Current Status of Knowledge and Research Perspectives in Korean Pear Genomics

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ABSTRACT The pear (*Pyrus* spp.) is most important fruit crop in the world. The genus *Pyrus* belongs to the subfamily Maloideae in the Rosaceae family and contains at least 22 primary species; however, only a few species, including *P. pyrifolia*, *P. ussuriensis*, *P. bretschneideri*, and *P. communis* have been utilized for fruit production. In Korea, awareness of the importance of the fruit industry and fruit tree breeding is low, and there is little support for genetic and genomic studies of fruit trees. In foreign countries, studies have focused on obtaining genomic information of fruit crops and the development of important agronomic trait-related molecular markers, providing a genomic framework for fruit tree breeding. Although Korea does not actively participate in research on the genomics of fruit trees, it is not far behind other countries in terms of technology and is therefore still competitive in research and development. The resequencing of 'Whangkeumbae' and 'Minibae' pears has been performed using the Illumina Hiseq 2000 platform as a part of the Biogreen 21 project, offering novel, rapid methods for identification of molecular marker, such as single nucleotide polymorphisms, insertion-deletions, and simple sequence repeats, through next-generation sequencing (NGS) technology. These NGS-based molecular markers are useful for genetic studies of Asian pears, e.g., for construction of genetic linkage maps, mapping of quantitative trait loci, and marker-assisted selection.

Keywords *Pyrus* spp., pear, next-generation sequencing, molecular marker

INTRODUCTION

The pear (*Pyrus* spp.) is one of the most economically important fruit crops worldwide, having been cultivated in Europe and Asia for at least 2000-3000 years, and is commercially grown in all temperate regions, including more than 50 countries (Bell *et al.* 1996). Approximately 25.2 million tons of pears, harvested from 1.6 million hectares, are produced worldwide each year, making pears the second most important fruit tree in Maloideae after apples (FAO 2013). In Korea, 302,731 tons of pears were produced from 13,127 hectares in 2014 (KOSTAT).

The genus *Pyrus* belongs to the subfamily Maloideae in the Rosaceae family, sharing a basic chromosome number of $x = 17$, which is indicative of a polyploid origin (Bell 1990). The genus *Pyrus* contains at least 22 primary

species; however, only a few species, including *P. pyrifolia*, *P. ussuriensis*, *P. bretschneideri*, *P. sinkiangensis*, and *P. communis* have been used for fruit production (Wu *et al.* 2013). According to its original distribution, the genus *Pyrus* is divided into two native groups: occidental and oriental pears (Bailey 1917). Of the occidental pears, *P. communis* is an important cultivated species and has been widely produced throughout Europe, North and South America, Africa, and Australia (Bell 1990; Yamamoto and Chevreau 2009). Of the oriental pears, there are four groups of highly cultivated species: *P. pyrifolia*, *P. ussuriensis*, *P. bretschneideri*, and *P. sinkiangensis* (Bao 2007). In Asia, *P. pyrifolia* is the main species cultivated in southern and central China, Japan, Korea, and countries of Southeast Asia. In northern China and Japan, *P. ussuriensis* and *P. bretschneideri* are grown for fresh pear production. Various

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Pyrus species are also used as rootstocks or as ornamentals (Yamamoto and Chevreau 2009).

Genetic characteristics of *Pyrus*

Most species of *Pyrus* are diploid ($2n=2x=34$), with a few triploids ($2n=3x=51$) or tetraploids ($2n=4x=68$). *Pyrus* varieties have highly heterozygous genetic backgrounds due to a gametophytic self-incompatibility controlled by a single *S* locus (Yamamoto and Chevreau 2009). Therefore, estimation of genetic diversity among *Pyrus* spp. is often challenging. Moreover, as with other self-incompatible woody perennials, pears have a juvenility; therefore, it is not easy for breeders to directly determine genes controlling target traits (Chevreau *et al.* 1997), and it is nearly impossible to obtain pure lines (Socias i company R 1998). Consequently, fewer genetic studies of pears have been performed than those of other major crops.

Genetic studies of *Pyrus* spp.

