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Genetic variation and conservation of *Changnienia amoena*, an endangered orchid endemic to China

A. Li and **S. Ge**

Laboratory of Systematic and Evolutionary Botany, Institute of Botany, Chinese Academy of Sciences, Beijing, China

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Abstract. *Changnienia amoena* is a diploid and self-compatible orchid endemic to China. This species is in great danger of extinction with its current distribution being highly fragmented and discontinuous. This study investigated the level and apportionment of genetic diversity of this species using RAPD technique. Based on 119 discernible DNA fragments generated by 16 primers, an intermediate level of genetic diversity was found at the species level with the percentage of polymorphic bands (*P*) of 76.5%, expected heterozygosity (*He*) of 0.194. However, the genetic diversity at the population level was significantly lower ($P = 37.2\%$, $He = 0.120$) compared with the average of other species with similar life history characteristics. A high level of population differentiation was detected with 43.8% variation resided among populations as measured by AMOVA. It is noteworthy that as much as 49.2% of the total diversity could be attributed to difference among populations when five populations from an area of approximately 60×25 km in the Xinning County were considered. These results, in conjunction with other evidence from pollination and ecological studies, suggest that the low within-population but high among-population variation in *C. amoena* is likely due to the small population sizes and local extinction because of habitat destruction and loss. The restricted gene flow is probably another factor contributing to the genetic structure found in *C. amoena*. Based on these findings, we proposed conservation managements for this endangered species, including habitat protection along with the protection of their pollinators, artificial pollination as well as *ex situ* conservation.

Key words: Conservation genetics, RAPDs, *Changnienia amoena*, endangered orchid.

Introduction

The Orchidaceae is one of the largest and most diverse families of flowering plants, including up to one tenth of all flowering plant species in the world (Dressler 1993). Since the years of Darwin, orchids have become one of the most popular groups of plants and have been featured in thousands of books and magazines. Their extraordinary species diversity, important evolutionary status, unique reproductive structures, and interesting interactions with pollinators have increasingly attracted studies on their phylogeny, population genetics, pollination and coevolution (Dressler 1993, IUCN/SSC Orchid Specialist Group 1996, Soliva and Widmer 1999, Sun and Wong 2001). It is now well known that biodiversity

is being lost globally at a rate that is faster than at any previous time in history (Heywood and Watson 1995). The situation is much worse for orchid species partly because most of them only survive in certain habitats and thus are susceptible to the habitat deterioration and fragmentation, and partly because they often bear spectacular flowers and thus experienced mass-collection for trade or by amateurs (IUCN/SSC Orchid Specialist Group 1996). Therefore, most orchid species are now considered to be at risk of extinction as a result, directly and indirectly, of human activities, and almost all of them are included in conservation lists (IUCN/SSC Orchid Specialist Group 1996).

Changnienia Chien is a monotypic genus of the tribe Calypsoeae, Orchidaceae (Dressler 1993), and is endemic to the eastern and central China (Fu 1992, Chen et al. 1999). *C. amoena* Chien, as the only species in the genus, is a diploid ($2n = 46$), self-compatible, perennial herb occurring at the altitude of 400–1500 m (Fu 1992, Xiong et al. 2003). Historical record indicated that this species had a relatively wide distribution throughout the hilly regions of subtropical areas in China (Fu 1992). Unfortunately, recent expeditions have revealed that many populations of *C. amoena* recorded previously have been extirpated and the current distributions are highly fragmented and discontinuous because of destruction and degradation of habitats by agriculture, silviculture, grazing and urbanization during the past decades (Fu 1992, Xiong et al. 2003, Sun et al. 2006). In addition to habitat loss, another threat to this species is the mass collection that has happened in many localities because its pseudobulbs are used as a medicine in the treatment of sores and snakebite (Fu 1992). Consequently, *C. amoena* was listed on the China Plant Red Data Book (Fu 1992) and now is in greater danger of extinction. As a taxonomically important group, *C. amoena* has received much attention and great efforts have been made to uncover its distribution, morphology, pollination biology and ecology (Wang et al. 1994, Chen and Tsi 1998, Xiong et al. 2003, Sun et al. 2006). However, population genetics of this species remains unknown, which makes it difficult to clarify the causes of its endangerment and to develop an effective conservation management.

