

Research paper

Genetic diversity and structure of the endemic and endangered species *Aristolochia delavayi* growing along the Jinsha RiverYu-Long Yu^{a, b}, Hui-Chun Wang^{b, c}, Zhi-Xiang Yu^d, Johann Schinnerl^e, Rong Tang^{b, c}, Yu-Peng Geng^{a, **}, Gao Chen^{b, f, *}^a School of Ecology and Environmental Science, Yunnan University, Kunming, 650091, China^b Yunnan Key Laboratory for Integrative Conservation of Plant Species with Extremely Small Populations, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, 650201, China^c University of Chinese Academy of Sciences, Beijing, 100049, China^d Sichuan Panzhihua Cycas National Nature Reserver Bureau, Panzhihua, 617000, China^e Department of Botany and Biodiversity Research, University of Vienna, Rennweg 14, A-1030, Vienna, Austria^f CAS Key Laboratory for Plant Biodiversity and Biogeography of East Asia, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, 650201, China

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ABSTRACT

The traditional medicinal plant, and endangered species *Aristolochia delavayi* (Aristolochiaceae) is an endemic species in China and occurs in the warm and dry areas along the Jinsha river. It is also a specific host of the larvae of *Byasa daemonius*, a vulnerable butterfly. In this study, 15 pairs of polymorphic microsatellite primers of *A. delavayi* were designed and screened based on the Simple Sequence Repeats (SSR) loci found by using the results of genome skimming. Based on these 15 SSR markers, the genetic diversity and structure of 193 individuals from ten natural populations were analyzed in detail. In comparison to other endemic and endangered plants in the region, the population of *A. delavayi* possess a relatively high genetic diversity ($H_e = 0.550$, $I = 1.112$). AMOVA analysis showed that 68.4% of the total genetic diversity was within populations and 31.6% of the variation occurred among populations. There was a significant genetic differentiation among natural populations of *A. delavayi* detectable, with low gene flow ($N_m = 0.591$). This might be attributed to geographical barriers and limited seed dispersal. To test the isolation by distance (IBD), we performed Mantel test, which showed a significant correlation between the geographic and genetic distances. In order to cope with the possible biases caused by IBD, we additionally performed Bayesian genetic cluster analyses and principal coordinate analysis (PCoA). The final cluster analysis revealed three groups with distinct geographical distribution. Habitat fragmentation and limited gene flow between these populations may be the main reasons for the current genetic structure. For conservation of this species, we suggest to divide its populations into three protection management units, with subsequent focus on the Yongsheng and Luquan populations which experienced a genetic bottleneck event in the past.

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1. Introduction

The perennial herb *Aristolochia delavayi* Franch. (Aristolochiaceae) is an endemic species in the dry-warm valleys along the Jinsha River in southwestern China (Chen et al., 2018; Yu et al., 2020). The plant species has a short single-flower flowering period (~3 days), but the duration of flowering within a population is approximate three months (mostly from June to September). It mainly reproduces in a sexual way, which relies on cross-pollination by pollinators, but the plant itself tends to increase

breeding opportunities through tillering (unpublished data). The fruits of *A. delavayi* are capsules, and its seeds are lacking elaiosomes. A previous study showed that seed dispersal mainly depends on gravity (Chen et al., 2015). Leaves of this plant species are locally used as a spice (Zhou et al., 1995; Sun et al., 2008) and also for medicinal purposes e.g. to strengthen the stomach, to increase the appetite and treat flatulence and malaria (Zhou and Yang, 1995). This plant species is also an important food source for the endangered butterfly species *Byasa daemonius* Alphéraky (Chen et al., 2015).

Aristolochia delavayi grows mainly in sparse vegetation in river valleys at an altitude of 1220–2250 m (Yang et al., 2014; He et al., 2017). The habitat in this area is fragile with high erosion rate, which makes vegetation restoration difficult (Zhong, 2000; Guan et al., 2013). Due to the habitat destruction by various human activities (e.g. hydropower construction, agricultural activities), and also the long-term overharvesting of this plant species, the populations of *A. delavayi* decreased gradually, so the species is on the verge of extinction (Yang et al., 2014; Chen et al., 2015). At present, *A. delavayi* is listed as a wild endangered species (EN) on the red list of higher plants in China and the red list of IUCN (Chen et al., 2015; Qin et al., 2017). It is also treated as a plant species with extremely small populations (PSESP) (Ma et al., 2013). To protect this species and to prevent extinction, protection measures are required.

Generally, before implementation of conservation, studying the genetic structure and diversity of a given species can help to develop scientific conservation strategies (Guan et al., 2013). The precipitous terrain and severe habitat fragmentation in the studied area may hinder gene flow among plant populations. Relevant studies revealed, the endemic taxa in this area generally show high genetic differentiation among populations, but the level of genetic diversity within populations is either high or low. This for example was shown on *Cycas panzhihuaensis* L. Zhou & S.Y. Yang (Xiao et al., 2020), *Musella lasiocarpa* (Franch.) C.Y. Wu (Ma et al., 2019), *Trilliaedoxa gracilis* W.W. Sm. & G. Forrest (Jia et al., 2016) and *Munronia delavayi* Franch. (Jia et al., 2014). By comparing the genetic differences between *A. delavayi* and other endemic plant species in this region, it is helpful to clarify the population history, its dynamics, and the effect of external environmental factors on the genetic pattern of the existing populations. Recent genetic analyses on *A. delavayi* was undertaken by Yang et al. (2014). They analyzed four populations using eight ISSR markers, which showed a high genetic diversity ($PPB = 84.71\%$). However, during our comprehensive field surveys in recent years, we found that the above-mentioned four populations are only located in the upstream part of the distribution area of *A. delavayi*, whilst the populations in the middle and lower part of the distribution area were not involved. Considering that *A. delavayi* is vulnerable, it is necessary to use a higher number of molecular markers to re-study and evaluate its population genetics comprehensively.

