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ORIGINAL PAPER

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Molecular markers derived from RAPD, SCAR, and the conserved 18S rDNA sequences for classification and identification in *Pyrus pyrifolia* and *P. communis*

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Abstract We generated RAPD, SCAR, and conserved 18S rDNA markers for classifying and identifying cultivars of *Pyrus pyrifolia* (Japanese pear) and *P. communis* (European pear). PCR amplification with selected specific primers—LCH327UP and LCH327DOWN—was performed using DNA extracted from 25 *P. pyrifolia* and *P. communis* cultivars. The 1,380-bp fragment was amplified from *P. communis* cvs. Beurre Giffard, Cascade, Conference, Clapp's Favorite, Packhams Triumph, and Winter Nelis. RAPD has only a dominant single band of 1,380-bp, however, SCAR has one or more band of the same size. Amplification involving sequence-specific primer pairs LCH346UP and LCH346DOWN resulted in a loss of polymorphism. The 1,190-bp fragment was amplified from all *P. pyrifolia* cultivars. The conserved sequences of the 18S rDNA fragment of 25 pear cultivars were amplified and analyzed with 42 restriction enzymes. Compared with *P. pyrifolia* cultivars, they lacked the restriction enzyme site of *Kpn*I and had one less *Rsa*I site. Cultivar Gamcheonbae had a specific *Pst*I restriction site, while cvs. Mansoo and Conference pear digested with *Alu*I showed a different presentation than other cultivars. For the Okusankichi and Shinil pears *Taq*I was best marker for identification in *P. pyrifolia*. These results can be adopted for identifying pear cultivars; to date there is no standard marker for identifying the cultivars of fruit trees in Korean fruit tree breeding programs.

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Introduction

Random amplified polymorphic DNA (RAPD) markers have been used for identification purposes and for the study of genetic relationships in apple (Koller et al. 1993), plum (Ortiz et al. 1997), and grapes (Lee et al. 1998). However, the sensitivity of RAPD banding patterns to reaction conditions makes them less useful markers for the routine analysis of large numbers of plants. Recently, a more reliable and specific PCR-based marker known as sequence characterized amplified regions (SCARs) has been developed. SCAR primers are longer than RAPD primers, and high annealing temperatures can be used to ensure specific priming (Paran and Michelmore 1993). Kesseli et al. (1994) studied the application of targeted and randomly generated markers (RFLP, RAPD, SCAR, AFLP and SSR) to crop breeding programs and the suitability of these markers in forest genetic research. In *Pyrus* species, both restriction fragment length polymorphism (RFLP; Teramoto et al. 1994) and RAPD markers (Oliveira et al. 1999) have been used for molecular typing. Kim et al. (2000) reported the generation of SCAR markers derived from RAPD markers for identification purposes in Japanese pear (*Pyrus pyrifolia*).

Sequences of fragments of the rRNA genes and intergenic spacers have led to new insights into the phylogeny of plants (Dubouzet and Shinoda 1999; Baldwin and Markos 1998) based on very extensive data, and the markers have proved valuable in species identification. Lee et al. (2001) compared the putative grafting affinity of grape using RAPD and 18S rDNA sequences for genetic linkage analysis.

The objective of the investigation reported here was to generate RAPD, SCAR, and 18S rDNA markers that can be used for classifying and identifying the cultivars of *P. pyrifolia* and *P. communis*.

Materials and methods

Plant materials

Pears were sampled from late June through to the first week of August in 1996 and 1997 from the field at the National Horticultural Research Institute, Suwon, Republic of Korea. The plant genotypes used for this study were 19 *Pyrus pyrifolia* and six *P. communis* cultivars. The pear cultivars and their origins are shown in Table 1. DNA was extracted using precipitation with polyvinylpyrrolidone (PVP) and salt followed by chloroform purification (Kim et al. 1997).

