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Genetic Relationships of *Pyrus* Species and Cultivars Native to East Asia Revealed by Randomly Amplified Polymorphic DNA Markers

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Abstract. A total of 118 *Pyrus* sp. (pear) and cultivars native mainly to east Asia were subjected to randomly amplified polymorphic DNA (RAPD) analysis to evaluate genetic variation and relationships among the accessions. Two hundred fifty RAPD markers were scored from 20 decamer primers. RAPD markers specific to species were identified. Clustering analysis revealed two divisions: one comprising cultivars of *P. communis* L., and the other including all accessions of *Pyrus* native to east Asia. The grouping of the species and cultivars by RAPD data largely agrees with morphological pear taxonomy. However, some noted incongruence existed between two classification methods. *Pyrus calleryana* Dcne. clustered together with *P. koehnei* Schneid., *P. fauriei* Schneid. and *P. dimorphophylla* Makino. *Pyrus betulaefolia* Bge. clustered with *P. ×hopeiensis* Yu and *P. ×phaeocarpa* Rehd. A noncultivated clone of *P. aromatica* Kikuchi et Nakai grouped with *P. aromatica* cultivars. *Pyrus hondoensis* Nakai et Kikuchi and cultivars of *P. ussuriensis* Max. formed a single group. Some accessions from Korea (named Korean pear) had species-specific RAPD markers and comprised an independent group. Most of the Chinese white pears clustered together with most of the Chinese sand pears. Based on the present results, the new nomenclature *P. pyrifolia* var. *sinensis* (Lindley) Teng et Tanabe for Chinese white pear was suggested. Most accessions of Japanese pears fell into one main group, whereas pear cultivars from Kochi Prefecture of Japan subclustered with some Chinese sand pears and one accession from Korea. Our results infer that some local Japanese pear cultivar populations may have been derived from cultivars native to Kochi Prefecture in Shikoku region, and that the latter may have been introduced from ancient China and/or Korea.

The genus *Pyrus*, with the common name pear, belongs to the subfamily Pomoideae, and the family Rosaceae. The basic *Pyrus* stock is believed to have arisen during the Tertiary period in the mountainous regions in western and southwestern People's Republic of China (China) (Rubtsov, 1944). From the geographical point of view, pears are traditionally divided into two native groups: Occidental pears and Oriental pears (Layne and Quamme, 1975; Lee, 1948; Rubtsov, 1944). The exact number of species in the genus *Pyrus* varies among taxonomists. According to Rubtsov (1944), the Occidental pears include over 20 species found in Europe, northern Africa, Asia Minor, Iran, central Asia, and Afghanistan; the majority of cultivars grown in these areas have originated primarily from *Pyrus communis*. The Oriental pears include 12 to 15 species, distributed from the Tian-Shan and Hindu Kush Mountains eastward to Japan. In a detailed taxonomic study of *Pyrus*, Challice and Westwood (1973) suggested 21 primary species and four geographic groups of species, of which 10 species native to east Asia were assessed. These east Asian pears are distributed primarily in China, Japan, and Korea.

China contains a majority of the most important pear species native to east Asia, and is also a world leader in pear production. According to ancient Chinese literature, the history of pear culture in China dates back at least 3000 years (Pu and Wang, 1963; Shen, 1980, Sun et al., 1983). Thirteen species originating in China have been classified (Yu, 1979). However, this classification system has generally been unrecognized by taxonomists and horticulturists outside of China. A preliminary survey indicated there are over 3000 pear cultivars in China (Pu and Wang, 1963; Shen, 1980). The commercial cultivars in China are derived principally from three species: *P. ×bretschneideri* Rehd. (Chinese white pear), *P. ussuriensis*, and *P. pyrifolia* (Burm.) Nakai, while minor cultivars have originated from *P. ×sinkiangensis* Yu, *P. ×phaeocarpa* and *P. pashia* D. Don (Pu and Wang 1963; Shen, 1980; Yu, 1979). Ancient cultivars of *P. communis* are even found in Xinjiang and Gansu provinces (Yuan and Du, 1980). Cultivars of Chinese white pears are grown extensively in China, especially in north China. 'Yali' and 'Laiyangcili (Cili, Tzu Li or Ts Li)' are the most widely known among this group. However, their origin has not been determined. Rubtsov (1944) and Kikuchi (1946) proposed an origin involving hybridization of *P. ussuriensis* and *P. pyrifolia*, and Kikuchi (1946) put this group of cultivars under the name of *P. ussuriensis* Max. var. *sinensis* Kikuchi. This nomenclature has not been accepted by Chinese taxonomists. Some researchers from Europe and the United States speculate that *P. ×bretschneideri* has originated from natural hybridization between *P. betulaefolia* and *P. pyrifolia* (Challice and Westwood, 1973). The Chinese sand pear, *P. pyrifolia*, occurs mainly in the Changjiang (Yangtze) River valley, and has been considered to be the same species as similar germplasm in Japan (Kikuchi, 1946, 1948). *P. ussuriensis* (Ussurian pear) grows not only in north China, but also in the Far East region of Russia and North Korea. This species is the hardiest among the genus *Pyrus* (Pieniazek, 1967; Pu and Wang, 1963). Its fruit usually becomes soft and edible after a ripening period, which is clearly different from the crisp flesh texture of Chinese sand pears and white pears (Pu and Wang, 1963).

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Except for a few cultivars of *P. aromatica*, the majority of pear cultivars in Japan are usually grouped into *P. pyrifolia* (Japanese pear or nashi). There are two different viewpoints about the origin of the native pear cultivars in Japan. Kikuchi (1948) proposed that japanese pear cultivars have been domesticated from wild *P. pyrifolia* which occurs in southern Japan. On the other hand, some researchers insisted that some local cultivars in Japan have come from ancient China and the Korean Peninsula (Kajiura et al., 1979; Shirai, 1929). It has been proven that fruit traits in pear cultivars distributed in the Kyushu area and the coast of the Japan Sea are much more similar to Chinese pear cultivars than to those in other areas of Japan (Kajiura and Suzuki, 1980). However, until now there has been little evidence to affirm that Japanese pear cultivars are closely related to pears in China and Korea. On the other hand, Japanese cultivars originating from the Kanto region may be related genetically to *P. aromatica* (Kajiura et al., 1983) or *P. hondoensis* (Kawata et al., 1995). Therefore, the taxonomic position of pear cultivars in Japan is yet to be determined.

It has been known that *P. ussuriensis*, *P. pyrifolia*, and *P. fauriei* occur in the Korean Peninsula. Pears grown in Korea were classified into many species by Japanese taxonomists (e.g., Uyeki, 1921, 1925). However, most of these so-called species should be treated as cultivars in the strict sense. It remains unclear how these types of pears are assigned to the known species.

