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Mating system patterns of natural populations of *Pinus koraiensis* along its post-glacial colonization route in northeastern China

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ABSTRACT. To understand the genetic mechanisms underlying the endangerment of *Pinus koraiensis*, we studied the mating system of 49 families of this species in 3 natural populations along its post-glacial colonization route across ~1500 km in northeastern China using the chloroplast simple sequence repeat technique. We analyzed 11 polymorphic loci with clear and repeating bands, and we calculated the multi-locus outcrossing rate (t_m), single-locus outcrossing rate, inbreeding index, and fixation index (F). Intra-population variation was not observed, but a large inter-population variation was observed in the outcrossing rate, and the t_m increased from 0.767 (the south population) to 0.962 (the north population) along the post-glacial colonization route. The t_m values within a population did not change with time over 2 consecutive years. The F values for the 3 populations were <0, which

indicates an excess of heterozygotes. The mean effective number of alleles, Shannon diversity index, and Nei's genetic diversity index did not show a south-north pattern. The north population had the highest outcrossing rate but the lowest genetic diversity. The average genetic differentiation of *P. koraiensis* populations was 0.1251, which was within the average range of woody plants with outcrossing and wind pollination. This study suggests that the current endangerment of *P. koraiensis* is not related to its genetic structure; perhaps it is mainly caused by man-made and natural disturbances such as deforestation and fire. Therefore, reducing disturbances and enhancing habitats, rather than the genetic aspects, play more important roles in the long-term protection of *P. koraiensis*.

Key words: Inbreeding depression; Outbreeding depression; Genetic diversity; Chloroplast simple sequence repeat; Endangered species

INTRODUCTION

The utilized plant mating system is a key biological factor affecting the genetic structure of a plant population (Ge, 1998). Mating systems of plant species present various evolutionary responses to natural selection, and the characteristics of mating systems affect the evolutionary rate of a species (Shaw et al., 1998). Studies on population-level mating systems promote a better understanding of population ecology, plant life history, and evolutionary biology. Plant mating systems can be studied by calculating the outcrossing and self-crossing rates of a population and their relative proportions of the generated offspring. Inbreeding depression is believed to be the main selection pressure in the evolution of a mating system, and the mating system determines whether inbreeding depression occurs (He and Ge, 2001).

Chloroplast simple sequence repeat (cpSSR) is a new high-efficiency molecular marker technique with the advantages of microsatellite markers and characteristics of chloroplast DNA. The cpSSR has important implications for the identification of closely related species and the description of genetic differences at the individual and population levels. Recently, this technique has been widely used in the genetic analysis of plant populations, phylogeny, and inter-population gene flow (Kaundun and Matsumoto, 2002). Many studies used this method to study genetic differentiation, pollen transmission, gene flow, and evolution history in *Pinus* species such as *P. resinos* (Walter and Epperson, 2001), *P. albicaulis* (Richardson et al., 2002), *P. pinaster* (Liu and Yang, 2007), *P. massoniana* (Liu and Yang, 2007), *P. yunnanensis* (Liu and Yang, 2007), and *P. taiwanensis* (Liu and Yang, 2007) because of the characteristics of the paternal heredity of the chloroplast genome of *Pinus* species. For *P. koraiensis* Sieb. et Zucc., the application of this method focused on the population genetic diversity (Shao et al., 2007), and the gene flow and mating system were not involved.

P. koraiensis, a protected tree species in China with important economic and ecological values, is a dominant species in the climax vegetation, mixed needle broad-leaved forests in the eastern mountainous region of northeastern China. Forest use and habitat fragmentation have led to rapid decline in the distribution areas of natural *P. koraiensis* populations. For example, the natural distribution of *P. koraiensis* in China decreased from 630,000 ha in 1984

to 180,000 ha in 2004 (State Forestry Administration of China, unpublished data). In addition to disturbance, we also need to understand the inbreeding and outcrossing depressions (Ge, 2000) to develop protection strategies for the endangered species. In this article, we studied the mating system of natural *P. koraiensis* populations using the cpSSR technique. We calculated the outcrossing and self-crossing rates of populations to infer whether inbreeding or outcrossing depressions exist in natural *P. koraiensis* populations. We aimed to explore the spatial and temporal patterns of the mating systems at the inter- and intra-population levels and their effects on the population genetic structure and offspring fitness to understand the mating mechanisms underlying the evolution process and reasons for that species being endangered.

