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Full Length Research Paper

Application of SRAP in the genetic diversity of *Pinus koraiensis* of different provenances

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The study applied sequence-related amplified polymorphism (SRAP) technique into genetic diversity of *Pinus koraiensis.* A total of 480 samples from 24 provenances were amplified by using selected 9 primer pairs. The band number amplified by each primer pair ranged from 24 to 33, with the molecular weight between 100 and 1,500 bp. In total 249 bands were observed, of which 143 were polymorphic (55.42%). No significant difference in the genetic diversity was observed among the provenances, and the maximum and minimum level was found in Dahailin and Bajiazi provenances, respectively. It indicated that no close relationship can be well established between genetic diversity and the location of provenance. The genetic variation mainly caused by the variation of intra population, accounting for 92.35% of the total genetic variation. Moreover, the gene flow of *P. koraiensis* between the provenances was 2.905, and the relative high gene flow can prevent efficiently gene drift. Based on the UPGMA cluster diagram, the 24 provenances may be divided with the genetic distance of 0.013 into three groups. The result is not completely consistent with the traditional division of provenances.

Key words: Genetic diversity, geographic provenances, *Pinus koraiensis*, sequence-related amplified polymorphism (SRAP).

INTRODUCTION

Pinus koraiensis, a national secondary protected plant in China, is a plus tree of zonal top vegetation--broadleaved mixed forests in eastern mountainous area in North-eastern China. The species belongs to tertiary relict plant. Its distribution extends to Changbai Mountain and the Korean Peninsula after multiple glacial actions, yearly moving to the Lesser Khingan Mountain. Currently, *P. koraiensis* forest is located between 35° - 52° north latitude and between 126° - 143° east longitude, mainly concentrating in Changbai Mountain, the Lesser Khingan Mountain, Wanda Mountain, and Changkuantsailing in Northeastern China, northern regions in North Korea, Japan and southern part of Russian Far East. *P. koraiensis* with the characteristics of slow growth and late sexual maturity has some difficulty in the tree renewal in natural forest. Thereby, once damaged, the re-vegetation for *P. koraiensis* forest is difficult. A study by Ma (1997) showed that the distributed area of natural *P. koraiensis* forest was sharply reducing in China. Although artificial *P. koraiensis* forest has been afforested largely in North-eastern China, some forest stands have a low production and quality due to a low seed quality from inferior provenances. Therefore, it is of great significance to provide fine provenances for successful afforestation of artificial *P. koraiensis* forest.

SRAP (Sequence-Related Amplified Polymorphism) is a new PCR-based marker, firstly raised by Li and Qurios (2001). Hence, the method is widely used to research on genetic diversity and species diversity (Riaz et al., 2001; Ferriol et al., 2003; Riaz et al., 2004; Budak et al., 2004; Lin et al., 2004). Up to now, researches on genetic diversity of *P. koraiensis* are mainly focused on only a few natural populations. They had no intensive representativeness in the range of *P. koraiensis* distribution (Yang et al., 1989; Xia et al., 2001; Feng et al., 2006). The study first attempts to introduce SRAP technique into the research on genetic diversity of *P. koraiensis* from 24 pro-

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Table 1. The site of 24 provenances of *Pinus koraiensis*.

Provenance	Longitude	Latitude	Provenance	Longitude	Latitude
Lushuihe	127°47′	42°28′	Yanshou	128°40′	45°30′
Helong	129°01′	42°30′	Chaihe	129°43′	44°45′
Bajiazi	129°07′	42°40′	Huanan	131°01′	46°10′
Baihe	128°08′	42°25′	Xiaobeihu	128°39′	44°15′
Dashitou	128°31′	43°19′	Hebei	130°02′	48°02′
Tongzigou	129°17′	43°34′	Wuying	129°18′	48°10′
Wangqing	130°09′	43°20′	Wuyiling	128°21′	49°01′
Linjiang	127°08′	41°48′	Tieli	128°10′	46°58′
Caohekou	123°54′	40°53′	Liangshui	128°51′	47°10′
Muling	130°31′	44°52′	Qinghe	129°03′	46°55′
Dongfanghong	133°06′	45°07′	Liangzihe	129°41′	47°03′
Dahailin	129°01′	44°28′	Zhanhe	127°47′	48°30′

venances in Northeastern China. The aim is to disclose the intra-provenance and inter-provenance genetic variation of *P. koraiensis*, and compare the level of genetic diversity in different provenances. It may provide theoretical base for selection breeding, provenance dividing and reasonable utilization of *P. koraiensis* resource.

