JSE Journal of Systematics and Evolution

Journal of Systematics and Evolution 50 (4): 305–315 (2012) doi: 10.1111/j.1759-6831.2012.00200.x

Research Article

Phylogeography of *Cyananthus delavayi* (Campanulaceae) in Hengduan Mountains inferred from variation in nuclear and chloroplast DNA sequences

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Abstract Phylogeographic studies on alpine plants endemic to the Hengduan Mountains of the southeastern Qinghai–Tibet Plateau are still limited in number. In this study, we used sequence variation of one nuclear gene (*ncpGS*, which encodes the chloroplastic glutamine synthetase) and in two chloroplast DNA segments to investigate the phylogeographic structure and population demographic history of *Cyananthus delavayi*, a narrow-range species endemic to this region. We identified eight chlorotypes and 16 nuclear genotypes in a survey of 10 populations sampled throughout the range of the species. The results of both phylogenetic and network analyses suggested that the genealogical relationships of both chlorotypes and nuclear genotypes showed a clear geographical correlation. High total genetic diversity, low levels of within-population diversity, and strong population differentiation (chloroplast DNA: $h_T = 0.827$, $h_S = 0.087$, $N_{ST} = 0.899$, $G_{ST} = 0.895$; nuclear DNA: $h_T = 0.910$, $h_S = 0.348$, $N_{ST} = 0719$, $G_{ST} = 0.618$) were identified. Based on the mismatch distribution analyses, no evidence of recent demographic population expansion was found for this species. Nested clade analyses of both chlorotypes and nuclear genotypes indicated that restricted gene flow resulting from isolation by distance and allopatric fragmentation were likely to have been the major processes that shaped their present-day spatial distribution. Our dating of the genetic divergences between three major geographic lineages suggested that the largest glaciation of the early Quaternary developed in the Qinghai–Tibet Plateau and mountainous isolation may have together led to deep intraspecific vicariance within this species.

Key words chloroplast DNA, *Cyananthus delavayi*, Hengduan Mountains, *ncpGS*, phylogeography, vicariance.

The Hengduan Mountains (HDM), the eastern Qinghai–Tibet Plateau (QTP), and their adjacent regions are together regarded as a global biodiversity "hotspot" (Myers et al., 2000). There are more than 8000 angiosperm species in the HDM, which is the distribution and diversity center for many alpine genera (Wu, 1988; Sun, 2002; Sun & Li, 2003). This high diversity appears to result from rapid radiation of species diversity because of the extreme topographic variation and complexity through allopatric speciation (e.g. Liu et al., 2006; Sun et al., 2012), in addition to the region's important role as the long-term refugium for a few species with ancient origins (Wu, 1988). The hypothesis of rapid diversification was also confirmed from phylogeographic studies of widely distributed species in the HMD and adjacent regions, showing that a high level of genetic differentiation was found for allopatric populations of numerous species (e.g. Ge et al., 2005; Xia et al., 2005; Chen et al., 2008a, 2010b; Wang et al., 2008a, 2011; Xu et al., 2010). Except for the limited gene flow imposed by the complex topography, the Quaternary climatic oscillations in this region (Herzschuh et al., 2009) may have further accelerated this trend through genetic drifts *in situ* due to repeated bottlenecks (e.g. Li et al., 2010; Cun & Wang, 2010). For example, the largest glaciation, which probably developed from 1.2 Ma and continued to 0.3 Ma in the QTP, was suggested to be responsible for the development of the deep intraspecific lineages distributed in the allopatric regions (Wang et al., 2009; Jia et al., 2011). In addition, topographic changes (e.g. development of the rivers and uplift of the mountains) in this region may have also caused the intraspecific and interspecific divergences through allopatric differentiation (Zhang et al., 2011b; Zhang & Sun, 2011; Yue et al., 2011). However, it remains unknown whether allopatric divergences appeared as a general evolutionary result for most species occurring there.

Received: 9 December 2011 Accepted: 15 April 2012
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In this study, we aimed to investigate the phylogeographic pattern of *Cyananthus delavayi* Franch. As one of the Sino-Himalayan endemic genera, *Cyananthus* diversified greatly in the HDM (Hong & Ma, 1991) and the phylogeographic pattern of a single species from this genus will provide important cues for understanding its species diversification. The species *Cyananthus delavayi* is a perennial herb with protandrous flowers and outcrossing breeding systems (Niu et al., 2011). It occurs in forest fringes and grassy calcareous slopes with altitudes ranging from 1950 m to approximately 4000 m. The genetic structures of plant populations are determined to a large extent by the movement of genes through pollen and seed dispersal. The contributions of these two forms of gene dispersal to the genetic structure of natural plant populations can be estimated using a combination of nuclear DNA (nDNA) and chloroplast DNA (cpDNA) markers (Viard et al., 2001; Fontaine et al., 2004; Avise, 2009). In our study, one biparentally inherited nDNA fragment and two maternally inherited cpDNA fragments were chosen for phylogeographic analyses. We found that this species had undergone extensive genetic differentiation at both DNA datasets along its allopatric distributions.

1 Material and methods

1.1 Population sampling

In total, 177 individuals were collected from 10 populations throughout the entire range of *Cyananthus delavayi* between August 2008 and October 2010. For each population, 15–25 randomly selected individuals were sampled at intervals of at least 30 m and immediately dried in silica gel. For the genealogical analyses, three other congeneric species were also collected as outgroups. Voucher specimens were deposited in the herbarium of the Kunming Institute of Botany (KUN), Chinese Academy of Sciences. The latitude, longitude, and altitude of each sampling site were recorded (Table 1).