Population genetic studies in conservation biology have been the subject of considerable discussion and have increasingly emphasized because assessment of the level and distribution of genetic diversity within species may not only contribute to knowledge of their evolutionary history and potential, but is also critical to their conservation and management (Hamrick and Godt 1996, Frankham et al. 2002). Nevertheless, studies on population genetics of orchid species are poorly represented in the literature although efforts have been made in recent decades toward this direction (Ackerman and Ward 1999, Wong and Sun 1999, Alexandersson and Ågren 2000, Ehlers and Pedersen 2000, Sun and Wong 2001, Li et al. 2002, Forrest et al. 2004, Hollingsworth et al. 2004). RAPD analysis is a popular method for estimating genetic diversity in plant populations with several advantages such as speed, low cost, and the use of small amounts of plant material (Huff et al. 1993, Ge et al. 1999, Nybom and Bartish 2000, Kingston et al. 2004). Because no sequence information for the target species is required, RAPD technique is especially suited to plant groups such as most orchids where little or no molecular genetic study has been conducted previously. As part of a large project that seeks to clarify the causes of the endangerment of *C. amoena* and to develop an effective and scientific protection plan, we conducted an investigation on the level and apportionment of genetic diversity in this species using RAPD technique. Such information should contribute to a better understanding of the genetic profile of this endangered species and help to develop strategies for its conservation and sustainable utilization.

Materials and methods

Sample collection. During 2000 and 2001, a total of 216 individuals representing 11 natural populations

of *C. amoena* were sampled across the main distribution of the species. These populations were sampled from Hunan (XN), Hubei (SNJ), Henan (JGS), Anhui (TTZ), and Jiangxi (LS) Provinces (Fig. 1). Of them, three populations (SNJ1, SNJ2, and SNJ3) were collected from Shennongjia (SNJ) of Hubei Province, while five populations (XN1-XN5) were sampled from Xinning (XN) of Hunan Province. The studied populations ranged from 26.45 N to 31.70 N in latitude and from 110.84 E to 115.95 E in longitude. Since the plant produces a single ovate-elliptic leaf and a solitary flower at the