At present, molecular genetic marker methodologies have been well developed which now allow performing sophisticated analyses. In particular, microsatellite markers have widely been used in the genetic research of wild natural populations and endangered species (Balloux and Lugon-Moulin, 2002; Chen et al., 2009; Tang et al., 2008; Wang et al., 2006; Yang et al., 2018). This kind of markers are rich in polymorphism, good in repeatability, mostly codominant, widely distributed in the genome, and reveal a large amount of information even within a small sample size (Jarne and Lagoda, 1996; Zhang and Hou, 2004; Habel et al., 2010; Zhang et al., 2019). In this study, SSR primers for *A. delavayi* were developed for the first time by using genome skimming technology. Based on these primers, we were able to analyze the genetic structure and its diversity of existing wild populations of this species. The obtained

results allowed us to analyze the internal causes of the species' endangerment and propose reasonable conservation strategies.

2. Materials and methods

2.1. Plant materials

During the population and habitat surveys of the past few years, molecular samples of 193 individuals from ten populations of *Aristolochia delavayi* were collected along the Jinsha River Basin in July 2018 and August 2019. Detailed information about the plant material is provided in Table 1. Most of the samples were collected in Yunnan Province, except the samples belonging to the population ML, which was collected from Sichuan province (Fig. 1). From each population were more than 20 individuals collected, with a few exceptions, such as DD (only two individuals) and WB (seven individuals) due to the rare occurrence of this plant species. Fresh and healthy leaves were dried with silica gel immediately after collection and stored at $-20\text{ }^{\circ}\text{C}$ prior DNA extraction. Voucher specimens of partial populations were deposited in the Herbarium of Kunming Institute of Botany (KUN), Chinese Academy of Sciences.

2.2. Laboratory protocols

Genomic DNA was extracted from the dried leaves using a modified CTAB method (Doyle and Doyle, 1987), and the quality and concentration of DNA were measured by using a spectrophotometer (Nanodrop - 1000). Using the genome skimming technology, the genomic DNA from two individuals in the DJ and LQ populations were used for genome skimming. The specific process is as follows: The sequencing was performed on the Miseq Benchtop Sequencer (Illumina X-Ten) using the 2×250 bp read mode. The obtained data were assembled using software SPAdes and the microsatellite sequences of two samples were compared by using the software QDD 2.1 Beta (Meglec et al., 2010). MISA software was used to design SSR primers based on the flanking sequence of the SSR loci.

The overall PCR reaction system volume was 20 μL , including 1 μL DNA template of 50–60 ng/ μL ; 2 \times Taq PCR MasterMix 10 μL , 0.3 μL of each primer at a concentration of 10 $\mu\text{mol/L}$; and 8.4 μL ddH₂O. The amplification procedure was set as follows: Pre denaturation at 95 $^{\circ}\text{C}$ for 3 min; denaturation at 95 $^{\circ}\text{C}$ for 30 s, annealing at appropriate temperature for 30 s, extension at 72 $^{\circ}\text{C}$ for 30 s, a total of 32 cycles; and the final extension step at 72 $^{\circ}\text{C}$ for 5 min. The annealing temperature (T_m) was determined according to the reference value of primer synthesis and optimized in the experiment. The PCR reaction was performed on the DNA Thermal Cycler (Applied Biosystems).

For the primer screening, the genomic DNA of four individuals from four populations were selected for PCR amplification. The PCR products were detected using 8% non-denaturing polyacrylamide gel electrophoresis. The gel was stained with silver and developed after electrophoresis, and the amplification was observed. The amplification effects of primers were compared, and polymorphic primers were screened for genetic analysis of all samples. The PCR products were separated and visualized using QIAxcel of capillary gel electrophoresis system (ABI PRISM 3730 XL, USA). The final banding data were read by GeneMarker v.2.2.

2.3. Data analysis

To detect linkage disequilibrium (LD) between the loci, we used the program GENEPOP v.4.7 (Rousset, 2008). The following parameters of genetic diversity at population and species level were calculated by the GenAlEx v.6.41 (Peakall and Smouse, 2006):

Table 1
Information on sampled populations of *Aristolochia delavayi*.

Population	Location	Longitude (E)	Latitude (N)	Altitude (m)	Sample size
HTX	Tiger Leaping Gorge Town	100.051503	27.186242	1860	21
SB	Jiangbian Village, Sanba Town	100.228188	27.316460	1858	29
DJ	Daju Town	100.237005	27.334241	1732	24
XZ	Xiazhuen Village, Sanba Town	100.159363	27.486931	1931	24
ML	Eya Town	100.358434	27.767007	1832	20
DD	Dadong Town	100.449564	27.163259	1584	2
HP	Huangping Town	100.443849	26.027998	2002	21
YS	Dongshan Town	100.969334	26.241000	1322	21
WB	Wanbi Town	101.179270	26.342586	1222	7
LQ	Zehei Town	102.755256	26.158288	1228	24

