Korean J. Plant Res. 20(6) : 563~569(2007)

Analysis of Genetic Relationship Among Native Pears Grown in Korea and Several Commercially Developed Cultivars from Two *Pyrus* Species Based on RAPD Analysis

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Abstract - RAPD analysis showed that all the OTUs of 'Sandolbae' were the same species because amplified band patterns of all samples generated by each of 5 random primers were identical. Even though there were different environmental conditions, all the "Chuiangne" trees from three different places were the same species, and also all the "Cheongshilli" trees were the same species too. No genetic variations were detected between native Korean pears grown in the habitats and in the research field. Because 212 polymorphic bands were generated by 9 primers selected through primer screening, they were possible to analyze genetic relationship among naturally growing three native Korean pears and nine cultivars of *Pyrus pyrifolia* and *P. communis*. Based on the RAPD analysis, three main groups were formed. The first group represented the Six *P. pyrifoia* cultivars, the second group was the three native Korean pears, and the last group was the three *P. communis* cultivars. Genetic distance between 'Wonwhang' and 'Chojuro' was closer than other cultivars in group 1 since dissimilarity index value between these two cultivars was 50.82. However, genetic distance between 'Niitaka' and 'Chojuro' was the most distant compared to the others in group 1. In group 2, 'Sandlobae' was genetically closer to 'Chuiangne' than 'Cheongshilli' because dissimilarity index value between 'Sandlobae' and 'Chuiangne' was smaller, 50.82, than the value between 'Sandlobae' and 'Cheongshilli' , 63.636. In group 3, 'Old Home' was genetically closer to 'Bartlett' than 'Kaiser Alexander (or Bosc)' . Group 3 composed of *P. communis* cultivars was genetically further than other two groups, *P. pyrifolia* cultivars and native Korean pears.

Key words - Genetic distance, Primer screening, *Pyrus*, RAPD analysis

Introduction

Genus *Pyrus* belongs to the subfamily Pomoideae of the family Rosaceae and has a basic chromosome number of $x = 17$ (Sax, 1931). The genus *Pyrus* is composed at least 22 species, which are distributed to Europe, temperate Asia and mountainous areas of northern Africa (Bell *et al.*, 1996). Kikuchi (1946) classified *Pyrus* species into three groups, small fruited species, large fruited species, and their hybrids. Among 22 primary species, the large fruited species are commercially cultivated in temperate zone and divided into three major species, *P. communis* L. (European pear), *P. bretschneideri* Rehd. or *P. ussuriensis* Max. (Chinese pear) and *P. pyrifolia* Nakai (Japanese pear: Nashi). *P. communis* L. is native to Europe, and is the main commercial species in Europe, North America, South America, Africa and Australia. *P. bretschneideri* is the main species in northern and central China. *P. pyrifolia* (Burm.) Nakai is the main species in Korea, Japan, southern and central China, and Taiwan, and recently in Europe and North America. Especially two species (*P. ussuriensis* Max. and *P. bretschneideri* Rehd.) are grown in colder area of China and Korea (Kim *et al.*, 2000). *P. communis* L. (European pear) and *P. pyrifolia* Nakai (Japanese pear: Nashi) are the most interesting *Pyrus* species for fruit production in Korea and Japan.

In Korea, there are many naturally growing native pears including 'Dolabe' , 'Sandlobae' , 'Cheongshilli' , and 'Chuiangne' (Chung and Ko, 1995; Ahn and Chung, 2002; Ahn *et al.*, 2002). 'Dolbae' tree was originated and named from naturally growing *P. pyrifolia* in Korea and used to develop many cultivars (Kim *et al.*, 2000; Ahn and Chung, 2002). 'Sandlobae' is known as a primary species of *P. ussuriensis* (Sax, 1931) and has been grown in Korea. However, 'Cheongshilli' and 'Chuiangne' are forma and variety of 'Sandlobae' respectively (Sax, 1931) (Table 1). In past 20 years, many cultivars have been developed only from *P. pyrifolia* or its forma and cultivated in Korea (Chung and Ko, 1995).