1.2 DNA extraction, amplification, and sequencing

Total genomic DNA was extracted from dried leaves using a modified CTAB protocol (Doyle & Doyle, 1987). Two cpDNA regions, *trnH-psbA* and *psbD-trnT*, were amplified with universal primers according to Hamilton (1999) and Shaw et al. (2007). The following parameters were used during polymerase chain reaction (PCR) amplification: 4 min at 94 °C, then 33 cycles each of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C, followed by a final extension of 7 min at 72 °C. In addition, 127 individuals representing 10 populations were surveyed for sequence variation in a region of the *ncpGS* gene that contains four introns, using the primers *cp*687f and *cp*994r designed by Emshwiller & Doyle (1999). This region has been used successfully in phylogenetic reconstruction of several genera, including legumes (Emshwiller & Doyle, 1999; Perret et al., 2003; Yockteng & Nadot, 2004) as well as in plant phylogeographic studies (Wallace et al., 2009). The PCR amplification parameters were as above, except that an annealing

Table 1 Details of sample locations, sample sizes, amount of each chlorotype (H1–H8) and nuclear DNA (nDNA) haplotype (H1–H16) in 10 populations of *Cyananthus delavayi*

Population	Locality (All in China)	Latitude, longitude, altitude (m)	cpDNA			nDNA		
			Haplotypes (no. of individuals)	π	*H*d	Haplotypes (no. of individuals)	π	*H*d
KM	Xishan, Kunming, Yunnan	102°37′17.9″N, 25°3′45.3″E, 1950	H2 (20)	0.00000	0.000	H4 (17)	0.00000	0.000
JC	Shizhongshan, Jianchuan, Yunnan	99°50′14.7″N, 26°21′32.9″E, 2434	H2 (19)	0.00000	0.000	H4 (11), H6 (4)	0.01061	0.405
CS	Cangshan, Dali, Yunnan	100°5′52.3″N, 25°39′52.2″E, 3985	H1 (20)	0.00000	0.000	H1 (17), H2 (2), H3 (1)	0.00038	0.198
ZD	Napahai, Zhongdian, Yunnan	99°39′46.6″N, 27°49′18.7″E, 3450	H7 (18), H8 (2)	0.00012	0.189	H2 (1), H13 (9), H14 (3), H15 (2), H16 (1)	0.00214	0.615
GZ	Xiaoxueshan, Zhongdian Yunnan	99°46′6.3″N, 28°17′54.3″E, 3875	H3 (14)	0.00000	0.000	H5 (5)	0.00000	0.000
DC	Yinming, Dongchuan, Yunnan	103°5′58.9″N, 26°15′14.9″E, 3360	H1 (10), H2 (3)	0.00071	0.385	H1 (1), H4 (2)	0.01099	0.533
YY	Xiaogaoshan, Yanyuan, Sichuan	101°42′44.9″N, 27°31′59.3″E, 3220	H4 (20), H6 (2)	0.00011	0.173	H7 (9), H8 (7), H11 (3), H12 (4)	0.00401	0.723
XD	Hebolu, Xide, Sichuan	102°31′30.7″N, 28°20′56.4″E, 2708	H4 (21)	0.00000	0.000	H7 (3), H8 (15)	0.00217	0.324
ML	Liangzishan, Muli, Sichuan	101°20′51.3″N, 28°0′17.7″E, 3810	H4 (15), H5 (1)	0.00008	0.125	H7 (4)	0.00000	0.000
MN	Shenrong, Mianning, Sichuan	101°58′34.9″N, 28°20′36.2″E, 2695	H4 (12)	0.00000	0.000	H7 (2), H8 (2), H9 (1), H10 (1)	0.00402	0.733

temperature of 55 °C was used. The PCR products were purified using the TIANquick Midi Purification Kit (Tiangen Biotech, Beijing, China) following the recommended protocol and sequenced by a commercial laboratory (Beijing Sunbiotech, Beijing, China). Sequences were aligned using CLUSTALX (Thompson et al., 1997), then alignments were double-checked manually.

Because *C. delavayi* is a diploid ($2n = 12$) species (our unpublished data, 2012), for *ncpGS* heterozygotes, two alleles should be sequenced simultaneously. We identified these two different haplotypes by using the haplotype subtraction method of Clark (1990). The phase program zipped in the software DnaSP version 5.0 software (Librado & Rozas, 2009) was used to extract haplotypes in the sequence alignment.

1.3 Data analyses

We used DnaSP version 5.0 software (Librado & Rozas, 2009) to extract chlorotypes and nuclear genotypes. All sequences were submitted to GenBank with accession numbers JQ313768–JQ313792. The geographical distribution of chlorotypes and nuclear genotypes was plotted on a relief map of China using ArcMap 9.3 (Esri, Redlands, CA, USA). Maximum parsimony analysis for both chlorotypes and nuclear genotypes was carried out using the program PAUP$_*$ version 4.0b10 (Swofford, 2002). Gaps were treated as missing data. Full heuristic tree searches were carried out with 100 replications of "random" sequence entries, tree bisection–reconnection branch swapping, the MulTrees option in effect, and using unweighted characters. Branch supporting values were assessed by bootstrap analysis with 1000 replicates of the full heuristic searches using the above settings. Genealogical relationships among chlorotypes and nuclear genotypes were also explored using the median-joining network method (Bandelt et al., 1999) as implemented in the program Network 4.5.0.2 (http://www.fluxus-engineering.com).

