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Genetic variation and genetic structure of the endangered species *Sinowilsonia henryi* Hemsi. (Hamamelidaceae) revealed by amplified fragment length polymorphism (AFLP) markers

H. Zhang, W.L. Ji, M. Li and L.Y. Zhou

College of Landscape Architecture and Arts, Northwest A & F University, Yangling, Shaanxi Province, China

Corresponding author: W.L. Ji
E-mail: jiwenli@nwsuaf.edu.cn

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ABSTRACT. Comprehensive research of genetic variation is crucial in designing conservation strategies for endangered and threatened species. *Sinowilsonia henryi* Hemsi. is a tertiary relic with a limited geographical distribution in the central and western areas of China. It is endangered because of climate change and habitat fragmentation over the last thousands of years. In this study, amplified fragment length polymorphism markers were utilized to estimate genetic diversity and genetic structure in and among *S. henryi*. In this study, Nei's genetic diversity and Shannon's information index were found to be 0.192 and 0.325 respectively, indicating a moderate-to-high genetic diversity in species. According to analysis of molecular variation results, 32% of the genetic variation was shown to be partitioned among populations, demonstrating a relatively high genetic divergence; this was supported by principal coordinate analysis and unweighted pair-group method with arithmetic average analysis. Moreover,

the Mantel test showed that there was no significant correlation between genetic and geographical distances. The above results can be explained by the effects of habitat fragmentation， history traits, and gene drift. Based on the results, several implications were indicated and suggestions proposed for preservation strategies for this species.

Key words: *Sinowilsonia henryi* Hemsi.; Genetic variation; Genetic structure; Amplified fragment length polymorphism (AFLP) marker

INTRODUCTION

Sinowilsonia Hemsi is a primitive monotypic genus in the family Hamamelidaceae, and *Sinowilsonia henryi* Hemsi is the only representative of this genus. Based on fossils found from Cretaceous to early Tertiary periods (Wolfe, 1973), it can be deduced that the plant was once widely distributed in warm and humid forests. Since *S. henryi* has a unique evolution history, studies of its genetic information can greatly improve our understanding of the occurrence and evolution of the flora in China. Due to habitat fragmentation and climate change, *S. henryi* can now only be found in the central and western regions of China-a transition area in between subtropical and warm temperate climate zones. *S. henryi* is mainly distributed in the relatively stable, mild, and moist hill and valley areas, altitudes of which range between 550 and 1500 m. Other species found together with *S. henryi* are *Euptelea pleiosperma, Broussonetia papyrifera, Alangium chinense, Dipteronia sinensis*. However, owing to rapid wildlife depletion and habitat fragmentation, *S. henryi* has been included in the red list of Critically Endangered Species in China (Fu, 1992) and the key protected species list in Shaanxi Province.

Genetic diversity is one of the important aspects of biological diversity, and it can provide effective guidance in designing conservation measures. Therefore, it should be a primary consideration in preserving endangered and threatened species (Hamrick and Godt, 1996). Evaluating the level of genetic diversity and genetic structure within species can provide more information about their phylogenetic processes and evolutionary history (Francisco-Ortega et al., 2000; Qiu et al., 2006). Retaining a sufficient level of genetic diversity in rare species is critical for maintaining stability of their genetic structures and for enhancing their ability to adapt to natural selection in changing environments (Honjo et al., 2004).

Godt and Hamrick (2001) concluded that genetic diversity is positively correlated with population size. Thus, endangered plants with narrow geographic distributions (which, in turn, lead to a lower level of genetic variation) are more susceptible to fluctuations in climate change and habitat fragmentation (Newman and Pilson, 1997; Hensen and Oberprieler, 2005).

Previous studies on *S. henryi* were focused mainly on morphology (Fu, 1993), and systematic biology (Fu and Gao, 1992; Zhang, 1999). Few studies on genetic variation and genetic structure have so far been carried out. Zhou et al. (2014) investigated the genetic diversity of *S. henryi* using inter-simple sequence repeat (ISSR) markers. The study showed that genetic variation mainly occurred among populations and a high genetic divergence existed. However, more comprehensive information on genetic relationships is needed to provide better protection strategies. Therefore, in this study, a different and more accurate method, i.e. amplified fragment length polymorphism (AFLP) markers were used and a different geographic scale was applied in order to investigate the genetic variation of *S. henryi*.

