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Population genetic structure of *Camellia nitidissima* (Theaceae) and conservation implications

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ABSTRACT. *Camellia nitidissima* Chi (Theaceae), with its golden-yellow flowers, is a famous ornamental species. Due to deforestation and collection of seedlings, its natural populations have receded greatly in recent decades. Genetic diversity and genetic differentiation of the twelve extant natural populations and one *ex situ* conserved population of *C. nitidissima* in China were analyzed using inter-simple sequence repeats (ISSR) markers. We found a low level of genetic diversity at both the species (*P* = 63.22%, Nei's genetic diversity H_T = 0.1561 and Shannon diversity H*sp* = 0.2490) and population levels (*P* = 18.77%, H_E = 0.0831 and H*pop* = 0.1188) and a relatively high degree of differentiation among populations (AMOVA analysis: 41.85%; Hickory θ_B: 0.4056) in naturally occurring populations. In contrast, the *ex situ* population contained higher genetic variability compared to each natural population. No significant correlation was found between genetic diversity and population size. Based on the results, we suggest that all the wild *C. nitidissima* populations should be protected *in situ*. For the *ex situ* conservation of the species in Guilin Botanical Garden, samples from Long'an County should be added to the existing collections.

Keywords: *Camellia nitidissima*; China; Endangered species; *Ex situ* conservation; ISSR fingerprinting; Small population size.

INTRODUCTION

The section *nitidissima* Chang of the genus *Camellia* (Theaceae) comprises 18 rare and endangered species occurring in a narrow range 20°32'-23°53' N, 104°-108° 56' E, and at the altitudes of 50-650 m, in Southwest China and North Vietnam (Zhang, 1996). The golden-yellow petals of the flowers have earned them the title "the queen of the Camellia family" (Liang, 1993). They represent valuable germplasm resources for cultivar breeding, especially for producing yellow flowers. With the big size, golden color, and the transparent waxy appearance of its flowers, *Camellia nitidissima* Chi is the most interesting species of the section. Although first discovered in Fangcheng County in 1933 and reported to the public in 1948, *C. nitidissima* received no attention from the public or horticulturists until the early 1960's when it was found again in Yongning County (Deng et al., 2000). *Camellia nitidissima* is a diploid shrub (2n = 30, Huang and Zhou, 1982), with a restricted distribution in the southwestern Guangxi Zhuang Autonomous Region in China and in the neighboring regions of North Vietnam. It grows under shady and moist evergreen broad-leaf forests dominated by *Canarium album*, *Dendrobenthamia hongkonensis*, and *Canstanopsis cuspidata*. Its big and colorful flowers (diameter: 1.2-2.3 cm) blossom from November to March and set fruits in the spring.

Due to deforestation of the regions where *C. nitidissima* grows and the over collection of its seedlings, its natural population has declined dramatically in recent decades. It is now classified as one of the most endangered plant species in China (Fu, 1992). In order to protect this valuable genetic resource, one natural reserve was established in Fangcheng in 1986. In addition to the *in situ* habitat preservation for rare and endangered plant species, *ex situ* conservation in botanical gardens plays an important role in conserving these plants. In the 1980s, Guilin Botanical Garden (GL) started an *ex situ* conservation program for this species and planted more than 1300 seeds and 100 seedlings collected from Yongning, Fangcheng, Dongxing, and Fusui Counties. Today, about 800 individuals are settled in the garden, and most of them blossom and set fruits.

