Conserv Genet (2011) 12:1539–1549
DOI 10.1007/s10592-011-0251-8

RESEARCH ARTICLE

The phylogeographic structure and conservation genetics of the endangered tree peony, *Paeonia rockii* (Paeoniaceae), inferred from chloroplast gene sequences

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Received: 6 July 2010 / Accepted: 19 July 2011 / Published online: 19 August 2011

Abstract The endangered species *Paeonia rockii* is the most important ancestral species of the cultivated tree peonies. These well-known ornamental plants are termed the 'King of Flowers' in China. In this study, we investigated the genetic diversity and phylogeographic structure of 335 wild samples from 20 populations throughout the entire distributional range of the species based on three chloroplast DNA sequences (*petB–petD*, *rps16–trnQ* and *psbA–trnH*). At those loci, high levels of genetic differentiation ($G_{ST} = 0.94$) and low levels of genetic variation ($\theta = 0.00185$) were detected. The intraspecific phylogeny revealed four groups, the western group, the Taibai mountain group, the northern group and the eastern group, which closely coincided with the geographic distribution of the species. A phylogeographic structure of this kind could result from a number of integrated factors, such as allopatric fragmentation, climatic fluctuations, increased abortion and declining germination of seeds, or lack of gene flow among populations, especially across the geographic barrier of the high Qinling Mountains, and it could also result from adaptive evolution. For conservation purposes, each extant population of *P. rockii* should be recognized as a conservation-significant unit, and a more stringent conservation strategy should incorporate in situ and ex situ methods.

Keywords *Paeonia rockii* · Conservation genetics · Phylogeographic structure · Chloroplast gene sequences · *petB–petD* · *rps16–trnQ* · *psbA–trnH*

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Introduction

The tree peony, known as the 'King of Flowers' in China, is of great ornamental and medicinal value and has been grown for approximately 1,400 years (Cheng et al. 2005). To date, more than 1,000 cultivars of tree peonies have been grown, but only one-third of those currently still cultivated are characterized by a colored flare (Li 2005). *Paeonia rockii* is endemic to the Qinling Range in central China (Hong 2010), along with three other tree peony species, *Paeonia jishanensis*, *Paeonia ostii* and *Paeonia cathayana.* These four *Paeonia* species constitute the ancestral group that has contributed significantly to the formation of the cultivated tree peonies (Cheng 2007). *P. rockii* is a deciduous shrub, 1.8 m in height and flowering from late April to early May. It has been divided into two subspecies, *P. rockii* ssp. *atava* and *P. rockii* ssp. *rockii* (Hong 1998; Hong and Pan 2005). According to historical records, *P. rockii* ssp. *atava* is distributed along the northern slope of the Qinling Range and further north, whereas *P. rockii* ssp. *rockii* can be found in the western and eastern parts of the Qinling Range as well as in the

Shenlongjia forest area of Hubei province at altitudes of 1,100–2,800 m (Cheng et al. 2005; Hong 2010).

In recent years, the distribution of *P. rockii* has decreased dramatically owing to human overexploitation and disturbance. The habitat of the species has also been reported to have dramatically declined. These declines have affected *P. rockii* populations (Hong 2010; Li 2005; Pei et al. 1995; Zhang et al. 2009; Zou et al. 1999). More than two-thirds of the distributional areas of *P. rockii* in the middle and south of Gansu province have disappeared. The Maxianshan and Maijishan populations (Yuzhong county, Tianshui city of Gansu province, respectively) are now extinct, and the species is nearly extinct on Taibai Mountain in the Qinling Range (Hong and Pan 2007; Li 2005). Previous studies have focused primarily on investigating germplasm, taxonomy (Li 2005; Hong 2010), and the biological traits causing the population declines of *P. rockii*, such as a low blooming rate, a low germination and rooting rate, and a higher level of pollen infertility or ovule abortion (Li 2005).

Thorough understanding of the levels and spatial partitioning of genetic polymorphisms will not only provide sufficient information for the conservation of this endangered species but will also furnish fundamental genetic information for the breeders of this outstanding ornamental plant. In recent years, molecular genetic methods have become increasingly popular. Given their haploid nature and a low frequency of genetic recombination, chloroplast (cp) DNA markers, which can only be transmitted by seeds and not by pollen, are suitable for phylogenetic reconstruction at various taxonomic levels, for conservation genetics, and for investigating phylogeographic structure (Comes and Kadereit 1998). These applications are informative because the genetic structure of cpDNA markers may be maintained for a longer period than that of nuclear (n) DNA markers (Arroyo-García et al. 2002). Moreover, a few cpDNA markers with moderate to high levels of genetic variation within and among tree species have been detected in some intergenic spacers (Yuan et al. 2010; Zhang et al. 2009). Therefore, using such informative sites will counterbalance the relatively slow evolution of cpDNA compared with that of nuclear markers.

In this study, three polymorphic cpDNA markers, *petB–petD*, *rps16–trnQ* and *psbA–trnH*, were analyzed in samples collected from the entire distributional range of *P. rockii* to investigate the population genetic structure and evolutionary history of this species and to provide information for developing effective and sustainable conservation plans. The objectives of this study were to understand the phylogeographic pattern exhibited by *P. rockii* and to examine the extent of genetic differentiation of the species. Furthermore, the results of this study may be helpful in improving the genetic basis for breeding cultivars, developing policies for the conservation for other wild tree peonies, and understanding both the genetic relationships among species in the section *Moutan* of the genus *Paeonia* and the overall phylogeographic pattern of the extant plants that are endemic to the Qinling Mountains or their vicinity.

