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ORIGINAL ARTICLE

Genetic diversity of *Rhododendron delavayi* var. *delavayi* (C. B. Clarke) Ridley inferred from nuclear and chloroplast DNA: implications for the conservation of fragmented populations

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Abstract *Rhododendron delavayi* Franch. is an ecologically and horticulturally important tree species distributed in a global biodiversity hotspot in southwest China, an area with high level of human-induced habitat degradation and fragmentation. In an attempt to explore the level of genetic diversity and population differentiation in the remnant populations of *R. delavayi* var. *delavayi* (C. B. Clarke) Ridley, we assessed patterns of chloroplast (trnS-trnG and trnL-trnF) and nuclear (*RPB2-i*) DNA variation in 327 individuals representing 17 natural populations from southwest China. Analyses revealed two phylogeographic groups, with similar circumscription between the two markers. We found moderate levels of genetic differentiation (cpDNA: $N_{ST} = 0.125$; $G_{ST} = 0.114$ and nuclear DNA: $N_{ST} = 0.261$; $G_{ST} = 0.152$). Both genomes demonstrate significant correlation between genetic and geographic distances (cpDNA: $r^2 = 0.248$, $p = 0.001$ and nuclear DNA: $r^2 = 0.250$, $p = 0.001$). The genetic diversity was positively associated with an increase in longitude. Populations from the eastern region of the Yungui plateau, representing potential refugial area for *R. delavayi* var. *delavayi,* registered higher haplotype diversity and allelic richness. The mismatch distribution analysis rejected rapid population expansion. The overall population expansion time for *R. delavayi* var. *delavayi* was estimated to be 0.208–0.624 mya. The complex landscape of southwest China and the human-induced fragmentation of the natural habitat have led to significant pairwise population differentiation and moderate genetic diversity (cpDNA: 0.626 and nuclear DNA: 0.506) and haplotypic richness (cpDNA: 4.01 and nuclear DNA: 2.589) in *R. delavayi* var. *delavayi*. Based on these findings, we recommend strategies for the conservation and sustainable management of *R. delavayi* var. *delavayi.*

Keywords *Rhododendron delavayi* var. *delavayi* · Genetic diversity · Habitat fragmentation · Yungui plateau · Chloroplast and nuclear DNA · Conservation

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Introduction

Biodiversity conservation is becoming increasingly challenging in the face of climate change and high levels of anthropogenic activities (Rands et al. 2010). In response to the increasing biodiversity loss and habitat degradation, areas with high endemism, species richness, and a high

number of threatened species have been designated as biodiversity hotspots (Myers et al. 2000; Mittermeier et al. 2005). China is one of the world's mega-diversity countries and is ranked third in terms of global plant biodiversity (Liu et al. 2003; López-Pujol et al. 2011). Three out of 34 biodiversity hotspots lie completely or partially inside the political boundary of China (Mittermeier et al. 2005). The south western provinces viz. Guangxi, Guizhou, Sichuan, and Yunnan posses the highest species diversity of all the provinces (Zhang and Ma 2008). Due to complex physiography and climate patterns, southwest China significantly contributed to floral diversity (Wu 1965; Ying 2001) with the occurrence of multiple refugia (Wang and Liu 1994; Wu and Wu 1998; Wang et al. 2009). This region is also the center of diversity for many economically and ecologically important species such as *Gentiana*, *Pedicularis*, *Primula* and *Rhododendron* (Milne et al. 2010; Li and Li 1993). Unfortunately, south west China also has the highest number of threatened species (Zhang and Ma 2008). In the past, this area has suffered tremendous pressure mainly due to excessive logging, conversion of forest into agricultural land, overgrazing, mining, and infrastructure development (Xu and Wilkes 2004; Yang et al. 2004; Pu et al. 2007). Reforestation programs have been implemented over the last few decades, but in the majority of logged natural forest areas, the monospecific plantations of exotic plants have completely replaced the native species (Xu and Wilkes 2004). The direct impact of habitat destruction is fragmentation and subsequent reduction of population sizes of several native species. Increased isolation of population and declining reproductive size have negative demographic and genetic consequences on the wild species (Frankham 2005). Habitat degradation and fragmentation are major threats to the native species along the region. Hence for the conservation and sustainable management of several native species in this biodiversity-rich area, major steps need to be taken. (Xu and Wilkes 2004; Yang et al. 2004).

Rhododendron L., which exhibits its highest concentration of species in northwest Yunnan and adjacent areas, is the largest genus (with more than 650 species) of the family *Ericaceae* in China (Kron et al. 2002). This woody genus is famous for its horticultural importance. *Rhododendron delavayi* Franch. (subgenus *Hymenanthes*, section *Ponticum* and subsection *Arborea*) is one of the most widely distributed species in southwest China, covering the region of the Yunnan and Guizhou (Yungui) plateau and the adjoining areas of Myanmar and northeast India (Fang et al. 2005; Chamberlain et al. 1996). Taxonomically, two varieties have been identified in this species, *Rhododendron delavayi* var. *peramoenum* (I. B. Balfour and Forrest) T. L. Ming with narrow leaves and reported from western Yunnan, NE India and Myanmar and *Rhododendron delavayi* var. *delavayi* (C. B. Clarke) Ridley with relatively broad leaves than former and shows extensive distribution throughout the range in China (Fang et al. 2005). These charismatic taxa grow in mixed broad-leaved forests and rocky slopes. Given an extensive occurrence of *Rhododendron delavayi* var. *delavayi* at an altitude range of 1,200–3,200 m, where excessive anthropogenic activities are prevalent, the remnant wild populations of this plant are highly isolated in several forest fragments, which remain at the top of mountains or along the edge of croplands. In this context, var. *delavayi*, is most likely susceptible to demographic stochasticity and the genetic effect of habitat fragmentation. Within the Sino-Himalaya region, human influence has impacted on the rhododendrons for many years. Tree rhododendrons are extensively used for charcoal, and as timber for furniture (Wangchuk 2011; Cox and Cox 1997). Habitat fragmentation and replacement of the natural forest of var. *delavayi* with pine trees have been reported before (i.e., Xu et al. 2007) and was also observed in several areas during specimen collection (personal observation of the authors). In general, long lived, woody trees with effective pollen and seed dispersal tend to have low effects of fragmentation (Hamrick et al. 1992). However, there is paucity of information on the effect of human-induced habitat destruction and fragmentation in *Rhododendron,* which is insect pollinated and seeds are dispersed by wind (Ng and Corlett 2000; Tian et al. 2011).

There have not been any species level studies carried out on the genetic structure and population differentiation of *Rhododendron* species in southwest China (Yungui plateau), despite it being regarded as a diversity hotspot for *Rhododendron* (Chamberlain. 1982; Milne et al. 2010). For the most part, previous studies on *Rhododendron* have focused on phylogeny, taxonomic identification, and natural hybridization using limited samples (Goetsch et al. 2005; Zhang et al. 2007b; Milne et al. 2010; Ma et al. 2010; Zha et al. 2010). The power of molecular markers in determining plant diversity has been confirmed by numerous studies (Holderegger and Abbott 2003). In this study, we used two chloroplast markers (trnL-F and trnS-G), and one nuclear marker (*RPB2-i*) to investigate population genetic structure and differentiation within and among populations of *Rhododendron delavayi* var. *delavayi*, that has extensive distribution in southwest China. The use of markers from different genomes can provide more information about the spatial distribution of genetic diversity and the level of population differentiation (Ikeda et al. 2008; Huang et al. 2011) which will then be more accurate to designate appropriate conservation priorities for specific populations and regions that need more attention.

This study was conducted to answer the following questions: (1) What are the levels of genetic diversity within and among populations of *Rhododendron delavayi* var. *delavayi* in southwest China? (2) How have historic and contemporary events influenced the current pattern of

Table 1 Population locations, numbers of sample size, the estimates of chloroplast and nuclear diversity within the 17 populations of *Rhododendron delavayi* var. *delavayi*

SN	Population	Locality/province	Longitude	Latitude	Altitude	*N*	Chloroplast				Nuclear			
							H_n	H_d	π	H_R	H_n	H_d	π	H_R
1	JY	Junzi shan, Yunnan	104.16	24.64	2,306	20	3	0.574	0.00079	1.80	4	0.574	0.00128	2.60
2	ZY	Zhujiang, Yunnan	103.92	25.89	2,370	20	5	0.758	0.00091	3.60	4	0.732	0.00269	2.80
3	WY	Guanpo, Yunnan	102.11	25.65	2,200	20	3	0.689	0.00068	2.00	2	0.268	0.00022	1.00
4	TY	Tanhua, Yunnan	101.23	25.96	2,100	18	3	0.629	0.00062	2.00	2	0.209	0.00017	0.99
5	CY	Zixi shan, Yunnan	101.40	25.03	2,250	18	4	0.723	0.00071	2.99	2	0.294	0.00024	1.00
6	XY	Mopan shan, Yunnan	101.99	23.94	2,500	18	4	0.660	0.00078	2.99	4	0.595	0.00253	2.88
7	HY	Huotou, Yunnan	103.01	24.31	2,330	20	3	0.679	0.00069	2.00	2	0.442	0.00036	1.00
8	WG	Yanchan, Guizhou	104.39	26.94	2,352	20	6	0.705	0.00105	4.20	4	0.721	0.0031	2.80
9	LG	Dafang, Guizhou	105.70	27.19	1,693	20	6	0.705	0.00105	4.87	5	0.790	0.00333	3.80
10	AY	Ailao Shan, Yunnan	101.03	24.54	2,627	20	3	0.532	0.0005	1.97	3	0.358	0.00108	1.94
11	NG	Shui dong, Guizhou	105.50	26.77	1,800	20	5	0.763	0.00123	3.91	4	0.737	0.00273	2.97
12	AS	Ai kao, Sichuan	101.60	27.18	2,500	20	2	0.100	0.00015	0.80	2	0.268	0.00152	1.00
13	NY	Ning Lang, Yunnan	100.89	26.85	2,700	18	3	0.307	0.00046	1.88	2	0.111	0.00054	0.89
14	LY	Laojun shan, Yunnan	99.82	26.64	2,700	19	3	0.292	0.00036	1.83	3	0.368	0.00083	1.84
15	EY	Luoping shan, Yunnan	99.86	26.00	3,000	20	4	0.363	0.00047	2.57	3	0.195	0.00057	1.60
16	DY	Wuliang shan, Yunnan	100.79	24.31	2,300	17	4	0.419	0.00047	2.88	3	0.228	0.00076	1.88
17	YY	Baotai shan, Yunnan	99.53	25.20	2,461	19	4	0.450	0.0006	2.68	3	0.368	0.00083	1.84

