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Detection of Low Genetic Variation in a Critically Endangered Chinese Pine, *Pinus squamata*, Using RAPD and ISSR Markers

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With only 32 individuals in the northeastern corner of Yunnan Province, China, Pinus squamata *is one of the most endangered conifers in the world. Using two classes of molecular markers, RAPD and ISSR, its very low genetic variation was revealed. Shannon's index of phenotypic diversity (*I*) was 0.030, the mean effective number of alleles per locus (*A_e*) was 1.032, the percentage of polymorphic loci (*P*) was 6.45, and the expected heterozygosity (*H_e*) was 0.019 at the species level based on RAPD markers. The results of ISSR were consistent with those detected by RAPD but somewhat higher (*$I = 0.048$, $A_e = 1.042$, $P = 12.3$, $H_e = 0.029$*). The genetic variation of the subpopulation on the southwest-facing slope was much higher than that of the subpopulation on the northeast-facing slope, which may be attributed to the more diverse environment on the southwest-facing slope. The genetic differentiation between the two subpopulations was very low. The between-subpopulation variabilities, Φ_{ST}, calculated from RAPD and ISSR data were 0.011 and 0.024. Because of the lack of fossil records and geological historical data, it was difficult to explain the extremely low genetic diversity of the species. We postulate that this ancient pine might have experienced strong bottlenecks during its long evolutionary history, which caused the loss of genetic variation. Genetic drift and inbreeding in post-bottlenecked small populations may be the major forces that contribute to low genetic diversity. Human activities such as logging may have accelerated the loss of genetic diversity in* P. squamata.

KEY WORDS: *Pinus squamata*; RAPD; ISSR; genetic variation; endangered species.

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INTRODUCTION

Pinus squamata X. W. Li is an extremely endangered species of pine recently described from the northeast corner of the province of Yunnan, in southwest China (Li, 1992). There are only 32 individuals in the wild. Because of its rareness and endangered status, it was listed as a first-class protected plant by the Chinese government in 1999. This species was locally used as timber or firewood. The wild population is close to the villages and its natural habitat was seriously threatened by local farming activities. Because of its general appearance, it was long considered a population of *P. bungeana* by foresters and was only described as a new species in 1992; however, this species can be easily distinguished from *P. bungeana* by its five-needled foliage. The systematic position of this species was also confusing. It was placed in a new series, Squamatae X. W. Li and Hsueh of sect. Parrya in subgenus *Strobus* when it was first published. Li and Zhu (1993) then treated it as a member of the Central American subsect. Balfourianae of their newly proposed subgenus *Parrya*. Price *et al*. (1998) suspected that this species might be a member of the subsect. Gerardianae of sect. Parrya or in its own subsection. Our recent molecular phylogenetic study based on five cpDNA sequences and nuclear ITS sequences confirmed that *P. squamata* should be included in subsect. Gerardianae of sect. Parrya (Zhang *et al*., 2003).

Although the community structure, wood anatomy, and phenology of *P. squamata* have been studied (Deng *et al*., 1995; Wang *et al*., 1998; Lu *et al*., 1999), 1ittle is known about its ecological properties as well as its genetic background. Conservation genetics has focused on rare and endangered species. Successful strategies for the maintenance of a species must include an understanding of the levels and distribution of genetic diversity (Hedrick, 2001). A lack of concern for the preservation of natural genetic diversity by conservation agencies is short-sighted (Hamrick and Godt, 1996).

The technique of random amplified polymorphic DNA, RAPD (Williams *et al*., 1990), has offered a promising marker system for use in the detection of genetic diversity in population genetics and conservation genetics (Fritsch and Rieseberg, 1996; Cruzan 1998). It has been successfully employed for that purpose in many species, such as the rare *Gentianella germanica* (Fischer and Matthies, 1998), the pines (Xia *et al*., 2001; S.-W. Lee *et al*., 2002), and wild rice (Qian *et al*., 2001).