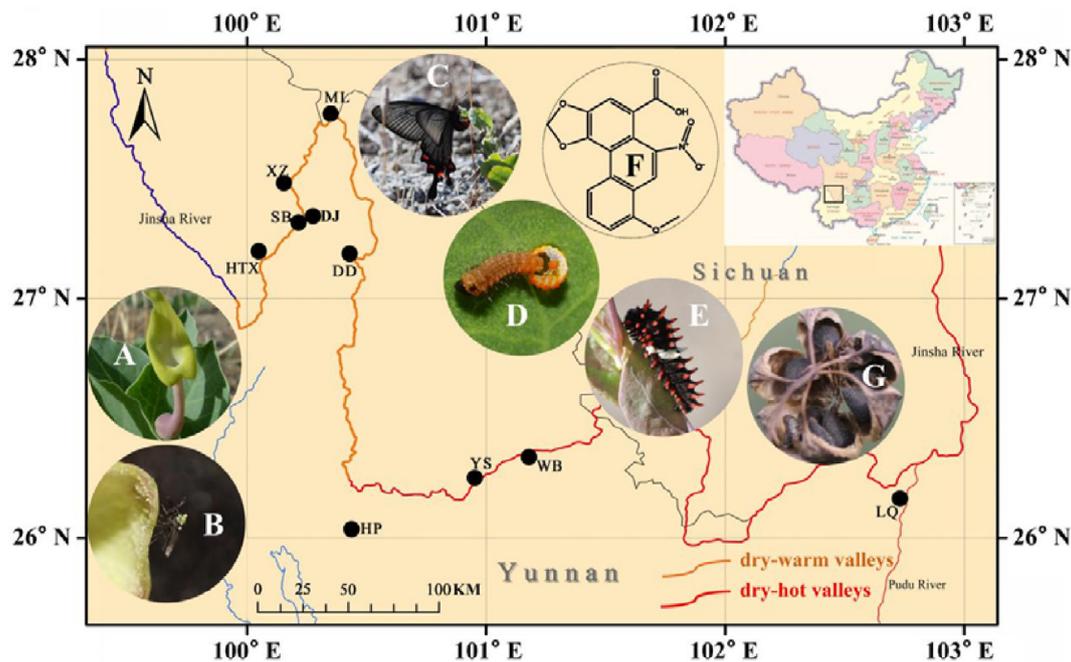


Fig. 1. Distribution of collected populations of *Aristolochia delavayi*. (A) A blooming flower (typical pollinator captive strategy in Aristolochiaceae, female flowering stage attracts pollinators and male flowering stage releases pollinators); (B) A pollinator carrying pollen crawls out of the split utricle; (C) A *Byasa daemonius* individual locates its spawning site based on the smell of *Aristolochia delavayi* leaves; (D) A newborn larvae leaving out of its shell (*Byasa daemonius*); (E) A larvae is feeding on *Aristolochia delavayi* leaf; (F) Aristolochic acid derivatives can be found in leaves and *Byasa daemonius* body parts (accumulated these compounds to repel its potential natural enemies); (G) Seed dispersal from dehiscent capsule by gravity. A, G pictures were taken by Gao Chen; B, D pictures were taken by Zhi Chen and C, E pictures were taken by Yang Niu.

Population sample sizes (N), number of alleles (N_a), effective number of alleles (N_e), Shannon's information index (I), observed (H_o) and expected (H_e) frequency of heterozygotes, percentage of polymorphic loci (PPL), and fixation index (F). The GenAlEx program was also used to calculate the inbreeding coefficient (F_{IS}) and gene flow (Nm) at population level.

The geographic distances between populations were calculated using the Franson CoordTrans Program v.2.3. Meanwhile, POPGENE v.1.32 was used to calculate the Nei's genetic distance between populations of *Aristolochia delavayi*. We established a geographic distance as well as a genetic distance matrix, and used Mantel test in GenAlEx v.6.41 to detect correlation between geographic and genetic distance (IBD) (Mantel, 1967; Smouse et al., 1986; Peakall and Smouse, 2006). The recent bottleneck effects were detected by using BOTTLENECK v.1.2.2. For this purpose, two evolutionary models, infinite alleles model (IAM) and two-phase model (TPM) (Wang et al., 2010), were selected, and 1000 iterations statistics were carried out by performing the Sign test and Wilcoxon sign-rank test, respectively.

Concerning the genetic structure, hierarchical analyses of molecular variance (AMOVA) were performed to assess the genetic

structure within and between populations by using Arlequin v.3.11 (Excoffier et al., 2005). Principal coordinate analysis (PCoA) was conducted using GenAlEx v.6.41 to test the genetic similarity of all the individuals in the included population (Peakall and Smouse, 2006). UPGMA cluster analysis based on the Nei's genetic distance between populations was performed by using MEGA v.7.0.14. Bayesian clustering method in Structure v.2.3.4 was used to cluster all individuals individually and determine subsequently the number of genetic groups (Pritchard et al., 2000). The parameters for the calculation of the ΔK value and analyze possible genetic structure were set as follows: The K value was set to 10, 10 replicates, using the admixture model and assumed allele frequencies correlated, followed by a burn-in of 1×10^6 iterations (Evanno et al., 2005).

3. Results

3.1. Development and screening of SSR primers

In this study, 3689 pairs of SSR primers were successfully discovered and designed based on the genome skimming technique, and 100 pairs of primers were randomly selected for

polymorphism primer screening. Finally, 15 pairs of SSR primers with good amplification effects and high polymorphism were selected and used for genetic analysis. The details of these primers are shown in Table 2.

3.2. Genetic diversity of the *Aristolochia delavayi*

The GENEPOP test showed that only a few detectable loci were linked in a few populations, but none of them were linked with other loci in more than three populations. Due to this result, the above-mentioned 15 pairs of SSR primers were used for the genetic analysis of *A. delavayi* (Wang et al., 2013) and the results of these analyses are presented in Tables 3 and 4. Overall, a total of 78.50 alleles (N_a) and 44.45 effective alleles (N_e) were detectable. Within these 15 pairs of SSR primers, 3–10 alleles and 2–6 effective alleles with an average of 5.23 alleles and 2.96 effective alleles could be detected (Table 3). The calculated observed heterozygosity (H_o) varied from 0.333 (DD) to 0.705 (SB), whilst the expected heterozygosity (H_e) varied from 0.233 (DD) to 0.677 (XZ). The calculated Shannon information index (I) ranges from 0.335 (DD) to 1.485 (SB). At population level, except the populations DD and WB, the percentages of polymorphic loci (PPL) in all other populations was 100% (Table 4). The calculated inbreeding coefficient (F_{IS}) value varied from –0.336 to 0.319, with an average value of 0.056. The average values of the genetic differentiation coefficient (F_{ST}) and the gene flow (N_m) among populations are 0.328 and 0.591, respectively.