Phylogenetic analysis of *Pyrus* varieties has traditionally depended on evaluations of morphological characteristics (Rehder 1915; Kikuchi 1948; Yuan and Du 1980). However, the taxonomy of the genus *Pyrus*, as evaluated using phenotypic identification, has not been fully determined due to the lack of wild populations, low morphological diversity, lack of differentiating characteristics among species, and widespread cross-ability. Moreover, morphological characteristics are influenced by environmental conditions (Winter and Kahl 1995; Mondini *et al.* 2009). In addition to morphological markers, several markers have been developed using secondary metabolism, such as isozyme markers, phenolic compounds, pollen ultrastructures, and sugar composition, in order to distinguish among pear species and cultivars (Challice and Westwood 1973; Westwood and Challice 1978; Kajiura *et al.* 1979; Lin and Shen 1983; Zou *et al.* 1986; Jang *et al.* 1992). However, the use of such markers is limited in that their expression is easily influenced by developmental stage and exposure to environmental influences. Moreover, the lack of informative markers limits the utilization of such markers.

Compared with morphological and biochemical markers, molecular markers are practically useful for cultivar identi-

fication and characterization because they are influenced by neither variable environmental conditions nor plant phenology and can discriminate among cultivars with similar phenotypes (Belaj *et al.* 2002). Recently, many researchers have attempted to evaluate genetic diversity in *Pyrus* spp. using several types of DNA markers, such as amplified fragment length polymorphisms (AFLPs), random amplification of polymorphic DNA (RAPD), and simple sequence repeats (SSRs).

Identification of AFLP markers is a highly sensitive method for detecting polymorphisms used in genetic studies and genetic engineering (Vos *et al.* 1995). In studies of the genetic diversity of pears, AFLP has several advantages over the RAPD technique, including the capacity for analysis of more loci and increased reproducibility of banding patterns. Dolatowski *et al.* (2004) and Monte-Corvo *et al.* (2000) studied the genetic relationships among pear cultivars using AFLP analysis.

RAPD is a polymerase chain reaction (PCR)-based molecular marker technique and was first reported in 1990 (Williams *et al.* 1990). In RAPD, DNA fragments are amplified in vitro with primers that are designed with random sequences. The arbitrary primers will bind somewhere in the sequence, but it is not certain exactly where (Schierwater and Ender 1993; Schlötterer 2004; Yamamoto and Chevreau 2009). RAPD has been successfully used for identification of the genetic relationships of pear species because of the advantages of being readily employed, requiring small amounts of genomic DNA (Oliveira *et al.* 1999; Kim *et al.* 2000; Teng *et al.* 2001, 2002; Kim and Ko 2004; Lee *et al.* 2004).

SSRs, also known as microsatellites, are tandem repeats of 2-6 nucleotides. SSR markers have several advantages over other molecular markers, including highly polymorphic features due to variations in length (repeat copy numbers), codominant inheritance, large numbers of alleles per locus, abundance in genomes, requirement for only a small amount of DNA for PCR, and suitability for automation (Weber and May 1989; Moore *et al.* 1991). More than 100 SSRs have been developed from European (*P. communis*) and Asian (*P. pyrifolia*) 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 applied

for evaluation of genetic diversity, cultivar identification, and construction of genetic linkage maps (Kimura *et al.* 2002; Yamamoto *et al.* 2002c, 2004; Bassil *et al.* 2005; Volk *et al.* 2006; Bao *et al.* 2007).

Single nucleotide polymorphisms (SNPs) are DNA sequence variations caused by single nucleotide changes (i.e., transitions, transversions, deletions, or insertions) among different members in the same species (Vignal *et al.* 2002). SNPs are highly abundant in plant and animal genomes, but their density varies dramatically from region to region in each genome (Weising *et al.* 2005). SNP markers have the advantages of being mostly bi-allelic and highly frequent in genomes. Although SNP polymorphism information content (PIC) is lower than that of SSR markers, tens, hundreds, or even thousands of SNPs can be easily used when required. In recent years, SNP markers have been identified in the pear genome sequence using next-generation sequencing (NGS). Yamamoto *et al.* (2011) developed SNP and SSR markers using random shotgun sequencing to obtain the genome sequence of the Japanese pear *P. pyrifolia* ‘Housui’. Wu *et al.* (2013, 2014) developed SNPs using a combination of BAC-by-BAC and NGS. Montanari *et al.* (2013) identified 1,096 SNP markers from three European pears (*P. communis*) using NGS. Terakami *et al.* (2014) developed a 1,536-SNP bead array without a reference genome sequence from expressed sequence tag (EST) analysis combined with NGS data of Japanese pears.

Genetic linkage maps in *Pyrus* spp.

