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Full Length Research Paper

Genetic diversity of *Chimonanthus grammatus* populations determined with inter-simple sequence repeats (ISSR) analysis: Implications for conservation

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Chimonanthus grammatus **is an endangered and national-protected medicinal plant species with highly restricted geographical distribution and disjunctly locates in the border area of Anyuan and Huichang county of Jiangxi province, China. In this study, inter-simple sequence repeats (ISSR) markers were used to analyzed genetic diversity of seven populations of *C. grammatus*, collected from the area of Anyuan (4 populations) and Huichang (3 population) county of Jiangxi province, China. Our results showed that ten primers produced 74 discernible bands (39 polymorphic), with a low genetic diversity at species level percentages of polymorphic bands (PPB = 52.7%, H = 0.157, I = 0.241) and at population level (*PPB* = 35.7%, *H* = 0.119, *I* = 0.177). A high population differentiation (G_{st} = 0.249) was detected among seven populations and significant association were found between genetic and geographical distances (r = 0.712, *p* < 0.001), suggesting that gene flow was restricted geographically. Analysis of molecular variance (AMOVA) also revealed that 26.4% of the ISSR variation resided among populations, while 73.6% resided within populations, and 21.8% of the ISSR variation resided among Anyuan and Huichang groups, while 78.2% resided within groups. Factors, such as gene flow, genetic drift and evolutionary history, might have important influence on genetic structure and diversity of *C. grammatus* populations. An understanding of the genetic diversity and population structure of *C. grammatus* can provide insight into the conservation and management of this species.**

Key words: *Chimonanthus grammatus*, genetic diversity, inter-simple sequence repeats (ISSR), conservation.

INTRODUCTION

The biodiversity on the earth is rapidly decreasing due to human activity, and many wild species are in danger of extinction. In this critical situation, maintenance of biodiversity is a global issue (Takeshi et al., 2007). The assessment of genetic diversity is the first step in evaluation the long-term conservation status of species in natural conditions (Gonzalez-Astorga and Castillo-Campos, 2004). The loss of genetic diversity within populations decreases adaptability to environmental changes, and increases extinction risk. Extinction of an individual population removes any unique biological characteristics that it may possess, influences biodiversity, and may ultimately lead to species extinction. Maintenance of genetic diversity is therefore an important factor for their conservation management (Hamrick and Godt, 1996). *Chimonanthus* Lindley is ancient angiosperm, whose pollen morphology characters are rare and unique, and it has been used as a traditional Chinese medicine for treatment of cough and rheumatic arthritis. Previous phytochemical studies on *Chimonanthus* revealed that the flower concrete was rich

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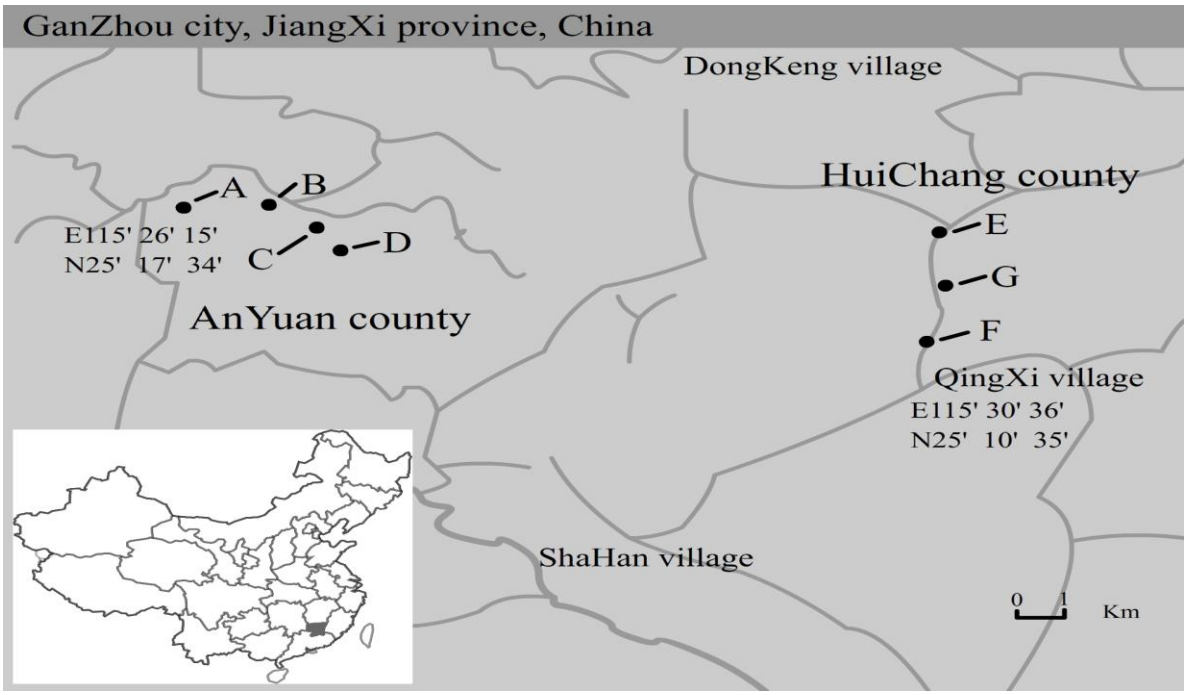