Haplotype diversity (H_d) and nucleotide diversity (π) were also determined using DnaSP version 5.0 (Librado & Rozas, 2009). To measure the level of genetic variation and estimate differentiations between populations, mean gene diversity within populations (h_S), total gene diversity (h_T), and two population differentiation parameters (G_{ST}, N_{ST}) were calculated using the program HAPLONST (Pons & Petit, 1996). Indirect estimations of the extent of gene flow among populations were made from G_{ST} values using the formula: $Nm = 0.25 \times (1–G_{ST})/G_{ST}$ (Wright, 1931), where Nm is the number of migrants per generation. Spatial genetic structures of chlorotypes and nuclear genotypes were analyzed using spatial AMOVA (SAMOVA) software (Dupanloup et al., 2002). The F_{CT} index of genetic differentiation among K initial groups was calculated to determine the best grouping of populations using values of K ranging from 2 to 8, with each simulation starting from 100 random initial conditions. The AMOVA was implemented using pairwise differences and haplotype frequencies with Arlequin version 3.0 software (Excoffier et al., 2005). Measures of DNA divergence between populations and groups (F_{ST}) were calculated, and their significance was tested using 10 000 permutations.

All indels were treated as point mutations and equally weighted with the other mutations. To assess geographical associations of haplotypes and infer the phylogeographic pattern of *C. delavayi*, nested clade analysis (NCA) based on both cpDNA and nDNA data was carried out using ANeCA software (Panchal, 2007) and a list of inference keys for the origin of the nested clades was generated.

The coalescence times (the most recent common ancestor, TMRCA) of all cpDNA haplotypes for *C. delavayi* were estimated using the program BEAST version 1.6.0 (Drummond & Rambaut, 2007). Three independent Markov chain Monte Carlo analyses were run for 1.0×10^7 generations under the HKY model, as inferred from MrModeltest 2.0 (Nylander, 2004). The program Tracer version 1.5 (Rambaut & Drummond, 2009) was used to compile and visualize the results from BEAST. No fossil records of *C. delavayi* are available to calibrate a cpDNA substitution rate for this species. Therefore, we assumed minimum and maximum values of a range of average mutation rates reported for synonymous sites of plant chloroplast genes, namely, 1 and 8.24×10^{-9} substitutions per site per year (s/s/y) (Richardson et al., 2001). These rates were then used for estimating TMCRA in BEAST under a strict molecular clock assumption (see Doc. S1).

To detect historical population dynamics of *C. delavayi*, mismatch distribution was estimated. The observed number of differences between pairs of chlorotypes and nuclear genotypes was compared to the theoretical distribution using a sudden (stepwise) expansion model (Rogers & Harpending, 1992) with the software DnaSP version 5.0 software (Librado & Rozas, 2009). A total of 1000 parametric bootstrap replicates were used to generate an expected distribution under a model of sudden demographic expansion (Rogers & Harpending, 1992). The sum of squared deviations (SSD) between observed and expected mismatch distributions were computed and P-values were calculated as the proportion of simulations producing a larger SSD than the observed SSD. The raggedness index and its significance were also calculated to quantify the smoothness of the observed mismatch distribution.

Neutrality tests using Tajima's *D*-test (Tajima, 1989) and Fu's F_S test (Fu, 1997) were implemented in Arlequin software to detect evidence of recent demographic expansion inferred from the nuclear loci data. The parameters of demographic expansion and spatial expansion were estimated using the methods of Schneider & Excoffier (1999) with Arlequin software.

2 Results

2.1 Chloroplast and nuclear DNA variations

The lengths of the aligned sequences of the *trnH-psbA* and *psbD-trnT* regions from *Cyananthus delavayi* were 394 bp and 1232 bp, respectively. Two *trnH-psbA* and seven *psbD-trnT* haplotypes were identified. The total alignment length of the combined *trnH-psbA* and *psbD-trnT* dataset was 1626 bp, including six indels and 16 substitutions. A total of eight chlorotypes (H1–H8) were detected across the 177 analyzed individuals (Table S1). Chlorotype frequencies for each population are presented in Table 1, and their geographical distribution is shown in Fig. 1. The haplotype diversity (H_d) and nucleotide diversity (π) for the combined dataset were estimated to be 0.755 and 0.00335, respectively.

The available length of aligned sequences of the *ncpGS* gene was 536 bp due to the poly (C) structure present at the 537 bp site. Twenty-eight polymorphic sites were detected, defining 16 nuclear genotypes

