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Genetic diversity and phylogenetic relationships among five endemic *Pinus* taxa (Pinaceae) of China as revealed by SRAP markers

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ABSTRACT

The genetic diversity and phylogenetic relationships among five endemic *Pinus* taxa of China (*Pinus tabulaeformis, P. tabulaeformis* var. *mukdensis, P. tabulaeformis* f. *shekanensis, Pinus massoniana* and *Pinus henryi*) were studied by SRAP markers. Using 10 SRAP primer pairs, 247 bands were generated. The percent of polymorphic bands (94.8%), Nei's genetic diversity (0.2134), and Shannon's information index (0.3426) revealed a high level of genetic diversity at the genus-level. At the taxon level, *P. tabulaeformis* f. *shekanensis* and *P. henryi* showed a higher genetic diversity than the others. The coefficient of genetic differentiation among taxa (0.3332) indicated a higher level of genetic diversity within taxon, rather than among taxa. An estimate of gene flow among taxa was 1.0004 and implied a certain amount of gene exchange among taxa. The results of neighbor-joining cluster analysis and principal co-ordinate analysis revealed that *P. tabulaeformis, P. tabulaeformis* var. *mukdensis* and *P. tabulaeformis* f. *shekanensis* were conspecific, which was in agreement with the traditional classification. Phylogenetic relationships analysis also indicated that *P. henryi* might be a distinct species closely related to *P. tabulaeformis*.

1. Introduction

The genus *Pinus* consists of approximately 100 species and widely distributed throughout the Northern Hemisphere. A total of 22 species and 10 varieties of the genus *Pinus* are distributed in China, and 11 of them are endemic (Song, 2009). In the present study, five endemic *Pinus* taxa from China (including *Pinus tabulaeformis* Carr., *P. tabulaeformis* var. *mukdensis* Uyeki, *P. tabulaeformis* f. *shekanensis* Yao et Hsü, *Pinus henryi* Mast. and *Pinus. massoniana* Lamb.) were analyzed. All five pines are geographically and phylogenetically closer to each other than to any other Chinese pines (Editorial board of flora of China, 1978). *P. tabulaeformis* is a dominant species of the coniferous forest in northern China (Chen et al., 2008). Its current distribution center lies between the Taihang Mountains of Shanxi province and the Ziwuling mountains of Shaanxi province, making up about 70% of the pine-forested area (Xu et al., 1993). *P. tabulaeformis* var. *mukdensis* is a variety of *P. tabulaeformis* (Editorial board of flora of China, 1978) and *P. tabulaeformis* f. *shekanensis* is a form of *P. tabulaeformis* (Le, 1957). *P. massoniana* is the most widely distributed pine species in China, and has expanded rapidly to reach an estimated area of 5.7 million hectares (Zhang et al., 2010). *P. henryi*, first described in 1902 by Masters, is a rare and endemic pine in China. There is a

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controversy regarding the taxonomic position of *P. henryi*. It was initially synonymized with *P. tabulaeformis* by Cheng (1930), then treated as a variety of *P. massoniana* (Wu, 1956; Kuan, 1983), and then regarded as a variety (Guan, 1982) or subspecies (Businsky, 1999) of *P.* tabulaeformis, and more recently identified as a distinct species (Zheng and Fu, 1978; Niu, 1990; Zhang et al., 1995; Zhao and Liu, 2010).

Molecular marker systems have been demonstrated to be useful tools for studying genetic diversity and phylogenetic relationships of species or populations (Liu et al., 2013; Xu et al., 2013; Liu et al., 2014). Sequence-related amplified polymorphism (SRAP) is a molecular marker system developed for selective amplification of open reading frames (Li and Quiros, 2001). These polymorphisms result mainly from various promoters, introns and spacers among different species and individuals. SRAP is a highly reproducible and informative technique for assessing genetic diversity in comparison with other PCR-based techniques (Li and Quiros, 2001; Gulsen et al., 2005). SRAP markers have been successfully used in genetic diversity, gene tagging, map construction, and phylogenetic studies in a wide range of plants (Zhou et al., 2011; Majid et al., 2012; Liu et al., 2013; Chen et al., 2014; Li et al., 2014).