The objectives of the study were: i) to estimate the genetic diversity in *S. henryi*; ii) to

analyze the genetic divergence and genetic structure among *S. henryi* populations; and iii) to provide suggestions for conserving and protecting the endangered species.

MATERIAL AND METHODS

Plant material

In this study, 96 individuals from 10 populations of *S. henryi* were randomly sampled during 2013 and 2014. The sampling regions included Henan, Shaanxi, and Gansu provinces. Each population had a sample size of 10 except for Baoji, which had six samples. Within the 10 populations, two (BJ, ZWY) were sampled from *ex situ*-cultivated individuals and the rest were sampled from naturally grown individuals.

Detailed information regarding locations and populations is provided in Table 1 and Figure 1.

In order to prevent duplication, the distance between two sampled individuals was at least 10 meters. Only young and healthy leaves were collected and placed in zip-lock plastic bags. After being dried in silica gel immediately, they were stored at 4°C in the laboratory until DNA extraction.

Table 1. Details of the S. henryi populations.

Pop Code	Longitude	Latitude	Altitude(m)	Sample size	Geographic localities
BJ	107°06'	34°21'	1372	6	Baoji, Shaanxi
HD	108°26'	33°26'	1475	10	Ankang, Shaanxi
HLG	112°08'	35°10'	951-960	10	Heilonggou, Jiyuan, Henan
HLT	112°17'	35°11'	617-630	10	Heilongtan, Henan
GS	106°07'	33°45'	1485-1490	10	Donggou, Huixian, Gansu
JSX	110°34'	33°20'	1034-1043	10	Jinsixia, Shangluo, Shaanxi
CCG	108°08'	33°37'	1200-1205	10	Chouchungou,Ningxi, Shaanxi
ZWY	108°20'	33°46'	1027	10	Botany Garden of Xian, Shaanxi
SBG	108°21'	33°47'	1277	10	Shibangou, Ningxi, Shaanxi
MH	108°18'	33°37'	1155	10	Muheqiao, Ningxi, Shaanxi

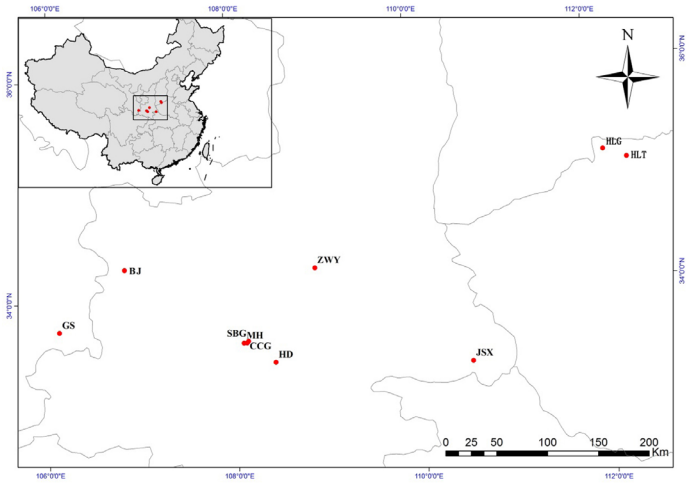

Figure 1. Geographic locations of the ten populations of *S. henryi*.

DNA extraction and polymerase chain reaction (PCR) amplification

Genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle, 1987). Extracted DNA was dissolved in 200 µL TE buffer [10 mM Tris-HCl, pH 8.0, 1 mM EDTA]. The purity of DNA was detected by 1.0% agarose gel electrophoresis and the concentration of DNA measured using a NanoDrop1000 spectrophotometer (Thermo Scientific, USA).

AFLP reactions were performed according to the method described by Vos (1995) with minor modifications; the restriction, digestion, and ligation steps were performed as one procedure. In this procedure, 200 ng of genomic DNA was incubated at 37°C for 5 h in a 25-µL reaction mix. The mix consisted of 5 U each of restriction enzymes *EcoR*I and *Mse*I bound to their respective adapters, 2 U T_4 DNA ligase, 10 mM ATP, 2.5 µL10X Tango buffer (10 mM Tris-HCl, pH 8.5, 10 mM $MgCl_2$, 100 mM KCl, 0.1 mg/mL BSA (TaKaRa Biotechnology Co. Ltd., Dalian, China)).

