Pear cpDNA was found to be a circular molecule with a total size of about 156 kb in which 2 inverted repeats of 24.8 kb divide the molecule into small (17 kb) and large (90 kb) single-copy regions. RFLP analysis was carried out on cpDNAs from 5 *Pyrus* species (*P. pyrifolia*, *P. ussuriensis*, *P. calleryana*, *P. elaeagrifolia* and *P. communis*) and 2 mutations, a recognition-site mutation and a length mutation (deletion), were found only in the cpDNA of *P. pyrifolia* cultivars. This information will make it possible to investigate the phylogenetic relationships between *Pyrus* species.

Iketani et al. (1998) examined polymorphism of chloroplast DNAs of 106 accessions of mainly East Asian accessions. Four haplotypes were observed with the combination of 3 independent restriction site mutations and all 4 types appeared in the oriental pear accessions. This suggests that the oriental species of *Pyrus* and occidental ones may have evolved independently. The distribution of four haplotypes in the East Asian pear was quite incongruent with the species or infrageneric classification using mainly morphological characters. Considering the high crossability and frequent occurrence of suspected interspecific hybrids in wild populations, the dis-accordance is inferred to be the results of the hybridization and introgression between species.

Nucleotide sequences at 6 noncoding regions of cpDNAs were identified for 8 pear varieties from 5 species (Kimura et al., 2003). A total of 38 mutations such as nucleotide substitutions, deletions and insertions were found in more than 5.7 kbp of nucleotide sequences. Nucleotide sequences at the *trnL-trnF* were revealed for 33 pear varieties and 8 mutations were identified. A cladogram obtained from the data showed that Asian pear varieties were divided into 6 groups and that intraspecific as well as interspecific diversities existed in Asian pears, whereas European pear varieties were identical with respect to the *trnL-trnF* region.

2.2 DNA Markers

2.2.1 DNA Marker Systems (Microsatellites, SNPs, AFLP, RAPD, etc.)

Microsatellites, or SSRs (simple sequence repeats), are polymorphic loci present in nuclear DNA that consist of repeating units of 1–4 base pairs in length. They are typically neutral and co-dominant, and show high degree of polymorphism and suitability for automation (Weber and May, 1989). SSR markers have several advantages over other molecular markers, which provide a more reliable method for DNA fingerprinting because of their co-dominant inheritance, large number of alleles per locus, and abundance in genomes. In addition, since SSR analysis is based on a PCR method, the technique is simple and only a small amount of DNA is required. More than 100 SSRs have been developed from European and Japanese pears (Yamamoto et al., 2002a, b, c; Sawamura et al., 2004; Fernandez-Fernandez et al., 2006; Inoue et al., 2007). These SSR markers have been used as molecular markers which have wide-ranging applications for the evaluation of genetic

diversity (Kimura et al., 2002, Volk et al., 2006, Bao et al., 2007), cultivar identification, and the construction of genetic linkage maps (Yamamoto et al., 2002c, 2004, 2007).

RAPD stands for random amplification of polymorphic DNA and is the segments of DNA that are amplified are random. The RAPD reaction is performed with arbitrary, short primers (8–12 nucleotides). No knowledge of the DNA sequence for the targeted gene is required, as the primers will bind somewhere in the sequence, but it is not certain exactly where. Its resolving power is much lower than targeted, species specific DNA comparison methods, such as SSRs. In recent years, RAPD technology has been used to characterize, and trace, the phylogeny of diverse plant species. RAPDs have also been widely used on pear genetic studies because RAPDs have the advantage of being readily employed, requiring small amounts of genomic DNA. RAPD markers have been successfully used for identification and genetic relationships of pear (Oliveira et al., 1999, Teng et al., 2001, 2002).

AFLP (amplified fragment length polymorphism) markers are a highly sensitive method for detecting polymorphisms used in the study of genetics and in the practice of genetic engineering, which was developed in the early 1990's by Keygene (Vos et al., 1995). AFLP uses restriction enzymes to cut genomic DNA, followed by ligation of complementary double stranded adaptors to the ends of the restriction fragments. A subset of the restriction fragments are then amplified using 2 primers complementary to the adaptor and restriction site fragments. The fragments are visualized on denaturing polyacrylamide gels either through autoradiographic or fluorescence methodologies. AFLP has several advantages over the RAPD technique, like a higher number of loci analyzed and a higher reproducibility of banding patterns on genetic diversity study in pear (Monte-Corvo et al., 2000).

