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Genetic diversity of the endangered Chinese endemic herb *Dayaoshania cotinifolia* (Gesneriaceae) revealed by simple sequence repeat (SSR) markers

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ABSTRACT

Dayaoshania cotinifolia W.T. Wang is a critically endangered perennial herb endemic to southern China. Simple sequence repeat (SSR) markers were employed to assess genetic diversity in two populations of *D. cotinifolia*. Eight primer pairs generated a total of 36 alleles, with a mean of 4.5 alleles per locus. The expected heterozygosity ($H_e = 0.416$) and observed heterozygosity ($H_o = 0.508$) indicated a moderate level of genetic diversity, though the genetic differentiation between the populations was low ($F_{st} = 0.014$), a result that was supported by a higher gene flow ($N_m = 18.000$). No severe bottleneck effect was detected in the two populations. Thus, the endangered status of this species is most likely due to anthropologic effects rather than a lack of genetic diversity. *In situ* conservation strategies should be promoted, and the sizes of the populations should be increased through artificial breeding.

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1. Introduction

Dayaoshania cotinifolia W.T. Wang, the sole representative of the monospecific genus *Dayaoshania* (Gesneriaceae) (Wang, 1983), is a perennial herb endemic to southern China where it has a very restricted distribution in the Dayaoshan Mountains. *D. cotinifolia* is a primitive diploid species in the Gesneriaceae family (Liu, 2003) and, thus, is well suited for expanding our understanding of the evolutionary history of this family. In addition, *D. cotinifolia* is a valuable wild flower resource (Li and Wang, 2004).

Many species of the Gesneriaceae family grow in special soils and under special conditions, thus their natural distribution is restricted to narrow and specific habitats. In general, the number and size of populations of these species are small, and large morphological differentiation occurs between populations of the same species (Wei et al., 2004). However, a population with these characteristics is often susceptible to habitat change, and the population size should rapidly decrease or even disappear when the environment becomes unfavorable (Wen and Li, 2005). *D. cotinifolia* individuals, which are characteristically small and have shallow root systems, are usually found under moist conditions in subtropical evergreen broad-leaved forests and are sensitive to changes in air humidity and topsoil moisture (Wang et al., 2008). Because of increasing anthropogenic disturbance, the number and size of *D. cotinifolia* populations have drastically decreased in recent decades. At present, only two populations are known to exist, Dumuqiao (DUQ) and Qingnianqu (QNQ), with a mere 1000 individuals

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(Wang et al., 2008); no more than 100 individuals have been found in the QNQ population. *D. cotinifolia* was listed among the 'first class protected key wild plants of China' in 1999 and has been registered as a critically endangered species on the China Species Red List (Wang and Xie, 2004). Given this serious situation, it is urgent that suitable protection strategies be implemented for *D. cotinifolia*.

However, since the description of *D. cotinifolia* in 1983 (Wang, 1983), there have been few related articles or studies. On the basis of field investigations of the habitat extent and biological and ecological characteristics of this species, Wang et al. (2008) analyzed the causes of endangerment and proposed a method of changing the endangered status to prevent extinction. Recently, Wang et al. (2011b) investigated the flowering phenology features and breeding system of *D. cotinifolia*. These authors postulated that an unstable pollination environment for reproduction is likely a major factor leading to the endangered status of *D. cotinifolia*, as self-pollination helps this species to adapt to an unstable environment during the blooming season. Because genetic diversity and genetic structure are the bases of a proper conservation strategy, it is necessary to study the population genetics of *D. cotinifolia* using suitable molecular markers.

Simple sequence repeat (SSR) markers are powerful tools for investigating the population genetics of wild species. Because they are unique in their abundance and random distribution throughout the eukaryotic genome, microsatellite markers are widely used to investigate genetic diversity, genetic structure, and gene flow within populations (White et al., 2002; Nybom, 2004; Yuan et al., 2012; Zhai et al., 2012). This report describes the use of SSR markers in a genetic diversity study of *D. cotinifolia*. The main objectives were to (a) characterize the level of genetic diversity in this critically endangered species, (b) reveal the gene flow between the populations, (c) detect bottlenecks in the populations, and (d) estimate the extent of inbreeding. On the basis of these results, feasible approaches for *D. cotinifolia* conservation are suggested.