Identification of pear cultivars and phylogenetic analysis of pear species have depended traditionally upon an evaluation of morphological characteristics (Kikuchi, 1948; Rehder, 1915; Yu, 1979; Yu and Kuan, 1963; Yuan and Du, 1980), which is a practiced skill and is made more difficult by the poor morphological diversity among pear species and cultivars. Therefore, some other markers such as phenolic compounds (Challice and Westwood, 1973; Kajiura et al., 1983), isozyme analysis (Jang et al., 1992; Lin and Shen, 1983), pollen ultrastructure (Westwood and Challice, 1978; Zou et al., 1986), and sugar composition (Kajiura et al., 1979) have been used to distinguish pear species and cultivars. The major drawback of these techniques is that their expression is influenced by the developmental stage and may also be vulnerable to environmental influence. In addition, they are limited by the number of informative markers.

Recently, DNA-based molecular markers have been used for cultivar and species identification in many plant species (e.g., Jan et al., 1999; Marquard et al., 1997). Attempts have been made to distinguish Asian pears with restriction fragment length polymorphism (RFLP) of nuclear DNA (Kawata et al., 1995) and chloroplast DNA (Iketani et al., 1998). Both studies have provided new information about east Asian pears. However, conclusive genetic relationships among Asian pear species and cultivars have not been established because of the limited number of entries of Chinese sand pears and relatively small degrees of polymorphism that was found in both studies. The polymerase chain reaction (PCR)-based randomly amplified polymorphic DNA (RAPD) technique developed by Williams et al. (1990) has been used for identification of plant species and cultivars because of simplicity, versatility, and ability to generate high rates of polymorphism. In related studies on pears, RAPD markers have been used to identify parentage (Banno et al., 2000) and a marker linked to the gene conferring susceptibility to black spot disease [*Alternaria alternata* (Fr.) Keissler] (Banno et al., 1999). Oliveira et al. (1999) and Monte-Corvo et al. (2000) have confirmed that RAPD is useful in pear cultivar identification and phenetic classification within the genus *Pyrus*. To our knowledge, no detailed research has been conducted to analyze genetic relatedness among pear species and cultivars native to east Asia using RAPD markers. Therefore, the objective of this study was to use RAPD markers to estimate genetic variation among pear species and cultivars native to east Asia and try to gain new understanding of genetic relationships among east Asian pear cultivars and taxa.

Materials and Methods

Plant material. Plant materials used in this study are listed in Table 1. Two white pear cultivars and all cultivars of Chinese sand pear were collected from the China Pear Germplasm Repository (CPGR), Research Institute of Pomology, Chinese Academy of Agricultural Sciences, located in Xincheng, Liaoning Province, China. Four accessions were from Gansu Pomology Institute (GPI), Gansu Academy of Agricultural Sciences, Gansu Province, China. Young pear leaves were taken in late May 1999 and lyophilized for 72 h. The lyophilized leaves were sealed in plastic bags packed with silicon gel, transported to Japan, and stored at –20 °C. Fresh leaves of Japanese pear cultivars and other accessions including three cultivars of *P. communis* were harvested from the pear germplasm collection at Tottori University (TU), Tottori, Japan, and stored at –80 °C until needed.

DNA extraction and purification protocol. In general, 3.5 g of fresh leaves or 1.0 to 1.4 g of lyophilized leaves were ground in liquid nitrogen using a mortar and pestle. The powder was then transferred into 50-mL centrifuge tubes with liquid nitrogen and frozen at –80 °C for sample storage before isolating DNA.

Total DNA was extracted following the protocol of Dellaporta et al. (1983), with modifications. Ground tissue was combined with 40 mL of washing buffer (0.1 M HEPES pH 8.0, 0.1% polyvinylpyrrolidone (PVP) (K-40), 1% 2-mercaptoethanol added just before use) and centrifuged at 4 °C and 21,400 *g*n for 5 min and the supernatant was discarded. This washing process was repeated three to five times until the aqueous phase showed a clearly greenish color. The pellet was resuspended in 10 mL

Table 1. *Pyrus* species and cultivars used in RAPD analysis.

Pyrus sp. or cultivar[z]	Origin[y]	Leaf source[x]
Cultivars of Chinese sand pear (*P. pyrifolia*)		
Baozhuli	Yunan Province	CPGR
Bingzili	Fujian Province	CPGR
Canqxixueli	Sichuan Province	CPGR
Chenjiadamali	Sichuan Province	CPGR
Cangwudashali	Guangxi Province	CPGR
Damali	Sichuan Province	CPGR
Fuyuanhuangli	Yunan Province	CPGR
Haidongli	Yunan Province	CPGR
Hengshanli	Taiwan	TU
Hongfenlili	Guizhou Province	CPGR
Hongpisuli	Sichuan Province	CPGR
Hongshaobangli	Sichuan Province	CPGR
Huishuijingaili	Guizhou Province	CPGR
Huobali	Yunan Province	CPGR
Kunmingmali	Yunan Province	CPGR
Mandingxueli	Fujian Province	CPGR
Qiangxiandashali	unknown	CPGR
Qiubaishali	Fujian Province	CPGR
Weiningdahuali	Guizhou Province	CPGR