Intersimple sequence repeat (ISSR) analysis is a novel technique applicable to genetic diversity studies (Gupta *et al*., 1994; Zietkiewicz *et al*., 1994). As a new class of genetic markers, ISSR overcomes some of the limitations of RAPD markers (Ratnaparkhe *et al*., 1998). In comparison with the RAPD method, ISSR is superior in terms of reliability (Fang *et al*., 1997; Nagaoka and Ogihara, 1997), because of the higher annealing temperature and the length of ISSR primers. It is expected that the genetic information revealed from this study using RAPD

Table I. Subpopulations of *P. squamata* for RAPD and ISSR Analyses

Subpopulation	Location	Environment	Population size	Sample size
Southwest-facing	103°00′E, 26°54′N Yangjiawan, Qiaojia Co.	1900 m alt, SW, gradient 43, mixed coniferous and broad-leaved forest	17	16
Northeast-facing	103°00′E, 26°54′N Fushan, Qiaojia Co.	1900 m alt, NE, gradient 45, broad-leaved forest	15	15

and ISSR markers will help to provide a framework for the development of a conservation program for this critically endangered species.

MATERIALS AND METHODS

Plant Material

Leaf samples were collected from 31 trees of *P. squamata* from two subpopulations on the southwest-facing and northeast-facing slopes about 0.5–1 km from each other, in Qiaojia County, the northeast corner of Yunnan province, southwest China (Table I; Fig. 1). Only one wild individual was not sampled; its needles were too high to be collected.

DNA Isolation

The protocol of CTAB total DNA isolation (Doyle and Doyle, 1987) was applied to isolate genomic DNA from silica-gel dried leaves. The DNA concentration was adjusted to 20 ng/μL for use in the polymerase chain reaction (PCR).

RAPD PCR Amplification

One hundred arbitrary primers from Operon Technologies Inc. (Operon B, C, J, S, and V kits) were used in the RAPD analysis. DNA amplification was performed in a T3 thermocycler (Biometra, Germany), programmed for an initial 240 s at 94°C, followed by 40 cycles of 15 s at 94°C, 45 s at 36°C, 90 s at 72°C, and a final 4 min at 72°C. Reactions were carried out in a volume of 20 μL containing 2.0 mmol/L $MgCl_2$ 0.5 μmol/L dNTP, 10× buffer, 2.5 μmol/L primer, 2 U *Taq* DNA, and 20 ng DNA template. Amplification products were analyzed by electrophoresis on 2.0% agarose gel buffered with 1× TAE, stained with ethidium bromide, and photographed under ultraviolet light. Molecular weights were estimated using Gene Ruler 100-bp DNA Ladder plus.

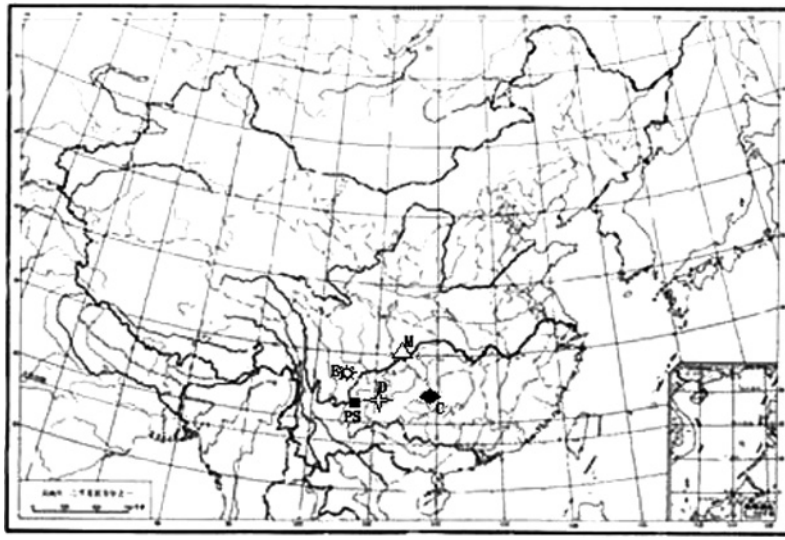

Fig. 1. The geographic location of *P. squamata* (PS) and adjacent locations of some living fossils, e.g., *Metasequoia glyptostroboides* (M), *Cathaya argyrophylla* (C), *Davidia involucrata* (D), and *Eucommia ulmoides* (E).