MATERIAL AND METHODS

Sites and plant materials

P. koraiensis is a tertiary relict tree species that expanded its distribution along a post-glacial colonization route from the refuge center in the Korean Peninsula northwards to Changbai Mountains, Lesser Khingan Mountains, and the Russian Far East. We selected natural *P. koraiensis* populations from south to north along the post-glacial expansion route in Baishilizi National Nature Reserve (south population, Kuandian, Liangning Province), Hongwei Forest Center (intermediate population, Lushuihe, Jilin Province), and Fenglin National Nature Reserve (north population, Yichun, Heilongjiang Province) in northeastern China (Figure 1 and Table 1).

Figure 1. Geographic locations of the natural *Pinus koraiensis* populations studied along the post-glacial colonization route in northeastern China.

Table 1. Population characteristics and site conditions.

Population	MAT (°C)	MAP (mm)	Coordinate	Altitude (m a.s.l.)	Community	Density (stem/ha)/DBH (cm) age (years) of *P. koraiensis*	Soil type
North (Fenglin)	-0.5	650	129°20'30"E, 48°03'54"N	550	*P. koraiensis Fraxinus mandschurica - Phellodendron amurense*	50-80/40-50/180-220	Dark brown forest soil
Intermediate (Lushuihe)	2.7	871	127°02'06"E, 41°52'49"N	700	*P. koraiensis - Tilia amurensis F. mandschurica*	60-70/50-130/200-300	Dark brown forest soil
South (Baishilizi)	5.3	1349	124°52'30"E, 40°53'12"N	950	*P. koraiensis - Quecus mongolica - F. mandschurica*	100-120/40-60/100-150	Mountain dark brown forest soil

MAT = mean annual temperature; MAP = mean annual precipitation.

To understand the inter-population variation, we selected 18 maternal individuals from the north (Fenglin) and south populations (Baishilizi) and 13 individuals from the intermediate population (Lushuihe) in September 2010 (Figure 1 and Table 1). The distance between any 2 individuals within a population was >300 m. Ten seeds were randomly selected from >20 cones collected from each maternal tree. A total of 490 offspring (seed embryos) were obtained. DNA was extracted from the seed embryos for further analysis. To study the time-dependent variation, we took samples from the 18 individuals in the north population in September 2011 (i.e., 2010 *vs* 2011).

DNA extraction

The seed embryo DNA was extracted using the traditional cetyltrimethylammonium bromide method (Clark et al., 1998). The DNA concentration and purity were examined by 0.8% agarose gel electrophoresis and ultraviolet spectrophotometer.

cpSSR-polymerase chain reaction (PCR) system

The amplification program was set according to Feng et al. (2010). PCR was carried out in a 20-µL volume containing 1X buffer, 50-120 ng template DNA, 2.0 mM Mg^{2+}, 0.2 mM of each dNTP, 0.5 µM reverse primer, 0.5 µM forward primer, and 0.4 U Taq polymerase.

An initial denaturation at 94°C for 5 min was followed by 35 cycles of 60 s at 94°C, 60 s at 35°C, and 2 min at 72°C and a final extension of 7 min at 72°C. Sequence data were obtained on a Gene Amp PCR System (ABI Cor., USA). dNTPs and Taq enzymes were provided by TaKaRa Biotechnology Co., Ltd. (Dalian, China).

Selection of primers

The cpSSR primers used in this study were obtained from published results for *Pinus* species such as *P. densiflora*, *P. pinaster*, *P. sylvestris*, and *P. contorta* (Provan et al., 1998; Marshall et al., 2002; Ribeiro et al., 2002; Dzialuk et al., 2009). A total of 70 pairs of cpSSR primers were synthesized for the first selection, and 47 pairs with significant main bands were selected. Finally, 9 pairs of cpSSR primers with high polymorphism were used (Table 2).

Table 2. cpSSR primer sequences.

