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## Genetic diversity and conservation evaluation of a critically endangered endemic maple, *Acer yangbiense*, analyzed using microsatellite markers

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### ABSTRACT

The newly discovered endemic maple *Acer yangbiense* of China has only five individuals left in the wild, and thus has been classified as a plant species with extremely small populations (PSESP). PSESP species call for emergency protection procedures, such as ex-situ conservation and reintroduction. Our objectives were to examine the genetic diversity of *A. yangbiense* and to evaluate former conservation strategies from a genetic point of view. Our results suggested that *A. yangbiense* was not genetically depauperate, but its genetic loss at a species level was obvious. A parentage analysis indicated a high selfing-rate in *A. yangbiense* and suggested the existence of a previously unknown wild individual. Former conservation strategies did not include all genetic variations of the wild population, and gene diversity of the ex-situ conserved seedlings is lower than that of the wild population. From our findings, we make suggestions to guide the subsequent protection of this species.

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## 1. Introduction

The critically endangered *Acer yangbiense* Y. S. Chen & Q. E. Yang (Aceraceae) has been recently described (Chen et al., 2003). After three separate in-depth investigations, only five individuals have been located, in a western valley of Cangshan Mountain, Yunnan Province, China. Three individuals are scattered across a sloping area mixed with farmlands and fallow lands (under daily grazing pressure) and two individuals are located in woody areas on the opposite hillsides. Three individuals have reached fertile maturity. The *A. yangbiense* distribution overlaps with a small village called Malutan, at 2200–2500 m altitude.

Based on all the available information and data from the surveys of its natural habitats, an IUCN assessment evaluated *A. yangbiense* as Critically Endangered [CR B1ab(v) + 2ab(v)] (Gibbs and Chen, 2009). Facing a very high risk of extinction, *A. yangbiense* is now listed in an emergency rescue plan for plant species with extremely small populations (PSESP) in China (Ma et al., 2013; Ren et al., 2012). This newly-initiated conservation action strategy focuses on PSESP and was approved by the

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Chinese government for immediate rescue of the most endangered plants before 2015. *A. yangbiense* was listed in the top 20 most endangered species in this PSESP plan (Ma et al., 2013). To address these aims in relation to *A. yangbiense*, in-situ conservation, seedling propagation, ex-situ conservation at the Kunming Botanical Garden (KBG) and reintroductions were made priorities.

In 2008 Dr. Chen (Institute of Botany, the Chinese Academy of Sciences) arranged for seeds of *A. yangbiense* to be collected and sent by a forest ranger to the Kunming Botanical Garden for ex-situ conservation. In total, 1606 seedlings were propagated and grown ex-situ in KBG. However, in order to avoid negative effects from starting with very small population sizes, such as loss of genetic diversity, and to evaluate the success of conservation actions, it is important to understand the genetic diversity of the natural population as well as these seedlings.

Although anthropogenic factors are considered to be the main cause of population extinction, genetic factors are also important (Lande, 1998). A decline in population size can cause random genetic drift, leading to allele loss (Eldridge et al., 2004; Hartl and Clark, 1997). Furthermore, a reduced population size increases the chance of inbreeding. In such cases, positive feedback between demographic and genetic decline can further reduce population size (Gilpin and Soule, 1986). Thus, inbreeding due to reduced population size lowers genetic diversity, the levels of which are positively correlated with population size in some cases (Ellstrand and Elam, 1993; Fischer and Matthies, 1998; Frankham, 1996; Linhart and Mitton, 1985; Vergeer et al., 2003). Population genetic analysis provides information on the levels of genetic diversity, which is essential for species conservation (Frankham et al., 2002). Therefore, assessing the level and distribution of genetic diversity are crucial for management and the development of effective conservation strategies for endangered species such as *A. yangbiense*.

Because microsatellites are unique in their abundance and codominant inheritance, they are powerful markers and are widely used to investigate genetic diversity, genetic structure and gene flow within populations (Nybom, 2004). For studies of contemporary gene flow as mediated by pollen and seeds, it can be employed for refined estimates of kinship and parentage (Nagamitsu et al., 2014; Schueler et al., 2003; Zane et al., 1999). Therefore 34 specific SSR primers for this research of *A. yangbiense* have been developed (Zhao et al., 2011).

In this study, the main objectives were: (1) to assess the genetic diversity of seedlings and natural population of *A. yangbiense*, (2) to assess the integrity of former conservations strategies from a genetic point of view and (3) to propose an effective conservation strategy for this species.

## 2. Materials and methods

### 2.1. Sampling methods and DNA extraction

A total of 83 samples of *A. yangbiense* were used for DNA analysis. Sampling included all five individuals from the natural population in Yangbi County (YB) and 78 samples chosen randomly from the 1606 ex-situ conserved seedlings (propagated from wild-collected seeds) in KBG. The locality information of the five wild samples (coded as Y01, Y02, Y03, Y04 and Y05) is shown in Fig. 1 and Table 1.