For RAPDs, each pear DNA RAPD reaction mixture [20 m*M* Tris-HCl (pH 8.0), 50 m*M* KCl, 0.1% Triton X-100, 37.5 μg/ml BSA (bovine serum albumin) and 1.5 m*M* $MgCl_2$] contained 0.018 U/μl DNA polymerase (AB *Taq* DNA polymerase; ABGENE, UK), 100 μM dNTP mix (dATP, TTP, dGTP, and dCTP), 0.2 μM primer, and approximately 10–20 ng total DNA in a final volume of 20 μl. The reaction mixture was overlaid with mineral oil (Sigma, St. Louis, Mo.), mixed, and centrifuged for 2 min at 2×10^3 *g*. The RAPD primers were obtained from the University of British Columbia (UBC; Vancouver, B.C.) and selected using the results of profiles based on the molecular phylogenetic tree (Kim et al. 2000a). Amplification of pear genomic DNA was performed in a PCR system PTC-100 thermal controller (MJ Research, Waltham, Mass.) programmed for one cycle of 5 min at 95°C (initial denaturation); 45 cycles of 15 s at 94°C (denaturation), 60 s at 37°C (annealing), 90 s at 72°C (extension); one final cycle of 10 min at 72°C (final extension). Reaction products were resolved by electrophoresis on 1.4% agarose gels. Each RAPD PCR analysis was repeated at least twice.

Construction of SCAR markers

Taq DNA polymerase-generated RAPD fragments were cloned and sequenced. Sequence-specific primers were designed by adding 8–15 bases to the 3′ and 5′ end of the original 10-mers in order to amplify SCARs, as described by Paran and Michelmore (1993).

Analysis of the conserved 18S rDNA

The conserved 18S rDNA sequence was amplified in 25 μl of reaction solution mixture [10 m*M* Tris-HCl (pH 8.3), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.2 m*M* each of dATP, dGTP, dCTP, and TTP, and 1.25 U *Taq* DNA polymerase (Takara Shuzo, Kyoto)] together with the templates of the plant DNA (0.2 μg) and the primers of the 18S rDNA (0.25 μM each). The primers used for PCR amplification and sequencing of the conserved 18S rDNA sequences are GenBank accession nos. AJ242597 (5′-GACTGTGAAACTGCGAATGG-3′) and AJ242598 (5′-TAAGTTTCAGCCTTGCGACC-3′). The PCR amplifications consisted of a preincubation at 95°C for 5 min followed by 35 cycles of 30 s at 94°C, 1 min at 55°C, and 2 min at 72°C. The PCR fragments were cloned directly into pGEM-T Easy vector using a TA cloning kit (Promega, Madison, Wis.) and plasmids digested with *Eco*RI to confirm the target DNA insertion. Sequence analysis was performed using DNASTAR (IBM PC) and BLAST (NCBI) programs.

Table 1 Cultivar name, scientific name, and origin of the 25 pear (*Pyrus pyrifolia* and *P. communis*) accessions used for RAPD analysis and analysis of the conserved sequences of 18S rDNA and the size, accession number, number of cutting enzymes, and site of the conserved 18S rDNA sequence in the pear cultivars