After the ligation reaction, the pre-amplification was then performed using non-selective nucleotides in a 20-µL reaction mix. The mix contained 5 µL ligated DNA, 0.3 µL primer *EcoR*I and *Mse*I (AoKe, Biotechnology Co. Ltd., Beijing, China), and 10 µL Premix Taq (TaKaRa Biotechnology Co. Ltd., Dalian, China). The reaction sequence was initial denaturation for 2 min at 94°C; followed by 24 cycles of denaturation at 94°C for 30 s, 56°C for 30 s, and 72°C for 60 s; and then 5 min final extension at 72°C. Subsequently, the products of pre-amplification were diluted with 10-fold ddH_2O and used as a template for selective amplifications.

Selective amplifications were then conducted using AFLP primers with two selective nucleotides. The reaction procedures included initial denaturation at 94°C for 4 min; 12 cycles of denaturation at 94°C for 30 s, then a reduction of the annealing temperature from 65°C (decreasing 0.7°C per cycle), and elongation at 72°C for 1 min; followed by 94°C for 30 s, 56°C for 30 s, and 72°C for 60 s for 23 cycles, concluding with elongation at 72°C for 5 min.

The mixture of 98% formamide loading buffer and the PCR product was heated at 95°C for 5 min, then immediately cooled in an ice bath. Bands were detected on 6% polyacrylamide gel electrophoresed at 1500 V for 2.5 h, then stained with 0.1% silver nitrate. Each PCR amplification and gel run was repeated again, and only clear and unambiguous fragments present in both runs were considered.

Data analysis

Genetic diversity

Six primer pairs (Table 2) that produced reproducible, bright, and clear bands were identified after screening 64 primer pairs. For all primer combinations used in the experiment, each AFLP amplified fragment was manually scored and converted into a binary matrix as present (1) or absent (0) based on unambiguous and clear bands. With the assumption that the populations were in Hardy-Weinberg equilibrium, software POPGENE version 1.31 (Yeh et al., 1999) was used to calculate the level of genetic variation. The calculated parameters of genetic diversity include the percentage of polymorphic loci (PPL), observed number of alleles (N_A), effective number of alleles (N_E), Nei's genetic diversity (H), Shannon's information index (I), and the coefficient of genetic differentiation (*Gst*). Nei's genetic distance and geographic distances (km) were also calculated.

Table 2. Amplifications and Polymorphism bands produced by six primer combinations.

Primer combinations	Amplification bands	Polymorphism bands	PPB(%)
E-ACG/M-CAT	65	61	93.85
E-ACT/M-CAG	47	44	93.62
E-AGC/M-CAA	59	55	93.22
E-ACG/M-CTT	55	52	94.55
E-ACT/M-CAC	56	53	94.64
E-AGG/M-CAT	69	66	95.65
Total	351	331	-
Mean	58.50	55.17	94.30

PPB: percentage of polymorphic bands.

Genetic structure

The analysis of molecular variance (AMOVA) was employed via GenAlEx version 6.5 (Peakall and Smouse, 2012) to analyze genetic hierarchy among and within populations, where 9999 permutations were used for significance tests. Genetic structure among populations of *S. henryi* was analyzed using different but complementary approaches, namely, PCoA, UPGMA-based genetic distance, and Bayesian model clustering.

PCoA grouped the individuals based on J parameters using NTSYSpc version 2.1 (Rohlf, 2000). UPGMA-based cluster analysis, which illustrated the relationships among populations, was performed using software package PowerMarker version 3.25 (Liu and Muse, 2005) and PHYLIP version 3.2 (Felsenstein, 1989) based on Nei's genetic distance. To assess the statistical support of each branch, a phylogenetic tree was created with 1000 replicates of bootstrapping. By using the STRUCTURE version 2.3.1 (Pritchard et al., 2000) based on the Bayesian clustering method, the number (K) and the clustering of the inferred natural populations were estimated, ignoring the original population information. Admixture mode and correlated allele frequencies were chosen in this modeling. To determine the optimal K value, 20 simulations were repeated for each K (from 1 to 10) with a burn-in of 100,000 and MCMC repetitions of 100,000. In the algorithm based on $\Delta K = M[|L(k+1)-2L(k)+L(k-1)|]/S[L(k)]$ (Evanno et al., 2005), M represents the mean for 20 runs and S represents the standard deviation. The Mantel test (Mantel, 1967) was conducted to determine the correlation between the genetic distance matrix and geographical distance matrix using GenAlEx.