N Number of individuals, H_n haplotype number, H_d haplotype diversity, π nucleotide diversity, H_R haplotypic richness with rarefaction equal to 16 individuals

genetic structure? (3) Does any phylogeographic differentiation exist across the remnant populations of var. *delavayi*? Finally, the implications of these findings are discussed with suggestions for the conservation and sustainable management of *Rhododendron delavayi* var. *delavayi* and its habitat.

Materials and methods

Population sampling of *Rhododendron delavayi* var. *delavayi*

In total, leaf samples of 327 trees originating from 17 populations covering almost the entire distribution range of *Rhododendron delavayi* var. *delavayi* within southwest China were collected. Wherever possible, 17–20 individuals of adult trees at least 100 m apart were sampled from each population. Identification of collected individuals was based on morphological characteristics such as corolla color, indumentum on the ventral leaf surface, style surface (*R. delavayi* var. *delavayi* has a smooth surface, while the hybrids have grains or short hairs on the surface) and hairy young shoots (Zhang et al. 2007b). The locations of each population included in this study are provided in Table 1. Leaves were dried in silica gel immediately after collection and stored at room temperature until DNA extraction.

DNA extraction, amplification and direct sequencing

Total DNA was extracted from silica gel-dried leaf tissue using the modified CTAB method (Doyle and Doyle 1987; Kobayashi et al. 1998). The quality and concentration of the extracted DNA was assessed by agarose gel electrophoresis with known concentrations of uncut lambda DNA (Takara, Dalian, China). Preliminary primer screening was conducted for chloroplast DNA (hereafter cpDNA) amplification and polymorphism in *R. delavayi* var. *delavayi* on five individuals each from all the collected population using five different primer pairs: *trn*H-*psb*A, *trn*S-G, *rp*l20-*rps*12, *psb*B-F (Hamilton 1999) and trnL-F (Taberlet et al. 1991). The two regions, the trnS-G and trnL-F revealed sequence variations within the individuals examined and were then used for a large-scale survey of haplotype variation in *R. delavayi* var. *delavayi*. Polymerase Chain Reaction (PCR) was performed using a PTC-100 thermocycler (MJ Research, Watertown, MA, USA), with a final volume of 50 ml containing 20 ng of template DNA, 10 mMTris-HCl, pH 8.3, 50 mMKCl, 2 mM MgCl2, 200 mM of each dNTP, 400 pmol of each primer and 1 U of Ex-taq (Takara, Kyoto, Japan) with the following temperature profile: an initial 2 min denaturing at 94 °C then 33 cycles of 1 min denaturing at 94 °C, 1 min primer annealing at 50 °C, and 1 min primer extension at 72 °C and a final 10 min extension at 72 °C. To obtain nuclear

DNA (nrDNA) polymorphisms, part of the nuclear gene *RPB2-i,* encoding a major RNA Polymerase II unit was amplified using primers 2F-3R (Goetsch et al. 2005) with the following temperature profiles: initial 2 min denaturing at 94 °C, then 35 cycles of 1 min denaturing at 94 °C, 45 s primer annealing at 54 °C, and 1 min 20 s primer extension at 72 °C and a final 10 min extension at 72 °C. The PCR products were cleaned using a purification kit (Takara) following the manufacturer's protocol. Direct sequencing was performed on both strands on an ABI PRISM 3730 Sequencer using the PCR primers. Nucleotides were edited and aligned in MEGA ver.5 (Tamura et al. 2011).

Data analysis

We resolved the heterozygotes in SNPs of the nuclear gene using PHASE 2.1.1 (Stephens et al. 2001), which uses a Bayesian statistical method to reconstruct haplotypes from population genotype data. This program helps in inferring the probabilities for each possible haplotype from a group of linked SNPs. A total of eight independent runs of 100 iterations each were performed, while other parameters were left as in default. Haplotypes were selected for each phased locus, which had highest probability as assessed by PHASE. For our subsequent haplotype analysis, we used only those alleles and genotypes resolved with >95 % posterior probability. The nucleotide diversity (π) (Nei and Li 1979), haplotype number for chloroplast, and haplotype diversity (H_T) was calculated using software DnaSP 5.1 (Librado and Rozas 2009). The phylogenetic relationships among nuclear and chloroplast haplotypes were estimated separately using the median-joining algorithm in NETWORK 4.6 (Bandelt et al. 1999) (available at http://www.fluxus-engineering.com). The two measures of population differentiation G_{ST} and N_{ST} were computed using the program PERMUT (Pons and Petit 1996) (available at http://www.pierroton.inra.fr/genetics/labo/Software/Permut) with 1,000 permutations. In the presence of significant phylogeographic structure, the value for N_{ST} will be higher than G_{ST} (Pons and Petit 1996), indicating the higher probability of detecting closely related haplotypes in the same geographic area. Using CONTRIB 1.02 software (Petit et al. 1998) the total and population haplotypic richness (allelic richness H_R) was calculated after rarefaction for each population containing more than 16 samples, (software available at http://www.pierroton.inra.fr/genetics/labo/Software/Contrib/). Furthermore, each population's contribution to total haplotype diversity (CT) and haplotypic richness (CTR) was determined, and each divided into two components; one (CS and CSR) for contribution from the population's own level of diversity and the other (CD and CDR) for its differentiation from other populations. ARLEQUIN 3.5 (Excoffier and Lischer 2010) was used for an Analysis of Molecular Variance (AMOVA) and to assess genetic differences between all pairs of population using an F_{ST} estimator. Spatial analysis of molecular variance was conducted to define groups of proximal population, using the software SAMOVA (Dupanloup et al. 2002) with 1000 simulated annealing steps. SAMOVA was run to identify the optimum number of groups of populations. The optimum number of groups (K) chosen has the highest differentiation among groups (F_{CT}) and does not have any single population as a group. To determine the significance between the genetic and geographical distances across the sampled landscape, Mantel test (Mantel 1967) was performed for both cpDNA and nuclear DNA sequences, with 10000 permutation tests using GenAlEx 6.5 (Peakall and Smouse 2012). To detect the tendency of change in diversity along the distribution area, a Spearman rank correlation test between total genetic diversity and longitude, and between haplotypic richness and longitude was performed among all population.

To understand the demographic changes in *R. delavayi* var. *delavayi,* two hypotheses of population expansion were tested in ARLEQUIN 3.5, the pairwise mismatch distribution analysis (Rogers and Harpending 1992) and statistical tests of neutrality, (Tajima 1989) *D* and (Fu 1997) *Fs*. Both of these analyses were conducted separately on the entire population and in the phylogeographic groups suggested by SAMOVA. A significant value of Tajima's *D* and a significantly large negative value for Fu's *Fs* indicate sudden population expansion. Tests were conducted under both demographic and spatial expansion models using a total of 10,000 parametric bootstrap replicates. Sums of squared deviations (SSD) between observed and expected mismatch distribution and the raggedness index were calculated. Population that experienced sudden expansion usually gives a unimodal distribution pattern, while population in demographic equilibrium presents a multimodal (non-unimodal) distribution pattern. The tau (τ) value obtained from the mismatch analysis was used to calculate the expansion time (t) using the equation $\tau = 2\mu t = 2mT\mu t$, where mT is the number of included nucleotides and μ is the cpDNA mutation rate. Given the lack of a substitution rate for the cpDNA genome of *Rhododendron*, the cpDNA synonymous substitution rate of $1.0–3.0 \times 10^{-9}$ substitutions per site per year found in seed plants was used (Graur and Li 2000) as the approximate evolutionary rates.