MATERIALS AND METHODS

Plant materials

Seeds of natural *P. koraiensis* forest from 24 provenances in Northeast China were collected by seed orchard of Lushuihe Forestry Bureau in Jilin Province in 1983 (Table 1), and cultured seedlings were planted in the provenance trial plot located at Hongguang Forestry Farm in 1986. A total of 20 maternal individuals were selected randomly from each provenance, and then one-year-old needles were collected in March 2006. The collected samples were stored at -40°C for further use.

DNA extraction

Genomic DNA was isolated from leaves using the modified CTAB method of Doyle and Doyle (1990). 0.2 g of ground fresh tissue was suspended in a pre-heated centrifugal tube with 750 µl CTAB. The suspension was mixed well, incubated at 65°C for 30 min, followed by centrifugation at 10,000 rpm for 3 min and supernatant fluid was abandoned. 750 µl CTAB was repeatedly added into the centrifugal tube, incubated at 65°C for 15 min followed by chloroform-isoamylalcohol (24:1) extraction twice and precipitation with 2/3 of the volume of isopropanol at -4°C. The pellet formed after centrifugation at 10,000 rpm for 10 min was washed with 95% ethanol twice. The DNA was then suspended in 50 µl deionized water. The DNA was stored at –20°C.

SRAP-PCR amplification

Each 20 µL reaction mixture consisted of 1 × buffer, 50 ng DNA templates, 2.0 mmol/L Mg^{2+}, 0.15 mmol/L dNTP, 0.15 µmol/L primers, and 1.5 U Taq enzyme. Amplification were carried out in a ABI 9700 Thermocycler with following PCR program: 5 min of denaturing at 94°C, five cycles of three steps: 1 min of denaturing at 94°C, 1 min of annealing at 35°C and 1 min of elongation at 72°C. In the following 30 cycles the annealing temperature was increased to 50°C, with a final elongation step of 7 min at 72°C and then storing at -4°C.

Amplification fragments were segregated on 6% denatured polyacrylamide gels with 7 mol/L urea, and 0.5 × TBE electrophoretic buffer. The gel was pre-run in 0.5 × TBE buffer at 85 W constant power (I = 60 mA, U = 2250 V) for 30 min. After loading samples, the gel was run at 65 W constant power (I = 60 mA, U = 2250 V) about 1.5 - 2 h until the xylene cyanol was to the 2/3 of the gel towards the bottom. After electrophoresis, the gel was stained by silver nitrate solution. The bands were scanned by Founder scanner U 430.

Selection of primers

According to the primer sequence provided by Li and Qurios (2001), Shanghai Sangon synthesized 8 forward primers numbering Me1-Me8, and 9 reverse primers numbering Em1-Em9. Out of 72 pairs of primers, 9 pairs of primers with clearly separated bands, stable amplification and rich polymorphism were selected. The sequence of primers selected was shown as Table 2.

Data analysis

On the basis of the band mobility and the absence (=0) or presence (=1) of binary data, the unclear and unidentified bands can be excluded. Then, POPGENE 32 software was used to calculate the single site of allele frequency, effective number of alleles, Nei's gene diversity index and Shannon's diversity index, etc. AMOVA 1.55 software was used to estimate the relative genetic differentiation (Gst) and gene flow (Nm) between provenances and MEGA 3.1 software for the analysis of the unweighted pair group method arithmetic average (UPGMA) over the 24 provenances of *P. Koraiensis*.

RESULTS

Amplification results of SRAP-PCR system

As shown in Figures 1 and 2, SRAP marker in *P. koraiensis* exhibited the remarkable characteristics of

Table 2. Sequence of SRAP primers.