High-density genetic linkage maps are useful for fundamental and applied genetic research. Such maps are usually developed for the purposes of determining the genetic control of specific traits (Folta *et al.* 2009).

In the genus *Pyrus*, genetic linkage maps have recently been shown to be sufficiently dense and saturated. *Pyrus* genetic linkage maps provide information regarding chromosomes that can be used to localized target genes, allow the identification of quantitative trait loci (QTLs), and application of marker-assisted selection (MAS) and marker-assisted breeding (Yamamoto *et al.* 2007). Several genetic linkage maps and QTL maps of *Pyrus* have been reported in the Japanese pear (*P. pyrifolia*) and European

pear (*P. communis*). Iketani *et al.* (2001) constructed a linkage map for the Japanese pear (*P. pyrifolia*) and were able to map pear scab and black spot resistance genes. Yamamoto *et al.* (2002b, 2007) exploited a high-density linkage reference map based on AFLP and SSR markers that could serve as a useful platform for mapping genes and QTLs in pears. Pierantoni *et al.* (2004, 2007) utilized two mapping populations, ‘Passe Crassane’ × ‘Harrow Sweet’ and ‘Abbè Fétel’ × ‘Max Red Bartlett’, to identify QTLs for pear scab resistance. Dondini *et al.* (2004) produced a linkage map of ‘Passe Crassane’ and ‘Harrow Sweet’ to identify QTLs for fire blight resistance. Sun *et al.* (2009) constructed a linkage map of the Chinese pears ‘Yali’ and ‘Jingbaili’ to identify QTLs for several vegetative growth traits. Zhang *et al.* (2013) constructed a genetic linkage map of interspecific hybrid pear ‘Bayuehong’ (European pear × Chinese pear) and the Chinese pear ‘Dangshansuli’ to identify QTLs for several fruit traits using AFLP, SSR, and sequence-related amplified polymorphism (SRAP) markers, along with the S locus for self-incompatibility. Using NGS technology, Montanari *et al.* (2013) developed SNP-based pear genetic maps in European pears (*P. communis*), consisting of 857 SNPs. Terakami *et al.* (2014) developed SNPs without a reference genome sequence from EST analysis combined with NGS data for the Japanese pear ‘Housui’, and 609 SNP loci were mapped to linkage groups in a genetic linkage map of ‘Housui’.

In genetic studies of pears, there is a lack of genetic resources, including molecular markers, making it difficult to construct genetic linkage maps and identify genes controlling target traits. Thus, revealing the location of trait-determining genes on linkage groups and obtaining applicable markers for MAS by the construction of genetic linkage maps and agronomic trait mapping is critical (Yamamoto *et al.* 2007; Folta *et al.* 2009; Kale *et al.* 2012; Wu *et al.* 2013).

NGS in *Pyrus* spp.

Previous methods for identification of molecular markers, such as SSRs, insertions and deletions (InDels), and SNPs, were time- and cost-intensive because of the requirement for preparation and sequencing of genomic libraries and the limited information available regarding nucleotide sequences

in the pear genome (Squirrell *et al.* 2003; Eujayl *et al.* 2004; Iniguez-Luy *et al.* 2008; Yamamoto and Chevreau 2009; Kale *et al.* 2012). However, recently developed NGS offer opportunities for high-throughput, time-saving, and cost-effective marker development (Mardis 2008; Morozova and Marra 2008). One of the primary advantages of NGS is the identification of sequence data from amplified single DNA fragments, avoiding the need for cloning of DNA fragments. However, the overall high cost of generating high-throughput sequence data remains a limiting factor of NGS technology, although the cost per base is still much lower than that of Sanger sequencing (Ansorge 2009). Another outcome is the capacity for accurate identification of sequences flanking molecular markers (e.g., SSRs, InDels, and SNPs) for use as locus-specific markers for downstream genotyping (Yang *et al.* 2012).

Our understanding of plant genetics and genomes has reached a new level owing to the use of molecular markers (Nguyen and Wu 2005). Advances in molecular genetics, particularly the development of molecular markers, has improved our capacity for analysis of plant genomes, genetic relationships between cultivars, evolutionary features, and genomic relationships, in addition to facilitating the mapping and tagging of significant trait loci in the genome (Russell *et al.* 2000; Sjakste *et al.* 2003; Hamza *et al.* 2004). With molecular markers, complicated trait loci have been identified and mapped in the genome, and molecular markers tightly linked to important target traits can be used for marker-assisted breeding (Young 1999).