Table 1. *Continued.*

Pyrus sp. or cultivar[z]	Origin[y]	Leaf source[x]
Xingyihaizili	Guizhou Province	CPGR
Yanzhouxueli	Zhejiang Province	CPGR
Yiwulizi	Zhejiang Province	CPGR
Zongbaoli	Fujian Province	CPGR
Cultivars of Chinese white pear		
Duanbajituili	Sichuan Province	CPGR
Enli	Shandong Province	TU
Hongxiaoli	Hebei Province	TU
Jizhuali	Unknown	TU
Jinhuali	Sichuan Province	CPGR
Lataili	Gansu Province	GPI
Laiyangcili	Shandong Province	TU
Lanzhoudongguoli	Gansu Province	GPI
Pingli	Hebei Province	TU
Pingguoli	Jinlin Province	TU
Pingzili	Hebei Province	TU
Qiubaili	Hebei Province	TU
Wowoli	Shandong Province	TU
Xinqingli	Unknown	TU
Xuehuali	Hebei Province	GPI
Yali	Hebei Province	TU
Yanbali	Hebei Province	TU
Zhuzuili	Unknown	TU
Cultivars of *P. ussuriensis* in China		
Beijingbaili	Beijing	TU
Jianbali	Liaoning Province	TU
Nanguoli	Liaoning Province	TU
Cultivars originated from Korea		
Cheongdangnobae	South Korea	TU
Hanheungli-Kou	North Korea	TU
Hanheungli-Otsu	North Korea	TU
Hoeryongbae	North Korea	TU
Happsilne	Central Korea	TU
Cultivars of *P. pyrifolia* in Japan		
Akaho	Kanagawa Pref.	TU
Akitatazawa-2 (UC)	Akita Pref.	TU
Amanogawa	Kochi Pref.	TU
Asahiryu	Niigata Pref.	TU
Awayuki	Unknown	TU
Chojuro	Kanagawa Pref.	TU
Doitsu	Unknown	TU
Edoya	Kanagawa Pref	TU
Gozennashi	Unknown	TU
Hakataao	Fukuoka Pref.	TU
Hakuteiryu	Niigata Pref.	TU
Hatsushimo	Unknown	TU
Heishi	Kanto Region	TU
Imamuraaki	Kochi Pref.	TU
Inugoroshi	Akita Pref.?	TU
Iwatemukaku (PA)	Iwate Pref.	TU
Kinchaku	Unknown	TU
Konpeito	Ishikawa Pref.	TU
Kosainashi	Unknown	TU
Kozo	Kanagawa Pref.	TU
Koyuki	Gunma Pref.	TU
Kunitomi	Niigata Pref.	TU
Meigetsu	Ishikawa Pref.	TU
Miyadani	Tottori Pref.	TU
Naganojisei (UC)	Nagano Pref.	TU
Nijisseiki	Chiba Pref.	TU
Okuroku	Kanagawa Pref.	TU
Okusankichi	Niigata Pref.	TU
Ohtazairai	Shimane Pref.	TU
Rokugatsu	Kanto Region?	TU
Ruisannashi	Niigata Pref.	TU
Saizounashi	Akita Pref.	TU
Sannashi	Iwate Pref.	TU
Sekaiichi	Saitama Pref.	TU
Sekiryu	Chiba Pref.	TU
Shikishima	Chiba Pref.	TU
Shimanezailai	Shimane Pref.	TU
Shimoichikoboku	Nara Pref.	TU
Shinchu	Kanagawa Pref.	TU
Sotoorishime (PA)	Akita & Yamagata	TU
Taihaku	Chiba Pref.	TU
Taihei	Kanagawa Pref.	TU
Tanponashi	Shimane Pref.	TU
Tosanishiki	Kochi Pref.	TU
Tsukushiinunashi	Kyushu Region	TU
Umajirou	Kochi Pref.	TU
Tottorijisei (UC)	Tottori Pref.	TU
Yokogoshi	Niigata Pref.	TU
Cultivars of *P. communis*		
Bartlett	England	TU
La France	France	TU
Passe Grassane	France	TU
Wild pears originating from east Asia		
P. ussuriensis	Northeast China	CPGR
P. pyrifolia[w]	South China	TU
P. betulaefolia-1	Northeast China	TU
P. betulaefolia-2	Gansu, China	GPI
P. betulaefolia-3	Ningxia, China	CPGR
P. betulaefolia-4[w]	Unknown, China	TU
P. hopeiensis	Hebei, China	CPGR
P. phaeocarpa	North China	TU
P. calleryana-1	South China,	TU
P. calleryana-2	Liaoning, China	CPGR
P. koehnei	South China, Taiwan	TU
P. fauriei	Korea	TU
P. hondoensis	Middle Japan	TU
P. aromatica	Northeastern Japan	TU
P. dimorphophylla-4	Mie Pref. Japan	TU
P. dimorphophylla-5	Mie Pref. Japan	TU
P. dimorphophylla-6	Mie Pref. Japan	TU

[z]Classification of species and cultivars originating from China is based on Pu et al. (1989), Pu and Wang (1963), and Yu (1979). Classification of species originating from Japan is based on Kikuchi (1948). UC = uncultivated and PA = *P. aromatica*.

[y]Geographic origin of cultivars in China is based on Pu et al. (1989), Pu and Wang (1963); those in Japan are based on Jang et al. (1992), Kajiura and Sato (1990), and Kikuchi (1948).

[x]Leaf sources are: CPGR = China Pear Germplasm Repository, Research Institute of Pomology, Chinese Academy of Agricultural Sciences; TU = Tottori University. The pear germplasm collection at Tottori University has been established mainly based on the extensive pear collection at the Fruit Tree Research Station, Ministry of Agriculture, Forestry and Fisheries, Tsukuba City, Ibaraki Prefecture, Japan. GPI = Gansu Pomology Institute, Gansu Academy of Agricultural Sciences, Gansu Province, China.

[w]Introduced from Oregon State Univ., Corvallis, Ore.

extraction buffer (0.1 M Tris-HCl pH 8.0, 50 mM EDTA pH 8.0, 0.5 M NaCl). Then, 1.0 mL of 20% sodium dodecyl sulfate (SDS) was added and mixed thoroughly by vigorous shaking. The extract was incubated at 70 °C for 15 min. The solution was then allowed to cool to about 25 °C, after which the extract was emulsified in one third volume of 5 M potassium acetate by gentle inversion, and incubated at 0 °C (on ice) for 20 min, followed by centrifugation at 4 °C and 21,400 *g*n for 30 min. The supernatant was poured into a clean 50-mL tube containing 1 volume of isopropanol and shaken gently at about 25 °C for about 30 min to precipitate the DNA. The DNA was hooked and put into a 15 mL tube (in most cases) or pelleted after a 5 min centrifugation at 1,300 *g*n. DNA was washed twice with 5 mL of 70% ethanol. The tube was air dried for 20 min. The DNA was redissolved in 1 to 3 mL Tris-EDTA buffer; then 2 to 5 µL of RNase (10 mg·mL^{-1}) was added, and the solution was incubated at 37 °C for 60 min and stored at 4 °C.

An aliquot of DNA-TE solution was added to a 1.5 mL tube and mixed with 1 volume of 25 phenol : 24 chloroform : 1 isoamylalcohol (PCI) (by volume) and centrifuged at 12,500 *g*n for 3 min. The upper phase was carefully transferred to a new 1.5 mL tube. This process was repeated tree to five times until the white layer disappeared between the aqueous phase and PCI phase. The sample was mixed with one tenth volume of 3 M sodium acetate and 2.5 volume of 100% ethanol and set at –80 °C for 20 min. The tube was centrifuged at 12,5000 *g*n for 20 min and drained. The pellet was washed with 70% ethanol, redissolved in TE buffer, and stored at –20 °C.

DNA concentration was determined by fluorometry using a fluorometer (DyNA Quant 200; Hoefer Scientific Instruments, San Francisco), following procedures supplied by the manufacturer. For use in PCR, the DNA was diluted with TE buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA) to 20 ng·µL^{-1} and stored at 4 °C until used for RAPD analysis.

DNA amplification. A series of optimization experiments were conducted in which concentrations of template DNA, primers, and *Taq* polymerase were varied to determine which conditions gave the strongest and most reproducible patterns. The repeatability of RAPD markers was tested by two methods: 1) each of two replicate DNA isolations from 'Nijisseiki', 'Yali', 'Pingguoli', and 'Tottorijisei' was used in a separated amplification reaction and 2) all reactions were repeated two or three times based on the results of the first kind of repeatability test.

