
Note

A Preliminary Study on Conservation Genetics of an Endangered Orchid (*Paphiopedilum micranthum*) From Southwestern China

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INTRODUCTION

Knowledge about genetic diversity and population genetic structure is a good baseline for formulating effective conservation plans, and can often provide novel, conservation-relevant insights (Avice, 1996; Geburek, 1997; Hamrick and Godt, 1996). An effective conservation strategy for a species can be made only after detailed population genetic information becomes available (Hamrick and Godt, 1996). Nevertheless, studies on population genetics of orchid species are relatively limited given that Orchidaceae is one of the largest families of flowering plants (Ackerman and Ward, 1999; Ehler and Pedersen, 2000; Wong and Sun, 1999). Among the vast variety of orchid species, the genus *Paphiopedilum* holds a special place in the minds of orchid growers for their exotic, large flowers on small plants (Cribb, 1998). Because of their spectacular flowers and the technical difficulties associated with their ex situ cultivation and propagation, many *Paphiopedilum* species are faced with increasing collection pressure. In addition, decades of agriculture, silviculture, grazing, and urbanization have caused habitat destruction and degradation, which has significantly decreased both the extent and density of their populations (Cribb, 1998). These threats have brought them to the edge of extinction in the wild (Cribb, 1998; Tsi *et al.*, 1999).

Paphiopedilum micranthum is one of the most northerly distributed species in the genus *Paphiopedilum* and is restricted to the karst limestone hills of southwestern China, including southeastern Yunnan, northern and western Guangxi, and southwestern Guizhou (Fig. 1) (Chen and Tsi, 1998; Lang *et al.*, 1999). Despite its

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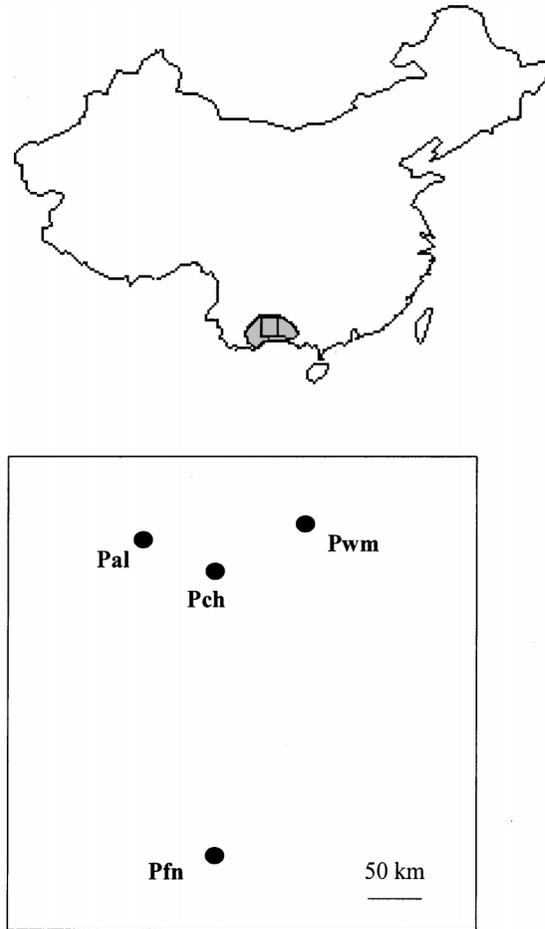


Fig. 1. Localities of *P. micranthum* populations sampled in this study. Codes for populations correspond to those in Table I. The geographic distribution of *P. micranthum* in China (shaded) is shown in the upper map of China.

remote habitat, *P. micranthum* has appeared in large quantities in cultivation since 1984 but is declining in the wild (Chen and Tsi, 1998; Cribb, 1998; Cribb *et al.*, 1999). Although efforts have been made to detect its distribution and the extent of its endangerment (Cribb *et al.*, 1999; Cribb and Luo, 1998; Tsi *et al.*, 1999), population genetics of this species remains unknown. As part of a larger project, we conducted a preliminary analysis of the level and apportionment of genetic diversity in *P. micranthum* using RAPD analysis. This information should contribute to

