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The population genetic structure and diversification of *Aristolochia delavayi* (Aristolochiaceae), an endangered species of the dry hot valleys of the Jinsha River, southwestern China

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Abstract: *Aristolochia delavayi* Franch. is an endangered species of the dry hot valleys of the Jinsha River, southwestern China. The genetic diversity and structure of the species and the occurrence of gene flow were investigated with eight inter-simple sequence repeat (ISSR) markers and four plastid loci (*matK*, *trnL-trnT*, *rps4-trnT*, and *trnC-rpoB*). The analysis of ISSR markers indicated that the genetic diversity of the species is relatively high (PPB = 84.71%). Similarly, a high gene diversity of H_T = 0.833 was found based on the four plastid loci. It is possible that this species previously maintained a large effective population size and that the current relict distribution of the species is a result of large-scale fragmentation and habitat destruction. The origin of the species at approximately 3.407 million years ago and the ensuing intraspecific divergence are generally consistent with the history of two major glaciations in this region. Conservation measures are urgently needed to increase the genetic diversity and population size of this species through both in- and ex-situ action.

Key words: Aristolochia delavayi, ISSR, phylogeographic, Hengduan Mountains, conservation, contraction.

Résumé : *Aristolochia delavayi* Franch. est une espèce menacée des vallées arides de la rivière Jinsha, au Sud-ouest de la Chine. La diversité et la structure de l'espèce sur le plan génétique, de même que l'existence de flux génique ont été examinés à l'aide de huit marqueurs ISSR et de quatre locus plastidaux (*matK*, *trnL-trnT*, *rps4-trnT* et *trnC-rpoB*). L'analyse des marqueurs ISSR a indiqué que la diversité génétique de l'espèce est relativement élevée (PPB = 84,71 %). De la même façon, une haute diversité (H_T = 0,833) a été trouvée sur la base des quatre locus plastidaux. Il est possible que cette espèce ait maintenu antérieurement une taille de population effective élevée et que la distribution actuelle des vestiges de cette espèce soit le résultat d'une fragmentation à large échelle et de la destruction de l'habitat. L'origine de cette espèce estimée à 3,407 Ma et la divergence intra-spécifique qui s'en est suivie sont généralement cohérentes avec l'historique des deux glaciations majeures dans cette région. Des mesures de conservation sont requises de manière urgente afin d'accroitre la diversité génétique et la taille de la population de cette espèce par des actions in situ et ex situ. [Traduit par la Rédaction]

Mots-clés : Aristolochia delavayi, ISSR, phylogéographique, monts Hengduan, conservation, contraction.

Introduction

Aristolochia delavayi Franch. is an endemic species of the dry hot valleys of the Jinsha River, southwestern China. The region contains a special and vulnerable ecosystem characterized by aridity, high temperatures, semi-savanna vegetation, and relatively few plant species. However, the dry hot valleys are especially rich in endemic genera (e.g., *Anemoclema*, *Nouelia*, *Musella*, *Ostryopsis*, *Trailliaedoxa*) and species (e.g., *Aristolochia delavayi*, *Cotinus nana*, *Mastixia microcarpa*, *Munronia delavayi*, *Terminalia franchetii*, *Vitex dulouxii*) (Jin et al. 1994; Li 1995; Jin 2002; Guan et al. 2013). The area is considered to have served as a refugium during the Quaternary glaciations (Zhang et al. 2009*b*; Li et al. 2011; Yang et al. 2012). Owing to natural, historical, and anthropogenic factors, this ecosystem has become seriously degraded, resulting in soil and water losses and significant difficulties in vegetation restoration (Jin et al. 1994; Li 1995; Zhong 2000). *Aristolochia delavayi* has a narrow distribution in the Jinsha River drainage in northwestern Yunnan and southwestern Sichuan provinces. Multiple field surveys only detected four populations (three populations had less than 50 individuals and one had 10 individuals; personal observation by Xun Gong) separated from each other by less than 200 km (Fig. 1). The species forms thickets on limestone mountain slopes in dry hot valleys ranging from 1640 to 2250 m a.s.l. (Ma 1989). The rarity of this long-lived perennial species is, most likely, a result of large-scale habitat destruction caused by human activities, and continued habitat alteration is likely to threaten the species further. First, the leaves and fruits of *A. delavayi* are used as flavorings by the local people when they cook mutton and beef. Second, the Jinsha River is one of the largest tributaries of the Yangtze River, originating from the Qinghai–Tibet plateau and flowing through the Hengduan Mountain region, which is rich in hydroelectric potential. Fourteen large hydropower stations are planned or under construction, including the Xiangjiaba and Xiluodu hydropower stations. These hydropower stations will flood the habitats of several endemic species that grow along the river banks. Furthermore, changes in the ecological environment will occur, including an increase in humidity and a decrease in the annual temperature. The direct and indirect extreme changes in habitats will severely threaten the survival of these endangered species. There is an urgent need to study and conserve the genetic diversity of