In the present study, SRAP markers were employed to assess the genetic diversity and phylogenetic relationships of *P. tabulaeformis, P. tabulaeformis* var. *mukdensis, P. tabulaeformis* f. *shekanensis, P. henryi* and *P. massoniana*. The main objectives of this study were to (1) examine the genetic diversity and phylogenetic relationships of the five pines; (2) assess the taxonomic status of *P. henryi*.

2. Materials and methods

2.1. Plant materials and DNA extraction

Five taxa of *Pinus* were analyzed in this study including *P. tabulaeformis, P. tabulaeformis* var. *mukdensis, P. tabulaeformis* f. *shekanensis, P. henryi* and *P. massoniana*. The locations of taxa and the sampled number are shown in Table 1. Fresh needles were sampled from individual adult trees from each population. The distances between sampled trees varied from 50 to 100 m depending on the population size, to ensure that the sampled trees truly represented their populations. To avoid degradation of plant tissues, all samples were labeled and kept in sealed bags with silica gel according to the method proposed by Sytsma et al. (1993) until DNA extracted.

Total genomic DNA of each plant was extracted from silica gel-dried needles using a DNA secure plant kit (TIANGEN Biotech Co., Ltd., Beijing, China). The quality and concentration of the DNA were determined by electrophoresis on 1.5% agarose gels.

2.2. SRAP-PCR amplification

Ten pairs of SRAP primers were used in the present study (Table 2), which were selected from 100 combinations of 10 forward and 10 reverse primers. The protocol for SRAP analysis was based on Li and Quiros (2001). Each 20 μL PCR reaction mixture consisted of 40 ng genomic DNA, 1× PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 mM for each primer and 1 unit of Taq polymerase. PCR cycling parameters included 4 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 35 cycles the annealing temperature was increased to 50 °C, and for extension, one cycle of 7 min at 72 °C. The amplification products were separated by 6% polyacrylamide gel electrophoresis (PAGE) and visualized by a simplified silver staining method previously described by Xu et al. (2002).

2.3. Data analysis

SRAP amplified fragments, with the same mobility according to their molecular weight (bp), were scored in terms of a binary code as present (1) or absent (0). Only those consistently reproducible bands were scored, and smeared and weak bands were excluded. The resulting 1/0 matrix was analyzed using POPGENE v.1.32 (Yeh et al., 1999), assuming Hardy–Weinberg equilibrium, to estimate genetic diversity parameters: the percentage of polymorphic loci (PPB), Nei's genetic diversity (H) (Nei, 1973), Shannon's information index (I) (Lewontin, 1972), the coefficient of genetic differentiation (Gst), and gene flow (Nm).

Table 1
Locations of the sampled *Pinus* taxa and the sampled number (N).

Taxa	Code	Location	N	Latitude (°N)/Longitude (°E)	Elevation (m)
P. tabulaeformis f. *shekanensis*	CS	Fuxian, Shaanxi	30	35.998/108.690	1316
P. tabulaeformis	YS	Huanglong, Shaanxi	30	35.632/109.772	1127
P. tabulaeformis var. *mukdensis*	HS	Anshan, Liaoning	30	40.960/123.147	294
P. massoniana	MS	Yangxian, Shaanxi	30	33.326/107.624	722
P. henryi	BS	Nanzheng, Shaanxi	30	32.857/106.586	1254

Table 2
Polymorphic analysis of *Pinus* taxa using different SRAP primers.

