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Phylogenetic Analysis of Pines Based on Chloroplast trnT-trnL Intergenic Spacer DNA Sequences

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Abstract

This study was conducted to distinguish the pines that are too similar to differentiate using conventional methods. *Pinus densiflora* and *Pinus sylvestris* have similar anatomical structure. They both have window-like pits and dentate ray tracheids, so it is not easy to distinguish the plants. We tried to find molecular markers by comparing chloroplast DNA sequences to differentiate the pines growing in Korea. We used *P. densiflora*, *P. densiflora* for. multicaulis, *P. sylvestris*, *P. rigida*, *P. rigitaeda*, *P. koraiensis*, and *P. bungeana* for this study. We found that the non-coding intergenic region of trnT(UGU) and trnL(UAA) genes have differences among the species. We designed a primer set to amplify the region efficiently and compared the PCR product sequences using CLC Workbench programs to find the polymorphism. We could distinguish the species using the sequences of the amplified region and the sequences were reproducible from the pines collected in Korea.

Key Words: Pines, trnT-trnL Intergenic Spacer, SNPs, Genetic diversity

Introduction

Genus *Pinus*, with 115 species, belongs to the family Pinaceae. The plants are evergreen trees, distributed in America, northern Eurasia and southeast Asia (Plomion et al. 2007). Several kinds of molecular markers have been developed to identify *Pinus* species. Tulsieram et al. (1992) developed isozyme markers to distinguish pines, but such markers could not distinguish many kind of pines. Restriction fragment length polymorphism (RFLP) was used to study *P. taeda* polymorphism during the early 1990s (Neale and Williams 1991). Researchers suggested that the RFLP marker had enough polymorphism for high-density genome mapping. Nevertheless, the method was used only for *P. taeda* (Devey et al. 1994) and *P. radiate* (Devey et al. 1996) because the method is labor and time intensive. During the mid-1990s, PCR-based methods, AFLP and RAPD, were developed and the analysis time was significantly reduced. These new techniques were adopted to analyze the tree genome including pines (Cervera et al. 2000). These markers have restrictions that can be applied for various purposes because they are dominant markers.

Chloroplast DNA is a haploid genome that is inherited paternally (or at least predominantly paternally) in *Pinus*

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(Wagner et al. 1992). Chloroplast simple sequence repeat (cpSSR) offers several advantages because it is a codominant marker (Vendramin et al. 2000). Therefore, it is used frequently for population genetics (Powell et al. 1996; Morgante et al. 1997; Echt et al. 1998). As the availability of DNA sequencing increases, the study of variation between individuals of a population becomes increasingly popular. For such studies, Single Nucleotide Polymorphism (SNP) is commonly used. SNP is a kind of point mutation and it is polymorphism of a single nucleotide. SNP is the most abundant variation and is easy to detect, so it is used for genetic map construction, quantitative trait locus (QTL) analysis, genetic variation detection, and germplasm searching (Shin et al. 2006).

In plants, many hybrids have developed between species or between genera, so with maternal or paternal markers, it is not easy to identify a species or trace the evolution of species. Nuclear DNA markers are good for tracing the evolution of species. Internal Transcribed Spacers (ITS) of nuclear rDNA are candidates for DNA markers, but plants have several thousand copies of the region in the genome so they are not a useful alternative (Alvarez and Wendel 2003).

Generally, the non-coding region of a genome evolves faster than the coding region. Therefore, the non-coding region provides more information for phylogenetic analysis because of the higher variation rate (Palmer et al. 1988; Clegg et al. 1991; Wang et al. 1999). The region is a good target for the intraspecific genetic marker for evolutionary research by amplification and sequencing (Taberlet et al. 1991). Recently, many researchers have been trying to use the non-coding region to establish a barcoding system. The trnL-trnF intergenic spacer, matK, ndhF, atpB, and rbcL are the main targets from the chloroplast (Kress et al. 2005). The trnT-trnF region of the chloroplast genome consists of one group I intron, one intergenic spacer, and one trnT exon. The group I intron is a well-conserved region encoding the self-splicing region of pre-tRNA (Michel and Dujon 1983; Cech 1988). In this study, we tried to find polymorphism from seven species or sub-species of pines using the intergenic spacer between trnT(UGU) and trnL(UAA) of chloroplast DNA. We analyzed the phylogenetic relationship between these species. All species or subspecies could be differentiated by the polymorphism.

