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Genetic Variation of Korean Pine (*Pinus koraiensis* Sieb. et Zucc.) at Allozyme and RAPD Markers in Korea, China and Russia

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Abstract

We studied and compared genetic variation of Korean pine (*Pinus koraiensis* Sieb. et Zucc.) from 12 natural populations in Korea, China, and Russian Far East using allozymes and random amplified polymorphic DNAs (RAPDs). Eighteen polymorphic allozyme loci and 38 polymorphic RAPD fragments were analyzed. The level of allozyme diversity (A = 1.95, P_{95} = 46.8%, H_o = 0.158, H_e = 0.169) and the degree of genetic differentiation (F_{ST} = 0.069) were comparable to those of other pines with similar life histories and ecological traits. Allozyme (H_e) as well as RAPD (Shannon's index) variation decreased from south (Korea) to north (Russia), providing an evidence for the hypothesis of Korean pine's northward migration. Differentiations among three different regions (Korea, China, and Russia) as well as among populations within regions were small. Substantial gene flow (N_m = 3.4) may be a partial explanation to this result. Clustering algorithms using various genetic distance measures showed some decisive geographic patterns at allozyme and RAPD level: the geographically close populations tended to be clustered together. On the other hand, two Chinese populations, Xobukho and Wangging, were grouped with the Russian populations rather than with the other Chinese populations. The Xiaoxing'anling and other mountains extended from north to south seemed to function as a barrier against gene flow between the Xobukho and Wangging (located east of the mountains) and the other Chinese *P. koraiensis* populations (located west of the mountains). The genetic diversities and differentiation estimated from RAPD data in Korean pine were congruent with those of allozymes.

Key words: Pinus koraiensis, allozymes, RAPDs, genetic variation, Korea, China, Russian Far East.

Introduction

The five stone pine species occur only in the Northern Hemisphere – one species in North America (*Pinus albicaulis*) and the other four in Eurasia and East Asia (*Pinus cembra*, *Pinus sibirica*, *Pinus pumila* and *Pinus koraiensis*). Taxonomically they fall under subsection *Cembrae* in section *Strobus*. They are characterized by having five needles in a fascicle, cones that do not open at maturity, and wingless seeds. CRITCHFIELD and LITTLE's (1966) maps show that the distribution of five

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species is mostly discrete, but there are overlapping distributions of Siberian stone pine (*P. sibirica*) and Japanese stone pine (*P. pumila*). Japanese stone pine also overlaps with Korean pine (*P. koraiensis*).

Korean pine (*P. koraiensis* Sieb. et Zucc.) occurs throughout Korea and eastern Manchuria into southeastern Siberia as well as on the islands of Honshu and Shikoku in Japan (CRITCHFIELD and LITTLE, 1966; MIROV, 1967). Unlike the Japanese stone pine, Korean pine grows in more maritime conditions and lower mountains, and it usually forms mixed forests with broadleaf trees and other conifers (POTENKO and VELIKOV, 1998; CAI et al., 2002). The natural distribution of Korean pine is not remarkably broad in the Korean peninsula. In South Korea, *P. koraiensis* is distributed more abundantly in the northeastern part and appears locally on the mountainous regions of high elevation, mainly from 600 to 1200 meter high. It is also mostly scattered on the hillsides of northern aspect and the mountain streams, containing a considerable soil depth and fertility (UYEKI, 1926; KIM, 1990).

Korean pine is of great interest for foresters because it occupies quite a vast territory and has ecological and economic values. It is one of the major forest-forming tree species in its habitat and produces good-quality timber and edible seeds. Accordingly, it has been subject to intensive harvesting and, consequently needs special breeding and conservation programs (WANG, 1995; YI, 2005). However, regardless of many silvicultural and breeding works, the genetic studies for this species are relatively few (POLITOV and KRUTOVSKII, 1994; KIM et al., 1994; POTENKO and VELIKOV, 1998; POTENKO, 2004 and references therein).

Allozymes and RAPDs each have advantages and disadvantages as genetic markers for assaying variation (FRANKHAM et al., 2002; AVISE, 2004). So by applying two different markers to the same set of samples, it is expected to get more reliable genetic information on target species. It also gives an opportunity to know whether genetic parameters estimated by one are comparable to those estimated by the other.

The objectives of the study reported here were (1) to characterize and compare genetic diversity and the genetic structure of Korean pines in three regions – Korea, China and Russia; (2) to compare the results with previous reports for other stone pines and (3) to examine the consistency of results from allozymes and RAPDs.

Materials and Methods

Enzyme extraction and allozyme procedures

Four natural populations in Korea, 5 in China and 3 in Russia were included in this study (*Fig. 1*). Populations were systematically chosen to bracket as much of the north-south range of the species.

For each population, cones were collected from trees with a minimum distance of 30 meter in the late summer during 2001–2003. The first goal was to sample thirty trees in each population. However, final samples were twenty-thirty because of a paucity of cone-bearing trees, cones bearing viable seeds and/or local physical constraints. Collected cones were dispatched to the Population Genetics Lab. at Korea University in Korea and seeds were extracted after the cones opened. Then seeds were stored at –70 °C until needed.

Figure 1. – The 12 sampled sites of *P. koraiensis*, 4 in South Korea, 5 in China and 3 in Russia. Names refer to those corresponding to the numbers in *Appendix 1*.