Number[a]	Cultivar name	Scientific name	Origin	The conserved sequence size of the 18S rDNA (bp)	GenBank accession no.	Number of cutting enzymes	Number of cutting sitess
1	Choju	*Pyrus pyrifolia*	Uncertain	1,063	AF179382	31	82
2	Chojuro	*P. pyrifolia*	Chance seedling	1,063	AF179383	33	83
3	Chuwhangbae	*P. pyrifolia*	Imamuraaki × Nijisseiki	1,065	AF179384	32	81
4	Gamcheonbae	*P. pyrifolia*	Okusankichi × Danbae	1,064	AF179385	33	84
5	Hosui	*P. pyrifolia*	(Kikusui × Yakumo) × Yakumo	1,066	AF179386	32	81
6	Imamuraaki	*P. pyrifolia*	Unknown	1,063	AF179387	32	82
7	Kimitzukawase	*P. pyrifolia*	Shinkozo × Doitsu	1,063	AF179388	32	82
8	Kosui	*P. pyrifolia*	Kikusui × Wasekozo	1,062	AF179389	31	81
9	Mansoo	*P. pyrifolia*	Danbae × Okusankichi	1,063	AF179390	33	84
10	Minibae	*P. pyrifolia*	Danbae × Kosui	1,061	AF179391	32	82
11	Miwhang	*P. pyrifolia*	Hosui × Okusankichi	1,063	AF179392	31	81
12	Niitaka	*P. pyrifolia*	Amanogawa × Imamuraaki	1,063	AF179393	32	85
13	Nijisseiki	*P. pyrifolia*	Chance seedling	1,063	AF179394	32	82
14	Okusankichi	*P. pyrifolia*	Seedling of cv. Wasesankichi	1,063	AF179395	32	83
15	Shinil	*P. pyrifolia*	Shinko × Hosui	1,062	AF179396	33	85
16	Shinsui	*P. pyrifolia*	Kikusui × Kimitzukawase	1,062	AF179397	32	81
17	Sunwhang	*P. pyrifolia*	Niitaka × Okusankichi	1,063	AF179398	32	80
18	Whangkeumbae	*P. pyrifolia*	Niitaka × Nijisseiki	1,063	AF179399	32	82
19	Yeoungsanbae	*P. pyrifolia*	Niitaka × Danbae	1,062	AF179400	32	82
20	Beurre Giffard	*P. communis*	Chance seedling	1,063	AF195617	31	80
21	Cascade	*P. communis*	Max Red Bartlett x Comice	1,063	AF195618	31	80
22	Conference	*P. communis*	Seedling of cv. Leon Leclerc de Laval	1,063	AF195619	36	74
23	Clapp's Favorite	*P. communis*	Flemish Beauty x Bartlett	1,063	AF195620	31	81
24	Packhams Triumph	*P. communis*	Uvedale St. Germain x Bartlett	1,063	AF195621	30	80
25	Winter Nelis	*P. communis*	Seedling selection	1,063	AF195622	31	80

[a] Accession numbers of pear cultivars by their scientific names and alphabetical order

Results

Systematic classification using markers by RAPD and SCAR

RAPD amplification of DNA from 25 accessions of two *Pyrus* species showed species-specific fragments as well as intraspecific polymorphisms (Figs. 1A, 2A). Two species-specific fragments were sequenced and 18- and 24-mer SCAR primers were designed. With the LCH327 SCAR primer pair [LCH327UP: 5′-ATACGGCGTCATGCCTTATTGCTTC-3′(T_m: 65°C); LCH327DOWN: 5′-ATACGGCGTCTAGCATTTTTATTTT-3′ (T_m: 65°C)] no product was found from *P. pyrifolia*, but a 1,380-bp fragment was amplified in all six *P. communis* cultivars. A comparison of RAPD and SCAR markers (Figs. 1, 2) showed some differences between the profiles with RAPD showing a dominant single band of 1,380 bp while SCAR had one or two bands.

Amplification involving primer pairs LCH346UP (5′-TAGGCGAACGGAAAAATTTTTCATA-3′, T_m: 65°C) and LCH346DOWN (5′-TAGGCGAACGATCTAT-AAAAAAAAG-3′, T_m: 65°C) was designed to amplify a species-specific RAPD fragment in *P. pyrifolia*. No amplification was obtained in the *P. communis* accessions, but a 1,190-bp fragment was recovered in all 19 *P. pyrifolia* cultivars. BLAST analysis showed that the amplified band obtained with the LCH346 SCAR primer pairs has a high homology (85–95%) with chloroplast DNA in other plants.