2. Materials and methods

2.1. Plant sampling

The two extant populations of *D. cotinifolia* are located in the Dayaoshan Mountains, and the two habitats are approximately 1000 m apart. Leaf samples of 30 or 31 randomly chosen individuals per population were collected (Table 1). For each individual, one medium-sized healthy leaf was chosen and dried rapidly in a Ziploc bag using at least a 10-fold greater volume of silica gel. The leaf tissue was then stored at ambient temperature until DNA extraction. The voucher specimens were deposited in the herbarium of Henan agricultural university (HEAC).

2.2. DNA extraction and microsatellite genotyping

Genomic DNA was extracted from the silica gel-dried leaves using a modified cetyltrimethylammonium bromide (CTAB) technique (Fang et al., 2009). The extracted DNA was dissolved in 100 μl TE buffer, and the quality and quantity of the DNA were determined using a UV-spectrophotometer (ND-2000, NanoDrop, USA). The DNA concentrations were adjusted to 30 ng/μl for all samples, and the samples were stored at −20 °C for subsequent use.

The genotypes of 61 individuals were determined using eight polymorphic microsatellite markers developed for this study (Zhang et al., 2011). PCR was performed in a 15 μl volume containing approximately 75 ng genomic DNA, 10 μM each of fluorescently labeled reverse and forward primers (FAM or HEX), and 1× PCR Mix (Tiangen Biotech, China). The microsatellites were amplified under the following conditions: 5 min initial denaturation at 95 °C, 36 cycles of 30 s at 94 °C, 30 s at 48–60 °C, and 1 min at 72 °C, and a final extension at 72 °C for 10 min. The amplified products were separated using an ABI 3730xl DNA Analyzer with GeneScanTM600 LIZ (Applied Biosystems, USA) as an internal size standard, and the sizes were determined using GeneMapper ver. 4.0 (Applied Biosystems, USA).

2.3. Data analysis

In each population, the number of alleles per locus (N_a), the observed and expected heterozygosity (H_o and H_e), and the deviation from the Hardy–Weinberg equilibrium (WHE) were analyzed using the computer program package Arlequin suite version 3.5 (Excoffier and Lischer, 2010).

Table 1
Genetic diversity parameters estimated at eight SSR loci in two *D. cotinifolia* populations.

Population	Location	Longitude (°E)	Latitude (°N)	Sample size	A[a]	Pa[b]	H_o[c]	H_e[d]	F_{is}[e]
DUQ	Dumuqiao, Guangxi	110°12.647′	24°06.303′	30	3.71	7	0.548	0.442	−0.256
QNQ	Qingnianqu, Guangxi	110°12.436′	24°06.045′	31	3.50	8	0.536	0.440	−0.220
Average				30.5	3.61	7.5	0.542	0.441	−0.238

[a] Observed number of alleles.
[b] The number of private alleles.
[c] Observed heterozygosity.
[d] Expected heterozygosity.
[e] Fixation index.

Wright's fixation index (F_{is}) (Wright, 1978), a measure of heterozygote deficiency or excess, was calculated using POPGENE (Yeh et al., 1999), and the significance was tested with 1000 permutations. In addition, Nei's unbiased measures of genetic distance, F_{st} and N_m (Nei, 1987), were estimated using POPGENE software. F_{st} is the coefficient of the genetic differentiation among populations under an infinite allele model (IAM). N_m, the average number of migrants between populations per generation, is derived from the formula $N_m = 0.25(1 - F_{st})/F_{st}$ (Slatkin and Marton, 1989).

The BOTTLENECK program was employed to evaluate whether the populations examined had experienced recent bottlenecks (Piry et al., 1999). Three models for locus evolution, namely, the infinite allele mutation model (IAM), the stepwise mutation model (SMM), and the two-phased model of mutation (TPM, under which 90% of the mutations were assumed to occur under the stepwise mutation model and 10% under the infinite allele model), were used for the analyses with the Sign test and Wilcoxon's signed rank test (Piry et al., 1999). The mode shift away from an L-shaped distribution of allelic frequencies was also tested using BOTTLENECK.