RESULTS

Genetic diversity and genetic distance

With six AFLP primers, 351 unambiguous and bright fragments were generated, whose lengths ranged from 100 to 1500 bp. Among these bands, 331 were polymorphic. The total number of fragments that each pairwise primer generated ranged from 44 (E-ACT/M-CAG) to 66 (E-AGG/M-CAT) with an average of 55 (Table 2). PPL varied within populations between 11.11% (BJ) and 43.30% (HD) with an average value of 32.49% (Table 3). The population JSX had the highest number of private bands (11), whereas no private band was found in the GS, HLT and SBG population. Under the assumption of Hardy-Weinberg equilibrium, the *H* and *I* values in species were 0.192 and 0.325 respectively. The parameters of genetic diversity in each population are shown in Table 3.

Table 3. Genetic diversity within all the 10 populations of *S. henryi*.

Pop code	N_A	N_E	*H*	*I*	PPL(%)
BJ	1.111	1.071	0.042 (0.123)	0.062 (0.180)	11.11
HD	1.433	1.221	0.135 (0.177)	0.208 (0.259)	43.30
GS	1.353	1.240	0.136 (0.197)	0.200 (0.283)	35.33
MH	1.365	1.272	0.151 (0.208)	0.218 (0.297)	36.47
HLG	1.430	1.258	0.155 (0.191)	0.232 (0.279)	43.02
HLT	1.405	1.220	0.136 (0.178)	0.207 (0.264)	40.46
JSX	1.308	1.216	0.121 (0.193)	0.177 (0.276)	33.77
CCG	1.353	1.246	0.138 (0.200)	0.202 (0.285)	35.33
ZWY	1.171	1.110	0.064 (0.148)	0.096 (0.216)	17.09
SBG	1.291	1.198	0.113 (0.186)	0.167 (0.268)	29.06
Mean	1.322	1.205	0.119 (0.180)	0.177 (0.261)	32.49
Species	1.954	1.270	0.192 (0.121)	0.325 (0.167)	95.44

N_A: observed number of alleles; N_E: effective number of alleles; *H*: Nei's gene diversity; *I*: Shannon's information index; PPL: the percentage of polymorphic loci; Values in brackets are standard deviations.

G_{ST} was 0.36, lower than that obtained by Zhou et al. (2014), which can be explained by different sampling strategies and different markers used. Gene flow (*Nm*) based on the formula $Nm = 0.5(1 - G_{ST})/G_{ST}$ was calculated as 0.86, indicating a low level of gene flow among populations. Genetic distances between paired populations based on AFLP (Table 4) data ranged from 0.051 for the least differentiated populations (SBG and CCG), to 0.142 for the most differentiated populations (JSX and GS).

Table 4. Nei's genetic distance (above diagonal) and geographic distances (km) (below diagonal) among populations of *S. henryi.*

	BJ	HD	GS	MH	HLG	HLT	JSX	CCG	ZWY	SBG
BJ	-	0.071	0.053	0.070	0.077	0.069	0.110	0.083	0.054	0.065
HD	152.04	-	0.099	0.093	0.110	0.090	0.133	0.101	0.085	0.085
GS	126.02	204.72	-	0.091	0.053	**0.071**	0.142	0.114	0.073	0.097
MH	130.88	24.53	195.97	-	0.107	0.111	0.132	0.077	0.089	0.057
HLG	468.39	407.03	582.44	401.95	-	0.052	0.133	0.117	0.075	0.102
HLT	476.45	414.21	**590.30**	409.35	8.2	-	0.117	0.107	0.082	0.093
JSX	322.14	193.79	398.12	203.12	265.38	270.46	-	0.123	0.119	0.117
CCG	133.02	22.95	197.83	2.17	400.83	408.22	201.13	-	0.086	0.051
ZWY	134.56	103.49	241.75	83.72	340.7	348.55	198.04	84.03	-	0.073
SBG	133.05	23.83	198.75	2.79	399.49	406.88	200.36	1.45	82.65	-

Genetic divergence

The AMOVA (Table 5) showed that 32% of the total genetic variance was present among populations, whereas 68% of the variance occurred among individuals within populations. AMOVA results were similar to the G_{ST}, demonstrating that a relatively high level of differentiation existed in *S. henryi*.

The results from the Mantel test showed positive but not significant correlation between the genetic and geographical distances ($r = 0.224$, $P > 0.05$).