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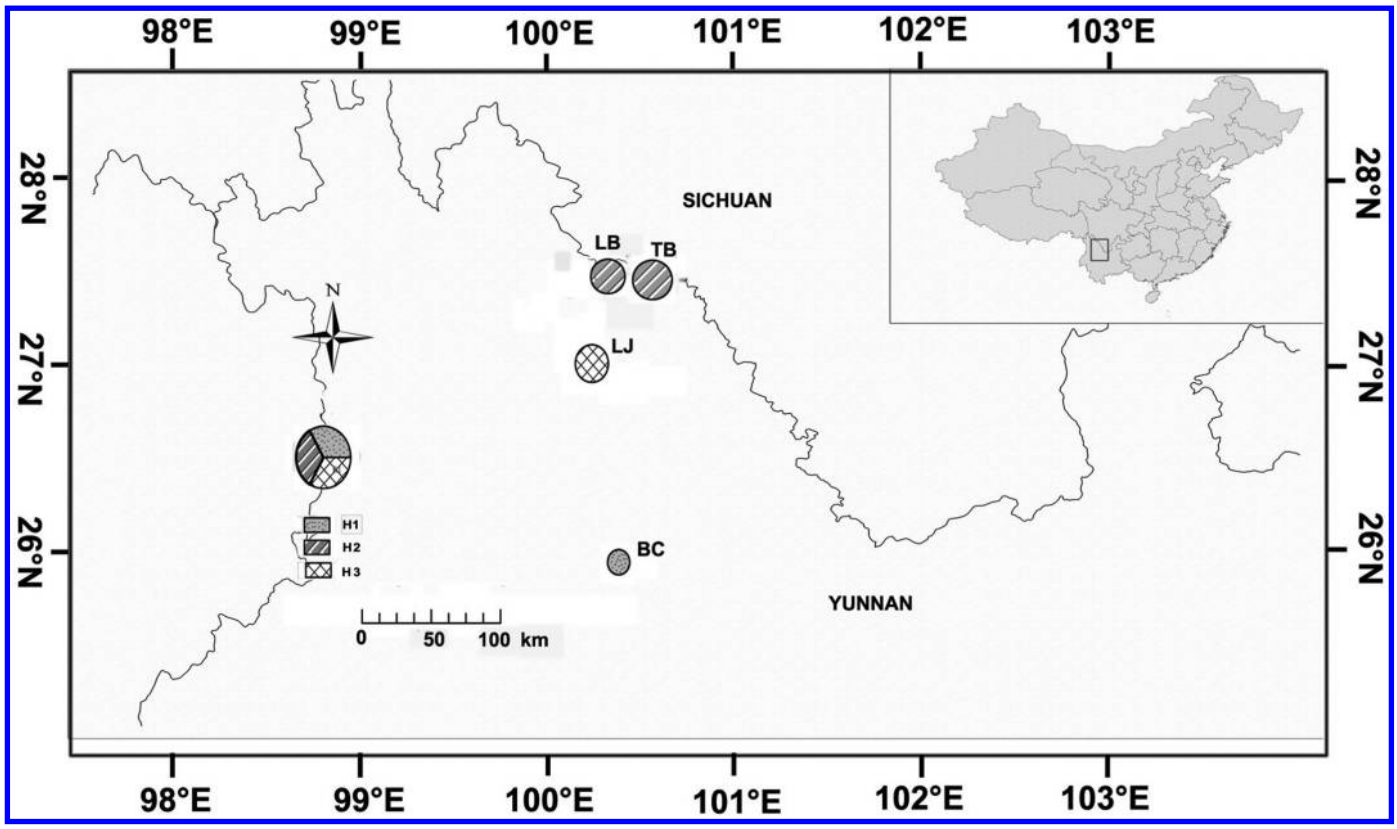
Fig. 1. Sampled populations of *Aristolochia delavayi* and the distribution of chloroplast DNA haplotypes. Pie charts show the proportions of haplotypes within each population. BC, Binchuan; LB, Sanjiangkou; LJ, Lijiang; TB, Linglang. The haplotype frequency map was constructed using ArcGIS 9.0.



species in the dry hot valleys of Jinsha River, especially the endemic species.

One of the main targets of conservation biology is to study the plight of threatened species, existing or potential species whose effective population sizes are small (Frankel and Soulé 1981). Threatened species are usually considered to have suffered from depauperization of their habitat and reductions of their genetic material (Drury 1974). Therefore, identifying ecological requirements, species diversity patterns and distribution ranges of habitats of interest is an important task in the development of conservation and restoration programs (Hacker and Gaines 1997; Otto et al. 2012). Declining populations are expected to possess low levels of genetic diversity because of the high probability of inbreeding within populations driven by random genetic drift (Ellstrand and Elam 1993; Hedrick 2001; Frankham 2005). In *A. delavayi*, given the small number and size of its populations, it is probable that genetic diversity will decrease. As long-term population viability is associated with the level of genetic variability (Schaal et al. 1991), the erosion of genetic diversity would eventually harm the health of populations and species (Luan et al. 2006). Genetic variation at the intraspecies level is a prerequisite for future adaptive change or evolution and has profound implications for species conservation (Schaal et al. 1998). Understanding genetic variation within and between populations is essential for the establishment of effective and efficient conservation practices for rare species. Several aspects of conservation biology, such as the loss of genetic diversity in conservation programs and the restoration of threatened populations, can only be addressed by detailed population genetics studies (Hamrick and Godt 1996). However, genetic variation in *A. delavayi* has not previously been estimated. In this study, the genetic diversity and structure and the phylogeographic patterns of the species were investigated based on both inter-simple sequence repeat (ISSR) markers and plastid sequence markers. The evolutionary factors that have influenced the spatial distribution of genetic diversity and the phylogeographic patterns of the species were considered in light of the genetic analysis. Effective and efficient measures for protecting *A. delavayi* are also suggested in this study.

Materials and methods

Plant materials

We conducted extensive field surveys throughout the entire range of *A. delavayi* from 2008 to 2012. However, only four populations were discovered and sampled (Fig. 1). Only 10 mature individuals were found from population Binchuan (BC), and less than 50 individuals were from each population of Lijiang (LJ), Sanjiangkou (LB), and Linglang (TB). In all, 10, 20, 19, and 20 mature individuals were sampled from populations BC, LJ, LB, and TB, respectively. The samples were dried with silica gel and stored at room temperature prior to DNA extraction. Voucher specimens were deposited at the Herbarium of the Kunming Institute of Botany, Chinese Academy of Sciences (KUN).

ISSR analysis

Total genomic DNA was extracted from dried leaves using a modified cetyltrimethylammonium bromide method (Doyle and Wolfe 1991). The purified total DNA was quantified by gel electrophoresis and its quality verified by spectrophotometry. The DNA was amplified with polymerase chain reaction (PCR) using ISSR primers from The University of British Columbia. Of 100 ISSR primers, 8 produced clear and reproducible bands and were selected for the subsequent experiment (Table 1). PCR amplification was performed in a total volume of 25 μL containing 20 ng

Table 1. Primers for polymerase chain reaction (PCR) amplifications, PCR annealing temperature (T_a), number of bands per primer, number and percentage of polymorphic bands in inter-simple sequence repeat analyses.