Figure 1. Map showing locations of *C. grammatus* and the study sites. A (N=29), B (N=30), C (N=30), and D (N=30) populations sampled from Anyuan county; E (N=30), F (N=32), and G (N=32) populations sampled from Huichang county. N: represented sample sizes in parentheses.

in essential oils (Ueyama et al., 1990), whereas the seeds and roots yielded alkaloids with antifungal bioactivity (Zhang et al., 2009) or antinociceptive. *Chimonanthus grammatus* is a transitional species between *sect Chimonanthus* and *sect Neochimonanthus* and also lies in unique systematic status during the evolutionary process of *Chimonanthus* (Zhang and Shen, 1999).

It is an evergreen shrub with traditional fragrant flower and peculiar fruit. The unique flowering time and long blooming period can make it as an ornamental plant. Also, chemical components of the species are rich as medicinal materials and volatilized factor is of great potential as a source of essential oil (Peng et al., 2002; Liu et al., 2011). However, it is an extremely endangered species with a highly restricted geographical distribution and disjunctly locates in the border area of Anyuan and Huichang county of Jiangxi province, China (Peng et al., 2002). Therefore, it is urgent to scientifically conserve this rare species. ISSR have been used in genomic fingerprinting, such as genetic diversity, phylogenetic analyses, evolutionary biology, and in several other fields (Shafie et al., 2011; Wang, 2010; Zhang et al., 2011). To date, studies on *C. grammatus* were confined to morphology, population distribution, community structure and biodiversity (Peng et al., 2002, 2005). To our knowledge, studies of genetic diversity and structure in the rare plant not only enhance our understanding of population dynamics, adaptation and evolution, but also provide useful information for biological conservation (Li et al., 2008; Rahimmalek et al., 2009; Gaiero et al., 2011). So it is necessary for using a sensitive and credible molecular technique to get genetic data of *C. grammatus*. Since no sequence information for the species is required, ISSR technique is especially suited to analyze the genetic diversity and population structure. The objectives of the present study were to: (a) describe the genetic diversity in *C. grammatus*; (b) to propose a conservation strategy based on genetic variation.

MATERIALS AND METHODS

Plant materials

The natural populations of *C. grammatus* are only distributed in the border area of Anyuan and Huichang County of Jiangxi province, China. According to the field survey information, seven wild populations of *C. grammatus* were sampled throughout its distribution range. Four populations (A, B, C and D) came from Anyuan County, and the other three populations were (E, F and G) from Huichang County. Twenty-nine to thirty-two plants were collected at random from each population at intervals of at least 3 m. A total of 213 individuals from the seven populations were included in the ISSR study. Details of experiment sites, population size and sampling number were given in Figure 1. About 5 to 10 g of fresh leaves was collected from individual and immediately stored in a sealed plastic bag containing about 50 g of silica gel which speeded up the drying process. The samples brought to the laboratory were stored at -70°C until deoxyribonucleic acid (DNA) extraction.

Table 1. Selected 10 ISSR primers used for DNA amplification of the 213 *C. grammatus* individuals from seven populations.