Traditionally, identification of pear cultivars was based on morphological or physiological aspects. In recent years,

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Table 1. The common and scientific names and their origin of 9 *Pyrus* cultivars and 3 native Korean pears used for RAPD analysis

No.	Common name	Scientific name	Origin
1	Whasanbae	*Pyrus pyrifolia*	Hosui × Okusankichi
2	Niitaka	*P. pyrifolia*	Amanogawa × Imamuraaki
3	Sandolbae	*P. ussuriensis* Maximowicz	Elementary species of *P. ussuriensis*
4	Kaiser Alexander	*P. communis*	Sport of cultivar Beurre Bosc
5	Whangkeumbae	*P. pyrifolia*	Niitaka × Nijisseiki
6	Chuiangne	*P. ussuriensis* var. acidula ,T. LEE	Sport of Sandolbae
7	Cheongshilli	*P. ussuriensis* var. ovoidea REHDER	Sport of Sandolbae
8	Bartlett	*P. commumis*	Chance seedling
9	Chojuro	*P. pyrifolia*	Chance seedling
10	Wonwhang	*P. pyrifolia*	Waseaka × Okusankichi
11	Old Home	*P. communis*	Chance seedling
12	Gamcheonbae	*P. pyrifolia*	Okusankichi × Danbae

biochemical markers like isozymes provided useful information for pear classification (Chung and Ko, 1995; Chevreau *et al.*, 1997) and many papers have described the value of molecular markers like RFLP (restriction fragments length polymorphism) and RAPD (random amplified polymorphic DNA) in taxonomical classification and cultivar-typing in fruit trees. Because the RAPD technique is a powerful tool for analyzing genetic relationships, it has been used for classification of cultivars, subspecies, or species of apple (Yae and Ko, 1995), plum (Ortiz *et al.*, 1997), lemon (Deng *et al.*, 1995), peach (Warburton and Bliss, 1996), and grapes (Lee *et al.*, 1998) by genetic distance. This technique is required very small amounts of genomic DNA and eliminates the need for blotting and radioactive detection to identify genetic relationships. For these reasons, RAPD technique was commonly used for identification and genetic relationships analysis among species, subspecies, or cultivars.

In *Pyrus* species, the taxonomical (Cheong, 1994) and palynological (Shim *et al.*, 1988) studies have been performed. In addition, isozyme pattern (Jang *et al.*, 1991), RFLP (Kawata *et al.*, 1995), and RAPD (Oliveira *et al.*, 1999) studies have been done to study genetic relationship among several cultivars developed from each *Pyrus* species. Nevertheless, no study has been done to identify genetic relationship among naturally growing native Korean pears and very little studies have been performed to determine genetic distance among groups of cultivars developed from three *Pyrus* species or between *Pyrus* species (Chevreau and Skirvin, 1992; Ye *et al.*, 1996; Chevreau *et al.*, 1997; Oliveira *et al.*, 1999).

The objective of this study was to investigate genetic relationship among naturally growing native Korean pears, 'Sandlobae', 'Cheongshilli' and 'Chuiangne', and also determine genetic distance among three native Korean pears and nine cultivars of *Pyrus pyrifolia* and *P. communis* using RAPD technique.

Materials and Methods

Plant materials

Leaf samples from 'Sandlobae', 'Cheongshilli', and 'Chuiangne' were collected from naturally growing pear trees in the native habitats. These leaf samples were also collected from three native Korean pear trees cultivated in the field of Pear Research Institute in Naju, Cheonnam. Leaves of each *Pyrus* cultivar used in this study shown in Table 1 were collected from each *Pyrus* cultivar trees cultivated in the field of Pear Research Institute in Naju, Cheonnam and used genetic relationship analysis by RAPD technique. Table 1 shows their common and scientific names and origins of 9 *Pyrus* cultivars and 3 native Korean pears used for this study.

Genomic DNA isolation

DNA was extracted from fresh newly expanded leaves. One gram of pear leaves were ground in liquid nitrogen and subsequently incubated 55℃ for 50 min with 15 mL of extraction buffer (NaCl 0.2M, Tris-HCl 0.1M, Sodium dodecyl sulfate 2%,

EDTA 0.1M, β-Mercaptoethanol 1%). The mixture was allowed to cool to room temperature (RT) and a 1:1 phenol-chloroform extraction was performed. The preparation was mixed by inversion to form an emulsion and then centrifuged at RT for 10 min (12,000 g) to separate the phases. Subsequently, DNA was precipitated from the aqueous phase by adding 2X volume of cold absolute ethanol. The pellet was washed with 70% (v/v) ethanol and dried on the air. The DNA was redissolved using 5 mL distilled water and diluted in distilled water at 5 ng$\mu\ell^{-1}$ for RAPD analysis. The concentration of DNA was determined by the spectrophotometer.