Primer	Sequence
ME1/EM9	TGAGTCCAAACCGGATA / GACTGCGTACGAATTTAG
ME3/EM1	TGAGTCCAAACCGGAAT / GACTGCGTACGAATTAAT
ME3/EM4	TGAGTCCAAACCGGAAT / GACTGCGTACGAATTTGA
ME3/EM6	TGAGTCCAAACCGGAAT / GACTGCGTACGAATTGCA
ME4/EM1	TGAGTCCAAACCGGACC / GACTGCGTACGAATTAAT
ME5/EM5	TGAGTCCAAACCGGAAG / GACTGCGTACGAATTAAC
ME5/EM9	TGAGTCCAAACCGGAAG / GACTGCGTACGAATTTAG
ME6/EM1	TGAGTCCAAACCGGACA / GACTGCGTACGAATTAAT
ME6/EM7	TGAGTCCAAACCGGACA / GACTGCGTACGAATTCAA

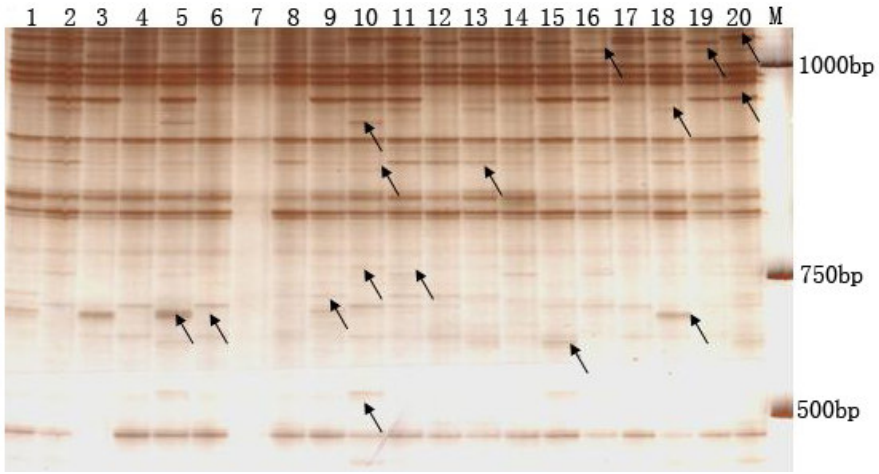

Figure 1. The bands of *Pinus koraiensis* amplified from the primer pair of ME6/EM1. Lanes 1 – 10 from Lushuihe provenance; lanes 11–20 from Qinghe provenance; M: marker. The arrows shows the polymorphic bands.

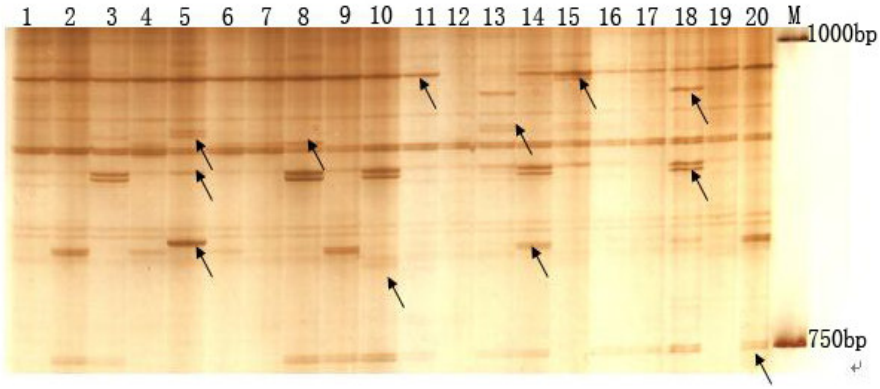

Figure 2. The bands of *Pinus koraiensis* amplified from the primer pair of ME3/EM1. Lanes 1 – 10 from Lushuihe provenance; lanes 11 – 20 from Qinghe provenance; M: marker. The arrows shows the polymorphic bands.

good stability and repeatability as well as clear bands. A total of 480 samples from 24 provenances were amplified by using selected 9 primer pairs. The band number amplified by each pair of primers ranged from 24 to 33, with the molecular weight between 100 and 1,500 bp. The maximum band number (33) was produced from the

Table 3. Parameters of 480 samples of *Pinus koraiensis* amplified with 9 pairs of primers.

