MAPPING CHARACTERIZATION OF *PINUS SYLVESTRIS* VAR. *SYLVESTRIFORMIS* BASED ON CHLOROPLAST DNA MICROSATELLITE MARKERS

Young Hee Joung [1,2] **& Mark S. Roh** [1]

[1] US Department of Agriculture, Agricultural Research Service, National Arboretum, Floral and Nursery Plants Research Unit, Beltsville, MD 20705, USA.
[2] Current address: Chonnam National University, School of Biological Science and Technology, Gwangju, 500-757, Korea.

Received January 3, 2005; accepted August 8, 2005

ABSTRACT

Molecular markers generated by random amplified polymorphic DNA (RAPD) and chloroplast DNA simple sequence repeats (cpDNA SSR) were used to document polymorphisms in *Pinus sylvestris* (*Ps*) var. *sylvestriformis* and to identify putative parental species that include *P. densiflora* (*Pd*) var. *densiflora* collected from Korea (*Pd*-K) and from China (*Pd*-C), and *Ps* var. *sylvestris* and *Ps* var. *mongolica*. *Pinus sylvestris* var. *sylvestriformis* occurs in a very limited region around Baihe, Jilin Province, China where it grows partly overlapping with the habitat of *Pd* var. *densiflora* and *Ps.* var. *sylvestris* varieties. Dendrogram constructed using RAPD markers showed that *Pd* var. *densiflora* (*Pd*-K and *Pd*-C) and *Ps* var. *sylvestriformis* grouped together in the major cluster and all other varieties of *P. sylvestris* clustered separately. *Pinus* var. *sylvestriformis* showed a closer affinity to *Pd*-K, rather than to *Pd*-C. In cpDNA SSR analysis, polymorphisms at nine loci (Pt1254, 15169, 26081, 30204, 36480, 45002, 63718, 71936, and 102584) were detected from all species. Most marker sizes overlapped between *Pd*-K and *Ps* var. *sylvestris* except loci at Pt15169 and Pt30204. Therefore, these two cpDNA SSR markers were used for paternity test of *Ps* var. *sylvestriformis*. The sequence analysis of cpDNA SSR locus at Pt30204 revealed that pollen source of *Ps* var. *sylvestriformis* are composed of *Pd*-K type which has polyT-C-polyA-polyG type cpDNA SSR and of *Ps* var. *sylvestris* which has polyT-polyA-polyG type without C between polyT and polyA and CTAT in SSR. At locus Pt15169, *Ps* var. *sylvestriformis* are composed of *Pd*-K type which does not have CTAT sequence in SSR and of *Ps* var. *sylvestris* type which has CTAT in SSR. This is the first report which confirms the existence of genetic variability in *Ps* var. *sylvestriformis* population based on the sequence analysis. Based on the presence of cpDNA SSR, *Ps* var. *sylvestriformis* appears to be an interspecific hybrid, and is the product of introgression involving *Pd*-K and *Ps* var. *sylvestris*. It is suggested to use the hybrid formula, *P. densiflora* × *P. sylvestris* for *Ps* var. *sylvestriformis* rather than an infraspecific taxon of either one or the other parental species.

Key words: Random amplified polymorphic DNA (RAPD); chloroplast DNA SSR markers; simple sequence repeats (SSR); interspecific hybrid; *P. densiflora* × *P. sylvestris*

INTRODUCTION

The genus *Pinus* includes approximately 100 species (FARJON 2001) and is one of the most widely distributed genera in the Northern Hemisphere. *Pinus sylvestris* (*Ps*) var. *sylvestriformis* (*Ps* var. *sylvestriformis*) occurs in a very limited region around Baihe, Jilin Province, China where it grows mostly at elevations ranging from 800 m to 1,600 m, partly overlapping with the habitat of *Pinus densiflora* (*Pd*) var. *densiflora*, which occurs below 900 m and with *Ps* var. *sylvestris* which has the most extensive continuous range of all species in this genus (BORATYŃSKI 1991). However, it does not overlap with *Ps* var. *mongolica,* the nearest populations of which occur in the northern part of Heilongjiang province, China (CHENG & FU 1978). *Pinus sylvestris* L. var. *sylvestriformis* (Taken.) W. C. Cheng & C. D. Chu was recognized as a variety (FU & MILLER 1999), and it is often treated as an intermediate between *Ps* var. *mongolica* Litv. and *P. densiflora* (Siebold et Zucc) (var. *densiflora*) based on morphological characters (CHENG & FU 1978; TAKENOUCHI 1942).

DNA fingerprinting techniques, such as random amplified polymorphic DNA (RAPD) (WILLIAMS *et al.* 1990; WELSH & MCCLELLAND 1990) have been widely used to identify unknown hybrids, discrimi-

nate cultivars, and understand the evolutionary consequences and their significance in ecological studies (LEWIS & SNOW 1992; KRAUSS 1997; SANZ-CORTÉS *et al.* 2001). RAPD markers have been shown to be mostly inherited in a biparental dominant manner (WILLIAMS *et al.* 1990; CARLSON *et al.*1991; RIESEBERG *et al.* 1993). However, for paternity or maternity tests or for phylogeographic studies, uniparentally inherited organellar-specific markers should be employed. A number of phylogeographic studies have been published that employ uniparental inherited organellar-specific markers (PETIT *et al.* 1997). In gymnosperms, chloroplast DNAs (cpDNA) are paternally transmitted while mitochondria DNAs (mtDNA) are maternally inherited (WAGNER 1992). POWELL *et al.* (1995, 1996) have demonstrated that the analysis of length polymorphisms of chloroplast simple sequence repeat (cpSSR) region in pines is a suitable method for studying cytoplasmic genome inheritance and monitoring gene flow and that cpSSR provided a higher resolution molecular technique as compared to PCR-RFLP analysis. Twenty cpSSR primer sets derived from *P. thunbergii* (WAKSUGRI *et al.* 1994) were applied to study of *Pinus* species (VENDRAMIN *et al.* 1996). The application of cpSSR markers has facilitated the resolution of evolutionary relationships in several conifer species for populations with little morphological variations in *P. resinosa* (ECHT *et al.* 1998), paternity and pollen movement in *P. radiata* (KENT & RICHARDSON 1997), and natural hybridization of the *P. halepensis* species complex (BUCCI *et al.* 1998).