Materials and Methods

Plant materials and DNA extraction

We collected leaves from the pine species *P. densiflora*, *P. koraiensis*, and *P. bungeana* in Chungbuk National University, Cheongju, Korea and *P. densiflora* for. multicaulis, *P. sylvestris*, *P. rigida*, and *P. rigitaeda* in Korea Forest Research Institute, Suwon, Korea (Table 1). DNA was extracted from 20 mg of leaf tissue using TissueLyser (Qiagen, Germany) and DNeasy plant mini kit (Qiagen, Germany) according to the manufacturer's instruction.

PCR amplification

Primers (primers a, b, c, and d) were designed from the intergenic spacer of trnT(UGU) and the trnL(UAA)3' exon (Taberlet et al. 1991). For more efficient PCR amplification of pine sequences, we designed two new primers (primers e and f) (Table 2). Primer locations are described in Fig. 1. The reaction mixture contained 100 ng pine genomic DNA, 10X PCR buffer, 2.5 mM MgCl2, 0.2 mM dNTP, 50 nM forward and reverse primers, and 1 unit of Taq DNA polymerase (Qiagen, Germany). The PCR pro-

Table 1. List of sampled species

No.	Taxon	Province	Repository	Number of samples
1	*P. densiflora*	Chungbuk, Cheongju	Chungbuk National University	15
2	*P. densiflora* for. *multicaulis*	Gyeonggi, Suwon	Korea Forest Research Institute	10
3	*P. sylvestris*	Gyeonggi, Suwon	Korea Forest Research Institute	6
4	*P. rigida*	Gyeonggi, Suwon	Korea Forest Research Institute	6
5	*P. rigitaeda*	Gyeonggi, Suwon	Korea Forest Research Institute	5
6	*P. koraiensis*	Chungbuk, Cheongju	Chungbuk National University	6
7	*P. bungeana*	Chungbuk, Cheongju	Chungbuk National University	6

Table 2. The primer names and sequences

Primer name	Marker	Primer sequence (5'→')	Tm	Reference
a	trnT(UGU)	CATTACAAATGCGATGCTCT	64.2	Taberlet et al., 1991
b	trnL(UAA)5' exonR	TCTACCGATTTCGCCATATC	66.2	Taberlet et al., 1991
c	trnL(UAA)5' exonF	CGAAATCGGTAGACGCTACG	70.3	Taberlet et al., 1991
d	trnL(UAA)3' exonR	GGGGATAGAGGGACTTGAAC	70.3	Taberlet et al., 1991
e	trnT(UGU) redesign	GATTCAATGACGCAATCCAG	57.5	*In this study*
f'	trnL(UAA)3' exon redesign	TCGTCCAACCATTTATTCCA	57.3	*In this study*

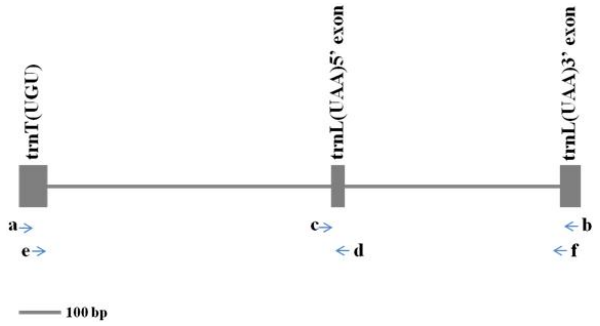

Fig. 1. Positions of primers used to amplify two non-coding regions between the trnT(UGU) and trnL(UAA) 3' exons of chloroplast DNA.