Materials and methods

Population sampling and DNA extraction

Three hundred and thirty-five wild individuals from 20 populations throughout the entire distributional range of *P. rockii* in China were sampled and investigated during the flowering period from 2007 to 2009 (Fig. 1; Table 1). Most of the extant natural populations had very few adult individuals and even fewer flowering individuals. The numbers of individuals belonging to these categories ranged from 0 to 15 within each population investigated, with the exception of the Neixiang (NX) population (Table 1). Because these populations are still declining owing to human overexploitation, only 2–30 individuals were collected from each population. To avoid the sampling of closely related plants and to obtain the greatest possible genetic representation, a minimum distance of 20 m was always maintained between the individuals sampled. The harvested leaves were stored in plastic bags with silica gel and kept at room temperature until analysis. The DNA was extracted following a modified CTAB method (Doyle and Doyle 1987). The DNA concentration was estimated either by agarose gel electrophoresis or using a photometer, and dilutions of 5–10 ng/μl were prepared.

cpDNA polymerase chain reaction amplification, nucleotide sequencing and sequence alignment

Based on Yuan et al. (2010) and Zhang et al. (2009), the *petB–petD* chloroplast region and two other cpDNA markers, *rps16–trnQ*, and *psbA–trnH*, were used for our analysis. We used the amplification primers for the *petB–petD* spacer, as described by Grivet et al. (2001), and the primers for *rps16–trnQ* and *psbA–trnH* spacers designed by Sang et al. (1997).

The polymerase chain reaction (PCR) was conducted in a 25 μl solution containing approximately 10 ng of genomic DNA, 0.5 U Taq polymerase (Bioline, Beijing, China), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 μM of each primer, and 1× PCR buffer (Tiangen, Beijing, China). Amplification was performed using a Biometra thermal cycler (Biometra, Göttingen, Germany) with the following cycling conditions: 95°C for 2 min, followed by 34 cycles of 30 s at 94°C, 30 s at 50°C, and 1 min at 72°C, and a final extension step of 10 min at 72°C. The PCR products