Morphological description of *Ps* var. *sylvestriformis* in relation to a presumed hybrid origin and possible parental taxa is not well documented. Some differences in branch-forming characteristics were observed from about 300-year old plants (Fig. 1). Some plants have short and densely spaced compact shoots resembling *Pd* var. *densiflora* while others have long shoots forming sparsely distributed terminal branches resembling *Ps* var. *sylvestris*. Hybrids in general displayed a mosaic of parental characters rather than solely intermediate ones (RIESEBERG & ELLSTRAND 1993). Molecular evidence of *Ps* var. *sylvestriformis* by PCR-RFLP indicated that *Ps* var. *sylvestriformis* had gene admixtures from both *Ps* var. *densiflora* and Ps var. *mongolica* based on allozyme analysis (SZMIDT & WANG 1993). However, correlations between morphological characters of *Ps* var. *sylvestriformis* with *Ps* var. *densiflora* and Ps var. *mongolica* was not possible, because a composite sample of *Ps* var. *sylvestriformis* was used from a population repre-

Figure 1. Morphological characters of *Ps* var. *sylvestriformis* resembling *Pd* var. *densiflora* (A), and *Ps* var. *sylvestris* (B).

sented by a bulked seed sample. *Pinus sylvestris* var. *sylvestriformis* has been referred to as *P. densiflora* Siebold & Zucc. f. *sylvestriformis* Taken and *P. sylvestris* L. var. *sylvestriformis* W. C. Cheng & C. D. Chu, both of which have been considered synonyms of *P. densiflora* (GREUTER *et al.* 2001). *Pinus densiflora* is widely distributed in Korea, but not readily observed within the range of *Ps* var. *sylvestriformis.*

The specific goals of this study were to examine the relatedness of *Ps* var. *sylvestriformis*, *Pd* var. *densiflora* and other *P. sylvestris* varieties. Because of the suggested introgressive nature of *Ps* var. *sylvestriformis* (SZMIDT & WANG 1993), *P. densiflora* collected from other regions, such as Korea, where *Ps.* var. *sylvestris* is not growing, were included. We examined biparentally inherited RAPD markers from the total genome, and maternally and paternally inherited mtDNA SSR and cpDNA SSR markers, respectively. Further, inheritance of cpDNA SSRs in *Ps* var. *sylvestriformis* was studied by analyzing the sequences to demonstrate the diversity and the relationship of this variety in comparison to *Pd* var. *densiflora* or other *P. sylvestris* varieties.

MATERIALS AND METHODS

Plant material

Pinus sylvestris var. *sylvestriformis* from Jilin Province, China, *Pd* var. *densiflora* from Korea (*Pd*-K) and China (*Pd*-C) and *Ps* var. *sylvestris* from northeastern part of China and Russia Far East were included in this study with other *P. sylvestris* varieties such as *Ps* var. *mongolica.* A total of 15 *P. densiflora*, of 12 *P. sylvestris* varieties, and of 20 *Ps* var. *sylvestriformis* accessions was made (Table 1). The materials were received from various germplasm repositories or botanical gardens in the United

Table 1. Accessions of *Pinus* germplasm[a].

Code	Accession No [b]	No of sample	Location	Altitude (m)	Latitude (N)	Longitude (E)
Pinus densiflora var. *densiflora*						
Pd-K1	NA55235	3	Kyong Gi, Daeyoupyong Island. Korea	100	38	126
Pd-K2	NA55207	3	Kyong Gi, Paekryong Island. Korea	120	38	124
Pd-K3	NA61727	6	Kangwon, Taeduck Mountain, Korea	1240	37	128
Pd-C	NA63011	3	Arboretum of Chinese Academy of Forestry	800	42	129
Pinus sylvestris var. mongolica						
Ps-M	MA87-124	3	Morris Arboretum, Mts. of NE China	?	42	128
Pinus sylvestris var. *sylvestris*						
Ps-SC1	NA37306	3	Heilongjiang, China		45	126
Ps-SC2	Ames2301	3	Mountains of NE China	700	46	127
Ps-SY	NA35362	1	Yugoslavia	950	45	20
Ps-SS	MA82-024	2	Morris Arboretum, Krasnoyarsk, Russia	?	55	95
Pinus sylvestris var. *sylvestriformis*						
Ps-C1	Cheng, 1999	1	Changbai Mt., China	?	42	128
Ps-C2	MA87-125	2	Morris Arboretum, Mts. of NE China	?	42	129
Ps-C3	NA68897	9	Baihe, Jilin (CBS 138)	800	42	128
Ps-C4	Roh, 1999	8	Baihe, Jilin	800	42	128

[a] Not all samples were used in RAPD. Only one sample from Pd-K1, Pd-K2, and Pd-K3. Refer Fig. 2 and 3 for samples used in RAPD. All samples were used in SSR analysis.
[b] NA and Ames, accession number of USDA National Arboretum; MA, accession number of Morris Arboretum.
[c] No information on the location.
[d] Sample of Psf-C1 was received as *P. densiflora* var. *sylvestriformis.* Psf-C2 samples of *Pinus sylvestris* var. *sylvestriformis* were propagated from seeds by Woody Landscape Plants Research Repository program and Psf-C4 were needles collected at Baihe.

States, or collected from the native population in China. DNA was extracted from needles using the CTAB method by DOYLE and DOYLE (1987).