Primers code	Primer sequences (5′-3′)	T_a (°C)	No. of bands per primer	No. of polymorphic bands	Polymophism (%)
840	$(GA)_8$YT	53	11	10	90.91
845	$(CT)_8$RG	52	7	5	71.43
856	$(AC)_8$YA	52	11	7	63.64
857	$(AC)_8$YG	52	13	12	92.31
888	BDB $(CA)_7$	50	13	11	84.62
889	DBD $(AC)_7$	52	11	10	90.91
890	VHV $(GT)_7$	52	11	10	90.91
891	HVH $(TG)_7$	52	8	7	87.50
Total			85	72	84.71

Note: Y = (C, T); R = (A, G); B = (C, G, T); D = (A, G, T); H = (A, C, T); V = (A, C, G).

template DNA, 2.0 μL 10 × PCR buffer, 2.2 mmol·L^{-1} $MgCl_2$, 1.5 mmol·L^{-1} dNTPs, 3% formamide, 300 nmol·L^{-1} primer, 0.5 U of Taq polymerase (Promega, Madison, Wisconsin, USA) and double-distilled water. PCR was programmed on an ABI Prism Reaction Dye Terminator Cycle with a hot bonnet (PerkinElmer, Waltham, Massachusetts, USA) as one cycle of denaturation at 94 °C for 5 min, 35 cycles of 30 s annealing at 50–53 °C and 1 min extension at 72 °C, followed by a 10 min extension at 72 °C. A negative control from which template DNA was omitted was included in every PCR protocol to test for contamination. Amplification products were electrophoretically separated on 1.5% agarose gels buffered with 1 × Tris–borate–EDTA. A DNA ladder was applied as a size marker (100–2500 bp). After staining with ethidium bromide for 30 min, DNA fragments were identified with image analysis software for gel documentation. Only those gels that showed consistent and clear bands were considered; those showing smeared or weak bands were excluded.

ISSR profiles were scored for each individual as the presence (1) or absence (0) of specific bands. All amplifications were repeated at least twice, and only reproducible and well-defined bands were considered for analysis. The software program POPGENE version 1.31 (Yeh et al. 1999) was employed to obtain the genetic diversity parameters, percentage of polymorphic bands (PPB), observed allele number per locus (A_o), effective allele number per locus (A_e), Nei's gene diversity (H_n) and Shannon diversity index (I) (Nei 1973). Genetic diversity parameters were calculated at both specific and population levels. Gene flow among populations (N_m) was estimated based on the mean coefficient of gene differentiation ($G_{st,}$), which were estimated using POPGENE (Yeh et al. 1999). Hickory version 1.1 (Holsinger et al. 2002) was applied to analyze inbreeding coefficient (F_{IS}). STRUCTURE version 2.3.1 was used to infer population structure and assign individuals to populations (Pritchard et al. 2000). To determine the optimal number of groups (K), STRUCTURE was used with K varying from 1 to 10, with five runs for each K value. Several studies have found that the posterior probability for a given K increases slightly even after the real K is reached (Dan et al. 2009). We used an ad hoc statistic, ΔK, to determine the true value of K (Evanno et al. 2005). The parameters were 100 000 burn-in periods and 10 000 Markov chain Monte Carlo repetitions after burn-in. In addition, a principal coordinate analysis and analysis of molecular variance (AMOVA) in GenAlEx 6.0 were performed to further examine the genetic relationships among the detected populations on the basis of the same ISSR data (Peakall and Smouse 2006).

Phylogeographic analysis

Four plastid regions, *matK*, *trnL-trnT*, *rps4-trnT*, and *trnC-rpoB* (Shaw et al. 2005), were selected for phylogeographic analysis after a screening of 32 loci. A PCR was conducted in a volume of 25 μL containing 10–30 ng genomic DNA, 0.5 μmol·L^{-1} of each primer, 10 mmol·L^{-1} Tris-HCl, 50 mmol·L^{-1} KCl, 200 μmol·L^{-1} each dNTP, 1.5 mmol·L^{-1} $MgCl_2$ and 1.5 U Taq polymerase at pH 8.2 using the following parameters: initial denaturation at 94 °C for 5 min followed by 32 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, extension at 72 °C for 2 min and a final extension at 72 °C for 10 min. PCR products were purified by electrophoresis in a 1.0% agarose gel using 1 × TAE buffer. The gel was stained with ethidium bromide and bromide, and the desired DNA band was cut and eluted using the Agarose Gel Purification Kit (QIAGEN, Valencia, California, USA). Sequencing reactions were performed with the same primers used in the amplification reactions, and the sequencing products were separated on an ABI 3730xl DNA analyzer (Applied Biosystems, Foster City, California, USA). The obtained sequences were aligned with ClustalX version 1.81 (Thompson et al. 1997) and then optimized manually. The sequences have been deposited in GenBank under the following inclusive accession numbers: KF974453–KF974474.

All four plastids were analyzed in combination because the chloroplast genome behaves as a single linked region. Genetic diversity was measured by haplotype diversity (H) (Nei and Tajima 1983) and by nucleotide diversity (π) for all samples and for each basin grouping using DnaSP 5.0 (Librado and Rozas 2009). G_{st} and N_{st} were used to estimate differentiation between populations, with G_{st} incorporating haplotype frequency and N_{st} additionally incorporating genetic differences. A comparison was made between G_{st} and N_{st} using a U-statistic. All of these parameters were calculated using the program HAPLONST (Pons and Petit 1996).