Primer code	Primer sequence	Suitable annealing T_m/°C	No of bands scored	No of polymorphic bands	% of polymorphic fragments
U811	$(GA)_8C$	57.2	9	7	77.8
U815	$(CT)_8G$	50.8	6	4	66.7
U817	$(CA)_8A$	53.7	7	4	57.1
U818	$(CA)_8G$	55.6	6	5	83.3
U823	$(TC)_8C$	52.1	7	1	14.3
U826	$(AC)_8C$	55.6	8	3	37.5
U827	$(AC)_8G$	55.6	9	3	33.3
U835	$(AG)_8YC$	55.6	9	5	55.6
U857	$(AC)_8YG$	55.6	8	5	62.5
U861	$(ACC)_6$	58.3	5	2	40.0

Genomic DNA extraction and inter-simple sequence repeat (ISSR) polymerase chain reaction (PCR) amplification

Genomic DNA of *C. grammatus* was isolated according to the modified Cetyl trimethylammonium bromide (CTAB) method (Zhao and Zhang, 2007). DNA was quantified by comparison with λDNA following electrophoresis in a 1% agarose gel. DNA was diluted to 20 ng/µl and stored at -20°C for ISSR amplification. One hundred primers (Biotechnology Laboratory, University of British Columbia, primer set # 9, Vancouver, BC, Canada: http://www.biotech.ubc.ca/services/naps/primers.pdf) were screened, and ten primers that could produce reproducible bands were used for ISSR analysis. PCR amplification was carried out in a total volume of 20 µl, containing 50 ng of template DNA, 1.5 mM $MgCl_2$, 0.15 mM Deoxyribonucleotide Triphosphates (dNTPs) (Promega, Madison, WI, USA), 600 nM primer (Shanghai shenggong, Shanghai) and 2 U Taq polymerase (Promaga Co). PCR reactions were carried out on a PTC-100^{TM} (MJ Research, USA), and programmed for an initial denaturation temperature at 94°C for 5 min, followed by 40 cycles 94°C for 1 min, 50.8 to 58.3°C for 45 s, 72°C for 2 min, and a finally 5 min at 72°C for final extension. Amplification products were electrophoresed on 2% agarose gels stained with ethidium bromide, and visualized by ultraviolet (UV) and recorded using UPV-8000 (Ultra-Violt Products Limited USA). Sizes of amplification products were estimated using a 100-bp DNA ladder (Li et al., 2008).

Data analysis

The amplified DNA fragments (bands) were scored as present (1) or absent (0), and the data matrix of ISSR banding patterns of all populations was assembled for further analysis. The following parameters were calculated in order to estimate genetic diversity: the percentage of polymorphic bands (*PPB*), Nei's (1978) genetic diversity (*H*) and Shannon index of diversity (*I*) (King and Schaal, 1989). Differentiation within and among *C. grammatus* populations was estimated by the coefficient of gene differentiation (G_{st}). All the calculations were performed using the program POPGENE version 1.31 (Yeh et al., 1999). The gene flow estimates (N_m) were calculated as $N_m = (1-G_{st})/4G_{st}$ (Slatkin and Barton, 1989), where N_m is the number of migrants per generation. AMOVA was performed using squared Euclidean distances (Excoffier et al., 1992) among all plants. Variance was apportioned to the following components: among populations and within populations.

The genetic analyses were performed with WINAMOVA v.1.55 (Excoffier, 1993). Input files for this program were generated using DCFA 1.1 (Zhang et al., 2002). Significance test were performed using 1000 permutations. To further investigate phonetic relationships among populations or among individuals, the binary ISSR matrix was used to calculated pairwise band similarity coefficients of Nei (1978). Clustering analysis of all populations and all individuals were performed using the unweighted pair group method with an arithmetic average (UPGMA) using NTSYS-pc 2.10e. In addition, in order to examine a correlation between genetic and geographical distances among populations, a Mantel test was performed using the program IBD v1.52 (Bohonak, 2002). The principle coordinate analysis (PCA) was performed on the Nei's (Nei, 1978) unbiased genetic distance matrix with the DECENTER and EIGEN programs using NTSYS pc2.1 (Rohlf, 2000).

RESULTS

Primer selection and ISSR polymorphism

Ten primers were selected for the identification of *C. grammatus* based on the number of amplification products, the quality of the profiles, the level of polymorphism, and the reproducibility of bands. A total of 74 clear and reproducible DNA fragments were obtained using 10 primers, with an average of 7.4 bands per primer (Table 1 and Figure 2). Size of the amplified products ranged from 300 to 1600 bp and the number of amplified products per primer ranged from 9 (U 811, U827and U835) to 5 (U861). The percentage of polymorphic bands varied from 83.3% (U818) to 14.3% (U823). The number of polymorphic bands per primer ranged from 1 to 7 (Table 1).