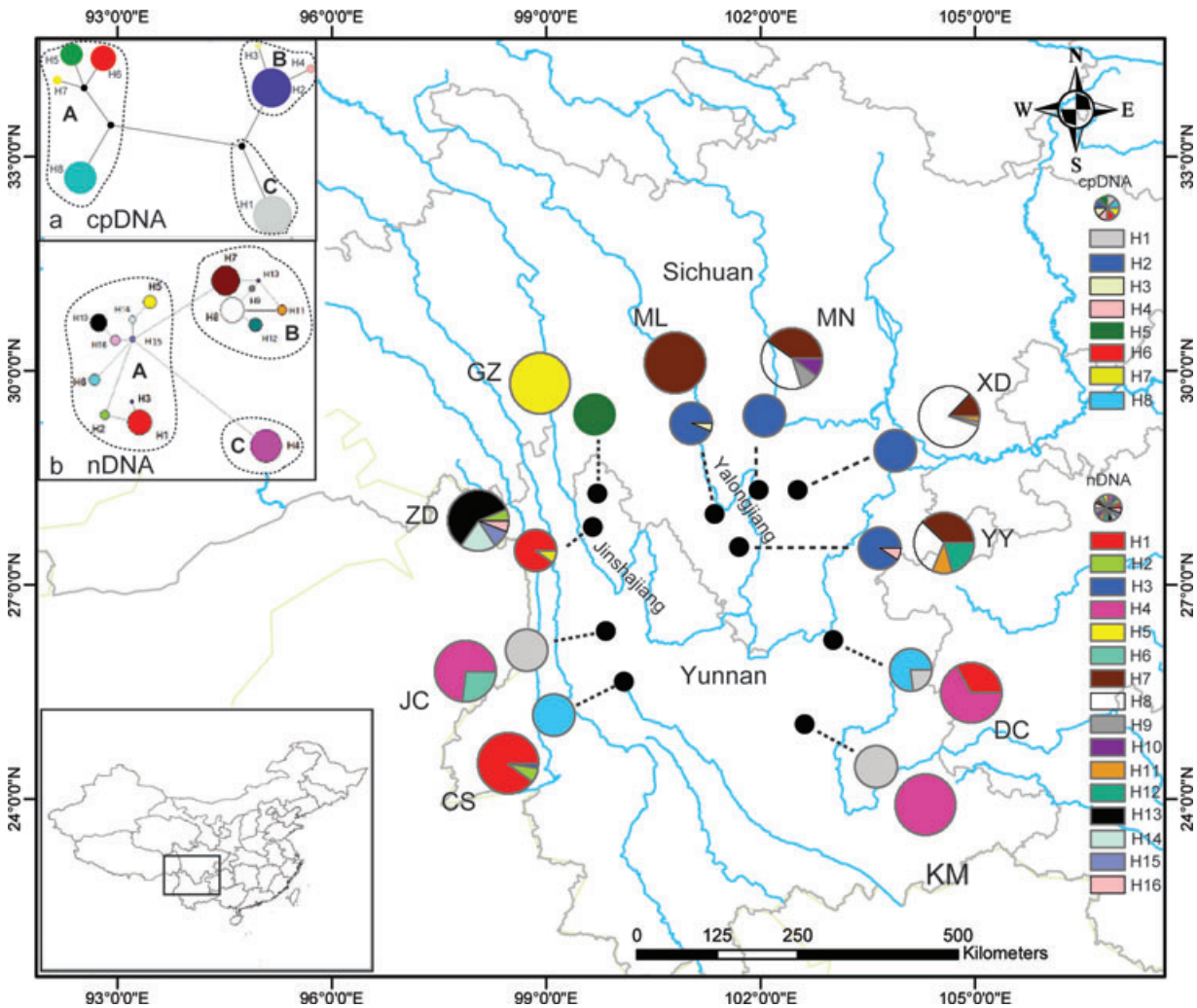

Fig. 1. Sample locations and distribution of chlorotypes (in small circles) and nuclear genotypes (in large circles) of *Cyananthus delavayi*, with networks of the chlorotypes (a) and nuclear DNA (nDNA) haplotypes (b) constructed using Network 4.5.0.2. The sizes of the circles in the network are proportional to the observed frequencies of the haplotypes. Frequency of chlorotypes and nDNA haplotypes in each population are indicated in pie charts. The map was constructed using ArcGIS 9.3. CS, Cangshan, Dali, Yunnan; DC, Yinming, Dongchuan, Yunnan; GZ, Xiaoxueshan, Zhongdian, Yunnan; JC, Shizhongshan, Jianchuan, Yunnan; KM, Xishan, Kunming, Yunnan; ML, Liangzishan, Muli, Sichuan; MN, Shenrong, Mianning, Sichuan; XD, Hebolu, Xide, Sichuan; YY, Xiaogaoshan, Yanyuan, Sichuan; ZD, Napahai, Zhongdian, Yunnan. cpDNA, chloroplast DNA.