Neutrality tests and mismatch distribution analyses were used to infer population demographic events. Tajima's D (Tajima 1989), Fu and Li's F^* (Fu and Li 1993), R_2 (Ramos-Onsins and Rozas 2002), and Mismatch distributions (Rogers and Harpending 1992; Harpending 1994) were calculated using DnaSP 5.0. The phylogenetic relationships among the cpDNA haplotypes were analyzed by the Maximum Parsimony (MP) analyses as implemented in PAUP*4.0b10 (Swofford 2002), and by Bayesian inference (BI) as implemented in MrBayes 3.1.1 (Ronquist and Huelsenbeck 2003), with gaps treated as missing data in all analyses. Heuristic searches with 1000 random addition sequence replicates, tree bisection-reconnection (TBR) branch swapping and the MULTREES option in effect were used for the MP analyses. All character states were treated as unordered and equally weighted. To evaluate the relative robustness of clades in the MP trees, a bootstrap analysis (Felsenstein 1985) was performed with 1000 replicates using the same options as above except that a maximum of 100 trees were saved per round. For Bayesian inference, one cold and three incrementally heated Markov chain Monte Carlo chains were run for 2.0 × 10^6 generations. Trees were sampled every 100 generations. The first 2000 to 5000 trees before stationarity were discarded as burn-in, and the remaining trees were used to construct majority-rule consensus trees using PAUP*. A haplotype network was constructed using TCS 1.21 (Clement et al. 2000). The geographical distribution of the different haplotypes identified was mapped using ArcMap 9.1 (ESRI, Redlands, California, USA).

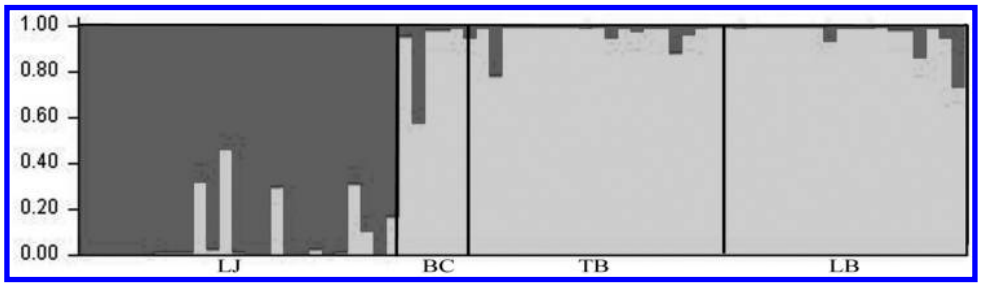
We also estimated phylogenetic relationships and divergence time by applying a coalescent analytical approach as implemented in BEAST 1.5.4 (Drummond and Rambaut 2007). Because there is no fossil record for the genus, we used the rate of 8.24 × 10^{-9} substitutions per site year, a mutation rate proposed for chloroplast non-coding regions in some herbaceous plants (Richardson et al. 2001; Yue et al. 2012). This coalescent-based approach estimates the posterior distribution for effective population size at intervals along a phylogeny, thereby allowing inferences of population fluctuations over time. The BEAST program was also used to create a Bayesian skyline plot. We ran 5 × 10^8 generations with a burn-in of 10^6 under the GTR model with estimated base frequencies, gamma shape distribution, proportion of invariant sites. All operations were automatically optimized. The effective

Table 2. Genetic variation within populations of *Aristolochia delavayi* indicated by inter-simple sequence repeat markers.

Population	A_o	A_e	H_n	I	PPB (%)
BC	1.271 (0.447)	1.183 (0.330)	0.105 (0.183)	0.154 (0.264)	27.06
LJ	1.659 (0.477)	1.453 (0.399)	0.256 (0.208)	0.375 (0.295)	65.88
LB	1.565 (0.499)	1.315 (0.343)	0.190 (0.193)	0.288 (0.280)	56.47
TB	1.659 (0.477)	1.484 (0.412)	0.268 (0.214)	0.388 (0.302)	65.88
Mean	1.538 (0.475)	1.359 (0.368)	0.205 (0.200)	0.301 (0.285)	53.82
Species level	1.847 (0.362)	1.514 (0.330)	0.304 (0.167)	0.455 (0.231)	84.71

Note: A_o, observed allele number per locus; A_e, effective allele number per locus; H_n, Nei's gene diversity; I, Shannon diversity index; PPB%, percentage of polymorphic bands. Values in parentheses are SD.

Fig. 2. Bayesian model-based clustering STRUCTURE analysis as inferred at $K = 2$ based on inter-simple sequence repeat data. The genotype of each individual accession is represented by a vertical line divided into black solid line segments, the lengths of which indicate the proportions of the genome attributed to the inferred clusters. BC, Binchuan; LB, Sanjiangkou; LJ, Lijiang; TB, Linglang.



sampling size (ESS) parameter was found to exceed 200, which suggests acceptable mixing and sufficient sampling. A Bayesian analysis of combined data was also used to estimate the divergence times of the species from the most recent common ancestor of all cpDNA haplotypes with BEAST software version 1.5.4 (Drummond and Rambaut 2007). To better understand the phylogeographical history of *A. delavayi*, three *Aristolochia* species (*Aristolochia tubiflora* Dunn, *Aristolochia grandiflora* Sw., and *Aristolochia fimbriata* Cham.) were included in the study as outgroups.

Results

Genetic diversity and genetic structure inferred from ISSR markers

In total, eight primer pairs produced 85 reliable ISSR bands from 69 individuals, of which 72 (84.71%) were polymorphic (Table 1). The PPB averaged 53.82%, with a range from 27.06% (BC) to 65.88% (LJ) at the population level (Table 2). Assuming Hardy–Weinberg equilibrium, the mean (SD) observed allele number per locus (A_o) was estimated to be 1.538 (0.475) within populations and 1.847 (0.362) at the specific level, the mean (SD) effective allele number per locus (A_e) was estimated to be 1.359 (0.368) within populations and 1.514 (0.330) at the specific level; the mean (SD) Nei's gene diversity (H_n) and Shannon diversity index (I) were 0.205 (0.200) and 0.301 (0.285), respectively, within populations and 0.304 (0.167) and 0.455 (0.231), respectively, at the specific level. The mean F_{IS} was estimated to be 0.5021. The STRUCTURE analyses estimated that the value of ΔK was 618.9, 437.2, and 226.6 for $K = 2$, $K = 3$, and $K = 4$, respectively, and less than 167 when K was greater than 4. Therefore, $K = 2$ best represented the data and showed a clear separation between LJ and the other three populations (Fig. 2). A two-dimensional principal coordinate analysis plot (Fig. 3) demonstrated that the first principal coordinate explained 34.49% of the total variation and separated the LJ populations from the BC, LB, and TB populations. The second principal coordinate (26.06% of total variation) separated most individuals from TB from those of BC and LB.

