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海岸林学会誌 (*Journal of the Japanese Society of Coastal Forest*) 1 (2): 1 − 5, 2002

Genetic variation of *Pinus densiflora* and *P. thunbergii* using molecular markers (II)

- Population differentiation of *P. densiflora* in Kangwon province of Korea − *

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Abstract: The population differentiation in seven natural populations of *Pinus densiflora* was studied based on RAPD markers. Four trees were sampled from each population. Six megagametophytic DNAs per tree were used as template DNAs for genotyping of a seed tree. Two hundred and fifty bands resolved in RAPD with twenty random primers were used to measure the genetic variation of population. The level of genetic diversity was relatively high (A=1.92, P_{95}=92.7, H_o=0.56, H_e=0.40) and the degree of genetic differentiation among populations (F_{ST}=0.135) was higher compared with those of other pines. The rate gene flow was estimated high (N_m=1.604). The mean values of genetic distance (0.083) was a very low differentiation and a close genetic relationship in *P. densiflora* of Korea. Especially, we could find statistically significant relationship between genetic and geographic distances (r= 0.8337, P<0.001). The results obtained suggests that RAPD markers are valuable for the estimation of genetic diversity and for the study of the divergence among population in *P. densiflora*.

Keywords *: Pinus densiflora, RAPD, Genetic diversity, Genetic differentiation, Genetic distance*

1 Introduction

Genetic markers are very important tools for investigating genetic variation of tree species in tree improvement and forest population genetics. In recent years, as DNA-based genetic markers have been developed, it is generally accepted that RAPD markers have the potential to overcome some of limitations of isozyme and will be a powerful tool for genetic studies and breeding work for several reason: 1) A large amount of polymorphism can be detected in any taxon, fast and precisely; 2) it can allow investigation of not only coding but also noncoding sequence variation; 3) it is usually possible to determine the mutation differences among DNA variants, which strengthen(Williams et al., 1990; Krutovskii et al., 1999; Dvorak et al., 2001; Gomez et al., 2001).

Isozyme markers have been applied extensively during the past 15 years in genetic variation studies of tree species. However, it has been considered to be inadequate for detecting polymorphism in natural populations because of the limitation in number of isozyme marker loci available (Kim, 1995; Lee et al., 1998; Aravanopoulos, 2001).

It is known that there are six distinctive geographic races of *P. densiflora* (Uyeki, 1928). On the other hand, genetic structure studies based on isozymes reports very little population differentiation in the species. Thus, it may be of worth to study genetic structure of the species using RAPD and find out if it produces the same results as the previous isozyme studies.

*This study was funded partly by Korea Research Foundation to Jae-Seon Yi (95/G5101/95-013-176).

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The objective of this study were to investigate genetic variation and structure of 7 natural populations of *P. densiflora* in Kangwon province of Korea and to detect the degree of differentiation among these populations based on RAPD marker analysis. In addition this study will provide basic information for the study of introgressive hybridization of *P. densiflora* and *P. thunbergii*, which are believed to hybridize easily in nature.

2 Materials and Methods

Cones were collected from four trees sampled in each of seven populations of *P. densiflora* (Fig. 1). DNA extractions were performed using a modified CTAB method (Doyle and Doyle, 1987).

Total DNAs were extracted from megagametophyte tissues (haploid tissue) of seeds. Six megagametophytic DNAs per tree were used as template DNAs in RAPD PCR for genotyping of a seed tree (Fig. 2).

3ng of genomic DNA were used for RAPD PCR in a 7 μℓ reaction containing 3.5mM Mgcl2, 1ng/㎖ BSA, 2mM dNTP's, 0.497 Unit of Taq DNA Polymerase, 4μ M primer (Operon Technologies, Alameda, Calif., USA) and 1.0x reaction buffer. The reaction was overlaid with one drop of mineral oil.