Table I. Genetic Variation Parameters of *Paphiopedilum micranthum* Based on RAPD Data

Population	Sample size	No. of polymorphic bands	<i>H</i>	<i>P</i> (%)	<i>I</i>
Pch	48	74	0.177	55.2	0.2687
Pwm	53	78	0.193	58.2	0.2907
Pal	43	52	0.123	38.8	0.1860
Pfn	17	38	0.090	28.4	0.1362
Mean	40.3	60.5	0.146	45.2	0.2204
Species	161	96	0.217	73.3	—

Note: *P*, percentage of polymorphic bands; *H*, expected heterozygosity; *I*, Shannon index of diversity.

a better understanding of the genetic profile of this endangered species and could then be used to develop strategies for its conservation and sustainable utilization.

MATERIALS AND METHODS

During November 1999 to August 2000, 161 individuals of *P. micranthum* were collected, representing four natural populations in southwestern China (Fig. 1). The location and number of sampled individuals of each population are listed in Table I. Because this species is rhizomatous and forms sizeable clumps of many growths in the wild (Tsi *et al.*, 1999), a single individual was randomly collected from each clump to prevent collecting ramets from a single genet. For a few clumps covering a large area, two individuals were randomly sampled.

Leaves were harvested and stored with silica gel in zip-lock plastic bags until DNA isolation. Total DNA was extracted as described previously (Ge *et al.*, 1999). Two hundred RAPD primers from Shengong Inc. were screened with two randomly selected individuals from two populations. Twelve primers (S13, S193, S195, S217, S250, S329, S346, S348, S351, S355, S370, S383) that produced clear and reproducible fragments were selected for further analysis. DNA amplification was performed in a Rapidcycler 1818 (Idaho Tech.), and commenced with a 2-min incubation at 94°C, followed by 38 cycles of 2 s at 94°C, 2 s at 50°C, 1 min at 72°C, and ended with a 7-min incubation 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 MgCl₂, 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.

RAPD bands were scored as present (1) or absent (0) for each DNA sample, and a matrix of the different RAPD phenotypes was assembled, and then used

for statistical analysis. Genetic diversity was measured by the percentage of polymorphic loci (P), which was calculated by dividing the number of polymorphic bands at population and species levels by the total number of bands surveyed. The P value is equivalent to the PPB value used by some authors (Ge *et al.*, 1999). The expected heterozygosity (H) and Shannon index of diversity (I) were also calculated using the POPGENE program (Yeh *et al.*, 1997).

A distance matrix was constructed by the RAPDistance program (Armstrong *et al.*, 1994), and the nonparametric Analysis of Molecular Variance (AMOVA) program Version 1.5 was used to describe genetic structure and variability among populations, as described by Excoffier *et al.* (1992).

RESULTS

A total of 131 bands ranging in size from 200 to 1400 bp was generated using 12 primers, corresponding to an average of 10.9 bands per primer. Of them, 73.3% (96 in total) were polymorphic among 161 individuals, i.e., the percentage of polymorphic bands (P) of this species was 73.3%. Every primer produced polymorphic bands when all of the four populations were considered. Genetic diversity varied greatly among populations with the P values ranging from 28.4% (Pfn) to 58.2% (Pwm). The expected heterozygosity (H) and Shannon index (I) showed the same trends (Table I).

To assess the overall distribution of genetic diversity, the AMOVA program was used to analyze the distance matrix given by the RAPDistance program. AMOVA showed highly significant genetic differentiation ($p < 0.001$) among populations. A large proportion of genetic variation (79.69%) resided within populations, whereas only 20.31% resided among populations (Table II).