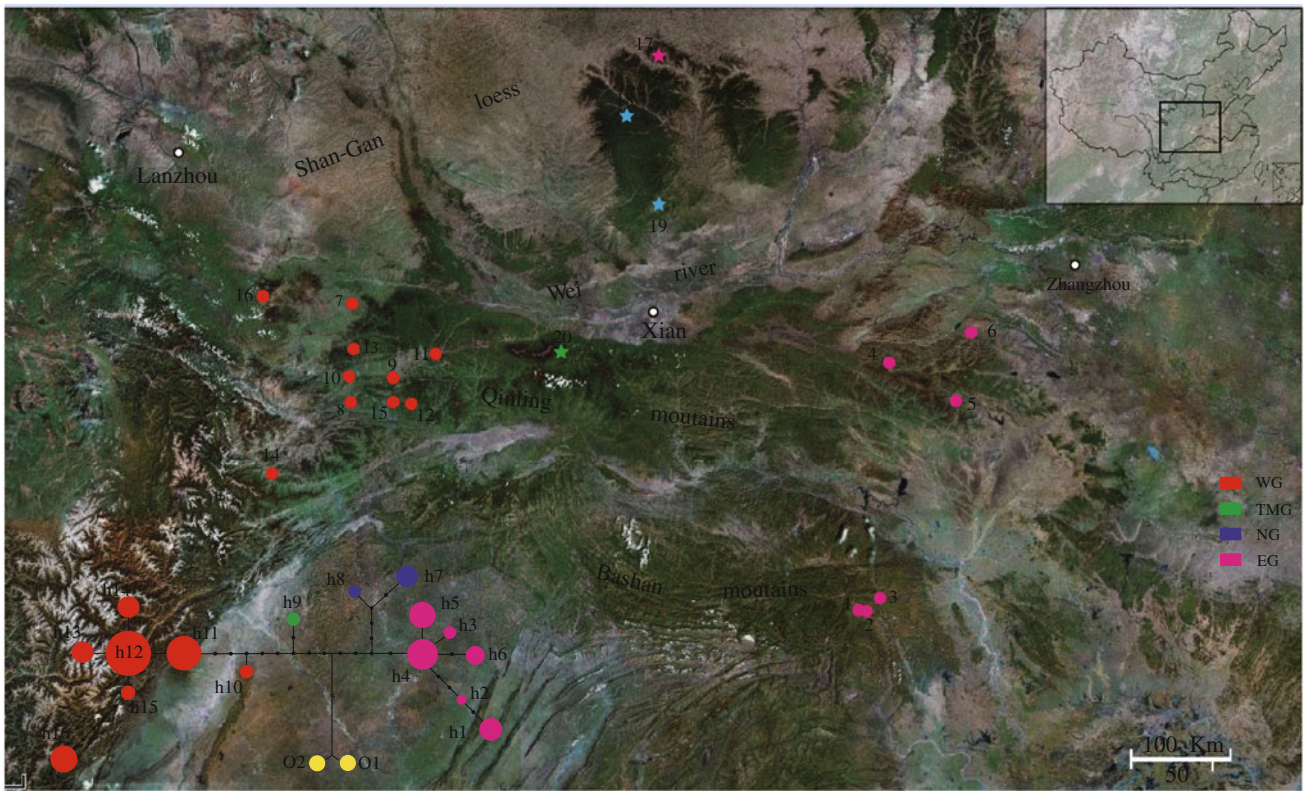

Fig. 1 Map showing locations of populations of *Paeonia rockii* sampled and a haplotype network (*bottom left*) based on three cpDNA (*petB–petD*, *rps16–trnQ* and *psbA–trnH*) intergenic spacers and constructed by TCS 1.2.1. The population names correspond to those in Table 1. The *pentagrams* indicate *P.* ssp. *atava*, and the *circles* indicate *P.* ssp. *rockii*. Haplotypes O1 (*P. delavayi*) and O2 (*P. ludlowii*) represent the outgroup. The sizes of the circles in the network (*bottom left*) are proportional to the observed frequencies of the haplotypes, and the *black dots* represent inferred nodes that were absent in the samples

were purified using a polyethylene glycol precipitation procedure following the manufacturer's protocols. The sequencing conditions were as follows: 30 cycles at 95°C for 30 s, 50°C for 30 s, and 60°C for 4 min. Both strands were sequenced on an AB 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA). The sequences from both strands were assembled using Sequencher version 4.6 (Gene Codes Corporation, MI, USA), then aligned and adjusted manually with Se–Al editor version 2.0 (Rambaut 1996). The gaps were treated as missing data, except for some indels which were coded (details in Table 2). All sequences were deposited into GenBank, with accession numbers JF496787–JF496855.

Data analysis

The levels of genetic diversity within and among populations and regions were characterized by the indices of segregating sites (S), the number of haplotypes (h), the haplotype diversity (H_d, Nei 1987) and the nucleotide diversity (θ, Nei 1987) using DnaSP version 5 (Librado and Rozas 2009).

The relationships among the populations of *P. rockii* were estimated by neighbor-joining (NJ) analysis based on Kimura's (Kimura 1980) two-parameter distance using MEGA version 5 software (Tamura et al. 2011), treating *Paeonia delavayi* and *Paeonia ludlowii* as outgroups. To evaluate clade support, 1,000 bootstrap replicates were performed using fast heuristic and TBR branch-swapping. A haplotype network resulting from combined cpDNA data was constructed using TCS version 1.21 (Clement et al. 2000), which implements statistical parsimony to connect haplotypes constrained by 95% confidence intervals. A phylogenetic tree of population haplotypes was constructed by Bayesian inference (BI) in MrBayes version 3.1.2 (Huelsenbeck et al. 2001; Rannala and Yang 1996), using *P. delavayi* and *P. ludlowii* as outgroups. The Markov Chain Monte Carlo (MCMC) algorithm was run for one million generations with four incrementally heated chains, starting from random trees and sampling one out of every 1,000 generations. The first 2,500 trees were discarded as burn-in, after which the chains appeared to have become stationary, and the remaining trees were used to construct Bayesian consensus trees.

Table 1 Locations, sample sizes and site coordinates of *Paeonia rockii* populations examined in this study

Species/population	Locality	Latitude (N)	Longitude (E)	Altitude (m)	Sample size	*h*	*S*	*Hd*	θ
1 BHC	Mt. Henchong, Baokang Co., Hubei prov.	31.7193	111.1207	1742	6	1	0	0	0
2 BHP	Changchongya, Baokang Co., Hubei prov.	31.7199	111.29	1377	6	1	0	0	0
3 DS	Dashui forestry farm, Baokang Co., Hubei prov.	31.688	111.3477	1599	22	2	5	0.485	0.00049
4 LC	Mt. Xuner, Luanchuan Co., Henan prov.	33.9313	111.2064	1100–1200	12	1	0	0	0
5 NX	Baotianman, Neixiang Co., Henan prov.	33.5131	111.8537	1348–1568	24	1	0	0	0
6 YS	Mt.Yang, Songxian Co., Henan prov.	34.1333	112.0855	1200	3	2	4	0.667	0.00095
7 DC	Dangchuan town, Tianshui city, Gansu prov.	34.5667	105.702	1393–1553	25	2	1	0.220	0.00009
8 JFM	Mt. Jifeng, Chengxian Co., Gansu prov.	33.6852	105.671	1724–1755	4	1	0	0	0
9 JL	Jialing town, Huixian Co., Gansu prov.	33.6874	106.1702	1200–1373	27	1	0	0	0
10 KZV	Kongzigou valley, Chengxian Co., Gansu prov.	33.8698	105.7024	1197–1429	13	1	0	0	0
11 LD	Zhangjia town, Liangdang Co., Gansu prov.	34.1531	106.5183	1505–1670	24	1	0	0	0
12 LY	Lueyang Co., Shaaxi prov.	33.593	106.1272	1332	3	1	0	0	0
13 MY	Mayan town, Hui Co., Gansu prov.	34.0543	105.7036	1350–1453	24	1	0	0	0
14 WX	Liujiaping town, Wenxian Co., Gansu prov.	33.0183	104.7431	1675	30	1	0	0	0
15 YP	Yanping village, Huixian Co., Gansu prov.	33.6696	106.2876	1557–1594	26	1	0	0	0
16 ZX	Zhangxian Co., Gansu prov.	34.6341	104.6736	1881–1942	26	1	0	0	0
P. rockii ssp. *rockii*					295	12	27	0.852	0.00157
17 GQ	Xiashi town, Guanquan Co., Shaanxi prov.	36.5762	108.9504	1376–1440	29	1	0	0	0
18 HS	Taibai town, Heshui Co., Gansu prov.	36.0026	108.6537	1312–1362	26	1	0	0	0
19 TC	Tongchuan city, Shaanxi prov.	34.9105	108.9347	1128	2	1	0	0	0
20 TM	Mt.Taibai, Baoji city, Shaanxi prov.	34.1572	107.8438	1400	3	1	0	0	0
P. rockii ssp. *atava*					60	4	14	0.585	0.00108
P. rockii					335	16	33	0.887	0.00185