Total genomic DNA of *Acer yangbiense* was extracted from dry leaf tissue ground in liquid nitrogen, using a modified cetyltrimethylammonium bromide (CTAB) extraction method (Doyle, 1987). The extracted DNA was dissolved in 50  $\mu$ l of Elution Buffer (Sangon Biotech, Shanghai, China) for SSR analysis.

### 2.2. SSR marker analysis

Nine nuclear microsatellite markers (coded as AY10, AY14, AY29, AY33, AY34, AY54, AY64, AY69 and AY74) were developed for this study (Zhao et al., 2011). Polymerase chain sequence reactions (PCRs) were performed in a 20  $\mu$ l reaction volume

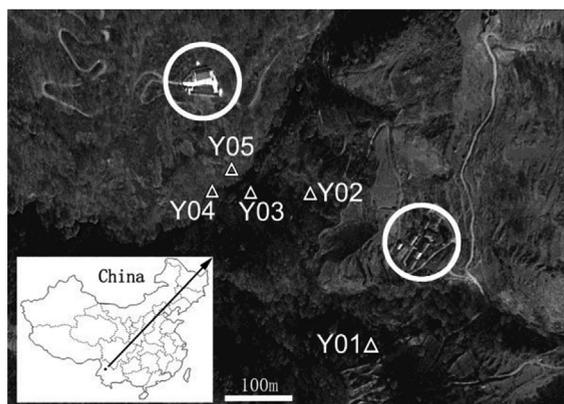


Fig. 1. The distribution of the *A. yangbiense* individuals. The white circles indicate the presence of buildings.

**Table 1**The locality information of the five wild individuals of *A. yangbiense* in Yangbi County.

Sample code	Elevation	North Latitude	East Longitude	Note
Y01	2427 m	25°44'56"	100°0'23"	fertile
Y02	2391 m	25°45'02"	100°0'20"	fertile
Y03	2308 m	25°45'02"	100°0'17"	
Y04	2327 m	25°45'02"	100°0'15"	fertile
Y05	2361 m	25°45'03"	100°0'16"	

containing 50–80 ng of template DNA, 10  $\mu$ l 2  $\times$  Taq PCR Master Mix (Tiangen, Beijing, China), and 20  $\mu$ M of each primer. The samples were amplified in a DNA Thermal Cycler (Applied Biosystems, Foster City, USA) programmed for denaturation of 3 min at 95 °C followed by 35 cycles of 45 s at 94 °C, 45 s at 51 °C–65 °C, 45 s at 72 °C; then final extension at 72 °C for 7 min. The products were loaded into QIAxcel Genetic Analyzer (QIAGEN Inc., Hilden, Germany), and the size of the products was determined using Bio Calculator V3.0.05.

### 2.3. Data analysis

Microsatellite alleles are assumed to be inherited in simple Mendelian fashion and thus parentage was first assessed by simple exclusion (Conrad et al., 2001). Maximum likelihood paternity assignment between three fertile wild individuals (Y01, Y02 and Y04) and KBG seedlings were analyzed by Cervus 3.0 (Marshall et al., 1998). The five wild individuals were treated as one population (YB), and the 78 seedlings were treated as another population (KBG). The mean number of alleles ( $A$ ), percentage of polymorphic loci (PPB), effective number of alleles ( $N_E$ ), observed ( $H_O$ ) and expected ( $H_E$ ) heterozygosities, inbreeding coefficient ( $F_{IS}$ ) and Nei's genetic distance were estimated using POPGENE 1.32 (Yeh et al., 1999). GenALEX 6.4 (Peakall and Smouse, 2006) was used to test for private alleles ( $A_P$ ). FSTAT V.2.9.3.2 (Goudet, 1995) was used to detect allelic richness ( $A_R$ ). An unweighted pair-group method with arithmetic means (UPGMA) tree was constructed based on Nei's genetic distance by NTSYS 2.1 (Rohlf, 1987).

## 3. Results

### 3.1. Parentage analysis of KBG seedlings

The genetic exclusion method revealed that the wild individual Y01 is the most likely both maternal and paternal plant for 43 individuals out of the 78 sampled seedlings, because all of their alleles can be found in Y01. Altogether, three foreign alleles were found in 35 seedlings, excluding all five wild individuals from being the paternal parent for these seedlings. Two foreign alleles were found in 19 seedlings on the site of AY10, with a frequency of 12.2% each, while a third foreign allele was found on the site of AY14 in 21 seedlings, with a frequency of 26.3%.