In the performed Bottleneck test, under the IAM hypothesis, both methods exhibited, that the population YS deviated from the mutation drift equilibrium, showing a significant heterozygosity excess. Under the TPM hypothesis, the sign test revealed that the population LQ deviated from the mutation drift equilibrium, exhibiting a significant heterozygosity excess (Table 5). This test indicated, that the above-mentioned two populations have recently experienced a bottleneck effect.

Table 2
Information of 15 pairs of polymorphic SSR primers for *Aristolochia delavayi*.

Primer No.	Primer sequence (5'–3')	Repeat motif	Fragment size (bp)	Tm (°C)
YYL-40	F:AAGCAGGATGTGGGTAATGG R:CCTACCCAACAAGGAAACGA	(CTT) ₁₀	304–325	55.4
YYL-42	F:CAAGGGAAGTGCACACATTG R:GGCTGACCTTAGCCTGAATG	(TAA) ₁₁	254–284	56.4
YYL-43	F:TTGCGATGCTAGAGAACACG R:ACGTACCAAGATGGCACTC	(AGA) ₁₁	175–205	56.4
YYL-45	F:AGAGGGGTAAGAGAAAGCGG R:GAATCCTGTACCAGCGGAGA	(GAA) ₁₂	246–267	57.4
YYL-47	F:CATGCATCAGGAGTTGTCT R:AGTTGCAGAGAAGGGAACGA	(AAG) ₁₂	140–164	55.4
YYL-48	F:GTATTACCACCATGGGGACG R:CGGAGCTCCATCTTCATCTC	(GAA) ₁₃	252–279	57.4
YYL-49	F:TAATCACCTGCTTCCTGCTG R:CTCTTGAGTACTGGGGCGAC	(GAA) ₁₄	306–330	57.4
YYL-54	F:CAGATTCGACGACGTCATGT R:GCCTACGTAATGATGCCCTT	(CGAT) ₅	246–278	56.4
YYL-59	F:ACACCCGTTTCGATTGAAG R:CTTGCTTTTGTGGTTGGGTT	(AGAA) ₅	193–205	53.8
YYL-68	F:CACGATCGGATCATCAACAC R:CTTTGTCTCTCCAGCTTC	(TGGA) ₆	215–247	56.4
YYL-69	F:AGATACATCGAATTGGGCG R:GGGATGGATTGGCTTAAGTTT	(TGTT) ₇	133–161	53.8
YYL-70	F:TCCACAGCCACTAAATTC R:TCGACGATCTCAAAATTC	(ATCT) ₇	163–199	54.6
YYL-78	F:TCGTGGAAGAACCAATTTTC R:CACGCCATGGAACACTACAG	(AATC) ₈	238–294	55.4
YYL-81	F:TAACGGGCAAACTGGAATC R:ATAGAAACCCGCAATCATGC	(TTTAT) ₅	195–215	53.8
YYL-86	F:CCTCACAAGGCCACAAGAAAT R:CAATTCTCAAACCGTCCAT	(AAATA) ₅	249–274	54.6

Table 3
Summary of genetic statistics and Wright's F-statistics of each loci in the wild population of *Aristolochia delavayi*.

Locus	Sample size	N_a	N_e	F_{IS}	F_{IT}	F_{ST}	N_m
YYL40	193	5.200	2.239	0.253	0.640	0.527	0.224
YYL42	193	6.300	3.664	–0.150	0.027	0.205	0.967
YYL43	193	7.400	4.146	0.004	0.143	0.217	0.902
YYL45	193	4.500	2.406	0.046	0.387	0.307	0.566
YYL47	193	4.800	2.556	–0.010	0.245	0.316	0.540
YYL48	193	5.100	3.032	0.046	0.204	0.258	0.718
YYL49	193	7.400	3.931	0.207	0.401	0.283	0.633
YYL54	193	4.600	2.248	0.199	0.435	0.364	0.437
YYL59	193	3.300	2.069	–0.083	0.391	0.429	0.333
YYL68	193	3.800	2.478	–0.336	–0.028	0.239	0.794
YYL69	193	4.700	2.338	0.319	0.616	0.446	0.311
YYL70	193	5.100	3.171	0.129	0.403	0.316	0.542
YYL78	193	9.600	6.391	0.048	0.164	0.172	1.206
YYL81	193	3.500	1.64	0.111	0.457	0.469	0.283
YYL86	193	3.200	2.139	0.058	0.382	0.378	0.412
Mean		5.233	2.963	0.056	0.324	0.328	0.591

Table 4
Summary of genetic statistics for *Aristolochia delavayi* at population level.

Pop	N	N_a	N_e	H_o	H_e	I	F	PPL
HTX	21	5.600	3.252	0.644	0.585	1.197	–0.056	100.00%
SB	29	7.733	4.071	0.705	0.673	1.485	–0.067	100.00%
DJ	24	6.200	3.309	0.608	0.613	1.289	0.014	100.00%
XZ	24	7.133	3.840	0.579	0.677	1.467	0.121	100.00%
ML	20	5.067	3.072	0.494	0.582	1.169	0.129	100.00%
DD	2	1.533	1.427	0.333	0.233	0.335	–0.417	53.33%
HP	21	4.200	2.080	0.386	0.433	0.829	0.151	100.00%
YS	21	4.933	2.948	0.581	0.579	1.127	0.012	100.00%
WB	7	4.000	2.736	0.667	0.556	1.046	–0.193	93.33%
LQ	24	5.933	2.897	0.530	0.566	1.180	0.144	100.00%
Mean		5.233	2.963	0.553	0.550	1.112	0.005	94.67%

Table 5
Bottleneck effect test of *Aristolochia delavayi* population based on IAM and TPM models, and two different detection methods.