RAPD analysis and clustering

PCR amplifications with a limited number of samples, but representing all taxa and accessions, were performed using Ready-To-Go PCR beads (Amersham Pharmacia Biotech., Piscataway, NJ) in a 25 µl reaction mix containing 10 ng of DNA and 5 pmol primers. Following a screening of 10-mer random primers (Operon Technologies Inc., Alameda, CA), nine primers (A07, A18, B05, B12, B20, C02, C04, C08, C15), that produced a high number of polymorphic bands, were used for further studies. DNA amplifications and gel separation were performed as described (JOUNG & ROH 2004). The molecular size was recorded based on DNA ladder using PCR marker P9577 (Sigma, St. Louis, MO, USA). The P-distances (a+b/a+b+c where a and b were the number of bands present in one sample but absent in the other, and c is the number of shared bands), were calculated from all samples (data not presented). Based on the p-scores, UPGMA and 1,000 replicates of bootstrap analyses were performed using the Molecular Evolutionary Genetic Analysis (MEGA) program (Version 1.0).

Simple sequence repeats (SSR) analysis

Eleven primer sets (Pt1254, Pt9383, Pt15169, Pt26081, Pt30204, Pt36480, Pt45002, Pt48210, Pt63718, Pt71936, and Pt102584) for chloroplast SSR analysis in Pinaceae (VENDRAMIN *et al.* 1996) and two primer sets for mitochondria SSR analysis (Mt SSR1 and Mt SSR2) were used (Table 2). Fragment amplification was performed in a 20 µl reaction containing 10 ng of DNA, each primer at 0.5 µM, all four dNTPs (each at 200 µM), 50 mM KCl, 10 mM Tris•HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% gelatin, and 0.25 unit of *AmpliTaq* Gold DNA Polymerase (PE Applied Biosystem, Inc., Foster City, CA). DNA amplifications were performed in a MJ Research PTC-100/96 thermocycler with the following profile: 94 °C (5 min), followed by 30 cycles of 94 °C (15 sec), 57 °C (30 sec) and 72 °C (40 sec). These cycles were followed by 5 min at 72°C. PCR products were separated and detected by capillary electrophoresis on a genetic analyzer (ABI Prism 310 Genetic Analyzer, PE Applied Biosystem, Inc.). PCR products from each species were cloned into the pCR Script 1vector (Stratagene, La Jolla, CA). At least two clones were sequenced for nucleotide sequence analysis from each cloning experiment.

Table 2. Oligonucleotide primer sequences for the amplification of 11 chloroplast and two mitochondrial microsatellites.

Code	Primer sequence (5' – 3') sense / antisense
Pt1254 [a]	CAATTGGAATGAGAACAGTAGG TGCGTTGCACTTCGTTATATAG
Pt9383	AGAATAAACTGACGTAGATGCCA AATTTTCAATTCCTTTCTTTCTCC
Pt15169	CTTGGATGGAATAGCAGCC GGAAGGGCATTAAGGTCATTA
Pt26081	CCCGTATCCAGATATACTTCCA TGGTTTGATTCATTCGTTCAT
Pt30204	TCATAGCGGAAGATCCTCTTT CGGATTGATCCTAACCATACC
Pt36480	TTTTGGCTTACAAAATAAAAGAGG AAATTCCTAAAGAAGGAAGAGCA
Pt45002	AAGTTGGATTTTACCCAGGTG GAACAAGAGGATTTTTTCYCATACA
Pt48210	CCGAGATTGATCCGATACCAG GAGAGAACTCTCGAATTTTTCG
Pt63718	CACAAAAGGATTTTTTTTCAGTG CGACGTGAGTAAGAATGGTTG
Pt719936	YYCATTGGAAATACACTAGCCC AAAACCGTACATGAGATTCC
Pt102584	TTCATGTAATTCCCAGATCCA CATTATGTGCGCGATAATTTC
MtSSR1 [b]	AACGATCTGCAGCTCAAATGG CGGCGAGACGCGGACATTAC
MtSSR2	CCATGAATGGAAGAAGGGTGC AGGACATTTCTCCGAAGCTCG

[a] From POWELL *et al.* (1995)
[b] MtSSR1 (intron part of NADH dehydrogenase subunit 1 gene.
MtSSR2 intergenic spacer of NADH dehydrogenase subunit 3 and ribosomal protein S12 gene.

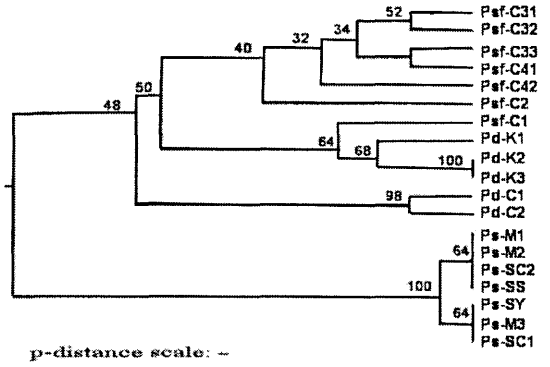

Figure 2. UPGMA analysis based on p-distance at $P<0.01$ level between populations of *Pinus* species. Bootstrap values are indicated in the dendrogram. For accession identity, see Table 1.