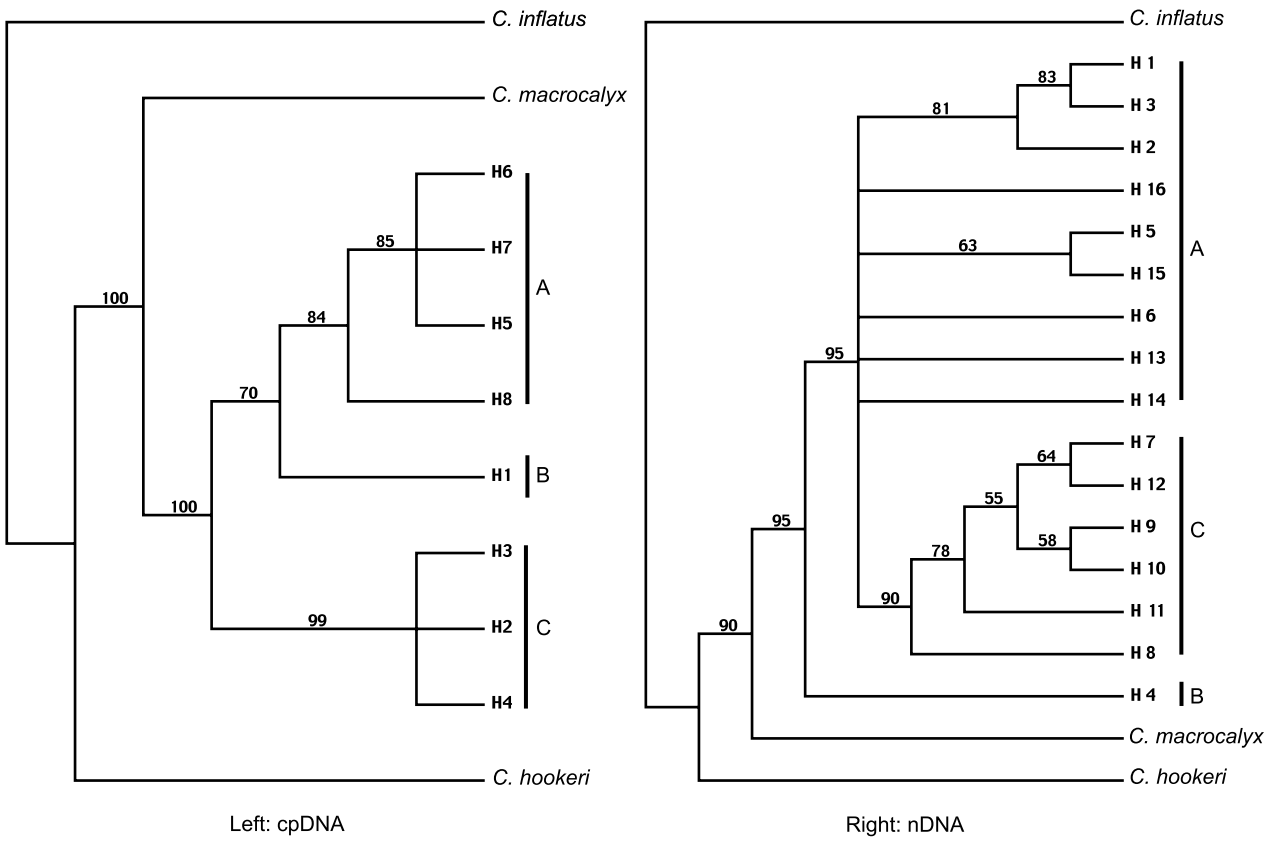

Fig. 2. Strict consensus trees of chlorotypes (left) and nuclear genotypes (right) of *Cyananthus delavayi.* Bootstrap support values are shown in the maximum parsimony (1000 replicates) analyses. A, Clade A; B, Clade B; C, Clade C.

(Table S2). Haplotype diversity (H_d) and nucleotide diversity (π) were estimated based on haplotype frequencies in each population, and their geographical distribution is presented in Table 1. Three of the nuclear genotypes identified (H4, H7, H8) were relatively common (found in $\geq$ 25% of the total populations), two haplotypes were shared by two populations, and 11 haplotypes were unique.

2.2 Phylogenetic analyses

Phylogenetic analysis indicated that three major clades were identified in all ingroup haplotypes for both markers (Fig. 2). Clade A comprised four populations (CS, ZD, GZ, DC), which were located at the Jinshajiang watershed; clade B comprised three populations (KM, JC, DC), which were mainly located at the Central Yunnan Plateau; and clade C comprised four populations (YY, ML, XD, MN) located at the Yalongjiang watershed. Among the three clades, only two haplotypes from locality DC occurred in both clades A and B. The two unrooted networks of both chlorotypes (Fig. 1: a) and nuclear genotypes (Fig. 1: b) also defined three haplotype clades, which were consistent with those identified from the topological structure of the maximum parsimony trees.

2.3 Population genetic diversity and structure

Examination of the chlorotype variation across all populations surveyed revealed that the total gene diversity ($h_T = 0.827$) was much higher than the within-population diversity ($h_S = 0.087$), and N_{ST} and G_{ST} were 0.899 and 0.895, respectively (Table 2). A permutation test showed that N_{ST} was higher than G_{ST}, but not significantly ($P > 0.05$), contradicting the null hypothesis of strong phylogeographic pattern (Rousset & Raymond, 1995; Pons & Petit, 1996). This result implies that if any phylogeographic structure was present, it was very weak. When nuclear genealogy variation was examined over all populations, the total genetic diversity ($h_T = 0.910$) based on nuclear haplotype variation was similar to that based on cpDNA, but the average within-population diversity ($h_S = 0.348$) was much higher. Consequently, both N_{ST} (0.719) and G_{ST} (0.618) were lower than those for cpDNA. A permutation test showed that G_{ST} and N_{ST} significantly differed ($N_{ST} > G_{ST}$, $P < 0.05$).

Table 2 Estimates of average gene diversity within populations (h_S), total gene diversity (h_T), interpopulation differentiation (G_{ST}), and number of substitution types (N_{ST}) for chlorotypes and nuclear genotypes within *Cyananthus delavayi*

Gene type	h_S	h_T	G_{ST}	N_{ST}
Chlorotype	0.087 (0.0413)	0.827 (0.0730)	0.895 (0.0501)	0.899 (0.1099)
Nuclear genotypes	0.348 (0.0918)	0.910 (0.0360)	0.618 (0.1050)	0.719 (0.1969)

Mean ± SE shown in parentheses.