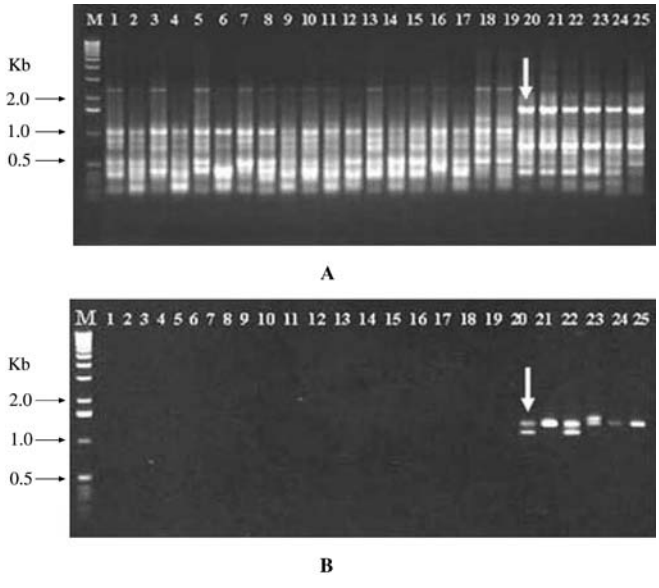

Fig. 1A, B Systematic classification of SCAR markers for 19 *Pyrus pyrifolia* and six *P. communis* cultivars obtained with the LCH327UP and LCH327DOWN primers. The 1,380-bp fragments were recovered in *P. communis* cultivars. The *arrow* indicates the location of the RAPD band. *Numbers above lanes* correspond to those in Table 1 (cultivar number). *M* 1-kb DNA ladder (GIBCO-BRL, Gaithersdurg, Md.). **A** RAPD profiles obtained with UBC 327 primer, **B** SCAR band obtained with LCH327UP and LCH327DOWN primers

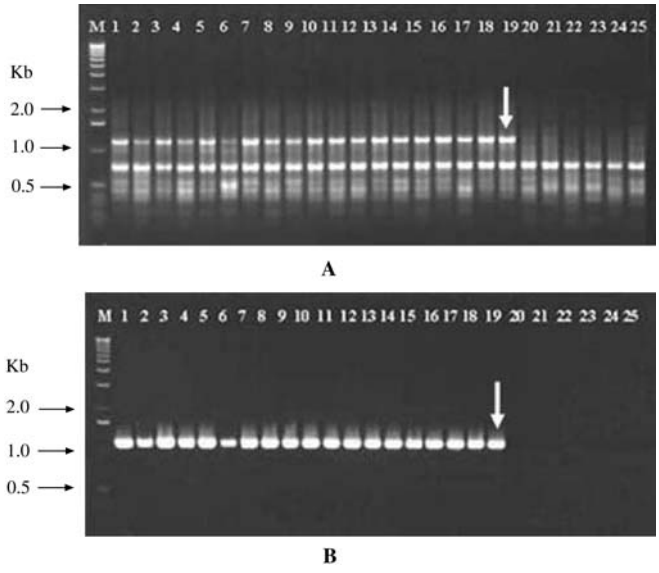

Fig. 2A, B Systematic classification of SCAR markers for 19 *P. pyrifolia* and six *P. communis* cultivars obtained with the LCH346UP and LCH346DOWN primers. The 1,190-bp fragments were recovered in *P. pyrifolia* cultivars. The *arrow* indicates the location of the RAPD band. *Numbers above lanes* correspond to those in Table 1 (cultivar number). *M* 1-kb DNA ladder (GIBCO-BRL). **A** RAPD profiles obtained with the UBC 346 primer, **B** SCAR band obtained with the LCH346UP and LCH346DOWN primers

Classification and Identification of markers from the conserved 18S rDNA sequences