Primer code	Forward/Reverse primer (5′- 3′)	No. of total bands	No. of polymorphic bands	Polymorphic bands (%)
M e1 + E m5	F: TGAGTCCAAACCGGATA R: GACTGCGTACGAATTAAC	35	35	100
Me1 + E m6	F: TGAGTCCAAACCGGATA R: GACTGCGTACGAATTAAC	37	36	97.3
M e3 + E m1	F: TGAGTCCAAACCGGAAT R: GACTGCGTACGAATTAAT	27	24	88.9
M e3 + E m4	F: TGAGTCCAAACCGGAAT R: GACTGCGTACGAATTTGA	19	19	100
M e4 + E m9	F: TGAGTCCAAACCGGACC R: GACTGCGTACGAATTACG	20	20	100
M e4 + E m10	F: TGAGTCCAAACCGGACC R: GACTGCGTACGAATTGCA	20	20	100
M e4 + E m1	F: TGAGTCCAAACCGGACC R: GACTGCGTACGAATTAAT	27	27	100
M e5 + E m1	F: TGAGTCCAAACCGGAAG R: GACTGCGTACGAATTAAT	15	10	66.7
M e5 + E m3	F: TGAGTCCAAACCGGAAG R: GACTGCGTACGAATTGAC	13	10	76.9
M e6 + E m1	F: TGAGTCCAAACCGGTAA R: GACTGCGTACGAATTAAT	23	22	95.7
Total	–	249	236	–
Average	–	24.9	23.6	94.8

The 1/0 matrix was transformed into the Nei & Li genetic distance matrix by FreeTree (Hampl et al., 2001). Using the Nei & Li genetic distance matrix, the phenograms were conducted with MEGA 4.0 (Tamura et al., 2007) via the neighbor-joining method. Nei's unbiased genetic distances were used to construct neighbor-joining tree (NJ tree) of five pines by MEGA 4.0. In addition, principal co-ordinate analysis (PCoA) in GenAlEx 6.5 (Peakall and Smouse, 2012) was employed to further examine the genetic relationships among detected taxa.

3. Results and discussion

3.1. SRAP polymorphism

Ten SRAP primer pairs (Table 2) were used to estimate the genetic relationships among five *Pinus* taxa. With 10 SRAP primer pairs and 150 samples from five *Pinus* taxa, 249 bands were scored, with an average of 24.9 bands per pair. Among these bands, 236 were polymorphic, with an average of 23.6 polymorphic bands per pair and 94.8% mean percentage of polymorphic band (Table 2). The results of each pair revealed high levels of polymorphism in the five pines, similar to previously published reports in *Pinus* species (Xu et al., 2008; Feng et al., 2009; Di and Wang, 2013).

3.2. Level of diversity and molecular variance

The genetic diversity analysis of *Pinus* taxa, by SRAP, gave the following results: 94.8% for the percentage of polymorphic bands (PPB), 0.2134 for Nei's genetic distance (H), and 0.3426 for Shannon's information index (I), which indicated a high level of genetic diversity at the genus-level. The genetic diversity of each taxon varied as follows: PPB from 50.0% to 75.7%, H from 0.1374 to 0.2063 and I from 0.2125 to 0.3215. The trend of H matched that of I. The trend in the genetic diversity indexes of each taxon was: *P. tabulaeformis* f. *shekanensis* (PPB = 75.7%, H = 0.2063, I = 0.3215) > *P. henryi* (PPB = 68.5%, H = 0.2048, I = 0.2761) > *P. tabulaeformis* (PPB = 62.4%, H = 0.1751, I = 0.2704) > *P. tabulaeformis* var. *mukdensis* (PPB = 61.9%, H = 0.1689, I = 0.2635) > *P. massoniana* (PPB = 50.0%, H = 0.1374, I = 0.2125) (Table 3).

Numerous examples in previous studies showed that species with a small geographic range generally maintain less genetic diversity than geographically widespread species (Gitzendanner and Soltis, 2000; Wu et al., 2004; Zheng et al., 2012). However, our data suggest that the taxa restricted to narrow populations (*P. tabulaeformis* f. *shekanensis* and *P. henryi*) were more diverse than *P. tabulaeformis* and *P. massoniana*, two species that are widely distributed. Hamrick and Godt (1989) concluded that the primary factors influencing the genetic diversity of plant populations include breeding system, distribution range, and habit. Plant species with higher genetic variation are usually characterized by long life span, wide geographic distribution, predominant outcrossing, anemophily, good fecundity, and a late stage of succession. Thus, the higher level of genetic diversity observed within *P. tabulaeformis* f. *shekanensis* and *P. henryi* may be related to their late stage of succession. Additionally, in this paper, each taxon, no matter its distribution, selected only in one population may be another reason.