DNA amplification

The primers used in RAPD analysis are 10-mer random primers provided by Operon Primer Kit (Operon Technolgies Co., Alameda, CA) and the sequences and GC content of 10-mer random primers are shown in Table 2. The PCR solution was 2X perfect-PreMix® (Takara, Japan). The solution was composed 0.5 units of Takara Ex Taq™ DNA polymerase, 0.4 mM of each dNTP and 4mM Mg^{2+}. The PCR reaction mixture contained PCR solution 10$\mu\ell$, 0.4μM primer and 25 ng template DNA. PCR reactions were performed in a Primus 96 thermal cycler (MWG AG Biotech, Milton Keynes, United Kingdom) programmed as follows: 5 min at 94℃ for initial denaturation, 50 cycles of 30s at 94℃ (denaturation), 30s at 35℃ (annealing), and 30s at 72℃ (extension). A final extension step at 72 ℃ for 10 min was followed. The amplification products were separated and visualized on 1.8% agarose gels stained with ethidium bromide.

Data analysis

Based on the bands recorded as present or absent, a binary matrix was obtained. The binary matrix was transformed into a similarity matrix by Nei and Li' s genetic index (1979) using an analytic program, Treecon (Van de Peer & De Wachter, 1993). From this matrix, a dendrogram was generated by cluster analysis, UPGMA method (Unweighted pair-group method with arithmatic average). A bootstrap analysis (Felsenstein, 1985; Sneath & Sokal, 1973) was also performed on the dendrogram as 100 replications.

Results and Discussion

Naturally growing 'Sandlobae' trees were found in two places, Gumdaebong, Jeongsunkun, Kwangwondo and Ansungsi, Kyeongkido. Naturally growing 'Chuiangne' trees were also found in two places, Hambacksan, Jeongsunkun, Kwangwondo and Ansungsi, Kyeongkido. However, naturally growing 'Cheongshilli' tree was only found in the area of Buseok Temple, Youngju, Kyeongbuk. These native Korean pear trees were cultivated in the field of Pear Research Institute in Naju, Cheonnam. RAPD analysis proved that all the OTUs of 'Sandolbae' in either two native habitats or the research field in Naju was the same species because amplified band patterns of all samples generated by each of 5 random primers (Operon Technolgies Co., Alameda, CA) were identical (data not shown). Even though there were different environmental conditions, all the "Chuiangne" trees from three different places were the same species, and also all the "Cheongshilli" trees were the same species too (data not shown). Currently, we do not know when and how these native Korean pear trees started to grow in the found areas and which native pear trees were collected and cultivated in the field of Pear Research Institute in Naju, Cheonnam. But, there was no genetic variation between

Table 2. The list of 9 random primers used in RAPD analysis among 9 *Pyrus* cultivars and 3 native Korean pears

Primer group	Number	Sequence (5 ' to 3')	CG content (%)
OPE	04	GTGACATGCC	60
	10	CACCAGGTGA	60
	19	ACGGCGTATG	60
	20	AACGGTGACC	60
OPAW	02	TCGCAGGTTC	60
	04	AGGAGCGACA	60
	05	CTGCTTCGAG	60
	09	ACTGGGTCGG	70
	15	CCAGTCCCAA	60

native Korean pear trees grown in the habitats and pear trees in the research field, so trees of each native Korean pear were identical no matter where they were growing. Naturally growing old and large 'Dolbae' tree was inhabited in Mount Kaya, Hapcheongun, Gyeongnam. Because leave condition collected from this native 'Dolbae' tree in the habitat as well as cultivated 'Dolbae' tree in the research field in Naju were not good, however, RAPD analysis was not performed. From this reason, it is uncertain that naturally growing native 'Dolbae' tree and cultivated 'Dolbae' tree in the research field are identical.

To determine genetic relationship among naturally growing three native Korean pears and nine cultivars of *Pyrus pyrifolia* and *P. communis* by RAPD technique, primer screening with 70 random primers (Operon Technolgies Co., Alameda, CA) was performed. In primer screening, PCR amplification with 9 random primers showed clear polymorphic bands (Fig. 1), so these 9 random primers (Table 2) were selected to determine genetic relationship among three native Korean pears and nine cultivars. For RAPD analysis, amplified DNA bands between 300 and 1800 bp in size were counted, but DNA bands below 300 bp were not counted because these bands were not certain to reproduce. Based on the fact that the band is present or absent, a binary matrix and dissimilarity index (Table 3) were obtained by the Nei and Li's method (1979) using an analytic program, Treecon (Van de Peer & De Wachter, 1993). From this matrix, dendrogram (Fig. 2) of 3 native Korean pears and 9 pear cultivars was generated by UPGMA cluster analysis of the similarity values given in Table 3.

