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Genetic diversity and population structure of *Ottelia acuminata* var. *jingxiensis*, an endangered endemic aquatic plant from southwest China

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ABSTRACT

O. acuminata var. *jingxiensis*, an endangered endemic species, is only distributed in rivers or streams in karst areas of southwest China. In this study, the genetic variation of 10 natural populations sampled from karst rivers was detected using 12 SSR markers. The results revealed a moderate level of genetic diversity within the populations (mean $H_E = 0.441$, $I = 0.781$) and population structure which significantly correlated with geographic distance ($r = 0.825$, $P < 0.01$). This pattern could be attributed to either restricted gene flow ($Nm = 0.341$) between the long-isolated populations or the occurrence of biparental inbreeding in closely distributed individuals ($F_{IS} = 0.362$). The analysis of molecular variance (AMOVA) revealed a high level of genetic variation among the populations ($F_{ST} = 0.423$) of *O. acuminata* var. *jingxiensis* and accounted for over 55% of the total genetic differentiation. Compared to gene flow, genetic drift is more likely to promote the current genetic structure because of low gene exchange among isolated habitats. Complete geographical isolation offers an opportunity for allopatric speciation at relatively small spatial scales that promotes high endemism. Our result indicated that it is likely that cryptic varieties exist in *O. acuminata*, such as Baishou (BS) and Luzhai (LZ) populations. Based on the available results, both *in situ* and *ex situ* efforts should be used to conserve this species.

1. Introduction

Ottelia, comprising of approximately 20 species, is the biggest genus of the aquatic monocotyledonous family Hydrocharitaceae (Cook and Urmi-Konig, 1984; Dandy, 1935). The genus *Ottelia* is mainly distributed in two centers of diversity, one in the tropical Africa (~13 species) and the other in Southeast Asia (~6 species) (Cook and Urmi-Konig, 1984). In southeastern Asia, the southwest region (Guangxi, Guizhou, Yunnan provinces) of China is a diversity center of *Ottelia*, comprising three endemic species, *O. sinensis*, *O. emersa* and *O. acuminata*. Within the species *O. acuminata*, five endemic varieties (i.e., *O. acuminata* var. *acuminata*, *O. acuminata* var. *jingxiensis*, *O. acuminata* var. *lunanensis*, *O. acuminata* var. *crispa* and *O. acuminata* var. *songmingensis*) that were established based on morphological characters have been recognized (He, 1991; Chen et al., 2017a,b). All varieties are predominantly outbreeding and bees are the most common pollinators; each variety produces a substantial amount of tiny seed, which easily drift in water as they are dispersed in the river systems (Li Z.Z., personal field observations). Excluding *O. acuminata* var. *acuminata*, each of the other varieties is confined to a limited range, such as a lake, a stream, or a small river ecosystem (Li, 1981).

Ottelia acuminata var. *jingxiensis*, distinguished from other varieties by its abundant male flowers in spathes, is only distributed in the rivers of Guangxi province (i.e., Jingxi, Debao, Luzhai, Du'an, and Yongfu counties) (Chen et al., 2017a,b). This variety is normally used to monitor changes in water quality due to its incredible sensitivity to water pollution. In recent years, increased water pollution and excessive human disturbance have greatly affected most of natural populations of this variety resulting in further deterioration and gradual extinction in many localities (Li Z.Z., personal field investigations during 2015–2017). In addition, the habitats of *O. acuminata* var. *jingxiensis* are in karst geological regions of southwest China, which has

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distinctive features, including high pH and calcium content. In particular, the great variation in hydrography (i.e., ecohydrological cycle) is different from other systems (Bonacci et al., 2009) and spatial segregation results in the limited dispersal, migration, and gene flow among populations of karst plant species. *Ottelia acuminata* var. *jingxiensis* is one of the typical endemic and endangered species, and has been listed in the Chinese Plant Red Book (Fu, 1992). Based on the current status, effective conservation and management programs of *O. acuminata* var. *jingxiensis* are urgently required to preserve the extant populations.

Up to now, only several studies on population genetics were done in several varieties of *O. acuminata*. Zhang et al. (2009) and Long et al. (2010) used inter-simple sequence repeats (ISSR) to estimate genetic diversity of *O. acuminata* in China. Zhang et al. (2009) indicated the polluted water did affect the genetic diversity of *O. acuminata*. Long et al. (2010) suggested high variation among populations owing to complex topography and restricted gene flow. For *O. acuminata* var. *crispa*, Li et al. (2016) investigated the genetic diversity among 11 populations based on amplified fragments length polymorphisms (AFLP); low genetic variation among populations was detected, possibly due to restricted gene flow. Zhai et al. (2018) studied the genetic diversity of representative populations of all five varieties of *O. acuminata* using 12 SSR markers and they revealed a high level of genetic variation among populations. However, in their study the sampling of each variety was limited and only two populations of *O. acuminata* var. *jingxiensis* were included. Chen et al. (2017a,b) reported the molecular divergence of *O. acuminata* including all five varieties using five chloroplast DNA (cpDNA) non-coding regions and nuclear ITS region. Although that study only included two representative populations of *O. acuminata* var. *jingxiensis* (DB and JX), two haplotype groups were found which indicated high divergence existed in *O. acuminata* var. *jingxiensis*. In view of previous studies, the genetic diversity and population structure of *O. acuminata* var. *jingxiensis* in southwest China remains largely uninvestigated.