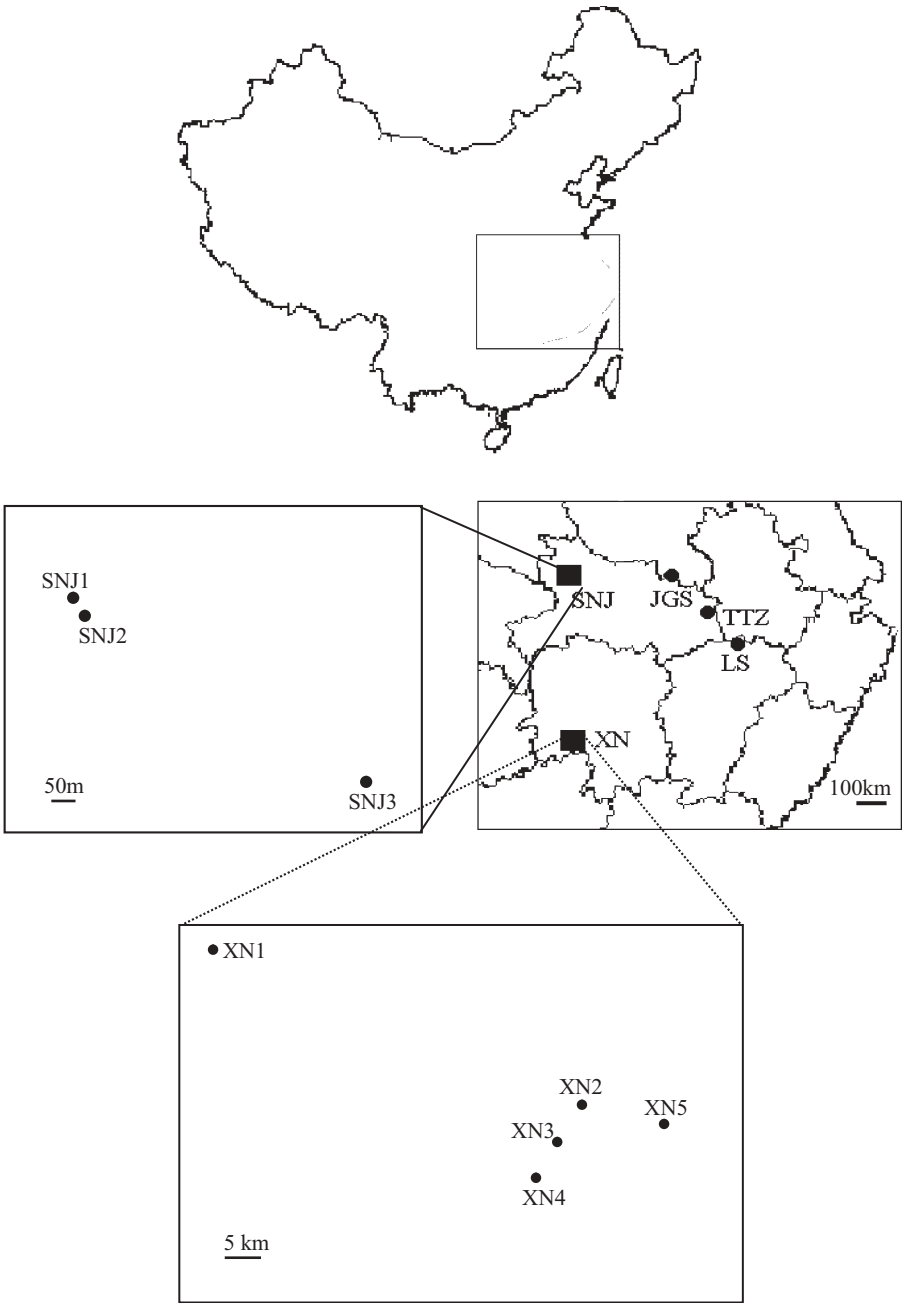

Fig. 1. Locations of the populations sampled. Alphanumeric codes of populations correspond to those in Table 1

top of 10–17 cm long stem, only 1/3 to 1/2 of the leaf was harvested from each individual in order to minimize the disturbance to their growth. Because there were less than 30 individuals in majority of the populations, all the individuals found were sampled unless more than one individuals grew within 1×1 m^2 where only one individual was sampled. Leaves were dried and stored with silica gel in zip-lock plastic bags until DNA extraction. The localities and sample sizes of the populations and their habitats are listed in Table 1.

DNA isolation and PCR amplification. Total DNA was extracted as described previously (Ge et al. 1999). Two hundred RAPD primers from Shengong Inc. (Shanghai, China) were screened with two randomly selected individuals from two populations. Sixteen RAPD primers (S14, S200, S219, S229, S249, S261, S288, S320, S326, S332, S337, S346, S348, S362, S363, and S375) that produced clear and reproducible fragments were selected for further analysis. DNA amplification was performed in a PTC-100^{TM} thermocycler (MJ Research Inc, USA), and commenced with 2 cycles of 90 s at 94 °C, 20 s at 36 °C, 40 s at 72 °C; followed by 42 cycles of 10 s at 94 °C, 10 s at 36 °C, 90 s at 72 °C; and ended with 7 min at 72 °C. Reactions were carried out in a volume of 10 μL containing 50 mM Tris-HCl (pH 8.3), 500 μg /mL BSA, 10% Ficoll, 1 mM Tartrazine, 2 mM $MgCl_2$, 200 μM dNTP, 1 μM primer, 5 ng of DNA template and 0.5 U *Taq* polymerase. Amplification products were resolved electrophoretically on 1.5% agarose gels run at 100 V in 1 × TBE, visualized by staining with ethidium bromide, and photographed under ultraviolet light. Molecular weights were estimated using a 100 bp DNA ladder.

Data analysis. RAPD bands were scored as present (1) or absent (0) for each DNA sample, and a matrix of RAPD phenotypes was used for statistical analysis. Genetic parameters including the percentage of polymorphic bands (*P*), expected heterozygosity (*He*), and Nei's subpopulation differentiation (*Gst*) were calculated using the computer program POPGENE (Yeh et al. 1997). A dendrogram of Nei's genetic distance was constructed using an unweighted paired group method of cluster analysis with arithmetic averages (UPGMA).