PCR amplification of 18S rDNA sequences resulted in products ranging from 1,061 bp to 1,066 bp in the 25 *Pyrus* accessions (Table 1, Fig. 3A). A panel of 33 restriction enzymes was used to identify internal polymorphisms between the rDNA sequences. *P. pyrifolia* cv. Gamcheonbae had 84 cutting sites with these 33 enzymes over the length of 1,064 bp, with one nucleotide insertion and unique cutting sites with *Bcl*I and *Pst*I(Fig. 3B) compared to other cultivars. Cultivar Mansoo had 84 cutting sites with the 33 enzymes, with one more cutting site of each of *Alu*I (Fig. 3C) and *Bst*XI and one less cutting site of *Hph*I along a length of 1,063 bp. With respect to *Taq*I digestion, cvs. Okusankichi and Shinil showed specific bands compared to the other cultivars (Fig. 3D). In *P. communis*, the amplification products of cvs. Beurre Giffard, Cascade, Conference, Clapp's Favorite, Packhams Triumph, and Winter Nelis were all 1062 bp. Compared with the *P. pyrifolia* cultivars, they lacked the restriction enzyme *Kpn*I site (Fig. 3E) and had one less *Rsa*I site (Fig. 3F). When sequence alignment was compared, a strong distinguishing point between *P. pyrifolia* and *P. communis* was a single sequence mutation of 55 bp.

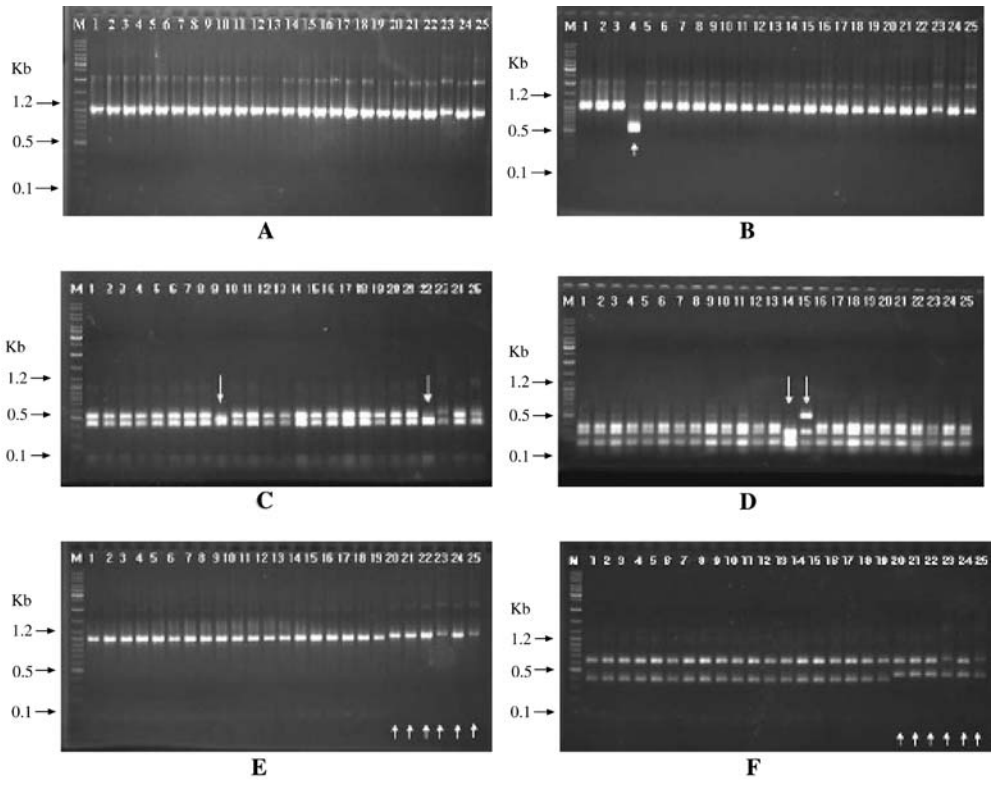

Fig. 3A–F Restriction enzyme digestion of amplified conserved sequences of 18S rDNA in 25 pear accessions (*P. pyrifolia* and *P. communis*). **A** The conserved 18S rDNA bands composed with Genbank accession nos. AJ242597 (5′-GACTGTGAAACTGCGAATGG-3′) and AJ242598 (5′-TAAGTTTCAGCCTTGCGACC-3′), **B** *Pst*I digestion, **C** *Alu*I digestion, **D** *Taq*I digestion, **E** *Kpn*I digestion, **F** *Rsa*I digestion. *Numbers above lanes* correspond to those in Table 1 (cultivar number). *Arrows* indicate differences in the sequences of the conserved sequences of 18S rDNA