Results

Sequence characteristics and haplotype variation

The two cpDNA regions (*trn*L-F and *trn*S-G) of *R. delavayi* var. *delavayi* were combined to a total 1,325 bp after

alignment. This included four polymorphic sites of which all were parsimony informative. These polymorphic sites were single-base substitutions and scattered along the sequences. The nuclear DNA (part of the nuclear gene *RPB2-i* sequences) of *R. delavayi* var. *delavayi* was aligned with a consensus length of 1,238 bp. This included 11 mutations of which nine were parsimony informative. A long indel of 239 bp in length was found at position 200 of the aligned matrix. A total of six cpDNA (DC1–DC6) (GenBank Accessions KJ489434 - KJ489445) and eight nuclear (DN1–DN8) (GenBank Accessions KJ489446 - KJ489453) haplotypes were detected among the 327 individuals, collected from 17 different natural populations of *R. delavayi* var. *delavayi* across its distribution range in southwest China (Table 1). All populations were polymorphic. The most frequent cpDNA haplotype, DC1 was observed 179 times (55 %) and occurred in every population. This was followed by DC2, observed 70 times (21 %) and was present in all except two (AY and LY) populations. Haplotype DC3 was also widespread, with 40 individuals (15 %). Haplotype DC5 occurred in very low frequency with a total of 19 individuals (5 %) occurring in 11 of the 17 populations. The haplotype DC6 was present in the three populations from Guizhou (WG, LG and NG), while haplotype DC4 was found only in the three populations from Guizhou (WG, LG, NG) and one eastern population of Yunnan (ZY). For nuclear DNA, the most frequent haplotype, DN2, distributed in all of the populations, was observed 192 times (65 %), followed by DN3 observed 54 times (18 %), distributed in all but two populations (AS and LY) (Fig. 1b). Haplotypes DN4 and DN5 were found only along the central and eastern regions of the sampled populations and with low frequency, while DN6 was only present in one population in Guizhou (LG). Similarly, haplotypes DC7 and DC8 were found only towards the western region of the distribution of the analyzed populations.

Genetic diversity and population structure

In *R. delavayi* var. *delavayi*, total cpDNA haplotype diversity (H_T) was 0.634, while the population haplotype diversity (H_d) ranged from 0.100 to 0.763. Total nucleotide diversity (π) ranged from 0.00015 to 0.00123 and the haplotypic richness (H_R) from 0.80 to 4.87 (Table 1). Among the 17 populations investigated, high levels of nucleotide diversity (0.00123) and haplotype diversity (0.763) were detected in the population of Na Yongshui Dong, Guizhou (NG). Other nearby populations in Guizhou, eastern and central Yunnan (WG, LG, HY, XY, CY and ZY) also exhibited high levels of nucleotide and haplotype diversity. These regions had relatively higher values of haplotypic richness (2.00–4.87). Although there was an apparent positive correlation between haplotype diversity and haplotypic richness, they were not equivalent. This was because some populations from the western regions (EY, DY and YY), albeit having low haplotype diversity (0.363–0.450), had values of haplotypic richness more than 2.00 (Table 1). The lowest level of nucleotide diversity (0.00015) and haplotypic richness (0.200) was detected in population AS, located in southwest Sichuan (Table 1). For nuclear DNA within population, haplotype diversity (H_d) ranged from 0.111 to 0.790, while total haplotype diversity (H_T) was 0.503. Total haplotype diversity and haplotypic richness (H_R) ranged from 0.00054 to 0.00333 and 0.89 to 3.80, respectively (Table 1).

A high level of nuclear nucleotide diversity (0.00333) and nuclear haplotype diversity (0.789) was detected in the population of Weining, Guizhou (WG). The two other populations from Guizhou (LG, NG) and eastern Yunnan (ZY) also had high nuclear haplotype and nucleotide diversity. Similar to cpDNA data, the populations with high nuclear haplotype diversity had higher values for haplotypic richness. The lowest level of nucleotide diversity (0.00054), and haplotypic richness (0.89) was detected in a northwest population from Ning Lang (NY) near the Yunnan-Sichuan border (Table 1). The contribution to the total genetic diversity and differentiation was different for different populations, as inferred for *R. delavayi* var. *delavayi*. Each population's contributions, with regard to the components of diversity (CS and CSR) and differentiation (CD and CDR), are shown in Fig. 2. Higher CS and CSR values in both cpDNA and nuclear DNA were identified in populations within group 1, as suggested by SMOVA (along the eastern regions of distribution, described below) (Table 2), where populations had relatively higher values of H_d and H_R (Table 1; Fig. 2) except population (JY). However, higher CD and CDR were not localized in any specific geographic region (Fig. 2). All the populations that showed positive contribution to haplotype diversity also showed positive contribution for haplotypic richness. However, when considering the contribution of divergence (CD and CDR) and diversity (CS and CSR) to the total contribution of diversity (CT and CTR), the positive contribution observed in the populations from western Yunnan LY, NY and EY and southwest Sichuan was not due to the diversity but due to the differentiation factor (Supplementary Tables S1 and S2). The populations from eastern Yunnan and Guizhou had strong positive diversity indices, indicating their highest contribution to total genetic diversity in *R. delavayi* var. *delavayi*.

The phylogenetic relationships among the chloroplast and nuclear haplotypes were addressed by a network analysis (Fig. 1a, b). Haplotypes from both markers showed a circular network and the relationship among the types unresolved. Although the N_{ST} (0.125) was higher than the

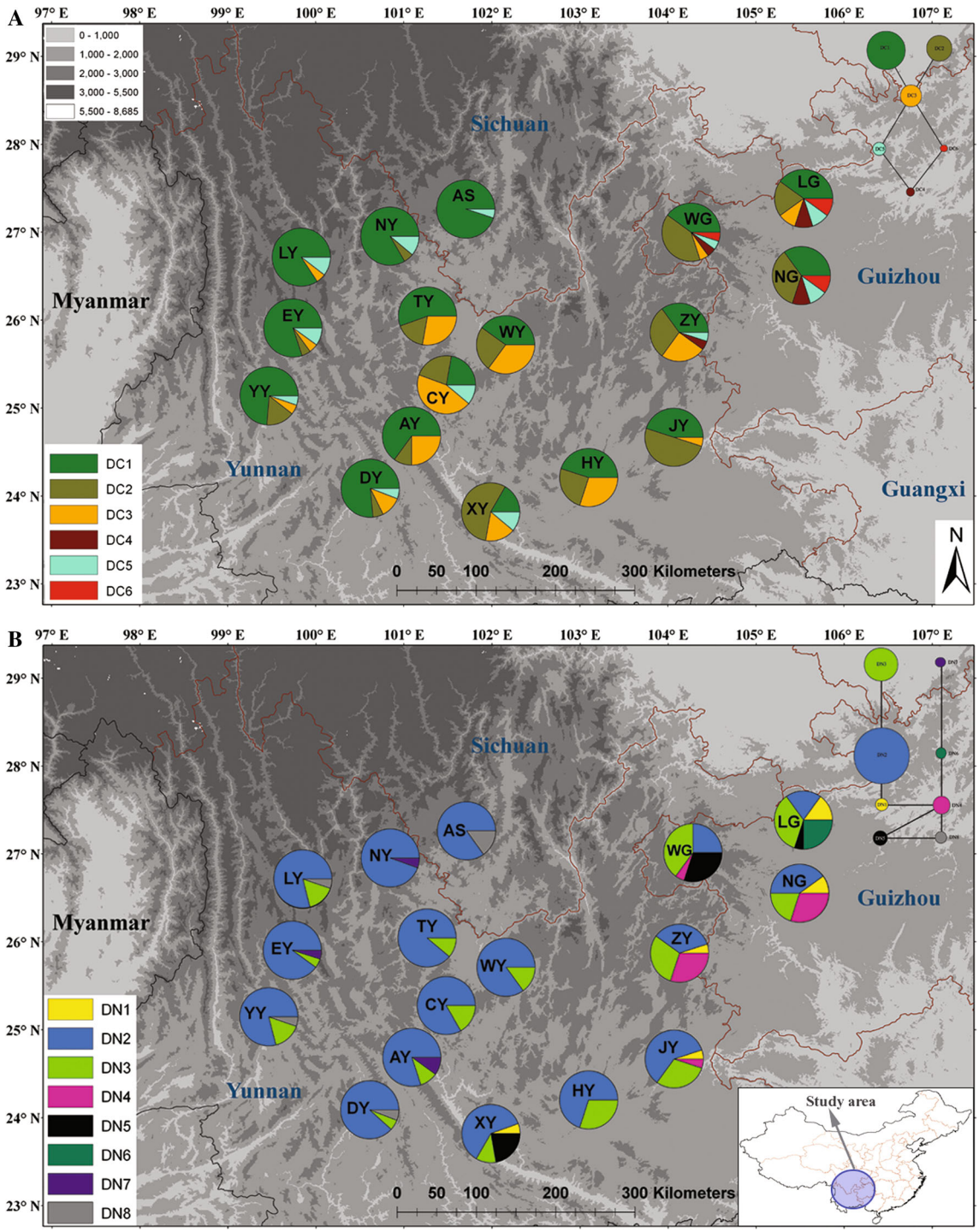

Fig. 1 Map showing spatial distribution of (**a**) chloroplast and (**b**) nuclear haplotypes in 17 sampled populations of *Rhododendron delavayi* var. *delavayi*. On each map, phylogenetic network of haplotypes is given separately at the *top right corner*. Study area is highlighted in the map of China presented at the *bottom right corner* of the figure. See Table 1 for detail