Extraction of enzymes and horizontal starch gel electrophoresis were performed according to CONKLE et al. (1982) with some modifications. Analyses were performed using seven haploid megagametophytes per mother tree and twelve enzyme systems were assayed (abbreviations and EC numbers in brackets): aconitase (ACO, 4.2.1.3), fluorescent esterase (FEST, 3.1.1.1), glutamate dehydrogenase (GDH, 1.4.1.3), glutamate-oxaloacetate transaminase (GOT, 2.6.1.1), isocitrate dehydrogenase (IDH, 1.1.1.42), leucine aminopeptidase (LAP, 3.4.11.1), malic dehydrogenase (MDH, 1.1.1.37), menadion reductase (MNR, 1.6.99.2), mannosephosphate isomerase (MPI, 5.3.1.8), phosphoglucose isomerase (PGI, 5.3.1.9), phosphoglucomutase (PGM, 2.7.5.1), and shikimate dehydrogenase (SKDH, 1.1.1.25).

DNA extraction and RAPD procedures

Total genomic DNA was extracted from seeds by a modified HUFF et al.'s (1993) method. Eight to nine megagametophytes per tree were mixed together and then DNA was extracted. PCRs (polymerase chain reactions) were carried out in a volume of 20 µL with final concentrations of 10 ng of template DNA; 0.2 mM each of the four dNTPs; 0.0025 % BSA (Sigma, USA); 2.5 mM

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$MgCl_2$; 1x PCR reaction buffer; 1 unit of Taq DNA polymerase (Advanced Biotechnique, UK) and 4 µL of 1.5 µM of primer. Amplifications were performed in a UNO-II thermocycler (Biometra, UK) using a period of 5 min of initial denaturation at 94 °C, followed by 55 cycles of 30 s of denaturation at 94 °C, 30 s annealing at 36 °C, 1 min of extension at 72 °C, and a final extension step of 10 min at 72 °C. Subsequent amplification products were electrophoresed using 1.8% agarose gels containing ethidium bromide fluorescence with a 1x TBE (tris-boric acid-ethylendiamine tetraacetic acid) pH 8.0 buffer for 2.5 h at 100 V and then photographed under UV light.

A total of 40 primers (UBC, Canada and Operon Technologies, USA) were screened using three representatives from each of the twelve populations. Five primers that gave clear and reproducible fragment patterns over multiple (at least four) amplifications were selected for final analysis: OPR-12 (ACAGGTGCGT), OPR-13 (GGACGACAAG), UBC-710 (GGTGCTGGGT), UBC-715 (CCACCACCCA), and UBC-744 (CCACCCACCA). In order to avoid biasing estimates of polymorphism, the selection of primers for band scoring was dependent only on the clearness and repeatability of RAPD fragments, not on the level of polymorphism.

Genetic Inference

For allozyme analysis, the number of loci and alleles were interpreted by drawing on the experience gained in our laboratory from studies of other pine trees and determined on the basis of previously reported studies on the allozyme inheritance of Korean pine (KIM et al., 1982; TOMARU et al., 1990). When several zones of activity were observed for a single enzyme, hyphenated alphabets following the enzyme abbreviation were used for identification. Eighteen loci were scored consistently and used in the statistical analysis.

Estimating genetic parameters

Allozymes

Genotypic and allelic frequencies were used to estimate genetic diversity (*A*: mean number of alleles per locus, P_{95}: proportion of polymorphic loci at 95% level, H_o and H_e: observed and expected heteozygosities) and WRIGHT's (1965) *F*-statistics (F_{IS}, F_{IT} and F_{ST}). The phenogram by the UPGMA clustering technique based on NEI's (1978) genetic distance was constructed. Deviations of genotype distributions from the Hardy-Weinberg expectations were tested by chi-square tests. LEVENE's correction (1949) was used to adjust for small sample size. Whenever cell size was less than 5, frequencies of the least common alleles were pooled (SOKAL and ROHLF, 1995). NEI's (1978) unbiased genetic distance coefficients between groups at two hierarchical levels [among regions (Korea, China, and Russia) and among populations within regions] were calculated. We also performed a hierarchical analysis of population differentiation using the formulation of WRIGHT (1978). In the analysis, F_{PT} (populations-total), F_{PR} (populations-regions), and F_{RT} (regions-total) were estimated along with the corresponding variance components. The computer program BIOSYS-1 (SWOFFORD and SELANDER, 1989) was used for all above-mentioned calculations. Degree of genetic isolation among populations was estimated by N_m, the number of migrants per generation. N_m was calculated by WRIGHT's (1951) method:

$$N_m = (1 - F_{ST}) / 4F_{ST}$$

where F_{ST} is the proportion of the total genetic diversity among populations.

RAPDs

For RAPD markers, RAPD phenotypic (presence-absence) data were used to calculate genetic parameters. For each tree, a binary vector of 1s (presence of a band) and 0s (absence of a band) for RAPD loci was created and then used for further analyses. The Shannon's index of phenotypic diversity (1948) using the POPGENE v.1.31 program (YEH et al., 1999) was calculated for estimating genetic diversity within populations. Degree of population differentiation was estimated by AMOVA using Arlequin V2.00 (SCHNEIDER et al., 2000). AMOVA was performed at 2 and 3 hierarchical levels. Genetic relationships among populations were reconstructed by UPGMA method (PHYLIP v3.5c; FELSENSTEIN, 1993) on the basis of pairwise Manhattan distance among populations computed by RAPDDIST v1.0 (BLACK, 1996).