Discussion

We set out to convert RAPD markers into SCARs in regions of interest. RAPD fragments were cloned and sequenced to provide longer primers (24-mers) for PCR. Paran and Michelmore (1993) reported that alleles are amplified from both parents in the basic population so that it is possible to convert a dominant RAPD locus into a codominant SCAR locus in several cases. Their conclusion suggests that the bands amplified with the LCH327UP and LCH327DOWN specific primers used in this investigation may be located in different alleles. The codominant SCAR marker detected with the LCH351UP (5′-CTCCCGGTGGGAAGTCTG-3′) and LCH351DOWN (5′-CTCCCGGTGGGGGTGGAA-3′) primer pairs amplified two or three bands in *P. pyrifolia* (Kim et al. 2000a). The size of the amplified bands differed in others; therefore it could be also applicable as a cultivar identification marker.

On the basis of BLAST analysis, the amplified band obtained with the LCH346UP and LCH346DOWN SCAR primer pairs has a high homology with chloroplast DNA in other plants, leading to the possibility that some differences exist in the chloroplast DNA between *P. pyrifolia* and *P. communis*. Chloroplast DNA has relatively conserved sequences in the species and is inherited maternally from one generation to next (Kim and Jansen 1998). In conclusion, the LCH327UP and LCH327DOWN primer pairs and LCH346UP and LCH346DOWN specific primer pairs can be useful markers to distinguish *P. pyrifolia* from *P. communis*.

With respect to molecular markers for identification purposes in *P. pyrifolia*, Kim et al. (2000a) reported the complete sequencing of six RAPD markers and the synthesis of 14 new sequence-specific primers for use under more stringent amplification conditions. The positive identification of SCAR markers for 19 *P. pyrifolia* cultivars was obtained with LCH332, LCH332-1.4, LCH350, LCH351, LCH384, and LCH387 specific primer pairs. The most useful primer pairs generated bands that may represent length variations. The LCH397UP and LCH397DOWN primers could be useful as negative marker for the identification of Imamuraaki pear cultivars in *P. pyrifolia*.

The limitations and uncertainties surrounding traditional sources of systematic evidence have created a continuing search for new and more robust sources of data. In this regard, recent advances in molecular biology have provided a variety of new tools applicable in systematic research. Most notably, restriction site analysis of chloroplast DNA (Palmer et al. 1988) and nuclear ribosomal genes (Hamby and Zimmer 1992) have been successfully applied in many studies over the past decade. Direct analysis of nucleic acid sequences has also increased in systematic research (Miyamoto and Cracraft 1991).

Our analysis of sequences of the conserved 18S rDNA has produced new markers for cultivar identification. Enzyme digestion with *Kpn*I or *Rsa*I could be used to classify markers in *P. pyrifolia* and *P. communis*. Kim et al. (2000b) previously analyzed the conserved sequence of 18S rDNA in pear cultivars. In this report, the same

group delimited the phylogenetically useful partitions present in 18S rDNA sequences among all major *P. pyrifolia* cultivars and characterized the amount and type of sequence variation present within this gene.

Historically, many important crop systems have suffered from a lack of genetic markers. However, we have shown here that the information obtained with four LCH327 and LCH346 SCAR sequence-specific primer pairs and the conserved sequences of 18S rDNA is sufficient for systematic classification and identification purposes in *Pyrus* species. Our results show that SCAR and 18S rDNA sequencing analysis could efficiently replace RAPD or RFLP analysis in the identification of pear cultivars.

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