ISSR (inter-simple sequence repeat) is a general term for a genome region between microsatellite loci. The complementary sequences to 2 neighboring microsatellites are used as PCR primers. Sequences amplified by ISSR-PCR can be used for DNA fingerprinting. Since an ISSR may be a conserved or non-conserved region, this technique is not useful for distinguishing individuals, but rather for phylogeographical analyses or maybe delimiting species. Sequence diversity is lower than in SSRs, but still higher than in actual gene sequences. Monte-Corvo et al. (2001) reported that ISSR analysis was used for cultivar identification and the determination of phylogenetic relationship in pears (*P. communis*).

A single nucleotide polymorphism (SNP) is a DNA sequence variation occurring when a single nucleotide – A, T, C, or G – in the genome differs between members of a species. For example, 2 sequenced DNA fragments from different individuals, AAGCCTA to AAGCTTA, contain a difference in a single nucleotide. Single nucleotide polymorphisms may fall within coding sequences of genes, noncoding regions of genes, or in the intergenic regions between genes. SNPs within a coding sequence will not necessarily change the amino acid sequence of the protein that is produced, due to degeneracy of the genetic code. It is believed that SNP will be the most efficient tool for comprehensive genetic studies in near future. Since nucleotide sequences of pear genome remain very limited, SNP markers are not frequently utilized for pear at this moment.

2.2.2 DNA Markers Associated with Interest Genes

Several phenotypic traits, including tree and production characters, fruit quality, disease resistance, pest resistance and adaptability, have been studied in pears. Since it is believed that a lot of phenotypic traits are controlled by polygenes or QTLs, it is not easy to develop DNA markers associated with specific genes and various characteristics. Associated molecular markers have been isolated for some specific phenotypic traits, some of which were identified based on genetic linkage maps. Recently, resistance to major diseases for pear cultivation, fire blight, pear scab and black spot were analyzed and genes (or QTLs) controlling resistances were identified in genetic linkage maps (Table 3). Other genes of interest for self incompatibility and fruit ripening were also investigated (Table 3).

Disease Resistance (Fire Blight, Pear Scab, Black Spot)

Fire blight caused by *Erwinia amylovora* (Burr.) Winsl. is the most serious disease in pears in North America and Western Europe. In the 19th century, breeding for fire blight resistance started to introgress resistance of Asian pears into European pears. Some interspecific hybrid cultivars between Asian and European pears showed more resistant to fire blight than European pears, but they were inferior fruit quality. Large scale evaluation for fire blight disease showed that resistance in *P. communis* is relatively rare with 5–10% being rated as at least moderately resistant. Dondini et al. (2004) designed 2 genetic linkage maps of European pears 'Harrow Sweet' (resistant) and 'Passe Crassane' (susceptible) with F1 progeny crossed between them. Different types of DNA markers including SSRs, MFLPs (microsatellite-anchored fragment length polymorphisms), AFLPs, RGAs (resistant gene analogs) and AFLP-RGAs were applied for construction of their genetic maps. The 'Harrow Sweet' map consisted of 156 loci, for a total length of 912 cM divided into 19 linkage groups. Four putative QTLs related to resistance against fire blight were identified in LGs 2, 4 and 9 (2 QTLs in LG 2) of the map of 'Harrow Sweet'. No QTLs were found for susceptible cultivar 'Passe Crassane'. About 50% of the total variance was explained by 4 QTLs, and 2 QTLs found in LG 2 showed large LOD values and high variance explained. This study will lead to identify associated DNA markers with fire blight resistance in order to develop MAS approach.