Table 3 Analysis of molecular variance of chlorotypes and nuclear genotypes for 10 populations of *Cyananthus delavayi*

Gene types	Source of variation	*d.f.*	*SS*	*VC*	*PV*	F_{ST}
Chlorotypes						0.97476
	Among regions	2	1227.686	10.19753	86.5	
	Among populations	7	157.304	1.29371	10.97	
	Within populations	165	49.102	0.29758	2.52	
	Total	174	1434.091	11.78882		
Nuclear genotypes						0.84544
	Among regions	2	736.546	4.12600	70.88	
	Among populations	7	128.074	0.79506	13.66	
	Within populations	242	217.717	0.89966	15.46	
	Total	251	1082.337	5.82072		

d.f., degree of freedom; F_{ST}, genetic differentiation index; *PV,* percentage of variation; *SS,* sum of squares; *VC,* variance components.

This result indicates that phylogeographic structure was significantly present in the nDNA (Table 2). The average values for gene flow (Nm) of cpDNA and nDNA were also calculated to be 0.03 and 0.15 individual per generation, respectively, across all 10 populations, indicating a relatively low level of gene flow.

The SAMOVA tests failed to uncover any reliable population genetic grouping from either the cpDNA or nuclear dataset because F_{CT} values fluctuated irregularly when the number of groups (K) was varied in the range 2–8. We therefore divided all the populations between three groups, in accordance with the phylogenetic trees. AMOVA tests based on cpDNA indicated that 86.5% of the total variance occurred between groups, approximately 10.97% of the total variance was between populations, and only 2.53% occurred within populations. The pairwise F_{ST} value between the three groups was 0.975. For nDNA variation, 70.88% of the total variation occurred between groups, 13.66% between populations, and 15.46% within populations. The pairwise F_{ST} value between the three groups was 0.845 (Table 3).

2.4 Intraspecific cladogram and phylogeographic inferences

Nested cladograms were constructed for both chlorotypes and nuclear genotypes (Fig. 3) using a network by linking the haplotypes in a hierarchical manner. Nested clade analysis nested the whole chlorotype parsimony network into three levels and the demographic inferences between them are listed in Table S3. For nDNA data, 17 one-step, six two-step, and three three-step clades were revealed. Clade 3–1 and clade 3–2 was separated by eight mutations, suggesting long independent evolutionary histories. Allopatric fragmentation was inferred within clade 3–1, whereas restricted gene flow due to isolation by distance was suggested to play an important role within clade 3–3 (Table S3).

2.5 Molecular dating and population's demographic history

The TMRCA analyses indicated that the divergences between three main cpDNA clades were estimated at approximately 0.86 Ma (95% confidence

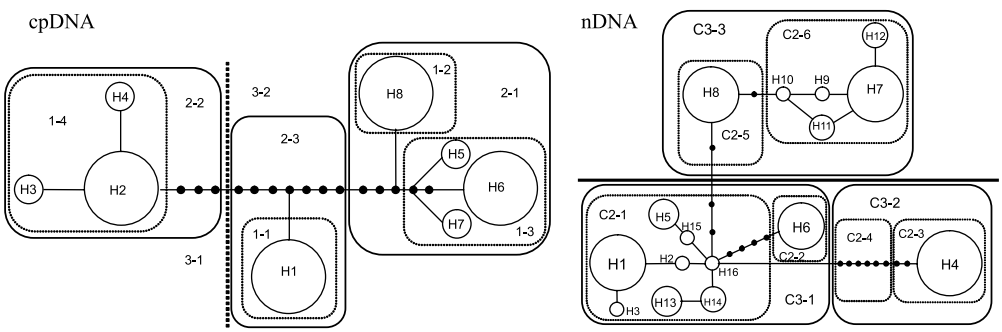

Fig. 3. Nested cladogram of the chlorotypes (H1–H8) (left) and nuclear genotypes (H1–H16) (right) of *Cyananthus delavayi*. Circles with numbers denote haplotypes. Dots represent putative haplotypes. Each branch represents one mutation. cpDNA, chloroplast DNA; nDNA, nuclear DNA.

