JSE Journal of Systematics and Evolution

Journal of Systematics and Evolution 52 (4): 458–465 (2014) doi: 10.1111/jse.12057

Research Article

Molecular phylogeography and conservation genetics of *Sladenia celastrifolia* inferred from chloroplast DNA sequence variation

[1,2]Xi LU [1,3]Li CHEN [1,2]Ya-Ping CHEN [1]Hua PENG*

[1](*Key Laboratory of Biodiversity and Biogeography*, *Kunming Institute of Botany*, *Chinese Academy of Sciences*, Kunming 650201, China)
[2](*University of Chinese Academy of Sciences*, Beijing 100049, China)
[3](*Southwest Forestry University*, Kunming 650224, China)

Abstract The genetic variation and structure of *Sladenia celastrifolia* Kurz, a species of conservation concern, were investigated. Analyses of two chloroplast DNA loci (*trnS-trnG* and *atpB-rbcL* intergenic regions) were carried out for 24 populations of *S. celastrifolia* and five haplotypes were identified. High levels of genetic differentiation ($G_{ST} = 1$, $F_{ST} = 1$) were detected, which may be a result of limited gene flow caused by geographic isolation. Analysis of molecular variance suggests that the existence of marked phylogeographical structure within the haplotype distribution is probably due to geographic barriers among populations. The haplotype network and mismatch distribution analyses did not detect any signals for recent population expansions in *S. celastrifolia*. Thus, it can be inferred that the species likely persisted *in situ* during climatic oscillations. Considering its genetic diversity and uniqueness, conservation strategies are further discussed for this species.

Key words chloroplast DNA, conservation, genetic structure, phylogeography, *Sladenia celastrifolia*.

Conservation genetics adopts the concepts and methods of genetics and applies them to problems in conservation biology. Its central goal is to understand the levels and partitioning of genetic variation across populations and geographical regions of endangered species, providing useful information for development of conservation strategies and management practices (Hedrick & Miller, 1992). Genetic structure and diversity of populations, which are heavily influenced by various evolutionary forces acting together through time and space, may therefore reflect historical as well as contemporary evolutionary events (Cruzan & Templeton, 2000). Phylogeographical methods, which provide effective tools for inferring the historical events that have shaped the evolution of populations and species, are useful for identification of evolutionarily significant units and management units for endangered species and can assist conservation efforts to preserve maximum genetic diversity within the target gene pool (Avise, 2000).

Plants of *Sladenia celastrifolia* Kurz (Sladeniaceae) are large trees, growing up to 5–18(–30) m tall. Usually, this species is found within monsoon evergreen broadleaved forests in wet valleys of the southern region, or at the edge of forests, also in wet valleys below semihumid evergreen broadleaved forests in the northern region, where the elevations ranges from 700 to 1900 m and there is ample sunlight and relatively high temperatures. It is monoecious and flowers open between May and June. Being an isolated relict species, *S. celastrifolia* is distributed primarily in Yunnan, southwestern Guizhou, and northwestern Guangxi Provinces in China, as well as in northeastern Myanmar, northern Thailand, and northern Vietnam (Min & Bruce, 2007). Populations of *S. celastrifolia* are similar to those of some other endangered species in China, such as *Liriodendron chinense* (Hemsl.) Sarg. and *Cathaya argyrophylla* Chun & Kuang, which have been largely altered by increasing human activities. Although *S. celastrifolia* has not been regarded as an endangered species until now, it has attributes typical of an endangered species according to the standards of International Union for the Conservation of Nature (IUCN) system (Frankham et al., 2002). Therefore, more attention should be paid to the conservation of this species (Chen & Peng, 2006).

Because chloroplast DNA (cpDNA) is maternally inherited and non-recombining in most angiosperms (Reboud & Zeyl, 1994), it has been widely used in population genetics and phylogeographic studies (Bain & Golden, 2003; Li et al., 2008, 2012; Yang et al., 2008; Ikuyo & Noriaki, 2009; Qiu et al., 2009b; An et al.,

Received: 28 March 2013 Accepted: 13 September 2013
* Author for correspondence. E-mail: hpeng@mail.kib.ac.cn. Tel.: 86-871-65223524. Fax: 86-871-65150227.