The optimized reaction conditions for RAPD in this study are as follows: the PCR mixture contained 2.7 µL 10× gene *Taq* universal buffer (15 mM Mg^{2+}) (Nippon Gene Co., Ltd., Toyama, Japan), 2.0 µL dNTPs (2.5 mM each dNTPs) (Nippon Gene Co., Ltd., Toyama, Japan), 1 µL 20 pmol primer, 0.25 µL *Taq* DNA polymerase (5 units/µL) (Promega), 0.5 µL template DNA (20 ng·µL^{-1}), and 13.55 µL water. The total volume, 20 µL, was overlaid with two drops of mineral oil. PCR amplification was performed in a thermocycler (model 480; Perkin/Elmer, Norwalk, Conn.). The thermalcycler was programmed to predenature DNA for 5 min, denature DNA at 95 °C for 1 min, anneal DNA to primers at 37 °C for 1.5 min, and polymerize DNA for 2 min at 72 °C. After 45 cycles, the program allowed a final extension of 5 min at 72 °C before maintaining at 4 °C.

Initially, forty-six 10-mer primers, 20 from kit A and 26 from kit 26 (Operon Technologies, Alameda, Calif.) were screened using the DNA from 'Nijisseiki', 'Nanguoli', and 'Baozhuli'. Primer selection was based on the ability to generate complex amplification patterns. Primers listed in Table 2 proved to be useful for producing valuable RAPD markers.

Following amplification, the RAPD products (10 µL) were

Table 2. List of the primers used in the RAPD analysis, their sequence, number of scorable polymorphic bands, and bands specific to species or cultivar.

Primer	Sequence (5'-3')	Scorable polymorphic bands (no.)	No. of bands specific to species or cultivar[z]							
			W	Duli	Uss	Cal	Aro	Chi	Cili	K
OPA-07	GAAACGGGTG	11	0	0	0	0	0	0	1	0
OPA-09	GGGTAACGCC	21	1	1	0	1	0	1	0	0
OPA-10	GTGATCGCAG	4	0	0	0	0	0	0	0	0
OPA-11	CAATCGCCGT	3	1	0	0	0	0	0	0	0
OPA-12	TCGGCGATAG	21	4	1	1	0	0	0	0	0
OPA-16	AGCCAGCGAA	15	1	0	0	0	0	0	0	1
OPA-18	AGGTGACCGT	10	3	0	0	0	0	0	0	0
OPA-19	CAAACGTCGG	17	2	0	0	0	0	0	0	0
OPA-20	GTTGCGATCC	8	1	0	0	0	1	0	0	0
OP-26-02	TGGATTGGTC	11	1	0	1	0	0	0	0	1
OP-26-05	GGAACCAATC	14	2	0	0	0	0	0	0	1
OP-26-08	TGGTAAAGGG	15	2	1	2	0	0	0	0	0
OP-26-13	GTTTTCGCAG	14	1	0	0	0	0	0	0	0
OP-26-15	GATCCAGTAC	11	0	0	0	1	1	0	0	0
OP-26-16	GATCACGTAC	10	1	0	1	0	0	0	0	0
OP-26-18	GATCTCAGAC	5	1	0	0	1	0	0	0	0
OP-26-20	GATCAATCGC	15	1	0	0	0	0	0	0	0
OP-26-22	GATCGCATTG	15	1	1	1	0	1	0	0	0
OP-26-24	GATCATAGCC	14	2	1	0	0	1	0	0	0
OP-26-25	GATCTAAGGC	16	2	1	0	0	0	0	0	1

[z]W = *P. communis*, Duli = Chinese name of *P. betulaefolia*, Uss = *P. ussuriensis*, Cal = *P. calleryana*, Aro = *P. aromatica*, Chi = Chinese sand pear and white pear, Cili = Laiyang Cili, and K = Korean pear.

loaded in 1.5% or 2.0% agarose gels, stained with ethidium bromide in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0), and separated by electrophoresis at 85 to 90 V for ≈3 h, and photographed on an ultraviolet transilluminator. The sizes of amplification products were determined by comparison with Lambda (λ) DNA digested with *Eco*RI and *Hind*III restriction enzymes.

Only strong and reproducible bands were scored as present (1) or absent (0) for calculating the Dice coefficients (Nei and Li, 1979) of similarity. Ambiguities were scored as missing data. A dendrogram was constructed based on the similarity matrix data by using the unweighted pair-group method with arithmetic average (UPGMA), using the NTSYS-pc program (Rohlf, 1998).

Following Marquard et al. (1997), average similarities of some representative accessions to main species or taxonomic groups were calculated to help understand the genetic relatedness between each accession and taxa. Morphologically ambiguous accessions (e.g., 'Pingli') were excluded from the calculation of average similarities. Because 'Laiyangcili', 'Wowoli', and 'Enli' shared very high similarities (≥0.987) each other and may come from the same lineage (see Results and Discussion), only 'Laiyangcili' was included in the calculation of average similarities.

Results and Discussion

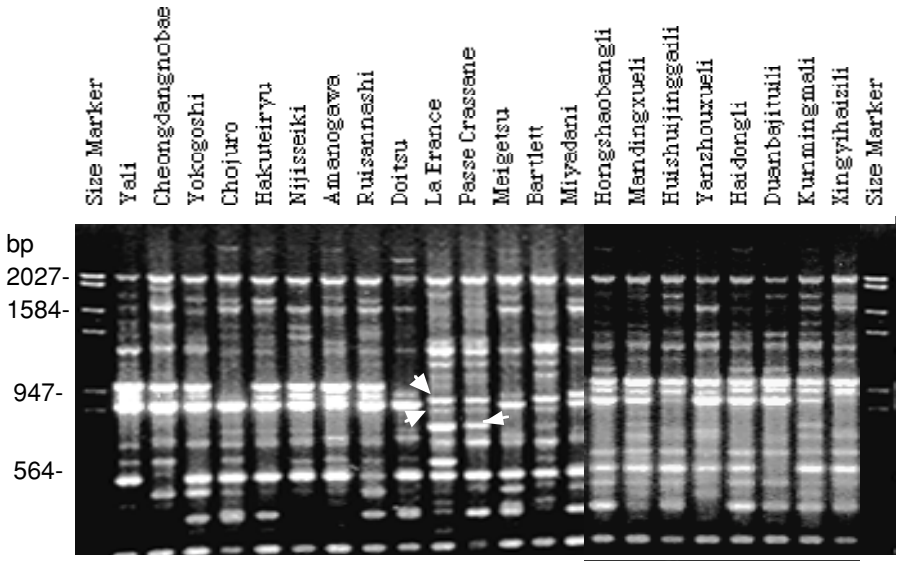
Characteristics of RAPD markers. Use of RAPD markers in some plants has resulted in poor levels of reproducibility. However, in this study, with careful optimization and strict control of PCR conditions, reproducible and bright RAPD bands were obtained (Fig. 1). Using 20 selected primers, 250 polymorphic RAPD markers were scored. The markers ranged in size from 300 to 2000 or rarely 2500 base pairs (bp), but were mostly 500 to 1600 bp. The number of markers scored for each primer ranged from 3 (OPA-11) to 21 (OPA-9 and -12), with an average of 12.5 per primer, which is in the scope reported by Oliveira et al. (1999) and Monte-Corvo et al. (2000) in separate studies related to pear identification using RAPD markers.