Results

Allozyme variation

All of the eighteen loci investigated were polymorphic. A locus was considered polymorphic when the frequency of the most common allele did not exceed 0.95 at least in a population. In most polymorphic loci, the most frequent allele was evident in each population (*Appendix 1*). On the other hand, populations sampled from three different regions (Korea, China, and Russia) were distinguished by differences in allele frequencies at some loci such as *Lap-A*, *Mnr-A*, and *Mdh-B*. At the locus *Lap-A*, all of the four Korean populations had allele 2 as the most frequent allele. In Chinese populations, allele 2 was the most frequent in 3 out of 5 populations, while allele 1 was the most frequent in 2 populations. All of three Russian populations had allele 1 as the most frequent allele, although the difference in frequencies between alleles 1 and 2 was small. At the locus *Mnr-A*, allele 2 was the most frequent in three of the 4 Korean populations, while allele 3 was the most frequent in all of three Russian populations and in four of the 5 Chinese populations. At the *Mdh-B* locus, allele 2 was the most common in all populations from different three regions, but its frequency increased with latitude. Allele frequencies at these three loci were significantly correlated with latitude at 5% level ($Lap\text{-}A_1$: r = 0.606, p = 0.037; $Lap\text{-}A_2$: r = –0.613, p = 0.034; $Mnr\text{-}A_2$: r = –0.595, p = 0.042; $Mnr\text{-}A_3$: r = 0.713, p = 0.009; $Mdh\text{-}B_2$: r = 0.785, p = 0.003).

Five of the 12 populations had a total of five private alleles (i.e., alleles found in only one population). Alleles, $Got\text{-}B_1$ and $Pgm\text{-}A_3$ were unique to Korea and Russia, respectively. The remaining three private alleles (*Idh-*

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Table 1. – Comparison of genetic variation at 18 allozyme loci in 12 natural populations of *P. koraiensis* in Korea, China and Russia.

Population	Mean sample size	Mean no. of alleles per locus (A)	% of polymorphic loci ($P_{0.95}$)	Heterozygosity	
				H_o	H_e
<Korea>					
[1] Mt. Chiri	27.8	2.2	38.9	0.145	0.168
[2] Mt. Palkong	26.0	1.8	38.9	0.165	0.191
[3] Mt. Taebaek	19.4	2.1	61.1	0.161	0.185
[4] Mt. Seorak	22.5	1.9	44.4	0.154	0.178
Mean of 4 Korean populations	***23.9***	***2.0***	***45.8***	***0.156***	***0.181***
<China>					
[5] Chohaku	20.3	1.9	44.4	0.148	0.169
[6] Losooha	22.3	1.9	61.1	0.178	0.189
[7] Wangging	21.7	1.9	44.4	0.182	0.164
[8] Xobukho	28.6	2.1	50.0	0.186	0.182
[9] Lyangsoo	28.5	2.1	50.0	0.167	0.162
Mean of 5 Chinese populations	***24.3***	***2.0***	***50.0***	***0.172***	***0.173***
<Russia>					
[10] Nizhnetambovsk	30.0	2.0	50.0	0.131	0.159
[11] Amur Kur-Umi	28.9	1.6	33.3	0.124	0.133
[12] Amur Komsomolsk	29.8	1.8	44.4	0.154	0.152
Mean of 3 Russian Populations	***29.6***	***1.8***	***42.6***	***0.136***	***0.148***
Total mean	***25.5***	***1.95***	***46.8***	***0.158***	***0.169***

A_1, *Pgm-*A_n, and *Skdh-*A_n) were observed in three different Chinese populations. On the other hand, four private alleles unique to Chinese and Russian populations in this study were observed in other Korean populations (KIM et al., 1994).

Levels of genetic diversity were low to moderate in all populations of Korean pine (*Table 1*).

The percentage of polymorphic loci varied from 33.3 (Amur Kur-Umi in Russia) to 61.1% (Mt. Taebaek in Korea and Losooha in China). The number of alleles per locus ranged from 1.6 (Amur Kur-Umi in Russia) to 2.2 (Mt. Chiri in Korea). Mean expected heterozygosity (H_e, unbiased estimate) was highest (0.191) in the Mt. Palkong from Korea and lowest (0.133) in the Amur Kur-Umi from Russia. These results showed that the level of genetic diversity observed in Russian populations was lower than those of the Korean and Chinese populations, while the genetic diversity parameters for the Korean and Chinese populations were similar. Actually, expected heterozygosity tended to decrease with the increase of latitude ($r = -0.719$, $p = 0.008$; *Fig. 2*).

Allelic richness was not greatly dependent upon sample size. The smallest sample (n = 20) from Mt. Taebak in Korea had nearly the greatest mean number of alleles, 2.1, compared to a value of 2.2 in the sample of Mt. Chiri in Korea (n = 28) and 2.0 at Nizhnetambovsk in Russia, which had the largest sample (n = 30).

Observed heterozygosity (H_o) ranged from 0.124 (Amur Kur-Umi in Russia) to 0.186 (Xobukho in China). In eight of 12 populations, observed heterozygosity was lower than expected heterozygosity. The heterozygote deficit was reflected in a mean (0.045) of Wright's F_{IS} (*Table 2*). However, the small mean value of F_{IS} (close to zero) reveals that the Korean pine populations studied here are generally in good agreement with the Hardy-Weinberg expectations. In fact, only eleven of 143 tests (7.7%) indicated significant deviations from the Hardy-Weinberg expectations. Two populations had an excess of heterozygotes (Lyangsoo in China and Amur Komsomolsk in Russia at the *Mnr-A*), while the other nine populations had a deficiency of heterozygotes (see *Appendix 1*).