AMOVA analysis showed a higher proportion of genetic differentiation within populations (67%) than among populations (33%) (Table 3). The level of gene flow (N_m) was estimated to be 0.576 ($N_m < 1$), indicating a low level of gene flow among populations.

Genetic diversity and genetic divergence inferred from chloroplast DNA

Four cpDNA regions (*matK*, *trnL-trnT*, *rps4-trnT*, and *trnC-rpoB*) in *A. delavayi* were aligned with a consensus length of 2885 bp. The analysis identified an indel at site 525 of *trnL-trnT* and two substitutions at sites 235 and 527 of *matK*, 167 and 331 of *rps4-trnT*, 312 and 398 of *trnC-rpoB*, and 227 and 490 of *trnL-trnT* (Appendix). An analysis of pooled sequences of four fragments allowed the identification of three haplotypes among all individuals examined.

The total nucleotide diversity (π_T) was inferred to be 0.00123 (Table 4). The total gene diversity (H_T) was estimated to be 0.833, and the within-population diversity (H_s) was 0.250. Significant population differentiation was observed, with $G_{st} = N_{st} = 0.700$. The R_2 test ($R_2 = 0.2210$, $P > 0.05$), as well as the Fu's test ($F = 2.172$, $P < 0.02$) and Tajima test ($D = 2.954$, $P < 0.01$), indicated *A. delavayi* experienced significant population reduction. This result was also supported by reconstructed Bayesian skyline plot (Fig. 4). The multimodal mismatch distribution also suggested this species has not experienced recent expansion (Fig. 5).

The geographical distribution of the haplotypes is shown in Fig. 1. An important characteristic of cpDNA diversity in *A. delavayi* is the nonrandom distribution of haplotypes. Each population contained only one haplotype (Table 4): haplotype 1 was in population BC, haplotype 2 was in population LJ, and haplotype 3 was in populations LB and TB. The TCS network (Fig. 6) of the three haplotypes was consistent with the Beast tree (Fig. 7). This pattern implies that haplotype 2 might be the ancestral form and the other two haplotypes are more recently derived.

No phylogeographic signal in the haplotype distribution was detected with a standard phylogeographic analysis because the G_{st} and N_{st} values were identical (0.700) and did not differ significantly. However, the haplotypes of *A. delavayi* suggested further inferences about current genetic structuring in this species and inferences about the history of the species. The dating analyses suggested that the most recent common ancestor of the clades of the species appeared approximately 3.407 million years ago (mya) (95% highest posterior density (HPD): 0.965–6.547 mya) and that two main divergence events occurred involving *A. delavayi*: the first one occurred at 0.479 mya (95% HPD: 0.358–2.367 mya) between population LJ and others, the second at 0.255 mya

Fig. 3. A two-dimensional plot of the principal coordinate analysis of inter-simple sequence repeat data showing the clustering of populations of *Aristolochia delavayi*. The first principal coordinates account for 34.49% of total variation. BC, Binchuan; LB, Sanjiangkou; LJ, Lijiang; TB, Linglang.

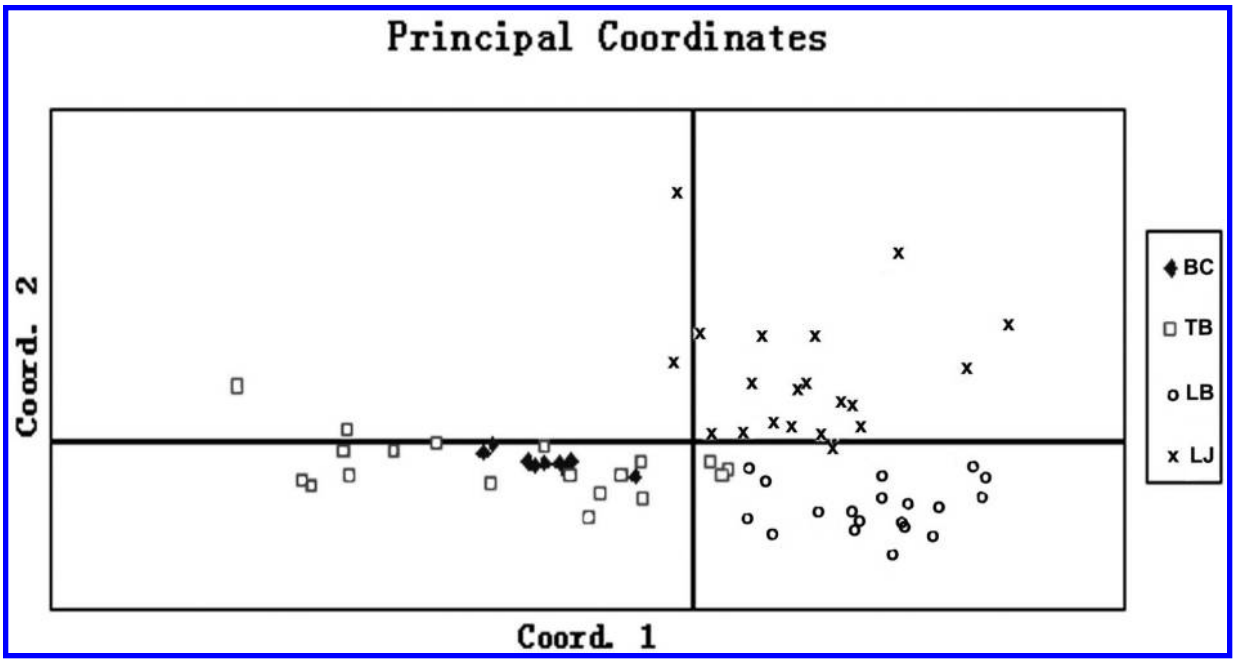


Table 3. Analyses of molecular variance (AMOVA) for *Aristolochia delavayi* by inter-simple sequence repeat markers.