ISSR PCR Amplification

One hundred primers from the University of British Columbia Biotechnology Laboratory primer set 9 were used in the ISSR analysis. DNA amplification was performed in 2.5 mmol/L $MgCl_2$, 0.5 μmol/L dNTP, 10× buffer, 1% formamide, 2.5 μmol/L primer, 1.5 U *Taq* DNA, and 20 ng genomic DNA per 20 μL reaction. The PCR cycles were as follows: initial denaturation was at 94°C for 5 min, followed by 35 cycles of 30 s at 94°C, 45 s at 49°C, 1.5 min at 72°C, and a final 7-min extension at 72°C. The PCR products were separated on 1.5% agarose gels buffered with 0.5× TBE, detected by staining with ethidium bromide, and photographed under ultraviolet light. Molecular weights were estimated using a 100-bp DNA ladder.

Data Analysis

Both RAPD and ISSR are dominant markers. Amplified fragments were scored for the presence (1) or absence (0) of homologous bands, and two different matrices of the RAPD and ISSR data were analyzed with POPGENE (Yeh *et al.*, 1997), assuming Hardy–Weinberg equilibrium. The following genetic diversity parameters were estimated: Shannon's index of phenotypic diversity (I), mean effective number of alleles per locus (A_e), percentage of polymorphic loci (P), and

expected heterozygosity (H_e). The coefficient of gene differentiation (G_{ST}) among subpopulations using dominant markers such as RAPD is generally upward biased when compared with those derived from genotypic data, especially when sample size is small in endangered species (Isabel *et al*., 1995, 1999); therefore, we described genetic structure and variability among the subpopulations of *P. squamata* by calculating the between-subpopulation variability, Φ_{ST}, using the nonparametric analysis of molecular variance (AMOVA) program version 1.55 (Excoffier *et al*., 1992), and partitioned the variation component among individuals within populations, among populations within regions.

RESULTS

Evaluation of Primers

Because RAPD PCR is sensitive to reaction parameters, we optimized the reaction conditions, as described in Materials and Methods section. Fourteen out of the 100 primers generated strong amplification products. They were B-13 (TTCCCCGCT), B-14 (TCCGCTCTGG), B-19 (ACCCCCCAAG), C-17 (TTCCCCCAG), J-01 (CCCGGCATAA), J-04 (CCGAACACGG), J-05 (CTCCATGGGG), J-06 (TCGTTCCGCA), J-14 (CACCCGGATG), J-17 (ACGCCAGTTC), S-09 (TCCTGGTCCC), S-18 (CTGGCGAACT), S-19 (GAGTCAGCAG), and V-06 (ACGCCCAGGT).

One hundred ISSR primers were screened with four randomly selected plants. After comparing the magnesium concentrations and annealing temperatures, 17 primers that produced clear and reproducible fragments were selected for further analysis. They were 807 $(AG)_8T$, 808 $(AG)_8C$, 811 $(GA)_8C$, 812 $(GA)_8A$, 813 $(CT)_8T$, 818 $(CA)_8G$, 820 $(GT)_8C$, 825 $(AC)_8T$, 828 $(TG)_8A$, 834 $(AG)_8YC$, 840 $(GA)_8YT$, 842 $(GA)_8YG$, 844 $(CT)_8RC$, 855 $(AC)_8YT$, 857 $(AC)_8YG$, 864 $(ATG)_5$, 886 $VDV(TC)_7$.