Primer	total number of bands	Number of polymorphic bands	Percentage of polymorphic loci
ME1/EM9	32	12	36.50%
ME3/EM1	28	20	71.43%
ME3/EM4	28	16	57.14%
ME3/EM6	24	15	62.50%
ME4/EM1	27	15	55.56%
ME5/EM5	24	13	54.17%
ME5/EM9	33	16	48.49%
ME6/EM1	25	19	76.00%
ME6/EM7	28	12	42.86%

primer pair of ME5/EM9. In total 249 bands were observed, of which 143 were polymorphic, percentage of polymorphic loci was 55.42%. The average band number amplified from each pair of primers was 27.7 bands, of which included 15.9 polymorphic bands, indicating an insignificant difference in the number of polymorphic loci amplified from the different pairs of primers (Table 3).

Genetic diversity

Significant difference in the percentage of polymorphic loci, ranging from 38.12 to 45.80%, was not found among different provenances, and the maximum and minimum percentages were found in Dahailin and Bajiazi provenances, respectively. The average percentage of all provenances was only 41.58%, lower than the mean value of the species (55.42%). Using POPGENE 32 software, it was easy to obtain some valuable indexes for estimation of population-level genetic diversity such as the effective number of alleles, Nei's gene diversity index and Shannon's diversity index of *P. koraiensis* from 24 provenances. The obtained effective number of alleles ranged from 1.1807 to 1.2416, Nei's gene diversity index between 0.1110 and 0.1438, Shannon's diversity index from 0.1691 to 0.2182. Moreover, the changes of the above three indexes were consistent with the percentage of polymorphic loci. The change tendency from low to high was Bajiazi, Liangshui, followed by Wuying, Helong, Wangqing, Tongzigou, Liangzihe, Dongfanghong, Linjiang, Lushuihe, Zhanhe, Tieli, Wuyiling, Muling, Dashitou, Hebei, Baihe, Caohekou, Chaihe, Huanan, Xiaobeihu, Yanshou, Qinghe, and Dahailin. The detailed values were shown as Table 4.

Integrating the discrete chart of percentage of polymorphic loci among the provenances in Figure 3, it can be intuitively found that the provenances such as Dahailin, Qinghe and Yanshou, with high percentage of polymorphic loci, were located at relative low latitudes, whereas the provenances with the low value such as Wuying, Liangshui and Bajiazi, had relative high latitudes. However, from the whole 24 provenances, negative correlation was not observed between the percentage of polymorphic loci and latitudes.

Genetic differentiation and gene flow

Based on the analysis by AMOVA 1.55 software on the genetic differentiation of *P. koraiensis* in the 24 provenances, the major genetic variation originated from intra provenances, accounting for 92.35% of the total population-level variations, while only 7.65% of variations occurred at inter provenances (Table 5).

Gene flow (Nm) is a major factor impacting genetic structure and genetic differentiation among populations. The gene flow of *P. koraiensis* among the provenances was 2.9049. Wright (1931) proposed that the gene can flow among the populations when $Nm>1$, resulting in the homogenization; when $Nm<1$, the populations can be strongly differentiated; once $Nm>4$, the populations would become a random unit (Wright, 1931). According to these criteria, high-level gene flow of *P. koraiensis* existed among provenances.

Genetic similarity

The genetic similarity is an important index for estimation of the genetic differentiation among provenances. Based on the analysis by POPGENE 32 software, the coefficient between two provenances ranged from 0.9590 to 0.9903. The minimum coefficient occurred between Helong and Tongzigou, the maximum occurred between Yanshou and Dongfanghong. The result suggested that there was a high genetic similarity of *P. koraiensis* between provenances, indicating a lower reproductive isolation among the provenances (chart omitted here).

UPGMA cluster analysis

Based on the UPGMA cluster diagram, the 24 provenances may be divided into three groups with the genetic

Table 4. The genetic diversity of *Pinus koraiensis* among 24 provenances.