3. Results

3.1. Genetic diversity

The characteristics of the eight microsatellite loci examined are shown in Table 2. In total, 36 alleles were detected, and the mean number of alleles per locus was 4.5. A significant deviation ($P < 0.05$) from HWE was detected in six of the eight loci, possibly as a result of dominant outcrossing. The genetic diversity parameters for each population, as based on the allelic frequencies, are presented in Table 1. A moderate level of genetic diversity was observed in each population, as indicated by the expected heterozygosity (H_e) estimates of 0.442 (population DMQ) and 0.440 (population QNQ), with an average of 0.441. The average observed heterozygosity (H_o) had slightly higher values than H_e, ranging from 0.536 (QNQ) to 0.548 (DMQ), with a mean value of 0.542. Consequently, the F_{is} calculated for each population, ranged from −0.220 (QNQ) to −0.256 (DMQ), with a mean of −0.238 (Table 1).

3.2. Genetic differentiation and gene flow between populations

Private alleles (Pa) reflect the specific evolutionary history of a population. A total of seven and eight private alleles were found in the DMQ and QNQ populations, respectively, revealing genetic differentiation between the two extant populations of *D. cotinifolia* (Table 1). However, Nei's unbiased measure of genetic distance between the populations was only 0.012. We also estimated the genetic variation between the populations using Wright's analysis of hierarchical F-statistics, and the mean value of F_{st} (0.014) was very low, suggesting that there is low genetic differentiation among the populations. The overall gene flow (N_m) between the populations was estimated to be 18.00, indicating that gene exchange between the populations was extensive (Table 2).

3.3. Demographic bottleneck

We detected two loci in population DMQ and three in population QNQ that differed significantly from the expectations of heterozygosity under mutation–drift equilibrium conditions (Table 3). In population DMQ, locus JT03 exhibited deficit heterozygosity ($P < 0.05$) under TPM and SMM, and locus JT34 showed an excess ($P < 0.05$) under IAM, SMM, and TPM. For population QNQ, locus JT34 also displayed excess heterozygosity ($P < 0.05$) under the three models, whereas loci JT03 and JT 21 showed a deficit under SMM. However, none of the three mutation models produced results suggesting bottlenecks in either population in the recent past ($P > 0.05$) (Table 4). There was an excess of heterozygosity resulting from many alleles

Table 2
Characterization of microsatellite markers used in this study.

Locus	Repeat motif	Size range (bp)	A[a]	H_o[b]	H_e[c]	F_{is}[d]	F_{st}[e]	N_m[f]
JT03	$(AG)_{14}$	424–446	12	0.525	0.610	0.129	0.051	4.644
JT12	$(TG)_9$	125–128	2	0.016	0.016	−0.017	0.008	29.500
JT14	$(TC)_{21}$	279–288	2	0.508	0.382	−0.335	0.002	168.500
JT17	$(TG)_{11}$	365–367	2	0.525	0.390	−0.349	0.000	689.500
JT21	$(TG)_{12}(AG)_9$	335–341	5	0.098	0.126	0.189	0.032	7.655
JT26	$(CA)_{19}(GA)_9$	379–403	7	0.475	0.658	0.277	0.014	18.156
JT29	$(TG)_{11}(AG)_4$	319–322	4	0.918	0.637	−0.450	0.002	126.500
JT34	$(GAT)_{10}$	183–186	2	1.000	0.504	−1.000	0.000	–
Average			4.50	0.508	0.416	−0.238	0.0137	18.000

[a] Observed number of alleles.
[b] Observed heterozygosity.
[c] Expected heterozygosity.
[d] Inbreeding coefficient.
[e] Population differentiation.
[f] Gene flow estimated from: $N_m = 0.25(1 - F_{st})/F_{st}$.

Table 3
Results of the heterozygosity expected under Hardy–Weinberg equilibrium at eight polymorphic loci for two populations of *D. cotinifolia*.