Although the morphology (Ye and Xu, 1992), ecology (Su, 1994; Huang, 2001), and condition for cultivation (Zhang and Huang, 1984) have been studied, the population genetic structure of this endangered species has never been examined extensively across the species range (Bin et al., 2005; Tang et al., 2006). Understanding the levels and

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distribution of genetic variation within species/populations not only aids in recovering the evolutionary history, it also helps in conservation and management of the species (Frankham et al., 2002). Several PCR-based fingerprinting techniques (e.g., RAPD, SSR, ISSR and AFLP) have been developed to study population genetic structures. Among them, the sensitivity of the inter-simple sequence repeat (ISSR) technique (Zietkiewicz et al., 1994) makes it a powerful tool for investigating genetic variation within species (Wolfe and Liston, 1998), and it has been successfully applied in the conservation genetics of many rare and endangered plant species. Amplifying ISSR markers does not require knowledge of the genome sequence. They are simple to use and generate data quickly, which makes them highly suitable for population genetics studies. Therefore, in this work, we estimated the genetic diversity and differentiation of twelve remaining natural populations and one *ex situ* population (GL) of the endangered *C. nitidissima* by using ISSR molecular markers, with a view to achieving more efficient conservation of this rare genetic resource and to ensure that most of its genetic variations are adequately preserved in the *ex situ* conservation projects. As it is governed by random genetic drift, like many other rare species, low levels of genetic diversity within populations, but high genetic differentiation among populations, would be expected.

MATERIALS AND METHODS

Sample collection

In August 2003, a total of 250 individuals were sampled from twelve geographically isolated natural populations of *C. nitidissima* across the entire range of distribution of the species in Guangxi, China (Figure 1; Table 1). These populations could be divided into two regions separated by about 117-148 km. Seven populations (A-G) were distributed around Fangcheng, and the other five populations (H-L) surrounded Nanning. Within each region, the populations were isolated from each other at a distance of 4 to 40 km, and their sizes varied from 32 to 206 plants (Table 1). In order to check the effectiveness of *ex situ* conservation of *C. nitidissima* in Guilin Botanical Garden, sixty individuals from this conserved population (GL) were also sampled. Fresh leaves of each selected plant were collected and dried quickly by using silica gels in the field. Vouchers were collected from each population and deposited at the herbarium of Guangxi Institute of Botany (IBK).

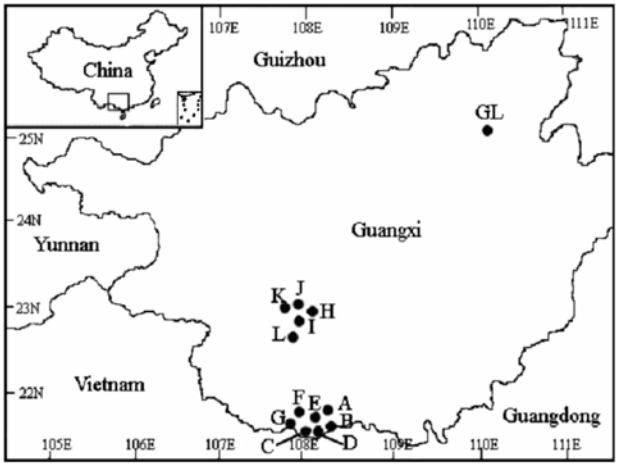

Figure 1. Locations of sampled populations of *Camellia nitidissima* in China.

Table 1. *Camellia nitidissima* populations in China and their genetic variability detected by ISSR analysis.

Code	Provenance	Altitude (m)	Longitude (E)	Latitude (N)	Ns[1]	N[2]	H_E	H*o*	*P* (%)
A	Fangcheng	165	108° 9’	21°52’	22	120	0.0948	0.1345	20.69
B	Fangcheng	20	108° 18’	21°43’	19	182	0.0804	0.1150	18.39
C	Dongxing	130	107° 58’	21°39’	20	37	0.0612	0.0874	13.79
D	Fangcheng	178	108° 07’	21°40’	20	63	0.0889	0.1279	20.69
E	Fangcheng	120	108° 03’	21°51’	20	206	0.0892	0.1285	20.69
F	Fangcheng	170	107° 56’	21°50’	22	39	0.0925	0.1319	20.69
G	Dongxing	410	107° 55’	21°41’	20	32	0.0664	0.0949	14.94
H	Yongning	240	107° 50’	22°57’	21	190	0.1033	0.1472	22.99
I	Yongning	380	107° 46’	22°53’	22	160	0.0970	0.1384	21.84
J	Long’an	230	107° 48’	22°58’	20	165	0.0513	0.0731	11.49
K	Long’an	250	107° 46’	22°56’	21	135	0.0677	0.0963	14.94
L	Fusui	280	107° 42’	22°52’	23	140	0.1048	0.1507	24.14
GL	*ex situ* conserved in Guilin Botanical Garden	178	110 °12’	25 °11’	60	800	0.1525	0.2273	41.38