G_{ST} (0.114) across the 17 populations, the difference was not significant. *RPB2-i* haplotypes showed a similar pattern with larger but not significant N_{ST} (0.261) than G_{ST} (0.152). The Mantel test detected a significant correlation between the genetic and geographic distances for both cpDNA ($r^2 = 0.248$, $p = 0.001$) and nuclear DNA ($r^2 = 0.250$, $p = 0.000$) (Fig. 3a, b) indicating that distant populations exhibit high pairwise genetic differentiation. The Spearman rank correlation test between nucleotide diversity (π) and longitude and, between haplotypic

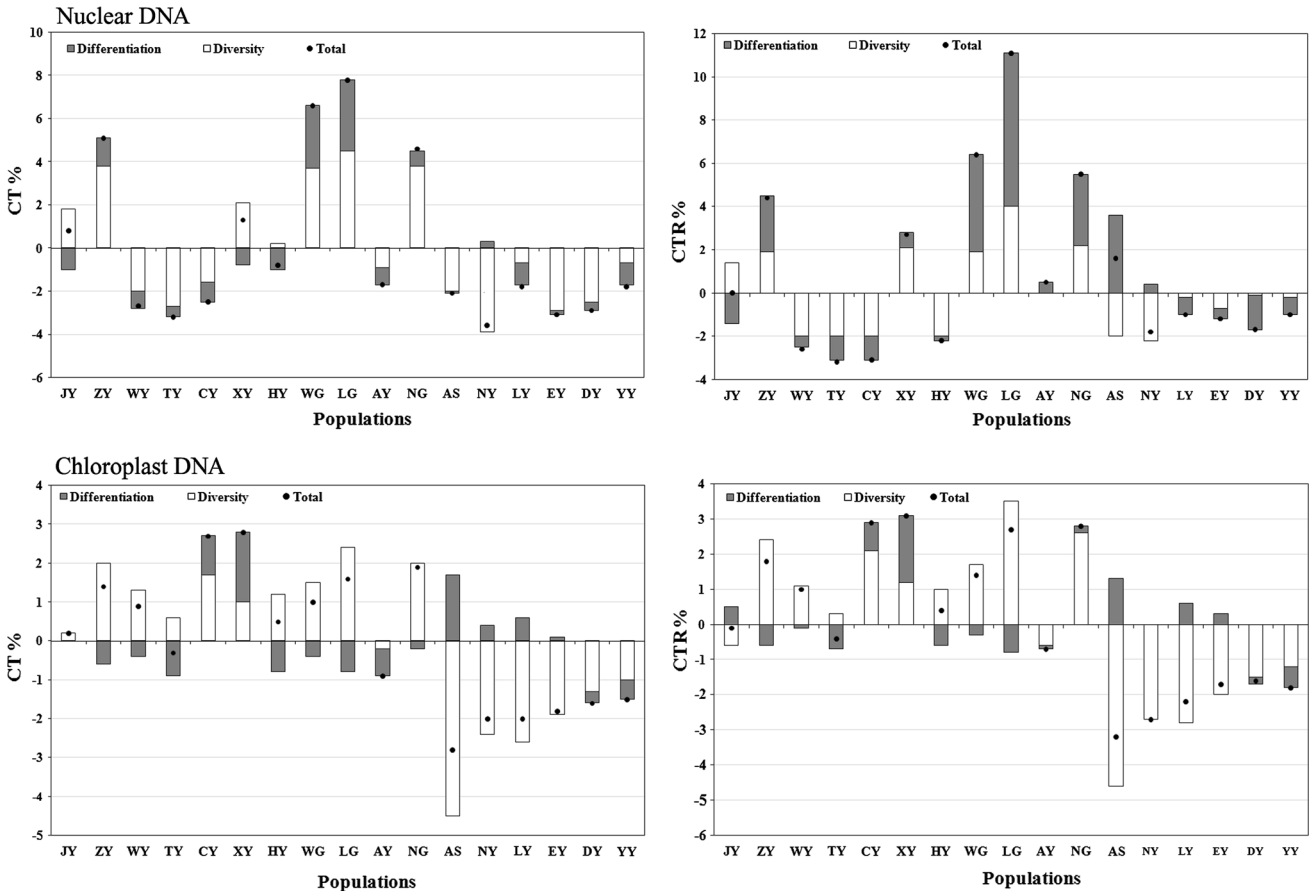

Fig. 2 The contribution to the total haplotype diversity (*CT*) and haplotypic richness of the 17 populations of *Rhododendron delavayi* var. *delavayi* using (**a**) nuclear and (**b**) chloroplast markers. The *grey* and *white bars* represent the contribution of diversity (CS and CSR) and differentiation (CD and CDR), respectively. Detail on population is given in Table 1

richness (H_R) and longitude, among the 17 studied populations showed significant positive correlation in cpDNA with a Spearman's rho (ρ) = 0.548 ($p = 0.02$) and $\rho = 0.463$ ($p = 0.007$), respectively, and also in nuclear DNA, with $\rho = 0.723$ ($p = 0.001$) and $\rho = 0.534$ ($p = 0.028$), respectively), suggesting that there is an increase in diversity with increasing longitude. This finding supports the fact that the eastern region of distribution harbors the maximum diversity and the diversity tends to decrease westward. The AMOVA analysis for cpDNA showed moderate levels of differentiation among all populations ($F_{ST} = 0.13104$, $p < 0.01$), with higher genetic variability within populations (86.9 %) compared to variations among populations (13.1 %). The SAMOVA of cpDNA suggested two groups ($K = 2$) to show the greatest value of F_{CT} (0.205, $p < 0.05$). The populations from central and eastern Yunnan and western Guizhou formed one group and those from western Yunnan and southwest Sichuan formed the other group (Table 2). The genetic structure was considerable at three levels i.e., between groups 20.6 % ($p < 0.05$), among populations within groups 1.22 % ($p < 0.05$) and within populations 78.18 % ($p < 0.05$). The AMOVA analysis on nuclear DNA data revealed a similar result as the cpDNA data with a modest value for F_{ST} (0.134, $p < 0.01$) with among- and within-populations variation being 9.91 and 90.09 %, respectively. The SAMOVA revealed two groups ($F_{CT} = 0.246$, $p < 0.05$), the populations from eastern Yunnan and western Guizhou formed one group and remaining formed the other (Table 2). Genetic diversity was partitioned; among populations within groups with 1.24 % ($p < 0.05$), between groups with 24.61 % ($p < 0.05$) and within populations with 75.63 % ($p < 0.05$). When the diversity indices were compared between the two groups as defined by SAMOVA for both organelle and nuclear markers, the group comprising the eastern regions of the Yungui plateau (east to central Yunnan and Guizhou) harbored more diversity in terms of number and richness (Table 2). The pairwise population differentiation (F_{ST}) was significantly larger when populations from eastern regions were compared with central and western populations. The F_{ST} values range from 0.102 to 0.603 and 0.117 to 0.349 in cpDNA and nuclear DNA, respectively (Table 3).

Table 2 Summary statistics, results of mismatch distribution analysis and neutrality tests for two groups suggested by SAMOVA done in 17 populations of *Rhododendron delavayi* var. *delavayi*

Parameters	Chloroplast			Nuclear		
	Group 1 (1–9, 11)	Group 2 (10, 12–17)	All population	Group 1 (2, 8, 9, 11)	Group 2 (1, 3–7, 10, 12–17)	All population
Sample size	194	133	327	99	227	327
Number of populations	10	7	17	5	12	17
Number of variable sites	4	3	4	10	10	10
Number of haplotypes	6	4	6	6	6	8
H_d	0.717	0.354	0.626	0.752	0.315	0.506
Nucleotide diversity	0.00088	0.00043	0.00077	0.00303	0.00093	0.00157
H_R	4.318	2.442	4.016	4.950	2.219	2.589
Tajima's D	1.263	0.056	1.095	2.399	0.708	0.787
Tajima's D *p* value	0.889	0.612	0.866	0.99	0.259	0.807
Fu's FS	0.694	0.077	0.695	5.886	1.775	2.333
Fu's FS *p* value	0.674	0.512	0.666	0.96	0.654	0.754
Mismatch distribution (demographic)	Non-unimodal	Non-unimodal	Non-unimodal	Non-unimodal	Non-unimodal	Non-unimodal
Demographic expansion (Tau)	1.404	0.242	1.6562	–	–	–
Demographic expansion (Mya)	–	–	(0.208-0.624)	–	–	–
Mismatch distribution (spatial)	Non-unimodal	Non-unimodal	Non-unimodal	–	–	–
Spatial expansion (Tau)	1.445	1.691	1.525	–	–	–
Spatial expansion (Mya)	–	–	(0.191–0.575)	–	–	–

Numbers in parentheses are the same as the population numbers in Table 1

H_d Haplotype diversity, H_R haplotypic richness with rarefaction equal to 16 individuals