h the numbers of haplotypes, *S* polymorphic sites, *Hd* haplotype diversity, θ nucleotide difference. Values based on cpDNA (*petB–petD*, *rps16–trnQ* and *psbA–trnH*) sequences. Co., county. prov., province

Table 2 Variable nucleotide sites of cpDNA (*petB–petD*, *rps16–trnQ* and *psbA–trnH)* sequences, haplotypes of the populations, and number of individual haplotypes for polymorphic populations

Haplotype	Populations	Nucleotide position in three combined cpDNA sequences (from alignment matrices)												
		petB–petD												
		6	24	39	387	433	447	549	708	720	995	996	1371	1455
h1	BHC BHP DS	C	A	T	T	C	G	A	C	T	G	T	G	A
h2	YS	C	A	T	T	C	G	A	C	T	G	T	G	A
h3	YS	A	G	C	T	C	G	A	C	T	G	T	G	A
h4	DS NX	A	G	C	T	C	G	A	C	T	G	T	G	A
h5	GQ	A	G	C	T	C	G	A	C	T	G	T	C	A
h6	LC	A	G	C	T	C	G	A	C	T	G	T	G	A
h7	HS	A	G	C	T	C	A	A	C	C	G	G	G	A
h8	TC	A	G	C	T	C	A	A	C	C	T	T	G	G
h9	TM	A	G	C	G	C	G	A	C	C	T	T	G	G
h10	LY	A	G	C	T	T	G	A	C	C	T	T	G	G
h11	DC KZV MY	A	G	C	T	T	G	A	C	C	T	T	G	G
h12	DC JL ZX	A	G	C	T	T	G	A	C	C	T	G	G	G
h13	LD	A	G	C	T	T	G	A	C	C	T	G	G	G
h14	YP	A	G	C	T	T	G	A	T	C	T	G	G	G
h15	JFM	A	G	C	T	C	G	A	C	C	T	G	G	G
h16	WX	A	G	C	T	C	G	C	C	C	T	G	G	G

Haplotype	Populations	Nucleotide position in three combined cpDNA sequences (from alignment matrices)												
		rps16–trnQ												
		153 1	155 3 5	170 3	179 7	179 8	180 8	183 9	192 5	194 7	199 6	199 3	208 7	248 1	255 2 0
h1	BHC BHP DS	C	T	C	A	A	A	G	G	1	G	G	1	T	A
h2	YS	C	T	C	A	A	A	G	G	1	G	G	1	T	A
h3	YS	C	T	C	A	A	A	G	G	1	G	G	1	T	A
h4	DS NX	C	T	C	A	A	A	G	G	1	G	G	1	C	A
h5	GQ	C	T	C	A	A	A	G	G	1	G	G	1	T	A
h6	LC	C	T	C	A	A	C	G	G	1	G	G	1	T	A
h7	HS	C	T	C	A	A	A	G	T	1	A	G	1	T	A
h8	TC	C	T	C	A	A	A	G	T	1	A	G	1	T	A
h9	TM	T	T	T	A	A	A	G	G	a	G	G	b	T	G
h10	LY	C	G	T	A	A	A	G	G	a	G	T	b	T	G
h11	DC KZV MY	C	G	T	A	A	A	G	G	a	G	G	b	T	G
h12	DC JL ZX	C	G	T	A	A	A	G	G	a	G	G	b	T	G
h13	LD	C	G	T	A	A	A	A	G	a	G	G	b	T	G
h14	YP	C	G	T	A	A	A	G	G	a	G	G	b	T	G
h15	JFM	C	G	T	A	A	A	G	G	a	G	G	b	T	G
h16	WX	C	T	T	T	T	A	G	G	a	G	G	b	T	G

Table 2 continued

Haplotype	Populations	Nucleotide position in three combined cpDNA sequences (from alignment matrices)					
		psbA–trnH					
		2	2	2	2	2	3
		7	7	8	9	9	0
		4	4	0	2	3	0
		2	4	7	0	2	4
h1	BHC BHP DS	T	G	c	A	G	1
h2	YS	T	G	1	G	G	1
h3	YS	T	G	1	G	G	1
h4	DS NX	T	G	1	G	G	1
h5	GQ	T	G	1	G	G	1
h6	LC	T	G	1	G	T	1
h7	HS	T	G	1	G	G	1
h8	TC	T	G	1	G	G	1
h9	TM	T	G	1	G	G	1
h10	LY	T	G	1	G	G	1
h11	DC KZV MY	G	A	1	G	G	1
h12	DC JL ZX	G	A	1	G	G	1
h13	LD	G	A	1	G	G	1
h14	YP	G	A	1	G	G	1
h15	JFM	G	A	1	G	G	1
h16	WX	G	A	1	G	G	d

Length polymorphisms absent (1) or present (a through d). a, GTAGA. b, 253-bp indel. c, GAAGA. d, ATTTT. Polynucleotide stretches (polyA or polyT) were excluded from the analysis. Population codes as in Table 1

Internodes with posterior probabilities ≥0.95 were considered statistically significant.