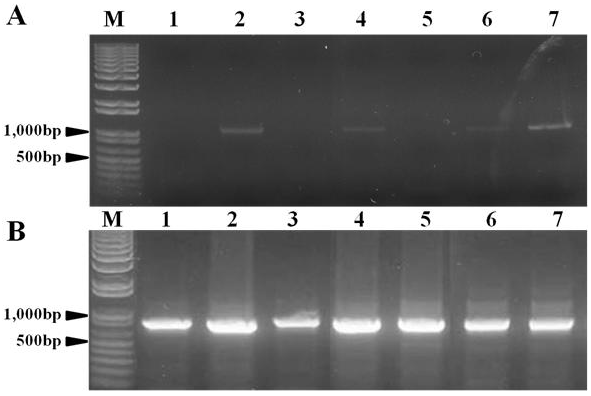

Fig. 2. PCR amplification of the trnT(UGU)- trnL(UAA)3' exon region of seven *Pinus* species on 1% agarose gel. Marker is 1 kb plus DNA ladder. A, primers a and b; B, primers c and d; 1, *P. densiflora*; 2, *P. densiflora* for. multicaulis; 3, *P. sylvestris*; 4, *P. rigida*; 5, *P. rigitaeda*; 6, *P. koraiensis*; and 7, *P. bungeana*.

gram of the T-gradient cycler (Biometra, Germany) was 94°C for 5 min, 35 cycles of 94°C for 30 s, 56°C for 45 s, and 72°C for 30 s followed by 72°C for 5 min. PCR products were separated using 1% agarose gel a nd stained with Gelstar (Lonza, USA).

Sequencing of PCR products

Sequencing reactions were performed in a MJ Research PTC-225 peltier thermal cycler using an ABI PRISM BigDye terminator cycle Sequencing kit with Ampli-taq DNA polymerase (FS enzyme), following the protocols supplied by the manufacturer. Single-pass sequencing was performed on each template using the PCR primers of both sides. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

Sequence alignment and phylogenetic analysis

DNA sequence was collected and analyzed using ClustalX and CLC Main Workbench programs (CLC bio, Denmark). From the seven pine species, the sequences with no gap were aligned using ClustalX (Thompson et al. 1997). The gaps generated after alignment were designated as missing characters and assigned the same weight for all characters. Phylogenetic analysis was performed using CLC Main Workbench and the phylogenetic tree was constructed using the maximum likelihood method.

Results and Discussion

To find the SNP markers for differentiation of seven *Pinus* species—*P. densiflora*, *P. densiflora* for. multicaulis, *P. sylvestris*, *P. rigida*, *P. rigitaeda*, *P. koraiensis*, and *P. bungeana*—the chloroplast non-coding region of the trnT(UGU)-trnL(UAA)3' exon region (Taberlet et al. 1991) was amplified. Four primers were synthesized according to Taberlet et al. (1991) and located on the trnT(UGU)-trnL(UAA)3' exon region (Table 2). Primer a was located

Table 3. *Pinus* species used in this study

Subgenus	Section	Subsection	Species	Accession[a]
Pinus[b]	*Pinus*	*Pinus*	*P. densiflora* Sieb. Et Zucc.	JX504689
			P. densiflora for. *multicaulis*	JX504684
			P. sylvestris L.	JX504686
	Trifoliae	*Australes*	*P. rigida*	JX504685
			P. rigitaeda	JX504688
Strobus	*Strobus*	*Cembrae*	*P. koraiensis* Sieb. Et Zucc.	JX504690
	Quinquefoliae	*Gerardianae*	*P. bungeana* Zucc.	JX504691

[a]Sequence of trnT(UGU)- trnL(UAA)3' exon region.
[b]Pinus classification is based on Gernandt et al. (2005).