The Gst was 0.3332, indicating that 33.32% of the genetic variance occurred among taxa and 66.64% was within taxon, which implied that the genetic diversity of the five pines analyzed mainly occurs within taxon, rather than among taxa. Nm

Table 3
Genetic diversity of five *Pinus* taxa revealed by SRAP markers.

Taxa code	PPB (%)	H	I
CS	75.7	0.2063	0.3215
YS	62.4	0.1751	0.2704
HS	61.9	0.1698	0.2635
MS	50.0	0.1374	0.2125
BS	68.5	0.2048	0.3128
Mean	63.7	0.1787	0.2761
Taxa level	94.8	0.2134	0.3426

Notes: PPB, percentage of polymorphic bands; H, Nei's genetic distance; I, Shannon's information index. Taxon codes are identified in Table 1.

among taxa was 1.0004, suggesting a high frequency of gene exchange among populations. The factors that affect the genetic structure of plant populations include the evolutionary history, mutation, genetic drift, mating system, gene flow, natural selection and life form (Zhang et al., 2012; Wang et al., 2014). Mating system and gene flow are the main explanations for the genetic structure of the five pines. In this research, mating system plays the most fundamental role in the evolution of species, which results in various genotypes after natural selection. Different mating methods can produce different ratios of heterozygous and homozygous genotypes. This results in a distribution of genetic variability within and among populations and affects the genetic structures (Wang et al., 2011). Typically outcrossing species maintain relatively more of their genetic diversity within populations rather than among populations (Wang et al., 2014), which agrees with our analysis of *Pinus*. Additionally, Hamrick and Godt (1989) found that if Nm > 1, then gene flow neutralizes the variance in genes caused by genetic drift. In this study, the gene flow (Nm) of the five pines was 1.0004. This result demonstrated that gene flow happens frequently among taxa, which suggests a low level of genetic variation among taxa.

3.3. Phylogenetic analysis

Based on the obtained distance matrix, Neighbor-joining tree (NJ-tree) was constructed to show the genetic relationship among five *Pinus* taxa (Fig. 1). Samples of *P. tabulaeformis, P. tabulaeformis* var. *mukdensis* and *P. tabulaeformis* f. *shekanensis* intimately and intricately clustered together, while samples of *P. henryi* and *P. massoniana* clustered within 2 well-differentiated groups in the NJ dendrogram (Fig. 1A). *P. massoniana* appeared to be the most genetically differentiated. *P. henryi* occupied an intermediate position, while *P. tabulaeformis* and *P. tabulaeformis* f. *shekanensis* were the most closely related (Fig. 1B).

Principal co-ordinate analysis (PCoA) was performed to provide a spatial representation of the relative genetic distances among individuals and to determine the consistency of differentiation among taxa defined by cluster analysis. The first 2