To further describe population structure and variability among populations without the

Table 1. Population number, size, locality and habitat of 11 populations of *C. amoena*

Population	Location	Sample size	Habitat
JGS	Jigongshan, Henan	8	500–600 m, under sparse bamboos or low trees
LS	Lushan, Jiangxi	25	On east-facing slope at 400 m under mixed shrubs and trees
SNJ1	Shennongjia, Hubei	13	On north-facing slope at 1300 m, under low trees
SNJ2	Shennongjia, Hubei	18	On north-facing slope at 1300 m, in degraded woodland
SNJ3	Shennongjia, Hubei	24	On north-facing slope at 1300 m, in degraded woodland
TTZ	Tiantangzhai, Anhui	25	950 m, under mixed shrubs and trees
XN1	Xinning, Hunan	17	On south-facing slope at 1100 m, under shrubs and scrubby trees
XN2	Xinning, Hunan	23	On north-facing slope at 1000 m, under sparse bamboos
XN3	Xinning, Hunan	23	On east-facing slope at 1100 m, under sparse bamboos
XN4	Xinning, Hunan	32	Mass collection by local people
XN5	Xinning, Hunan	20	On north-facing slope at 1100 m, under bamboos along roads

Table 2. Genetic diversity of *C. amoena* based on RAPD data*

Population	Sample size	*P* (%)	He
JGS	8	31.9	0.110
LS	25	36.1	0.124
SNJ1	13	39.5	0.133
SNJ2	18	32.8	0.110
SNJ3	24	35.3	0.130
TTZ	25	48.7	0.136
XN1	17	24.4	0.083
XN2	23	43.7	0.148
XN3	23	42.0	0.125
XN4	20	40.3	0.119
XN5	20	34.4	0.096
Average	19.6	37.2	0.120
(SD)		(6.60)	(0.019)
Species	216	76.5	0.194

**P*, percentage of polymorphic bands; *He*, expected heterozygosity

assumption that the populations are in Hardy-Weinberg equilibrium, the non-parametric Analysis of Molecular Variance (AMOVA) was performed with the RAPDistance program version 1.04 (Armstrong et al. 1994) and WINAMOVA program version 1.5 (Excoffier et al. 1992), where the variation was partitioned among individuals within populations, among populations within regions, and among regions.

Results

Sixteen RAPD primers produced 119 bands ranging in size from 250 to 1500 bp with 91 polymorphic ones, i.e. the percentage of polymorphic bands (*P*) at the species level was 76.5%. Each individual sampled gave a unique RAPD band profile, indicating that no samples shared the same genotype. Genetic diversity varied greatly among populations with the *P* values ranging from 24.4% (XN1) to 48.7% (TTZ). The expected heterozygosity (*He*) showed a similar trend, i. e. the lowest value was also found in XN1 (0.083) but the highest was found in XN2 (0.148), followed by TTZ (0.136) (Table 1). The mean values of *P* and *He* at the population level were 37.2%, and 0.120, respectively. The value of *He* was 0.194 at the species level.

To assess the overall distribution of genetic diversity, the AMOVA program was used to analyze the distance matrix obtained by RAPDistance program. Table 3 shows the AMOVA results with different variation partitions. It is apparent that highly significant ($p < 0.001$) genetic differentiations exist among regions, among populations within regions as well as within populations ($p < 0.001$). Of the total genetic diversity, 14.99% was attributable to regional divergences, 30.85% to population differentiation within regions, and 54.17% resided within populations. When considered at the regional and population levels separately, 30.12% of the total genetic variation was distributed among five regions whereas 43.75% of the total genetic variation resided among 11 populations (Table 3). These results suggest that high level of genetic differentiation

Table 3. Analysis of molecular variance (AMOVA) for *C. amoena* populations using RAPD

Source of variance	d.f.	SSD	MSD	Variance component	Percentage (%)	P-value
Variance among groups	4	13.356	3.339	0.0417	14.99	< 0.001
Variance among populations within groups	6	11.005	1.834	0.0858	30.85	< 0.001
Variance within populations	205	30.890	0.151	0.1507	54.17	< 0.001
Variance among populations	10	24.361	2.436	0.1172	43.75	< 0.001
Variance within populations	205	30.890	0.151	0.1507	56.25	< 0.001
Variance among groups	4	13.356	3.339	0.0856	30.12	< 0.001
Variance within groups	211	41.895	0.199	0.1986	69.88	< 0.001

occurred both among regions and among populations in this species. Nei's *Gst* value (0.375) showed a similarly high level of population differentiation.