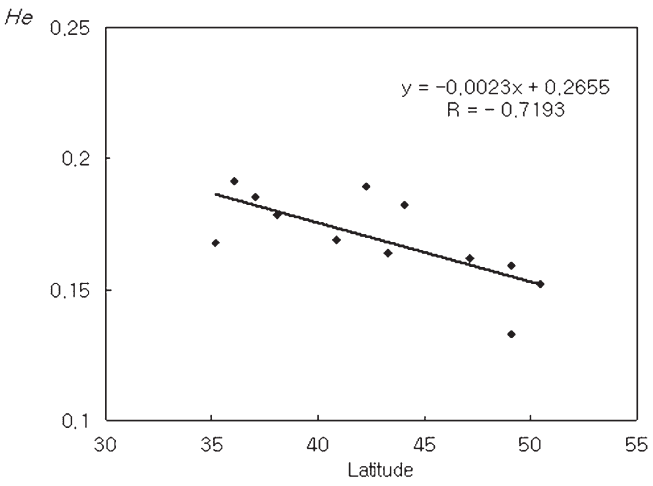

Figure 2. – The correlation of expected heterozygosity with latitude for 12 natural populations of *P. koraiensis*.

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Diversity among populations at species level, F_{ST}, was 0.069, which means that 93% of the observed variation was within populations. Hierarchical Wright's *F*-statistics (*Table 3*) partitioned the total genetic differentiation (F_{PT} = 0.041) into among- (F_{RT} = 0.009) and within-region (F_{PR} = 0.033) components. This indicates that the degree of genetic differentiation among regions is lower than that of among populations within regions.

Table 2. – *F* statistics for 18 polymorphic loci in 12 natural populations of *P. koraiensis*.

Locus	F_{IS}	F_{IT}	F_{ST}
Acon-A	-.037	-.010	.026
Fest-B	.071	.120	.053
Gdh-A	.108	.152	.049
Got-A	.026	.055	.029
Got-B	-.025	-.002	.023
Got-C	.065	.097	.034
Idh-A	-.018	-.006	.045
Lap-B	-.053	.174	.090
Mdh-A	.092	.148	.044
Mdh-B	.109	.070	.077
Mdh-C	-.008	-.022	.016
Mdh-D	-.038	.000	.053
Mnr-A	-.056	.072	.066
Mpi-A	.007	.217	.113
Pgi-B	.089	.184	.104
Pgm-A	-.009	.082	.090
Skdh-A	.047	.089	.044
Skdh-B	.201	.237	.045
Mean	.045	.111	.069

Table 3. – Hierarchical variance components and F-statistics among three regions.

Comparison		Variance Component	F_{XY}
X	Y		
Populations – Total		.13170	.041
Populations – Region		.10318	.033
Region – Total		.02852	.009

Estimates of hierarchical NEI's (1978) unbiased genetic distances (*Table 4*) ranged from 0.003 among populations within Korea to 0.018 between Korean and Russian regions. Of three regions, Russia was most distinctive from the other regions.

To better visualize the results, a dendrogram produced by the UPGMA clustering technique is presented in *Fig. 3*. This dendrogram showed some decisive regional trends; all of the four Korean populations and the three Chinese populations (Lyangsoo, Losooha and Chohaku) showed a tendency of clustering into the same group, while Russian populations made up a separate group with the other two Chinese populations (Xobukho and Wangging).

Indirect estimate of gene flow between populations was substantial. *Nm* calculated from Wright's F_{ST} was 3.4 migrants per generation.

RAPD variation

The five primers used in the present study revealed 42 reproducible RAPD loci. Of the 42 putative RAPD loci examined, 38 loci were polymorphic in at least one population. Estimates of Shannon's index ranged from 0.257 (Nizhnetambovsk in Russia) to 0.326 (Xobukho in China) with a mean value of 0.293 (*Table 5*).

The estimates of Shannon's index (*Fig. 4*) tended to decrease from south (Korea) to north (Russia) (r = –0.509, p = 0.09), which is similar to the results of allozyme variation (*Fig. 2*).

Results from AMOVA gave a similar pattern of population and regional differentiation observed from the hierarchical Wright's *F* analysis at isozyme level; around 10% of the RAPD variation was distributed among populations within regions and 3% was distributed among regions. The rest (around 87%) of the total genetic variation was within populations (*Table 6*).

The UPGMA dendrogram (*Fig. 5*) showed the very similar tendency to that from isozyme analysis (*Fig. 3*); the Korean populations were clustered together, and then grouped with the three Chinese populations. The Russian populations and the other two Chinese populations (Xobukho and Wangging) were clustered together.

Table 4. – NEI's (1978) genetic distance within and among three regions by hierarchical design (Ranges in parentheses).

Region	No. of Populations	1	2	3
[1] Korea	4	.003 (.002-.006)		
[2] China	5	.008 (.001-.020)	.010 (.001-.022)	
[3] Russia	3	.018 (.003-.029)	.010 (.002-.017)	.006 (.003-.009)

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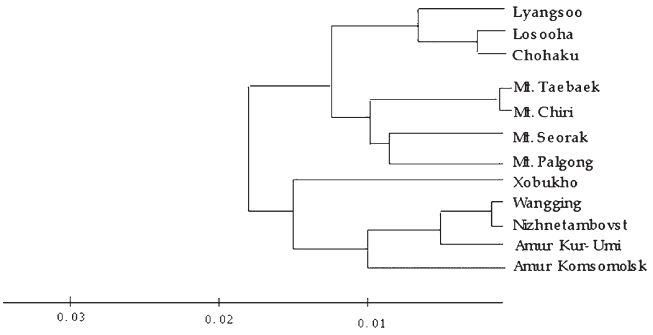

Figure 3. – Dendrogram constructed by the UPGMA method based on NEI's (1978) unbiased genetic distance coefficient from allozyme data.