Genetic diversity of *C. grammatus* populations

Shannon indices at the ISSR level ranged from 0.095 to 0.222, with an average value of 0.177. Nei's gene diversity (*H*) ranged from 0.060 to 0.154; with an average of 0.119 (Table 2). The PPB for this species was 52.7%. At the species level, the Nei's index and Shannon index were 0.157 and 0.241, respectively. At the population

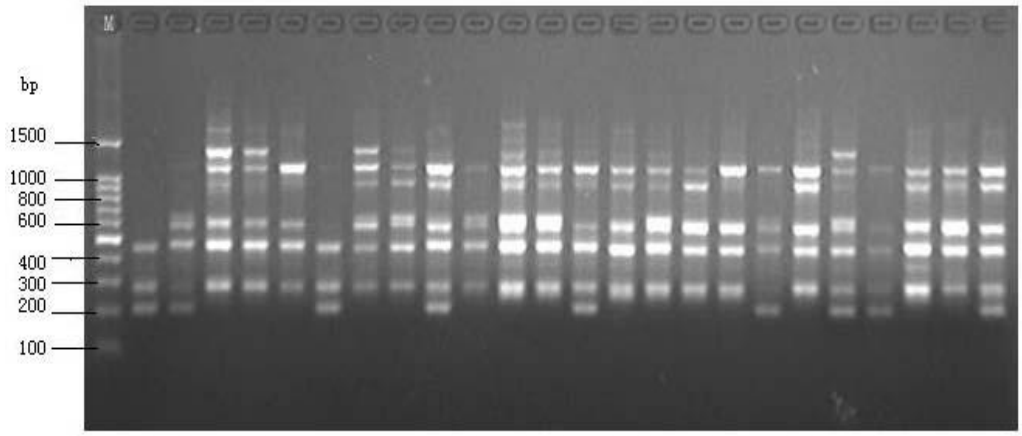

Figure 2. Map showing ISSR profiles of 24 samples from *C. grammatus* population using primer U811. M: 100 bp DNA ladder marker.

Table 2. Genetic diversity within seven populations of *C. grammatus*.

Population	N	No of bands	PPB (%)	H	I
A	29	74	40.5	0.134	0.201
B	30	74	41.9	0.142	0.212
C	30	74	37.8	0.126	0.190
D	30	74	37.8	0.154	0.222
E	30	74	35.1	0.096	0.148
F	32	74	35.1	0.118	0.174
G	32	74	21.6	0.060	0.095
Population level		74	35.7	0.119	0.177
Species level	213	74	52.7	0.157	0.241

N: sample size; PPB: Percentage of polymorphic band; H: Nei's genetic diversity; I: Shannon's information index.

level, PPB values ranged from 21.6 to 41.9%. Genetic diversity of four populations from Anyuan County exhibited higher than that of three populations from Huichang County (Table 2).

Genetic relationship within and among *C. grammatus* populations

The coefficient of genetic differentiation between populations (G_{st}) was 0.249, indicating 24.9% genetic variation between *C. grammatus* populations. The number of migrants (N_m) was estimated to be 0.755. AMOVA analysis further revealed significant ($P < 0.001$) genetic differences among and within the seven populations of *C. grammatus*. 26.4% of the total genetic diversity was distributed among populations and the rest 73.6% resided within populations. 21.8% of the ISSR variation resided among Anyuan and Huichang groups, while 78.2% resided within regions (Table 3). Population pairwise relationships showed the lowest genetic distance occurred between population E and G (0.0208), and the highest genetic distance between population A and E (0.0875) (Table 4). While the opposite trend was showed in the genetic identity, that is, the lowest genetic identity was observed between population A and E, and the highest genetic identity between population E and G (Table 4). Based on Nei's (1978) genetic distance, a cluster analysis UPGMA indicated that four populations (B, C, D and A) from Anyuan county clustered together as a clade and three populations (E, G and F) from Huichang county clustered as another clade (Figure 3). Association among 213 samples of *C. grammatus* by PCA was consistent with the result of UPGMA (Figure 4). A mantel test showed significant correlation between genetic and geographic distance ($r = 0.712$, $p < 0.001$) from 1000 randomizations.

DISCUSSION

Genetic diversity of populations

A lower level of genetic diversity at species level of

Table 3. Analysis of molecular variance (AMOVA) for genetic differentiation within/among populations.

