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High regional genetic differentiation of an endangered relict plant *Craigia yunnanensis* and implications for its conservation

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ABSTRACT

Of the genus *Craigia*, widespread in the Tertiary, only two relict species survived to modern times. One species is now possibly extinct and the other one, *Craigia yunnanensis*, is severely endangered. Extensive surveys have located six *C. yunnanensis* populations in Yunnan province, southwest China. Using fluorescent amplified fragment length polymorphism (AFLP), the genetic diversity and population structure of these populations were examined. It was found that genetic diversity of *C. yunnanensis* was moderate at the species level, but low at regional and population levels. Analysis of population structure showed significant genetic differentiation between Wenshan and Dehong regions, apparently representing two geographically isolated for long time refuges. There are also clear indications of isolation between populations, which, together with anthropogenically caused decline of population size, will lead to general loss of the species genetic variation with subsequent loss of adaptive potential. To conserve the genetic integrity of *C. yunnanensis*, we recommend that ex-situ conservation should include representative samples from every population of the two differentiated regions (e.g. Wenshan and Dehong). The crosses between individuals originated from different regions should be avoided because of a high risk of outbreeding depression. As all the extant populations of *C. yunnanensis* are in unprotected areas with strong anthropogenic impact, there is no alternative to reintroduction of *C. yunnanensis* into suitable protected locations.

1. Introduction

Genetic variation within and among natural populations is important for the species future adaptive changes and evolution (Frankham et al., 2010; Frankham, 2012). Knowledge of genetic variation of endangered species can provide critical information needed to understand the evolutionary history of populations and identify short- or long-term risks for the species (Avise and Hamrick, 1996). Knowledge of extent and structure of genetic variation of endangered species is essential for designing efficient conservation practices (Hedrick and Miller, 1992; Hamrick and Godt, 1996a,b; Woodruff, 2001).

The genus *Craigia* W. W. Smith & W. E. Evans (Tiliaceae) was widespread throughout the Northern Hemisphere, and abundant across Europe, North America and East Asia during the Tertiary period (Jin et al., 2009; Kvaček et al., 2002), but only two *Craigia* species existed in modern time: *C. yunnanensis* W. W. Smith & W. E. Evans (distributed in southern China and northern Vietnam), and *Cragia kwangsiensis* H. H. Hsue (endemic to China). In the IUCN (International Union for Conservation of Nature) Red List, *C. yunnanensis* is listed as 'endangered' (http://www.iucnredlist.org/details/32335/0) and *C. kwangsiensis*is listed as 'critically endangered' (http://www.iucnredlist.org/details/32395/0). *C. kwangsiensis* known only from type and not found in the wild since 1975 apparently got extinct due to deforestation (Tang et al., 2007). *C. yunnanensis* is a deciduous canopy tree occupying limestone mountainous forests. As a result of deforestation most of the species habitat was destroyed and the remaining habitat is severely fragmented. Survey and mapping of the remaining populations is the first step of a conservation program to prevent extinction of this

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species, followed by a carefully designed action plan (Corlett, 2016; Volis, 2016).

Based on all the available information and our extensive survey of the natural habitats of *C. yunnanensis*, six small, remnant populations were located in Yunnan province (Gao et al., 2010). No material could be found in Guizhou or Guangxi, indicating that the species may already be extinct in these provinces. Its status in Vietnam is currently unknown due to travel restrictions and logistical limitations. The survey also revealed that in order to grow economically important plants such as tea, *Amomum tsaoko* and *Cunninghamia lanceolata*, locals chop down *C. yunnanensis* or change its habitat into farmlands. Facing a very high risk of extinction, *C. yunnanensis* is now listed as a plant species with extremely small populations of China (PSESP) in the national-level *Implementation Plan of Rescuing and Conserving China's PSESP* (2010–2015). A conservation strategy focusing on PSESP and aimed at rescuing the most endangered plants was approved by the Chinese government in 2009 (Ma et al., 2013; Ren et al., 2012). Conservation of *C. yunnanensis* based on this strategy must include seed collection, seedling propagation, ex-situ conservation at the Kunming Botanical Garden (KBG) and reintroductions.

Knowledge of extent and structure of genetic variation in *C. yunnanensis* may have important implications for management of the species germplasm collection, in-situ/ex-situ conservation and later reintroduction (Hoban and Schlarbaum, 2014; Li et al., 2008; Volis, 2015; Yang et al., 2015). This analysis is a preliminary step for selecting breeding material and establishing conservation strategies for *C. yunnanensis*. Here, we use AFLP (amplified fragment length polymorphism) to study extent and structure of genetic variation in *C. yunnanensis*. AFLP is commonly used in population genetic studies of rare and endangered species (Bensch and ÅKesson, 2005; Juan et al., 2004; Nybom, 2004). The results of this study can be useful for setting an appropriate conservation strategy for *C. yunnanensis*.