Certain amplified bands were found to be specific to a given species, i.e., they were present in (or absent from) only one species but absent from (or present in) the remaining species. These bands could be used for species identification (Table 2, Fig. 1). The bands specific to *P. communis* were observed in RAPD profiles with most primers and bands specific to *P. betulaefolia*, *P. ussuriensis* (including *P. hondoensis*), *P. aromatica*, *P. calleryana* (including *P. koehnei*, *P. fauriei*, and *P. dimorphophylla*), and some pear cultivars from the Korean Peninsula occurred with only some primers. Using primer OPA-9, the unique band near 400 bp was observed for 18 Chinese cultivars of sand pears and white pears (42% of total), which infers that both Chinese white pear and sand pear cultivars share a common progenitor species. However, this specific band was not found in the cultivars of Japanese pears used in this study. A cultivar specific band was found in 'Laiyang Cili' and its nearest relatives.

Genetic relationships among and within *Pyrus* species. Similarity values of accessions, estimated by Dice's coefficient (Nei and Li, 1979), ranged from 0.284 for *P. dimorphophylla*-1 and 'La France' to 0.993 for 'Enli' and 'Wowoli'. Species and cultivars native to east Asia had the lowest affinity to the cultivars of *P. communis* (Table 3). The accessions, on the other hand, generally had the highest affinity to the taxa to which they have been assigned, based on morphological traits. However, the boundary of Chinese white pear and sand pear was ambiguous (Table 3). The dendrogram resulting from the UPGMA cluster analysis is shown in Fig. 2. The dendrogram clearly separated all accessions into two divisions at the 0.37 level of similarity. The first division included all accessions of pears native to east Asia and was divided further into 11 major groups, and the second division was formed by a single group of three cultivars of *P. communis*, which is in agreement with the studies of Kawata et al. (1995), Iketani et al. (1998), Oliveira et al. (1999), and Monte-Corvo et al. (2000), who also divided *Pyrus* into the occidental group and the oriental group using RFLP or RAPD markers. These results support the traditional view that genus *Pyrus* consists of two geographic species groups: Occidental pears and Oriental pears (Layne and Quamme, 1975; Lee, 1948; Rubtsov, 1944).

Pea pears are endemic to east Asia and characterized by their small fruit with a diameter of ≈1 cm. In this study, they were separated into two main groups: *P. calleryana* group (Group I) and *P. betulaefolia* group (Group XI) (Fig. 2). Species in both groups had distant affinities to the large-fruited species (Table 3). Group I included *P. calleryana* and its relatives. These species shared the same 3 species-specific RAPD markers (Table 2). Because they have some resemblance to each other morphologically, these pea pears were treated as varieties of *P. calleryana* by Rehder (1940). In this group, *P. calleryana*-1 and *P. koehnei* branched at a similarity of 0.750 and had a close affinity, which is related with their geographic origins (Table 1). Yu (1979) classified *P. koehnei* as *P. calleryana* Dcne. var. *koehnei* (Schneid.) Yu. In addition, the *P. fauriei* clone and the *P. dimorphophylla* clone showed distant relationships with the *P. calleryana*, which supports the view that *P. fauriei* and *P. dimorphophylla* should be treated as independent species (Challice and Westwood, 1973;

Fig. 1. RAPD profiles for the 22 pear cultivars using primer OPA-18. Arrows indicate markers specific to *P. communis*.



Westwood, 1968). Genetic polymorphism was observed within *P. dimorphophylla* (Fig. 2), which reflects the observed morphological polymorphism.

Pyrus betulaefolia is another pea pear species important for its extensive use as a pear rootstock in east Asia. It is distributed from east to west in North China (Pu and Wang, 1963; Yu, 1979). Samples from different regions showed some genetic variation (Fig. 2). *Pyrus betulaefolia* clustered together with *P.* ×*hopeiensis* and *P.* ×*phaeocapa* (Group XI) (Fig. 2). *Pyrus* ×*phaeocarpa* was presumed to be a hybrid between *P. betulaefolia* and *P. ussuriensis* (Challice and Westwood, 1973; Yu, 1979; Yu and Kuan, 1963), and the origin of *P.* ×*hopeiensis* may involve *P. betulaefolia* and *P.* ×*phaeocarpa* (Yu, 1979; Yu and Kuan, 1963). *Pyrus* ×*hopeiensis* and *P.* ×*phaeocarpa* were found to share some markers with *P. betulaefolia* and *P. ussuriensis* (Table 2). These data support the hypothesis that *P. betulaefolia* and *P. ussuriensis* are involved in the ancestry of *P.* ×*hopeiensis* and *P.* ×*phaeocarpa*.

'Pingguoli' (apple-like pear) is one of the leading cultivars in China. The origin and classification of this cultivar are obscure. It is said that this cultivar was introduced into Jilin Province, China, from North Korea in 1921. In 1998, three stock trees of this cultivar were still alive in China (Teng, unpublished data). In the literature, 'Pingguoli' has been assigned either to *P.* ×*bretschneideri* (Pieniazek, 1967; Pu et al., 1989) or to *P. pyrifolia* (Pu and Wang, 1963; Yu, 1979; Zou et al., 1986), and is believed to be a hybrid between *P. pyrifolia* and *P. ussuriensis* (Pu and Wang, 1963). In the present study, it clustered together with some cultivars native to Korea (Group II) (Fig. 2), which genetically supports a geographic origin in North Korea. Cultivars of this group shared some common RAPD bands with *P. ussuriensis*, which infers that they have some relationships with *P. ussuriensis*. Markers specific only to these cultivars were found with some of the primers (Table 2). On the other hand, this group branched distantly from the majority of Chinese white pears or sand pears and japanese pears (Fig. 2) and had low affinities to other Asian large-sized pears (Table 3). Morphologically and physiologically, 'Pingguoli' belongs to neither typical *P. pyrifolia* (or *P.* ×*bretschneideri*) nor typical *P. ussuriensis* (Pu and Wang, 1963; Zou et al., 1986). Zou et al. (1986) reported that the round pollen grains of 'Pingguoli', which are different from those of other pear

Table 3. Average similarity (affinity) of some pear accessions to the taxonomic groups of genus *Pyrus*. Averages were computed from the genetic similarity (Dice coefficient; Nei and Li, 1979) matrix.