Amplification was performed in a MJ Research 100 DNA Thermal Cycler with 40 cycles of 92℃ for 1 min, 40℃ for 1 min and 72℃ for 2 mins. Amplified products were analysed on a 2.0% agarose gel for 2.5 hrs at 180 volts in 1x TAE buffer (0.4 M Tris base, 9.5 μ M ethidium bromide, 1.14% glacial acetic acid, and 1mM Na-EDTA). Gel images were captured with the program provided by SciTech Pty Ltd (Plenty Rd, Preston South, Vic, Australia), printed, and stable

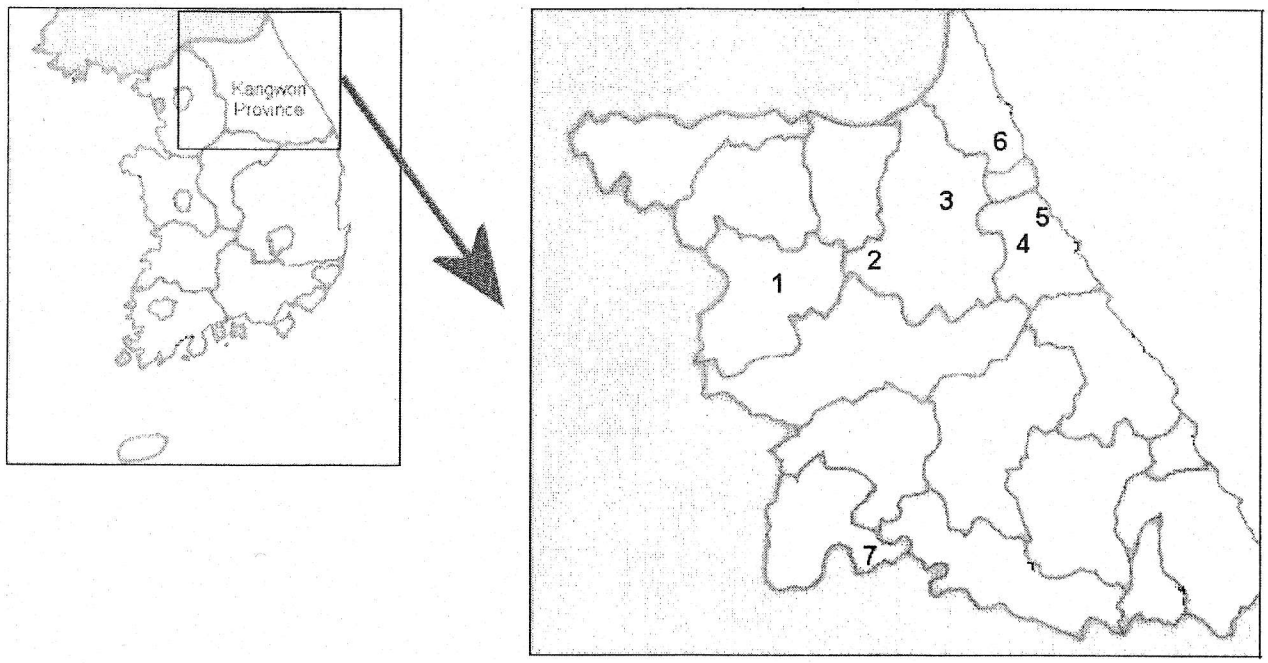

Figure 1: Location of the seven populations of *P. densiflora* in Korea

1. Hyoja-Dong, Chunchonsi 2. Sinnam-Myun, Inje-Gun 3. Buk-Myun, Inje-Gun 4. Seo-Myun, Yangyang-Gun 5. Sonyang-Myun, YangYang-Gun 6. Toseong-Myun, Goseong-Gun 7. Synrim -Myun, Wonju-Si.

heterozygous markers were scored.

In order to identify primers that detect polymorphism among seeds from a single tree, 30 primers were used in PCR with DNA from six seeds. Of 30 primers, 20 primers were finally chosen and used to score for segregation of the polymorphic bands among 168 seeds. 250 bands were scored in RAPD PCR with 20 primers. Usually, products below 500 bp or above 2000 bp gave faint and non-reproducible bands, hence most of the scored products are in the range 0.5～2.0 kb.

Allele frequencies were analysed using the POPGENE (population genetic analysis, ver. 1.31: Yeh and Yang, 1999) computer program. For each population, average number of alleles per locus (A), percentage of polymorphic loci (P_{95}: 95% criterion), observed heterozygosity (H_o), and expected heterozygosity were computed from the frequencies of alleles at 250 loci.

For assaying the populations genetic structure the fixation indices (F_{IS}, F_{IT} and F_{ST}) were used (Wright, 1978). F_{IS} and F_{IT} measure the deviation of genotype frequencies from Hardy–Weinber proportions in the populations and in the total population, respectively, whereas F_{ST} measures the degree of genetic differentiation among populations. In addition, the amount of gene flow among populations (N_m) was calculated from Wright's F_{ST}. Finally, the relationships among the populations were visualized through the construction of dendrogram by the unweighted pair–group method with arithmetic means(UPGMA; Nei, 1978).