Pear scab, caused by two species of *Venturia*, *V. nashicola* and *V. pirina*, is one of the most serious diseases of Asian and European pears, especially Japanese pear. *Venturia nashicola* infects Asian pears throughout their natural range, and *V. pirina* occurs in most regions where European pears are grown. The species are classified in the same genus as *V. inaequalis*, which causes apple scab. *Venturia nashicola* is pathogenic only on Asian pears and is not pathogenic on European pears (Bell et al., 1996, Ishii et al., 2002). In contrast, Japanese and Chinese pears are generally resistant to *V. pirina* (Bell et al., 1996, Ishii et al., 2002). None of the major commercial Japanese pear cultivars are resistant to scab disease caused by *V. nashicola* (Ishii et al., 1992, Bell et al., 1996), but no scab symptoms were observed on the indigenous Japanese pear cultivar 'Kinchaku', some Chinese pears, and many European

Table 3 DNA markers associated with interest genes and their positions in genetic linkage map

Characters	Gene symbol	Gene source	Associated DNA markers (gene name, QTL name)	Linkage group No.	References
scab resistance to *V. nashicola*	*Vnk*	Kinchaku	STS-OPW2, STS-CT/CTA	1	Terakami et al. (2006)
fire blight resistance		Harrow Sweet	HS2A (QTL)	2	Dondini et al. (2004)
		Harrow Sweet	HS2B (QTL)	2	Dondini et al. (2004)
		Harrow Sweet	HS4 (QTL)	4	Dondini et al. (2004)
		Harrow Sweet	HS9 (QTL)	9	Dondini et al. (2004)
black spot susceptibility	*A*	Osa Nijisseiki	CMNB41/2350	unknown	Banno et al. (1999)
	Ani	Osa Nijisseiki	CH04h02, CH03d02	11	Terakami et al. (2007)
	Ana	Nansui	CH04h02, CH03d02	11	Terakami et al. (2007)
self-incompatibility	*S*	Japanese pear, European pear	S-RNase (gene name)	17	Yamamoto et al. (2002)
fruit skin color		Niitaka	OPH-19-425	unknown	Inoue et al. (2006)
fruit storage		Japanese pear	ACC synthase (gene name)	unknown	Itai et al. (2003)

pears. Inheritance analysis indicated that resistance of 'Kinchaku' is controlled by a single dominant gene (Abe and Kotobuki, 1998). Genetic linkage maps of the Japanese pear cultivars 'Kinchaku' and 'Kousui' were constructed using RAPD markers (Iketani et al., 2001). The 'Kinchaku' map consisted of 120 loci in 18 linkage groups covering a length of ca. 770 cM, in which 2 disease-related genes associated with resistance to pear scab and susceptibility to black spot (caused by *Alternaria alternata*) were mapped. The resistance gene *Vnk* of the Japanese pear 'Kinchaku' against pear scab disease was identified in the central region of LG 1 (Terakami et al., 2006). Six DNA markers (one SSR Hi02c07 and 5 STSs converted from AFLP and RAPDs) showed tight linkages to *Vnk*, being mapped with distances ranging from 2.4 to 12.4 cM. The SSR CH-Vf2, which was isolated from a BAC clone of the contig containing the apple scab gene *Vf*, was mapped at the bottom of linkage group 1 in 'Kinchaku', suggesting that the *Vnk* and *Vf* loci are located in different genomic regions of the same homologous linkage group.

Black spot disease, caused by *Alternaria alternata* (Fr.) Keissler Japanese pear pathotype, is one of the most serious diseases in Japanese pear cultivation. Large amount of costs and labors are required for bagging and spraying of fungicide in order to prevent infection of this disease (Kozaki, 1973). The AK-toxin, which is specifically produced by *A. alternata* Japanese pear pathotype, causes the necrosis, early leaf fall, and decrease of yield for Japanese pears (Nakashima et al., 1985). Many major Japanese pear cultivars 'Nijisseiki', 'Shinsui', and 'Nansui' show susceptibility to this disease, and the susceptibility to black spot is controlled by single dominant gene designated as *A* (Kozaki, 1973). Banno et al. (1999) tested 250 RAPD primers to screen a pair of bulked DNA samples derived from open-pollinated progeny of Japanese pear 'Osa Nijisseiki' to identify markers linked to the susceptible *A* gene. One RAPD marker CMNB41 was identified to show linkage to the susceptibility gene at a genetic distance of 3.1 cM. Iketani et al. (2001) reported that the susceptibility to black spot was identified in the genetic linkage map of 'Kinchaku'. More recently, the susceptibility genes to black spot disease of the Japanese pear cultivars 'Osa Nijisseiki' (designated as *Ani*) and 'Nansui' (*Ana*) were genetically identified and mapped in their genetic linkage maps, locating at the top region of a linkage group 11 (Terakami et al., 2007). Two SSR markers CH04h02 and CH03d02 showed tight linkages to *Ani* and *Ana*, using a genome scanning approach (GSA, Patocchi et al., 2005). This information about the position and molecular markers linked to the disease resistance genes will be useful for marker-assisted selection and for pyramiding resistances in pear breeding programs.