		Average affinity to									
Species	Accession	*P. betulaefolia*	*P. calleryana*	Chinese sand pear	Japanese pear	Chinese white pear	*P. ussuriensis*	*P. hondoensis*	*P. aromatica*	Korean pear	*P. communis*
P. betulaefolia	*P. betulaefolia*-1	0.750	0.470	0.463	0.420	0.432	0.420	0.477	0.440	0.400	0.330
P. phaeocarpa	*P. phaeocarpa*	0.620	0.570	0.570	0.545	0.600	0.547	0.467	0.530	0.550	0.320
P. fauriei	*P. fauriei*	0.480	0.570	0.529	0.496	0.539	0.510	0.504	0.540	0.490	0.310
P. dimorphophylla	*P. dimorphophylla*-5	0.520	0.660	0.507	0.475	0.527	0.470	0.492	0.500	0.520	0.300
P. calleryana	*P. calleryana*-1	0.594	0.826	0.560	0.545	0.570	0.509	0.522	0.548	0.510	0.356
P. koehnei	*P. koehnei*	0.528	0.690	0.565	0.539	0.573	0.512	0.504	0.553	0.537	0.371
P. hondoensis	*P. hondoensis*	0.470	0.492	0.503	0.472	0.524	0.589	1.000	0.569	0.539	0.366
P. aromatica	*'Iwatemukaku'*	0.495	0.566	0.630	0.602	0.637	0.586	0.597	0.702	0.642	0.389
P. ussuriensis	'Nanguoli'	0.448	0.509	0.588	0.558	0.608	0.616	0.642	0.624	0.594	0.379
Chinese white pear	'Lataili'	0.524	0.616	0.738	0.695	0.729	0.621	0.556	0.671	0.645	0.358
	'Laiyangcili'	0.476	0.515	0.665	0.630	0.709	0.532	0.522	0.616	0.596	0.365
	'Jinhuali'	0.479	0.579	0.739	0.658	0.736	0.548	0.519	0.622	0.495	0.318
	'Yali'	0.468	0.555	0.694	0.676	0.706	0.552	0.544	0.651	0.628	0.407
Korean pear	'Pingguoli'	0.474	0.496	0.618	0.582	0.617	0.580	0.510	0.601	0.732	0.396
P. pyrifolia	*P. pyrifolia*	0.480	0.610	0.679	0.653	0.682	0.560	0.518	0.670	0.630	0.390
from China	'Cangwudashli'	0.442	0.523	0.674	0.668	0.688	0.565	0.479	0.624	0.613	0.338
	'Cangxixuli'	0.520	0.576	0.661	0.631	0.645	0.537	0.439	0.607	0.603	0.400
	'Hongfenli'	0.446	0.533	0.632	0.581	0.622	0.493	0.417	0.546	0.575	0.342
	'Huishuijingaili'	0.441	0.563	0.703	0.672	0.728	0.566	0.497	0.652	0.617	0.385
	'Qianxiandashali'	0.446	0.532	0.679	0.673	0.702	0.535	0.540	0.630	0.607	0.415
	'Yiwulizi'	0.467	0.579	0.708	0.704	0.712	0.601	0.559	0.662	0.632	0.399
from Korea	'Hanheungli-Ots'	0.460	0.547	0.658	0.687	0.690	0.591	0.541	0.637	0.581	0.363
from Japan	'Amanogawa'	0.465	0.539	0.680	0.702	0.692	0.586	0.489	0.628	0.583	0.361
	'Chojuro'	0.400	0.470	0.630	0.716	0.630	0.540	0.482	0.620	0.560	0.330
	'Imamuraaki'	0.423	0.500	0.665	0.725	0.692	0.543	0.466	0.625	0.580	0.366
	'Inugoroshi'	0.476	0.574	0.691	0.746	0.704	0.560	0.493	0.656	0.588	0.394
	'Kosainashi'	0.448	0.594	0.677	0.691	0.686	0.559	0.534	0.663	0.609	0.343
	'Nijisseiki'	0.416	0.521	0.636	0.723	0.660	0.527	0.466	0.593	0.577	0.369
	'Shimoichikoboku'	0.468	0.590	0.676	0.668	0.676	0.577	0.517	0.644	0.637	0.368
	'Tanponashi'	0.455	0.530	0.670	0.742	0.688	0.549	0.481	0.634	0.575	0.365
	'Tosanishiki'	0.470	0.602	0.675	0.714	0.674	0.546	0.531	0.637	0.604	0.386
	'Tsukushiinunashi'	0.435	0.530	0.650	0.675	0.670	0.611	0.504	0.634	0.625	0.373
	'Umajiro'	0.429	0.497	0.619	0.628	0.618	0.521	0.489	0.588	0.544	0.374
P. communis	'La France'	0.320	0.344	0.381	0.357	0.354	0.336	0.354	0.388	0.372	0.707

species, could be transmitted to its progenies. Based on the above facts, 'Pingguoli' and other cultivars in Group II should be treated as an independent species. Here, the name 'Korean pear' is suggested to represent those pears tentatively.

'Beijingbaili', 'Nanguoli', and 'Jianbali', well-known cultivars of *P. ussuriensis*, clustered together with *P. hondoensis* (Group X), which suggests a close relationship between these two species. Moreover, RAPD markers specific to *P. ussuriensis* were also present in *P. hondoensis* (Table 2). Based on morphological traits, *P. hondoensis* was once classified as a variety of *P. ussuriensis*. On the other hand, these Ussurian cultivars grouped separately from wild *P. ussuriensis* (Group III). A similar result was reported by Kajiura et al. (1983), who found that flavonol aglycone (a kind of flavonoid) existed in wild *P. ussuriensis* and a majority of its cultivars, but not in 'Beijingbaili' and 'Nanguoli', and proposed that the origin of these two latter cultivars may involve hybridization with other pears in China. It is interestingly noted that 'Pingli' and 'Hongxiaoli', morphologically classified as *P.* ×*bretschneideri*, clustered together with *P. ussuriensis* (Group III). They may be hybrid cultivars involving *P. ussuriensis*, because some markers specific to *P. ussuriensis* were also observed in RAPD profiles for 'Pingli' and 'Hongxiaoli'.