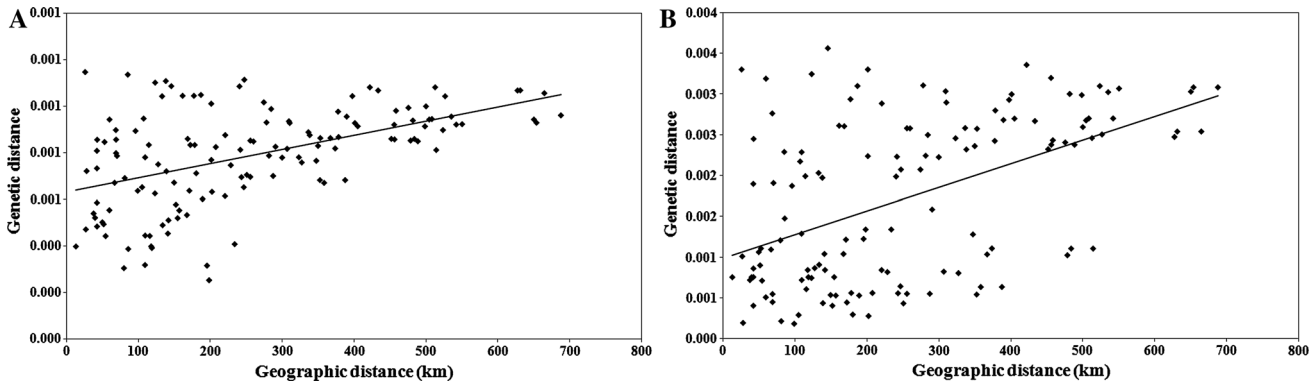

Fig. 3 *Scatterplot* showing the relationship between genetic distance and geographic distance of 17 populations of *Rhododendron delavayi* var. *delavayi*. **a** Chloroplast DNA and **b** Nuclear DNA

Demographic expansion of *Rhododendron delavayi* var. *delavayi*

The mismatch distribution analysis for both cpDNA and nuclear DNA revealed a non-unimodal distribution, rejecting the null model of rapid population expansion (Fig. 4a, b). The mismatch analysis performed separately in the two groups suggested by SAMOVA was also not unimodal. The calculated values of Tajima's *D* and Fu's *Fs* statistics in both cpDNA and nuclear DNA were positive but not significant. When the test was performed individually in each group defined by SAMOVA, the result was significantly positive for nuclear DNA in only one group, while the rest showed non-significant positive values (Table 2). These results suggest that there was no rapid population expansion in *R. delavayi* var. *delavayi*. With the

Table 3 Pairwise comparison of F_{ST} estimates based on nuclear (above diagonal) and chloroplast markers (below diagonal) between 17 populations of *Rhododendron delavayi* var. *delavayi*

	JY	ZY	WY	TY	CY	XY	HY	WG	LG	JY	NG	AS	NY	LY	EY	WY	YY
JY	0.000	0.100	0.036	0.045	0.025	0.063	0.009	**0.122**	**0.186**	0.011	0.148	0.021	0.036	0.026	0.020	0.003	0.026
ZY	0.005	0.000	**0.271**	**0.265**	**0.256**	0.019	**0.255**	0.025	0.020	**0.130**	0.045	0.072	**0.199**	**0.165**	**0.205**	**0.163**	**0.165**
WY	0.016	0.035	0.000	0.049	0.055	**0.208**	0.013	**0.282**	**0.349**	0.030	**0.318**	0.105	0.033	0.019	0.001	0.003	0.019
TY	0.072	0.018	0.017	0.000	0.045	0.199	0.052	**0.277**	**0.342**	0.025	**0.310**	0.094	0.013	0.019	0.012	0.008	0.019
CY	0.082	0.031	0.004	0.081	0.000	**0.195**	0.005	**0.266**	**0.334**	0.026	**0.304**	0.100	0.035	0.024	0.003	0.002	0.024
XY	0.038	0.036	**0.102**	**0.222**	0.057	0.000	**0.209**	0.018	0.029	0.069	0.011	0.003	**0.123**	0.100	**0.131**	0.089	0.100
HY	0.013	0.027	0.050	0.033	0.017	**0.122**	0.000	**0.261**	**0.334**	0.059	**0.310**	**0.137**	**0.117**	0.003	0.073	0.060	0.003
WG	0.024	0.040	0.013	0.034	0.009	0.020	0.011	0.000	0.007	**0.157**	0.014	0.097	**0.223**	**0.182**	**0.227**	**0.183**	**0.182**
LG	0.024	0.040	0.013	0.034	0.009	0.020	0.011	0.053	0.000	**0.207**	0.026	**0.152**	**0.277**	**0.251**	**0.285**	**0.247**	**0.251**
JY	**0.155**	0.092	0.055	0.036	**0.174**	**0.328**	0.028	**0.105**	**0.105**	0.000	**0.168**	0.006	0.033	0.028	0.034	0.033	0.028
NG	0.033	0.026	0.023	0.067	0.009	0.038	0.028	0.035	0.035	**0.132**	0.000	0.095	**0.235**	**0.209**	**0.244**	**0.201**	**0.209**
AS	**0.430**	**0.373**	**0.382**	**0.246**	**0.502**	**0.603**	**0.340**	**0.354**	**0.354**	**0.157**	**0.353**	0.000	0.015	0.010	0.024	0.012	0.010
NY	**0.270**	**0.210**	**0.205**	0.080	**0.315**	**0.442**	**0.168**	**0.204**	**0.204**	0.021	**0.211**	0.001	0.000	0.019	0.052	0.044	0.019
LY	**0.327**	**0.254**	**0.251**	**0.118**	**0.360**	**0.493**	**0.214**	**0.251**	**0.251**	0.047	**0.251**	0.008	0.051	0.000	0.032	0.049	0.056
EY	**0.259**	**0.194**	**0.184**	0.060	**0.294**	**0.430**	**0.148**	**0.194**	**0.194**	0.004	**0.202**	0.016	0.053	0.046	0.000	0.046	0.032
WY	**0.228**	**0.163**	**0.145**	0.024	**0.262**	**0.403**	**0.110**	**0.165**	**0.165**	0.027	**0.178**	0.041	0.047	0.036	0.052	0.000	0.049
YY	**0.143**	**0.109**	0.087	0.010	**0.207**	**0.325**	0.055	**0.104**	**0.104**	0.034	**0.131**	0.085	0.022	0.008	0.027	0.043	0.000

Bold values are significant at $p < 0.05$

SSD values between the observed and the expected mismatches, and Harpending's r indices being significant, the overall population expansion time for *R. delavayi* var. *delavayi* was calculated based on the estimated *tau* (τ) value. The demographic and spatial expansion times for *R. delavayi* var. *delavayi* across the sampled populations along southwest China range between 0.208–0.624 and 0.191–0.575 mya, respectively.

Discussion

Genetic diversity and differentiation

Out of the five cpDNA regions (*trn*H-*psb*A, *trn*S-*trn*G, *rpl*20-*rps*12, *psb*B-*psb*F and *trn*L-*trn*F) assessed in *Rhododendron delavayi* var. *delavayi*, sequence polymorphisms were only obtained in *trn*S-*trn*G, and *trn*L-*trn*F. These two regions have been widely used to investigate the population diversity and differentiation of several taxa (Petit et al. 2005; Qiu et al. 2011) including *Rhododendron* (Li et al. 2012). As in other studies (Li et al. 2010; Zecca and Grassi 2013), the intron region of nuclear gene *RPB2-i* was also found to be informative for studying the pattern of diversity in *R. delavayi* var. *delavayi.* The cpDNA and nuclear DNA diversity estimates obtained in *R. delavayi* var. *delavayi* from SW China revealed a moderate level of haplotype diversity. The cpDNA total haplotype diversity ($H_T = 0.635$) is only slightly lower than the mean for cpDNA as reported by Petit et al. (2005) in 170 species of angiosperms ($H_T = 0.670$). The observed estimates of H_T are slightly lower, but are nonetheless comparable to 27 taxa for which values were available (Supplementary Fig. 1). Among congeners, estimated total diversity in *R. delavayi* var. *delavayi* was lower than in *R. pseudochrysanthum* (0.879) (Huang et al. 2011), *R. formosanum* (0.857) (Huang et al. 2011) and *R. simsii* (0.749) (Li et al. 2012). The high value for *R. pseudochrysanthum* is explainable because Huang et al. (2011) included the entire species complex in their study. Concerning the nuclear haplotype diversity, there are no reports for comparison that have used the same low-copy nuclear sequences to address the genetic diversity. When compared with other studies based on low-copy nuclear DNA sequences along the studied geographic region, *R. delavayi* var. *delavayi* had a relatively low haplotype diversity (0.506) (e.g., *Camellia taliensis*, $H_T = 0.836$, Liu et al. 2012; and *Hippophae rhamnoides*, $H_T = 0.811$, Jia et al. 2012). The pattern of distribution of genetic diversity was very similar in cpDNA and nuclear DNA, where the majority of haplotype diversity was present within a few populations. Populations along the eastern and central region of the sampled distribution (WG, LG, HY, XY, CY and ZY) had higher chloroplast haplotype diversity, where the nuclear diversity was only higher towards the eastern region (WG, LG, NG, ZY and XY). The populations in Guizhou and central-eastern Yunnan had relatively high haplotype diversity and similar results have been found in *Pyrus*