in the trnT(UGU) region, and primers b and c were located on the trnL(UAA)5' exon in forward and reverse direction, respectively. Primer d was located on the trnL(UAA) 3' exon (Fig. 1). Primer combinations (a+b, a+d, c+d) were used for the amplification of *Pinus* DNA. Combinations a+b and c+d did not show the amplification. Combination a+d showed a single amplicon of about 1,000 bp, but the amplifications were not good enough for sequencing analysis (Fig. 2A). Therefore, we designed new primer sets (primers e and f) based on the sequences of the pine trnT(UGU)-trnL(UAA)3' exon region amplified by the primer combination a+d. The region was efficiently amplified by the new primer combination e+f (Fig. 2B). Using trnT(UGU)(primer e) and trnL (UAA)3' (primer f) exon reverse primers, six to fifteen samples from each seven species or subspecies (Table 1) were amplified, purified, and sequenced by the Sanger method. The trnT(UGU)-trnL(UAA)3' exon region was 995～1095 bps long and the sequence was deposited at GenBank (Table 3). After the sequence quality analysis, the sequence was aligned using ClustalX and CLC Main Workbench. From the aligned sequences, we found 113 points of polymorphic sites, including SNP and insertion/deletion (INDEL) (Table 4). The sequences of the same species or sub-species were identical. Seven species were differentiated by the sequence alignment, with each species having a unique sequence from the sequenced region.

The maximum likelihood tree was constructed using CLC Main Workbench. Seven species of pines formed 3 clades (Fig. 3): clade 1 includes *P. densiflora*, *P. densiflora* for. multicaulis, and *P. sylvestris*; clade 2 has *P. rigida*, and *P. rigitaeda*; and clade 3 has *P. koraiensis* and *P. bungeana*.

Pines are economically important for their high productivity of pulp and resins, such as terpene and pitch. *P. densiflora* is a typical pine, used for various purposes because of its good wood quality. *P. densiflora* and *P. sylvestris* look very similar because of their anatomical structure of window-like pits and dentate ray tracheids.

DNA barcoding is a method used to differentiate species, identifying plants using relatively short fragments of DNA sequence. This technique is being used for evaluation of the diversity of nature. The technique can be performed using small parts of the organism, so it overcomes the limitation of morphological classification (Hollingsworth et al. 2009). In this technique, non-coding regions of DNA are particularly important, as they tend to have the most variation (Fazekas et al. 2008). DNA barcoding technology is being used for various purposes in the tree industry. It is used for analysis of heterogeneity of trees and cultivar identification markers for walnuts and chestnuts (Marinoni et al. 2003; Kim et al. 2012b), and for *Brasenia schreberi* in South Korea (Kim et al. 2012a).

In this study, we have investigated the genetic variation of *Pinus* species based on DNA sequence variation. We designed a new set of PCR primers that can effectively amplify the chloroplast trnT(UGU)-trnL(UAA)3' exon inter space. By sequence analysis, we could differentiate the seven species of pines tested. If we can develop a method that we can use to extract the DNA from processed wood, we can use the markers to identify the tree species used for wood processing (Rachmayanti et al. 2006).

P. densiflora, *P. densiflora* for. multicaulis, and *P. sylvestris* were grouped to clade 1 (Fig. 3). *P. densiflora* and *P. syl-*

Table 4. Placement of phylogenetic informative SNPs and Indels in the aligned sequences from seven *Pinus* species

Gene region	trnT(UGU)-trnL(UAA) 3'exon																																								
Species	9	58	92	103	135									144	221	258	271	274																							297
P. densiflora	G	A	C	T	A	G	A	A	G	T	A	A	G	G	T	G	A	G	G	A	G	A	G	A	A	G	G	G	G	A	G	A	G	A	A	G	G	G	G	A	G
P. densiflora form. *multicaulis*	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
P. sylvestris	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	–	–	–	–
P. rigida	·	·	·	C	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	T	·	·	·	T	·	·	·	·	·	·	·	·	·	·	G	–
P. rigitaeda	·	·	·	C	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	·	·	·	T	·	·	·	T	·	·	·	·	·	·	·	·	·	·	–	–
P. koraiensis	T	G	A	·	–	–	–	–	–	–	–	–	–	–	G	A	T	C	·	·	·	·	·	·	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
P. bungeana	T	G	A	·	–	–	–	–	–	–	–	–	–	–	G	A	T	C	·	·	·	·	T	·	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