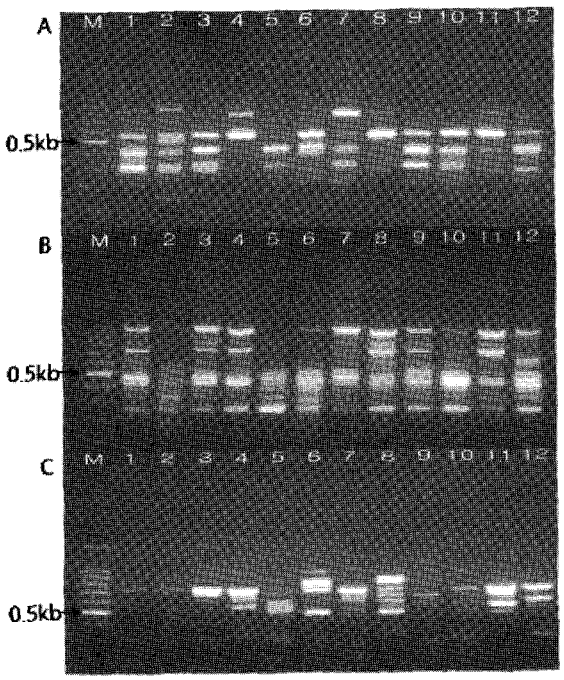

Fig. 1. DNA profiles obtained from 3 native Korean pears and 9 *Pyrus* cultivars with three primers, OPAW-09, 0PE-04, OPE-19. Numbers correspond to those in Table 1. M: 100bp DNA ladder. A: OPAW-09, B: 0PE-04, C: OPE-19.

Each of 9 random primers produced clear polymorphic DNA profiles. A total of 212 polymorphic bands were generated by 9 primers. As Chevreau *et al.* (1997) mentioned that pear is a high polymorphic species in their early paper, the relatively large number

Table 3. Dissimilarity index (matrix) among 3 native Korean pears and 9 *Pyrus* cultivars generated by Nei and Li's (1979) dissimilarity coefficients using UPGMA as the clustering method

No.[z]	1	2	3	4	5	6	7	8	9	10	11	12
1	0											
2	55.263	0										
3	72.621	74.798	0									
4	62.5	72.727	73.831	0								
5	58.73	69.231	74.981	62.264	0							
6	76.471	77.143	54.412	75.862	78.947	0						
7	78.667	76.623	63.636	78.462	81.252	68.116	0					
8	73.529	74.286	70.37	51.724	75.439	74.194	71.014	0				
9	60	75	73.855	63.333	69.492	71.875	71.831	59.375	0			
10	63.077	67.164	74.231	56.364	66.667	62.712	72.727	66.102	50.82	0		
11	60.526	74.359	73.303	63.636	78.462	65.714	71.429	45.714	63.889	61.194	0	
12	68.116	69.014	70.976	66.102	65.517	65.079	65.714	58.73	60	56.667	54.93	0

[z] Numbers correspond to those in Table 1.

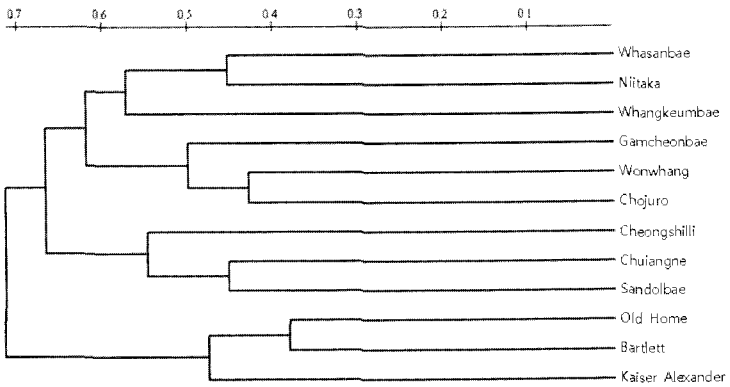

Fig. 2. Dendrogram of 3 native Korean pears and 9 pear cultivars generated by UPGMA cluster analysis of the similarity values given in Table 3.

of polymorphic bands was produced with seven primers, OPAW-02, 05, 09 and 15, and OPE-04, 19 and 20. Kim *et al.* (2000) also showed that many polymorphic bands were generated by various random primers with *P. pyrifoia* cultivars in their paper. Fig. 1 shows the representative polymorphic profiles obtained with three primers, OPAW-09, OPE-04 and OPE-19. The amplified polymorphic bands between 300 and 1800 bp shows different intensities among 9 pear samples. Yang and Quiros (1993) suggested that band intensity might reflect differences in the copy number of the amplified sequence among different genotypes. Demeke *et al.* (1992) considered such bands to be useful for polymorphic markers.