Population	Mutation-drift equilibrium test model			
	Infinite allele model (IAM)		Two-phase model (TPM)	
	Sign test	Wilcoxon test	Sign test	Wilcoxon test
HTX	0.28867	0.33026	0.51241	0.89038
SB	0.38354	0.15143	0.21489	0.56140
DJ	0.54858	0.52448	0.58928	0.48871
XZ	0.58743	0.10699	0.42055	0.35913
ML	0.05394	0.05536	0.53515	0.38940
DD	0.12492	0.31250	0.18390	0.31250
HP	0.56735	0.63867	0.28262	0.27686
YS	0.04278 ^a	0.02155 ^a	0.16179	0.30280
WB	0.16491	0.24121	0.49945	0.76086
LQ	0.55134	0.89038	0.01079 ^a	0.08325

Note: P-value is the test of heterozygosity excess.

^asignificant with $P < 0.05$.

3.3. Genetic differentiation and genetic structure

The AMOVA results (Table 6) indicated significant differences among populations of *Aristolochia delavayi*, because 31.62% of the variation existed among populations and 68.38% occurred within populations. The genetic differentiation coefficient (F_{ST}) at species level is 0.316, which is considered as significant, i.e. $0.25 < F_{ST} < 1$. This indicates a significant genetic differentiation among populations (Wright, 1978). The performed Mantel test resulted in a significant positive correlation between the genetic distances of the studied populations and its geographic distance ($P = 0.01 < 0.05$ and $r = 0.561 > 0.5$) (Fig. 2), which also indicates the presence of IBD in the population structure. The result of principal coordinate analysis indicated, that the analyzed ten natural populations can be roughly grouped into three groups (Group A includes populations HTX, SB, DJ, XZ, ML, DD, Group B includes YS, WB, LQ and Group C includes HP) (Fig. 3). This result was supported by the performed UPGMA cluster analysis based on genetic distance (Fig. 4). Structural analysis showed, a ΔK is at the maximum, when $K = 6$. This indicated that all populations can be assembled in six groups. These results further refine the grouping results of the above-mentioned methods. Among all analyzed samples, six populations collected in Northwestern Yunnan consist of three groups; the three populations in Central Yunnan clustered in two groups, and the Huangping population was still remained separately (Fig. 5).

4. Discussion

4.1. Genetic diversity of *Aristolochia delavayi*

Genetic variation of species is the premise of local adaptation and evolution. It is also considered as an important parameter to determine the priority of population conservation in the protection of endangered plants (Schaal et al., 1998; Laikre et al., 2010; Zhao

and Gong, 2015). So, understanding the genetic status of *Aristolochia delavayi* provides a rational basis for the evaluation of conservation work and formulating of effective protection measures. Young et al. (1996) showed that genetic variation of population eroded with reduced remnant population size. However, our analysis results showed that the expected and observed heterozygosity of *A. delavayi* at species level are 0.550 and 0.553, respectively. These values are higher than reported values from other endemic or endangered taxa in this region, such as *Nouelia insignis* Franch. ($H_e = 0.149$, $H_o = 0.216$) (Luan et al., 2006), *Buddleja crispa* Benth. ($H = 0.314$, $I = 0.485$) (Zhang et al., 2015), *Cycas hongheensis* S.Y. Yang & S.L. Yang ex D.Y. Wang ($H_e = 0.435$, $H_o = 0.403$) (Zhao and Gong, 2015), *T. gracilis* W.W. Smith et Forrest ($H_s = 0.489$) (Jia et al., 2016), *Amorphophallus albus* P.Y. Liu & J.F. Chen ($H_e = 0.504$, $H_o = 0.528$) (Tang et al., 2020), *C. panzhihuaensis* L. Zhou & S.Y. Yang ($H_e = 0.328$, $H_o = 0.189$) (Xiao et al., 2020). Our calculated result is consistent with the result reported by Yang et al. (2014), indicating that the species possess relatively high genetic diversity. Moreover, this indicates that the recent population size reduction only affects little the genetic variation of the species. According to Loveless and Hamrick (1984) and Nybom and Bartish (2000), the factors which affect the genetic variation of species generally include population history, population size, reproduction pattern, breeding system, genetic drift, gene flow, natural selection, geographical distribution, etc. The reduction of the population size cause genetic drift effects, but drift has only a little effect on genetic variation in limited generations (Young et al., 1996). The recent habitat fragmentation event may be below the fragmentation threshold, which probably not cause the loss of genetic variation (Prober and Brown, 1994). We assume that in the past few decades, with the rapid reduction of the population size, the genetic drift effects are not yet accumulated, and the species may retain its genetic variation by its sexual as well as asexual reproduction.

4.2. Genetic differentiation and structure

The results of AMOVA analysis showed that the genetic variation of *Aristolochia delavayi* occurred mainly within the population, but there was a significant genetic differentiation among the populations observable. The high percentage of genetic diversity within the population may be due to the retention of genetic resources prior the population reduction and to the outcrossing of sexual reproduction within the population. For genetic differentiation among populations, it is generally considered to be caused by restricted gene flow (Loveless and Hamrick, 1984; Slatkin, 1985). The results of this study showed, that the level of gene flow among the population is generally low ($N_m = 0.591 < 1$). This reduced gene flow may be related to the limited reproductive characteristics of the plant itself.