2012; Jia et al., 2012). Furthermore, chloroplast genomes are particularly sensitive to the effects of fragmentation as a result of their small effective population sizes and restricted seed-mediated gene dispersal. Thus, chloroplast-specific markers are of great use in identifying genetic bottlenecks and founder effects, as well as in measuring genetic drift (Petit et al., 1997). In the present study, we sampled 220 individuals from 24 natural populations across the range of *S. celastrifolia* and selected two cpDNA sequences (*trnS-trnG* and *atpB-rbcL*) to explore the genetic and phylogeographic structure of the species. The major goals of this study were to elucidate: (i) how genetically polymorphic *S. celastrifolia* is; (ii) whether relatively high levels of genetic differentiation among populations or between geographical regions exist; and (iii) what factors are involved in the shaping of population genetic structure for this species.

1 Material and methods

1.1 Population sampling

During 2010–2012, 220 individuals from 24 populations of *Sladenia celastrifolia* were collected in Yunnan, Guangxi, and Guizhou Provinces, China. Due to difficulties in obtaining materials from other countries, we sampled only the species ranged in China. The collection sites covered almost the entire geographical distribution area of the species except for some highly cultivated or urbanized locations where *S. celastrifolia* was not found. For each population, 10–20 individuals (spaced at least 10 m apart) were sampled, with a few exceptions, such as in localities JD (only five individuals) and LC (seven individuals) (Table 1). Fresh leaves were dried with silica gel and stored at room temperature until DNA extraction. Voucher species for all populations were deposited in the Herbarium of the Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, China (KUN).

1.2 DNA extraction, polymerase chain reaction (PCR) amplification, and sequencing

Total genomic DNA was extracted from approximately 50 mg silica gel-dried leaf material per sample, following the CTAB method (Doyle & Doyle, 1987) with some modifications. Two cpDNA regions were amplified and sequenced, the *trnS-trnG* intergenic region (Hamilton, 1999) and the *atpB-rbcL* intergenic region (Chiang et al., 1998), using the primers reported in the references above. The PCR reactions were carried out in total volumes of 25 μL containing 20 ng template DNA, 2.5 μL 10× PCR buffer, 2 μL $MgCl_2$ (25 mmol/L), 0.5 μL dNTP mix (2.5 mmol/L), 1 μL each primer (5 pmol/L), and 0.3 μL (1 unit) Taq DNA polymerase. DNA amplification was carried out in a T1 thermocycler (Biometra, Göttingen, Germany), with an initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 52 °C for 1 min, extension at 72 °C for 1.5 min, and a final extension of 7 min at 72 °C. Amplification products were purified directly using a PCR product purification kit (Shanghai Sangon Biological Engineering Technology & Service Corporation Ltd., Shanghai, China). Cycle sequencing reactions were carried out with the primers described above, in both directions by standard methods, and analyzed on an ABI 3770 automated sequencer (Applied Biosystems, Foster City, CA, USA) at Shanghai Sangon Company.

1.3 Data analysis

Sequences were aligned by ClustalX 1.81 (Thompson et al., 1997) and manually corrected using BioEdit 7.0.9 (Hall, 1999). DnaSP version 5.0 (Librado & Rozas, 2009) was used to calculate: (i) nucleotide diversity per site (π) (Nei, 1987); (ii) haplotype diversity (H_d) (Nei & Tajima, 1983); and (iii) Tajima's D (Tajima, 1989) and Fu and Li's D^* and F^* (Fu & Li, 1993) statistics to assess the likelihood that the DNA sequences have evolved in a neutral manner with significance tests (1000 simulations) (significant, $P < 0.05$).