Primers	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
ccSSR-3	CCAAAAGCTGACATAGATGTTA	TTTCATTCGGCTCCTTTATG
ccSSR-14	GGGTATAATGGTAGATGCCC	GCCGTAGTAAATAGGAGAGAAA
ccSSR-15	GCTTATGACCTCCCCCTCTATGC	TGCATTACAGACGTATGATCATTA
Pt79951	CTTTTGTTTTTCAACAATTGCA	ACATCTATCTCCCATATCGGC
PCP1289	TCCTGGTTCCAGAAATGGAG	TAATTTGGTTCCAGAATTGCG
PCP45071	ACTGGTCTGATCGACCCAAT	TTCTACACTTGCGGAAACCC
PCP79987	TTTTCAACAATTGCATTTACCG	GGCGGGATAGGAGTCTTTTC
10F/RR	CAGAAGCCCAAGCTTATGGC	CGGATTGATCCTAACCATAC
69F/R	TTTCGGGCTCCACTGTTATC	CGTACTCAATTTGTTACTAC

Product examination

Amplified fragments were segregated on 6% denatured polyacrylamide gels with 7 M urea and 0.5X Tris-boric acid-ethylenediaminetetraacetic acid electrophoretic buffer. After electrophoresis, the gel was stained with silver nitrate solution. The bands were scanned using a Founder scanner U 430.

Data analysis

The MLDT program was used to evaluate the multi-locus (t_m) and single-locus (t_s) outcrossing rates, standard deviations, inbreeding index (t_m - t_s), and fixation index (F) according to the mixed mating model (Ritland, 1990). The POPGENE 32 software (Yeh et al., 1999) was used to calculate Nei's gene diversity index, observed number of alleles, Shannon's diversity index, effective number of alleles, and genetic differentiation.

RESULTS

Differences in inter-population mating systems

Nine cpSSR primers (Table 2) were used for the amplification of 490 offspring samples from 49 individual families of 3 populations. A total of 11 polymorphic loci with clear and repeating bands were obtained, and their fragment lengths ranged from 130 to 500 bp (Figure 2).

Analysis of the 11 polymorphic loci (Figure 2) revealed that both the t_m and t_s values had a consistent changing pattern (Table 3) that followed the following decreasing order: the north population > the intermediate population > the south population. For example, the t_m values decreased from 0.962 (north population) to 0.767 (south population) (Table 3). The 3 populations had a low inbreeding level and an F value of less than zero (Table 3), indicating the existence of excess heterozygotes. Based on the F values calculated for the 3 populations (Table 3), the south population had the mating pattern that was closest to random mating.

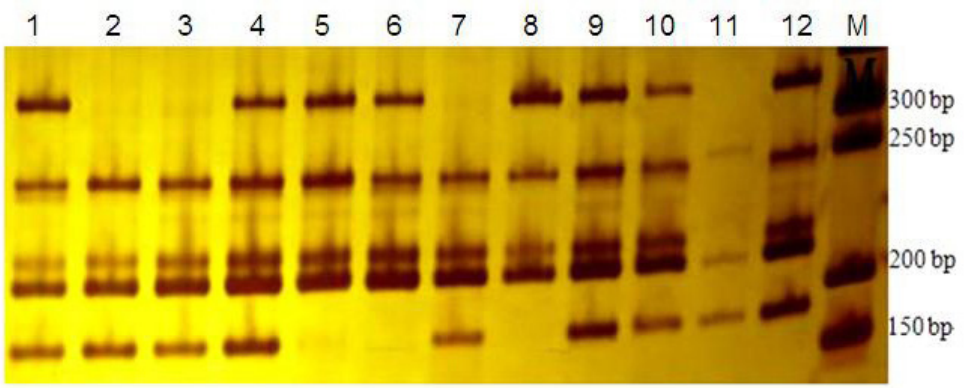

Figure 2. Amplification of the partial samples from the south population (Baishilizi, Figure 1, Table 1) by the primer 69F/R. *Lane M*: marker.

Table 3. Comparison of mating systems between the three *Pinus koraiensis* populations (mean values ± SD, N = 18 for the north and the south population, and N = 13 for the intermediate population).

Population	Multi-locus outcrossing rate (t_m)	Single-locus outcrossing rate (t_s)	Inbreeding index (t_m - t_s)	Fixed index (F)
North (Fenglin)	0.962 ± 0.000	1.116 ± 0.160	-0.154 ± 0.010	-0.300
Intermediate (Lushuihe)	0.804 ± 0. 023	0.778 ± 0.011	0.026 ± 0.000	-0.300
South (Baishilizi)	0.767 ± 0.060	0.771 ± 0.035	-0.004 ± 0.031	-0.077

Mating system distribution in different individuals

Statistical significance analysis showed that there was no significant difference in the t_m and t_s values among individuals within a population (Table 4). The t_m and t_s values of the 13 individuals in the intermediate population (Lushuihe) ranged from 0.5 to 1.0 (accounting for 31% of the total outcrossing rates for the 13 individuals) and from 1.0 to 1.5 (38% of the total rates), respectively. The t_m and t_s values of the 18 individuals in the north (Fenglin) population were in the range of 0.5 to 1.0 (39% of the total rates) and 1.0 to 1.5 (33%), respectively. The t_m of the south (Baishilizi) population was in the range of both 0.5 to 1.0 and 1.0 to 1.5, each accounting for 39% of the total rate for the 18 individuals, whereas the t_s values were in the range of 1.0 to 1.5, accounting for 50% of that of the 18 individuals. The t_m and t_s values had a similar normal distribution for each of the 3 populations (Figures 3 and 4).