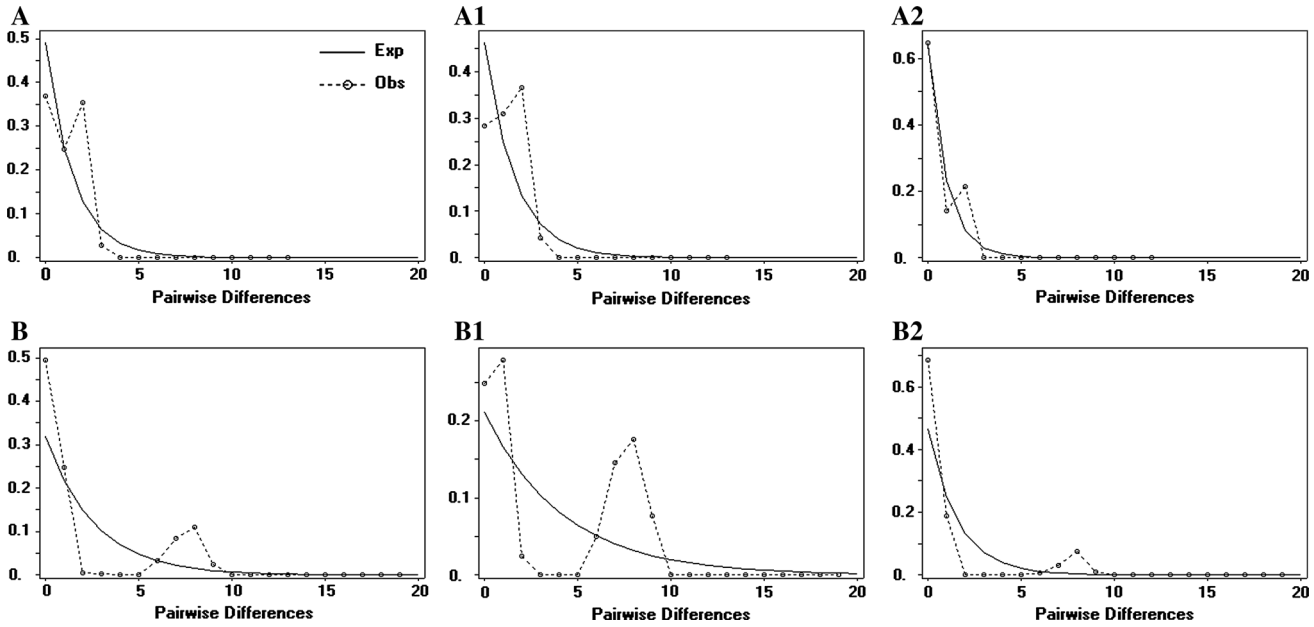

Fig. 4 Mismatch distribution analysis for 17 populations of *Rhododendron delavayi* var. *delavayi* using chloroplast and nuclear markers. **a** Chloroplast all populations (*A1*) chloroplast SAMOVA group 1 (*A2*) chloroplast SAMOVA group 2. **b** Nuclear all populations (*B1*) nuclear SAMOVA group 1, (*B2*) nuclear SAMOVA group 2

pashia (Liu et al. 2013). Although Milne et al. (2010) in their phylogenetic study of Rhododendron sub genus *Hymenanthes* have shown high diversity of Rhododendrons in Hengduan mountain region, our population genetic study of *R. delavayi* var. *delavayi* revealed high diversity in populations distributed in the central-eastern part of Yunnan. This region along southwest Guizhou that has been reported to have a high richness of *Rhododendron* species is popularly known as "One hundred miles of natural *Rhododendron* communities", (Zhang et al. 2007a; Chen et al. 2008).

The genetic differentiation of cpDNA and nuclear DNA present in *R. delavayi* var. *delavayi* populations was supported by the existence of two distinct groups with F_{CT} values (0.205 and 0.246, respectively) and F_{ST} (0.228 and 0.233, respectively). The two major population groups identified by nuclear DNA in *R. delavayi* var. *delavayi* are spatially not in complete harmony with those registered by cpDNA. The differences might lie in their different modes of inheritance, with chloroplasts DNA being uniparental and often maternal in angiosperm [including in *Rhododendron delavayi* (Zhang et al. 2007b, c)], and nuclear DNA biparental with recombination of genes and regions (Petit et al. 2005). Nevertheless, with markers of both genomes, one of the groups suggested by SAMOVA consisted of populations from eastern and central regions and the other group consisted of western region highlighting the fact that there is difference in haplotype composition between populations from east to west. The estimated genetic differentiation of *R. delavayi* var. *delavayi* for cpDNA ($G_{ST} = 0.114$, $N_{ST} = 0.125$) and nuclear DNA ($G_{ST} = 0.152$, $N_{ST} = 0.261$) was not very high when compared with other species of angiosperms (Petit et al. 2005). The lower values of G_{ST} suggest that the populations as a whole are not widely differentiated and most of the variation was partitioned within populations, a common phenomenon observed in long living woody plants (Hamrick et al. 1992). The low level of genetic differentiation must have resulted from the widespread distribution of certain haplotypes (cpDNA-DC1, DC2 and DC3; nuclear DNA-DN2 and DN3).

Beyond these general observations, it was found that populations with the highest pairwise genetic differentiation correspond well with the level of haplotypic diversity between compared populations [Tables 1 and 3)]. For cpDNA, all the western populations (AS, NY, LY, EY, DY, and YY) exhibited the highest population differentiation, while populations of Ghuizou (LG, WG and NG) and northeast Yunnan (ZY) with high nuclear haplotype diversity showed significant population differences in comparison with the other populations. Mantel test and Spearmans' rank correlation test showed significant differences among populations along longitude which support the findings that diversity tends to decrease with westward movement along the sampled distribution.

In most angiosperms, genetic differentiation values are generally higher for chloroplast DNA than nuclear DNA because cpDNA is maternally inherited and only travels

through seeds (Petit et al. 2005). In case of *R. delavayi* var. *delavayi*, this was not that evident because values of G_{ST} and N_{ST} for both cpDNA and nuclear DNA were low and not significant, with lower among population variation in both nuclear DNA (9.91 %) and cpDNA (13.1 %). The most likely rationale could be that the seeds of *Rhododendron* are not very far dispersed by wind and cannot travel more than 30–80 m distance within or nearby the same population (Ng and Corlett 2000; Tian et al. 2011). Population differentiation increases due to restricted gene flow among populations. Distribution of common haplotypes (both cpDNA and nuclear DNA) in all sampled populations and moderate genetic differentiation observed in *R. delavayi* var. *delavayi* suggest that this species must have had a more continuous distribution with closer and connected populations in the recent past. Due to destruction of habitat and complex landscape, few haplotypes should have been restricted in the western and the eastern distribution ranges. The recent habitat destruction and forest encroachment practices (Xu and Wilkes 2004; Pu et al. 2007; Yang et al. 2004) might have removed several natural strands of *R. delavayi* var. *delavayi* resulting the extinction of low-frequency haplotypes and the increased genetic differentiations among natural populations. The presence of comparatively higher haplotypes in the populations that are located inside the protected areas viz. ZY, LG, NG and XY is suggestive of an impact of habitat destruction on the spatial distribution of haplotype diversity and differentiation.

Demographic expansion

The mismatch distribution analyses for both cpDNA and nuclear DNA lineages were non unimodal, suggesting that *R. delavayi* var. *delavayi* did not experience rapid population expansion. The non-star like haplotype network and positive values of both Tajima's *D* and Fu's *Fs*, over the whole region and within the region as defined by SAMOVA, are also consistent with the absence of rapid population expansion (Slatkin and Hudson 1991). The population expansion time for *R. delavayi* var. *delavayi* across the sampled populations along SW China was estimated to be between 0.208 and 0.624 mya. The demographic expansion time for *R. delavayi* var. *delavayi* overlaps with the expansion time estimated for *Sphaeropteris brunoniana* (Wang and Guan 2011) and *Pyrus pashia* (Liu et al. 2013) distributed in southwest China.

Periodic fluctuations in climate and the complex topography in northwest Yunnan and the adjacent regions, might have caused periods of isolation and subsequent expansion of populations (Qiu et al. 2011). During recent glaciations, *R. delavayi* var. *delavayi* most likely persisted in the localized populations along central and eastern regions of the Yungui plateau, forming localized glacial refugia. Similar refugial status has also been suggested by phylogeographical studies such as *Cathaya argyrophylla* (Wang and Ge 2006), *Cupressus* spp. (Xu et al. 2010), *Nouelia insignis* (Gong et al. 2011), *Pyrus pashia* (Liu et al. 2013) and *Taxus wallichiana* (Gao et al. 2007). The Yungui plateau and its adjacent regions have long been believed to be an important center of origin for the East Asiatic flora (Wang 1992) and a refugial area for many plant species in China (Ying 2001; Wang et al. 2009, 2013). As a general rule, the populations in refugia have higher levels of genetic diversity and private haplotypes than migratory populations (Avise 2000). When considering both cpDNA and nuclear DNA, the populations WG, LG and NG along the eastern region and population XY along the center region have relatively higher values of haplotypic richness and haplotype diversity, indicating that these populations might have been preserved during Quaternary glaciations. Similar results have been inferred for *Pyrus pashia* (Liu et al. 2013). Therefore, the present distribution pattern of haplotypes in *R. delavayi* var. *delavayi* are the results of the combined effect of historical climatic events, effects of complex topography, and the impact of forest clearance and habitat destruction in the study area.

Conservation implications

The genetic profile uncovered in this study has not only provided important insights into the evolutionary history, but is also critical for the conservation and management of *R. delavayi* var. *delavayi* in SW China and adjacent regions. The authors' personal observations and correspondence with elders in each field site indicated that human activity has caused fragmentation and substantial reduction of natural populations of *R. delavayi* distributed along Yungui plateau. *R. delavayi* var. *delavayi,* one of the tallest woody rhododendron, with large flowers that last for 1–3 months, attracts many pollinators (insects such as bees and bumblebees) (Ma et al. 2010; Zhang et al. 2007b), and plays an important role in maintaining the ecology of the region (Gibbs et al. 2011). Despite the wider importance of *R. delavayi* in providing ecological, economic and recreational services, no special measures have yet been devised for the conservation and management of this species.