To determine the population structure of 24 sampled populations, spatial analysis of molecular variance of haplotypes was carried out in SAMOVA 1.0 (Dupanloup et al., 2002). This program implements a simulated annealing approach to gather geographically homogenous populations and maximally differentiate them from each other within defined groups of populations (K). The simulated annealing process was repeated for 1000 replications. An F_{CT} value was given for every calculation. We carried out these analyses for the range of $2 \leq K \leq 10$. We used the program Arlequin version 3.5 (Excoffier et al., 2005) to carry out an analysis of molecular variance (AMOVA), and thus to estimate genetic variation within populations, among populations within groups (as identified by SAMOVA), and between groups. Permut (Pons & Petit, 1996) (available at http://www.pierroton.inra.fr/genetics/labo/Software/Permut/) was used to calculate within-population diversity (H_S), total diversity (H_T), and the level of population differentiation (G_{ST}, N_{ST}) at the species level.

Genealogical haplotype networks (with 95% most parsimonious connection limits) were constructed using TCS version 1.21 (Clement et al., 2000). We used a

Table 1 Geographic location and sample sizes of 24 populations of *Sladenia celastrifolia* sampled in this study

Code	Population locality (all in China)	Code	*n*	Latitude (N)	Longitude (E)	Altitude (m)
1	Jingdong, YN	JD	5	24°2850′	100°4409′	1780
2	Zhenyuan, YN	ZY	10	24°0921′	101°0540′	1810
3	Xinhua, YN	XH	10	24°0651′	101°5158′	1920
4	Yangbi, YN	YB	10	25°4147′	99°5727′	1750
5	Luquan, YN	LQ	10	25°4036′	102°2342′	1820
6	Mengla, YN	ML	10	21°3250′	101°2944′	1250
7	Ailaoshan, YN	ALS	10	24°2710′	101°1703′	1860
8	Menghai, YN	MH	10	21°5723′	100°2449′	1080
9	Yimen, YN	YM	10	24°4006′	102°0814′	1910
10	Eshan, YN	ES	10	24°0942′	102°2352′	1800
11	Fengqing, YN	FQ	10	24°3850′	100°0604′	1394
12	Mojiang, YN	MJ	10	22°5150′	101°0861′	1352
13	Jiuzhou, YN	JZ	9	25°4456′	99°1347′	1200
14	Dachaoshan, YN	DCS	10	24°2256′	100°2348′	1120
15	Lincang, YN	LC	7	24°0511′	100°0410′	1350
16	Yongping, YN	YP	10	25°3432′	100°1344′	1200
17	Caojian, YN	CJ	9	25°3925′	99°0735′	1800
18	Baihualing, YN	BHL	7	25°0620′	99°0818′	1800
19	Qiubei, YN	QB	10	24°0400′	103°3739′	1750
20	Zhemi, YN	ZM	10	22°4136′	102°3955′	1850
21	Dadieshui, YN	DDS	10	24°3951′	103°1159′	1700
22	Jinzhongshan, GX	JZS	8	24°4001′	104°5140′	1250
23	Huazizhai, GX	HZZ	10	24°2353′	104°3835′	1515
24	Canggeng, GZ	CG	5	24°4449′	104°4453′	1150

Voucher species for all populations were deposited in the Herbarium of Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, China (KUN). GX, Guangxi Province; GZ, Guizhou Province; *n*, sample size; YN, Yunnan Province.

pairwise mismatch distribution to test for population expansion by the DnaSP program. Phylogenetic relationships among cpDNA haplotypes were evaluated by neighbor-joining (NJ) analyses using MEGA 5.0 (Tamura et al., 2011). In the analysis, we chose the K2P model (Kimura-2 parameter), and bootstrap consensus values were calculated using 1000 replicates.