In *Pyrus* species, whole-genome sequencing has been performed for European pears (*P. communis*) and Chinese pears (*P. bretschneideri*) using NGS technology. Wu *et al.* (2013) reported the draft genome of the pear cultivar ‘Suli’ using a combination of BAC-by-BAC and NGS. A 512.0 Mb sequence corresponding to 97.1% of the estimated genome size of the pear was assembled, and density genetic maps consisting of 2,005 SNPs anchored 75.5% of the sequence to all 17 chromosomes. Chagné *et al.* (2014) presented a draft assembly of the genome of the European pear cultivar ‘Bartlett’. This genome consisted of 142,083 scaffolds and covered a total of 577.3 Mb, representing most of the expected 600 Mb *Pyrus* genome. The sequencing data of ‘Bartlett’ pears are available for

identification of the genetic control of target traits and for enhancing marker-assisted breeding in *Pyrus*.

Genome-wide association studies (GWASs) in *Pyrus*

GWASs are a powerful approach to identifying novel loci influencing human diseases (Klein *et al.* 2005). GWASs allow the detection of causal genes or QTLs based on the association between genome-wide markers and phenotypes caused by linkage disequilibrium (LD) between markers or between causal genes and QTLs (Iwata *et al.* 2013). With the development of high-throughput genotyping technologies, such as DNA chips (Gupta *et al.* 2008) and NGS (Davey *et al.* 2011), in addition to associated statistical methods, new genomics-based strategies, such as GWAS, are possible. GWASs are suitable for QTL detection, particularly in long-lived perennials (Oraguzie *et al.* 2007). However, GWASs are just beginning to be applied to fruit tree species, with only a few species having been analyzed to date (Myles *et al.* 2011). Yamamoto *et al.* (2014) conducted GWASs to detect significant associations between 162 markers and nine agronomic traits, including harvest time, resistance to black spot disease, firmness of flesh, fruit size, fruit shape in the longitudinal section, acid content, total soluble solid content, number of spurs, and tree vigor, which are important in pear production. GWASs using the 76 Japanese pear cultivars have detected four markers showing significant associations with three traits: resistance to black spot disease, harvest time, and the number of spurs. In this previous study, significant associations were detected, despite the use of only a few markers and samples for GWAS.

Because few GWASs have been performed in *Pyrus* species, GWASs will become a powerful tool for identification of associated DNA markers for many important phenotypic traits and will be the most efficient tool for improving pear breeding programs.

Genetic studies of the Asian pear (*Pyrus* spp.) in Korea

In Korea, genetic studies of the Asian pear (*Pyrus* spp.) have been performed as a part of the Biogreen 21 project. ‘Whangkeumbae’ and ‘Minibae’ were chosen as the representative cultivars of *P. pyrifolia* and hybrids in Korea. The genomes of ‘Whangkeumbae’ and ‘Minibae’

were resequenced using the Illumina Hiseq 2000 platform. In ‘Whangkeumbae’ and ‘Minibae’, an average of 173.2 million high-quality two-paired-end (PE) reads was generated, corresponding to an average of 17.4 Gb of sequence (Table 1). To align the reads to the reference genome, the Burrows-Wheeler Aligner (BWA) program (Li and Durbin 2009) and Bowtie aligner (Langmead *et al.* 2009) were applied. The generated sequences of ‘Whangkeumbae’ and ‘Minibae’ were aligned to the pear reference genome, i.e., the Chinese pear ‘Suli’ (*P. bretschneideri*), and the length was 512 Mb (Wu *et al.* 2013), with an average of 74.3% of all PE reads aligning. The reads-mapping region of ‘Whangkeumbae’ and ‘Minibae’ was about 444 Mb, around 23% smaller than reference genome.

The SNPs, InDels, and SSRs detected between ‘Whangkeumbae’ and ‘Minibae’ using NGS data are shown in Table 2. For SNP detection, NGS data for ‘Whangkeumbae’ and ‘Minibae’ were mapped to the reference genome, and a mapped read depth of three or more SNPs was selected through SNP validation and selection. The numbers of total SNPs were 2,711,000, and 2,746,000 in ‘Whangkeumbae’ and ‘Minibae’, respectively. Of all SNPs of each cultivar, the numbers of homozygous

and heterozygous SNPs were 1,318,000 and 1,393,000 in ‘Whangkeumbae’ and 1,206,000 and 1,540,000 in ‘Minibae’, respectively. Polymorphic SNPs were selected based on the same SNP position, and the difference between each cultivar and polymorphic SNPs was 2,510,000. In terms of the distribution of polymorphic SNPs in the intergenic and genic regions, polymorphic SNPs occupied 73.83% of intergenic regions and 26.17% of genic regions.