Provenance	Percentage of polymorphic loci	Effective number of alleles	Nei's gene diversity index	Shannon's diversity index
Lushuihe	40.87%	1.1895	0.1162	0.1788
Qinghe	44.77%	1.2265	0.1363	0.2081
Huanan	43.40%	1.2105	0.1265	0.1941
Chaihe	43.16%	1.2014	0.1235	0.1904
Wuyiling	41.28%	1.2097	0.1264	0.1925
Helong	39.66%	1.1862	0.1135	0.1744
Yanshou	44.49%	1.2189	0.1313	0.2008
Dahailin	45.80%	1.2416	0.1438	0.2182
Liangzihe	40.68%	1.2103	0.1244	0.1884
Dashitou	41.88%	1.1984	0.1193	0.1832
Dongfanghong	40.79%	1.1875	0.1117	0.1719
Tongzigou	40.09%	1.2109	0.1246	0.1884
Zhanhe	40.95%	1.1975	0.1187	0.1816
Xiaobeihu	43.64%	1.2139	0.1293	0.1981
Hebei	42.37%	1.2112	0.1282	0.1959
Baihe	39.83%	1.2266	0.1307	0.1951
Linjiang	40.85%	1.2172	0.1273	0.1920
Tieli	40.95%	1.2066	0.1241	0.1886
Liangshui	38.33%	1.1867	0.1130	0.1722
Muling	41.48%	1.1944	0.1189	0.1827
Wangqing	42.42%	1.2187	0.1290	0.1952
Caohekou	42.98%	1.1922	0.1189	0.1850
Wuying	39.13%	1.1954	0.1174	0.1785
Bajiazi	38.12%	1.1807	0.1110	0.1691

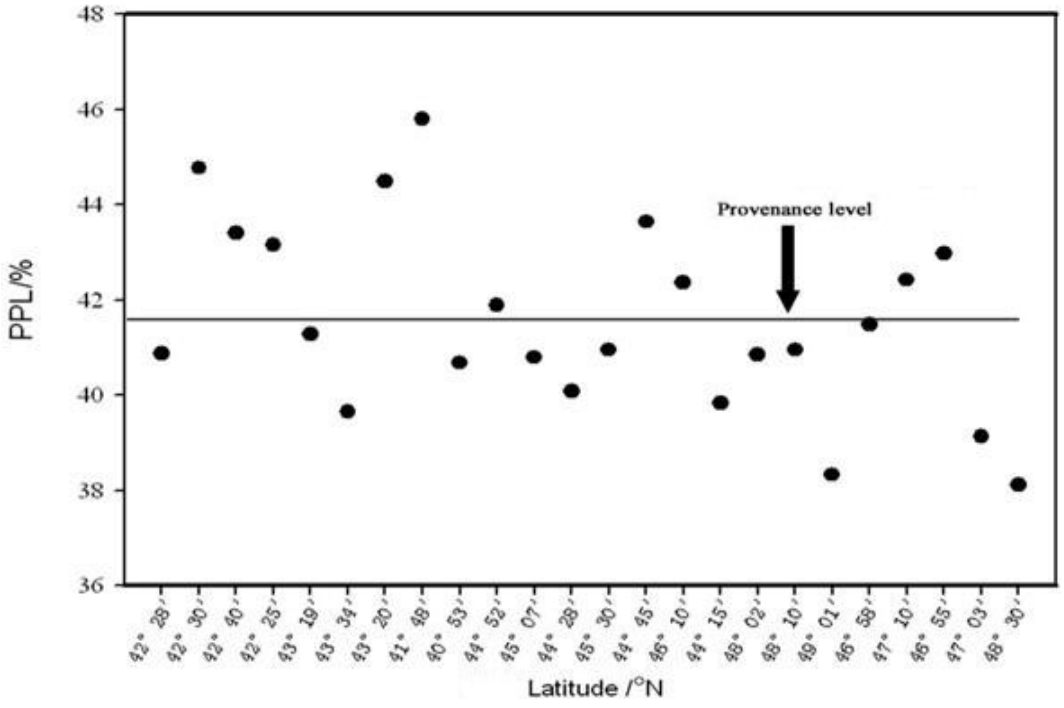

Figure 3. Discrete chart of percentage of polymorphic loci among the 24 provenances located at different latitudes.

Table 5. The genetic differentiation of *Pinus koraiensis* in the 24 provenances.