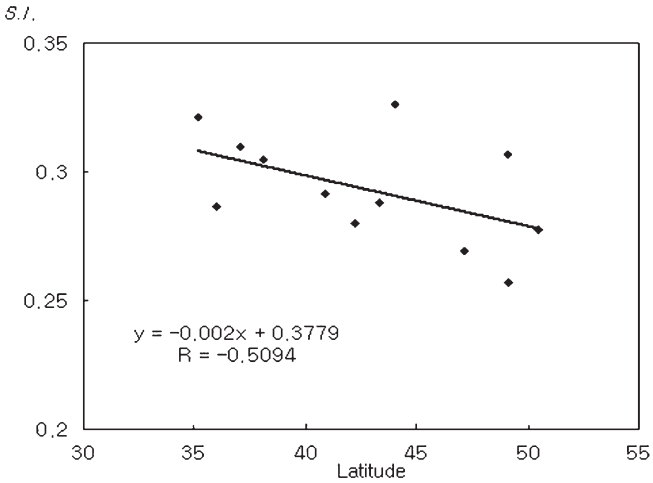

Figure 4. – The correlation of Shannon's index with latitude for 12 natural populations of *P. koraiensis*.

Discussion

The genetic variation maintained by *P. koraiensis* at the population level (H_e = 0.169, *Table 2*) was slightly higher than that reported previously for gymnosperms (H_{ep} = 0.151 from 102 entries) in general and pines (H_{ep} = 0.136 from 93 entries) in particular (HAMRICK et al., 1992). When the mean expected heterozygosity of *P. koraiensis* was compared to those of other pine species in the subsection *Cembrae* to which *P. koraiensis* belongs, it was lower than those of *P. pumila* (H_e = 0.255, GONCHARENKO et al., 1993a; H_e = 0.223, TANI et al., 1996; H_e = 0.249–0.334, LEDIG, 1998) and those of *P. albicaulis* (H_e = 0.204, LEDIG, 1998). And it was similar to and/or slightly higher than those of *P. sibirica* (H_e = 0.158, POLITOV and KRUTOVSKII, 1994; H_e = 0.176, GONCHARENKO et al., 1993b, H_e = 0.147–0.185, LEDIG, 1998) and those of *P. cembra* (H_e = 0.109, POLITOV and KRUTOVSKII, 1994). It is generally known that genetic diversity within populations is influenced mainly by the geographic distribution of the species, mating system, the methods of seed dispersal, and the methods of reproduction (HAMRICK and GODT, 1989). Of these, in woody plants, geographic range is known to be most highly correlated with genetic diversity (HAMRICK et al., 1992). Namely, species with restricted ranges and discontinuous distribution often have low genetic diversity compared with more widespread species with similar biology and life history traits do. The above comparisons are in good agreement with these general trends. Among 5 stone pines, *P. pumila* with the most widespread geographic range showed the highest level of genetic diversity.

Of Korean pines in different three regions, the Korean populations showed a relatively high polymorphism,

Table 5. – Comparison of genetic diversity in 12 natural populations of *P. koraiensis* in Korea, China and Russia based on RAPD markers.

Population	Shannon's index (*S.I.*)	
	Mean	S.D.*
<Korea>		
[1] Mt. Chiri	0.3214	0.090
[2] Mt. Palkong	0.2868	0.086
[3] Mt. Taebaek	0.3098	0.092
[4] Mt. Seorak	0.3045	0.089
Mean of 4 Korean populations	***0.3056***	***0.089***
<China>		
[5] Chohaku	0.2918	0.083
[6] Losooha	0.2802	0.094
[7] Wangging	0.2882	0.087
[8] Xobukho	0.3264	0.084
[9] Lyangsoo	0.2694	0.088
Mean of 5 Chinese populations	***0.2912***	***0.087***
<Russia>		
[10] Nizhnetambovsk	0.2568	0.065
[11] Amur Kur-Umi	0.3069	0.074
[12] Amur Komsomolsk	0.2776	0.082
Mean of 3 Russian Populations	***0.2804***	***0.074***
Total mean	***0.2933***	***0.084***

S.D.: standard deviation

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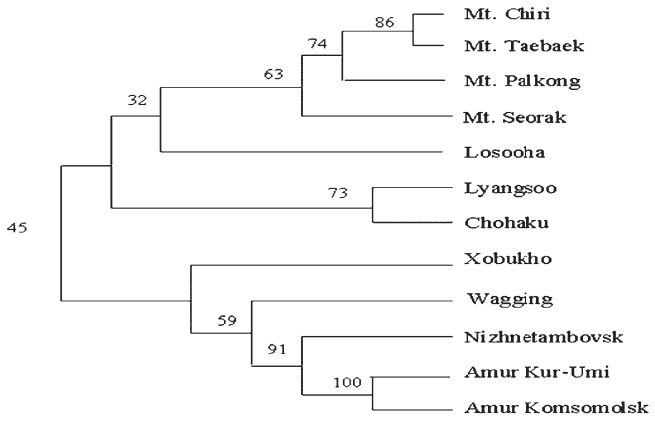

Figure 5. – Dendrogram constructed by the UPGMA method. Figures at the node denote confidence limits estimated on the basis of 1000 bootstrapped. Only confidence limits of higher than 50% were indicated.

while the Russian populations exhibited the lowest level of genetic diversity (*Table 1*). If the Korean pine in the Russian Far East are peripheral isolates, their genetic diversity might be lower than that for the central populations due to the influence of random drift caused by small population size; severe directional selection in ecologically marginal habitats which may reduce variation; or founder effects during species migration. Actually, so far, several allozyme studies have shown lower genetic diversity in marginal populations (FURNIER and ADAMS, 1986; LEDIG, 2000). The low level of genetic diversity in Russian Far East stone pines was also reported in elsewhere. According to POLITOV and KRUTOVSKII (1994), the H_e averaged over 3 Eastern Far Russian populations was 0.131. On the other hand, KIM et al. (1994) reported the H_e value of 0.208 in 8 Korean *P. koraiensis* populations, which is slightly higher than that of Korean populations (H_e = 0.181) and much higher than that of Russian populations (H_e = 0.148) investigated here. NEISHTADT (1957) hypothesized that the Korean pine came to the Far Eastern region from the south as late as postglacial times (the Holocene) and, previously, during the Pleistocene, *P. koraiensis* grew only south of the present border of Russia. Our results support this hypothesis. Namely, genetic diversity had a tendency to decrease from south (Korea) to north (Russia), suggesting that some diversity was lost during founder events as the Korean pine dispersed northward (*Fig. 2* and *Fig. 4*).