[1]sample size; [2]population sizes; H_E: Nei’s genetic diversity; H*o*: Shannon’s information index; *P*: percentage of polymorphic loci.

Total DNA extraction and ISSR analysis

Total DNA was extracted following the CTAB method described by Doyle (1991). One hundred ISSR primers from the University of British Columbia primer set nine (the Michael Smith Laboratories, University of British Columbia, primer set #9, Vancouver, BC, Canada: http://www.michaelsmith.ubc.ca/services/NAPS/Primer_Sets/Primers.pdf) were initially screened for PCR amplifications. Eleven of them (UBC # 808, 834, 835, 836, 840, 841, 848, 855, 857, 866, 880) that consistently generated clear and reproducible banding patterns were selected for further analysis. PCR and gel electrophoresis were carried out as described by Ge et al. (2003). Only those bands that showed consistent and unambiguous amplifications were scored. Smeared and weak bands were excluded.

Data analysis

ISSR profiles were scored for each individual as having a specific band present (1) or absent (0). POPGENE 1.31 (Yeh et al., 1999) was used to compute the percentage of polymorphic loci (*P*), Nei's genetic diversity (H_E) (Nei, 1973), and Shannon diversity ($Ho = -\Sigma p_i \log_2 p_i$), where *pi* was the frequency of the fragment recorded. Shannon diversity was calculated at two levels: the average diversity within populations (H*pop*), and the total diversity (H*sp*). The correlation between population size and genetic diversity parameters was calculated with the Spearman rank correlation coefficient.

Components of variance within and between populations were estimated using an analysis of molecular variance (AMOVA) that was performed by Arlequin 2.000 (Schneide et al., 2000). Pairwise genetic distances (Φ_{ST}) among the twelve populations were obtained from the AMOVA. For each analysis 1,000 permutations were performed to obtain significance levels. Genetic structure was also examined using a Bayesian approach (Holsinger et al., 2002). We estimated θ_B, a Bayesian derived analogue of F_{ST}, using Hickory's default values, burn-in (50,000), sampling (250,000) and thin (50), to specify the prior distributions. Gene flow was estimated indirectly using Wright's (1931) formula: $Nm = 0.25(1-F_{ST})/F_{ST}$, where F_{ST} was from the AMOVA analysis.

In order to test the correlation between genetic (*D*) and geographical distances (in km) among populations, a Mantel test was performed using TFPGA (Miller, 1997) with 5000 permutations. A neighbor-joining (NJ) dendrogram based on pairwise F_{ST} (Φ_{ST}) comparisons between populations was constructed using the PHYLIP programs NEIGHBOR and CONSENSE (Felsenstein, 1993). The topology of the dendrogram shown in Figure 2 was assessed by a bootstrap test with 1000 replicates.

RESULTS

For the twelve natural populations, eleven primers generated 87 DNA bands with molecular sizes ranging from 230 to 1600 bp. Of these bands, 55 (63.22%) were polymorphic at the species level. The percentages of polymorphic loci (*P*) ranged from 11.49% (J) to 24.14% (L) within the twelve natural populations, with an average of 18.77%. The average genetic diversity was 0.0831 within populations (H_E) and 0.1561 at the species level (H_T) (Table 1). Population L had the highest level of variability (*P*: 24.14%, H_E: 0.1048) while population J had the lowest (*P*: 11.49%, H_E: 0.0513). The Shannon diversity indices also revealed the lowest estimate (0.0513) in J and the highest (0.1048) in L (Table 1). The mean value of Shannon diversity was 0.1188 at thepopulation level (H*pop*) and 0.2490 at the species level (H*sp*) (Table 1). The *ex situ* conserved population GL had higher genetic variability than any of the twelve natural populations, but less than that at the species level (*P*: 41.38%, H_E: 0.1525, H*o:* 0.2273) (Table 1). No significant correlation was found between population size and genetic diversity ($r_s = 0.31$, $p = 0.154$ for H_E or H*pop* and $r_s = 0.32$, $p = 0.154$ for percentage of polymorphic loci).