RESULTS AND DISCUSSION

Pinus densiflora and *Ps* var. *sylvestriformis* (Psf-C2, -C3, -C4), which grow sympatrically in a localized area, belonged to one major cluster in dendrogram constructed from RAPD markers (Fig. 2). One sample of *Ps* var. *sylvestriformis* (Psf-C1) clustered together with *P. densiflora* from Korea (*Pd*-K) could have been misidentified. All other *Ps* var. *sylvestriformis* samples, whether collected from the wild or from cultivated seedlings, clustered together. All samples of other *P. sylvestris* varieties, including *Ps* var. *mongolica* (Ps-M) and *Ps* var. *sylvestris* from Yugoslavia (Ps-SY), China (Ps-SC1 and -SC2), and Russia Far East (Ps-SS) clustered closely together and showed a low diversity. Low nucleotide diversity at the *pal1* locus in the widely distributed *P. sylvestris* has been reported (DVORNYK *et al.* 2002). The species-specific band sharing of *Ps* var. *sylvestriformis* and *Pd*-K with primer A 18 (Pd-1-A 18) and with primer C 04 (Pd-1-C 04 and Pd-2 - C 04) was clearly evident (Fig. 3). *Pinus sylvestris* species specific bands (Ps-1, Ps-2 and Ps-3 with primer A 18 and Ps-1 with primer C 04 were not shown in *Ps* var. *sylvestriformis*. Variance in banding patterns of one *Pd-C* accession (Pd-C, Fig. 2) suggested that *Pd-C* is not homogenous and could be attributed to hybridization with *Ps* var. *sylvestris* or *Ps* var. *mongolica* (Fig. 3). It may be possible that *Pd*-C accessions used in this study are different from *P. densiflora* used in the previous study that resulted from an introgression of *Ps* var. *mongolica* isozyme alleles (SZMIDT & WANG 1993). In our study, one *Pd*-C accession no. 2 had a band, when amplified with a primer A-18, that was also observed in *Ps* var. *sylvestriformis* (Ps-2 – A 18) and *Pd*-K (Pd-1 – A 18 band) (Fig. 3). Therefore, more germplasm of *Pd*-C should be included in the future to investigate the parentage of *Ps* var. *sylvestriformis*. During the visit to the site of *Ps* var. sylvestriformis, *Pd*-C was not observed growing in the region.

Based on the RAPD analysis the genetic diversity of *Ps* var. *sylvestriformis* and further its maternity and paternity cannot be assessed, because UPGMA tree only indicates similarity, but not phylogenetic relationships. Therefore, using specific SSR primer sets (VENDRAMIN *et al.* 1996), cpDNA SSR analysis was performed, which is inherited uniparentally in gymnosperms (POWELL *et al.* 1995, 1996) and for paternally in *Pinus* (BRIKY 1988; NEALE & SEDEROFF 1989; PROVAN *et al.* 2001). For maternity test, specific primer sets for mitochondria DNA (mtDNA), which is inherited maternally (WAGNER 1992), were used (Table 2). In contrast with the chloroplast genome, the complete mitochondria genome has not been sequenced yet in *Pinus*, and only a few sequenced genes in the mitochondrial genome that have SSR region. Therefore, two SSR regions, the intron part of NADH dehydrogenase subunit 1 gene and intergenic spacer between NADH dehydrogenase subunit 3 and ribosomal protein S12 gene, were used to test maternity. However, there were no polymorphisms based on fragment size variants produced by these two mtDNA specific primer sets and all fragments had the same nucleotide sequences (data not presented).

Chloroplast DNA polymorphisms based on fragment size variations were detected from all species by 11 primer sets and these variations were detected within the same species in most loci (Table 3). Especially, at eight loci (Pt1254, 15169, 26081, 30204, 36480, 45002, 63718, and 102584), size variations were detected between *Pd* var. *densiflora* from Korea (*Pd*-K) and from China (*Pd*-C) (Table 3). At loci Pt1254, 15169, 30204, 36480, 63718, and 102584, fragment length differences between *Pd*-K and *Pd*-C were bigger than between *Pd*-K and *Ps* var. *sylvestris*. Even at loci Pt63718 and 102584, the same size of SSR fragments, 88 bp and 128 bp, respectively, were amplified in all *Pd*-K and *Ps* var. *sylvestris* except *Pd*-C (Table 3). These results suggested that all *Ps* var. *sylvestris* collected from China, Russia Far East, and Yugoslavia was very close to each other, that SSR fragments of *Pd*-C were out of range of those analyzed in *Ps* var. *sylvestriformis* SSR, and that fragment length variations of *Pd*-C is clearly different from those of *Pd*-K and of *Ps* varieties (Table 3, 4, Fig. 4, 5). Therefore, *Pd*-C was not considered as paternal source of *Ps* var. *sylvestriformis*. No variances in cpDNA SSR were detected in any *Ps* varieties, and therefore, one population of *Ps* var. *mongolica* was

Table 3. Fragment length variants (bp) of 11 SSR loci of *Pinus*. *Pinus sylvestris* var. *mongolica* is included in *P. sylvestris var. sylvestris* because there were no differences in fragment sizes.

Primer set	*P. densiflora* var. *densiflora*		*P. sylvestris* var. *sylvestris*	*P. sylvestris* var. *sylvestriformis*		
	Korea	China		Psf-C1	Psf-C2	Psf-C3, C4[a]
Pt1254	67–68[b]	73–75	67–70	67	67–68	67–68
Pt9383	85–86	85–86	85–86	85	85–86	85–86
Pt15169	122–123	116–117	125–127	128	125	122–129
Pt26081	109–111	107–108	110–111	111	110–111	110–111
Pt30204	139–140	145–147	141–145	143	142–143	139–144
Pt36480	144	147	143–144	144	143–144	143–144
Pt45002	167–168	165–166	167–169	169	168–169	168–169
Pt48210	85–86	86–87	85–86	86	85–86	85–86
Pt63718	88	89	88	88	88	88
Pt719936	145–149	146–147	142–145	143	143	143–148
Pt102584	128	129	128	128	128	127–128

[a] Combined samples of Psf-C3 and Psf-C4. Refer to Table 1 for other abbreviations.
[b] Minimum – maximum fragment size at each marker.

Table 4. Fragment length variants (bp) and repeated sequence of two SSR loci.