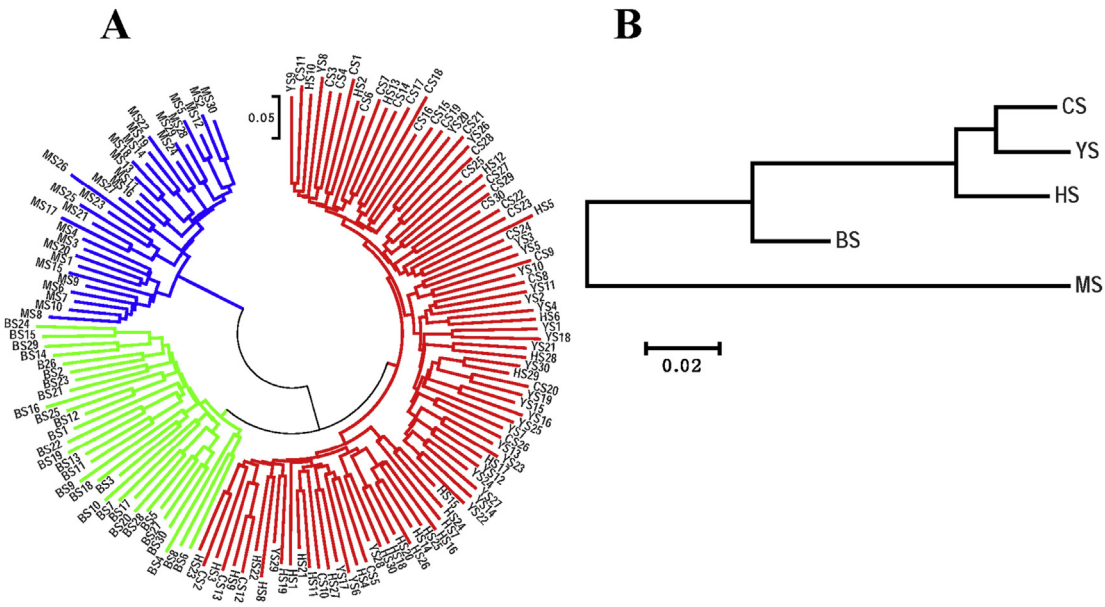

Fig. 1. Neighbor-joining (NJ) diagram derived from SRAP markers of: A, 150 individuals (5 taxa); B, five *Pinus* taxa. Individual or taxon codes are identified in Table 1.

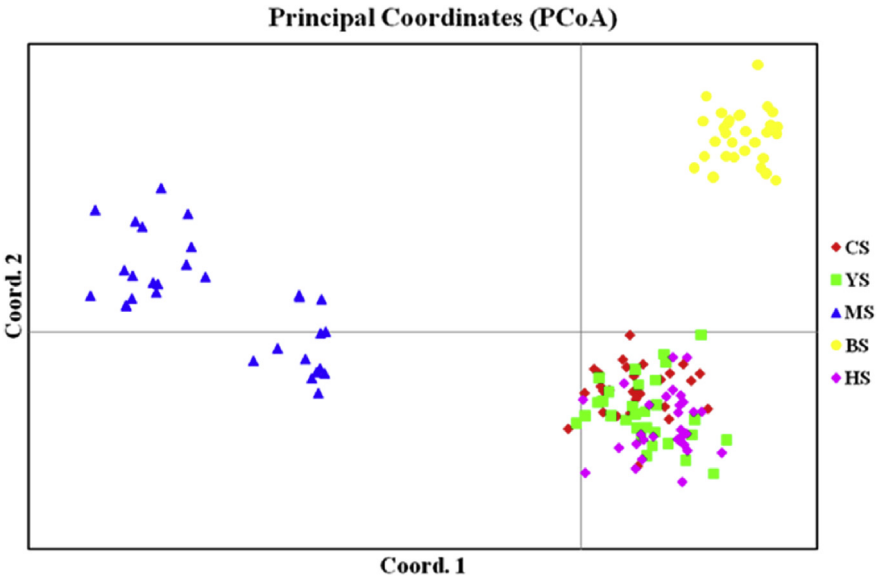

Fig. 2. Distribution of 150 individuals (5 taxa) of *Pinus* in the plane of the first two principal coordinates. The first and second axis extracted 52.47% and 16.18% of the total genetic variance, respectively. Taxon codes are identified in Table 1.

principal components explained 41.5% and 17.34% of the total variation, respectively, while 72.0% was explained by the first 3 components. PCoA clustering based on SRAP data revealed that three groups existed in the investigated five pines, which were group I: *P. tabulaeformis, P. tabulaeformis* var. *mukdensis* and *P. tabulaeformis* f. *shekanensis*; group II: *P. henryi* and group III: *P. massoniana* (Fig. 2).

The results of neighbor-joining cluster analysis and PCoA analysis a revealed that there were distinctly phylogenetic relationships among five investigated taxa of *Pinus. P. tabulaeformis, P. tabulaeformis* var. *mukdensis* and *P. tabulaeformis* f. *shekanensis* grouped into one cluster and distinctly separated from *P. massoniana* and *P. henryi* (these two taxa also differentiate from each other) indicated that *P. tabulaeformis, P. tabulaeformis* var. *mukdensis* and *P. tabulaeformis* f. *shekanensis* were conspecific, which was in agreement with the traditional classification (Le, 1957; Editorial board of flora of China, 1978). These results also indicated that *P. henryi* might be a distinct species rather than a subspecies of *P. tabulaeformis* or *P. massoniana*, although it is closely related to *P. tabulaeformis.*

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