2 Results

2.1 Chloroplast variation and haplotype distribution

Of eight DNA regions surveyed for this study (*trnL-F*, *rpl32-trnL*, *trnQ-*$^{5'}$*rps16*, *trnH-psbA*, *trnS-trnG*, *atpB-rbcL*, internal transcribed spacer, and external transcribed spacer), only two (*atpB-rbcL* and *trnS-trnG*) showed polymorphisms in *Sladenia celastrifolia* populations. The cpDNA *atpB-rbcL* and *trnS-trnG* matrix was 1392 bp in length. A total of five haplotypes (H1–5) were identified in the 24 sampled populations, based on eight substitutions in these cpDNA regions (Table 2). All haplotype sequences were deposited in the GenBank database under the accession numbers KC898238–KC898242, for *atpB-rbcL*, and KC898243–KC898247, for *trnS-trnG*. Haplotype frequencies in each population and geographic distribution are presented in Table 1 and Fig. 1. Haplotypes H1 (from localities ALS, DCS, FQ, JD, LC, LQ, YM, and ZY) and H3 (from localities CG, DDS, ES, HZZ, JZS, MJ, QB, and XH) showed widespread distributions, both comprising eight populations. Haplotype H2 occurred in populations BHL, CJ, JZ, YB, and YP; H4 was scattered in populations MH and ML. In addition, H5 only occurred in population ZM (Table 1; Fig. 1). All populations had only one haplotype, and the total haplotype diversity (H_d) and the nucleotide diversity (π) were 0.734 and 0.00248, respectively.

2.2 Population genetic and phylogeographic structure

Genetic diversity analysis of *S. celastrifolia* revealed that total genetic diversity (H_T) across all populations was 0.757, and none of the 24 populations surveyed showed cpDNA polymorphism (Table 3).

Table 2 Variable sites from the aligned sequences of the two chloroplast DNA spacers in five haplotypes (H1–5) of *Sladenia celastrifolia*

Haplotype	*atpB-rbcL*			*trnS-trnG*				
	1 5 7	2 7 5	5 0 3	1 1 3	1 3 4	2 1 3	2 2 9	2 6 0
H1	A	C	C	G	G	C	A	A
H2	A	C	C	G	G	A	T	A
H3	C	C	G	A	T	A	T	C
H4	A	C	G	G	G	C	A	A
H5	C	A	G	A	G	C	T	C

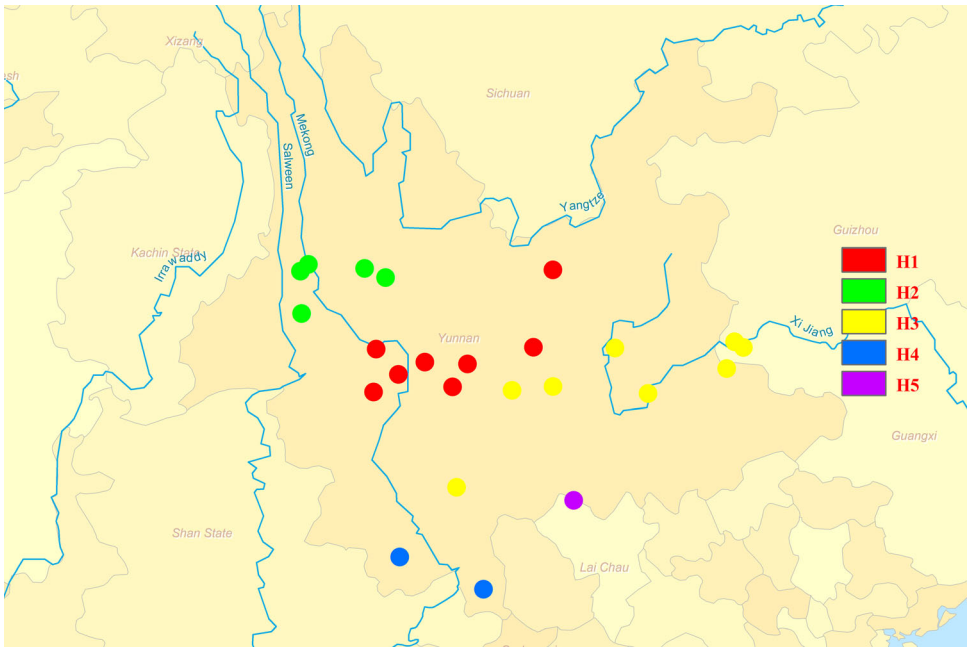

Fig. 1. Geographic distribution of five chloroplast DNA haplotypes (H1–5) in 24 populations of *Sladenia celastrifolia* in southwestern China. Map constructed using ArcGIS 9.3.