Population	Locus	N[a]	H_o[b]	IAM[c]		TPM[d]		SMM[e]	
				H_e[f]	Prob[g]	H_e	Prob	H_e	Prob
DMQ	JT3	60	0.475	0.678	0.058	0.760	0.006[h]	0.782	0.001[h]
	JT14	60	0.364	0.213	0.256	0.239	0.316	0.252	0.330
	JT21	60	0.033	0.215	0.227	0.237	0.161	0.255	0.133
	JT26	60	0.685	0.616	0.365	0.72	0.246	0.738	0.177
	JT29	60	0.621	0.462	0.184	0.575	0.443	0.602	0.489
	JT34	60	0.508	0.215	0.020[h]	0.250	0.024[h]	0.254	0.018[h]
	JT17	60	0.381	0.207	0.211	0.245	0.304	0.251	0.302
QNQ	JT3	62	0.649	0.665	0.360	0.757	0.068	0.779	0.026[h]
	JT12	62	0.032	0.206	0.245	0.240	0.142	0.255	0.133
	JT14	62	0.405	0.210	0.207	0.239	0.242	0.242	0.262
	JT17	62	0.405	0.211	0.203	0.244	0.259	0.254	0.292
	JT21	62	0.211	0.464	0.106	0.574	0.014[h]	0.602	0.005[h]
	JT26	62	0.625	0.463	0.174	0.574	0.414	0.604	0.503
	JT29	62	0.662	0.460	0.114	0.572	0.242	0.595	0.319
	JT34	62	0.508	0.199	0.021[h]	0.239	0.028[h]	0.246	0.024[h]

[a] Sample size of alleles.
[b] Observed heterozygosity.
[c] Infinite allele mutation model.
[d] Stepwise mutation model.
[e] Two-phased model of mutation.
[f] Expected heterozygosity.
[g] Probability.
[h] Indicate evidence for bottleneck.

when using both the Sign test and the Wilcoxon test (Table 4) for both populations. Additionally, our data revealed that alleles occurring at low frequencies (<0.1) were the most abundant frequency class of allele in both populations of *D. cotinifolia* (Fig. 1, Fig. 2). Therefore, no recent genetic bottleneck was detected in the studied populations.

4. Discussion

4.1. Genetic diversity in D. cotinifolia

The amount of genetic diversity within populations is a fundamental parameter in evolutionary and conservation biology. High levels of genetic variation are expected to increase the potential of populations to respond to selection and to maintain the health of individuals. The results of the present study showed that the genetic diversity of *D. cotinifolia* was similar to the average values of short-lived perennial plants ($H_e = 0.55$, $H_o = 0.53$) and species with a mixed breeding system ($H_e = 0.60$, $H_o = 0.51$) but higher than that of endemic-distribution species ($H_e = 0.42$, $H_o = 0.32$) (Nybom, 2004).

Species that grow in a variety of habitats often harbor a high level of genetic diversity. The habitats in which *Primula merrilliana* Schltr. were distributed were different with regard to soil type, microclimate category, moisture condition, and light intensity, and these various environments supported the high genetic diversity of this species (Zhang and Chen, 2003). In contrast, species within homogenous habitats usually possess relatively low genetic diversity. For example, *Typha latifolia*, which lives under homogenous aquatic conditions, harbored a low level of genetic diversity ($H_e = 0.18$–0.31) (Tsyusko et al.,

Table 4
Tests for bottleneck effects under three models of microsatellite evolution.

Population	Exp. H exc.[a]	H exc.[b]	H def.[c]	Probability		Interpretation
				Sign test	Wilcoxon test[d]	
Infinite allele model						
DUQ	3.40	5	2	0.200	0.578	No deviation
QNQ	4.00	5	3	0.360	0.383	No deviation
Two-phased model of mutation						
DUQ	3.61	4	3	0.536	1.000	No deviation
QNQ	4.23	5	3	0.428	0.843	No deviation
Stepwise mutation model						
DUQ	3.65	4	3	0.548	0.813	No deviation
QNQ	4.21	5	3	0.420	0.844	No deviation

[a] Expected number of loci with excess heterozygosity.
[b] Observed number of loci exhibiting heterozygosity excess.
[c] Observed number of loci with heterozygosity deficiency.
[d] Two tails for H excess or deficiency.