Source of variation	d. f.	SSD	MSD	Variance component	Total variance (%)	P-value
Among regions	1	130.9973	130.997	1.2060	21.8	<0.001
Within regions	211	912.6741	4.325	4.3255	78.2	<0.001
Among populations	6	268.5458	44.758	1.3475	26.4	<0.001
Within populations	206	775.1256	3.7630	3.7627	73.6	<0.001

Table 4. Nei's genetic identity (above diagonal) and genetic distance (below diagonal) among seven *C. grammatus* populations calculated by using Nei's unbiased measures.

Population	A	B	C	D	E	F	G
A	****	0.9660	0.9369	0.9431	0.9163	0.9256	0.9292
B	0.0345	****	0.9683	0.9488	0.9387	0.9313	0.9389
C	0.0652	0.0322	****	0.9604	0.9570	0.9436	0.9369
D	0.0586	0.0526	0.0404	****	0.9475	0.9598	0.9435
E	0.0875	0.0632	0.0440	0.0539	****	0.9778	0.9794
F	0.0773	0.0712	0.0580	0.0410	0.0224	****	0.9638
G	0.0735	0.0630	0.0652	0.0582	0.0208	0.0369	****

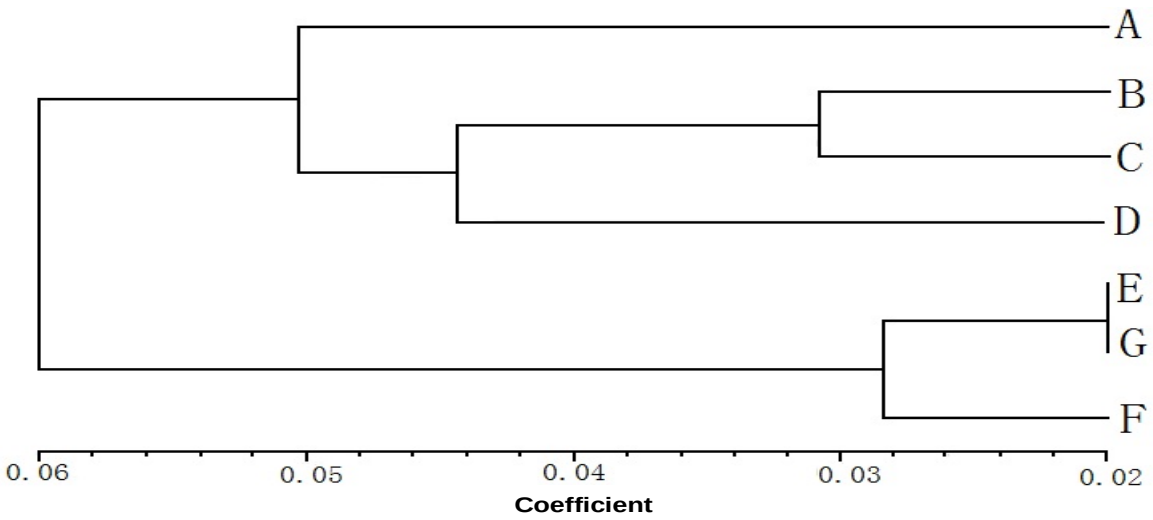

Figure 3. UPGMA dendrogram for seven populations of *C. grammatus* based on Nei's genetic distance using ISSR markers.

C. grammatus (*P* = 52.7%, *H* = 0.157) was detected in this study, as compared with the mean value (*P* = 64.7%, *H* = 0.257) of 220 genus, 662 other species (Hamrick and Godt, 1992). An average of Nei's index (*H* = 0.119) at population level was lower than the mean value (*H* = 0.22 or 0.23) of many species (Nybom, 2004). This revealed that the level of genetic diversity of *C. grammatus* was low not only at species level but also at population level. The low genetic diversity of *C. grammatus* might due to the breeding system, restricted geographic attribution, gene flow, gene drift, selfing, as well as natural selection. Hamrick and Godt (1990) reported that breeding system is mainly affects genetic diversity both among and within populations. In general, out crossing species commonly have higher levels of genetic diversity and lower differentiation between populations than selfing and clonal plants (Rossetto et al., 1995).