Source of variation	df	Sum of squares	Variance component	Percentage of variance	*P* value
Among population	3	177.584	59.195	33	<0.01
Within population	65	419.982	6.461	67	<0.01

(95% HPD: 0.051–1.241 mya) between population BC and populations TB and LB.

Discussion

Genetic diversity

The results of genetic diversity and structure will supply key information to develop efficient preservation strategies for species with small populations (Bevill and Louda 1999). Generally speaking, species having small geographic ranges and small population sizes tend to maintain lower genetic diversity than those of geographically widespread species (Hamrick and Godt 1989). However, certain endangered species are known to have a high level of genetic diversity and weak genetic differentiation (Gitzendanner and Soltis 2000; López-Pujol et al. 2002; Wang et al. 2004; Luan et al. 2006). The critically endangered *A. delavayi* represents a similar example, with unexpectedly high levels of genetic diversity (PPB = 84.71% indicated by ISSR markers; H_T = 0.833 indicated by four plastid loci) compared with most endemic species (PPB = 40%, H_T = 0.096) (Hamrick and Godt 1989).

High genetic diversity in rare plants has been attributed to a number of factors (Hedrick 2011), e.g., a recent decrease in population size plus insufficient time for isolation, or extensive and recurrent gene flow (Crawford 1983). Population genetic theory predicts that larger populations tend to maintain higher allelic diversity (Ellstrand and Elam 1993; Yao et al. 2007). The high species-level genetic diversity revealed by both nuclear ISSR and plastid sequence markers suggests that *A. delavayi* once had a large effective population size. The current relict distribution of this species may be attributed to large-scale fragmentation following the Pliocene climate oscillation and the uplift of the Hengduan Mountain region.

Local people use the leaves and fruits of *A. delavayi* as flavorings when they cook mutton and beef. Recent overharvesting and habitat destruction have aggravated the fragmentation of this species. Each of four extant populations consists of fewer than 50 individuals, and no more than 200 individuals in total survive in the wild according to our field observations. In addition to these two sites, specimens of *A. delavayi* were collected from Sandai, Dayao County, Yunnan Province, and Eya, Muli County, Sichuan Province, during 2008–2012. We failed to find any individuals at these two sites despite multiple attempts. Most likely, this species is permanently extirpated in these locations. Because few young seedlings are detected in its wild populations (personal observation by Xun Gong), this species may suffer from reproductive failure through low seed productivity and (or) seed germination rates. At the same time, this species was inferred to have a high inbreeding rate (F_{IS} = 0.5021). Accordingly, this endemic and rare species of the dry hot valleys of the Jinsha River should be assigned a high priority for protection.

Population differentiation

Both nuclear ISSR and plastid sequence data revealed significant genetic differentiation among the four populations and low gene flow among the populations. The ISSR data suggested the separation of the LJ population from the other three populations and a low among-population gene flow of N_m = 0.576. The four plastid loci revealed significant population differentiation, with $G_{st} = N_{st}$ = 0.700 and each population was fixed for a single haplotype. In general, the breeding system of flowering plants significantly affects population genetic differentiation (Hamrick and Godt 1996), and typical animal-pollinated and outcrossing plant species tend to show high population differentiation (Frankham et al. 2002). Although no experiments addressing this issue have been conducted with *A. delavayi*, the self-pollinated flowers of other studied *Aristolochia* species fail to set fruit, and cross-pollinated flowers have an extremely high fruit set (Sakai 2002). All investigated *Aristolochia* species are pollinated by flies or butterflies (Sakai 2002). According to the observations of Gao Chen

Table 4. Sample locations, sample size, cpDNA haplotype frequencies, and nucleotide diversity (π) in four populations of *Aristolochia delavayi*.

Locality	Code	Latitude (N)	Longitude (E)	Elevation (m a.s.l.)	*N*	Haplotypes (frequencies, %)	π
Binchuan, Yunnan	BC	25°56.300′	100°24.433′	1700	10	H1 (100)	0.00000
Lijiang, Yunnan	LJ	27°17.770′	100°11.946′	1900	20	H2 (100)	0.00000
Sanjiangkou, Yunnan	LB	27°45.967′	100°22.983′	1640	19	H3 (100)	0.00000
Linglang, Yunnan	TB	27°45.983′	100°25.600′	2250	20	H3 (100)	0.00000
Total					69		0.00123

Fig. 4. Bayesian skyline plot based on the cpDNA for the effective population size fluctuations throughout time. *x* axis: time (years ago); *y* axis: the effective population size × generation time (black line, median estimations; area between gray lines, 95%).