Self Incompatibility

Most pear cultivars show self-incompatibility and the proposition of pollinizers inter-planted in the orchard is a requirement to get an economic crop from most of the cultivars (Sanzol and Herrero, 2002). In *Pyrus*, gametophytic self-incompatibility is controlled by a single locus, the S-locus. The S-locus harbors a multi-allelic gene, which encodes for S-RNase that blocks incompatible-tube

growth through the style (Ushijima et al., 1998). In Japanese pear, cDNAs encoding S1- to S9-RNase have been isolated and sequenced (Sassa et al., 1997, Ishimizu et al., 1998, Takasaki et al., 2004). Ishimizu et al. (1999) established a PCR-RFLP system for S-genotype assignment in Japanese pear. Molecular techniques were used for the identification of S-genotypes in European pears (Sanzol and Herrero, 2002; Zuccherelli et al., 2002; Zisovich et al., 2004).

Recently, candidates of pollen S (S locus F-box brothers, SFBB) were identified in apple and pear (Sassa et al., 2007). Three SFBB genes were isolated in each of the Japanese pear S4 and S5 haplotypes. These SFBB genes in Japanese pear show S haplotype-specific sequence polymorphism, which can be used as CAPS markers for identifying self-incompatibility genotypes.

The self-incompatibility locus (S locus) was mapped in the Japanese pear 'Housui' and the European pear 'Bartlett' in their genetic linkage maps at the bottom of the linkage group 17 (Yamamoto et al., 2002c). The position of S locus in both pear and apple was identified in the same homologous LG 17 (Maliepaard et al., 1998).

Other Phenotypic Traits

Ethylene production drastically varies during fruit ripening in cultivated Japanese pears. Climacteric-type fruits exhibit a rapid increase in ethylene production and show a low storage potential. Non-climacteric fruits show no detectable ethylene production and their fruit quality is maintained for over a month in storage. Itai et al. (1999, 2003a) cloned three ACC (1-aminocyclopropane-1-carboxylate) synthase genes (*PPACS1, 2, 3*) and showed that fruit storage potential was closely related to ethylene production and expression of ACC synthase genes during fruit ripening. It was identified that *PPACS1* was expressed in cultivars with high ethylene production, while *PPACS2* was specifically expressed in cultivars of moderate ethylene production. These two ACC synthase genes (*PPACS1, 2*) were identified as RFLP markers, differentiating to high, moderate and low ethylene production. Furthermore, CAPS markers were established, converted from RFLP markers (Itai et al., 2003b), which will be utilized for selection of Japanese pear cultivars with enhanced post-harvest storage ability.

Since pears are mainly served as fresh fruits and must have an attractive appearance, the fruit color is one of the most important factors contributing to appearance. In Japanese pears, yellow-green and brown russet fruits are preferred for consumers. Inoue et al. (2006) reported the RAPD marker linked to major genes controlling the fruit skin color in Japanese pear. Two F1 progenies from the cross of 'Kousui' × 'Kinchaku' and 'Niitaka' × 'Chikusui' segregated by fruit skin color were used for bulked segregant analysis. After 200 random primers were screened against bulks, the 425-bp band produced with OPH-19 primer (OPH-19-425) was selected in association with green bulks. The recombination rate between OPH-19-425 and the green skin phenotype was 7.3%.