Table 1: Genetic variability at 250 loci in 7 populations of *Pinus densiflora* (standard deviation in parentheses)

	Population	A	P_{95}	H_0	H_e
1	Hyoja-Dong, Chooncheon-Si	1.90 (0.30)	90.0	0.59 (0.31)	0.38 (0.16)
2	Sinnam-Myun, Inje	1.89 (0.31)	89.2	0.64 (0.32)	0.39 (0.16)
3	Buk-Myun, Inje-Gun	1.92 (0.27)	92.4	0.64 (0.31)	0.40 (0.14)
4	Seo-Myun, Yangyang	1.91 (0.28)	91.2	0.68 (0.31)	0.41 (0.15)
5	Sonyang-Myun, YangYang-Gun	1.95 (0.21)	95.2	0.73 (0.27)	0.43 (0.12)
6	Toseong-Myun, Goseong-Gun	1.96 (0.20)	96.0	0.68 (0.27)	0.43 (0.12)
7	Synrim-Myun, Wonju,-Si	1.93 (0.26)	92.8	0.54 (0.30)	0.37 (0.14)
	Mean	1.92	92.7	0.56	0.40

A is the number of alleles per locus; *P95* is the percentage of polymorphic loci at 95% level; *Ho* is the observed heterozygosity; *He* is the unbiased expected heterozygosity, as proposed by Nei (1973).

3 Results and Discussion

A total of 250 bands were scored in RAPD PCR with 20 primers. The mean number of alleles per locus ranged from 1.89 to 1.96, and averaged 1.92. The proportion of polymorphic loci ranged from 89.2% to 96.0%, with an average of 92.7%. The observed heterozygosity was from 0.54 to 0.73, with an average of 0.56. The mean value of expected heterozygosity was higher and amounted to 0.40, with variation from 0.37 to 0.43 (Table 1).

When the level of genetic diversity of *P. densiflora* was compared to those of other pine species, it was higher than those of *P. wallichiana* (A=1.85, P_{95}=54.8, H_o=0.163, H_e=0.168, Lee et al., 1998), of *P. sylvestris* (A=3.2, P_{95}=68.8, H_o=0.242, H_e=0.283, Puglisi and Attolico, 2000), and of *P. Koraiensis* (A: 1.93, P_{95}: 49.2, H_o: 0.182, H_e: 0.183, Potenko and Velikov, 1998).

The F_{IS} and F_{IT} values of Wright (1978) measure deviations from the Hardy-Weinber expectations across all populations for each locus at the population and the whole species level, respectively (Table 2). The value of F_{IS} varied among 250 loci with a mean -0.602. This means that there is 60.2% excess of heterozygosity relative to Hardy-Weinberg expectations within a population. Assuming the entire species as one large random mating population, the total fixation index, F_{IT} was larger than F_{IS} in all loci. The mean of F_{IT} over all loci was -0.386, which indicates 38.6% of hetrozygosity excess as a whole. The population substructuring was indicated by an overall F_{ST} value of 0.135. Thus, the total percentage of genetic variation present in the population explained by among population differences is 13.5%. This value is very high compared to those of other pine species and allozyme study (Szmidt and Wang, 1993; Kim and Lee, 1995; Lee et al., 1998; Potenko and Velikov, 1998) and greatly similar to the result of a previous study based on RAPD in *P. densiflora* (Kim, 1995).

Also, the estimated gene flow based on F_{ST} was relatively high (N_m=1.604). This result reflects the long distance of pollen dispersion in *Pinus* species, but it is discorddant with the level of F_{IS}.

Table 2: Wright's F-statics and gene flow for the 250 loci

Locus	F_{IS}	F_{IT}	F_{ST}	N_m*
B01P1-01	-0.6000	-0.4583	0.0885	2.5735
B01P1-02	-0.6000	-0.4583	0.0885	0.5735
B01P1-03	-0.7524	-0.6970	0.0316	7.6563
.	.	.	.	.
.	.	.	.	.
.	.	.	.	.
B01P20-248	-0.6604	-0.5795	0.0487	4.8816
B01P20-249	-0.6970	-0.5174	0.1058	2.1128
B01P20-250	-0.6000	-0.4359	0.1026	2.1875
Mean	-0.6024	-0.3863	0.1349	1.6037

* N_m = Gene flow estimated from $F_{ST} = 0.25(1-F_{ST}) / F_{ST}$.

