Discordance Between Geographical Distribution and Genetic Relationship Among Populations of Japanese Red Pine in Korea Revealed by Analysis of I-SSR Markers

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Abstract

Level and distribution of genetic diversity in 8 populations of Japanese red pine in Korea were estimated using I-SSR variants. A total of 80 I-SSR variants were observed in the analyzed 150 individuals, which revealed DNA fingerprints-like individual specific amplicon profiles for all of them. Relatively higher level of genetic diversity within populations was observed in 8 populations of Japanese red pine (mean of 0.450) than in other tree species. From the results of AMOVA, majority of genetic diversity was allocated within populations (93.42%) resulting in a moderate degree of population differentiation (Φ_{ST} = 0.066). The observed distribution pattern of I-SSR variants among 8 populations was coincided with the typical patterns for the long-lived woody species. Genetic relationships among the populations, reconstructed by UPGMA and Neighbor-Joining methods, revealed 2 genetic groups. The populations of Gangwon-Uljin and Chungnam-Taean turned out to be the most closely related despite a distant location between them. The overall genetic relationships among the 8 populations, reconstructed by both methods, were not coincided with geographic distances. The discrepancy between genetic relationships and geographical distribution among the populations suggests that the analyzed populations might have undergone random changes in genetic composition due to some kinds of disturbances. Results obtained in this study suggests that more careful approach should be made in preparing strategy for gene conservation of Japanese red pine in Korea. More information on countrywide molecular population genetic status of Japanese red pine will be helpful to prepare more reasonable strategy for gene conservation of the species in the country.

Key words: Pinus densiflora, Japanese red pine, I-SSR markers, Genetic diversity, AMOVA, Population differentiation, Genetic relationships.

Introduction

Japanese red pine (*Pinus densiflora* Sieb. et Zucc.), which belongs to the subsection Sylvestres of the subgenera Pinus (diploxylon or hard pines), is widely distributed in Korea, China (Shandong, Jiangsu), Japan (Honshu, Shikoku, Kyushu), and Southern Ussuriland (MIROV, 1967; LEE, 1987). In Korea, Japanese red pine is one of the most representative trees, and holds an important position in forest ecology and forestry. According to fossil records, this species is known to be an ancient tree of the Korean peninsula (MIROV, 1967; EARLE, 1999). It occurs naturally within pure stands in different sites of various soils and climatic conditions (LEE, 1987). However, taken into account its high wood quality, this species has been extensively used as materials for building construction, ship building, furniture, coffins, firewood, and different valuable by-products (LEE, 1987; FRI, 1999). This high frequent use has devastated the Japanese red pine forests in Korea. In addition, from the 16th century to the middle of 20th century, the repeated invasions by neighboring countries had made natural forests undergo severe damage. Therefore, Korean government have made tremendous efforts to reforest this pine in every place of the country over several centuries (reviewed in FRI, 1999), as a result, Japanese red pine forests have been retrieved its former position. Accordingly, for the more effective uses and management of this species in the future, we should make an effort to understand population genetic architecture of the current forests by broadening the knowledge on the level and distribution of genetic diversity.