2.3 Linkage and Physical Maps

2.3.1 Genetic Linkage Maps in European Pears and Japanese Pears

High-density genetic linkage maps are very useful for fundamental and applied genetic research. Linkage maps enable studies of the genome structure, the localization of interest genes, identification of quantitative trait loci (QTLs), and conduction of marker-assisted selection (MAS) and marker-assisted breeding (MAB). Several genetic linkage maps of pears have been reported in the European pear (*P. communis*) and the Japanese pear (*P. pyrifolia*) (Table 4). Recent pear genetic linkage maps contain linkage groups corresponding to its basic chromosome number (n = 17), and are sufficiently dense and saturated. In order to conduct MAS and to evaluate genome structures, it will be necessary to construct pear genetic maps covering entire genome regions with a large number of DNA markers.

Iketani et al. (2001) reported the construction of RAPD-based genetic maps of the Japanese pear varieties 'Kinchaku' and 'Kousui'. The former map consisted of 120 loci in 18 linkage groups covering a length of ca. 770 cM. The map of 'Kousui' contains 78 RAPD loci in 22 linkage groups extending 508 cM. The resistance to pear scab disease (*Vn*) and the susceptibility to black spot disease (*A*) were identified in the genetic map of 'Kinchaku' and several RAPD markers were found to show significant linkages to pear scab resistance and black spot susceptibility.

Partial genetic linkage maps of the European pear cultivars 'Passe Crassane', 'Harrow Sweet', 'Abbe Fetal' and 'Max Red Bartlett' were constructed using apple SSRs, showing 3 linkage groups 10, 12 and 14 (Pierantoni et al., 2004). Dondini et al. (2004) reported on 2 genetic linkage maps that were made of the European pears 'Passe Crassane' and 'Harrow Sweet'. The former map included 155 loci for a total length of 912 cM organized in 18 linkage groups. The 'Harrow Sweet' map consisted of 156 loci, for a total length of 930 cM divided into 19 linkage groups. Hemmat et al. (2003) suggested that many apple SSRs would be useful for genetic mapping in European pears in a preliminary experiment.

Integrated high-density genetic linkage maps were constructed for the European pear cultivar 'Bartlett' and 'La France', and the Japanese pear cultivar 'Housui' based on AFLPs, SSRs (from pear, apple and Prunus), isozymes, and phenotypic traits (Yamamoto et al., 2002c, 2004, 2007). The map of 'Bartlett' consisted of 447 loci including 58 pear SSRs, 60 apple SSRs and 322 AFLPs, which covered 17 linkage groups over a total length of 1,000 cM with an average distance of 2.3 cM between markers. Another genetic linkage map of 'La France' contained 414 loci including 66 pear SSRs, 68 apple SSRs and 279 AFLPs, on 17 linkage groups encompassing a genetic distance of 1,156 cM. Both maps consisted of more than 400 loci and covered 17 linkage groups, which corresponded to the basic chromosome number of pear (n=17). Both maps were well aligned using a total of 97 SSR markers in the 17 linkage groups. The map of 'Housui' contains 180 loci including 110 AFLPs, 64 SSRs (29 pear, 29 apple, 6 *Prunus* SSRs) on 20 linkage groups encompassing a genetic distance of 995 cM (Yamamoto et al., 2004). Three linkage groups or chromosomal regions could not be established because no