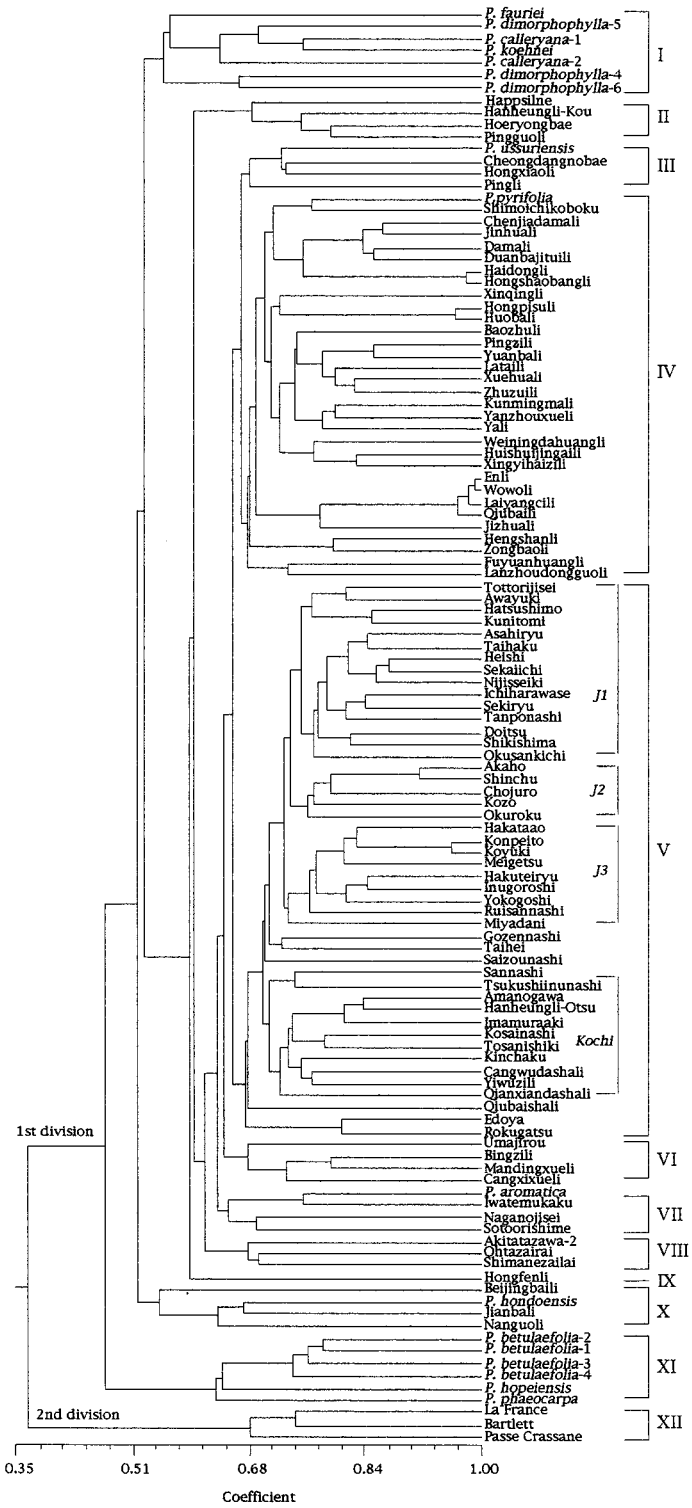


Fig. 2. Dendrogram of 118 pear species and cultivars resulting from UPGMA cluster analysis based on Dice's similarity coefficient (Nei and Li, 1979).

Pyrus aromatica grows wild in Iwate, Aomori, and Akita Prefecture, Japan (Kikuchi, 1948). It clustered together with its cultivars 'Iwatemukaku' and 'Sotoorishime', and an unidentified accession, 'Naganojisei' (Group VII in Fig. 2). 'Sotoorishime' bears fruit with smooth skin (green), which is different from the russet fruit of wild *P. aromatica* and 'Iwatemukaku'. Genetically, 'Sotoorishime' was closer to Japanese pears than the two latter types (data not presented), which may infer that this cultivar is not a pure cultivar of *P. aromatica*, but a hybrid with *P. pyrifolia*. Cultivars in this group shared some RAPD markers with *P. ussuriensis* or *P. hondoensis*, but also had their own species-specific RAPD markers (Table 2).

Group IV is comprised of Chinese white pears and sand pears, including wild *P. pyrifolia*, and 'Shimoichikoboku', a semicultivated pear clone grown in Nara Prefecture in Japan (Fig. 2). It should be noted that 'Shimoichikoboku' clustered together with wild *P. pyrifolia* native to China, and showed the closest affinity to the latter (a similarity of 0.763), rather than other Japanese accessions. Taking into consideration the flourishing trade and cultural exchanges between Nara and China during ancient times, it can be hypothesized that 'Shimoichikobuku' or its progenitors were probably introduced from ancient China. If this accession is native to Nara Prefecture of Japan, the present result would mean that wild *P. pyrifolia* in both China and Japan is identical. Surprisingly, in this group Chinese white pears and sand pears did not cluster further into separate subgroups based on their presumed taxa (Table 1). In contrast, the subgroup usually was formed by a combination of Chinese white pears and sand pears. This clustering could very well represent the true genetic relationship of those clones to each other, as it is based on a rather large number of molecular markers that directly reflect genetic differences at the DNA level. Accessions of Chinese white pears generally had the same affinities to the *P. pyrifolia* group as to Chinese white pear group (Table 3). Previous studies have indicated that Chinese white pears resemble Chinese sand pears in both leaf morphology and fruit texture (Kikuchi, 1948; Pu and Wang, 1963; Yu, 1979), and peroxidase isozymic patterns (Lin and Shen, 1983). All of these data indicate that wild *P. pyrifolia* should be a common progenitor species of both Chinese sand pear and white pear cultivars. Their common RAPD markers (Table 2) further confirmed a close relationship between Chinese white pears and sand pears.

We did not find any RAPD markers specific to *P. betulaefolia* that were present in any accessions of Chinese white pears. This finding does not support the view that *P. betulaefolia* is one of the progenitor species of Chinese white pear (Challice and Westwood, 1973). Except for 'Hongxiaoli' and 'Pingli', cultivars native to the north part of Hebei Province, China, and which are hybrids

involving *P. ussuriensis* (Table 2, Fig. 2), the other accessions of Chinese white pears used in this study were not found to share species-specific RAPD markers with *P. ussuriensis*. It may be appropriate to conclude that the majority of Chinese white pear cultivars originated directly from *P. pyrifolia*. In China, *P. pyrifolia* arose in the Changjiang River valley and bears fruit with either russet skin or smooth green or yellow skin (Kikuchi, 1948; Pu and Wang, 1963; Yu, 1979). The results presented above would infer that through borealization, some *P. pyrifolia* (very probably those with smooth fruit skin) have acquired winter hardiness and evolved as new ecotypes, from which cultivars of Chinese white pears have arisen. Taxonomists usually can not classify distinctly the cultivars of Chinese pears with smooth fruit skin, which are located in the geographic zone of overlap between sand pears and white pears. This is another reason to believe that our inference about the origin of Chinese white pears is reasonable. In the northern part of Hebei Province and southern Liaoning Province, where the distribution of *P. ussuriensis* and white pears overlap, some white pears have hybridized with *P. ussuriensis* and formed types that closely resemble *P. ussuriensis*. So-called wild *P. ×bretschneideri* grown in northern Hebei Province most probably has no relationship with Chinese white pear cultivars prevailing in North China, because morphological traits of Chinese white pear cultivars differ very much from those of so-called wild *P. ×bretschneideri*, according to the description of Kikuchi (1946). Until recently, boundaries of cultivated species of *Pyrus* originating from China were poorly understood by researchers external to China. As a result, species names have been misapplied in the taxonomic and agronomic literature. Different authors have often described similar and/or identical genotypes under different names. Based on the above facts, we assign cultivars of Chinese white pears a new name: *P. pyrifolia* var. *sinensis* (Lindley) Teng et Tanabe, which reflects the status of Chinese white pears more exactly than does *P. ussuriensis* var. *sinensis* Kikuchi.