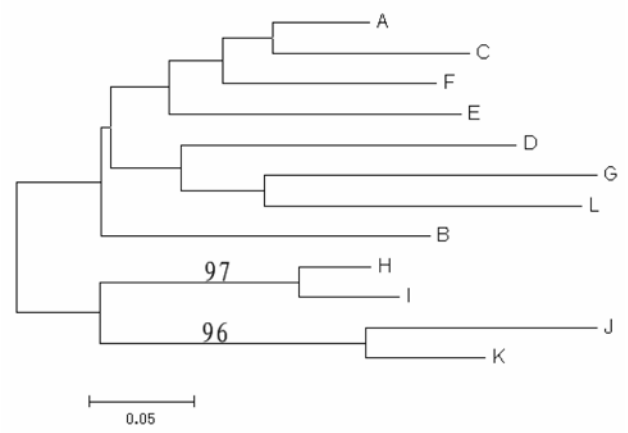

Figure 2. The neighbor-joining (NJ) dendrogram constructed based on population pairwise genetic distances. Branch length is proportional to genetic distance. Numbers at nodes indicate bootstrap values in percentage (> 50%).

AMOVA indicated that 11.83% of the molecular variation was attributable to regional divergences (between Fangcheng and Nanning), 30.02% to population differentiation within regions, and 58.15% to differences among individuals within populations (Table 2). According to the Bayesian analysis of population structure, the best model was obtained with the free model (DIC = 1309.79, lower than the $f = 0$ model with DIC = 1354, the full model with DIC = 1356, $f = 0$ model with DIC = 4433). The mean θ_B in the free model was 0.4056 (SD = 0.0256). The estimated gene flow among populations was $Nm = 0.347$.

Two clusters were identified (Figure 2), one consisting of populations of the Fangcheng region and population L of the Nanning region and the other including the remaining four populations of the Nanning region (from Yongning and Long'an Counties). Populations H and I (97%) and J and K (96%) were closely clustered as indicated by bootstrapping. Mantel tests show a low correlation

Table 2. AMOVA results for the twelve natural populations of *Camellia nitidissima*.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	*P*-value
Among regions	1	104.779	0.57989	11.83	< 0.001
Among populations within regions	10	334.607	1.47119	30.02	< 0.001
Within populations	238	678.261	2.84984	58.15	< 0.001
Total	249	1117.648	4.90091		

P-values are the probabilities of having a more extreme variance component than the observed values by chance alone. Probabilities were calculated by 1000 random permutations of individuals across populations.

between genetic and geographical distances among populations ($r = 0.5114$, $p = 0.002$).

DISCUSSION

Genetic variation and genetic structure

As the primary components of evergreen broadleaf forest in East Asia, like most *Camellia* species, *C. nitidissima* is rather restricted to a small area in southwestern Guangxi Province of China and the neighboring region of Vietnam. Compared to the average of angiosperms obtained with dominant markers (RAPD, AFLP, ISSR) (Nei's genetic diversity at population level: 0.22-0.23; Nybom, 2004), a low level of genetic diversity was detected in *C. nitidissima*, a result consistent with Tang et al.'s (2006) observations. This can be interpreted as a consequence of restricted, scattered distributions of *C. nitidissima*, as a strong association exists between geographical range and genetic diversity (Hamrick and Godt, 1989), although exceptions are also common (Gitzendanner and Soltis, 2000). Comparing eleven pairs of endemic and widespread congeners for genetic variability, Karron (1991) found that rare species had significantly lower levels of genetic variation than their widespread congeners.