Table 4 Characteristics of genetic linkage maps in pear

Cultivar names	Species	Population	No. of linkage group	No. of loci	Map distance (cM)	Marker types	References
Kousui	*Pyrus pyrifolia*	Kinchaku × Kousui F1 (82 progeny)	22	78	508	RAPD	IKetani et al. (2001)
Kinchaku	*Pyrus pyrifolia*	Kinchaku × Kousui F1 (82 progeny)	18	120	768	RAPD	IKetani et al. (2001)
Housui	*Pyrus pyrifolia*	Bartlett × Housui F1 (63 progency)	16	307	1,054	SSR, AFLP	Yamamoto, unpublished
Passe Crassane	*Pyrus communis*	Pause Crassane × Harrow Sweet (99 progeny)	18	155	912	SSR, MFLP, AFLP, RGA	Dondini et al. (2004)
Harrow Sweet	*Pyrus communis*	Pasue Crassane × Harrow Sweet (99 progeny)	19	156	930	SSR, MFLP, AFLP, RGA	Dondini et al. (2004)
Bartlett	*Pyrus communis*	Bartlett × Housui F1 (63 progeny)	17	447	1,004	SSR, AFLP	Yamamoto et al. (2007)
La France	*Pyurs communis*	Shinsei × (Housui × La France) (55 progeny)	17	414	1,156	SSR, AFLP	Yamamoto et al. (2007)

SSR and AFLP markers of these regions showed segregating alleles (fragments) for 'Housui'.

2.3.2 Genome Structure Between Species Based on Linkage Maps in PYRUS

Genetic linkage maps have been constructed for European pear cultivars 'Bartlett' and 'La France' (Yamamoto et al., 2004, 2007), 'Passe Crassane' and 'Harrow Sweet' (Dondini et al., 2004), and 'Passe Crassane', 'Harrow Sweet', 'Abbe Fetel' and 'Max Red Bartlett' (Pierantoni et al., 2004) (Table 4). Japanese pear maps were reported for 'Kinchaku' and 'Kousui' (Iketani et al., 2001), and 'Housui' (Yamamoto et al., 2002c, 2004). However, the saturated high-density genetic linkage maps of pear were constructed only for European pear cultivars 'Bartlett' and 'La France' (Yamamoto et al., 2007). At this moment, it is rather difficult to compare genome structure between species based on linkage maps in *Pyrus*. Yamamoto et al. (2007) described that all 17 linkage groups of 'Bartlett' and 'La France' could be connected together by using a total of 97 SSR loci with at least 1 SSR locus per linkage group. The linkage groups 10 and 14 of both maps were well consolidated by 10 and 11 anchor loci, respectively (Fig. 2). The positions and linkage groups of commonly mapped SSR loci were well conserved between 'Bartlett' and 'La France' except for a very few exceptions which may have been due to multi-locus SSRs, indicating that the genome structure is very well conserved within European pears.

Two saturated (or high-density) maps were published in apple (Maliepaard et al., 1998, Liebhard et al., 2003). The first one was based on the F1 progeny of a cross between the apple cultivars 'Prima' and 'Fiesta' (Maliepaard et al., 1998), and another one was based on 267 F1 progeny from a cross of 'Fiesta' × 'Discovery' (Liebhard et al., 2002, 2003). When pear genetic linkage maps of 'Bartlett' and 'La France' were compared with the maps of 'Discovery' and 'Fiesta', 66 apple SSR loci could be successfully identified into the same homologous linkage groups between pear and apple. Moreover, their positions within linkage groups were identified in almost the same regions between pear and apple. Since genetic relatedness within *Pyrus* is much closer than that between apple and pear, it is believed that genome structure should be very well conserved within *Pyrus*.

About 10–20% of SSR markers in pear are multi-loci as well as in apple (Yamamoto et al., 2004, Liebhard et al., 2002, 2003). Liebhard et al. (2002, 2003) pointed out duplication patterns of multi-locus SSRs in the linkage group pairs 1–7, 4–12, 5–10, 9–17, 12–13, 12–14 and 1–3. In pear, duplication of the linkage group pairs 2–5, 9–17, 5–10, 3–14, 2–15, 1–3, 10–17, 8–15, 13–16 and 12–14 were revealed by multi-locus SSRs between 'Bartlett' and 'La France'. Duplication of 1–3, 5–10, 12–14 and 9–17 were commonly observed in apple and pear. This information suggests that at least 2 homologous chromosomes or genomic regions exist in pear genome. Recent molecular genetic studies have provided supporting evidence that the subfamily Maloideae originated from autopolyploidy or hybridization between closely related members of a single lineage, with species of the Spiraeoideae subfamily being the most probable parental lineages (Morgan