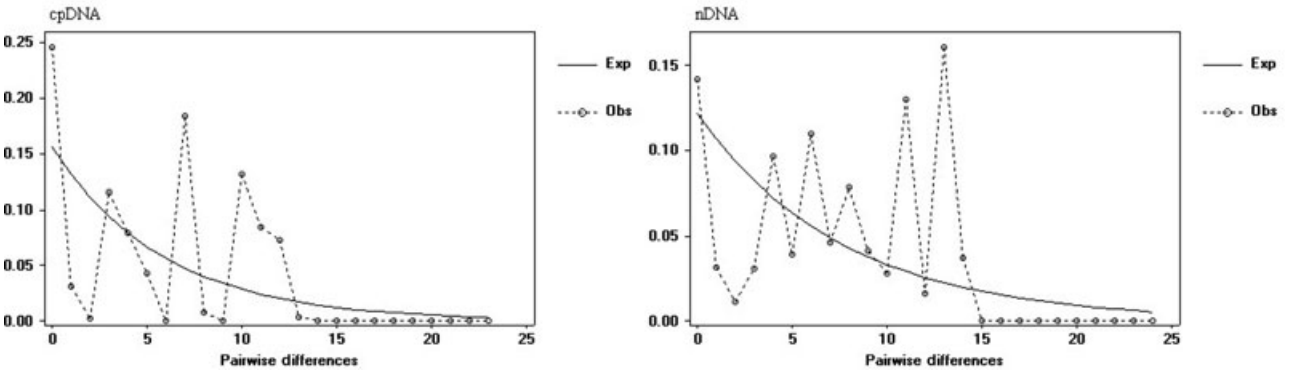

Fig. 4. Distribution of the number of pairwise nucleotide differences for chlorotypes and nuclear DNA (nDNA) haplotypes in *Cyananthus delavayi*. Dashed line shows observed values (Obs). Solid line represents expected values (Exp) under a model of sudden (stepwise) population expansion (Rogers & Harpending, 1992). cpDNA, chloroplast DNA.

interval, 0.3–2.65 Ma) and 0.49 Ma (95% confidence interval, 0.14–1.55 Ma). The observed mismatch distribution of pairwise nucleotide differences for all cpDNA and nDNA haplotypes differed from that predicted under a model of sudden range expansion (Fig. 4), as the shapes of the functions were not unimodal. This deviation was also supported by tests of neutrality based on the *ncpGS* locus, which showed that Tajima's *D* and Fu's *F*s values were mostly positive but always non-significant at both within-population and within-group levels (Table S4). These results rejected the hypothesis that there has been recent demographic population growth in this species.

3 Discussion

3.1 Genetic diversity and gene flow

In this study, both maternally inherited (cpDNA) and biparentally inherited (nDNA) markers revealed a high level of genetic diversity in *Cyananthus delavayi* at the species level ($h_T = 0.827/0.91$). This result is consistent with several previous phylogeographic studies in the HDM, such as, *Dipentodon sinicus* Dunn. ($h_T = 0.90$; Yuan et al., 2008), *Primula secundiflora* Franch. ($h_T = 0.97$; Wang et al., 2008b), *Ligularia tongolensis* (Franch.) Hand.-Mazz. ($h_T = 0.86$; Wang et al., 2011), *Terminalia franchetii* Gagnep. ($h_T = 0.78$; Zhang et al., 2011b), and *Primula poissonii* Franch. ($h_T = 0.791$; Song et al., 2011). Furthermore, these values are much higher than the mean value of cpDNA diversity ($h_T = 0.67$), which was detected by various cpDNA markers in 170 plant species (data compiled by Petit et al., 2005). However, plants with narrow distribution ranges have low genetic diversity (Hamrick et al., 1989). *Cyananthus delavayi* is narrowly distributed and its population size is relatively small. The high level of genetic diversity of *C. delavayi* in this study may result from its outcross breeding system (our unpublished data, 2012). As summarized by Hamrick & Godt (1996), outcrossing species usually have higher levels of genetic diversity than selfing plants. As a gynodioecious species, *C. delavayi* may have accumulated and maintained the high level of genetic diversity at the species level.

However, we found that the average haplotype diversity within each population was very low ($h_S = 0.087/0.348$) and genetic differentiation between populations was extremely high ($G_{ST} = 0.895/0.618$). It is commonly acknowledged that the level of genetic differentiation between populations is likely to be higher for maternally inherited cpDNA markers than for biparentally inherited nuclear genes because of the differences between seed and pollen migration parameters (Ennos, 1994; Raspé et al., 2000). In addition, this differentiation is also affected by life histories (Hamrick et al., 1992) and ecological traits of the studied species (Hewitt, 2004; Newton et al., 1999). The inefficient seed dispersal ability of *C. delavayi* may partly account for this high genetic differentiation. This species disperses its seeds in the vicinity of the parent plants by gravity when the capsules are dry and dehiscent. In fact, the gene flow (N_m) estimated based on the materially inherited cpDNA (0.03) did show a low level of dispersal ability. Our NCA analyses of both cpDNA and nDNA data also suggested that restricted gene flow with isolation by distance and allopatric fragmentation were likely the major processes that shaped the present-day spatial distribution of haplotypes (Table S4, Fig. 3). In addition, high levels of genetic differentiation have been widely found for numerous species distributed in the HDM and adjacent regions, e.g. *Megacodon stylophorus* (C. B. Clarke) H. Smith. ($G_{ST} = 0.807$; Ge et al., 2005), *Rhodiola alsia* (Fröd.) S. H. Fu ($G_{ST} = 0.703$; Xia et al., 2005), *Primula secundiflora* ($G_{ST} = 0.82$; Wang et al., 2008b), *Tsuga dumosa* (D. Don) Eichler

(G_{ST} = 0.95; Cun & Wang, 2010), *Stellera chamaejasme* L. (G_{ST} = 0.982; Zhang et al., 2010), *Sinopodophyllum hexandrum* (Royle) T. S. Ying (G_{ST} = 0.79; Li et al., 2011), and *Primula poissonii* (G_{ST} = 0.916; Song et al., 2011). Therefore, such a low gene flow and high genetic differentiation between populations was mainly attributed to the complex topography and high environmental heterogeneity in the HDM. These complex habitats usually promote a high level of genetic differentiation between regional populations (Till-Bottraund & Gaudeul, 2002).