Source of variation	df	SSD	MSD	Proportion		Significance testing (P)
				Absolute	%	
Inter-provenances	23	786.33	34.19	1.54	7.65	> 0.05
Intra-provenances	216	4061.20	18.80	18.80	92.35	> 0.05
Total	239			20.34	100	

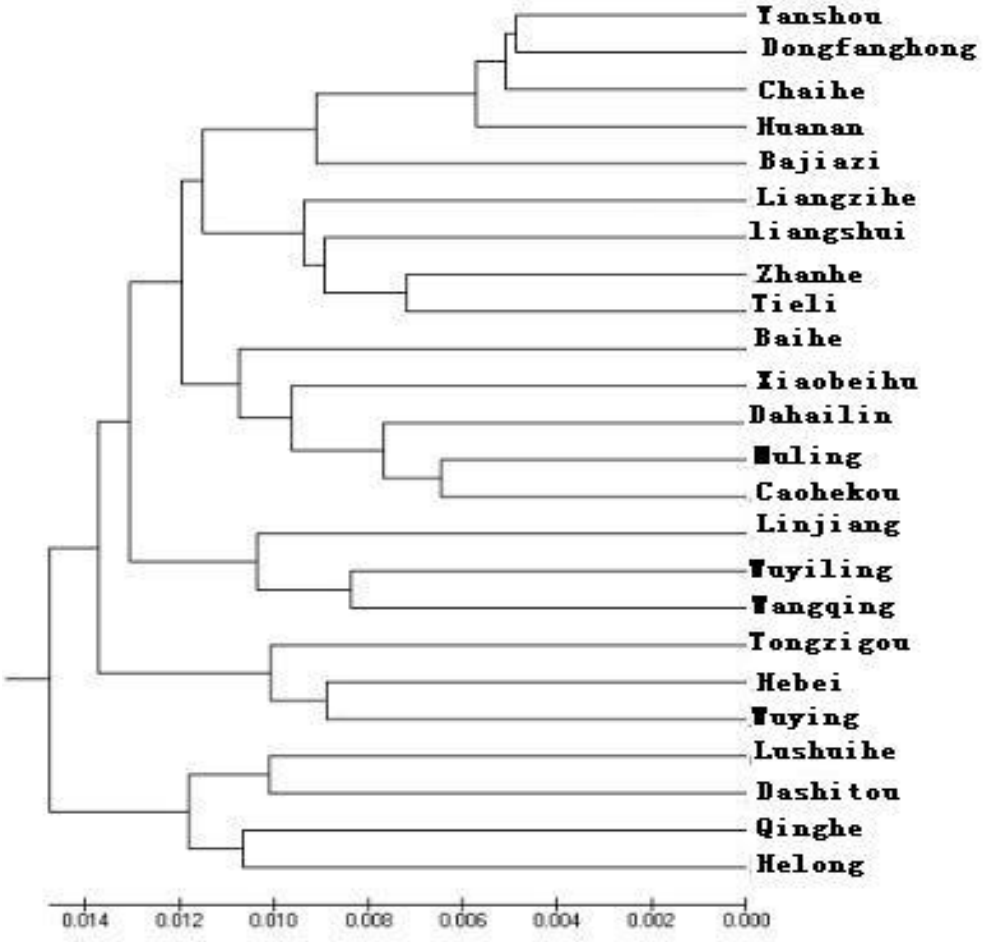

Figure 4. Genetic-distance based on dendrogram of *Pinus koraiensis* among the 24 provenances.

distance of 0.013. One group included Yanshou, Dongfanghong, Chaihe, Huanan, Bajiazi, Liangzihe, Liangshui, Zhanhe, Tieli, Baihe, Xiaobeihu, Dahailin, Muling, Caohekou, Wuyiling, Wangqing, and Linjiang; another group, Tongzigou, Hebei and Wuying; the third group, Lushuihe, Qinghe, Helong and Dashitou.