The results of the analysis of variation in the chloroplast and nuclear genome reported in this study revealed local genetic differentiation and geographic structure among populations in SW China. Particularly, high level of genetic diversity was found in eastern and central population located in the potential refugial sites along east Yungui plateau. Criteria for selecting populations for conservation must include the uniqueness and level of genetic diversity, especially with regard to its allelic composition (Petit et al.

1998). When considering the widely accepted criteria required for the recognition of management units (MU) for conservation, the populations having significant divergence of allele frequencies at nuclear and organelle loci should be considered (Moritz 1994). We propose that the two phylogeographic groups of *R. delavayi* var. *delavayi* could be treated as different MUs. Two groups of populations containing representative genetic diversity as identified by SAMOVA are recommended as units for conservation. The populations of Guizhou (WG, LG and NG) located in the first conservation unit, and the population XY of central Yunnan, are the populations that contribute most to the total diversity of *R. delavayi* var. *delavayi*. This fact highlights the importance of preserving large area to maintain genetic diversity. Along the western region of distribution (SAMOVA group two of both chloroplast and nuclear DNA), no particular population of high diversity could be suggested as priority. But, the entire group of population could be considered the second conservation unit to maintain the genetic integrity of this area.

The majority of population shared common haplotypes; however, the presence of private haplotypes and a gradient in allelic richness between sites show restricted inter-population gene flow among regional populations. Populations that form a clear phylogeographic group may contain different adaptive variations as a consequence of experiencing subsequent effects of selection. Therefore, seed transfer between these regions, and selecting source material for planting into or near the potential refugia sites, should be done with caution. High genetic diversity was found in population located in the protected areas (NG, JY, WG, XY and ZY). Unfortunately, some of the protected areas were found to be highly disturbed with very few seedlings and adult trees. Improved management policies are necessary to maintain effective population sizes of *R. delavayi* var. *delavayi* and to restore its natural habitats both inside and outside of protected areas. Direct actions such as controlled forest clearance and logging activities, regulated pine forestation practices and, more importantly transplantation of seedlings into natural habitats should be carried out to restore populations of *R. delavayi* var. *delavayi* and its genetic diversity. Further, studies based on hypervariable markers like microsatellites can be useful to obtain a better understanding of the fine-scale spatial patterns of genetic diversity and levels of gene flow in and among the extent populations of *R. delavayi* var. *delavayi* in SW China and adjacent areas.

Conclusions

The combined analysis of chloroplast and nuclear markers revealed moderate levels of genetic diversity in *R. delavayi* var. *delavayi* distributed in SW China. A marked difference among eastern and western population was observed in terms of allelic richness and population differentiation. The impacts of complex landscape and habitat fragmentation, in addition to the degradation of natural habitats over the last few decades have contributed substantially in shaping the present genetic pattern and observed moderate level of genetic diversity in *R. delavayi* var. *delavayi*. Increased fragmentation restricted the gene flow among population and that, consequently increased population differentiation, and reduced population size eventually led to genetic depauperation of the populations. Therefore, it is clear that management strategies should consider habitat restoration, remnant diversity preservation and maintenance of effective population sizes of *R. delavayi* var. *delavayi* in SW China.

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References

Avise J (2000) Phylogeography: the history and formation of species. Harvard University Press, Cambridge

Bandelt H, Forster P, Rohl A (1999) Median-joining networks for inferring intraspecific phylogenies. Mol Biol Evol 16:37–48

Chamberlain DF (1982) A revision of *Rhododendron* II. Subg Hymen Notes R Bot Gard Edinb 39:209–486

Chamberlain DF, Hyam R, Argent G, Fairweather G, Walter KS (1996) The genus *Rhododendron*, its classification and synonymy. Royal Botanic Garden Edinburgh, Oxford

Chen X, Xie B, Wu H, Huang L (2008) *Rhododendron* species and communities in Guizhou (China). Acta Hort (ISHS) 769:339–342

Cox P, Cox K (1997) Encyclopedia of *Rhododendron* species. Glendoick Publishing, Perth

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf material. Phytochem Bull 19:11–15

Dupanloup I, Schneider S, Excoffier L (2002) A simulated annealing approach to define the genetic structure of populations. Mol Ecol 11(12):2571–2581. doi:10.1046/j.1365-294X.2002.01650.x

Excoffier L, Lischer HEL (2010) Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Mol Ecol Res 10:564–567

Fang MY, Fang RY, He MY, Hu LZ, Yang HB, Chamberlain DF (2005) Flora of China, *Rhododendron*, vol 14. Science Press, Beijing

Frankham R (2005) Genetics and extinction. Biol Conserv 126(2):131–140. doi:10.1016/j.biocon.2005.05.002

Fu YX (1997) Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. Genetics 147(2):915–925

Gao LM, Möller M, Zhang XM, Hollingsworth ML, Liu J, Mill RR, Gibby M, Li DZ (2007) High variation and strong phylogeographic pattern among cpDNA haplotypes in *Taxus wallichiana* (Taxaceae) in China and North Vietnam. Mol Ecol 16(22):4684–4698. doi:10.1111/j.1365-294X.2007.03537.x

Gibbs D, Chamberlain D, Argent G (2011) The red list of *Rhododendrons*. Botanical Gardens Conservation International Richmond, UK

Goetsch L, Eckert AJ, Hall BD (2005) The molecular systematics of *Rhododendron* (Ericaceae): a phylogeny based upon RPB2 gene sequences. Syst Bot 30(3):616–626. doi:10.1600/036364405477382170

Gong X, Luan SS, Hung KH, Hwang CC, Lin CJ, Chiang YC, Chiang TY (2011) Population structure of *Nouelia insignis* (Asteraceae), an endangered species in southwestern China, based on chloroplast DNA sequences: recent demographic shrinking. J Plan Re 124(2):221–230. doi:10.1007/s10265-010-0363-0

Graur D, Li W (2000) Fundamentals of molecular evolution, 2nd edn. Sinauer Associates, Sunderland

Hamilton M (1999) Four primer pairs for the amplification of chloroplast intergenic regions with intraspecific variation. Mol Ecol 8(3):521–523

Hamrick J, Godt M, Sherman-Broyles S (1992) Factors influencing levels of genetic diversity in woody plant species. New For 6:95–124

Holderegger R, Abbott RJ (2003) Phylogeography of the arctic-alpine *Saxifraga oppositifolia* (Saxifragaceae) and some related taxa based on cpDNA and ITS sequence variation. Am J Bot 90(6):931–936. doi:10.3732/ajb.90.6.931

Huang C, Hung K, Hwang C, Huang J, Lin H, Wang W, Wu P, Hsu T, Chiang T (2011) Genetic population structure of the alpine species *Rhododendron pseudochrysanthum* sensu lato (Ericaceae) inferred from chloroplast and nuclear DNA. BMC Biol Evol 11(1):108

Ikeda H, Senni KEI, Fujii N, Setoguchi H (2008) Consistent geographic structure among multiple nuclear sequences and cpDNA polymorphisms of *Cardamine nipponica* Franch. et Savat. (Brassicaceae). Mol Ecol 17(13):3178–3188. doi:10.1111/j.1365-294X.2008.03821.x

Jia DR, Abbott RJ, Liu TL, Mao KS, Bartish IV, Liu JQ (2012) Out of the Qinghai-Tibet plateau: evidence for the origin and dispersal of Eurasian temperate plants from a phylogeographic study of *Hippophaë rhamnoides* (Elaeagnaceae). New Phytol 194(4):1123–1133. doi:10.1111/j.1469-8137.2012.04115.x

Kobayashi N, Horikoshi T, Katsuyama H, Handa T, Takayanagi K (1998) A simple and efficient DNA extraction method for plants, especially woody plants. Plant Tis Cult Biotech 4(2):72–80

Kron KA, Judd WS, Stevens PF, Crayn DM, Anderberg AA, Gadek PA, Quinn CJ, Luteyn JL (2002) Phylogenetic classification of *Ericaceae*: molecular and morphological evidence. Bot Rev 68(3):335–423. doi:10.1663/0006-8101(2002)068[0335:pcoema]2.0.co;2

Li X, Li J (1993) A preliminary floristics study on the seed plants from the region of Hengduan Mountain. Act Bot Yun 15:217–231

Li M, Liang J, Li Y, Feng B, Yang ZL, James TY, Xu J (2010) Genetic diversity of Dahongjun, the commercially important "Big red mushroom" from southern China. PLoS One 5(5):e10684. doi:10.1371/journal.pone.0010684

Li Y, Yan HF, Ge XJ (2012) Phylogeographic analysis and environmental niche modeling of widespread shrub *Rhododendron simsii* in China reveals multiple glacial refugia during the last glacial maximum. J Syst Evol 50(4):362–373. doi:10.1111/j.1759-6831.2012.00209.x

Librado P, Rozas J (2009) DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25(11):1451–1452. doi:10.1093/bioinformatics/btp187

Liu J, Ouyang Z, Pimm SL, Raven PH, Wang X, Miao H, Han N (2003) Protecting China's biodiversity. Science 300(5623):1240–1241. doi:10.1126/science.1078868