Based on the dissimilarity index (Table 3) and the dendrogram (Fig. 2), genetic relationships of twelve samples were determined. From these two results, three main groups were formed. The first group that was formed represented the Six *P. pyrifoia* cultivars including 'Whasanbae', 'Niitaka', 'Whangkeumbae', 'Chojuro', 'Wonwhang', and 'Gamcheonbae'. The second group was the three native Korean pears including 'Sandlobae', 'Cheongshilli', and 'Chuiangne'. The last group was the three *P. communis* cultivars; 'Kaiser Alexander (or Bosc)', 'Bartlett', and 'Old Home' (Fig. 2). As Oliveira *et al.* (1999) mentioned that *Pyrus* species are self-incompatible and have great genetic variability, these three groups should be separated each other in terms of genetic relationship. According to the dendrogram in Fig. 2, group 1 is clustered furthermore into two subgroups. The first subgroup is the three cultivars, including 'Whasanbae', 'Niitaka', and 'Whangkeumbae' and the second subgroup is the three cultivars, including 'Chojuro', 'Wonwhang', and 'Gamcheonbae'. This result supports the previous study (Kim *et al.* 2000).

Table 3 shows the dissimilarity index value of 3 native Korean pears and 9 *Pyrus* cultivars. The value of dissimilarity index means that genetic distance between two samples is far if dissimilarity index value is close to 100, but genetic distance is close each other if dissimilarity index value is close to zero (Nei and Li, 1979). According to the dissimilarity index, genetic distance between 'Wonwhang' and 'Chojuro' was closer than other cultivars in group 1 since dissimilarity index value between these two cultivars was 50.82. However, genetic distance between 'Niitaka' in subgroup 1 and 'Chojuro' in subgroup 2 was the most distant compared to the others in group 1 and their dissimilarity index value was 75.

In group 2 in Fig. 2, 'Sandlobae' was genetically closer to 'Chuiangne' than 'Cheongshilli' because dissimilarity value between 'Sandlobae' and 'Chuiangne' is 50.82, but dissimilarity index value between 'Sandlobae' and 'Cheongshilli' was 63.636 (Table 3). This result indicates that genetic variation of 'Cheongshilli' from 'Sandlobae' has occurred more than 'Chuiangne'. According to Sax (1931), 'Cheongshilli' is a forma of 'Sandlobae' and 'Chuiangne' is a variety of 'Sandlobae' (Table 1). This implied that genetic variation between a variety and its origin species is smaller than variation between a forma and its origin species.

Three European cultivars, 'Kaiser Alexander (or Bosc)', 'Bartlett', and 'Old Home' belong to group 3 (Fig. 3). In group 3, 'Old Home' was genetically closer to 'Bartlett' than 'Kaiser Alexander (or Bosc)' and dissimilarity value between 'Old Home' and 'Bartlett' was 45.714. Group 3 composed of *P. communis* cultivars were genetically further than other two groups, *P. pyrifolia* cultivars and native Korean pears, *P. ussuriensis*.

Exceptional dissimilarity index value was found between 'Wonwhang' and 'Kaiser Alexander (or Bosc)'. The dissimilarity

index value between two cultivars should be bigger than 54.364 because 'Wonwhang' is a *P. pyrifoia* cultivars classified into group 1 and 'Kaiser Alexander (or Bosc)' is a *P. communis* cultivars classified into group 3 (Table 3). Currently, it is incomprehensible why the dissimilarity index value between these two cultivars is smaller than the expected value. However, there could be one possible explanation about this result. Since only two kinds of random primers, OPE and OPAW in Table 2, were used for RAPD analysis, this may mislead into unexpected result like this. Therefore, it is necessary to perform further RAPD analysis with various random primers to minimize this kind of an error. Additionally, three representative *Pyrus* species, such as 'Dolbae' (*P. ussuriensis*), 'Sandolbae' (*P. pyrifolia*), and a primitive species of *P. communis* should be compared each other through RAPD analysis in order to study genetic variation among different *Pyrus* cultivars, but 'Dolbae' tree known as an elementary species of *P. pyrifolia* was not included in this study. Nevertheless, RAPD assay directly reflects the structural differences of genome and it is not affected by environmental factors. So, it is a very useful technique to study phylogeny of species and identify various cultivars.

Acknowledgements

This research was supported by Chung-Ang University Grant in 2004.

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(Received 20 November 2007 ; Accepted 21 December 2007)