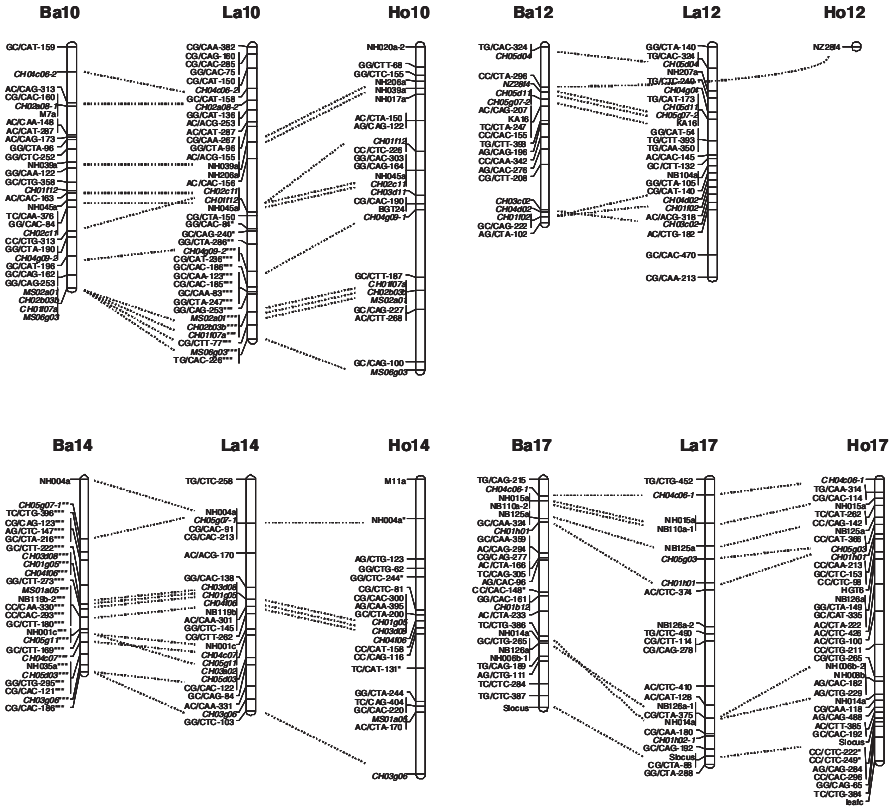

Fig. 2 Comparative linkage groups 10, 12, 14 and 17 of pear cultivars 'Bartlett' (Ba), 'La France' (La) and 'Housui' (Ho) (Yamamoto et al., 2007, unpublished data). The SSR markers and S locus observed in both maps are indicated by the *dotted lines*

et al., 1994, Evans and Campbell, 2002). Linkage groups identified by multi-loci SSRs might reflect the polyploid nature of pear. It will be interesting to evaluate the genome structure of polyploid origin using detailed DNA markers.

2.3.3 Physical Map

Physical maps are particularly important when searching for interest genes by positional cloning strategies and for DNA sequencing. Physical mapping is the process of determining how DNA contained in a group of clones overlap without having to sequence all the DNA in the clones. Once the map is determined, we can use the clones as a resource to efficiently contain stretches of genome in large quantity. This type of mapping is more accurate than genetic maps. However, it appears that no or few information is available for physical map in pear.

2.4 Association Mapping

Variations in the DNA sequences of humans can affect how humans develop diseases, respond to pathogens, chemicals, drugs, etc. However, their greatest importance in biomedical research is for comparing regions of the genome between cohorts. The study of single nucleotide polymorphisms is also important in crop and livestock breeding programs. Genetic association studies including linkage disequilibrium (LD) are performed to determine whether a genetic variant is associated with a disease or trait: if association is present, a particular allele, genotype or haplotype of a polymorphism or polymorphism(s) will be seen more often than expected by chance in an individual carrying the trait. In pears, it appears that no or few information is available for association mapping. This approach will become a powerful tool to identify associated DNA markers for many important phenotypic traits controlled by polygenes or QTLs. Association mapping will lead to development of DNA markers associated with interest genes and characteristics such as tree and production characters, fruit quality, disease resistance, pest resistance and adaptability would be controlled by polygenes or QTLs in pears.

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