Accession type[a]	Primer set Pt30204		Primer set Pt15169	
	PCR fregment size (bp)	repeat type	PCR fragment size (bp)	repeat type
Pinus densiflora var. *densiflora*				
Korea (Pd-K1,2,3)	139–140	(T)4C(A)8–9(G)9–10	122–123	(C)9(T)9–10A(T)9–10
China (Pd-C)	145–147	(T)4C(A)10(G)13–15	116–117	(C)7–8(T)7–8A(T)8
Pinus sylvestris var. *sylvestris* (Ps)				
	141–145	(T)3–4(A)9–10(G)11–14	125–127	(C)8–10(T)9–10A(T)8–9+CTAT
Pinus sylvestris var. *sylvestriformis*				
Psf-C1 (Ps type)	143	(T)3(A)11(G)12	125	(C)8(T)10A(T)9+CTAT
Psf-C2 (Ps type)	142–143	(T)3(A)11(G)11–12	128	(C)11(T)10A(T)9+CTAT
Psf-C3, 4 (Pd-K type)	139–141	(T)4C(A)9(G)9–10	122–123	(C)8–9(T)9–10A(T)9–10
Psf-C3, 4 (Ps type)	139–141	(T)3–4(A)10(G)11–13	125–129	(C)8–11(T)9–10A(T)8–9+CTAT

[a] Refer Table 1 for other abbreviations.

included with *Ps* var. *sylvestris* (data not presented). Based on the result presented in this paper, *Pd*-C included in this study and in the previously study (SZMIDT & WANG 1993) are different from each other and comparison to understand whether which one of these two populations represent the true species should be performed. No comparisons of *Pd* var. *densiflora* between Korean populations and Japanese and Chinese populations were done in both studies and further studies are required.

Most marker sizes overlapped between *Pd*-K and *Ps* var. *sylvestris* except loci at Pt15169 and Pt30204. Therefore, only these two cpDNA SSR markers were used for the genetic diversity and paternity test of *Ps* var. *sylvestriformis*. At locus Pt15169, a putative open reading frame (ORF) between ATP synthase A chain (atpI) and ribosomal protein S2 (rps2) gene, *Pd*-K produced 122 and 123 bp and *Ps* var. *sylvestris* produced 125, 126 and 127 bp (125-127 bp), and *Ps* var. *sylvestriformis* resulted

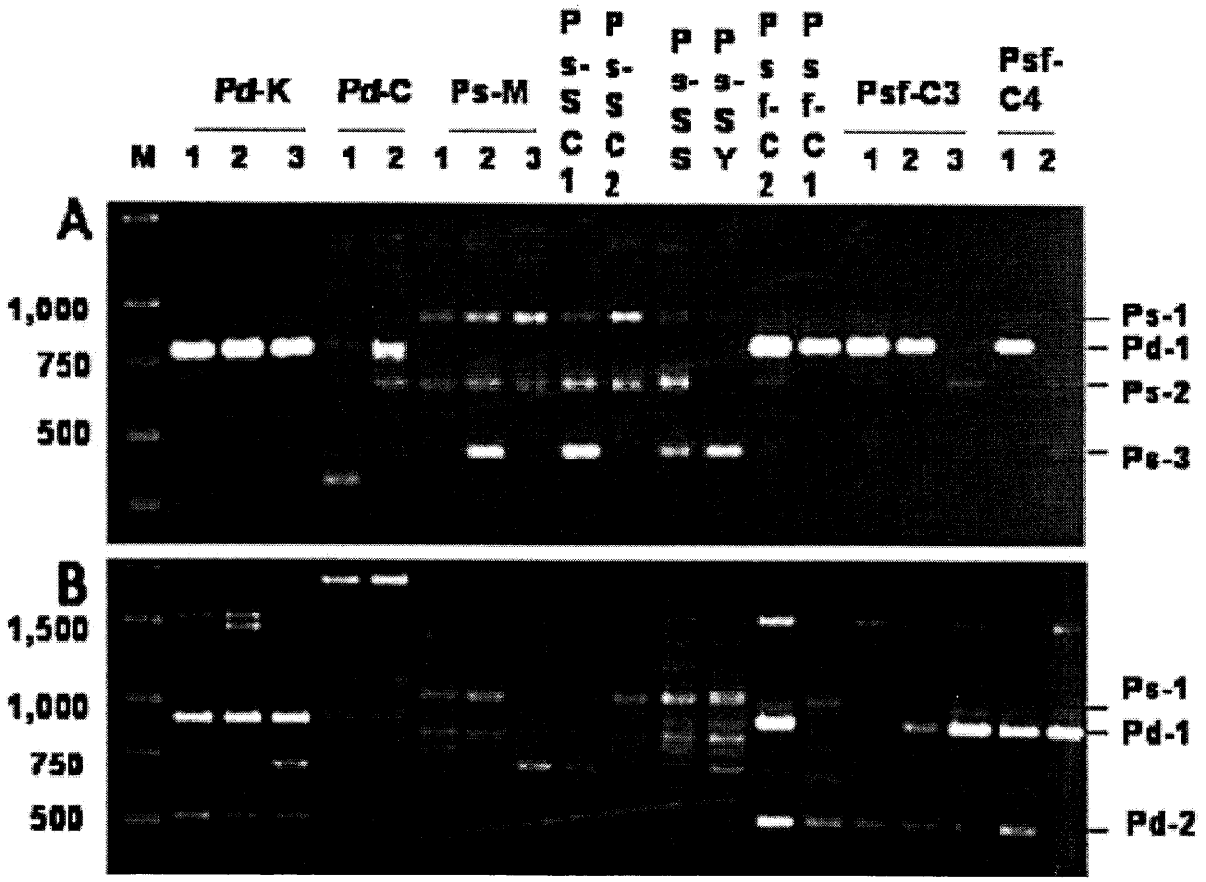

Figure 3. RAPD banding patterns using primer A 18 (A) and C 04 (B) of *Pinus* species. Only one sample of each from Pd-K1, Pd-K2, and Pd-K3 was selected, and randomly selected samples from other accessions were amplified. M : PCR size marker (P9577, Sigma) Species specific bands are indicated. Refer to Table 1 for abbreviations.