Most of inter-simple sequence repeat (I-SSR) markers have been known to be dominant markers in terms of phenotype shown on the gel (i.e., presence vs. absence of PCR product of the same size). Therefore, the phenotype of heterozygous state is undistinguishable from that of homozygous state with presence of PCR product in diploid genome. Such undistinguishable genotypic states in diploid genome restrict application of the I-SSR markers to the various estimation of population genetic statistics, such as G-statistics and F-statistics. However, Mendelian inheritance of I-SSR variants has been reported in the analysis of the large number of megagametophytes (i.e., haploid genome) from a single tree of Douglas-fir and sugi families (TSUMURA *et al.,* 1996), and *Abies koreana* (HONG *et al.,* 1998), which verified the I-SSR variants as informative genetic markers. Additionally, in spite of the above-mentioned disadvantage of the I-SSR markers, there are a couple of advantages of I-SSR markers that characterize I-SSR markers to be eligible for population genetic studies. Because SSRs are known to be distributed over the whole genome of plant and animal species [e.g., over 50,000 copies of SSR motifs of $(AG)_n$ and $(CT)_n$ in pines; reviewed in TSUMURA *et al.,* 1996], multiple amplification products could be generated by a single PCR according to the number of priming sites which should be located inversely oriented within the range of successful PCR (approximately <3kb) on the alternate DNA strands. Furthermore, large numbers of I-SSR markers could be generated by using various kinds of primers consisted of simple sequence repeats (SSRs) motifs with a couple of additional arbitrary nucleotides at the 3'-end. The characteristic features of I-SSR enable the PCR-amplified I-SSR markers to be suitable for analyzing genetic status of the populations (SAGHAI *et al.,* 1994; GODWIN *et al.,* 1997; HONG *et al.,* 2000, 2001, 2003; JOSHI *et al.,* 2000; YANG *et al.,* 1996; PARSONS *et al.,* 1997; SALIMATH *et al.,* 1995).

Although I-SSR markers could be analyzed for population genetic study, estimation of different population genetic statistics should be unavoidable on account of the dominat phenotype of the variants. A statistical approach of analysis of molecular variance (AMOVA) was initially introduced as an extension of the analysis of genetic frequencies (COCKERHAM, 1973; LONG, 1986; WEIR and COCKERHAM, 1984) for molecular haplo-

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types in an essentially haploid system. The typical input for AMOVA consisted of a matrix of pairwise Euclidean distance between all multisite haplotypes and files containing the frequency of those haplotypes within each populations. Components of variance of the genetic distance attributable to differences between groups, between populations within groups, and between individuals within populations were estimated from this matrix using AMOVA (EXCOFFIER *et al.,* 1992). Therefore, such variance components represent the estimates of the genetic diversity, which could be partitioned into between groups, between populations within groups, and between individuals within populations.

In the present paper, the extent and distribution of I-SSR variants were estimated in the eight populations of Japanese red pine in Korea, which were also compared with other previous results obtained by analyzing other genetic marker systems. This study might provide valuable information for the establishment of efficient conservation practices on Japanese red pine in Korea.

Materials and Methods

Plant Material and DNA Extraction

Open-pollinated seeds were collected from 150 individual trees, representing eight populations that are located within the native range of Japanese red pine in South Korea (*Fig. 1*). For each population, 17–20 trees were selected which were spacing a minimum distance of 50m in order to decrease the risk of genetic uniformity. Collected seeds were stored at –20 °C until DNA extraction. Ten megagametophytes were pooled from each individual tree. Total DNA was extracted from the pooled samples by the modified CTAB method (HONG *et al.,* 1993). The amount of DNA was indirectly quantified by agarose gel electrophoresis with known quantity standard of uncut λ-DNA.

PCR Amplification and Electrophoresis

Polymerase chain reactions (PCR) were carried out in a volume of 20μl with final concentration of 10 ng of template DNA; 1x PCR reaction buffer, 1.75 mM of $MgCl_2$, 0.2 mM of dNTPs, 0.0025% BSA, 0.6 unit of Taq DNA polymerase (Advanced Biotechnologies Ltd., UK) and 0.4 μM of I-SSR primer. Amplifications were performed in a Primus 96 plus thermocycler (MWG AG Biotech, Germany) using a period of 5 min of initial denaturation at 94 °C, followed by 45 cycles of 30 sec of denaturation at 94 °C, 30 sec of annealing at 52 °C, 1 min of extension at 72 °C, and a final extension step of 10 min at 72 °C. PCR products were separated by electrophoresis in 2% (w/v) agarose gel prepared with 1X TBE buffer (pH 8.0) containing ethidium bromide (125 ng per 250ml of gel mixture). A 100 bp marker (GeneRuler™ 100bp DNA Ladder Plus, MBI Fermantas, Lithuania) was used a reference for sizing the amplocon obtained. After electrophoresis, gel was photographed over UV trans-illuminator. For semi-automated analyses, pipetting and gel loading was performed using RoboSeq® 4204S Automated Biosystem (MWG AG Biotech, Germany). Six I-SSR primers that gave clear and reproducible fragment patterns were selected for final analysis: UBC #809 (5'-AGAGAGAGAGAGAGAGG-3'), #811 (5'-GAGAGAGAGAGAGAGAC-3'), #818 (5'-CACACACACACACACAG-3'), #826 (5'-ACACACACACACACACC-3'), #846 (5'-CACACACACACACACA(AG)T-3'), and #873 (5'-GACAGACAGACAGACA-3').