During the performed field work, it was observed that the sexual propagation strategy of *Aristolochia delavayi* relies on the participation of pollinators. The main pollinators are some small flies belonging to the families Ceratopogonidae and Chironomidae, which basically have weak flying abilities and low pollination

Table 6
Analysis of molecular variance (AMOVA) within/among *Aristolochia delavayi* populations.

Source of variation	df	Sum of squares	Variance components	Percentage of variation [%]	F_{ST}
Among groups	1	200.41	0.78	12.28	
Among populations within groups	8	401.98	1.23	19.34	
Within populations	376	1641.18	4.36	68.38	0.32 ^a
Total	385	2243.56	6.38	100	

Note: df means degree of freedom; grouping of populations: Yunnan northwestern group (populations HTX, SB, DJ, XZ, ML and DD) and Yunnan central group (populations HP, YS, WB and LQ).

^aextremely significant with $P < 0.01$.

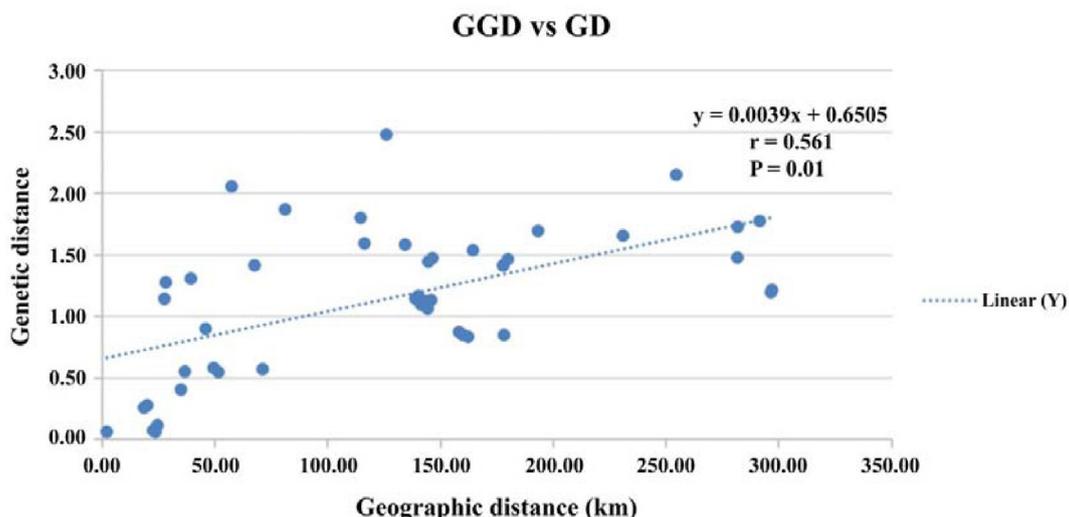


Fig. 2. Correlation analysis of geographic distance (GGD) and genetic distance (GD) of ten populations of *Aristolochia delavayi* (Mantel test).

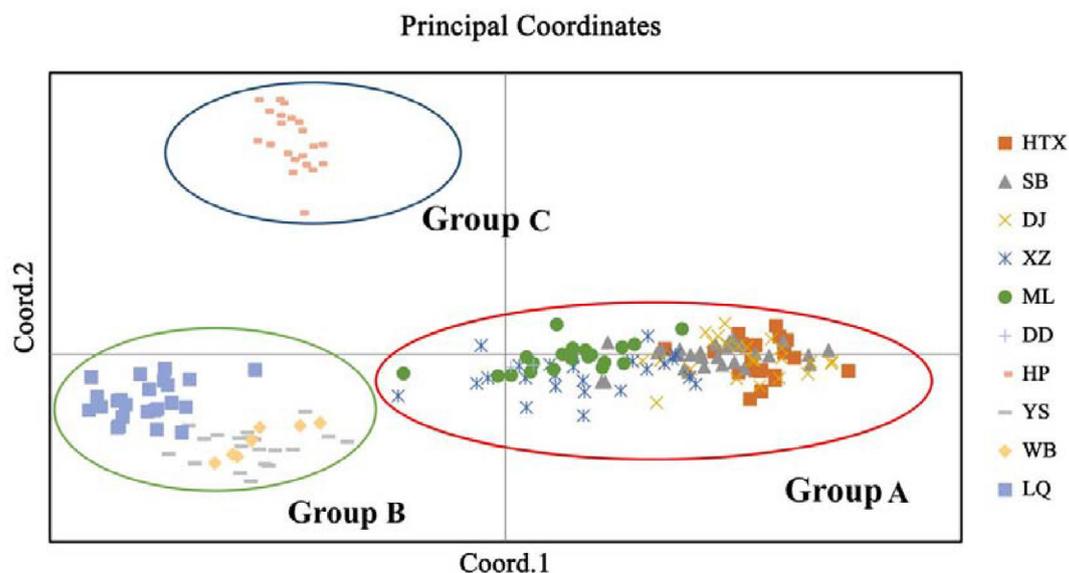


Fig. 3. The result of Principal Coordinates Analysis (PCoA) of *Aristolochia delavayi* populations. The first and second axis explained 34.13% and 25.23% of the total genetic variance, respectively.

efficiency. The seed dispersal only depends on gravity with a limited dispersal distance, and the seeds are prone to dry under this climatic circumstance. In addition, the active geological structure and erosion in the area caused major rivers in the region which formed valleys with a steep and rugged topographies. This became an obstacle for gene exchange among most plant species in the region as reported previously (Yue et al., 2012; Zhang et al., 2015; Tang et al., 2020). At the same time, the fragmentation of habitat caused by human activities became another obstacle for the gene flow of the studied plant species. Presumably, the restricted pollen and seed dispersal mechanisms of *A. delavayi* and its relative geographical isolation may be the main reasons for the observed genetic differentiation among populations of this species. Though the limited number of populations, the present outcrossing reproductive strategy may enable sufficient gene exchange among individuals, which maintain a high genetic diversity within the population.