A hierarchical analysis of population subdivision was performed using an analysis of molecular variance (AMOVA) as implemented in ARLEQUIN version 3.10 (Excoffier et al. 2005). Two measures of population differentiation, G_{ST} and N_{ST}, and the test statistic U, comparing the values of N_{ST} and G_{ST}, were calculated using HAPLONST (Pons and Petit 1996). Gene flow within and among regions or populations was approximated as *Nm* (analogous to $M = (1/Fst - 1)/2$) (Slatkin 1993), where Nm is a measure of the extent of gene flow in an island model at equilibrium. *Fst* (Hudson et al. 1992) was estimated in DnaSP version 5 (Librado and Rozas 2009).

Results

Genetic diversity of cpDNA of *P. rockii*

The total combined length of the aligned sequences of the three cpDNA markers (*petB–petD*, *rps16–trnQ* and *psbA–trnH*) was 3074 bp (excluding outgroups) or 3,094 bp (including outgroups). A total of 33 variable sites with a potential informative character value (PIC = number of substitutions and indels/total number of characters) of 1.07% were found, including 29 nucleotide substitution sites and four indels. Of the three cpDNA markers we investigated (Table 2), *petB–petD* was 1,458 bp (PIC = 0.89%) and included 13 substitution sites; *rps16–trnQ* was 993–1,241 bp with a PIC of 0.97% and included 12 substitution sites and two related indels, with lengths of 5 and 253 bp; and *psbA–trnH* was 365–375 bp with a PIC of 1.64% and included four substitution sites and two indels, with lengths of 5 bp and either 20 or 27 bp of inverted repeats of indels. The inverted repeats were not considered for calculating sequence divergence or reconstructing phylogeny (Sang et al. 1997).

The chloroplast spacer is A/T rich with an average content of 65.5%. This finding is consistent with the nucleotide composition of most noncoding spacers and pseudogenes and reflects low levels of functional constraints (Li 1998). In all, 16 haplotypes were identified from 335 individuals of *P. rockii* and plotted on a relief map of central China (Fig. 1; Table 1). The number of haplotypes (h) and haplotype diversity (H_d) identified in *petB–petD*, *rps16–trnQ*, *psbA–trnH* and all three cpDNAs combined were 11, 9, 5 and 16, and 0.843, 0.724, 0.638 and

0.887, respectively. The nucleotide diversity (θ) values, identified in *petB–petD*, *rps16–trnQ*, and *psbA–trnH* separately and then combined, were 0.0014, 0.00225, 0.00262 and 0.00185, respectively. Of 20 populations of *P. rockii*, 17 were observed to be monomorphic and only three polymorphic. H_d was highest for population YS (0.667), followed by DS (0.485), DC (0.220), and BHC, BHP, LC, NX, GQ, HS, TC, TM, LY, LD, YP, JL, JFM, KZV, MY, ZX, and WX (all 0) (Table 1). θ was also highest for YS (0.00095), followed by DS (0.00049), DC (0.00009), and the other 17 populations (all 0) (Table 1). At the subspecies level, the number of haplotypes and the haplotype diversity in *P.* ssp. *rockii* and *P.* ssp. *atava* were $h = 12$ and 4, and $H_d = 0.852$ and 0.584, respectively. These values were higher for ssp. *rockii* than for ssp. *atava*, whereas the θ value of ssp. *atava* (0.00108) was lower than that of ssp. *rockii* (0.00157) (Table 1).

Phylogeographic structure and genetic differentiation of *P. rockii*

The NJ approach clustered the *P. rockii* populations into four genetically distinct clades. The first clade, the western group (WG), clustered with a bootstrap value of 100%, included the 10 populations LY, DC, JL, YP, LD, MY, ZX, KZV, JFM and WX from the western Qinling Mountains. The second clade, the Taibai Mountain group (TMG), included only one population, TM, from the northern slopes of the Qinling Mountains. The third clade, the northern group (NG), clustered with a bootstrap value of 99%, included only two populations, HS and TC, from the Ziwuling forest area of the northern Qinling Mountains. Similarly, the fourth clade, the eastern group (EG), clustered with a bootstrap value of 96%, included the seven populations (GQ, NX, LC, YS, DS, BHC and BHP) from the eastern Qinling Mountains and the eastern Bashan Mountains, as shown in Fig. 2a. Nearly the same four-group pattern could be inferred from the haplotype network (Maximum Parsimony, MP) (Fig. 1, lower left) and the Bayesian phylogenetic tree (Fig. 2b). No common haplotype occurred among the four major groups, suggesting that the four clades were truly genetically distinct. The long branch representing the WX population was found in all three phylogenetic analyses.