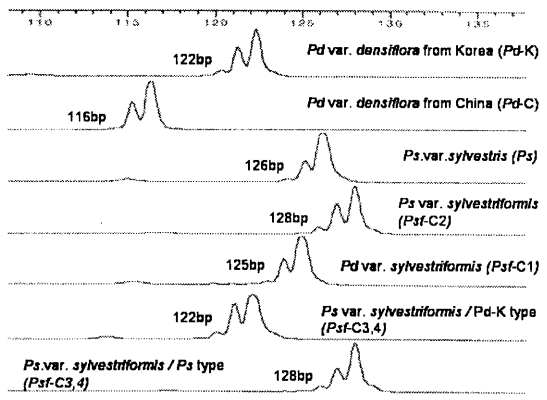

Figure 4. DNA band patterns of SSR analysis of *Pinus* species using Pt15169 primer set.

in various SSR length, the shortest was 122 bp and the longest was 129 bp (Table 3, 4 and Fig. 4). Fragment length variation of *Pd*-C is clearly different from those of *Pd*-K and of *Ps* varieties (Table 3). This confirms that *Pd*-C examined in this study are different from those used in other studies or may not be reasonably pure representatives of the taxa or introgressed with *Ps* variety as stated above. During a visit in 1999 and 2002 to the Mt. Changbai area, *Ps* var. *sylvestriformis* was not easily observed sympatrically distrib uted with *Pd*-C, and it was not possible to add more *Pd*-C germplasm in this present study.

In our study, a total of 20 individual samples of *Ps* var. *sylvestriformis* were subjected to cpDNA SSR. At locus 15169, six samples of *Ps* var. *sylvestriformis* (Psf-C3-1, 3, 5, 7 and Psf-C4-3, 6) produced the same fragments as observed in *Pd*-K, while 12 others (Psf-C3-2, 4, 6, 8, 9 and Psf-C4-1, 2, 3, 4, 5, 7, 8) produced the same fragments as observed in *Ps* var. *sylvestris* (data not presented). To confirm these results, sequence analyses of all cpDNA SSR fragments were performed. The sequence analyses results revealed that the *Pd* and *Ps* var. *sylvestris* fragments were quite different. *Pd*-K had polyC–polyT-A-polyT type in SSR, \\while *Ps* var. *sylvestris* had polyC–polyT-A-polyT and CTAT in SSR (Table 4 and Fig.4). Only *Ps* var. *sylvestris*

had the CTAT sequence in the SSR (Fig. 5). In *Ps* var. *sylvestriformis*, the six samples (Psf-C3-1, 3, 5, 7 and Psf-C4-3, 6) showed the *Pd*-K type cpDNA SSR sequence while all other samples showed the *Ps* var. *sylvestris* type.

At locus Pt30204, which is the intergenic space between ATP-dependent protease proteolytic subunit (clpP) gene and ribosomal protein S12 (rps12) gene, *Pd*-K produced 139 and 140 bp, *Ps* var. *sylvestris* produced 141-144 bp, and *Ps* var. *sylvestriformis* resulted in 139-144 bp (Table 3, 4 and Fig. 4). We could expect that the pollen parent of some of the *Ps* var. *sylvestriformis* sample, which showed 139-140 bp length SSR, were from *Pd*-K types and others which showed over 141-bp length SSR were from *Ps* var. *sylvestris*. The result of the sequence analyses indicated that the pollen parents' sources of *Ps* var. *sylvestriformis* may include both *Pd*-K types and *Ps* var. *sylvestris* (Table 3 and Fig. 4). At locus Pt30204, six of *Ps* var. *sylvestriformis* (Psf-C3-1, 3, 5, 7 and Psf-C4-3, 6) products showed *Pd*-K type cpDNA SSR while others were of the *Ps* type. This result was same as locus Pt 15169. Therefore, the pollen parent source of Psf-C3-1, 3, 5, 7 and Psf-C4-3, 6 was apparently of the *Pd*-K types while that of others was apparently *Ps* var. *sylvestris*. Differences in fragment sizes and sequence of *Ps* var. *sylvestriformis* at loci Pt15169 and Pt30204 suggest that more than one paternal parent sources were involved in hybridization of *Ps* var. *sylvestriformis*. In the previous report, estimation of cpDNA admixtures in *Ps* var. *sylvestriformis* was not possible because a composite sample of this variety was used SZMIDT & WANG 1993). Therefore, it is not clear whether *Ps* var. *sylvestriformis* is currently reverting to its putative sympatric parent of Pd-C types growing in the north-eastern part of China or *Pd*-K types growing in the northern part of Korea or *Ps* var. *sylvestris* or represents a stabilized introgressant. These answers could be answered by examining each individual of *Ps* var. *sylvestriformis*. Further the difference in the fragment sizes found in *Ps* var. *sylvestriformis* which were regarded as *Ps* var. *sylvestris* type were not the same as those found in *Ps* var. *sylvestris*. This could be attributed to the variable SSR in *Ps* var. *sylvestris* and resolved when more samples of *Ps* var. *sylvestris* are included to find the exact matching size of *Ps* var. *sylvestriformis* with those of *Ps* var. *sylvestris*

From these cpDNA SSR analyses, it is evident that individuals within *Ps* var. *sylvestriformis* have paternally inherited chloroplasts either from *Pd*-K or *Ps* var. *sylvestris*. The results of our study clearly demonstrated that *Ps* var. *sylvestriformis* harbors two different species, i.e., one morphologically resembling *Pd*-K and another possibly *Ps* var. *sylvestris*. There is no report documenting whether *Ps* var. *sylvestriformis* shows solely intermediate form between *P. densiflora* and *Ps var. sylvestris* or mosaic patterns of two species. Generally, hybrids are described to display a mosaic of parental and intermediate characters rather than solely intermediate ones (RIESEBERG & ELLSTRAND 1993). Any correlation between cpDNA SSR and morphology