Table 4. Statistical significance analysis of multi-locus (tm) and single-locus (ts) outcrossing rates among individuals within a population.

Outcrossing rate	North (Fenglin)	Intermediate (Lushuihe)	South (Baishilizi)
t_m	0.78^{ns}	1.05^{ns}	0.97^{ns}
t_s	1.07^{ns}	1.17^{ns}	0.98^{ns}

'ns' means non-significant difference (P > 0.05) in t_m or t_s among individuals within a population.

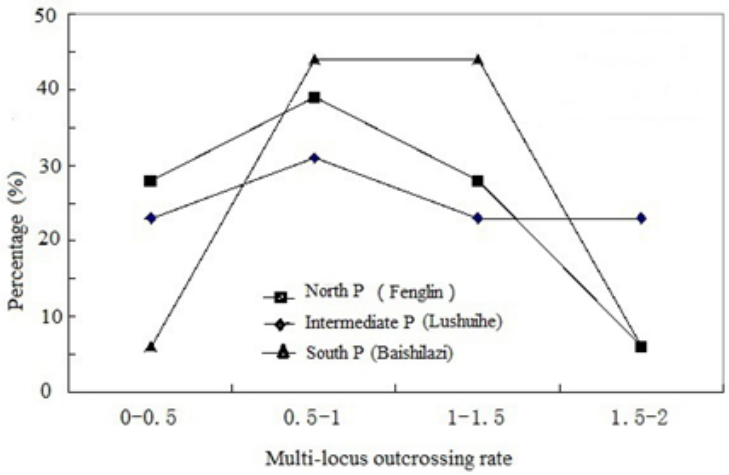

Figure 3. Distribution of the multi-locus outcrossing rate in different individuals of *Pinus koraiensis* populations (P).

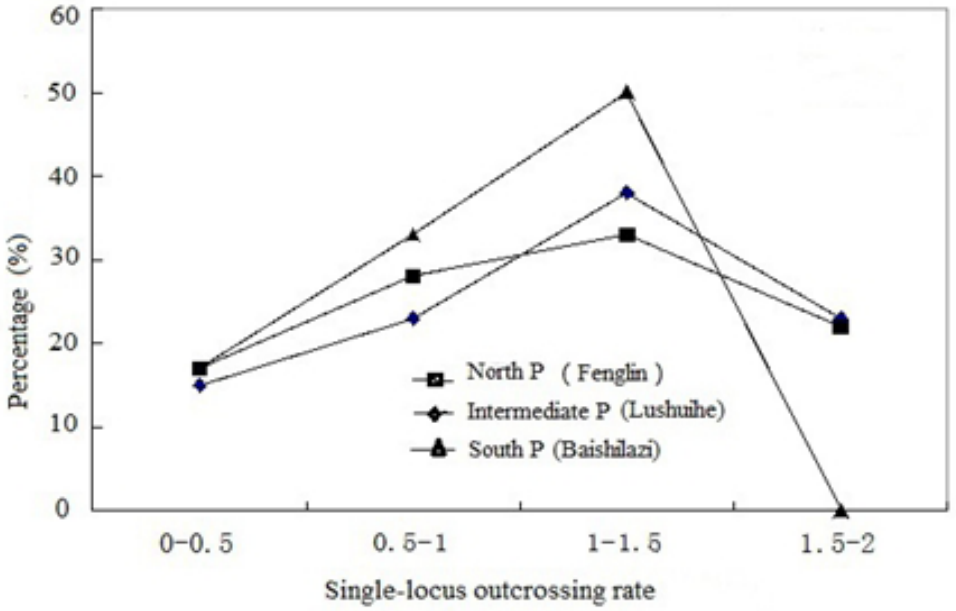

Figure. 4. Distribution of the single-locus outcrossing rate in different individuals of *Pinus koraiensis* populations (P).