To further detect the population structure on a local scale, we conducted a similar AMOVA investigation on five populations in Xinning County (XN1–XN5) because these populations were sampled from a limited area of approximately 60×25 km^2. The result indicated that a large proportion of the total genetic diversity (49.17%) existed among populations ($p < 0.001$), suggestive of significant high level of population differentiation even within a small area.

The UPGMA phenogram based on Nei's unbiased genetic distance matrix was shown in Fig. 2. It is obvious that population XN4 was distinct while the remaining populations formed two clusters. The first cluster consists of populations from two regions (SNJ and TTZ), while the second includes populations from the remaining three regions (XN, LS, JGS). It is noteworthy that the five populations from Xinning regions (XN1-XN5) occurred in different clusters, indicating that there is no trend that genetic distance increases with geographic separation. To investigate a possible correlation between genetic relationships and geographic distances, we compared Nei's unbiased genetic distance matrix with a corresponding geographic distance matrix. The two matrices were not significantly correlated ($r = 0.243$, $P = 0.925$), which is concordant with the AMOVA analysis in which high genetic differentiations were found among populations at both regional and local levels (Table 3).

Discussion

Genetic diversity. In recent decades, population genetic structures of a large number of plant species have been investigated using allozyme and RAPD techniques (Hamrick and Godt 1989, Bussell 1999, Nybom and Bartish 2000), as well as other molecular markers. In their recent reviews of RAPD-based studies of natural plant populations, Nybom and Bartish (2000) demonstrated that taxonomic status, successional stages, and particularly breeding system had a highly significant impact on within population diversity, in good agreement with the conclusion by Hamrick and Godt (1989)

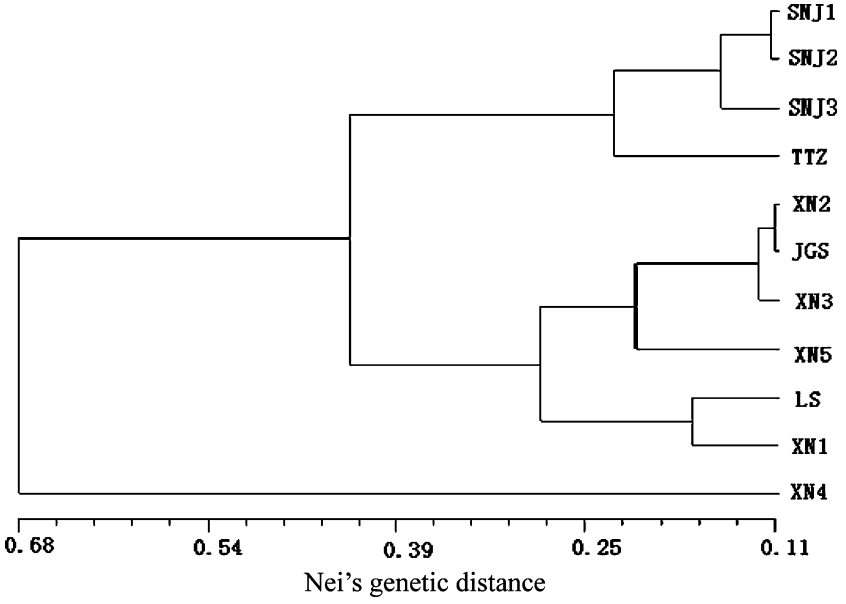

Fig. 2. Dendrogram of Nei's genetic distances among 11 populations of *Changnienia amoena*