In contrast, POTENKO and VELIKOV (1998) reported a high mean values of H_e (H_e = 0.183) in 19 Russian Far East populations and did not find a decline of genetic variation from south to north in Russian *P. koraiensis* populations. In a succeeding study, POTENKO (2004) postulated not a single but a small number of centers of genetic variation in different locations of Russian Far East. From these results, he hypothesized the long-term existence of the Korean pine in the Russian Far East region, i.e., *P. koraiensis* might grow earlier than the Holocene. Several well-dated pollen records also showed the widespread occurrence of pines in Northeast China before the last glacial maximum (c. 18000 BP) (LIU, 1988). Possibly, *Pinus* species was not completely eliminated from the North China, but survived in small populations in favorable habitats throughout the cold and dry stage (KREMENETSKI et al., 1998). For a better understanding of this issue, however, we need to study the genetic variation of the North Korean as well as the Japanese *P. koraiensis* populations using the same markers. An intensive, range-wide sampling including key areas from evolutionary time should be also important for future research as employed in the study of other conifers (LI and ADAMS, 1989). Additionally, further studies such as tracing the migration route of Korean pine using mitochondrial and/or chloroplast DNA markers will give more detailed answers to this issue.

The mean F_{IS} value across all polymorphic loci was 0.045 (*Table 2*), indicating a slight deficit (4.5%) of observed heterozygotes relative to the Hardy-Weinberg ratio. Likewise, the mean F_{IT} value (0.111) showed a heterozygote deficiency in the whole of Korean pine. KIM et al. (1994) and POTENKO and VELIKOV (1998) also reported the deficiency of heterozygotes in the Korea and the Russian Far East *P. koraiensis* populations, respectively. The mating system of Korean pine may provide a partial explanation for the excess of homozygotes. Outcrossing rate (t = 0.914) of Korean pine estimated by an indirect method [Allard et al., 1968: $t = (1 - F_e)/(1 + F_e)$, where F_e is the equilibrium fixation index which substituted for F_{IS} in this study] suggests that the Korean pine is not a completely outcrossing species although most of pollination (over 90%) is outcrossing. POLITOV and KRUTOVSKII (1994) reported that t_s (single-locus estimate of outcrossing) and t_m (multi-locus estimate of outcrossing) for Russian Far East *P. koraiensis* were 0.936 and 0.974, respectively. Korean pines bear large and wingless heavy seeds and so it is highly proba-

Table 6. – Analysis of molecular variance of *P. koraiensis* based on RAPD markers.

Source of variation	d.f.	% of variance component
Variation between groups	2	3.40
Variation among populations within groups	9	10.08
Variation among individuals within populations	299	86.52

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ble that individuals in close proximity are closely related because of the limited seed dispersal from mother tree. Consequently, there might be a chance of inbreeding among neighboring trees. This hypothesis is supported by HONG et al.'s (2001) findings of the genetic patchy in a *P. koraiensis* population. In contrast, most conifers bearing small and light seeds have shown a random distribution of allozyme genotypes in space (KNOWLES, 1991; LEONARDI et al., 1996).

Individual *P. koraiensis* population maintained a high proportion of the variation apparent in the species, as is characteristic of most conifers. More than 93% of genetic variation resided within each population (*Table 2*). In general, woody plants with widespread distributions, outcrossing mating systems, widely dispersed pollen and/or seed such as *P. koraiensis* tend to have more genetic diversity within populations and less variation among populations than species with other combinations of traits (HAMRICK et al., 1992). The mean F_{ST} value for *P. koraiensis* (0.069) is comparable to that of pines in general (G_{ST} = 0.065; HAMRICK et al., 1992) and, in particular, slightly higher than those of other stone pines such as *P. sibirica* (G_{ST} = 0.041, GONCHARENKO et al., 1993b; G_{ST} = 0.025, KRUTOVSKII et al., 1994), and *P. pumila* (G_{ST} = 0.043, GONCHARENKO et al., 1993a; G_{ST} = 0.021, KRUTOVSKII et al., 1994). On the other hand, the F_{ST} value for this study is higher than those for *P. koraiensis* from other studies: F_{ST} = 0.059 in Korea (KIM et al., 1994); G_{ST} = 0.021 in Russian Far East (KRUTOVSKII et al., 1994); and F_{ST} = 0.015–0.018 in Russian Far East (POTENKO and VELIKOV, 1998; POTENKO, 2004). These differences between the present study and the other studies may be due to the fact that the present study includes more widely distributed populations. Nevertheless, the genetic differentiation among different regions investigated here was relatively small. Effective gene flow, typical of most wind-pollinated conifers, would tend to decrease genetic heterogeneity among regions as well as among populations within regions. Wright's estimate of gene flow (N_m = 3.4) was moderate or small relative to those of other pines (LEDIG, 1998), but still substantial for such widely separated populations in *P. koraiensis*. It is not reliable that the N_m value observed here is the current number of immigrants exchanged, but reflects at least recent as well as past contact, perhaps through a chain of populations.