Data Analysis

Variants of the I-SSR amplicon were recorded as the presence (1) versus the absence (0) of the same size on the gel. The Shannon's index (S.I.; SHANNON, 1948) was calculated using the POPGENE 1.31 program (YEH *et al.,* 1999) to estimate the distribution of I-SSR amplicon variants among the categories of presence or absence within population. Degree of genetic differentiation among populations was estimated at 2 and 3 hierarchical levels using AMOVA v1.55 (EXCOFFIER *et al.,* 1992) on the basis of genetic distance calculated by Euclidean metric of EXCOFFIER *et al.* (1992). Genetic relationships among populations were reconstructed by UPGMA and Neighbor-Joining methods (phylip v3.5c; FELSENSTEIN, 1993) on the basis of pairwise Manhattan distance (WRIGHT, 1978) between populations computed by RAPDDIST v1.0 (BLACK, 1996). Statistical test for the topology of each node was performed with 100 bootstrapped samples prepared by RAPDDIST v1.0 (BLACK, 1996).

Results and Discussion

Based on I-SSR PCR analyses, a total of 80 variants were observed in 8 populations of Japanese red pine by using 6 I-SSR primers [UBC #809 (12 variants), 811 (11), 818 (14; *Fig. 2*), 826 (17), 846 (16), 873 (10)]. On the basis of pooled phenotypic data of the observed 80 amplicon variants, all the analyzed 150 individuals revealed DNA fingerprints-like individual specific amplicon profiles. Relatively high level of genetic

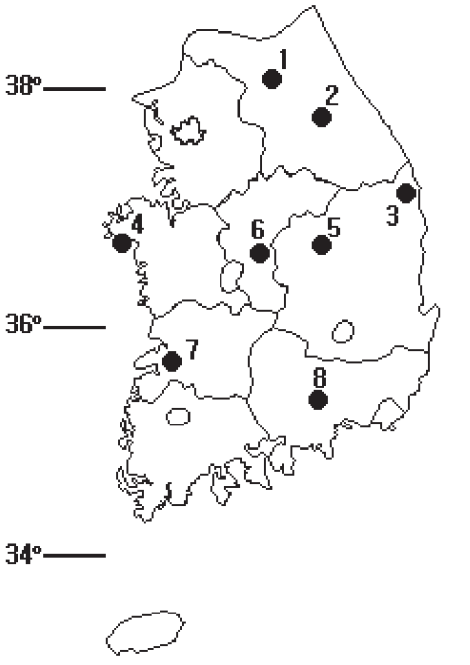

Figure 1. – Location of sampled populations. Population names refer to those corresponding to the numbers in *Table 1.*