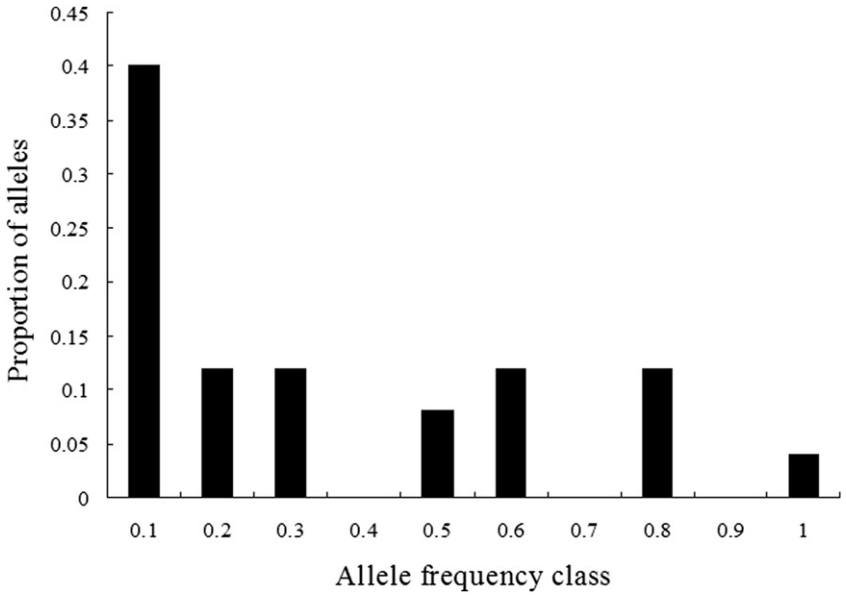

Fig. 1. Allelic frequency distribution for all polymorphic microsatellite loci examined for the Dumuqiao population (DMQ). An L-shaped distribution was obtained. The values along the *x*-axis represent the maximum value for each respective allele frequency class (e.g., the first class represents alleles with frequencies between 0 and 0.1 and the second, between 0.1 and 0.2).

2005). Our study showed that *D. cotinifolia* possessed a moderate level of genetic diversity ($H_e = 0.416$, $H_o = 0.508$), even though its habitat was unique and simple. Similar results have been found for other species, such as *Primulina tabacum* Hance (Ni et al., 2006), *Taihangia rupestris* Yu et Li (Wang et al., 2011a), and *Titanotrichum oldhamii* (Wang et al., 2004). Although *P. tabacum* only grows with other calciphilous and shade-tolerant plants around the entrance to karst caves at an elevation of approximately 300 m, a high level of genetic diversity remains, both at the population and species levels ($P = 85.6$, $H_e = 0.339$, Is = 0.495). *T. rupestris* plants grow mostly in small crevices on the faces of shaded cliffs at elevations ranging from 600 to 1500 m a.s.l., and a relatively high genetic diversity was detected at the species levels (PPB = 80.43; $h = 0.2479$; $I = 0.3785$), even though the environmental conditions on these cliffs are much more stable than non-cliff habitats (Colas et al., 2001;