Population genetic theory predicts that small populations tend to lose genetic variation because of the effects of genetic drift. However, in some investigations, levels of genetic diversity were not correlated with population size (i.e., Greimler and Dobeš, 2000; Lei and Mutikainen, 2005; Mathiasen et al., 2007). As *C. nitidissima* is a rare and endangered species, its natural populations are generally small, eleven of the twelve populations have fewer than 200 individuals (Table 1). Despite the low levels of genetic diversity at the species level, no significant correlation was found between population size and genetic diversity in this study. The population size of *C. nitidissima* has only fallen in recent decades due to deforestation and destructive collection of seedlings; the current fragmented populations are the remants of a larger former population. According to our demographic study (Wei et al., unpublished data), the populations of *C. nitidissima*, as a long-lived shrub, are dominated by adults with DBH greater than 2 cm. Seedlings with DBH shorter than 1 cm were rare. In the present study, the genetic variation of sampled adult *C. nitidissima* trees may reflect a structure established prior to the destruction that has recently occurred.

The *ex situ* conserved GL population has a higher level of genetic diversity than any of the twelve natural populations, and it contains all the DNA bands that the natural populations have. According to the sampling record, the plants cultivated in the garden cover the range of the species in China except for Long'an County. When we combined the genetic data of J and K populations with GL together, the genetic diversity as a whole exceeded that of the natural populations at the species level (H_T and H*sp*: 0.1701 and 0.2571 vs. 0.1561 and 0.2490), demonstrating that the *ex situ* conservation in the Guilin Botanical Garden could be improved through supplemental materials from Long'an County.

The genetic structure of plant populations reflects the interactions of various long-term evolutionary processes, such as shifts in distribution, habitat fragmentation, population isolation, mutation, genetic drift, mating system, gene flow, and selection (Schaal et al., 1998). The breeding system is one of the most powerful explanatory variables for genetic diversity within and among populations of plant species (Hamrick and Godt, 1989). Inbreeding species are generally characterized by high levels of genetic differentiation among populations while outbreeding ones tend to retain considerable variability within populations. In this study, about 41% of genetic variation was partitioned between populations in *C. nitidissima*, comparable with that of species with a mixed breeding system (Φ_{ST}: 0.40) and gravity seed dispersal (Φ_{ST}: 0.45) (cf. Nybom, 2004). Significant genetic differentiation was also reported by Bin et al. (2005) (Φ_{ST}: 0.5752) and Tang et al. (2006) (Φ_{ST}: 0.539).

High genetic divergence among populations suggests limited gene flow ($Nm = 0.347$), which in turn increases the probability of inbreeding. Although no comprehensive studies on its mating system and seed dispersal have been done, *C. nitidissima* was found to be mainly pollinated by bees of a short flight range (Cheng et al., 1994). Moreover, long distance seed dispersal is unlikely to be efficient because it's the species' big, heavy seeds (1.73-2.16 cm long and 1.94-2.5 cm in diameter, 2.3-3.5 g in weight). In con-

trast to this limited gene flow in *C. nitidissima*, bird-pollination contributes to a strong gene flow in *C. japonica*, for which a low degree of allozyme differentiation between populations in Japan and Korea was detected (Chung and Chung, 2000). Apparently, limited gene flow is one factor contributing to *C. nitidissima's* high level of population differentiation.

Conservation consideration

Threatened and endangered species that possess small-sized populations are more prone to extinction than those with large, stable populations. According to IUCN criteria, *C. nitidissima* populations are mostly endangered. The goals of conservation are to avert the genetic deterioration of the species, to preserve the species' potential for adaptation to both short- and long-term environmental variation, and thereby reduce the chances of extinction. Genetic impoverishment, coupled with demographic stochasticity, is expected to increase the risk of local extinction in small populations (Hanski and Gilpin, 1991). Although a natural reserve for *C. nitidissima* has been established in Fangcheng, one of the primary distribution areas of the species in China, germplasm of the other seven populations, especially populations H and L with the highest observed and expected genetic diversities (Table 1) has never been included. Results of the present study indicate that representation of the species' gene pool could be substantially improved through inclusion of additional populations.