For the detection of InDels, NGS data for ‘Whangkeumbae’ and ‘Minibae’ were mapped to the reference genome, and a mapped read depth of three or more InDels was selected through InDel validation and selection. The number of total InDels was 197,000 for both ‘Whangkeumbae’ and ‘Minibae’. The numbers of insertions and deletions were 88,000 and 109,000 in ‘Whangkeumbae’ and 88,000 and 109,000 in ‘Minibae’, respectively. Polymorphic InDels were selected based on the same insertion and deletion positions, and the difference between each cultivar and polymorphic InDels was 149,000. In terms of the distribution of polymorphic InDels in the intergenic and genic regions, polymorphic InDels occupied 73.67% of intergenic regions and 26.33% of genic regions.

SSRs were detected in the consensus sequence, which was mapped to the reference genome, and sequences of 2-6

Table 1. Illumina Hiseq raw data and reads-mapping region using pear reference genome^{z)} in ‘Whangkeumbae’ and ‘Minibae’.

Cultivar	Number of reads	Average length (bp)	Total length (bp)	Genome coverage ^{y)}	Reads-mapping region ^{x)}
Whangkeumbae	187,595,914	100	18,759,591,400	36.64×	444,371,531
Minibae	158,913,318	100	15,891,331,800	31.04×	444,569,932

^{z)}Pear reference genome: *Pyrus bretschneideri*.

^{y)}Genome coverage: Total length of cultivar / total length of reference genome.

^{x)}Reads-mapping region: (Reads-mapping region / total length of pear reference genome)×100.

Table 2. Comparative analysis of SNPs, InDels, and SSRs between ‘Whangkeumbae’ and ‘Minibae’.

Cultivar	Whangkeumbae	Minibae
Number of total SNPs	2,711,000	2,746,000
Number of Polymorphic SNPs	2,510,000	
Number of total InDels	197,000	197,000
Number of Polymorphic InDels	149,000	
Number of total SSRs	75,000	75,000
Number of Polymorphic SNPs	1,200	

nucleotides in length that were repeated more than five times were selected by SSRs. Polymorphic SSRs were based on differences in the repeated lengths of SSR-generating regions between each cultivar. The number of total SSRs for ‘Whangkeumbae’ and ‘Minibae’ was 75,000, with validation of 1,200 polymorphic SSRs. Thus, these results provide novel, rapid methods for identification of molecular markers using NGS technology. These NGS-based molecular markers will be useful for genetic studies of Asian pears (*Pyrus* spp.), including for construction of genetic linkage maps, QTL mapping, and MAS in pear breeding efforts.

CONCLUSION

The importance of the fruit industry and fruit tree breeding has not been recognized fully in Korea, and support for genomic researcher of fruit trees has been poor. Foreign countries are concentrating their efforts on the acquisition of genomic information of fruit crops and the development of important trait-related molecular markers, with the goal of establishing bases for precise fruit tree breeding. Although Korean scientists are not actively researching the genomes of fruit trees, the technologies available in Korea are sufficiently advanced that the country can still be considered competitive in terms of research and development technologies.

In the first phase of the Biogreen 21 project, we performed resequencing in Asian pears (*Pyrus* spp.) and developed genome-based molecular markers. The second phase, which involves GWAS analysis using a core collection of pear germplasms, is currently under way. The latest genotyping by sequencing (GBS)-based analysis technology can reduce the cost and time of DNA sequence analysis and enhance the efficiency of genetic mapping and the development of molecular markers related to useful traits (Thompson 2014). In addition, GWAS analysis using genotype data developed in GBS and data on useful traits based on phenotypes can overcome difficulties in the genetic analysis of fruit crops through identification of useful genes and development of related molecular markers, thereby enhancing the efficiency of the identification and

application of genes related to agriculturally important characters (Huang 2010; Iwata 2013). Through these methods, we may be able to improve our understanding of genetic phenomena in specific fruit crops and among all fruit crops and to enhance the competitiveness of domestic fruit tree breeding through high-efficiency and precise breeding.

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