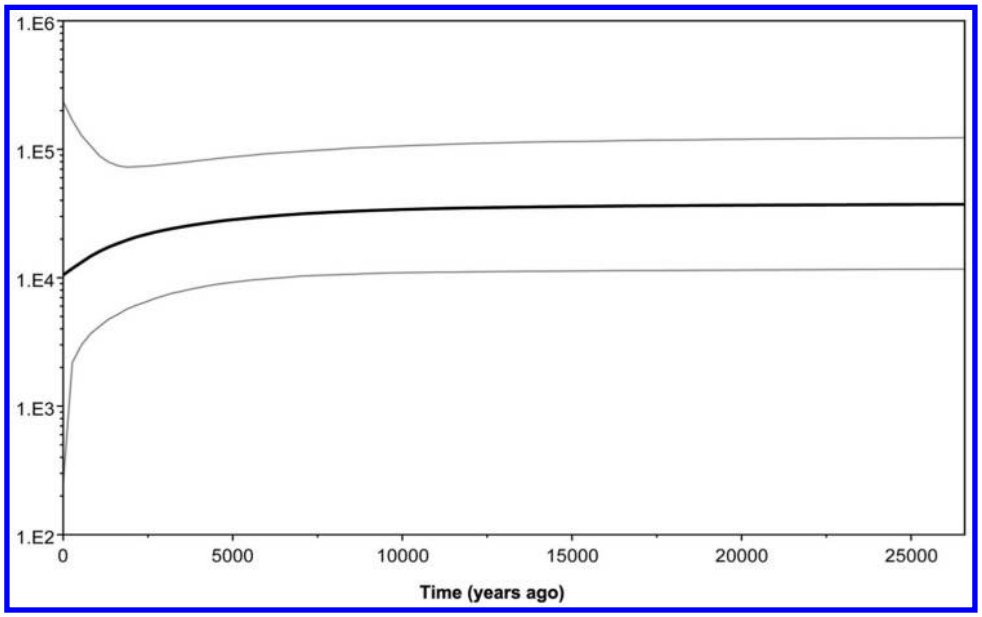
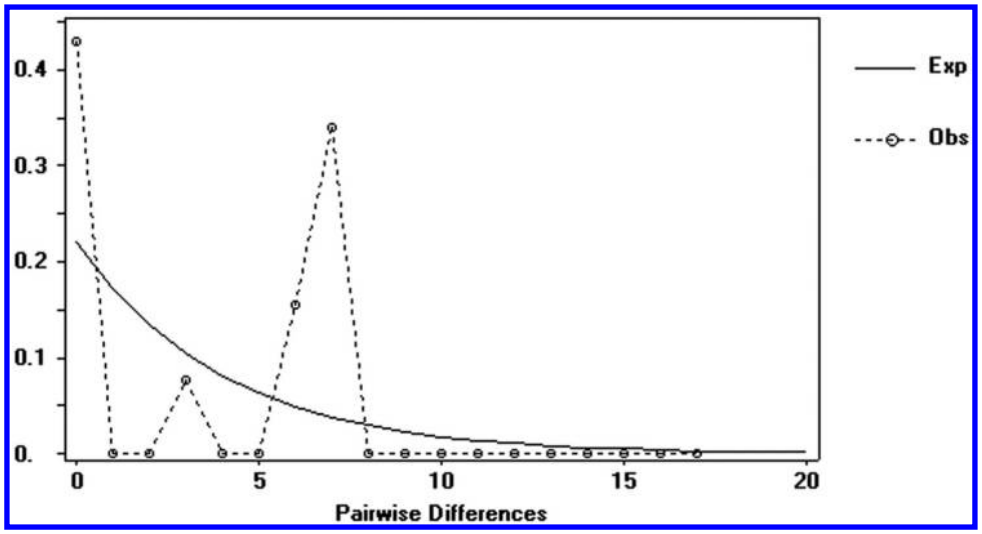


Fig. 5. Mismatch distribution of the *Aristolochia delavayi* chloroplast DNA region in the overall population sampled. The dashed line represents observed values (Obs) and the solid line shows expected values (Exp).



(personal communication, 2013), *Byasa daemonius* is the only observed pollinator of *A. delavayi*. The relatively high level of genetic differentiation and low level of gene flow among populations of *A. delavayi* strongly suggest that genetic drift might play an important role in the observed population differentiation. As *A. delavayi* is known to be insect pollinated, long-distance pollen dispersal is possible in light of the gene flow of $N_m = 0.576$ estimated from the nuclear ISSR markers. This species lacks efficient seed dispersal mechanisms, and the seeds are most likely dispersed by gravity within a short distance from their maternal plant. This suggestion is consistent with the observations that each population is fixed for a single haplotype indicated by the plastid markers. The limited dispersal of seeds and pollen in *A. delavayi* substantially reduces gene flow among populations and increases matings between individuals

Fig. 6. Network of the three haplotypes of *Aristolochia delavayi*. Circle size is proportional to haplotype frequency; 0 represents missing haplotype (not sampled or extinct).

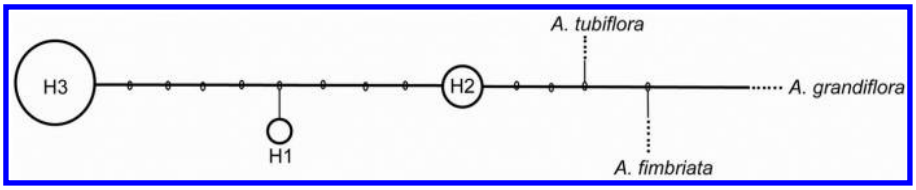
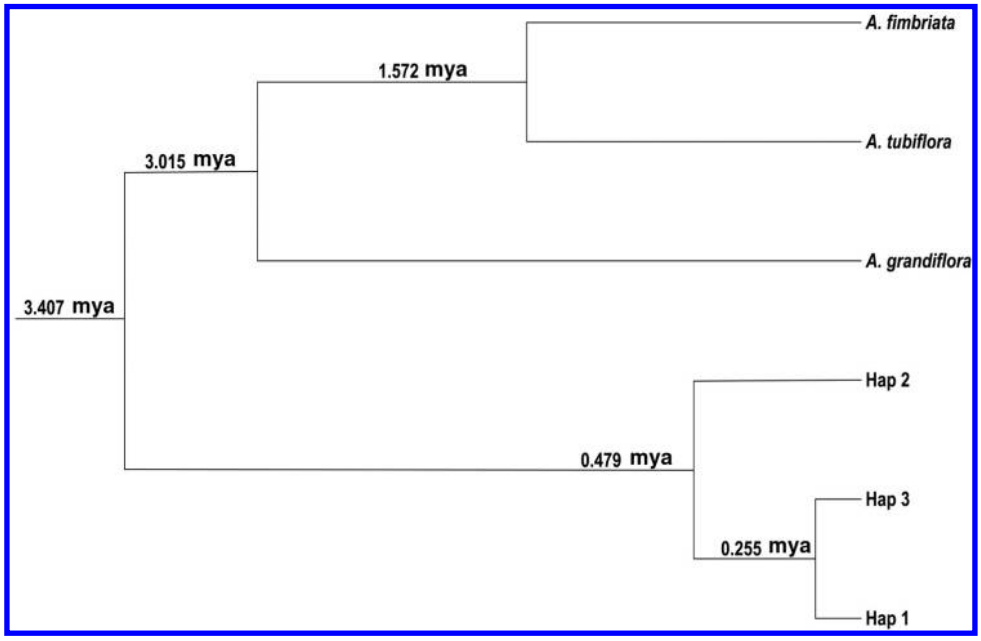