Genetic structure

The phylogenetic tree (Figure 2) obtained by UPGMA divided the 10 populations into four clusters. Three populations (CCG, SBG, and MH) from Ningxi area in Shaanxi province clustered

together with population HD with high bootstrap values. The second cluster comprises GS, HLT, and HLG populations, which belong to two different provinces. The third cluster includes BJ and ZWY, and the last cluster only contains JSX, which is clearly differentiated from the others.

Table 5. Analysis of molecular variance (AMOVA) of *S. henryi* based on amplified fragment length polymorphism (AFLP) data.

Source of variation	d.f.	Sum of squared deviation	Variance component	Percentage of variance	Φ	P value
Among Pops	9	1184.785	11.230	32%	0.319	0.001
Within Pops	86	2065.767	24.021	68%		
Total	95	3250.552	35.251	100%		

d.f.: degree of freedom; Φ: the proportion of the total variance. P values: the probabilities of having a more extreme variance component than the observed values alone. Probabilities were calculated by 1000 random permutations of individuals across populations.

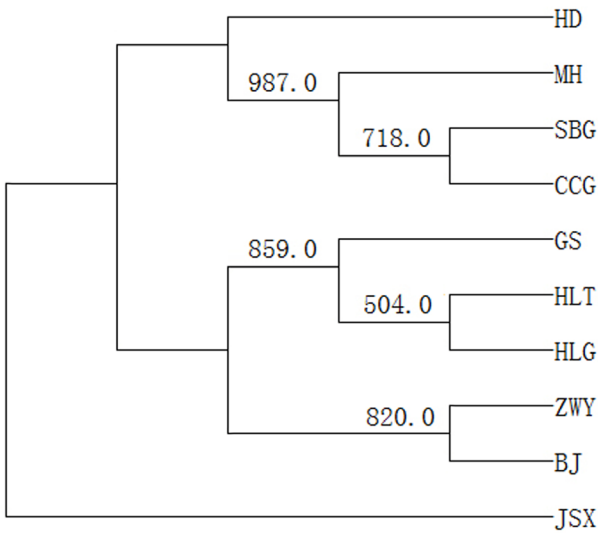

Figure 2. Genetic relationship of the ten populations using UPGMA based on Nei's genetic distance. Only bootstrap values greater than 50% were marked.

The result of PCoA (Figure 3) was relatively conformable with the UPGMA cluster analysis, which provided additional evidence for high-level genetic divergence among populations. The entire population was divided into two groups along coordinates 1. One group included CCG, SBG, MH, JSX, and part of HD, all of them belonging to Shaanxi province. The remaining populations comprised the other group.

As shown in Figure 4, the STRUCTURE analysis demonstrated the likelihood distribution mean of L(K) at the real K in A, the distribution of DeltaK at the real K in B, and that all the ten populations shared two gene pools in C. The first gene pool contained five populations (MH, CCG, SBG, JSX, and HD) from Shaanxi Province, while there were some individuals from different locations in population HD. The second gene pool contained the remaining five populations (BJ, GS, HLG, HLT, and ZWY).

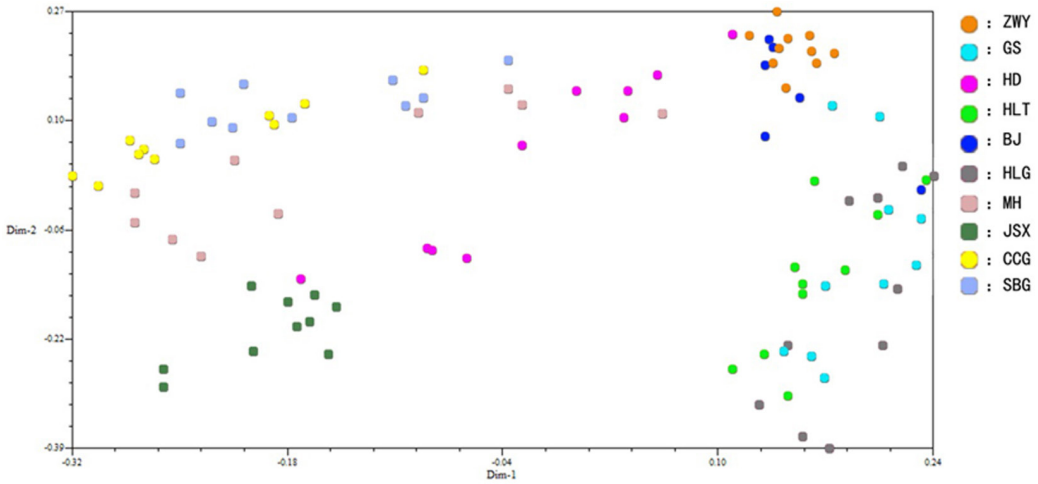

Figure 3. Principal coordinate analysis (PCoA) plot of the individuals of *S. henryi* based on the two coordinate axes.