According to the traditional division of provenances, the natural *P. koraiensis* forests in China can be divided with the mountainous regions and latitude populations into three sub-regions, southern, central and northern. The southern sub-region mainly refers to Changbai Mountain. The central sub-region refers to Changkuant-sailing and Wandashan Forest Area. The northern sub-region refers to Lesser Khingan Mountain. On the basis, the 24 provenances can also be divided into three groups. The southern sub-area includes Lushuihe, Helong, Bajiazi, Baihe, Dashitou, Tongzigou, Wangqing, Linjiang, and Caohekou. The central sub-area includes Muling, Dongfanghong, Dahailin, Yanshou, Chaihe, Huanan, and Xiaobeihu. The northern sub-area includes Hebei, Wuying, Wuyiling, Tieli, Liangshui, Qinghe, Liang-zihe, and Zhanhe. However, the dendrogram in Figure 4 showed that two provenances in the same subarea may belong to different groups based on the genetic distance.

Table 6. Allele frequencies with significant difference among the 24 provenances (part results).

Provenance	Allele frequency									
	ME1-EM9-5-0	ME1-EM9-12-0	ME1-EM9-20-0	ME3- EM1-3-1	ME3-EM1-5-0	ME3-EM1-19-1	ME3-EM4-1-0	ME3-EM6-18-0	ME5-EM5-10-0	ME5-EM9-18-0
Dongfanghong	0.3162	0.3162	0	0.6838	0.3162	0.1056	0.5477	0	0.5477	0.8367
Liangshui	0	0.6325	0.3162	0.2254	0	0.3675	0.5477	0.5477	0.7746	0.5477
Wangqing	0.5477	0.5477	0.3162	0.4523	0.3162	0.2929	0.6325	0.4472	0.4472	0.5477
Hebei	0.4472	0.5477	0.3162	0.4523	0.4472	0.1633	0.3162	0.3162	0.7071	0.7746
Liangzihe	0	0.5477	0	0.2929	0	0.2254	0.3162	0.5477	0.3162	0.4472
Wuyiling	0	0.4472	0.3162	0.3675	0	0.2254	0.3162	0.3162	0	0.7746
Helong	0.5477	0.3162	0.3162	0.4523	0.3162	0.1633	0.5477	0.3162	0.6325	0.3162
Dashitou	03162	0.3162	0.4472	0.1633	0.3162	0.5528	0.4472	0.5477	0.6325	0.4472
Qinghe	0.3162	0.3162	0.4472	0.4523	0.3162	0.2254	0	0	0.5477	0.7746
Dahailin	0	0	0.4472	0.5528	0.4472	0.1633	0.3162	0	0.7071	0.5477
Huanan	0.6325	0.4472	0.3162	0.4523	0.4472	0.1056	0.4472	0.3162	0.6325	0.5477
Chaihe	0.6325	0.4472	0.4472	0.0513	0	0.1056	0.4472	0.6325	0	0
Zhanhe	0	0.4472	0.3162	0.4523	0.3162	0.2254	0.7746	0.4472	0.8367	0.5477
Tongzigou	0.3162	0.4472	0	0.1056	0	0.5528	0.7071	0.3162	0.7746	0.5477
Yanshou	0.4472	0.3162	0.7071	0.1633	0	0.1056	0.3162	0.4472	0	0
Xiaobeihu	0.3162	0.4472	0.3162	0.4523	0.3162	0	0.5477	0.4472	0.3162	0.7746
Tieli	0.4472	0.4472	0.3162	0.5528	0.5477	0.1056	0	0.3162	0	0.7071
Wuying	0	0.4472	0.3162	0.5528	0.4472	0.2929	0.7071	0.3162	0.6325	0.6325
Lushuihe	0	0	0.3162	0.3675	0	0.2254	0.6325	0.3162	0.5477	0.5477
Linjiang	0.3162	0.4472	0.4472	0.2254	0.4472	0.2254	0.7071	0.6325	0.8367	0.4472
Baihe	0.3162	0.4472	0.3162	0.3675	0.3162	0.4523	0	0.3162	0.6325	0.6325
Muling	0.4472	0.5477	0.4472	0.1633	0.3162	0.2929	0.5477	0.4472	0.5477	0.5477
Caohekou	0.4472	0.5477	0.4472	0.1633	0.4472	0.2254	0.3162	0.4472	0.3162	0
Bajiazi	0.5477	0	0.3162	0.4523	0.7071	0	0.6325	0.3162	0.4472	0.6325

For instance, both Helong and Tongzigou provenances belong to the southern subarea, but the genetic distance between them was large among the 24 provenances. It meant that two provenances traditionally divided into the same subarea may not have a high genetic similarity. The above finding disagrees with the previous reports that provenances in the same mountainous region have a relative high similarity of genetic structure due to the low geographic isolation.