Fig. 7. Chronogram from coalescence analyses of the three chloroplast DNA haplotypes in *Aristolochia delavayi* detected from the *matK*, *trnL-trnT*, *rps4-trnT*, and *trnC-rpoB* regions. mya, million years ago.



within a small range, ultimately promoting interpopulation differentiation.

Demographic history

The results of this study imply an origin for *A. delavayi* at approximately 3.407 mya (95% HPD: 0.965–6.547 mya) and a subsequent divergence into three clades at 0.479 mya (95% HPD: 0.358–2.367 mya) and 0.255 mya (95% HPD: 0.051–1.241 mya). These two divergence events are generally consistent with two major glaciations in the Qinghai–Tibet Plateau and adjacent areas, the Naynayxungla glaciation (0.72–0.50 mya) and the Guxiang glaciation (0.30–0.13 mya) (Yang et al. 2012). These events may represent the principal causes of the intraspecific differentiation of this species. Furthermore, the intraspecific differentiation of *A. delavayi* may have been profoundly affected by the complex topography in the Hengduan Mountain region. The region is characterized by steep mountains (mean elevation > 5000 m a.s.l.) with narrow gorges and an alpine plain (Zhang et al. 2009*a*). During the Pleistocene, the Hengduan Mountains provided a refuge for many temperate taxa, which rapidly evolved in the ever-changing habitat of the uplifted plateau during postglacial periods (Zhang et al. 2009*b*). The high levels of genetic differentiation and the unique phylogeographic structure of *A. delavayi* could be explained by restricted gene flow caused by both the complex topography of the Hengduan Mountain region and the historical climatic oscillations. Such an inference is also supported by the high population differentiation documented by cytoplasmic DNA markers in other plants distributed in this region (Chen et al. 2010, 2011; Cun and Wang 2010; Li et al. 2009, 2011; Wang et al. 2008, 2011*a*, 2011*b*; Yuan et al. 2008; Yang et al. 2012).

Bayesian skyline plot and R_2 analyses performed in the study implied that *A. delavayi* experienced contraction, and mismatch distribution analyses suggested this species has not experienced demographic expansion. Anthropogenic habitat loss and fragmentation have been implicated in the endangerment and extinction of many species (Tilman et al. 1994; Hughes et al. 1997; Sih et al. 2000; Chiang et al. 2009).

Conservation strategy

The risk of extinction is higher for rare and endangered species with small populations than for species with large and stable populations, especially if gene flow among populations is rare (Frankham et al. 2002). Small populations are more susceptible to a loss of genetic diversity through genetic drift and inbreeding. Only four extant *A. delavayi* populations were found in the field in this study, and each population consisted of less than 50 individuals. Furthermore, our molecular data revealed low genetic differentiation and gene flow among populations. Based on the high genetic diversity of the species and the low number of individuals, conservation measures are urgently needed to increase the population size of this species.

Both the uniqueness of a population and its diversity level, especially in terms of its allelic composition, must be considered in the selection of populations to be conserved (Doyle and Wolfe 1991). Populations TB and LB share one plastid haplotype, and each population BC and population LJ of *A. delavayi* harbors a unique plastid haplotype, accordingly, they should be considered as three separate "evolutionarily significant units" (Moritz 1994), allowing for the preservation of specific, locally adapted genotypes in response to future biotic and (or) abiotic environmental

change. Despite the endangered status of *A. delavayi*, all four populations are located outside reserves. Additionally, another important factor that should be considered in designing conservation strategies for this species is hydropower construction in the Jinsha River drainage. According to field investigations, no populations will be flooded after the construction of the nearest hydropower station. However, the ecological environments in the vicinity of these populations will be subject to marked changes, such as an increase in humidity and a decrease in the annual temperature range. These changes could increase the probability of extinction of *A. delavayi*. Given the current severe habitat loss from human disturbance, habitat conservation that allows the remaining representatives of the species to survive will be extremely important for the conservation of *A. delavayi*. Ex situ conservation measures, including seed collections and storage in germplasm bank, transplantations to nearby reserves and (or) plant gardens and reintroductions to suitable habitats near the current wild populations are necessary for the recovery of the wild populations. When ex situ conservation is carried out, as many populations as possible should be protected because only four populations exist, most of which contain a private haplotype.

In addition, artificial translocation of individuals among populations will increase the gene flow and decrease the inbreeding depression among individuals separated by long distances (Storfer 1999; Epps et al. 2005). The exchange of individuals among genetically distinct populations is an alternative strategy to improve genetic diversity. However, the adaptive differences between populations of *A. delavayi* are unclear, and it is better to apply such measures to populations with low genetic differentiation, i.e., LB and TB.

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Appendix

Table A1. Chloroplast DNA sequence polymorphisms detected in *matK*, *trnL-trnT*, *rps4-trnT*, and *trnC-rpoB* regions of *Aristolochia delavayi*, in which three haplotypes were identified (H1–H3).

	matK		*trnL-trnT*			*rps4-trnT*		*trnC-rpoB*	
	235	527	227	490	525	167	331	312	398
H1	A	A	C	G	—	T	G	A	A
H2	C	G	T	T	TATA	A	T	A	A
H3	C	A	C	G	—	T	G	G	T