Genetic Variability

All estimates of genetic variation based on both RAPD and ISSR were very low in *P. squamata* (Table II). The 14 primers of RAPD produced a total of 93 bands in the 31 individuals of the two subpopulations studied. Of the 93 loci surveyed, only six were polymorphic in the population. At the subpopulation level, the percentage of polymorphic loci was 6.45 for the southwest-facing subpopulation and 2.15 for the northeast-facing subpopulation. The 17 primers of ISSR produced a total of 73 bands in the 31 sampled individuals of the two subpopulations. Of the 73 loci surveyed, only nine were polymorphic in the population. At the subpopulation level, the percentage of polymorphic loci per subpopulation was

Table II. Genetic Variability at Subpopulation and Species Levels Detected by RAPD and ISSR

Subpopulation/species level	I	A_e	P (%)	H_e
RAPD				
Southwest-facing subpopulation	0.036	1.045	6.45	0.025
Northeast-facing subpopulation	0.014	1.017	2.15	0.009
Species level	0.030	1.032	6.45	0.019
ISSR				
Southwest-facing subpopulation	0.060	1.065	12.3	0.039
Northeast-facing subpopulation	0.018	1.018	4.11	0.011
Species level	0.048	1.042	12.3	0.029

12.3 for the southwest-facing subpopulation and 4.11 for the northeast-facing subpopulation.

Genetic Structure

The total genetic variance was primarily distributed in the two subpopulations. Based on RAPD, variance within subpopulations was 98.87% and variance among subpopulations was 1.13%. Based on ISSR, variance within subpopulations was 97.57% and variance among subpopulations was 2.43%. The estimate of Φ_{ST} was 0.011 based on RAPD and 0.024 based on ISSR, indicating a very low level of genetic differentiation between the two subpopulations.

DISCUSSION

Genetic Variation

A primary objective of conservation genetics is to estimate the level and distribution of genetic variation in endangered species (Fritsch and Rieseberg, 1996). Accurate estimates of genetic diversity are very useful for optimizing sampling strategies of conserving and managing tree genetic resources (Hamrick *et al*., 1991). Results from the present study using RAPD and ISSR markers revealed the very low level of genetic diversity in *P. squamata*, which is much lower than the genetic diversity reported in most conifers, regardless of the markers used (Wang *et al*., 1996; Bucci *et al*., 1997; Ge *et al*., 1998; Delgado *et al*., 1999; Li *et al*., 1999; Yu *et al*., 2000; Xia *et al*., 2001; S.-W. Lee *et al*., 2002), and higher than only *P. resinosa* (red pine) revealed by RAPD and isozyme markers (Mosseler *et al*., 1991, 1992).

Genetic diversity maintained in a plant species is influenced by specific characteristics of the species (Hamrick and Godt, 1989). As a group having long life-history traits associated with wind-pollinating behavior, most conifers

are among the most genetically variable of plant species (Hamrick *et al*., 1992). In reviewing a few pines that have been studied, a general pattern of high genetic diversity seems to be emerging, except for red pine. For red pine, it was hypothesized that a historical genetic bottleneck during glacial episodes of the Holocene was the main reason for low polymorphism in this species. The highly fragmented metapopulation structure of the red pine and its ability to self-pollinate may have resulted in a further loss of genetic variation.

Because of poor knowledge of the fossil and historical distribution of *P. squamata*, in addition to its very small distribution at present, it is difficult to explain the historical factors that may have contributed to the low genetic variation of *P. squamata*. Based on our recent molecular phylogenetic analysis (Zhang *et al*., 2003), *P. squamata*, together with two archaic pines *P. gerardiana* and *P. bungeana*, is basal to all other members of the subgenus *Strobus* or to those of the sect. Strobus, which indicates that *P. squamata* is an ancient species that underwent a long evolutionary history. Qiaojia County is not far from the areas where many relict species occur (often referred to as living fossils, e.g., *Metasequoia glyptostroboides*, *Cathya argyrophylla*, *Davidia involucrata*, and *Eucommia ulmoides* (Hu, 1980; Ying *et al*., 1994) (Fig. 1). The floristic influence of central China is conspicuous in northeastern Yunnan (Li, 1995; Li and Li, 1997). In fact, *Davidia involucrata* is also distributed in Qiaojia. Hu (1980) referred to the flora of this part of central China as the Metasequoia flora, which was thought to be spared from the direct effects of the repeated Pleistocene continental glaciation. The population of *P. squamata* might have experienced a similar geological history. The present population may be the descendant that survived the continental glaciation.