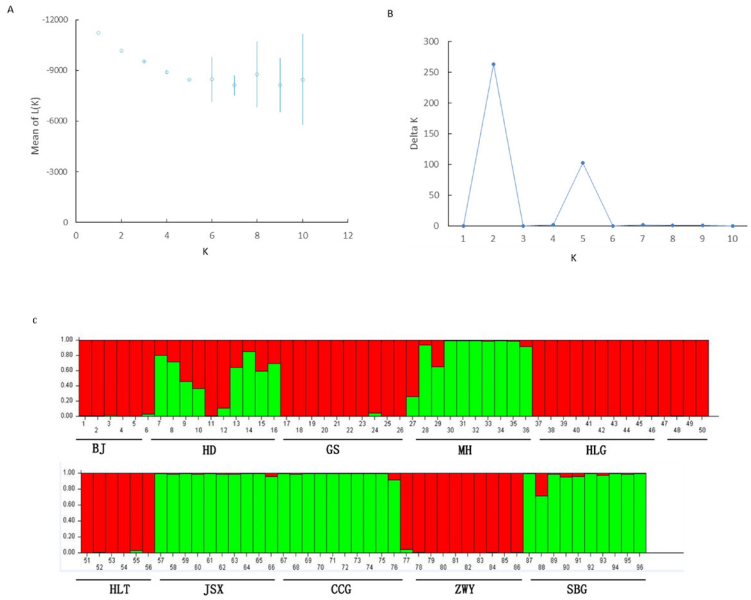

Figure 4. A. The likelihood distribution L (K) at the real K. **B.** The distribution of DeltaK at the real K. **C.** STRUCTURE analysis of all the 96 individuals. Each single bar refers to each single individual in a population.

DISCUSSION

Genetic diversity

Estimating the level of genetic diversity in endangered species accurately is crucial for designing conservation and management strategies for them. In general, endemic species with

narrower geographical distributions have lower genetic diversity than those with wider geographical distributions, mainly due to genetic drift and inbreeding depression in small population sizes (Ellstrand and Elam, 1993). However, some studies reveal that endemic and endangered species can still harbor relatively high levels of genetic diversity (Rossetto et al., 1995; Yu et al., 2014). Based on the results of AFLP analysis, *I* and *H* values were 0.325 and 0.192 respectively, which showed that the endangered species *S. henryi* maintained a moderate-to-high level of genetic variation.

Hamrick and Godt (1996) concluded that evolution history and biological traits can significantly influence the level of genetic diversity and the genetic structure in species. The moderate-to-high level of genetic variation of *S. henryi* is probably on account of the following factors.

First, information gained from fossils found from the Cretaceous to early Tertiary periods indicates that *S. henryi* was once widely distributed in warm and humid forests (Wolfe, 1973). It can be deduced from this that, starting from the ancestral plants, genetic variability accumulated over centuries during the long process of evolution. That is why, despite the loss of habitat during the last several decades due to human activities and climate change during Quaternary glacial age, the genetic diversity of *S. henryi* is still at a moderate-to-high level.

Second, the biological traits of species, especially their breeding system played an important role in maintaining the genetic diversity at the species level. *S. henryi* has the biological features of monoecism, unisexual flowers, and dichogamy. In theory, plants capable of cross-pollination tend to have higher genetic diversity (Hamrick and Godt, 1990). In addition, perennial species show a slower loss of genetic diversity than short-lived species (Feyissa et al., 2007).

In this study, the level of genetic diversity within species (PPL = 95.44%) was evidently higher than that within populations (PPL = 32.49%). It is highly probable that the main cause of the low population-level genetic diversity was inbreeding within the population. Results from several studies (Hamrick and Godt, 1989; Nybom and Bartish, 2000) indicated that genetic variation of inbreeding species among populations accounted for about 50%, whereas the outcrossing species accounted for about 20% of the total genetic variation. In the present study, the AMOVA showed that the value of Φ was 0.32, indicating that a mixed-mating system existed in this species. Owing to a lack of the pollinators and limited pollen dispersal distance, inbreeding occurs frequently in many plant species with prolonged periods of small population size to ensure reproduction success and to maintain the stability of populations, even though this pattern could lead to inbreeding depression (McCall et al., 1994). Prolonged periods of small population size can also lead to gene drift, founder effect, and genetic bottlenecks, which can also be the factors causing low genetic diversity within populations (Barrett and Kohn, 1991).