Dynamics of mating systems

The t_m and t_s values for the north (Fenglin) population were 0.966 and 0.993 in 2011 and 0.962 and 1.116 in 2010, respectively, showing a non-significant difference in the t_m values but a significant difference in the t_s values between the 2 consecutive years.

Genetic diversity and differentiation

The 3 populations had the same patterns of effective number of alleles, Shannon diversity index, and Nei's diversity index: they followed the decreasing order of intermediate population (Lushuihe) > south population (Baishilizi) > north population (Fenglin) (Table 5). The north population had the highest outcrossing rate but the lowest genetic diversity (Table 5).

The mean total genetic diversity (H_T) of *P. koraiensis* populations was 0.1260. The intra-population genetic diversity (H_S) was 0.1102, and the inter-population genetic diversity (D_{ST}) was 0.0158. The average genetic differentiation was 0.1251. The H_S and D_{ST} accounted for 87.5 and 12.5% of H_T, respectively.

Table 5. Genetic diversity of the three *Pinus koraiensis* populations.

Populations	Numbers of samples	Averaged numbers of alleles	Averaged effective numbers of alleles	Shannon diversity index	Nei diversity index
North (Fenglin)	180	1.5000[a]	1.1058[a]	0.1481[a]	0.0835[a]
Intermediate (Lushuihe)	130	1.5000[a]	1.3009[b]	0.2568[a]	0.1727[a]
South (Baishilizi)	180	1.4444[a]	1.1887[ab]	0.1963[a]	0.1241[a]

Mean values are given. Different letters in the same column indicate significant difference at $P < 0.05$ level.

DISCUSSION

Inbreeding and outcrossing depression

This study found that the outcrossing rate of *P. koraiensis* populations ranged from 0.767 to 0.962 (Table 3), which is at the medium level of data published for *Pinus* species. The outcrossing rate was 0.451-0.522 for *P. bungeana* (Li and Gu, 2004), 0.864 for natural *P. tabuliformis* populations (Zhang et al., 2000), 0.950 for *P. sylvestris* (Korshikov and Demkovich, 2010), 1.098 for *P. massoniana* (Tan et al., 2012), and 1.020 and 1.000 for *P. strobus* and *P. contorta*, respectively (Beaulieu and Simon, 1995).

The genetic basis of inbreeding depression is considered as heterozygote vigor and homozygosis of deleterious recessive genes (Charlesworth and Charlesworth, 1999). According to Wright (1969), $-1 < F < 0$ indicates an excess of heterozygotes and a deficiency of homozygotes. The Wright F values obtained in this study (-0.300 to -0.077; Table 3) showed that an excess of heterozygotes and a deficiency of homozygotes existed in all 3 of the populations, indicating that the homozygosis of deleterious recessive gene occurs with a low probability; therefore, there is a low probability of inbreeding depression.

Genetic differentiation has been used to judge whether outcrossing depression occurs. Offspring fitness will decline when genotype differences of parents exceed a certain threshold (Ge, 2000). Similarly, Becher et al. (2006) stated that outcrossing depression occurs only when the genetic differentiation exceeds a certain threshold. This study found that the average genetic differentiation value was 0.1251, accounting for 12.5% of the total genetic diversity. This result is in agreement with our previous results (G_{ST} = 0.1468; Feng et al., 2009). Hamrick et al. (1992) reported that the inter-population gene variation for most wind-pollinated and cross-pollinated woody plants accounts for 10% of the total gene variation. The genetic differentiation of the *P. koraiensis* populations is at a medium level, suggesting that outcrossing depression may not be possible in this species.

Stebbins et al. (1957) concluded that the mating system more strongly influences the plant genetic structure than any other life-cycle factor. There is a common view that the mating system affects population genetic diversity mainly by influencing heterozygosity and the coefficient of genetic differentiation (Ge, 1998). In this study, we found that the inter-population genetic variation was 12.5%, which was similar to that reported by Hamrick et al. (1990), who found that the inter-population variation accounted for 51% of the total genetic variation of self-mating species, whereas it only accounted for 12% of the genetic variation of outcrossing species. Our results suggest that *P. koraiensis* has an outcrossing-dominated mixed mating type.

Stability of the mating system

Previous studies have found that the mating system of plant species is dynamic. For example, the outcrossing rate may have large variations among populations of the same species, among different individuals within a population, or in the same individual in consecutive years (Hamrick, 1990). The mating system is influenced not only by genetic factors but also by various environmental factors such as altitude, temperature, humidity, population size, and density (Neale and Adams, 1985). Carneiro et al. (2011) also found that the felling period has impacts on the mating system and pollen transmission of *Hymenaea courbaril* populations.