Molecular markers (e.g. ISSR and SSR), which have been widely utilized to evaluate population genetic differentiation and population genetic structure, are still effective in contemporary genetic research of aquatic species. Simple sequence repeats (SSR) are among the most popular methods for estimating genetic diversity due to their codominant inheritance and high polymorphism (Yue et al., 2011), for example, in *Nymphoides peltata* (Liao et al., 2013), *Nelumbo nucifera* (Yang et al., 2012), and *Glehnia littoralis* (Wang et al., 2016). In the present study, we employed 12 SSR markers to assess the genetic diversity of *O. acuminata* var. *jingxiensis* in southwest China. Individuals were sampled in 10 natural populations from across the entire known distribution zones in China. The objectives of this study were to 1) assess the amount of the genetic differentiation among populations and, 2) detect the geographic pattern of genetic variation of the isolated populations in the karst river systems. The genetic information from our study will be incorporated into the conservation program of *O. acuminata* var. *jingxiensis* to provide invaluable insights for its conservation together with other endangered hydrophytes in karst areas of southwest China.

2. Material and methods

2.1. Sample collection

During June to August 2017, a total of 196 individuals from 10 populations of *O. acuminata* var. *jingxiensis* were collected from Guangxi province, China (Table 1; Fig. 1). Considering the availability and the size of the populations fresh leaves of 12 to 53 individuals were randomly sampled in each population. In order to avoid sampling of clones, attention was paid to ensure that only individuals at least 10 m apart were sampled. Each leaf was placed into a small envelope containing silica gel for immediate desiccation. Voucher specimens were deposited in the herbarium of the Wuhan Botanical Garden (HIB), Chinese Academy of Sciences.

2.2. DNA extraction, PCR amplification and sequencing

Total DNA was isolated from the silica-dried leaf using the MagicMag Genomic DNA Micro Kit (Sangon Biotech Co., Shanghai, China) according to the manufacturer's protocol and genotyped using 12 polymorphic SSR loci from our previous study (Li et al., 2017). All forward primers were labeled with a fluorescent dye FAM (Applied Biosystems, Foster City, CA, USA). SSR-PCR amplifications were performed on a PTC-100 thermocycler (Bio-Rad, Hercules, CA, USA) in 20 μL reaction volumes using the protocol described in Li et al. (2017). PCR products were checked electrophoretically on 2.0% (W/V) agarose gels at 100 V, stained with ethidium bromide. Then unambiguous amplified products were estimated on an ABI prism 3730xl and scored manually according to an internal DNA standard (Rox-500, Life Technologies) in GENEMAPPER v.4.0 (Applied Biosystems, Foster City, CA, USA).

2.3. Data analysis

Linkage Disequilibrium (LD) and deviations from Hardy-Weinberg equilibrium (HWE) were tested using GENEPOP 4.2 (Rousset, 2008). For each SSR locus, POPGENE 1.31 (Yeh et al., 1997) and CERVUS 3.0 (Kalinowski et al., 2007) were used to estimate the total number of alleles (*Na*), effective number of alleles (*Ne*), observed heterozygosity (H_o), the expected heterozygosity (*He*), Shannon' s information index (*I*) and the Polymorphic Information Content (PIC) of each SSR marker. For estimates of population-level genetic variation, the average number of alleles (N_A), the effective number of alleles (N_E), the private allele (A_P), the observed (H_O) and expected (H_E) heterozygosity, inbreeding coefficient (F_{IS}), Shannon' s information index (*I*), and the pairwise F_{ST} between populations were estimated using GenALEx 6.5 (Peakall and Smouse, 2012). Gene flow (*Nm*) among populations was estimated using the expression $Nm = (1-F_{ST}) / 4F_{ST}$ (Slatkin and Barton, 1989). ARLEQUIN 3.5 (Excoffier and Lischer, 2010) was used to perform the analysis of molecular variance (AMOVA), which estimated the relative level of genetic variation within populations (F_{ST}), among populations (F_{SC}) and among groups (F_{CT}) which were defined by STRUCTURE analysis in this study. The significance of all the values was calculated with 1000 permutations.

The detection of population genetic structure was implemented in STRUCTURE 2.3.1 (Pritchard et al., 2000) using a Bayesian clustering approach. This approach infers the genetic structure by assigning samples into distinct clusters (*K*) based on the Hardy-Weinberg principle (Gao et al., 2007). Under the admixture model with the correlated allele frequencies among populations option, the number of *K* was set to vary from 2 to 10. Ten independent simulations of each *K* were implemented with a burn-in of 50,000 and a run length of 500,000 Markov Chain Monte Carlo (MCMC) steps. The most likely value of *K* was chosen using Structure Harvester following ΔK approach which uses the second-order rate of alteration in the likelihood partition (Evanno et al., 2005). The program CLUMPP 1.1.2 (Jakobsson and Rosenberg, 2007) was used to further adjust a consistent value of *K* with 10,000 repetitions based on the Greedy algorithm. Bar plots depicting the resulting population genetic structure were generated using DISTRUCT 1.1 (Rosenberg, 2004).

To further appraise the genetic relationships between populations, the software TreeFit (Kalinowski, 2009) was applied to calculate Nei's genetic distance (D_A; Nei et al., 1983) and construct a neighbor-joining (NJ) tree based on D_A matrix with 1000 permutations. Based on a matrix of pairwise geographical and genetic distances, the relationship between geographical scale and population structure was tested using GenAlEx 6.5 (Peakall and Smouse, 2012) with 9999 permutations. The geographic distance matrix was obtained from the straight line distances between populations.

A history of a recent population bottleneck was tested with BOTTLENECK 1.2.02 (Piry et al., 1999). Wilcoxon's signed rank tests were