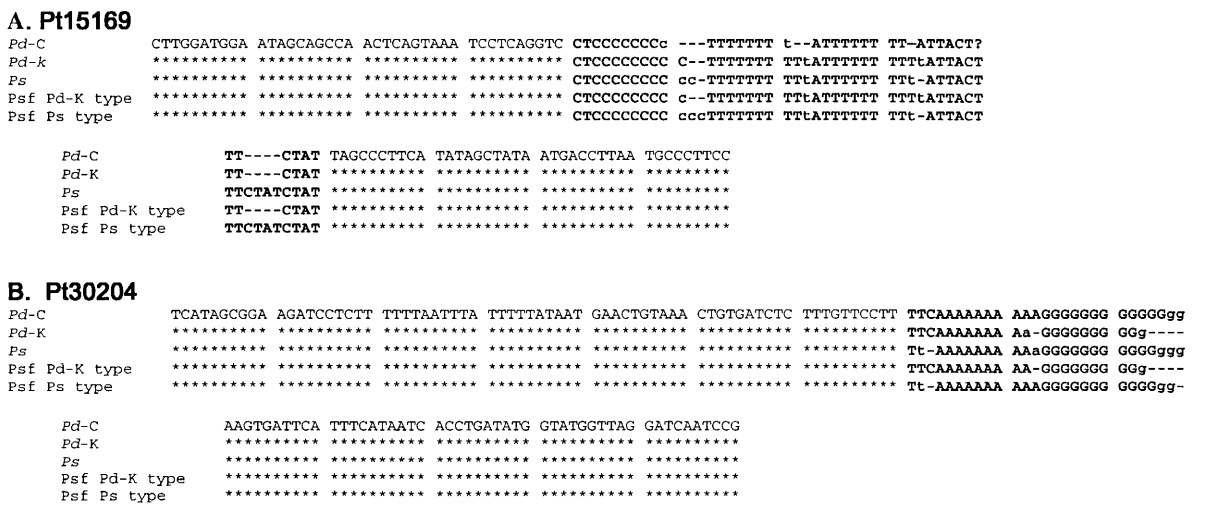

Figure 5. Aligned nucleotide sequences indicating polymorphism among *P. densiflora* var. *densiflora* from Korea (*Pd*-K) and China (*Pd*-C), *Ps* var. *sylvestris* (Ps) and *Ps* var. *sylvestriformis* (Sf-K, *Pd*-K type and Sf-S, *Ps* type) at loci Pt15169 (putative ORF between ATP synthase CF0 A chain gene and ribosomal protein S2 gene) (A) and Pt30204 (intergene spacer between ATP-dependent protease proteolytic subunit gene and ribosomal protein S12 gene) (B). Bold letter, SSR sequence; Small letter, variable sequence in intra species; −, gaps that indicate variation between aligned sequences; * identical sequence.

in the populations of *Ps* var. *sylvestriformis* were not investigated in our study. Needles and seeds of more *Ps* var. *sylvestriformis* will be collected to further study the correlation between morphology and cpDNA SSR.

CONCLUSION

The clustering based on the RAPD analysis suggested that *Ps* var. *sylvestriformis* had closer affinity to *Pd*-K than to *Pd*-C and *Ps* var. *sylvestris* and other varieties. The repeat type of cpDNA SSR in *Pd*-K and also in *Ps* var. *sylvestris* that is present in *Ps* var. *sylvestriformis* suggests that a hybridization has occurred, that both *Pd*-K types and *Ps* var. *sylvestris* are considered as the paternal source, and genetic diversity of *Ps* var. *sylvestriformis* indicating that the population is composed of two distinct groups closely related to *Pd* var. *densiflora* and and *Ps* var. *sylvestris*. No attempts have been made in this study to correlate between cpDNA SSR and morphology in the populations of *Ps* var. *sylvestriformis*. Further studies are required to see whether individuals of *Ps* var. *sylvestriformis* which have *Ps* var. *sylvestris* cpDNA SSR are approaching *Ps* var. *sylvestris* morphologically, while those that have *Pd*-K type cpDNA SSR are similar to *Pd* var. *densiflora*. Further, *Ps* var. *sylvestriformis* germplasm should be collected from various aged plants, for example, about 300-, 100-, and 15 to 20-year old plants that are growing in the same location, to investigate the introgressive nature of this variety.

Based on the data presented, it is suggested that *Ps* var. *sylvestriformis* is the result of multiple hybridizations involving both Pd-K types and *Ps* var. *sylvestris* as the parents, and thus consists of introgressant between these two taxa. Following the International Code of Botanical Nomenclature (GREUTER *et al.*, 2000), it is suggested to use the hybrid formula, *P. densiflora* × *P. sylvestris*, rather than an infraspecific taxon of one or the other parental species, i.e., *P. densiflora* Siebold & Zucc. f. *sylvestriformis* Taken or *P. sylvestris* L. var. *sylvestriformis* W. C. Cheng & C. D. Chu .

ACKNOWLEDGEMENTS

We thank North America-China Plant Exploration Committee member institutes for the interest and making the germplasm available to this study. This project was supported in part by Germplasm Evaluation Program supported by USDA, ARS, National Program. Also, appreciation is due to Ms. C. S. Zheng for providing samples for this study. Arrangements for M. S. Roh's visit to Baihe, Jilin by Chinese Academy of Agricultural Science, Institute of Vegetable and Flowers are greatly appreciated. Arrangements made by Drs. R. Schnell and A. Meerow, USDA, ARS, Miami, for the use of laboratory facilities and review of the manuscript are greatly appreciated. We also thank Drs. Richard Whitkus, Jerry W. Leverenz, Knub Ib Christensen, and John Wiersema for their critical reading of this manuscript and helpful comments.

REFERENCES

BORATYŃSKI, A. 1991: Range of natural distribution. *In*: Giertych, M., and Mátyás, C. (eds.) Genetic of Scots pine, Akademia Kiadó, Budapest, pp. 19–30.

BRIKY, C. W. 1988: *In*: Plant Evolutionary Biology. Chapman and Hall, London, (eds. Gottlieb, L. D., Jain, S. K.) pp. 23–53.

BUCCI, G., ANZIDEI, M., MADAGHIELE, A. & VENDRAMIN, G.G. 1998: Detection of haplotypic variation and natural hybridization in *halepensis*-complex pine species using chloroplast simple sequence repeat (SST) markers. *Mol. Ecol.* **7**: 1633–1643.

CARLSON, J.E., TULSIERAM, L.K., GLAUBITZ, J.C., LUKE, V.W.K., KAUFFELDT, C. & RUTLEDGE, R. 1991: Segregation of random amplified DNA markers in F_1 progeny of conifer. *Theor. Appl. Gene.* **83**: 194–200.