Therefore, interpopulation differentiation was maximal in this species ($G_{ST} = 1$). To assess the hierarchical genetic variation and make lineage demographic inferences, SAMOVA was further applied. The highest F_{CT} value (0.833) was obtained when we defined $K = 2$, suggesting the division of the five haplotypes into two groups, group 1 (H1, H2, and H4) and group 2 (H3 and H5). The AMOVA analysis showed that all variations were distributed among populations and none within population ($F_{ST} = 1$, $P < 0.01$) (Table 4).

2.3 Phylogenetic and genealogical relationships of cpDNA haplotypes

Phylogenetic relationships were reconstructed among cpDNA haplotypes of *S. celastrifolia*, and the NJ tree was obtained based on cpDNA sequence polymorphism (Fig. 2). Two clades (I and II) were identified in the NJ tree and clade II received moderate support. Clade I comprised three haplotypes (H1, H2, and H4), and clade II included two haplotypes (H3 and H5). The haplotype network obtained from the TCS 1.21 analysis was largely consistent with that from SAMOVA and NJ tree (Fig. 3).

Table 3 Genetic diversity, differentiation parameters, and results of neutrality tests for chloroplast DNA in sampled *Sladenia celastrifolia* populations in southwestern China

H_S	H_T	G_{ST}	N_{ST}	Tajima's D	Fu and Li's D^*	Fu and Li's F^*
0	0.757	1	1	3.409**	1.202	2.374*

*$P < 0.05$; **$P < 0.02$.

2.4 Demographic analysis

Significantly positive Tajima's D ($D = 3.409$, $P < 0.01$) and Fu and Li's F^* values ($F^* = 2.374$, $P < 0.05$) were obtained (Table 3). The mismatch distributions based on the cpDNA haplotypes dataset for all populations were multimodal and inconsistent with the bell-shaped curve, indicating that a recent population expansion in this species is unlikely (Fig. 4).

3 Discussion

3.1 Genetic diversity and population structure

In this study, moderate genetic diversity based on the cpDNA sequences was revealed for *Sladenia celastrifolia*. In the two cpDNA sequences, we detected eight substitutions and five haplotypes for the 24 populations of *S. celastrifolia*. Nucleotide diversity at the species level ($\pi = 0.00248$) was higher than in other endangered plants for which one or several cpDNA regions have been sequenced, for example, $\pi = 0.00140$ of *Dysosma versipellis* (Hance) M. Cheng ex T. S. Ying (Berberidaceae) (Qiu et al., 2009a). Additionally, a striking feature of *S. celastrifolia* is the marked interpopulation differentiation and low within-population diversity. Although haplotype diversity at

Table 4 Analysis of molecular variance for 24 populations of *Sladenia celastrifolia* in southwestern China

Source of variation	*d.f.*	Sum of squares	Variance components	Percentage of variation	Fixation index
Among groups	1	272.492	2.58874	83.36	$F_{CT} = 0.83^*$
Among populations with groups	22	103.904	0.51664	16.64	$F_{SC} = 1^*$
Within populations	196	0.000	0.00000	0.00	$F_{ST} = 1^*$
Total	219	376.395	3.10538	NA	NA

d.f., degrees of freedom; NA, not applicable. $^*P < 0.01$.

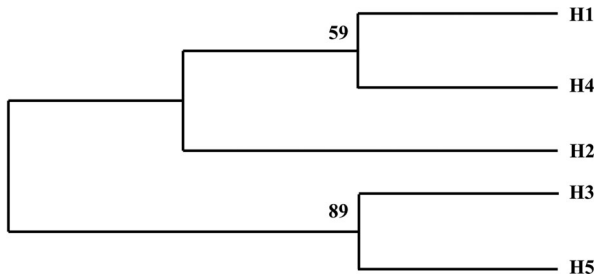

Fig. 2. Neighbor-joining tree obtained from five chloroplast DNA haplotypes (H1–5) of *Sladenia celastrifolia*. Bootstrap values based on 1000 replicates are indicated above branches.