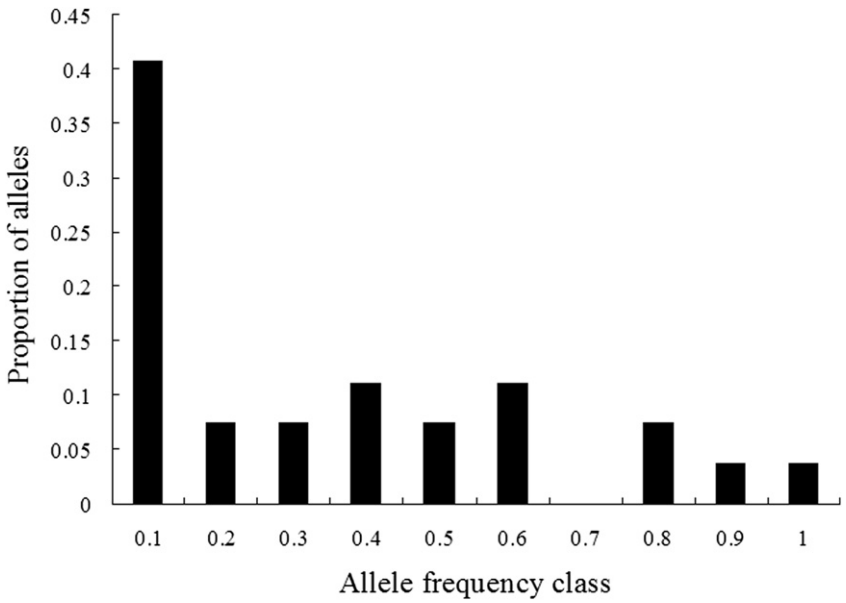

Fig. 2. Allelic frequency distribution for all polymorphic microsatellite loci examined for the Qingnianqu population (QNQ). An L-shaped distribution was obtained. The values along the *x*-axis represent the maximum value for each respective allele frequency class (e.g., the first class represents alleles with frequencies between 0 and 0.1 and the second, between 0.1 and 0.2).

Larson et al., 2000). The main factors that contribute to the moderate genetic variability of *D. cotinifolia* were found to be its perennial and outcrossing characteristics. The perennial characteristic maintains the genetic mutations that occur in *D. cotinifolia* populations, whereas the outcrossing characteristic disperses these mutations.

4.2. The bottleneck and gene flow

Low levels of heterozygosity have been considered to be evidence for bottlenecks in populations known to have undergone severe demographic decline (Houlden et al., 1996). The number and size of *D. cotinifolia* populations have drastically decreased in past decades. *D. cotinifolia* is now a critically endangered plant, but the moderate level of observed heterozygosity suggests that the two extant populations of this species may have maintained a large effective population size and may not have experienced a severe loss of genetic variability. This result is consistent with the lack of recent bottleneck events in the *D. cotinifolia* populations. Although limited loci were used in this study, our results provide evidence for the demographic history of *D. cotinifolia*. Based on the Sign test and the Wilcoxon signed-rank test, there was no evidence of recent bottleneck events in *D. cotinifolia*, and the mode-shift test showed a normal L-shaped distribution pattern of the allele frequencies in the two populations.

The inbreeding coefficient is affected by gene flow and the breeding system: a high level of gene flow reduces inbreeding probability and also decreases genetic differentiation among populations. The negative value of the inbreeding coefficient ($F_{is} = -0.238$) indicated that outcrossing was predominant in *D. cotinifolia*. Even in the smallest population, DUQ (100 individuals), the inbreeding coefficient ($F_{is} = -0.256$) was a significant departure from zero, a result that was supported by the higher H_o (0.542) than H_e (0.441) and the extensive gene flow ($N_m = 18.000$). In this study, the N_m values among the loci were very different, which may be related to differences in the selection pressure on these loci.

Dominant outcrossing in *D. cotinifolia* was confirmed by the study of the breeding system. The breeding system of *D. cotinifolia* consists of outcrossing with partial self-compatibility (Wang et al., 2011b). The showy flowers of this species attract the pollinator Chalcididae sp., increasing outcrossing probability. In addition, the stigma is notably higher than the anther during pollen dissemination, which makes this species easily receptive to allogamy.

4.3. Implications for conservation

In summary, the endangered status of this species is most likely due to habitat damage from human activity and the difficult establishment of new populations because of its special environmental requirements rather than a lack of genetic diversity (Wang et al., 2008). Thus, *in situ* conservation strategies should be preferred, such as preventing illegal removal for horticultural and medicinal uses and protecting the entire environment in which *D. cotinifolia* grows. In addition, the sizes of the populations should be increased by artificial breeding. The fruit-setting ratio through artificial xenogamy reached 86.67%, whereas open pollination was only 28.00% (Wang et al., 2011b). Moreover, seed germination was largely accelerated by gibberellin treatment (Wang et al., 2008). Therefore, we can produce a large number of *D. cotinifolia* individuals through artificial breeding and can then transplant them in fields to enlarge the population size. Finally, *ex situ* conservation strategies should be adopted to establish new populations in other areas, even though previous efforts were unsuccessful.

In this study, eight polymorphic microsatellite loci revealed a moderate level of genetic diversity in *D. cotinifolia*. Although the sizes of its populations have drastically decreased in past decades, *D. cotinifolia* did not undergo a severe recent bottleneck. Extensive gene flow reduced the inbreeding probability and decreased the genetic differentiation between populations. *In situ* conservation strategies should be preferred, and artificial breeding should also be adopted to enlarge the population size.

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