Single site of allele frequency

A total of 143 polymorphic loci from the 24 provenances were obtained by amplification of 9 primer pairs. Each locus contained a pair of allele, remarked as 0 and 1. Through comparing the obtained 286 alleles, about 110 alleles among different provenances showed significant difference, indicating multi-alleles of different provenances may be undergone natural selection (Table 6).

DISCUSSION

SRAP is a novel molecular marker technique designed to amplify open reading frames (ORFs), which is the essential components of gene sequences. Obviously, SRAP can obtain precisely large numbers of information on the diversity of genetic resource. However, the novel technique is rarely introduced into the researches on forest tree genetic diversity, especially needle-leaved

tree species. Thereby, estimating genetic diversity of *P. koraiensis* at the level of species is still incomparable. The present study shows that percentage of polymorphic loci and average number of polymorphic loci of each primer pair amplified of *P. koraiensis* are medium level as compared with those of reported in other species (Li and Zhang, 2005; Wang et al., 2006; Li et al., 2006; Han et al., 2007, Li et al., 2007; Liu et al., 2007; Zhang et al., 2007; Wang et al., 2008; Ortega et al., 2007; Zhang et al., 2008; Guo and Luo, 2006). This result is consistent with those reported by Xia et al. (2001) and Feng et al. (2006). For a large population with random mating, genetic diversity is mainly caused by gene mutation and gene recombination, involving comprehensive factors such as population lifetime, effective size, structure, chromosomal property and structure, and breeding regime (Stebbins, 1950). As an ancient species, *P. koraiensis* has accumulated a large number of variations during long-term evolution. Moreover, from the life period, *P. koraiensis* belongs to the species of longevity, outcrossing and K strategy with anemophily. All the above features are of benefit to improve gene recombination. Therefore, it can infer a higher genetic diversity in *P. koraiensis*. But taking into consideration the limitation of genetic recombination by later sexual maturity of natural *P. koraiensis* forest (80 - 120 years) and low harvest of the seeds, the genetic diversity of the species can be affected negatively to some extent.

Keeping genetic diversity is the key in multi-generation improvement of forest tree, fine provenances should have extensive genetic base and significant genetic gain. However, neither the percentage of polymorphic loci nor diversity index has significant difference among the 24 provenances. In productive practice, when provenances have large difference in genetic diversity, selection breeding must be first confirmed to select large natural population rich in genetic diversity, and then to select good individuals. At present, selection breeding of *P. koraiensis* forest can ignore the limitation of geographic location and directly select the individuals with advanced growth traits.

Breeding system plays a key role in influencing the pattern of genetic variation at the population level, especially the genetic diversity within and among populations. According to the statistical results of 449 plants (gymnosperm and angiosperm populations) from 1968 to 1988, Hamrick and Godt (1990) calculated that 90% genetic variation of outcrossing species occurred at intra populations; only 10% genetic differentiation occurred at inter populations. Yang et al. (1989) analyzed the frequency (G_{ST} = 0.027) of polymorphic loci of 4 populations distributed in major *P. koraiensis* forest in Northern China, by using isozyme technique; 97.30% of total diversity came from the variation of intra population. Feng et al. (2006) reported the relative genetic differentiation (G_{ST} = 0.2700) through analyzing genetic structure of 4 large natural *P. koraiensis* forest by ISSR technique, and found that 72.99% of total genetic diversity was caused by the variation of intra population. The present study (G_{ST} = 0.0765; 92.35%) is slightly higher than that (approximate 90%) reported by Hamrick and Godt (1990). The reason of slightly higher G_{ST} may be caused by the morphological character of *P. koraiensis*; the tall plants benefit due to reduced resistance to pollen movement in air. This is because the extine of *P. koraiensis* has air chamber, is bigger in length and width than other pine. Also, a long-distant pollen flow enhances the gene recombination (Zu et al., 2000). Moreover, lack of effective geographic isolation may also contribute to the improvement of gene homogenization among provenances; *P. koraiensis* forest are mainly distributed at low mountainous upland in Northern China, with a closer distance between provenances.

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