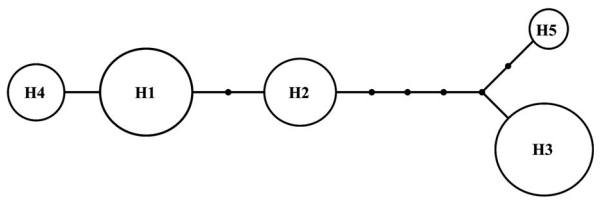

Fig. 3. Statistical parsimony network of the five chloroplast DNA haplotypes (H1–5) of *Sladenia celastrifolia*. The size of each circle is proportional to the haplotype frequency. Each solid line represents one mutational step that interconnects two haplotypes. The small black dots indicate hypothetical haplotypes.

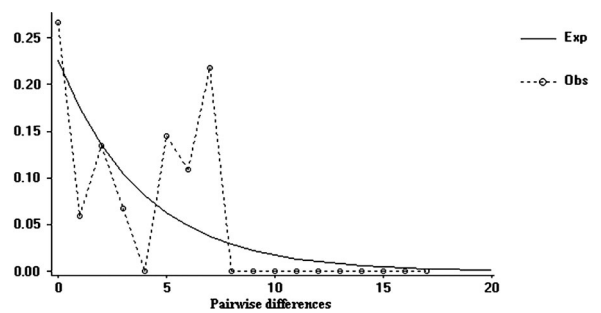

Fig. 4. Mismatch distribution of *Sladenia celastrifolia* populations based on chloroplast DNA data showing observed (Obs) (dotted line) and expected (Exp) (solid line) pairwise nucleotide site divergences obtained with the DnaSP program.

the species level was not low or even moderate, population subdivision for cpDNA was extremely large ($G_{ST} = 1$, $F_{ST} = 1$) in *S. celastrifolia* compared with other seed plants for maternally inherited markers (Huang et al., 2002; Gao et al., 2007). There are three reasons for the marked interpopulation differentiation of *S. celastrifolia* populations. The first is the high proportion of abortive seeds. The seed germination rate of *S. celastrifolia* is only 0.3% and so gene flow among populations is restricted (Li et al., 2001). Second, differentiation might be attributed to long-term habitat fragmentation caused by geographic isolation among populations. *Sladenia celastrifolia* is usually found in wet valleys at the edge of forests, where there is sufficient sunlight and the temperature is relatively high. Populations are isolated from each other because of the highly fragmented habitats. Ancestral population fragmentation often leads to the formation of smaller populations. In small-sized populations, the effects of genetic drift are more obvious and genetic variation within populations easily decreases, leading to high genetic differentiation among populations and no variation within populations (Young et al., 1996; Templeton et al., 2001). Finally, high genetic differentiation among populations may mainly be ascribed to the limited amount of gene flow through both seeds and pollen, which is hindered by geographic and anthropogenic barriers. In China, the distribution areas of *S. celastrifolia* are spread among several mountains, such as Mt. Jiaozi, Mt. Ailao, and Mt. Wuliang. The southern MJ (H3) population is isolated from northern LC and ZY (H1) populations by Mt. Ailao and Mt. Wuliang, whereas the QB (H3) population is separated from LQ and YM (H1) populations by Mt. Jiaozi. The result was supported by NJ analyses (Fig. 2).

3.2 Demographic history

Tremendous global climatic oscillations together with local climatic changes were ascribed to the uplift of the Qinghai–Tibet Plateau. Particularly during the Quaternary glaciations, several glacial–interglacial cycles finally resulted in the expansion and contraction of many species' habitats (Axelrod et al., 1996; Harrison et al., 2001; Abbott & Brochmann, 2003; Hewitt, 2004; Yang et al., 2012). Climate changes during the last glacial maximum had especially dramatic effects on the distribution ranges and genetic structure of many plants (Harrison et al., 2001). Because of its extremely complex topography and

climate, the Yunnan Plateau was less affected by cold air from Siberia during glacial periods than other regions in China, but the climatic oscillations of glacial ages, and interglacial or postglacial ages, also led to extinction, or changed the distribution and evolution of many plants (Winkler & Wang, 1993; Zheng, 2000). During the last glacial maximum, population numbers of cold-tolerant plants, such as *Picea* and *Abies*, increased, while that of subtropical plants like *Dacrydium* and *Podocarpus* decreased or disappeared from the Yunnan Plateau (Li, 1998).