Even in the absence of severe and prolonged bottlenecks, the small population size of *P. squamata* may have resulted in low genetic diversity, because genetic drift, increased inbreeding, and reduced gene flow will all decrease genetic diversity in small and isolated populations. A positive correlation between actual population size and genetic diversity has been found in several plant species (Godt *et al*., 1996; Fischer and Matthies, 1998), with some exceptions (Ellstrand and Elam, 1993; Schmidt and Jensen, 2000). Westemeier *et al*. (1998) also documented concurrent declines in population size and fitness as well as an overall reduction in genetic diversity in a remnant population of greater prairie chickens (*Tympanuchus cupido pinnatus*) in southeastern Illinois.

As its trunk is gray-white and straight, *P. squamata* is very conspicuous in the forest, and because the wild population is close to a village, logging and farming activities may be another reason for low genetic variation. C. T. Lee *et al*. (2002) demonstrated substantial genetic erosion caused by logging in one of the regenerated stands corresponding to its extremely low tree density for *Scaphium macropodum*. More than a dozen stumps of *P. squamata* remaining in the community bear witness to the effects of logging.

Comparing the genetic variation in the two subpopulations, that of the subpopulation on the southwest-facing slope is much higher than that of the subpopulation on the northeast-facing slope. This pattern may be attributed to the more divergent habitat on the southwest-facing slope. Wang *et al*. (1996) draw the same conclusion that populations in divergent environments have higher genetic variation than populations in uniform ones.

Distribution of Genetic Variation among Subpopulations

The present distribution of genetic diversity within species is influenced by both natural history and evolutionary history (Hamrick and Godt, 1996). Pines are wind pollinated, and different populations can exchange their genes through pollen dispersal, so the pines always display little genetic differentiation among populations (mean $G_{ST} = 0.073$; see Hamrick *et al*., 1992). The differentiation among subpopulations ($\Phi_{ST} = 0.011$ for RAPD, $\Phi_{ST} = 0.024$ for ISSR) of *P. squamata* is also very low, as in most other pines (Hamrick *et al*., 1992). This indicates that there was frequent gene flow between the two subpopulations of *P. squamata*, separated from each other by about 0.5–1 km.

Implications for Conservation

Knowledge of the level and distribution of genetic variation is a prerequisite for the establishment of effective and efficient conservation practices (Ge *et al*., 1998). The generally low level of genetic variation of *P. squamata* apparently results from its past evolutionary processes, as well as some recent losses due to human activities, and the among-population difference is mostly in allele frequencies rather than in allele composition. This pattern of population structure in *P. squamata* has important conservation implications. Loss of individuals at certain locations may not cause immediate loss in genetic diversity, but more damage may occur in terms of long-term genetic consequences due to reduced numbers of individuals and smaller population size. The conservation of endangered species depends on maintaining a substantial population size (Carson, 1990). Given the extremely limited number of individuals of *P. squamata*, it is necessary to protect all the existing subpopulations and individuals *in situ* in order to preserve as much genetic variation as possible. On the other hand, with knowledge of its genetic architecture available, an appropriate strategy for sampling and propagation could be formulated when *ex situ* conservation is carried out. Although a small number of *ex situ* individuals can preserve most of the genetic variation in this endangered pine, it is strongly recommended that seeds for propagating should be collected from different subpopulations to preserve as much genetic variation as possible.

Hedrick and Kalinowski (2000) pointed out that populations of some endangered species have become so small that they have lost genetic variation and

appear to have become fixed for deleterious genetic variants (genetic load). To avoid extinction from this genetic deterioration, some populations may benefit from the introduction of individuals from related populations or subspecies for genetic restoration, i.e., elimination of deleterious variants and recovery to normal levels of genetic variation. It is necessary to carry out investigations in adjacent areas to find other potential populations and then perform controlled crosses between genetically distinct populations to restore and to enrich genetic backgrounds of the extremely endangered pine.

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