CHENG, W.-C. & FU, L.-G. 1978: Chinese Flora. *Sci. Press* **7**:203–280 [in Chinese].

DOYLE, J.J. & DOYLE, J.L. 1987: A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.* **19**: 11–15.

DVORNYK, V., SIRVIÖ, A., MIKKONEN, M. & SAVOLAINEN, O. 2002: Low nucleotide diversity at the *pal1* locus in the widely distributed *Pinus sylvestris*. *Mol. Biol. Evol.* **19**: 179–188 .

ECHT, C.S., DEVERNO, L.L. ANZIDEI, M. & VENDRAMIN, G.G. 1998. Chloroplast microsatellites reveal population genetic diversity in red pine, *Pinus resinosa* Ait.*Mol. Ecol.* **7**: 307–316.

FARJON, A. 2001. World checklist and bibliography of conifers. 2nd Ed. Richmond, Surrey, U.K.: Royal Botanic Gardens. 309 pp.

FU, L., LI, N. & MILL, R,R. 1999: Pinaceae. *In*: Flora of China, Vol. 4, Science Press (Beijing, China) and Missouri Bot. Garden (St. Louis, USA). (eds. Wu, W., and Raven, P. H.) pp.11–41.

GREUTER, W., MCNEILL, J., BARRIE, F. R., BURDET, H.-M., DEMOULIN, V., FILGUERAS, T.S., NOCOLSON, D.H., SILVA, P. C., SKOG, J.E., TREHANE, P., TURLAND, N.J. & HAWKSWORTH, D.L. 2001: International Code of Botanical Nomenclature (St. Louis Code). Regnum Vegetabile 138. Koeltz Scientific Books, Königstein.

JOUNG, Y.H. & ROH, M. S. 2004: Paternity determination of *Ornithogalum* seedlings using DNA markers. *J. Hort. Sci. & Biotech.* **79**: 316–321.

KENT, J. & RICHARDSON, T.E. 1997: Fluorescently labeled, multiplexed chloroplast microsatellites for high-throughput paternity analysis in *Pinus radiata*. *New*

Zealand J. For. Sci. **27**: 305–312.

KRAUSS, S.L. 1997: Low genetic diversity in *Persoonia mollis* (Proteaceae), a fire-sensitive shrub occurring in fire-prone habitat. *Heredity* **78**: 41–49.

LEWIS, P.O. & SNOW, A.A. 1992: Deterministic paternity exclusion using RAPD markers. *Mol. Ecol.* **1**:155 –160.

NEALE, D.B.& SEDEROFF, R.R. 1989: Paternal inheritance of chloroplast DNA and maternal inheritance of mitochondrial DNA in loblolly pine. *Theor. Appl. Genet.* **77**: 212–216.

PETIT, R.J., PINEAU, E., DEMESURE, B., BACILIERI, R., DUCOUSSO, A. & KREMER, A. 1997: Chloroplast DNA footprints of postglacial recolonization by oaks. *Proc. Nat. Acad. Sci. U.S.A.* **94**: 9996–10001.

POWELL, W., MOGANTE, M., MCDEVITT, R., VENDRAMIN, G.G. & RAFALSKI, J.A. 1995: Polymorphic simple sequence repeat regions in chloroplast genomes: Applications to the population genetics of pines. *Proc. Nat. Acad. Sci.* **92**: 7759–7763.

POWELL, W.L, MACHRAY, G. C. & PROVAN, J. 1996: Polymorphism revealed by simple sequence repeats. *Trends in Plant Sci.***1**: 215–222.

PROVAN, J., POWELL, W. & HOLLINGSWORTH, P.M. 2001: Chloroplast microsatellites: new tools for studies in plant ecology and evolution. *Trends Ecol. Evol.* **16**: 142–147.

RIESEBERG, L.H. & ELLSTRAND, N.C. 1993: What can molecular and morphological markers tell us about plant hybridization? *Critical Rev. Plant Sci.* **12**: 213 –242.

SANZ-CORTÉS, F., BADENES, M. L., PAX, S., ÍÑIGUES, A. & LLACER, G. 2001: Molecular characterization of Olive cultivars using RAPD markers. *J. Amer. Soc. Hort. Sci.* **126**: 7–12.

SZMIDT, A,E. & WANG, X-R. 1993. Molecular systematics and genetic differentiation of *Pinus sylvestris* (L.) and *P. densiflora* (Sieb.et Zucc.). *Theor. Appl. Genet.* **86**: 159 –165.

TAKENOUCHI, M. 1942: A preliminary report on the conifers of Manchukuo. *J. Jap. For. Soc.* **24**: 113 –129.

VENDRAMIN, G.G., LELLIM L., ROSSI, P. & MORGANTE, M. 1996: A set of primers for amplification of 20 chloroplast microsatellites in Pinaceae. *Mol. Ecol.* **5**: 595–598.

WAGNER, D. B. 1992: Nuclear, chloroplast, and mitochondrial DNA polymorphism as biochemical markers in population genetic analyses of forest trees. *New Forests* **6**: 373–390.

WAKASUGI, T., TSUDZUKI, J., ITO, S., SHIBAT, M. & SUGIURA, M. 1994: Loss of all ndh genes as determined by sequencing the entire chloroplast genome of the black pine *Pinus thunbergii. Proc. Natl. Acad. Sci. U.S.A.* **91**: 9794–9798.

WANG, X-R., SZMIDT, A.E. & LINDGREN, D. 1991: Allozyme differentiation among populations of *Pinus sylvestris* L. from Sweden and China. *Hereditas* **114**: 219–216.

WELSH, J. & MCCLELLAND, M. 1990: Fingerprinting genomes using PCR with arbitrary primers. *Nucl. Acids Res.* **18**: 7213–7218.

WILLIAMS, J.G., KUBELIK, A.R., LIVAK, K.J., RAFALSKI, J.A. & TINGEY, S.V. 1990: DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucl. Acids Res.* **18**: 6531–6535.