Gene region	trnT(UGU)-trnL(UAA) 3'exon																																								
Species	298																	315	316														330	335				339	355	370	419
P. densiflora	A	G	A	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	G	G	G	G	A	G	T	A	G	G	G	G	A	G	T	G	A	G	T	A	C	G	T
P. densiflora form. *multicaulis*	·	·	·	·	–	–	–	–	–	–	–	–	–	–	–	–	–	–	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
P. sylvestris	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
P. rigida	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	·	A	·	·	A	G	G	·	·	A	·	·	·	A	·	·	·	·	·	·	A	·
P. rigitaeda	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	·	A	·	·	A	G	G	·	·	A	·	·	·	A	·	·	·	·	·	·	A	·
P. koraiensis	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	·	·	·	·	·	A	·	A
P. bungeana	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	G	T	A	G	A	·	A

Gene region	trnT(UGU)-trnL(UAA) 3'exon																												
Species	703	722	731									740	761				765	777				781	804					809	825
P. densiflora	A	A	G	A	–	–	T	G	T	T	A	G	–	–	–	–	–	–	–	–	–	–	–	G	–	–	–	–	G
P. densiflora form. *multicaulis*	·	·	·	·	–	–	·	·	·	·	·	·	–	–	–	–	–	–	–	–	–	–	A	·	–	–	–	–	·
P. sylvestris	·	·	·	·	–	–	·	·	·	·	·	·	–	–	–	–	–	–	–	–	–	–	A	·	–	–	–	–	·
P. rigida	·	·	·	·	–	–	·	·	·	·	·	T	–	–	–	–	–	T	G	A	A	T	A	·	–	–	–	–	A
P. rigitaeda	·	·	·	·	–	–	·	·	·	·	·	T	–	–	–	–	–	T	G	A	A	T	A	·	T	A	A	G	A
P. koraiensis	C	G	T	·	–	–	·	T	·	·	·	T	A	C	T	G	G	T	G	A	A	T	A	·	–	–	–	–	·
P. bungeana	C	G	T	·	–	–	·	T	·	·	·	T	A	C	T	G	G	T	G	A	A	T	A	·	–	–	–	–	·

[a]Dashes represent alignment gaps; Dots indicate the same nucleotide as the reference *P. densiflora*.

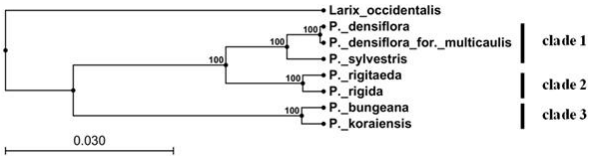

Fig. 3. Phylogenetic tree of trnT(UGU)-trnL(UAA)3' exon region sequences of seven *Pinus* taxa. The tree was made using the maximum likelihood method of CLC Main Workbench program. Bar indicates distance.

vestris were grouped together from previous studies (Wang et al. 1999). This result means that these two species are genetically very close. *P. densiflora* for. multicaulis is also grouped together with *P. densiflora*, showing that they are very closely related species. Clade 2 includes *P. rigida* and *P. rigitaeda*. *P. rigitaeda* differs in many sites from *P. rigida*, even though it is a hybrid species of *P. rigida* and *P. taeda*. Clade 3 includes *P. bungeana* and *P. koraiensis*. *P. bungeana* has five leaflets in a bundle and *P. koraiensis* has only three. In the analysis of DNA sequence, *P. bungeana* and *P. koraiensis* are very close. We suppose that these two species are phylogenetically close.

P. densiflora for. multicaulis is a subspecies of *P. densiflora* and has the shape of an umbrella. Grafted *P. densiflora* is sold as *P. densiflora* for. multicaulis because the market price of *P. densiflora* for. multicaulis is higher than for *P. densiflora*. The markers developed in this report could be used for the differentiation of *P. densiflora* for. multicaulis from grafted *P. densiflora*. Gernandt et al. (2005) classified the pines using rbcL and matK regions. Our grouping is similar to their result because we both group *P. densiflora*, *P. densiflora* for. multicaulis, and *P. sylvestris* together. Our grouping of *P. rigida* and *P. rigitaeda* is different from their result, possibly because of the usage of markers. The trnT(UGU)-trnL(UAA)3' exon inter space region could be a good candidate for differentiation of seven species of pine. The marker, based on the chloroplast genome, can be used for identification of the tree species from processed wood.

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