The performed Mantel test detected isolation by distance (IBD) in the population structure of the studied species, which indicated that geographic barriers played an important role in the formation of the present genetic structure of populations, but there may be potential biases in further cluster analysis (Perez et al., 2018). Therefore, we have performed principal coordinate analysis. In this study, the results of principal coordinate analysis and UPGMA clustering are consistent, showing a clear geographic regionality. The ten populations of *Aristolochia delavayi* are assembled in three groups, which are clustered in Northwest Yunnan, Central Yunnan, and Huangping areas, respectively. Bayes cluster analysis further refined the above grouping results. The genetic clustering results of the three methods all disclosed the separation of the Huangping population. This indicates a distant genetic relationship with the other studied populations. The geographical distribution of this population is rather isolated, far away from the Jinsha River and its tributaries.

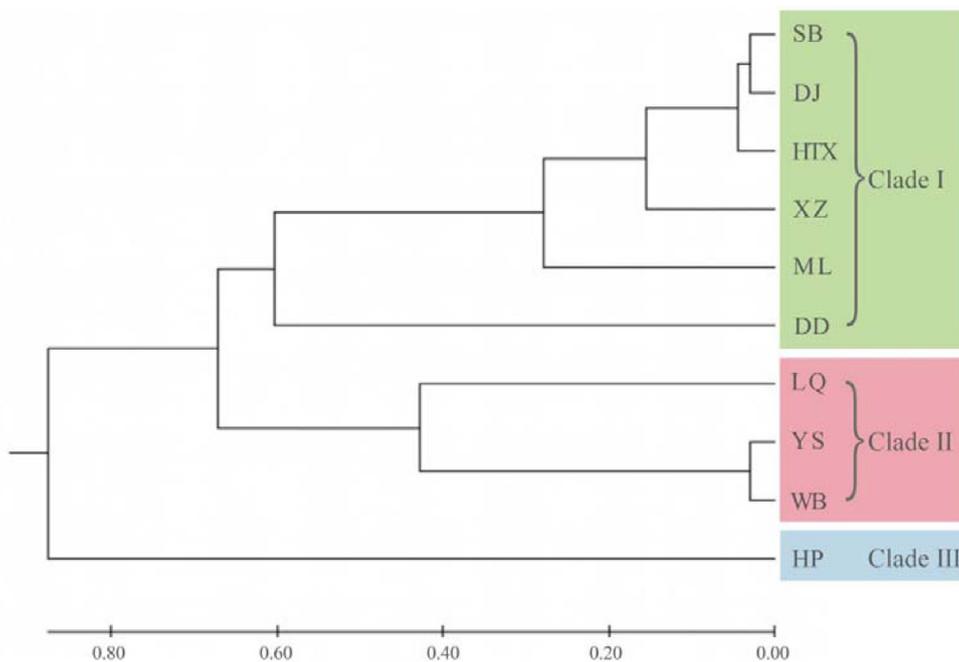


Fig. 4. UPGMA dendrogram based on Nei's genetic distance. Population codes see Table 1.

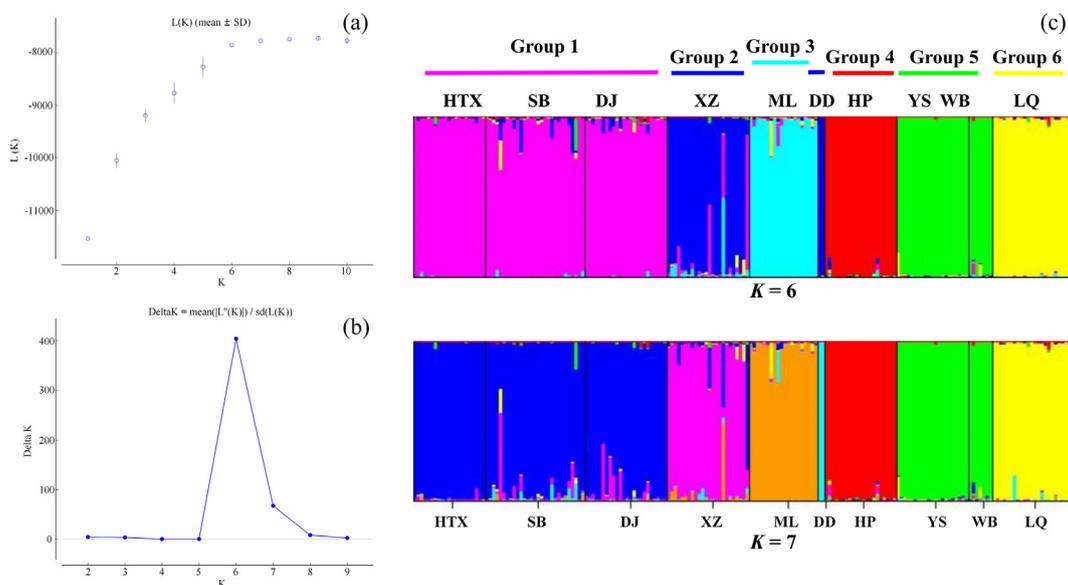


Fig. 5. Genetic structure of *Aristolochia delavayi* inferred by Bayesian clustering of SSR data. (a) Plot of mean posterior probability $\ln(K)$ values in the range of K from 1 to 10; (b) The corresponding ΔK statistics calculated according to Evanno et al. (2005); (c) Bar plots showing assignment probabilities from structure analysis when $K = 6$ and $K = 7$, the same color in the figure represents the same cluster. Population codes see Table 1.

Compared to other populations, there were no obvious differences in the main taxonomic characteristics (flower and leaf) of *A. delavayi* in Huangping population. But the different plant size (Fig. 6), leaf scent (Yu et al., 2020), and diverged genetic data suggest that this population could be a subspecies or variety of *A. delavayi*. But further efforts are required to validate this.