When the temperature decreased, plants tended to migrate towards lower latitudes or altitudes and with the increase of the temperature they migrated backward. However, we failed to detect such a pattern of population expansion in *S. celastrifolia*. As shown in Fig. 3, the widespread haplotypes (H1, H2) might represent ancient polymorphism rather than recent gene flow and expansion (Schaal et al., 1998). In addition, the haplotype network is not a star-like phylogeny, which usually indicates population expansion as evidenced in many species (Hwang et al., 2003; Yang et al., 2008; Novaes et al., 2010). Based on mismatch distribution analysis (Fig. 4) and neutrality tests (Table 3) of the cpDNA dataset, we found no evidence for population expansion, but did record effects that match the signature of past genetic bottlenecks in present-day genetic patterns.

According to these results, it can be inferred that populations of *S. celastrifolia* survived *in situ* when the climate changed during the glacial ages, rather than migrating to suitable habitats and backwards in the interglacial (or postglacial) age. The Yunnan Plateau in southwestern China, with mountains and rivers compressed within a narrow geographic mosaic, is characterized by physical environmental heterogeneity. The numerous geographic barriers among populations of *S. celastrifolia* can be ascribed to the environmental complexity of the Yunnan Plateau. It is difficult for different populations to retreat to common refugia during interglacial or postglacial periods and so they remain separate. Due to climatic oscillations, population sizes may be shrinking and some populations may even become extinct in some places (Xie et al., 2012). In our study, multiple missing haplotypes detected in the cpDNA network (Jakob & Blattner, 2006) suggest that *S. celastrifolia* populations experienced severe extinctions and/or stronger genetic drift, resulting in the loss of chloroplast haplotypes. Owing to agriculture, urbanization, and human overexploitation, habitat destruction and degradation has become a severe problem. *Sladenia celastrifolia* is isolated in small areas as small-sized populations. Our field observations indicate that many populations have been largely altered by increasing human activities.

3.3 Conservation

Population size is the most important factor of the five criteria for listing species as endangered under the IUCN system (Frankham et al., 2002). Based on our field observations, almost all extant *S. celastrifolia* populations face a serious threat of extinction due to stochastic processes, limited number of individuals, and high divergence. Increasing the population size and genetic diversity of *S. celastrifolia* should become the principal conservation activities. Among all the extant populations, there are less than 10 individuals in populations JD, JZ, LC, CJ, BHL, JZS, and CG. In these populations, we need to enlarge their population sizes for *in situ* conservation with high priority.

Along with climatic changes, marginal populations in the distribution range are important for the long-term conservation of genetic diversity. Due to phylogenetic history and the current evolutionary trajectory, these populations are the most prone to extinction (Hampe & Petit, 2005). Population ZM is located at the edge of the range of *S. celastrifolia* and possesses the private haplotype H5. This population contains less than 20 individuals and should be given more attention.

Considering the current severe habitat loss and fragmentation from human disturbance, habitat conservation will be very important for the conservation of *S. celastrifolia*. Almost all of the populations are located outside of reserves. Therefore, local nature reserves need to be established, especially for the small-sized populations that are seriously disturbed. *Ex situ* conservation strategies include germplasm collections, botanical gardens (Kunming, Beijing), or reintroductions at appropriate areas with similar habitats, are also necessary measures to preserve the genetic resources of *S. celastrifolia*. If *ex situ* conservation is put in practice, samples should be collected from as many populations as possible, given that a large portion of genetic diversity exists among rather than within populations. Populations JD, JZ, LC, CJ, BHL, JZS, and CG should be given high priority as they are either small-sized populations or contain a private polymorphism (H5).

Although *S. celastrifolia* has not been protected by law in China, it is essential that people be aware of the importance of preservation. Effective education and information dissemination are absolutely necessary. More genetic diversity work and field studies are required to ensure better protection of this species. Additionally, to obtain a better understanding of the factors that have influenced the evolutionary history of

flora in southwestern China, studies on a wide range of different species endemic in this region are urgently needed.

Acknowledgements Thanks to Diana D. Jolles for polishing the language of this article. This project was supported by the National Natural Science Foundation of China (Grant Nos. 31100164 and 30900075).

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