Data from group IV show a close relationship between some pear cultivars that originated in the same geographic location. 'Enli', 'Wowoli' and 'Laiyangcili', which originated in Shandong Province in China were tightly subclustered together with 'Qiubaili', a cultivar grown in Hebei Province, where is adjacent to Shandong Province, and distantly related to the large main group of Chinese white pears and sand pears. 'Enli', 'Wowoli', 'Laiyangcili', and 'Qiubaili' share a common RAPD band (Table 2). Morphologically, they have specific characteristics, such as fruit skin covered with many large russetted lenticels, and thick coriaceous leaves, which make them clearly distinguishable from other white pears (Kikuchi, 1948). These morphological and genetic results suggest a common lineage among these cultivars. 'Chenjiadamali' and other cultivars, all native to Sichuan Province (Table 1), subclustered closely. 'Hengshanli', a cultivar extensively grown in Taiwan, which is said to have been introduced from southern China (Lin et al., 1991), subclustered with 'Zongbaoli' (similarity of 0.792), which is native to Fujian Province.

Most Japanese pear cultivars fell into Group V (Fig. 2). Among all accessions of Japanese pears, 'Kopeito' and 'Koyuki', which originated from different regions (Table 1), had the highest affinity, with a similarity of 0.957. This finding is consistent with the result from an isozyme analysis in a separate study (Jang, 1992), that reported the same pattern of peroxidase isozymes for these two cultivars. These results suggest that these two cultivars have a relatively similar genetic background, and may have arisen in the same region. 'Nijisseiki', which originated in Chiba Prefecture, is one of the most well-known Japanese cultivars. It had the highest affinity (similarity of 0.859) to 'Heishi', a cultivar originating from the Kanto Region, and clustered with 'Sekaiichi' and 'Taihaku', also from the Kanto region. Previous research has suggested that Japanese cultivars native to the Kanto region, including 'Nijisseiki', may be related genetically to *P. hondoensis* (Kawata et al., 1995) or *P. aromatica* (Kajiura et al., 1983). However, we did not find RAPD markers from *P. hondoensis* or *P. aromatica* that were present in 'Nijisseiki' or other cultivars of Japanese pears (Table 2).

Within Group V, several subclusters were identified (Fig. 2). Some cultivars subclustered according to their origin. 'Akaho', 'Shinchun', Chojuro', 'Kouzou', 'Okuroku', and 'Taihei' native to Kanagawa Prefecture clustered together (J2 subgroup). Most cultivars from Kochi Prefecture also subclustered. However, most others did not subcluster according to their geographic distribution. For those cultivars native to Kanagawa Prefecture, it appears likely that there was relatively little widespread movement before recent times. For other Japanese pear cultivars, widespread movement would have been more common.

It was said that wild *P. pyrifolia* was once distributed in the Shikoku and southern Kyushu regions of Japan, where the climate was warmer than in other areas (Kikuchi, 1948). However, cultivation of Japanese pears flourished in Niigata and Gunma Prefectures in North Japan. Most of the native cultivars of Japanese pears originated from Kanagawa, Niigata and Chiba Prefectures, which is incongruent with distribution of wild Japanese pears (Kikuchi, 1948). If cultivars of Japanese pears have been derived from wild *P. pyrifolia* grown in Japan, primitive cultivars in these areas should have been introduced from Kochi Prefecture and other areas where wild *P. pyrifolia* grows. In this study, some accessions from Kochi Prefecture and 'Tsukushiinunashi' from the Kyushu region subclustered in Kochi subgroup (Fig. 2). 'Ichiharawasa' from Kochi Prefecture was included in the J1 subgroup. Some accessions native to areas outside of Kochi Prefecture, such as 'Taponashi' in the J1 subgroup, and 'Inugoroshi' in the J3 subgroup, shared high similarities with some cultivars in the Kochi subgroup (data not presented). These data suggest that germplasm native to Kochi Prefecture is related to other populations of Japanese pear cultivars.

Most accessions of Japanese pears showed much higher affinities to other cultivars of the same taxa (Japanese pear group) than to cultivars of Chinese sand pears, although both kinds of pears belong to the same species, *P. pyrifolia* (Table 3). Results of our study suggest that intrapopulation genetic variation within Japanese pears is smaller than that within Chinese sand pears, which supports the view of Kikuchi (1948) that Japanese pears are genetically more homogeneous than Chinese pears.

Three of the five cultivars native to Kochi Prefecture were clustered with some Chinese sand pear cultivars and one Korean sand pear cultivar (Fig. 2). These Chinese sand pear cultivars showed similar affinities to both the Chinese sand pear group and the Japanese pear group (Table 3). In addition, 'Umajiro', a cultivar originating from Kochi, clustered into Group VI with Chinese sand pears, two cultivars from Fujian Province, and one cultivars from Sichuan Province. These results suggest that at least some old cultivars of Japanese pears may have been introduced from ancient China or Korea. To clarify relationships among cultivars of *P. pyrifolia* grown in China, Korea, and Japan, further studies will be needed using large samples from Korea,

and from Zhejiang and Fujian Provinces of China.

'Akitatazawa-2', 'Ohtazairei', and 'Shimanezailai' fell outside the main group of Japanese pears and formed an independent group (Group VIII). These three cultivars had distant affinities to other Japanese pear cultivars (Fig. 2). Some RAPD markers common to *P. communis* were also found in 'Shimanezailai', which means this cultivar may be a hybrid between Japanese pear and *P. communis*.

'Hongfenli', a Chinese sand pear from Guizhou Province was found to be distantly related to other cultivars of *P. pyrifolia* from both China and Japan (Table 3) and clustered independently as Group IX (Fig. 2), which reflects its genetic uniqueness from other cultivars of *P. pyrifolia*. For this reason, it may be a useful source of genetic diversity.

In summary, results herein indicate that the RAPD technique is useful in distinguishing species and cultivars of the genus *Pyrus*. RAPD markers specific to species or cultivar were identified. The grouping of the species and cultivars based on RAPD data agrees to a large extent with pear taxonomy based on morphological traits. New findings from this study will help to establish correct phylogenetic relationships in the genus *Pyrus* native to east Asia and to clarify the origin of large-fruited species of *Pyrus*, especially Chinese white pears and Japanese pears.

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