Populations BJ and ZWY showed a lower genetic diversity than the wild populations in this study. A possible explanation for this is that the process of domestication and cultivation decreased the genetic variation. Similar phenomena can also be found in other endangered species such as *Spondias purpurea* (Miller and Schaal, 2006) and *Eucommia ulmoides* (Yao et al., 2012).

Gene flow and genetic divergence

Genetic structure among populations can be influenced by several elements, such as mating systems, gene drift, gene flow, mutation, evolution, and natural selection (Schaal et al., 1998). In the current study, obtained value *Gst* (0.36) was higher than the average value for outcrossing plant species (*Gst* = 0.22). Both the results of AMOVA and the *Gst* value suggested a relatively high genetic divergence. This might be caused by the limited gene flow (*Nm* = 0.86), which indicated that the number of migrants per generation is below 1 (Grant, 1991).

During field investigations, the seeds only scattered around the parent tree since *S. henryi* does not have a favorable dispersal structure, and seed dispersal mainly depends on gravity. It also had a low rate of seed germination (Zhang et al., 2011), which resulted in a low quantity of seedling. *S. henryi* has the characteristic of wind pollination，the effective pollination distance being limited. These factors greatly limited the gene flow among populations.

The main geographical barriers for *S. henryi* include mountains, rivers, and severe habitat fragments, which limit the gene flow and lead to a high genetic structure. The estimated gene flow from our data was 0.86, and for each generation it was not sufficient to compensate the effects of genetic drift; ultimately, this would lead to a continuous divergence among populations (Wright, 1951). It also confirmed the assumption that the populations adjacent to each other (such as CCG, SSG, and MH) tend to cluster together.

Genetic structure

This evident genetic structure found by AMOVA and *Gst* was well supported by data from PCoA and UPGMA. As shown in Figure 2, the four populations CCG, SBG, MH, and HD in Shaanxi province have relatively small genetic distances between each other, whereas the genetic distances between JSX and any of the above four populations are evidently larger. The possible explanation for this can be that JSX, being part of a natural conservation area, with a unique valley ecosystem with high mountains and deep valleys, was quite isolated. As shown in Table 4, the population of GS was genetically close to HLG despite being over 500 km apart from it.

It can be interpreted that natural selection or gene drift has a more significant impact on the genetic structure pattern than gene flow. Nonetheless, since there was no distinct difference in the sampled populations, we can conclude that natural selection plays a less significant role, whereas gene drift caused by fragmentation may be the main factor leading to this genetic structure pattern in *S. henryi* populations. Similar results were also found in the endangered species *Liparis japonica* (Chen et al., 2013), where two populations were genetically similar even though the distance between them was about 400 km.

Bayesian analysis (STRUCTURE) showed that the optimal number of inferred group for all 96 individuals was 2, showing that the correlation between genetic relationship and spatial distribution was weak. The Mantel test also validated these results. We concluded that the natural populations in our study originated in the distant past from two different genetic populations or geographic areas. However, further research is needed to investigate the genetic evolution of *S. henryi* to confirm this hypothesis.

Conservation

The genetic variation found in our study about *S. henryi* can help in designing conservation strategies to enhance the genetic diversity in endangered species.

In situ conservation is usually the most effective strategy for conserving endangered species. The results of our research showed that genetic diversity was high within species, and that a high degree of divergence existed among populations. Therefore, relevant departments should strengthen the protection of local natural environments for wild populations to protect them from extinction, as *S. henryi* is vulnerable to environment changes.

Ex situ populations showed considerable low-level gene diversity in our results. Several factors can be considered in order to improve gene variety: Collecting seeds or transferring

seedlings from different populations to appropriate places with relatively stable, mild, and humid environment is crucial to improve gene flow among populations. However, with respect to population genetic structure, outbreeding depression might influence the already weakened structure when transferring individuals from other populations (Sagvik et al., 2005). Therefore, preferably, geographically neighboring groups should be chosen when transferring the plants.

Conflicts of interest

The authors declare no conflict of interest.

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