Parentage of the seedlings was deduced using Cervus 3.0. Confidence level indicates the level at which Cervus assigned parentage to the most likely parent. Cervus calculates an LOD score as the natural logarithm of the product across all loci of likelihood ratios comparing a potential mother or father to an individual drawn at random. The individual with the highest positive LOD score is the most likely father of an offspring. Three fertile wild individuals (Y01, Y02 and Y04) were candidate parents. The Results are shown in Table 2.

The parent pair analysis results with sexes known showed that 24 seedlings at 90%–95% confidence intervals (LOD = 0.66), 43 seedlings at 88.59% (LOD = 0.09) and all seedlings at 85% (LOD < 0) were assigned maternity of the wild individual Y01. No seedlings were assigned paternity at 75%–95% confidence intervals, but at the confidence levels of 70% the father of two

**Table 2**Result of paternity analysis of *A. yangbiense* seedlings in KBG.

Confidence (%)	Critical LOD	Assignments		Assignment rate	
		Observed	(Expected)	Observed	(Expected)
Result of parent pair analysis (sexes known) for mother in different confidence					
90.00	0.66	24	(59)	31%	(75%)
88.59	0.09	43	(67)	55%	(86%)
85.00	–999.00	78	(78)	100%	(100%)
Result of parent pair analysis (sexes known) for father in different confidence					
70.00	0.73	2	(51)	3%	(65%)
65.00	0.57	41	(63)	53%	(81%)
Result of paternity analysis (mother known) in different confidence					
75.00	0.64	31	(63)	0%	(80%)
74.00	0.34	43	(66)	55%	(85%)
70.00	–2.56	77	(73)	99%	(93%)

seedlings were determined as Y01 and the parentage of 41 seedlings was determined as Y01 at a confidence level of 65% (LOD = 0.57). When the paternity analysis was conducted with an identified maternal tree, 31 seedlings could be assigned paternity at 75% confidence level (LOD = 0.64) and 43 seedlings at 74% (LOD = 0.34), however the only putative paternal tree was also Y01. In the dendrogram (not shown) generated from the distance values using UPGMA, all the seedlings and a wild individual (Y01) were separated from the clusters of other four wild individuals (Y02, Y03, Y04 and Y05), suggesting that the seedlings were all mothered by the wild individual Y01 and that the other four wild individuals had no genetic input into the seedlings.

### 3.2. Genetic diversity of *A. yangbiense*

Genetic diversity parameters for the natural population (YB) and the seedlings (KBG) are presented in Table 3. In total 23 alleles were amplified by the nine SSR markers, with an average of 2.56 alleles per locus. Six private alleles were found in YB and three in KBG. The percentage of polymorphic loci of natural population and seedlings were  $PPB = 88.89\%$  and  $PPB = 77.78\%$  respectively. Both the observed and expected heterozygosity (Nei's genetic diversity) were higher in the natural population ( $H_O = 0.4889$  and  $H_E = 0.4272$ ) than in the seedlings ( $H_O = 0.3048$  and  $H_E = 0.3670$ ). The inbreeding coefficient ranged from  $F_{IS} = -0.166$  for the natural population to  $F_{IS} = 0.170$  for the seedlings.

## 4. Discussion

### 4.1. Unexpected genetic donor of KBG seedlings

The genus *Acer* (maples) contains great variability in reproductive systems (De Jong, 1976). *Acer yangbiense* is morphologically andromonoecious but actually monoecious, as two kinds of flowers were found during our observations: staminate and morphologically hermaphroditic. Our observations showed that the morphologically hermaphroditic flowers were functionally female because the anthers were incapable of shedding pollen, and they were located on different branches of the trees from the staminate flowers (herkogamy).

Maximum likelihood analysis (Cervus 3.0) indicated that Y01 is likely to be the maternal plant of all 78 seedlings, which supports the information from the local forest ranger, who said that Y01 was the seed provider. The genetic exclusion method suggests that the selfing rate reached 55.1%, which is high compared with other maples (Gleiser et al., 2008; Kikuchi et al., 2009). It has been observed that in cases of monoecious maples (*Acer crataegifoliu*, *Acer hookeri*, *Acer maximowiczii*, *Acer rubescens* and *Acer tschonoskii*) self-pollination always occurs (De Jong, 1976). The sex expression of *A. yangbiense* has not yet been fully studied, but the high selfing-rate indicates duration of a synchronous anthesis of male and female flowers and lack of foreign pollen competition.