DISCUSSION

In recent decades, population genetics of a large number of plant species have been investigated mainly by allozyme and RAPD techniques (Bussell, 1999; Hamrick and Godt, 1990). Comparative data showed that RAPDs often detected much

Table II. Analysis of Molecular Variance (AMOVA) for *Paphiopedilum micranthum* Using RAPDs

Source of variance	df	SSD	MSD	Variance component	Percentage	P value ^a
Variance among populations	3	4.6025	1.534	0.0537	20.31	<0.001
Variance within populations	96	20.2505	0.211	0.2109	79.69	<0.001

^aSignificance tests after 3000 permutations.

higher genetic diversity than allozyme data. For example, Liu and Fournier (1993) studied the genetic diversity of two *Populus* species using allozyme and RAPDs. They found that in *P. tremuloides*, RAPDs and allozyme showed 100% and 77% polymorphic loci, respectively. In *P. grandidentata*, the polymorphism detected by allozyme (29%) was much lower than that detected by RAPDs (87%). In recent studies on Chinese populations of wild rice (*Oryza rufipogon*), genetic diversity revealed by RAPD (35%, Ge *et al.*, 1999) was also significantly higher than that revealed by allozyme (25.6%, Gao *et al.*, 2000). Similarly, using allozyme and RAPD techniques, Wong and Sun (1999) investigated the population genetics of a self-compatible orchid species (*Goodyera procera*). They found that there existed much higher diversity detected by RAPDs than that by allozymes at both species and population levels (RAPDs: $P = 97.0\%$, $H = 0.293$ at species level, and $P = 55.1\%$, $H = 0.181$ at population level; allozyme: $P = 33.3\%$, $H = 0.151$ at species level, and $P = 21.8\%$, $H = 0.073$ at population level). Given the fact that consistently higher diversity was revealed by RAPD analysis, we chose to use this method in our study.

Wong and Sun (1999) concluded that *G. procera* possessed relatively low genetic diversity because of the lower allozyme diversity in this species compared with many other orchid species. In comparison with *G. procera*, *P. micranthum* possesses lower RAPD diversity, particularly at population level (see the above and Table I) despite the fact that a large number of individuals (up to 53) were surveyed for each population in this study. Bussell (1999) reviewed RAPD studies on population genetics of 38 plant species and indicated that the average genetic variance component among populations of the 30 outbreeding species given by AMOVA was 14.4%. Although *Paphiopedilum* species were predominantly insect-pollinated outcrossers (Cribb, 1998), *P. micranthum*, with among-population diversity being 20.3% (Table II), maintains significantly higher population differentiation than other outcrossing species.

The relatively low genetic diversity and high genetic differentiation occurring in *P. micranthum* are most likely to be a result of habitat fragmentation. Recent investigations indicated that the habitats suitable for orchid species in China have been seriously destroyed and fragmented (Chen and Tsi, 1998; Tsi *et al.*, 1999). As a result, the number and sizes of the extant populations have decreased greatly, which in turn has led to further loss of genetic diversity and alternation of population genetic structure (Ellstrand and Elam, 1993). In this study, population Pfn shows the lowest diversity which can be explained by its being farthest away from other populations. This may also be due to the relatively small sample size of this population. Overall, however, the lower RAPD diversity occurring in this species is more likely to be a result of habitat fragmentation that caused genetic drift and limited gene flow among populations. In addition, overcollection of wild individuals is also an important factor for the rarity and endangered status of orchid species, particularly for those *Paphiopedilum* species (Tsi *et al.*, 1999).

Field investigation and ecological studies indicated that *P. micranthum* often reproduces by clonal growth (Cribb *et al.*, 1999; Tis *et al.*, 1999). Therefore, with both sexual and asexual reproductive strategies the species has sufficient ability to propagate successfully in the wild as long as habitats are no longer disturbed seriously and overcollection is forbidden. In this regard, in situ conservation will be important and practical for this species. When ex situ strategies are needed, sampling should be able to cover all the populations across its distribution though the species is broken up or disjunct at present (Cribb, 1998). In addition, once a sampling strategy is formulated, much attention should be paid to the among-population variation because of its significant genetic structure as indicated by this study. Detailed studies of the reproductive biology and population demography of this species are currently under way and should yield valuable information for the conservation management of *P. micranthum*.