3.2 Deep intraspecific divergences

Both phylogenetic and Network analyses recovered three distinct clades within *C. delavayi* (Figs. 2, 3). Clades A and C were located at the two sides of Shaluli Mountain, with clade B at the Central Yunnan Plateau. These allopatric divergences were also supported by AMOVA analyses that around 86.5% of the total variance occurred between these three clades. These regional divergences may reflect the long-term blocks of gene flow posed by both geographical and climatic isolation (Avise, 2000). Our dating of these three clades suggested that their divergences occurred between 0.86 Ma and 0.49 Ma, although these results should be taken with extreme caution due to the lack of palaeogeographic data and fossil records. However, available evidence suggests that the Shaluli Mountains were covered by ice sheets during the Quaternary glacial stages (Xu et al., 2004; Cao & Zhao, 2007). In fact, in the QTP and adjacent regions, even in China, the largest glaciation started around 1.2 Ma, reached its maximum between 0.8 and 0.6 Ma, and continued its range until 0.17 Ma after the penultimate glaciations (0.3–0.13 Ma) (Zhang et al., 2000; Zheng et al., 2002). In the QTP, the ice sheet was five to seven times larger than today (Shi, 2002). It is highly likely that glaciers and cold climates had continued in the high mountains in the QTP and adjacent HDM regions even during the interglacial warm stages. In addition, the valleys containing several major rivers in the HDM may have been deepened and the mountains there may have also been uplifted at this stage (Shi, 2002). This geographic isolation, as well as this continuous climate block in the mountain tops, may together have resulted in the long divergence between regional populations of *C. delavayi* distributed on each side of Shaluli Mountain as well as those on the Yunnan Plateau.

It is interesting that the deep intraspecific divergences in the allopatric distributions were widely recorded in both plant and animal species in the HDM and adjacent QTP regions (Su et al., 2001; Wang et al., 2008a, 2008b; Wang et al., 2009; Chen et al., 2010b; Song et al., 2011; Wang et al., 2011; Zhang et al., 2011a). The dated timescales of the deep intraspecific divergences varied greatly, but mostly between 0.3 and 1.2 Ma, fitting well within the development of the largest glaciation evidenced by geological studies in China (Zhang et al., 2000; Shi, 2002). These dating differences may result from the following three factors. First, most dating had no calibration points with correct fossils. Because the mutation rate may differ between species, all estimations should have unavoidable errors. Second, the initialization of the largest glaciation as well as possible geological changes may differ at different places. Finally, these species have different habits and other biological characters, which may further affect their evolution even under the same climatic and geological events.

Because these deep lineages were dated to occur far before the Last Glacial Maximum, they should have maintained in multiple refugia at this stage, as did many other species (e.g. Wang et al., 2008a, 2008b, 2009; Chen et al., 2010a; Jia et al., 2011). However, at the end of the Last Glacial Maximum, we failed to detect the large-scale range or population expansion evident in other species (e.g. Cun & Wang, 2010; Jia et al., 2011; Wang et al., 2011) and the following hybridizations between different intraspecific lineages (e.g. Wang et al., 2009). Mismatch analyses of both cpDNA and nDNA sequences showed a non-unimodal pattern (Fig. 4) and tests of neutrality (Tajima's *D*, Fu's *F*s), based on nDNA data, suggested no expansion (Table S4). This may be due to the fact that this species is distributed in strictly preferred habitats and all extant populations are relatively small.

The deep intraspecific lineages in the allopatric distributions recovered here for *C. delavayi* may partly mirror the rapid species diversification in the HDM and other QTP regions. Most congeneric species occurring there should have experienced a similar evolutionary history if their divergences had occurred at the same time with these intraspecific lineages. In fact, the dating of species diversification in a few genera revealed a largely consistent timescale for the divergence of some closely related species between the late Pliocene and the Quaternary (Liu et al., 2002, 2006; Sun et al., 2012). Therefore, these findings together highlight that geographical isolation caused by both climatic oscillations and orogenic activities promoted plant diversification in the HDM and adjacent regions.

Acknowledgements We sincerely thank Prof. Jian-Quan LIU, Dr. Ying-Xiong QIU, and two anonymous reviewers for comments and suggestions to improve the manuscript. We thank Dr. Hong-Guang ZHA and Dr. Yong-Hong ZHANG for assistance with the molecular

techniques and helpful comments on this manuscript. We are also grateful to Dr. Yang NIU, Mr. Zhuo ZHOU, and Mr. Bo XU for help with the field survey and leaf collection. This study was supported by a National Natural Science Foundation of China and Yunnan Natural Science Foundation Joint Project (Grant No. U1136601), the 100 Talents Program of the Chinese Academy of Sciences (No. 2011312D11022), and the National Natural Science Foundation of China (Grant No. 31061160184 and No. 40930209 to Prof. Hang SUN; 30960501 to Prof. Zi-Gang QIAN).

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Supporting Information

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S1. BEAST XML input-files generated by BEAUti for calculating the most recent common ancestor (TMRCA) of chlorotypes.
Table S1. Sequences of the eight identified chlorotypes.
Table S2. Sequences of the 16 identified nuclear genotypes.
Table S3. Nested contingency analysis of geographical associations and phylogeographical inferences made from nested haplotype analysis of *Cyananthus delavayi* based on chloroplast DNA and nuclear DNA data.
Table S4. Results of the neutrality test for seven populations of *Cyananthus delavayi* with intrapopulation divergence based on nuclear DNA.

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