2. Materials and methods

2.1. Study species and sampling

C. yunnanensis (Tiliaceae) is a deciduous canopy tree. It is a predominantly outcrossing with partial self-compatibility species. The major pollinator is a blowfly (*Chrysomyia megacephala*). The flowering time is from middle August to late September. Fruit of *C. yunnanensis* is samara containing about 6 seeds (around 34 mg per seed). Upon maturation in early December, the fruits are dispersed by wind and gravity. On the bare soil, the seeds could germinate easily. The experiments (Gao et al., 2010) showed that for some populations, germination capacity in canopy gaps was significantly higher than that in closed canopy, indicating that canopy removal might promote regeneration from seed. Small seedlings and resprouts are abundant but very few of them reach the sapling stage.

Sampling was done in six populations representing two disjunct regions of Yunnan province, China (Fig. 1). Four populations, W-FD (Fadou county), W-LH (Lianhuatang town), W-ML (Malipo county) and W-MG (Maguan county), are from Wenshan prefecture (southeastern Yunnan); two populations, D-JD (Jiangdong town) and D-HG (Huguo town) are from Dehong prefecture (south-western Yunnan). Wenshan and Dehong are more than 600 km apart. Locality information of the six wild populations is provided in Table 1. The number of reproductive individuals in each population was counted and in total 105 reproductive individuals were sampled (Table 1). There were only 11 reproductive individuals in the W-MG population, and 10 of them were sampled for the AFLP analysis.

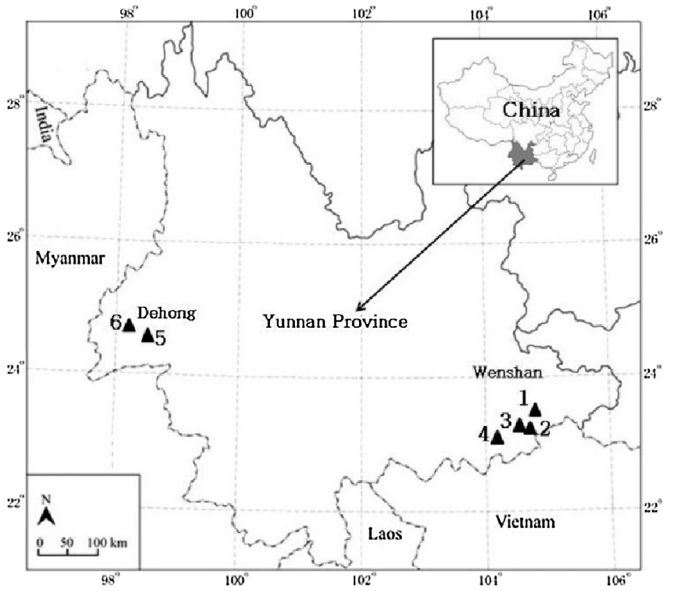

Fig. 1. The geographic locations of six studied *C. yunnanensis* populations. Numbers correspond to the population codes in Table 1.

2.2. AFLP analysis

Total genomic DNA was extracted using a modified CTAB method (Doyle, 1987). AFLP analysis was performed following Vos et al. (1995) with minor modifications. 200 ng samples of genomic DNA were digested with *Eco*RI and *Mse*I (New England Biolabs), followed by ligation of appropriate adapters. Pre-selective PCR amplifications were performed using the primer pair *Eco*RI + 1 and *Mse*I + 1. For selective PCR amplification, three *Eco*RI + 3/*Mse*I + 3 primer combinations (*E*-ACR/*M*-CAG, *E*-ACA/*M*-CAG and *E*-AAG/*M*-CTC) were chosen among 64 selective primer pairs available from Perkin–Elmer AFLP Selective Amplification Start-Up Module. PCR products were separated on a 6% poly acrylamide gel (acrylamide:bisacrylamide = 19:1) and stained using the silver nitrate method.

2.3. Data analysis

AFLP bands between 100 and 500 bp in the poly acrylamide gels were scored manually as present (1) or absent (0), and transformed into a binary data matrix. The following genetic diversity parameters were calculated using POPGENE version 1.31 (Yeh et al., 1999): the percentage of polymorphic loci (PPL), expected heterozygosity (H_E) and Shannon's information index ($I_S = -P_i \log_2 P_i$) (Lewontin, 1972). The relationship between population size (the number of reproductive individuals detected in each population, Table 1) and genetic diversity (estimated as PPL, H_E and I_S) was analyzed via Pearson's product moment correlation using SPSS statistical package version 15.0 (SPSS, Inc., Chicago, IL, USA).

Nei's genetic distance between populations (Nei, 1978), the coefficient of genetic differentiation (G_{ST}) and gene flow (Nm) were also estimated in POPGENE and a dendrogram was constructed from Nei's genetic distance with the unweighted pair-group method of averages (UPGMA) using the TFPGA software version 1.3 (Miller, 1997). Using the same software, a Mantel test was performed to calculate the correlation between the Nei's genetic distance and geographical distances. Analysis of molecular variation (AMOVA) within/among populations and between geographic regions was calculated using Arlequin 3.1 (Excoffier et al., 2005). The