Conserving the genetic diversity of rare and endangered species and their evolutionary potential is one of the long-term goals of *ex situ* conservation. Appropriate sample collections are the key to creating *ex situ* reserves. For *C. nitidissima*, Tang et al. (2006) suggested "more individual plants from each population but fewer populations" as the *ex situ* sampling strategy. However, based on the observed high genetic differentiation in *C. nitidissima*, we suggest an *ex situ* sampling strategy of fewer individual plants from every population. The high genetic diversity harbored in the GL population verified the effect of this strategy as it has a wide source from Yongning, Fangcheng, Dongxing, and Fusui Counties.

Botanical gardens have played important roles in the *ex situ* conservation of rare and endangered plants. Nevertheless, because of limited spatial and financial resources, most *ex situ* conservation project in Chinese botanical gardens ignore the sampling strategy. Generally only a very few individuals from each species are cultivated, completely neglecting the need to conserve the genetic diversity range (Li et al., 2002). Inevitably, much genetic diversity is lost, subsequently increasing inbreeding chances in the *ex situ* population. Fortunately, about 800 *C. nitidissima* individuals were conserved in the Guilin Botanical Garden. The high genetic diversity in the GL population indicates that the reintroduction of *C. nitidissima* from this garden is plausible from a genetic diversity perspective.

As with most endangered species, habitat loss and destructive human collection of seedlings are the main threats to *C. nitidissima*. Although the plants in the natural reserves are shielded from destruction, the risk of extinction cannot be reduced (Hensen and Wesche, 2006). In the long term, the small population size of *C. nitidissima* will drive this species to an "extinction vortex." Reintroduction to increase population size and genetic diversity is thought to be the most effective method for population recovery (Falk et al., 1996). For *C. nitidissima,* the reintroduction from *ex situ* conserved populations is needed in the future.

Genetic diversity and differentiation of neutral markers often provide useful information on the demographic events that occurred in natural populations (such as bottlenecks, rapid expansion, and migration). Nevertheless, due to the lack of correlation between genetic variability of neutral markers and variation of genes coding quantitative traits, molecular-marker studies have contributed little to our understanding of natural selection and adaptation in forest-tree populations (González-Martínez et al., 2006). In this study, only spatial demographic processes was measured using neutral markers, and whether the *ex situ* population can represent the quantitative and adaptive genetic variation remains unclear. For *C. nitidissima*, the extant twelve populations of two regions (Fangcheng and Nanning) may have evolved into locally adapted ecotypes. Therefore, a detailed population genomics study by using candidate gene markers (e.g. SNPs or EST-based markers) is needed for re-evaluating the *ex situ* conservation of this valuable genetic resource.

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金花茶的種群結構（茶科）及其保育意義

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採用 ISSR 分子標記技術，對金花茶的 12 個自然種群和 1 個遷地保育種群的遺傳多樣性水準和群體遺傳結構進行了研究。分析結果表明：12個自然種群的多態位點百分率為 63.22%。在物種水準上，種群總的 Nei's 基因多樣性指數和 Shannon 資訊多態性指數分別為 0.1561 和 0.2490。在種群水準上，期望雜合度和 Shannon 資訊多態性指數分別為 0.0831 和 0.1188。金花茶具有相對較低遺傳多樣性水準；且種群內的遺傳多樣性水準也較低。基因分化係數為 0.4185，金花茶遺傳變異主要存在於種群內。與自然種群相比，遷地保育的種群的遺傳多樣性水準高於單個自然種群但低於全部種群的遺傳多樣性水準，根據研究結果，我們建議必須對所有的自然種群進行保護。

關鍵詞：瀕危植物；遷地保育；ISSR；小種群；*Camellia nitidissima*。