The results of the three phylogenetic analyses (Figs. 1, 2) indicated the presence of two clades, one including WG and TMG and a second including NG and EG. TMG and NG were identified as *P.* ssp. *atava*, whereas all individuals belonging to WG and EG (except for GQ, identified as *ssp. atava*) were identified as *P.* ssp. *rockii*. Most of the clades from the NJ and Bayesian trees and the haplotype network clearly coincided with the geographic distribution pattern of the population. GQ was the sole exception. The populations separated by smaller geographic distances were more likely to be placed in the same clade by this analysis than the populations separated by greater geographic distances (Figs. 1, 2). Therefore, the analysis also suggested that *P. rockii* is significantly differentiated throughout its range in and near the Qinling Mountains.

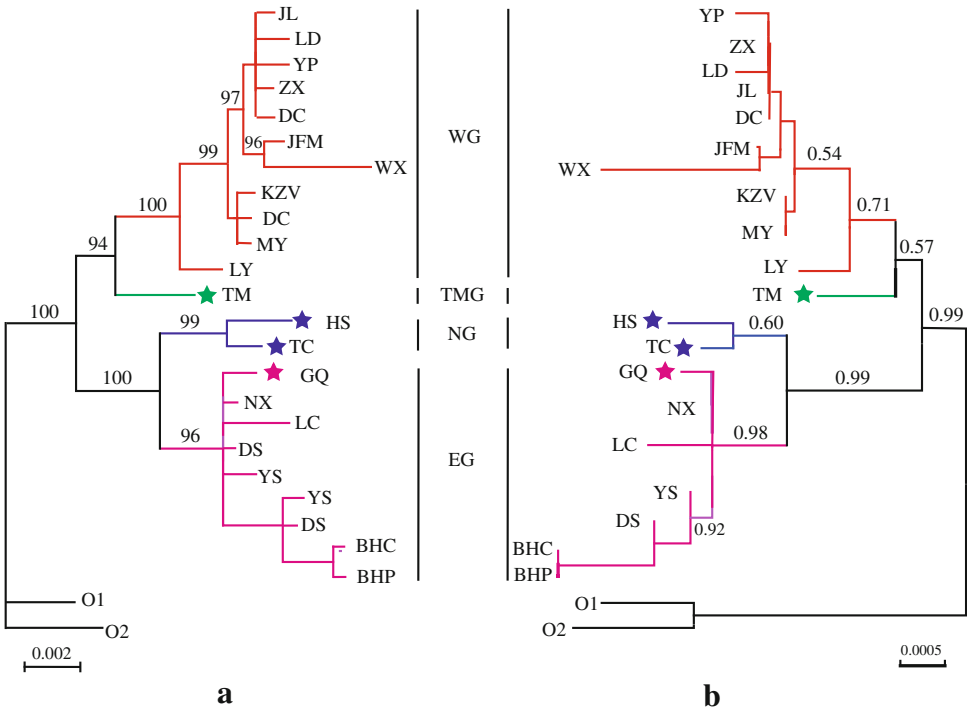

Fig. 2 A population NJ tree (**a**) and a Bayesian inference phylogenetic tree (**b**) of the chlorotypes of *Paeonia rockii* based on cpDNA (*petB–petD*, *rps16–trnQ* and *psbA–trnH*) intergenic spacers using MEGA 5 and MrBayes 3.12 software, respectively. O1 (*P. delavayi*) and O2 (*P. lodlowii*) represent the outgroup. The *numbers* appearing at the branches are bootstrap values (**a**) and posterior probabilities (**b**). *Pentagrams* indicate *P.* ssp. *atava*. Population codes are as in Table 1

Table 3 Results of the analysis of molecular variance (AMOVA) in the cpDNA (*petB–petD*, *rps16–trnQ* and *psbA–trnH*) sequence for 20 populations of *P. rockii*

Source of variation	d.f.	Sum of squares	Variation components	Variation (%)
Among four groups	3	21722.749	119.82106	96.86***
Among populations within groups	16	966.201	3.66967	2.97
Within populations	315	67.731	0.21502	0.17
Total	334	22756.681	123.70574	

d.f. degrees of freedom. *** $P < 0.001$

Table 4 Pairwise comparisons of estimated *Nm* (below diagonal) and *Fst* (above diagonal) between four groups (WG, TMG, NG and EG) of *Paeonia rockii* based on cpDNA (*petB–petD*, *rps16–trnQ* and *psbA–trnH*) sequences

	WG	TMG	NG	EG
WG		0.813	0.883	0.808
TMG	0.115		0.982	0.882
NG	0.066	0.009		0.780
EG	0.119	0.067	0.14	

The test of the phylogeographic structure of the haplotypes showed that the difference between N_{ST} (0.97) and G_{ST} (0.94) was significant ($U = 1.72$, $P < 0.01$). This result implies a population subdivision in which closely related haplotypes are more likely to occur in the same geographical area than less closely related haplotypes. The *Nm* values of 0.009–0.14 and the *Fst* values of 0.78–0.982 indicated high levels of genetic differentiation among the four groups (Table 4). The AMOVA analysis showed that the differences among the four groups (EG, TMG, NG and EG) explained 96.86% of the total cpDNA variation. In all, 2.97% of the total variation occurred among the populations within each region, whereas only 0.17% occurred within each population (Table 3).