based on allozyme data. Wong and Sun (1999) and Sun and Wong (2001) have studied four orchid species and found that the RAPD diversity varied greatly both at the species level ($Ps = 49.3 \sim 97.0\%$, $Hes = 0.144 \sim 0.293$) and at the population level ($Pp = 2.8\% \sim 55.1\%$, $He = 0.011 \sim 0.181$; the subscript s and p are used to indicate the measures at the species and population levels, respectively, and hereafter). The result of our previous study on an endangered orchid species (*Paphiopedilum micranthus*) ($Ps = 73.3\%$, $Hes = 0.210$; $Pp = 45.2\%$, $Hep = 0.146$) (Li et al. 2002) also fell within this range. The present study indicates that *C. amoena* has comparable and intermediate RAPD diversity ($Ps = 76.5\%$, $Hes = 0.194$; $Pp = 37.2\%$, $Hep = 0.120$). As a self-compatible but pollinator-dependent outcrosser, however, the genetic diversity of *C. amoena* is relatively low in comparison with other species with similar life history characteristics, because the RAPD diversity was significantly higher in outcrossing species ($Hep = 0.208$) than in inbreeding species ($Hep = 0.091$) (Nybom and Bartish 2000).

Although a relatively small number of RAPD studies have been conducted on wild orchid species, evidence available so far suggests that most orchid species have relatively low genetic diversity, especially at the population level (Sun and Wong 2001, Li et al. 2002). In this regard, the low genetic diversity occurred in *C. amoena* populations is not unexpected and could be attributed to several factors. One of the main factors is most likely to be the small population sizes and local extinction because of habitat destruction and loss. Our field investigations indicate that population sizes of *C. amoena* are small, with individuals from a few to 30 in most populations. In addition, the habitats of *C. amoena* are basically under secondary shrubs or woods that are frequently interfered directly by mass collection and indirectly by logging, grazing and agriculture. In this case, local extinction may happen repeatedly as in our field survey no plant of *C. amoena* was found in some locations where herbarium records exist. Theoretically, reductions in population size and local extinction cause genetic bottlenecks and enhance genetic drift, which in turn has led to further loss of genetic diversity (Ellstand and Elam 1993, Frankham et al. 2002). In an allozyme study on two closely related terrestrial orchids, Sun (1996) found that polymorphism and allelic diversity were the most affected by population size. Consequently, as indicated by Vucetich et al. (2001), RAPD diversity in small populations with high extinction and recolonization rates would be more influenced by drift and founder events.

Another factor that may contribute to the low genetic diversity in *C. amoena* is the restricted gene flow, again probably the result of habitat fragmentation. As pointed out by Sun and Wong (2001), gene flow appears to be much more restricted in wild orchids than in other plants. In *C. amoena*, both AMOVA and Nei's coefficient of subpopulation differentiation (*Gst*) showed that high genetic differentiations exist among populations, even in a small area of Xinning (see discussion below). Population isolation in conjunction with the small population sizes would lead to relatively low genetic diversity in *C. amoena*.

Population genetic structure. In plants, the distribution pattern of their genetic variation can be influenced by various life-history traits, with breeding system having a particularly significant effect (Hamrick and Godt 1996, Bussell 1999, Nybom and Bartish 2000). Bussell (1999) summarized the RAPD data of 35 species and found that on average 19.3% of total genetic diversity resides among populations for 29 outbreeding species while 62.5% of total diversity resides among populations for six inbreeding species. In comparison, significantly higher population differentiation (43.8%) exists in *C. amoena* than the average in other outcrossing species. It is noteworthy that as high as 49.2% of total diversity was due to differences among populations in an area of approximately $60 \times 25\ km^2$ in the Xinning County. This high among population diversity is also showed by the UPGMA phenogram. For example, the two populations (XN2 and JGS) from different regions were genetically

similar despite being separated by seven hundreds of kilometers, but populations from the same region in Xinning (between XN4 and any other XN populations) differed genetically even though separated by less than a few kilometers (Figs. 1 and 2).

Changnienia amoena is a self-compatible, nonrewarding, bumblebee-pollinated orchid species (Wang et al. 1994, Sun et al. 2006). A deceptive species generally disperses its pollen and seeds over long distances and thus would result in high outcrossing rates (Ferdy et al. 2001). Therefore, *C. amoena* is expected to have low population differentiation. Apparently, the marked differentiation among populations in this species would result from factors other than its breeding system. We have reasons to assume that small population sizes and lack of gene flow among populations due to habitat fragmentation as mentioned above are likely the major factors contributing to the present pattern of population structure in *C. amoena*. Evidence showed that the habitats suitable for most orchid species in China have been seriously destroyed and fragmented (Chen and Tsi 1998). As a result, the number and sizes of the extant populations of many orchids like *C. amoena* have decreased greatly, leading to alternation of population genetic structure in addition to the loss of total genetic diversity. Based on the studies on orchid species with different breeding systems, Wong and Sun (2001) indicated that in addition to the breeding system, genetic drift in small populations of orchids might play an important role in determining the amount of genetic variation within populations and genetic differentiation among populations.