Hence, the relatively large inter-population variation in the outcrossing rate (0.767 for the south population to 0.962 for the north population) that was observed in this study (Table 3) may be a result of environmental variations among the 3 populations across a large scale of 1500 km (Figure 1 and Table 1). Moreover, the outcrossing rates of the populations showed an increasing trend along the evolutionary expansion route from south to north; this implies that natural selection pressure constantly enhances the outcrossing level in the evolution process of *P. koraiensis*. Shaw et al. (1998) stated that the evolution and changes in the population mating system are profoundly influenced by new selective pressures. Generally, inbreeding can improve the probability of homozygosis of recessive gene, which may lead to a loss of detrimental genes in a population, although these lost genes might be able to adapt to a changing environment under global environmental change (Charlesworth and Charlesworth, 1995). Therefore, an inbreeding population has a short lifetime compared to an outcrossing population (Holsinger, 2000). *P. koraiensis* is a typical species with long longevity; it tends to select the strategy to improve the outcrossing rate in its evolution.

We did not observe significant differences in the outcrossing rate among individuals within a population, which showed a normal distribution (Figures 3 and 4). This result may be attributed to the consistent ecological and evolutionary consequences within a population, leading to a stable mating system with small intra-population variation. Similarly, Wang et al. (2012) found that the t_m values of 15 *Tsoongiodendron odorum* individuals ranged from 0.885 to 0.999, with a non-significant difference. Tan et al. (2012) also found a non-significant difference in the t_m values (ranging from 1.033 to 1.200) of 8 individuals obtained from a seed orchard of *P. massoniana*. However, De-Lucas et al. (2008) showed that there was a significant difference in the t_m values among *P. pinaster* individuals growing across areas in the Mediterranean region, which may be co-determined by abiotic factors across areas and biotic factors such as florescence synchronism, tree height, and canopy size among individuals in different populations (Tamaki et al., 2009).

The north (Fenglin) population showed a similar t_m value in 2010 (0.966) as it did in 2011 (0.962). It seemed that there is non-significant variation in the mating system of *P. koraiensis* across a short time series. This result agrees with the findings by Zhang et al. (2009), who also found similar values of outcrossing rates (1.200 and 1.072) in *P. massoniana* seeds collected in 2 consecutive years. Burczyk (1998) showed that the outcrossing rates of *P. sylvestris* in a clonal seed orchard were 0.976, 0.966, and 0.962 in 3 consecutive years. Zhang et al. (2004) investigated the outcrossing rates of *P. tabulaeformis* in a clonal seed orchard before and after thinning conducted in 1994, and they found that the outcrossing rate was 0.975 in 1984 and 0.962 in 1993 (before thinning), and 0.795 in 1996 and 0.801 in 2000 (after thinning). Our results indicate that *P. koraiensis*, like other *Pinus* species, has a non-significant variation in the outcrossing rate in a short time sequence. Unfortunately, long-term variations in the outcrossing rate of *Pinus* species are unavailable. Thus, investigations of long-term variations in the outcrossing rate of *P. koraiensis* trees are needed to reach a more accurate conclusion. Our results did not show differences in the t_m values, but there was a significant difference in the t_s values across 2 consecutive years, which suggests the use of t_s values to study the time-dependent mating system of a population. Similarly, Brown et al. (1985) found that the outcrossing rate had a small impact on polymorphic locus estimates but a large impact on single locus estimates.

Li and Gu (2004) found that the endangerment of *P. bungeana* was mainly caused by a high inbreeding rate in the mating system, while the endangered status of *P. ponderos* and

Cathaya argyrophylla was attributed to a low genetic diversity (Allendorf et al., 1982; Mosseler et al., 1992). This study found that the current endangerment of *P. koraiensis* did not seem to be related to its genetic structure; perhaps, the endangerment was mainly caused by man-made and natural disturbances such as deforestation and fire disaster. Therefore, reducing disturbance and enhancing habitats, rather than genetic aspects, play more important roles in the long-term protection of *P. koraiensis*. Moreover, the genetic variation of *P. koraiensis* exists among individuals within a population (Table 5), which suggests that it is also needed to protect and preserve the diversity within a population. For instance, collecting seeds from many different possible individuals within a population may help to maintain that genetic diversity in species to keep pace with global changes in the future (Wang, 1998).

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