Discussion

Genetic diversity of cpDNA and conservation strategy for *P. rockii*

The results of this study revealed a low genetic diversity at the population level and a higher genetic diversity ($Gst = 0.94$) at the species level, compared with the mean value of cpDNA diversity detected by various markers in 138 plant species ($Gst = 0.65$) (Petit et al. 2005). The level of genetic variation ($\theta = 0.00185$) was found to be lower than that of other endangered shrubs, e.g., *Dunnia sinensis* ($\theta = 0.0022$, Ge et al. 2002) and *Tetraena mongolia* ($\theta = 0.00447$, Ge et al. 2011). The relatively high haplotype diversity ($H_d = 0.887$) is consistent with the results from previous studies of the genetic diversity of different tree peony species or cultivars (Meng and Zheng 2004; Pei et al. 1995; Yuan et al. 2002; Zou et al. 1999).

Of 20 *P. rockii*, populations, 17 were found to be monomorphic, whereas only three were polymorphic. The finding that each of 17 populations carried only a single haplotype indicates that the endangered status of this species is the result of its extremely small effective population size, limited gene flow and isolation by geographic barriers. Our field observations from April 2006 to 2009 indicated that most of the extant populations are found only in remote and undisturbed forest areas and that the overall population sizes have become very small. As reported by numerous studies (Cheng et al. 1997, 2005; Hong 2010; Jing and Zheng 1999; Li 2005), the area of distribution and the number of both individuals and populations of *P. rockii* have decreased steadily during recent decades because of habitat change owing to natural factors and human overexploitation. *P. rockii* has been listed as a rare and endangered taxon in the Red Book of Chinese Plant Species (Fu 1992). However, its numbers have continued to decrease, and some populations have even disappeared completely. Some specific biological traits of *P. rockii* also contribute to the small population size. For example, a plant must reach almost 7 years of age on average before its first flowering, an adult plant flowers at most once every 2 years, and only 30% of adult plants flower. Also, high levels of infertile pollen and ovule abortion (up to 80%), a low germination rate, a rooting rate of only 4.4%, and a seed dormancy of up to 4 years (Cheng et al. 2005; Li 2005) may have impacted the growth of *P. rockii* populations. *P. rockii* is primarily pollinated by insects (Hong 2010, Li 2005), and gene flow between populations via pollen would be limited by the migratory capacity of the pollinators. In addition, the seeds are primarily dispersed by gravity (author's unpublished data) or by rats (Hong 2010). As a result, seed dispersal is probably limited to short distances. Other factors, including large seeds, leathery seed capsules, increased seed abortion and decreased seed germination, have also constrained the dispersal of *P. rockii* (Luo et al. 1998). To some extent, the high diversity found at the species level could also be the consequence of high diversity in the past.

The type of genetic profile found by this study also appears to be critical for the conservation of *P. rockii*. Owing to the low genetic diversity within populations and the small population sizes of this species, almost all extant populations of *P. rockii* are facing a serious threat of extinction caused by stochastic processes. Both theoretical studies and conservation practices indicate that extinction is more likely to result from genetic drift when the effective population size is small (Frankham 2005; Lande 1988). The International Union for the Conservation of Nature identified the effective population size as the most important of the five standards used to evaluate the threatened status of a species (Frankham et al. 2002). A more important consideration is that the potential ability of *P. rockii* to compete with other species and recolonize new habitats is considered very low because of unusually low fertility and survival rates, in addition to the deterioration and fragmentation of suitable habitat for this species (Cheng et al. 1997, 2005; Jing and Zheng 1999). The application of a more stringent local conservation strategy is therefore one of the most important measures needed for the conservation of this species. In addition to the in situ activities currently employed, ex situ conservation strategies should be given a high priority to offset the deterioration and fragmentation of habitats and the possible damage from local digging activities. In view of the fact that we found most populations of *P. rockii* to have a single haplotype, the loss of any one of the extant populations would correspond to a loss of genetic diversity that could likely never be regained. Continuous habitat destruction and fragmentation would inevitably result in smaller and more isolated populations and further increase the risk of population extinction (Ledig et al. 2002). Given high levels of genetic differentiation, lower genetic diversity within populations and reciprocal monophyly between almost all populations, an effective strategy to conserve this species should treat each population in *P. rockii* as a different evolutionarily significant unit for conservation. Conservation management policies that include reintroduction and remigration should be adopted to produce new self-sustaining populations of *P. rockii* within its original area of distribution.