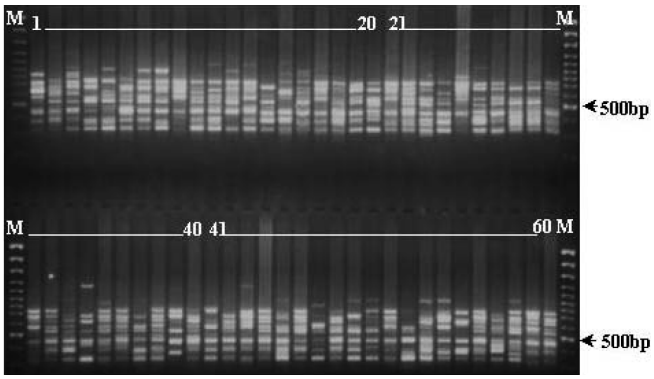

Figure 2. – Example of I-SSR amplicon profiles. I-SSR PCR was performed with UBC primer #818. 'M' denotes DNA size markers of 100bp ladder. Lane 1-20, Gangwon-Uljin population; Lane 21-40, Gangwon-Hongcheon population; Lane 41-60, Gangwon-Yeongwol population.

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Table 1. – Phenotypic diversity within population.

Population	Individuals	S.I.
1. Gangwon-Hongcheon	17	0.421
2. Gangwon-Yeongwol	19	0.472
3. Gyeongbuk-Uljin	19	0.442
4. Chungnam-Taean	20	0.451
5. Gyeongbuk-Mungyeong	20	0.411
6. Chungnam-Boeun	18	0.428
7. Jeonbuk-Buan	19	0.495
8. Gyeongnam-Haman	18	0.483
Average		0.450

Table 2. – Results of AMOVA for 8 populations. Groups were voluntarily designated on the basis of genetic relationship among populations observed in this study.

Source of variance	d.f.	Component Variance
Among populations	7	6.58%
Within populations	142	93.42%
Among groups	1	0.96%
Among populations within groups	6	6.03%
Within populations	142	93.01%

$\Phi_{ST} = 0.0699 : \Phi_{SC} = 0.0609 : \Phi_{CT} = 0.0096$
Φ_{ST}: genetic differentiation among populations
Φ_{SC}: genetic differentiation among populations within groups
Φ_{CT}: genetic differentiation between 2 genetic groups

diversity within populations was observed ranging from 0.411 (Gyeongbuk-Mungyeong) to 0.495 (Jeonbuk-Buan) with the mean of 0.450 (*Table 1*). The corresponding parameters in other tree species are as follows: *Torreya nucifera* : 0.353 (Hong *et al.,* 2000), *Ginkgo biloba* : 0.379 (Hong *et al.,* 2001), and *Rubus coreanus* : 0.242 (Hong *et al.,* 2003). Of the 8 populations, the populations of Gyeongbuk-Uljin and Chungnam-Taean turned out to be genetically most closely related (*Fig. 3*) showing similar level of genetic diversity within population (0.442 and 0.451, respectively).

The result of AMOVA with 8 populations revealed that majority of genetic diversity was allocated within populations (93.42%), which resulted in moderate degree of population differentiation ($\Phi_{ST} = 0.066$, *Table 2*). When AMOVA was performed at 3 hierarchical levels with 2 genetic groups designated on the basis of the results of genetic relationships among 8 populations, very low level of genetic differentiation was observed between groups (Φ_{CT}=0.0096). This observation suggests that majority of genetic differentiation is allocated among populations within each group and that voluntary grouping on the basis of genetic relationships is genetically unjustified. Observed degree of population differentiation in Japanese red pine ($\Phi_{ST} = 0.066$) was comparable with that reported for *Ginkgo biloba* (0.057; Hong *et al.,* 2001), *Fraxinus rhynchophylla* (0.066; Cho *et al.,* 2002), and *Fraxinus chiisanensis* (0.04; Cho *et al.,* 2002), but somewhat lower than those of *Torreya nucifera* (0.094; Hong *et al.,* 2000), *Fraxinus mandshurica* (0.10; Cho *et al.,* 2002), and *Rubus coreanus* (0.185, Hong *et al.,* 2003). In general, long-lived woody species maintain less than 10% of their genetic diversity among populations (Hamrick and Godt, 1990). The distribution pattern of genetic diversity among populations of Japanese red pine was coincided with the typical one for the long-lived woody species.