Table 3: Matrix of Nei's genetic distance (bellow diagonal) and genetic identity (above diagonal) among 7 populations of *Pinus densiflora*

Population	1	2	3	4	5	6	7
1	***	0.9335	0.9264	0.9248	0.9027	0.9146	0.8486
2	0.0688	***	0.9407	0.9462	0.9244	0.9210	0.8682
3	0.0764	0.0611	***	0.9507	0.9614	0.9547	0.8751
4	0.0782	0.0553	0.0506	***	0.9616	0.9444	0.8892
5	0.1024	0.0786	0.0394	0.0392	***	0.9730	0.8840
6	0.0893	0.0822	0.0463	0.0572	0.0273	***	0.8875
7	0.1642	0.1413	0.1334	0.1174	0.1233	0.1194	***

Population 1 ~ 7: As in Table 1.

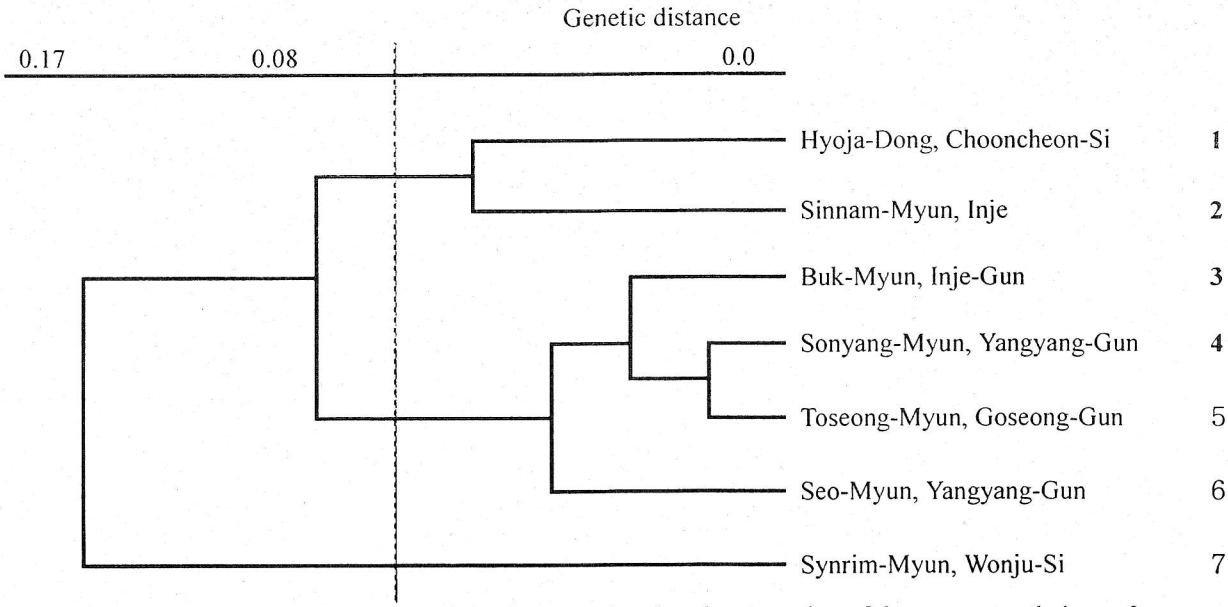

Figure 2: UPGMA-derived dendrogram showing the clustering of the seven populations of *Pinus densiflora* based on the genetic distance of Nei (1978).

The genetic distance of Nei (1978) were low between seven populations of *P. densiflora* and averaged 0.083. The largest value (0.164) was detected between Choonchon (Pop. 1) and Wonju (Pop. 7) population, both of which pertain to different population groups (Table 3). In addition, the genetic identity values among pairs of populations range from 0.849 to 0.973.

The similarity among *P. densiflora* populations can be seen in the UPGMA dendrogram, where total populations cluster at below a genetic distance of 0.17 (Fig. 2). Seven populations could be classified into 3 groups: Namely, group Ⅰ is Wonju population, group Ⅱ is Chooncheon and Inje (Sinnam-Myun) population, group Ⅲ is composed of Inje, Goseong, and 2 Yangyang populations. Namely, geographically close populations showed a tendency of clustering into the same group. Especially, we could find statistically significant relationship between genetic and geographic distances ($r= 0.8337$, $P<0.001$).

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〔Received October 2 ,2001, Accepted December 20,2001〕