4.3. Conservation suggestions

Aristolochia delavayi is an important species in the traditional medicinal and as spice, and its essential oil is essential for the

development and application of antibacterial drugs (Li et al., 2013). At the same time, this species is an important host of the endangered butterfly *Byasa daemonius*. To sustain this plant species may effectively promote the gradual recovery of the *B. daemonius*, and may also avoid cascading effects (Koh, 2004; Brodie et al., 2014; Chen et al., 2015). Over the past few decades, the habitat destruction and overharvesting by human activities have led to a rapid decline in the population sizes. At present, only ten isolated populations of *A. delavayi* were found and most of them comprise of only few individuals. Therefore, the wild resource of the plant species needs effective protection urgently.

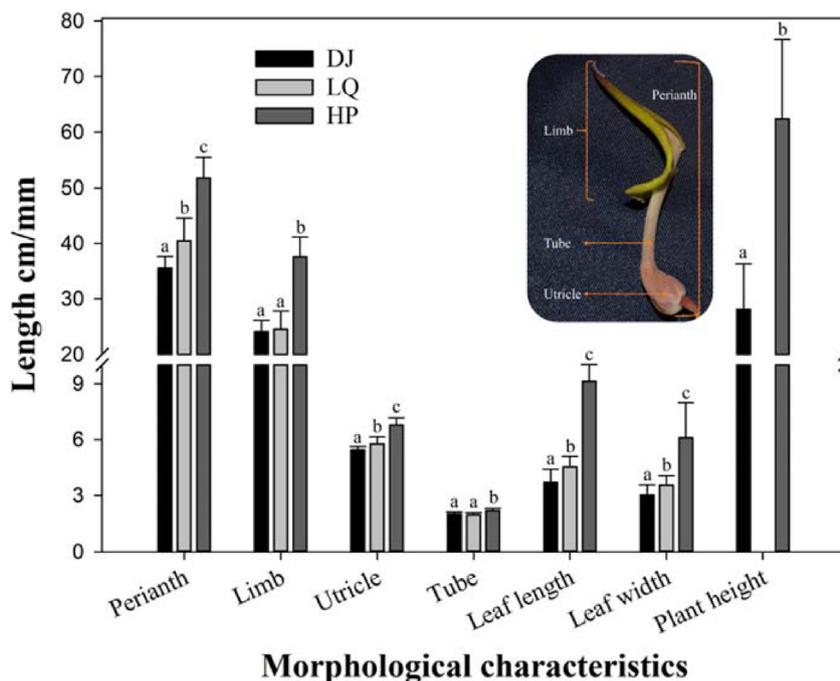


Fig. 6. Comparison of flower and leaf morphological characteristics of *Aristolochia delavayi* from different populations. The two populations of DJ (Daju) and LQ (Luquan) are the farthest apart in geographical distribution, HP (Huangping) population is far away from the Jinsha River, a main distribution area of *A. delavayi*. Except for the leaf length, width and plant height in cm, the other features are in mm. Sample sizes of each population: flowers ($n = 32$) and leaves ($n = 90$). a, b and c represent the significant difference ($P < 0.05$). The plant height data of LQ is lacking because of the plants have been destroyed before measurement.

Because of the rarity of wild populations of *Aristolochia delavayi* and its significant genetic differentiation among the populations, it is necessary to protect the present populations. In particular, the two populations (YS and LQ), which have recently experienced genetic bottlenecks, would need special attention. For example, intensify the implementation of *in situ* protection may maintain the existing population size, and improve their population adaptability.

In view of the uniqueness of Huangping population from other populations, it is recommended to perform *in situ* conservation measures to sustain the current genetic resources for future research and utilization. For the Dadong population, due to the limited number of individuals found within this population, it is recommended to conduct first a thorough survey in order to assess the size of the existing population. Afterwards propagation of these plants, *ex situ* conservation should be implemented. Additionally, re-introduction of these cultivated individuals should be performed in order to increase the number of individuals within this population. If *ex situ* conservation measures are implemented, the pollination and seed dispersal processes should be taken into account, otherwise limited pollinators and seed dispersers may impact the reproduction systems negatively (Tang et al., 2019).

According to the genetic differences of the populations of *Aristolochia delavayi* in different regions, it is recommended to divide them into three protection units. Due to the low gene flow among populations, measures such as artificial pollination and seed dispersal may be taken into account. This would ensure and, as a consequence, increase the gene flow between populations. These measures should be carried out within the same protection unit in order to avoid outbreeding depression (Tallmon et al., 2004; Barmentlo et al., 2018).

Remarkably, a large number of *Aristolochia zhongdianensis* J.S. Ma individuals are mixed with *A. delavayi* in the population SB. Our morphological data indicated that some suspected hybrids occurred in this population (unpublished data). Previous studies

exhibited, that in two species showing hybridization phenomenon, gene introgression tends to dilute genetic variation within a small population (Levin et al., 1996; Wolf et al., 2001). Therefore, further research is needed to reveal possible gene introgression between *A. delavayi* and *A. zhongdianensis* in the population SB. This would clarify the direction of gene flow, and the trends of genetic variation of the population. Such studies may also provide a scientific guidance for reasonable and successful protection.

Author contributions

Yu-Long Yu and Gao Chen collected plant materials, and Zhi-Xiang Yu assisted in the field population survey. Yu-Long Yu independently performed the experiments, analyzed the data and wrote the manuscript. Hui-Chun Wang and Johann Schinnerl provided assistance in the language modification and polish of the manuscript. Rong Tang provided useful suggestions for the writing of the article. Gao Chen and Yu-Peng Geng designed and supervised the study, and also revised the manuscript. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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