No geographic patterns were obvious in the isozymes assayed here with the exception of few loci such as *Lap-A*, *Mnr-A*, and *Mdh-B*, which showed a tendency of selection (cline). These may suggest the action of selection on *LAP-A*, *Mdh-B*, and *Mnr-A* or closely linked segments of the genome. However, clustering algorithms using various genetic distance measures showed some decisive geographic patterns (*Fig. 3* and *Fig. 5*). Namely, the geographically close populations tended to be clustered together. On the other hand, two Chinese populations, Xobukho and Wangging, were not grouped with the other adjacent Chinese populations like Lyangsoo and Losooha, but clustered with the Russian populations. This grouping pattern is congruent with the results of RAPDs. The Xiaoxing'anling, Changguangcaling and other mountains extended from north to south would have functioned as a barrier against gene flow between the Xobukho and Wangging and the other Chinese *P. koraiensis* populations (*Fig. 1*). This may be also a reason why NEI's (1978) genetic distance between populations within China is larger than that between populations within Korea and Russia. It is even larger than that between the Chinese and the Korean groups (*Table 4*).

The estimated genetic diversities and differentiation from RAPD data in Korean pine are congruent with those of allozymes (*Table 5* and *Table 6*). It is reported that plant populations exhibiting low/high allozyme variability also exhibit low/high RAPD diversity (LIU and FURNIER, 1993; LEE et al., 2002 and references therein). That means that the gross pattern of genetic variation of the RAPDs is similar to that of allozyme markers.

In woody plants, RAPDs generally show similar or higher levels of polymorphism than isozyme markers (AAGAARD et al., 1998; PENG et al., 2003) Our present measurements of genetic diversity based on Shannon's index prevent us from comparing them directly with the genetic diversity parameters (expected heterozygosity, H_e) from the allozyme markers. Several studies have shown that some factors such as the dominant nature of RAPD markers can overestimate or underestimate the level of genetic diversity and the degree of genetic differentiation when the RAPDs are used for diploids, especially for diploid outcrossing organisms (LIU and FURNIER, 1993; LYNCH and MILLIGAN, 1994). So they suggested that caution should be taken when estimating population genetics parameters using RAPD markers and codominant allozyme markers still appear most suitable for many types of population genetic studies in plants. More detailed discussion on this issue can be found in elsewhere (ISABEL et al., 1995; PEAKALL et al., 1995; LEE et al., 2002; TORRES et al., 2003).

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Appendix 1. – Allele frequencies for 18 polymorphic loci in 12 natural populations of *P. koraiensis.*

Locus	Population					
	[1] Mt. Chiri	[2] Mt. Palkong	[3] Mt. Taebaek	[4] Mt. Seorak	[5] Chohaku	[6] Losooha
Acon-A						
1	.037	.000	.000	.000	.048	.000
2	.963	1.000	1.000	1.000	.952	1.000
Fest-A						
1	.000	.000	.000	.000	.000	.020
2	.983	.982	1.000	.979	.952	.940
3	.017	.018	.000	.021	.048	.040
Gdh-A		+				
1	.017	.036	.000	.000	.095	.058
2	.983	.964	1.000	1.000	.905	.942
Got-A						
1	.021	.000	.025	.000	.000	.000
2	.979	1.000	.975	1.000	1.000	1.000
Got-B						
1	.069	.000	.000	.000	.000	.000
2	.931	1.000	1.000	1.000	1.000	1.000
Got-C						
1	.125	.222	.182	.262	.167	.208
2	.857	.778	.818	.690	.833	.792
n	.018	.000	.000	.048	.000	.000
Idh-A						
1	.000	.000	.000	.000	.000	.000
2	1.000	1.000	1.000	1.000	1.000	1.000
Lap-A			+			
1	.304	.429	.409	.348	.275	.320
2	.661	.571	.591	.652	.725	.680
n	.036	.000	.000	.000	.000	.000
Mdh-A						
1	1.000	1.000	.932	1.000	1.000	1.000
2	.000	.000	.068	.000	.000	.000
Mdh-B						
1	.103	.179	.105	.000	.000	.096
2	.621	.536	.763	.545	.833	.731
3	.276	.286	.132	.455	.167	.173
Mdh-C						
1	.034	.000	.024	.021	.048	.037
2	.966	.982	.929	.979	.952	.944
3	.000	.018	.048	.000	.000	.019
Mdh-D						
1	.982	.893	.909	.891	.952	.815
n	.018	.107	.091	.109	.048	.185
Mnr-A		+				
1	.161	.074	.068	.000	.143	.000
2	.411	.463	.364	.525	.333	.519
3	.339	.370	.500	.450	.429	.481
4	.089	.093	.068	.025	.095	.000
Mpi-A						
1	1.000	1.000	.886	.979	.938	1.000
2	.000	.000	.114	.021	.063	.000
Pgi-B						
1	.207	.393	.100	.196	.119	.214
2	.793	.607	.800	.761	.881	.786
3	.000	.000	.100	.043	.000	.000
Pgm-A						
1	.000	.036	.000	.000	.000	.038
2	1.000	.964	1.000	1.000	1.000	.904
3	.000	.000	.000	.000	.000	.000
n	.000	.000	.000	.000	.000	.058
Skdh-A						
1	.036	.019	.033	.050	.048	.000
2	.089	.278	.133	.175	.262	.288
3	.857	.704	.833	.750	.667	.712
4	.018	.000	.000	.025	.000	.000
n	.000	.000	.000	.000	.024	.000
Skdh-B				+		
1	.000	.000	.042	.056	.000	.000
2	.980	1.000	.917	.944	1.000	.960
3	.020	.000	.042	.000	.000	.040

n: Null allele; [1–4] Populations located in Korea, [5–6] Populations located in China; Note: Deviations of genotype frequency from Hardy-Weinberg expectations are indicated by a minus sign (significant heterozygote excess) or by plus sign (significant heterozygote deficit); X^2, $p < 0.05$.