Phylogeographic structure and the hypothesis for its formation in *P. rockii*

The results of the phylogenetic analyses using a NJ tree, a Bayesian tree and a haplotype network showed that the population genetic structure of *P. rockii* consisted of four well-defined groups: WG, TMG, NG, and EG. This population differentiation was primarily the result of isolation by geographic distance and decreased gene flow, as revealed by the phylogenetic analysis (Fig. 1), the analysis of the difference between N_{ST} and G_{ST}, the AMOVA analysis, and the comparison with both *Nm* and *Fst*. The pattern of clades indicated by the three phylogenetic analyses was nearly coincident with the pattern of geographic distribution of populations. In this pattern, the populations separated by smaller geographic distances are grouped together, rather than those at greater geographic distances (Figs. 1, 2). The phylogeographic structure was also examined using a test of haplotype variation, which showed that the difference between N_{ST} (0.97) and G_{ST} (0.94) was significant ($U = 1.72$, $P < 0.01$). This finding indicated that population subdivision resulted because more closely related haplotypes were more likely to co-occur in the same areas than less closely related haplotypes. The AMOVA analysis showed that the differences among the four groups (EG, TMG, NG and EG) explained 96.86% of the total cpDNA variation. The differences among populations within the region explained 2.7% of the total variation. Only 0.17% of the total variation was explained by the differences within populations (Table 3). The estimated *Nm* values of 0.009–0.14 and the estimated *Fst* values of 0.78–0.982 also indicated high levels of genetic differentiation between the four groups (Table 4). The Qinling Mountains extend more than 1,500 km from west to east. These mountains and the Wei river partition the natural distribution pattern of *P. rockii* in a way that corresponds to the four distinct groups identified by the molecular analysis: WG to the west, TMG in the middle, NG to the north, and EG to the east of the mountains. The finding that this geographic distribution pattern corresponds to the genetic structure of *P. rockii* indicates that the genetic evolution and differentiation of this species has most likely been strongly influenced by the geography of the Qinling Mountains (Fig. 1).

The phylogeographic structure observed for *P. rockii* could be attributed to a number of integrated factors, such as habitat fragmentation, climatic fluctuations, decreased seed germination and ripening capacity, and lack of gene flow among populations. Adaptive evolution in this species may also have helped shape its phylogeographic structure. Allopatric fragmentation of the distribution area and long-distance dispersal are two possible factors that might produce isolation among different groups of a species inhabiting a large geographic range. This pattern occurs in many plant species (Printzen et al. 2003). In the case of *P. rockii*, allopatric fragmentation is assumed to be the main force driving the differentiation of this species. Most species in southern temperate areas and tropical areas of the northern hemisphere have been divided into several different groups of populations at some point in their evolutionary past. These divided groups then evolved in isolation, and gene flow among them was further restricted during the glacial cycles of the Quaternary (Hewitt, 2000). However, glaciation had

no serious impact in most parts of eastern Asia, including the mainland of China. The phylogeographic structure of *P. rockii* is likely to have been influenced by climatic fluctuations to some extent. In particular, cold climatic conditions might have been responsible for the southward or southeastern movement of the northern populations to the eastern Qinling Range and further to the Funiu and Xuner Mountains in Henan. This scenario may possibly be supported by the finding that the GQ population was located to the north of Qinling, but this population clusters with the populations of the eastern groups (e.g., NX), as revealed by the phylogenetic trees (Fig. 1) and the haplotype network (Fig. 1).

The first major clade, WG and TMG, does not share any haplotypes with the second major clade, NG and EG (Figs. 1, 2). The absence of shared haplotypes between these two main clades means that neither the ancestral population of *P. rockii* nor the migratory routes of the species can be inferred with any confidence. The lack of fossils and the absence of a stable sister clade for *Paeonia* (Paeoniaceae) impede the dating of the intraspecific evolutionary history of *P. rockii*. Moreover, cpDNA analysis estimates only the contribution of seed movement to total gene flow, not the pollen gene flow, because cpDNA is maternally inherited and has haploid characteristics. Owing to these limitations, caution is required if the phylogeographic pattern of *P. rockii* is to be explained. Indeed, it is very difficult to evaluate the impact of human activities on the phylogeographic structure of *P. rockii*. Intensified human activities have certainly increased the rate of extinctions and the likelihood of endangered status for populations of *P. rockii*. Therefore, we suggest that it is necessary to continue investigating wild populations using molecular markers (e.g., nuclear markers) to further evaluate the genetic structure of this species.

This study included considerations of geographic distribution, phylogenetic trees based on cpDNA and morphotypic variation. Based on these considerations, we conclude that the intraspecific phylogeny of *P. rockii* has evolved to include four main groups: WG, TMG, NG and EG (Figs. 1, 2). The individuals in all of the populations belonging to the TMG and NG were identified as *P. rockii* ssp. *atava*, whereas the individuals of the EG and WG populations (except for GQ as *P.* ssp. *atava*) were identified as *P. rockii* ssp. *rockii*. This kind of phylogeographic structure could be the result of a number of integrated factors, including allopatric fragmentation, climatic fluctuations, the increased abortion and declining germination of seeds, and lack of gene flow among populations. Such structure could also result from adaptive evolution in *P. rockii*. These results may be further interpreted by considering the geographic barrier represented by the high Qinling Mountains, extending farther than 1,500 km from the west to the east. In the light of this information, the results of the study indicate that much of the genetic evolution and differentiation that are evident within this species have been caused by low genetic diversity and small population sizes. Accordingly, almost all extant populations of *P. rockii* are facing a serious threat of extinction owing to stochastic processes. Each of these populations should be treated as a conservation significant unit. Therefore, a more stringent local conservation strategy should be undertaken by including both in situ and ex situ approaches to conserve every population of *P. rockii*.

Acknowledgments We are grateful to three anonymous reviews and Dr. Rehan Naeem for critically reviewing the previous manuscript, providing insightful comments, and great help in English revision. We also deeply thank Mr. Jiajue Li, Dr. Jia Wang, Mrs. Yaling Tian, Mr. Jianhong Wang and the administrative staff of Gansu Forestry system for their help during the sample collection and field work. This study was funded by the Key Project of Science and Technology of State Forestry Administration of China (2006–40).

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