Successful pollinations from the other two fertile trees (Y02 and Y04) were not discovered in any samples. This does not match the expectation that natural pollination might have introduced pollens from them. Random sampling did not include all seedling genotypes therefore this might be a reason for not detecting Y02 and Y04 as parents. Additionally, pollen interference (high contribution rate of specific male parent) may have affected pollinator preferences well. The foreign alleles on sites AY10 and AY14, indicating undiscovered fertile adults, provided hope that there are more adult trees in the wild. Though the great ability for interspecific hybridization was observed in monoecious maples (De Jong, 1976), the possibility was excluded by the anthesis period of *A. yangbiense* which occurs in February, earlier than the five other maple species (with anthesis in April or May) found near the population.

### 4.2. Genetic diversity

The gene diversity of the *A. yangbiense* wild population (YB:  $H_E = 0.4272$ ) was slightly higher than studies of other endemic species ( $H_E = 0.42$ ) and lower than long-lived perennial species ( $H_E = 0.68$ ) estimated based on SSR markers (Nybom, 2004). The lowest genetic diversity among the SSR data from maples was found in *Acer skutchii* ( $H_E$  ranged from 0.054 to 0.247 in different populations), a rare endemic maple with five isolated populations in Central America, with climatic change after the last glacial epoch most likely to be the cause of its decreasing population size (Lara-Gomez et al., 2005). Another study on six maple species showed a diversity of  $H_E = 0.39$  (Guarino et al., 2008). Considering that only five individuals were sampled in

**Table 3**  
Statistics of genetic diversity for *A. yangbiense*.

Pop. Code	A	A <sub>p</sub>	A <sub>R</sub>	PPB (%)	H <sub>O</sub>	H <sub>E</sub>	F <sub>IS</sub>
YB	2.11	6	2.11	88.89	0.4889	0.4272	-0.166
KBG	1.89	3	1.66	77.78	0.3048	0.3670	0.170
total	2.56	—	1.68	100.00	0.3159	0.3866	-0.0325

Note: A, mean number of alleles; A<sub>p</sub>, private allele; A<sub>R</sub>, allelic richness; PPB, percentage of polymorphic loci; H<sub>O</sub>, observed heterozygosity; H<sub>E</sub>, expected heterozygosity; F<sub>IS</sub>, coefficient of inbreeding.

our research, it could be safely assumed that they are remnants of a population with relatively high diversity which was endemic and narrowly distributed in the evergreen broad-leaved forest (elevation below 2900 m) of Cangshan Mountain.

Before leaves even start to sprout, the flowers of *A. yangbiense* appear early in the spring, and thus have less competition with other flowering plants, which ensures sufficient bee visits (fieldnotes). The negative  $F_{IS}$  values of the YB population suggested that in the original population of *A. yangbiense* a high level of gene flow reduced the probability of inbreeding and outcrossing was predominant; therefore a reproduction barrier probably did not contribute to the threat to this species. According to interviews with local residents, people started to immigrate into this valley about one hundred years ago and their villages and farmlands (elevation 2200 m–2500 m) gradually replaced the original forest. That Y01 and Y03 were sprouting from old stumps also suggests that recent human disturbance was the main cause of threat to *A. yangbiense*.

#### 4.3. Genetic loss and evaluation of former conservations strategies

At the species level, genetic loss was indicated by the considerably low average number of alleles per locus ( $A = 2.56$ ). Several other maple species have been studied using microsatellite markers. *A. skutchii* ( $A = 2.6$ ) has a slightly higher number of alleles per locus than *A. yangbiense* (Lara-Gomez et al., 2005), with the highest number observed in *Acer mono* ( $A = 12.63$ ) (Kikuchi et al., 2009); a survey of six *Acer* species in Italy showed  $A = 4.42$  using six loci (Guarino et al., 2008). At the individual level, Y01 has three unique alleles that were discovered in seedlings. However, six alleles found in the other four wild individuals (Y02, Y03, Y04 and Y05) were not inherited by the randomly sampled seedlings. It is possible that there are offspring of Y02 or Y04 in the ex-situ conserved seedlings propagated in 2008, which were not sampled in this research. It is also possible that the six alleles were not included in the gene pool of all ex-situ seedlings, which case 26% of the genetic variations of *A. yangbiense* have not been conserved by former conservation strategies.

#### 4.4. Implications for conservation

Future preservation of the species *A. yangbiense* cannot afford the loss of any of the surviving individuals. Therefore forest rangers have been assigned to patrol the area at least once per week. Necessary measures, such as in vitro culture and collecting seeds of Y02 and Y04, which could also cover the remaining genotypes, must be taken to save all genetic variations. This species is not genetically depauperate, but its growth environment has been damaged and will be difficult for the population to recover. The overlap of *A. yangbiense* with Malutang village makes human disturbance such as farming around them and grazing by animals difficult to forbid. To save this species in the wild, artificial pollination and reintroduction of genetically diverse seedlings to the surrounding forests could be more beneficial than allowing for the slow process of natural recovery. Additionally, seedlings with new genotypes should be conserved ex-situ as well.

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