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Appendix 1. – (Continued)

Locus	Population					
	[7] Wangging	[8] Xobukho	[9] Lyangsoo	[10] Nizhnetam – bovsk	[11] Amur Kur-Urmi	[12] Amur Komsomolsk
Acon-A						
1	.000	.017	.017	.000	.000	.000
2	1.000	.983	.983	1.000	1.000	1.000
Fest-A						
1	.023	.074	.000	.017	.034	.000
2	.977	.926	1.000	.983	.914	1.000
3	.000	.000	.000	.000	.052	.000
Gdh-A						
1	.045	.000	.034	.000	.000	.117
2	.955	1.000	.966	1.000	1.000	.883
Got-A						
1	.023	.038	.000	.000	.000	.000
2	.977	.962	1.000	1.000	1.000	1.000
Got-B						
1	.000	.000	.000	.000	.000	.000
2	1.000	1.000	1.000	1.000	1.000	1.000
Got-C						
1	.250	.086	.069	.133	.190	.017
2	.750	.845	.914	.867	.810	.983
n	.000	.069	.017	.000	.000	.000
Idh-A						
1	.000	.000	.017	.000	.000	.000
2	1.000	1.000	.983	1.000	1.000	1.000
Lap-A					+	
1	.568	.300	.793	.533	.574	.519
2	.364	.700	.207	.450	.426	.426
n	.068	.000	.000	.017	.000	.056
Mdh-A						
1	1.000	.964	1.000	1.000	1.000	1.000
2	.000	.036	.000	.000	.000	.000
Mdh-B						
1	.000	.000	.052	.017	.000	.000
2	.818	.733	.741	.817	1.000	.933
3	.182	.267	.207	.167	.000	.067
Mdh-C						
1	.023	.033	.034	.017	.017	.000
2	.955	.967	.966	.967	.983	1.000
3	.023	.000	.000	.017	.000	.000
Mdh-D						
1	.909	.966	.828	.950	1.000	.883
n	.091	.034	.172	.050	.000	.117
Mnr-A			-		+	-
1	.000	.050	.019	.000	.121	.183
2	.412	.300	.327	.383	.345	.183
3	.588	.633	.442	.567	.534	.633
4	.000	.017	.212	.050	.000	.000
Mpi-A						
1	1.000	.881	1.000	.917	.966	.883
2	.000	.119	.000	.083	.034	.117
Pgi-B						
1	.091	.117	.103	.050	.000	.000
2	.909	.867	.810	.950	1.000	1.000
3	.000	.017	.087	.000	.000	.000
Pgm-A						
1	.045	.140	.054	.083	.276	.050
2	.955	.860	.946	.867	.724	.867
3	.000	.000	.000	.050	.000	.083
n	.000	.000	.000	.000	.000	.000
Skdh-A				+		
1	.000	.000	.052	.033	.000	.000
2	.159	.283	.086	.333	.190	.400
3	.841	.700	.845	.633	.810	.600
4	.000	.017	.017	.000	.000	.000
n	.000	.000	.000	.000	.000	.000
Skdh-B		+	+			
1	.023	.033	.069	.000	.000	.000
2	.864	.967	.931	.967	1.000	.983
3	.114	.000	.000	.033	.000	.017

n: Null allele; [7–9] Populations located in China, [10–12] Populations located in Russia; Note: Deviations of genotype frequency from Hardy-Weinberg expectations are indicated by a minus sign (significant heterozygote excess) or by plus sign (significant heterozygote deficit); X^2, $p < 0.05$.

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Variation in Reproductive Phenology in a *Pinus radiata* D. Don Seed Orchard in Northern Spain

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Summary

Reproductive phenology was studied in a *Pinus radiata* seed orchard, located in northern Spain. Timing of flowering was determined on the basis of data recorded by visual observations made in 2000, 2001 and 2002. The genetic and environmental factors affecting female and male phenology, as well as reproductive synchronization, were studied. The dates of beginning of the receptive phase and pollen shedding varied greatly from year to year but the variation on the sum of degree-days was low. In general, the flowering periods of the different clones overlapped. The clonal differences in the phenology of receptivity and pollen shedding were in most cases statistically significant. The time needed to reach flowering stages was under strong genetic control. Genetic control was stronger for the female than the male flowering process. However, correlations between years were stronger for male than for female flowering phenology. The male flowering clones that best synchronized with the females appeared to be those that started flowering earlier. The phenological overlap index varied greatly among clones, whether male or female, and also among years.

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Key words: flowering receptivity, pollen shedding, reproductive synchronization, flowering phenograms, cumulative growing degree-days, phenological overlap index, SYNCHRO.SAS programme, clonal repeatibility, progeny test, genetic variation, genetic parameters, quantitative traits.

Introduction

Radiata pine is one of the most commonly planted exotic tree species in Galicia (northwest Spain). It occupies almost 60.000 ha (4% of Galician forests) with an annual volume increment estimated at around 10^6 $m^3 \cdot year^{-1}$ (XUNTA DE GALICIA, 2001). Together with *Pinus pinaster* (324.000 ha) and *Eucalyptus globulus* (290.000 ha), it accounts for 90% of the Galicia forestry Industry. The main use for *Pinus radiata* timber is in the furniture industry and the trees are grown on a short rotation of some 16 to 30 years, depending on the fertility of the site. Production is approximately 600 x 10^3 m^3 per year, making planting of these species a very attractive investment (DANS DEL VALLE, 1999). The demand for improved seed has warranted active breeding programmes.

Genetic improvement of *P. radiata* stock in Galicia was initiated in 1992 and has included phenotypic mass selection in plantations and use of this material for seed production in clonal seed orchards. A clonal radiata pine

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