The percentage of total genetic variation among *C. grammatus* populations (G_{st}) was 24.9%, which is relatively higher than the mean G_{st} value (0.129) reported in the 220 genus, 662 other species (Hamrick and Godt, 1992). Studies by Ellstrand and Elam (1993) also

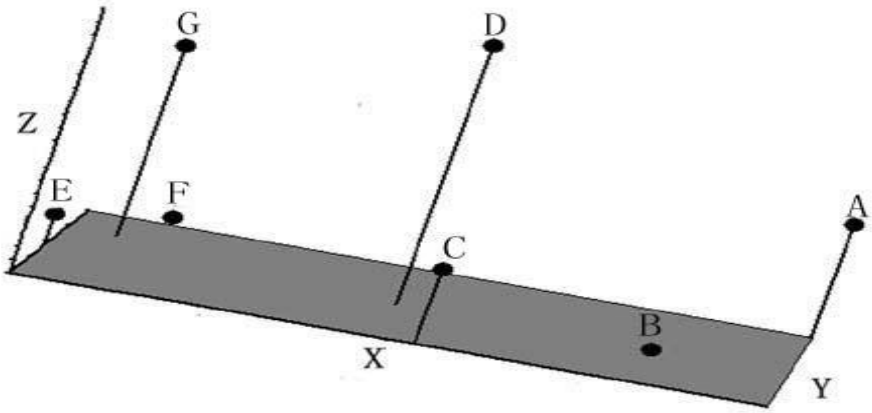

Figure 4. Associations among 213 samples of *C. grammatus* revealed by principal component analyses (PCA).

demonstrated that G_{st}>0.1 indicated a high level of genetic differentiation. AMOVA analysis further revealed a highly genetic differentiation (26.4%) among the seven populations of *C. grammatus* and a highly genetic differentiation (21.8%) among regions (Table 3).

The highly genetic differentiation was mainly affected by the geographic range, which was explained by the Nei's genetic identity and genetic distance (Table 4). Furthermore, seeds of *C. grammatus* are entrapped by pericarp and dispersed by gravity. Lack of effective mechanisms for long-distance dispersal of seeds may also play an important role in shaping the low level of diversity and the observed genetic structure (Wallace, 2002). Addition, the mantel test also showed that there was significant correlation (r = 0.712, p<0.001) between the geographical distance matrix and the pairwise genetic distance matrix of *C. grammatus* populations. This indicated that gene flow may have occurred only between closely neighboring populations. The mating system is the most critical factor to affect the genetic diversity (He and Liu, 2003). At present, there have been no comprehensive studies on *C. grammatus*' mating system, it was found that mating system of *Chimonanthus nitens* Oliv. and *Chimonanthus praecox* were facultative hybridization, with a small part of self-pollination which needs pollinators, and the seed set rate of plants by xenogamy was higher than one of plants by self-pollination (Zhou et al., 2003, 2006). *C. grammatus* is quite similar to *C. nitens* Oliv., with the low seed set and producing small amounts of viable seeds. In addition, the percentage of total genetic variation among *C. grammatus* populations (G_{st} = 24.9%) is between the average of out crossing plant species (G_{st} = 10%) and the average of selfing plant species (G_{st} = 51%) (Hamrick and Godt, 1990). The gene flow (N_m = 0.755) of *C. grammatus* is highly restricted. According to these facts, it is very possible that mating system of *C. grammatus* belongs to facultative hybridization.

The conservation program of *C. grammatus*

In recent years, *C. grammatus* have declined rapidly in number of individuals. The natural habitat of *C. grammatus* existed in a very restricted region. Therefore, it is quite necessary to carry out relevant strategies for conservation management. Maintenance of genetic variation is considered essential for the long-term survival of a species. The information obtained in this study provides valuable baseline data of population genetics to address conservation concerns for this threatened species. (1) Conservation by preserving as many populations as possible is the best way to maintain genetic diversity. (2) Conservation must pay more attention to restoring the suitable habitat based on the biological characteristics and ecological traits and to restoring the efficient population size to minimize the loss of the genetic variation. (3) Make more researches on asexual reproduction, because *C. grammatus* is the low seed set and does produce small amounts of viable seeds. Further studies on pollen and seed dispersal are needed to understand the mating system of *C. grammatus.* (4) Replacement of the lost population at certain locations can be achieved by transplanting seedlings from different populations, increasing the seed germination, breaking the dormancy period and so on to artificially increase the gene flow among populations and maintain the sufficient genetic richness.

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