The limited pollen and seed dispersal in *C. amoena* is probably another contributing factor to its high level of population differentiation because the characteristics of pollen and seed dispersal profoundly influence the genetic structure of natural plant populations (Hamrick and Godt 1989, Alexandersson and Ågren 2000). Although orchids have seeds that are well suited for wind dispersal (Dressler 1993), no relevant data is available for *C. amoena*. Preliminary studies of reproductive biology of the species showed that fecundity of this plant is rather low, with the fruit set varying from 2.3% to 26.98% (Xiong et al. 2003, Sun et al. 2006). Based on our observations during three consecutive years, pollen transfer in *C. amoena* was mainly restricted within populations, and the transfer among populations was rare or nonexistent in some locations partly because of lack of pollinators (Sun et al. 2006 and unpublished). These features inevitably reduce gene flow among populations. Although the pattern of high population differentiation may also imply the adaptation of *C. amoena* populations to local environment, this needs to be further investigated.

Implications for conservation management. The population genetic structure of *C. amoena* can be summarized as low within- but high among-population diversity, most likely resulting from small population sizes, flow dispersal ability and limited gene flow mainly due to long-term habitat fragmentation and loss. As a distinctive evolutionary lineage in a monotypic genus and being indanger of extinction, *C. amoena* deserves conservation consideration. As pointed out by many authors, knowledge about genetic diversity and population genetic structure is the baseline for formulating effective conservation plans, and can often provide novel, conservation-relevant insights (Avise and Hamrick 1996, Hamrick and Godt 1996, Geburek 1997). This study, in conjunction with our pollination and ecological investigations, has a number of implications for the development of conservation strategies for *C. amoena*.

Firstly, habitat protection is the top priority for the species given the fact that its suitable habitats have been seriously destructed and fragmented due to forest clearance, exploitation and agricultural practice in recent decades. Most orchid species including *C. amoena* have habitat preference and pollinator dependence (IUCN/SSC Orchid Specialist Group 1996). Our recent study on *C. amoena* pollination showed that its reproductive success

largely depends on the availability and frequency of bumblebees' visits and different species of bumblebees occur in different regions (Sun et al. unpublished). Therefore, habitat protection will ensure the species' coexistence with other organisms like fungi and pollinators on which orchids depend in their life cycles. In such case, mass collection can be prohibited simultaneously.

Secondly, in order to maintain the total genetic diversity of the species, more populations need to be protected to maximize the coverage of entire distribution of the species because most of them are very divergent as evidenced by high population genetic differentiation even on a local scale. This is particularly challenging in comparison with many other plant species (Sun and Wong 2001). In addition, those populations either with distinct genetic composition or with high diversity (e.g. XN4, XN2 and TTZ) should be given top priority whenever *ex situ* or *in situ* conservation strategies are taken.

Thirdly, many investigations indicated that reproduction in orchids was pollinator limited (Zimmerman and Aide 1989). The low fecundity of *C. amoena* is most likely a result of lacking pollinator's visits because the pollinator populations (bumblebees) for *C. amoena* are inadequate in many places (personal observations). Therefore, nearby pollinator populations should also be protected when *in situ* conservation program is initiated. In addition, artificial pollination may be helpful for the recovery of this species. On the other hand, artificial transplantation between populations or regions, which were proposed for many other endangered plants (Avise and Hamrick 1996, Kingston et al. 2004), should be practiced with caution because of the pollinator specificity of *C. amoena* as mentioned above.

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Address of the authors: Ang Li and Song Ge (e-mail: gesong@ibcas.ac.cn), Laboratory of Systematic and Evolutionary Botany, Institute of Botany, Chinese Academy of Sciences, Xiangshan, Beijing 100093, P. R. China.