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REFERENCES

- Ackerman, J. D., and Ward, S. (1999). Genetic variation in a widespread, epiphytic orchid: Where is the evolutionary potential? *Syst. Bot.* **24**:282.
- Armstrong, J. S., Gibbs, A. J., Peakall, R., and Weiller, G. (1994). *The RAPDistance Package*. Available at <ftp://life.anu.edu.au/pub/software/RAPDistance> or <ftp://lite.anu.edu.au/molecular/software/rapd.html>.
- Avise, J. C. (1996). Introduction: The scope of conservation genetics. In Avise, J. C., and Hamrick, J. L. (eds.), *Conservation Genetics*, Chapman and Hall, New York, pp. 1–9.
- Bussell, J. D. (1999). The distribution of random amplified polymorphic DNA (RAPD) diversity amongst populations of *Isotoma petraea* (Lobeliaceae). *Mol. Ecol.* **8**:775.
- Chen, S. C., and Tsi, Z. H. (1998). *The Orchids of China*, Chinese Forestry Publisher, Beijing.
- Cribb, P. J. (1998). *The Genus Paphiopedilum*, Natural History Publications, Borneo.
- Cribb, P., and Luo, Y. B. (1998). *Paphiopedilum malipoense* in China. *Orchid Rev.* **106**:343.
- Cribb, J., Luo, Y. B., McGough, N., and Siu, L. P. (1999). *Paphiopedilum micranthum* in South-west China. *Orchid Rev.* **107**:149.
- Ehler, B. K., and Pedersen, H. A. (2000). Genetic variation in three species of *Epipactis* (Orchidaceae): Geographic scale and evolutionary inferences. *Biol. J. Linn. Soc.* **69**:411.
- Ellstrand, N. C., and Elam, D. R. (1993). Population genetic consequences of small population size: Implications for plant conservation. *Annu. Rev. Ecol. Syst.* **24**:217.
- Excoffier, L., Smouse, P. E., and Quattro, J. M. (1992). Analysis of molecular variance inferred from metric distances among DNA haplotypes: Application to human mitochondrial DNA restriction data. *Genetics* **131**:479.
- Gao, L., Ge, S., and Hong, D. Y. (2000). Allozyme variation and population genetic structure of common wild rice, *Oryza rufipogon* Griff. in China. *Theor. Appl. Genet.* **101**:494.
- Ge, S., Oliveira, G. C. X., Schaal, B. A., Gao, L. Z., and Hong, D. Y. (1999). RAPD variation within and between natural populations of the wild rice *Oryza rufipogon* from China and Brazil. *Heredity* **82**:638.

- Geburek, K. (1997). Isozymes and DNA markers in gene conservation of forest trees. *Biodiver. Conserv.* **6**:1639.
- Hamrick, J. L., and Godt, M. J. W. (1990). Allozyme diversity in plant species. In Brown, A. H. D., Clegg, M. T., Kahler, A. L., and Weir, B. S. (eds.), *Plant Population Genetics, Breeding and Genetic Resources*, Sinauer Associates, Sunderland, MA, pp. 43–63.
- Hamrick, J. L., and Godt, M. J. W. (1996). Conservation genetics of endemic species. In Avise, J. C., and Hamrick, J. L. (eds.), *Conservation Genetics*, Chapman and Hall, New York, pp. 281–304.
- Lang, K. Y., Chen, S. C., Luo, Y. B., and Zhu, G. H. (1999). *Flora Reipublicae Popularis Sinicae*, Tomus 17, Beijing.
- Liu, Z., and Furnier, G. R. (1993). Comparison of allozyme, RFLP, and RAPD markers for revealing genetic variation within and between trembling aspen and bigtooth aspen. *Theor. Appl. Genet.* **87**:97–105.
- Tsi, Z. H., Luo, Y. B., Cribb, P. J., McGough, N., Siu, G., and Chau, L. (1999). A preliminary report on the population size, ecology, and conservation status of some *Paphiopedilum* species (Orchidaceae) in southwest China. *Lindleyana* **14**(1):12–23.
- Wong, K. C., and Sun, M. (1999). Reproductive biology and conservation genetics of *Goodyera procera* (Orchidaceae). *Am. J. Bot.* **86**:1406.
- Yeh, F. C., Yang, R. C., Boyle, T. B. J., Ye, Z. H., and Mao, J. X. (1997). *POPGENE, the user-friendly shareware for population genetic analysis*, Molecular Biology and Biotechnology Centre, University of Alberta, Edmonton, Alberta, Canada.