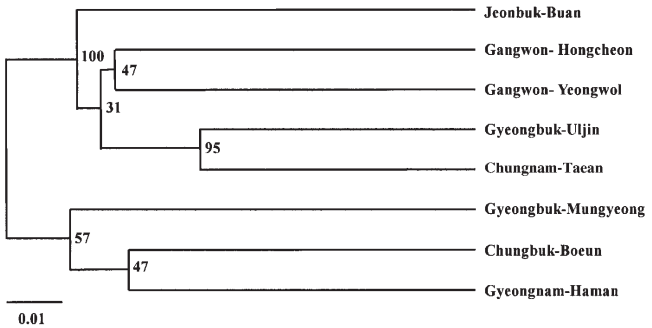

Figure 3. – Dendrogram reconstructed by UPGMA. Figures at the node represented confidence interval estimated from 100 bootstrapping. Cheonbuk-Buan population was used to root the tree.

Genetic relationships among the populations were reconstructed by UPGMA (*Fig. 3*) and Neighbor-Joining methods (figure not shown). Two genetic groups were observed in both analyses. Within a group of 5 populations, the populations of Gangwon-Uljin and Chungnam-Taean showed a high probability of sharing the same hypothetical ancestor, where the 95% confidence limit was estimated from the replicated analyses with 100 pseudo-replicate data sets prepared by bootstrapping. In general, if natural populations originated from the same hypothetical ancestors and underwent stable evolution, geographically close populations should show the closest genetic relationship. However, overall genetic relationships among the 8 populations, reconstructed by both methods, were not coincided with geographic distances among them. For example, the shortest genetic distance was observed between Gyeongbuk-Ulgin and Chungnam-Taean (0.0983), between which geographic distance is relatively far. The discrepancy between geographical distribution and genetic relationships among the analyzed populations suggests that they might have undergone the random changes in genetic composition due to some kinds of disturbances (i.e., logging, natural fire, artificial fire for preparing forest ground for cultivation, and afforestation etc.). These random changes might result in random genetic drift induced by the drastic changes in population size and transplantation from other populations for reforestation. Therefore, although high level of confidence limit (i.e., 95%) was observed for grouping of populations of Gangwon-Uljin and Chungnam-Taean, it might be induced by a chance of sampling for reforestation rather than sharing the same hypothetical ancestors for both populations. Some archival evidences for such postulation could be found in Pibyonsa Tungnok, the record for Pibyonsa that was the highest ruling organ at the end of the Chosun Dynasty (1616-1892 A.D.). Several records about warning against severe logging of Japanese red pines for ship building at Anmyon, which is located near Taean, as well as about encouraging reforestation of the exploited forests with seeds collected from superior Japanese red pines have been found in the archives from the 17th century (NIKH, 2001). In the light of both the presented results and historical records, the origination of the current Japanese red pine forest at Chungnam-Taean might be presumed that it might be reforested with the seeds or seedlings collected from the Japanese red pine forest at Gangwon-Uljin. However, in order to corroborate such presumption, further study should be done involving a larger number of populations from the whole country.

Observations on the distribution of I-SSR variants, degree of genetic differentiation, and genetic relationships among the populations of Japanese red pine in Korea suggest that more careful approach should be made in preparing strategy for gene conservation of Japanese red pine in Korea. For example, although Gangwon-Uljin and Chungnam-Taean populations are located far from each other, conserving only one of them might be enough for saving genetic variation in both populations because of their nearly identical gene pool resulting from a probable reforestation of Chungnam-Taean population with the seeds or seedlings collected from Gangwon-Uljin population since the 17th century. In order to prepare more reasonable

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strategy for gene conservation of Japanese red pine in Korea, more information on molecular population genetic status of the species should be provided throughout the country.

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