Liu Y, Yang SX, Ji PZ, Gao LZ (2012) Phylogeography of *Camellia taliensis* (Theaceae) inferred from chloroplast and nuclear DNA: insights into evolutionary history and conservation. BMC Biol Evol 12(1):92

Liu J, Sun P, Zheng X, Potter D, Li K, Hu C, Teng Y (2013) Genetic structure and phylogeography of *Pyrus pashia* L. (Rosaceae) in Yunnan province, China, revealed by chloroplast DNA analyses. Tree Genet Genom 9(2):433–441. doi:10.1007/s11295-012-0564-x

López-Pujol J, Zhang FM, Sun HQ, Ying TS, Ge S (2011) Centres of plant endemism in China: places for survival or for speciation? J Biogeogr 38(7):1267–1280. doi:10.1111/j.1365-2699.2011.02504.x

Ma Y, Milne RI, Zhang C, Yang J (2010) Unusual patterns of hybridization involving a narrow endemic *Rhododendron* species (Ericaceae) in Yunnan, China. Am J Bot 97(10):1749–1757. doi:10.3732/ajb.1000018

Mantel N (1967) The detection of disease clustering and a generalized regression approach. Cancer Res 27:209–220

Milne R, Davies C, Prickett R, Inns L, Chamberlain D (2010) Phylogeny of *Rhododendron* subgenus *Hymenanthes* based on chloroplast DNA markers: between-lineage hybridisation during adaptive radiation? Plant Syst Evol 285(3–4):233–244. doi:10.1007/s00606-010-0269-2

Mittermeier RA, Gil P, Hoffmann M, Pilgrim J, Brooks T, Mittermeier CG, Lamoreux J, Fonseca AB (2005) Hotspot revisited: earth's biologically richest and most endangered terrestrial ecoregions. Conservation International, Washington

Moritz C (1994) Defining 'evolutionarily significant units' for conservation. Trends Ecol Evol 9(10):373–375. doi:10.1016/0169-5347(94)90057-4

Myers N, Mittermeier RA, Mittermeier CG, da Fonseca GAB, Kent J (2000) Biodiversity hotspots for conservation priorities. Nature 403(6772): 853–858. doi:http://www.nature.com/nature/journal/v403/n6772/suppinfo/403853a0_S1.html

Nei M, Li WH (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc Nat Acad Sci USA 76(10):5269–5273

Ng SC, Corlett RT (2000) Comparative reproductive biology of the six species of *Rhododendron* (Ericaceae) in Hong Kong, South China. Can J Bot 78(2):221–229. doi:10.1139/b99-181

Peakall R, Smouse PE (2012) GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research-an update. Bioinformatics 28(19):2537–2539. doi:10.1093/bioinformatics/bts460

Petit RJ, El Mousadik A, Pons O (1998) Identifying populations for conservation on the basis of genetic markers. Conserv Biol 12(4):844–855. doi:10.1111/j.1523-1739.1998.96489.x

Petit RJ, Duminil J, Fineschi S, Hampe A, Salvini D, Vendramin GG (2005) Comparative organization of chloroplast, mitochondrial and nuclear diversity in plant populations. Mol Ecol 14(3):689–701. doi:10.1111/j.1365-294X.2004.02410.x

Pons O, Petit RJ (1996) Measuring and testing genetic differentiation with ordered versus unordered alleles. Genetics 144(3):1237–1245

Pu YS, Zhang ZY, Pu LN, Hui CM (2007) Biodiversity and its fragility in Yunnan, China. J For Res 18(1):39–47. doi:10.1007/s11676-007-0008-x

Qiu YX, Fu CX, Comes HP (2011) Plant molecular phylogeography in China and adjacent regions: tracing the genetic imprints of Quaternary climate and environmental change in the world's most diverse temperate flora. Mol Phylogen Evol 59(1):225–244. doi:10.1016/j.ympev.2011.01.012

Rands MRW, Adams WM, Bennun L, Butchart SHM, Clements A, Coomes D, Entwistle A, Hodge I, Kapos V, Scharlemann JPW, Sutherland WJ, Vira B (2010) Biodiversity conservation: challenges beyond 2010. Science 329(5997):1298–1303. doi:10.1126/science.1189138

Rogers AR, Harpending H (1992) Population growth makes waves in the distribution of pairwise genetic differences. Mol Biol Evol 9:552–569

Slatkin M, Hudson RR (1991) Pairwise comparisons of mitochondrial DNA sequences in stable and exponentially growing populations. Genetics 129:555–562

Stephens M, Smith NJ, Donnelly P (2001) A new statistical method for haplotype reconstruction from population data. Am J Hum Genet 13:978–989

Taberlet P, Gielly L, Puatou G, Bouvet J (1991) Universal primers for amplification of three non-coding regions of chloroplast DNA. Plant Mol Biol 17:1105–1109

Tajima F (1989) Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123:585–595

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28(10):2731–2739. doi:10.1093/molbev/msr121

Tian X, Ma Y, Zhang C, Zhang J, Tang G (2011) Recent advances of reproductive biology in *Rhododendron* L. J Nanj Fores Uni 35(3):124–128

Wang WT (1992) On some distribution patterns and some migration routes found in the eastern Asiatic region. Acta Bot Sin 30:1–24

Wang HW, Ge S (2006) Phylogeography of the endangered *Cathaya argyrophylla* (Pinaceae) inferred from sequence variation of mitochondrial and nuclear DNA. Mol Ecol 15(13):4109–4122. doi:10.1111/j.1365-294X.2006.03086.x

Wang ZJ, Guan KY (2011) Genetic structure and phylogeography of a relict tree fern, *Sphaeropteris brunoniana* (Cyatheaceae) from China and Laos inferred from cpDNA sequence variations: implications for conservation. J Syst Evol 49(1):72–79. doi:10.1111/j.1759-6831.2010.00108.x

Wang XP, Liu K (1994) Theory and practice of biodiversity in China. China Environmental Press, Beijing

Wang J, Gao P, Kang M, Lowe AJ, Huang H (2009) Refugia within refugia: the case study of a canopy tree (*Eurycorymbus cavaleriei*) in subtropical China. J Biogeogr 36(11):2156–2164. doi:10.1111/j.1365-2699.2009.02165.x

Wang JF, Gong X, Chiang YC, Kuroda C (2013) Phylogenetic patterns and disjunct distribution in *Ligularia hodgsonii* Hook. (Asteraceae). J Biogeogr. doi:10.1111/jbi.12114

Wangchuk S (2011) Fuelwood consumption and production in alpine Bhutan: a case study of resource use and implication for conservation and management in Wangchuck Centennial Park. Msc dissertation, The University of Montana Missouls, Montana

Wu CY (1965) On the tropical affinities of Chinese flora. Chi Sci Bull 1:25–33

Wu ZY, Wu SG (1998) A proposal for a new floristic kingdom (realm), the Asiatic kingdom, its delineation and characteristics Floristic characteristics. In: Zhang A, Wu S (eds) Floristic characteristics and diversity of East Asian plants China. China higher education Press, Beijing

Xu J, Wilkes A (2004) Biodiversity impact analysis in northwest Yunnan, southwest China. Biodiv Conserv 13(5):959–983. doi:10.1023/b:bioc.0000014464.80847.02

Xu J, Yang Y, Fox J, Yang X (2007) Forest transition, its causes and environmental consequences: empirical evidence from Yunnan of Southwest China. Trop Eco 48(2):137–150

Xu T, Abbott R, Milne R, Mao K, Du F, Wu G, Ciren Z, Miehe G, Liu J (2010) Phylogeography and allopatric divergence of cypress species (*Cupressus* L.) in the Qinghai-Tibetan plateau and adjacent regions. BMC Biol Evol 10(1):194

Yang Y, Tian K, Hao J, Pei S, Yang Y (2004) Biodiversity and biodiversity conservation in Yunnan, China. Biodiv Conserv 13(4):813–826. doi:10.1023/B:BIOC.0000011728.46362.3c

Ying TS (2001) Species diversity and distribution pattern of seed plants in China. Biodiv Sci 9:393–398

Zecca G, Grassi F (2013) RPB2 gene reveals a phylodemographic signal in wild and domesticated grapevine (*Vitis vinifera*). J Syst Evol 51(2):205–211. doi:10.1111/j.1759-6831.2012.00215.x

Zha HG, Milne RI, Sun H (2010) Asymmetric hybridization in *Rhododendron agastum*: a hybrid taxon comprising mainly F1 s in Yunnan, China. Ann Bot 105(1):89–100. doi:10.1093/aob/mcp267

Zhang YB, Ma KP (2008) Geographic distribution patterns and status assessment of threatened plants in China. Biodiv Conserv 17(7):1783–1798. doi:10.1007/s10531-008-9384-6

Zhang D, Chen X, Geng F, Li Z, Zhang R (2007a) One hundred miles of natural *Rhododendron* communities. Paper presented at the SNA Research Conference

Zhang J, Zhang C, ZK W, Qiao Q (2007b) The potential roles of interspecific pollination in natural hybridization of *Rhododendron* species in Yunnan, China. Biodiv Sci 15:658–665

Zhang JL, Zhang CQ, Gao LM, Yang JB, Li HT (2007c) Natural hybridization origin of *Rhododendron agastum* (Ericaceae) in Yunnan, China: inferred from morphological and molecular evidence. J Plant Res 120:457–463