Botanical Journal of the Linnean Society, 2008, **157**, 657–671. With 2 figures

Genetic variation in the endangered aquatic fern *Ceratopteris thalictroides* (Parkeriaceae) in China: implications from RAPD and ISSR data

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Received 28 March 2006; accepted for publication 20 February 2008

The genetic diversity within and among 13 populations of the endangered aquatic fern *Ceratopteris thalictroides* from five regions of China was investigated using random amplification of polymorphic DNA (RAPD) and intersimple sequence repeat (ISSR) markers. The percentages of polymorphic bands were 61% for RAPD and 65% for ISSR. The genetic diversity revealed by RAPD and ISSR varied greatly among populations, with the percentages of polymorphic bands ranging from 15% to 39% for RAPD and from 20% to 39% for ISSR. Analysis of molecular variance showed that 36% (RAPD) and 34% (ISSR) of variability was partitioned among populations. The results indicate that *C. thalictroides* possesses an intermediate level of genetic diversity at the species level and a low level of genetic differentiation among populations. The Mantel test showed no significant correlation between genetic distance and geographical distance (RAPD, $r = 0.53$; ISSR, $r = 0.18$) and, similarly, a very poor fit between the two markers ($r = 0.24$). A number of causes, including inbreeding and clonal growth, may have led to the low genetic diversity within populations. A high gene flow via spore dispersal in an earlier period when *C. thalictroides* was widely distributed in China is a plausible reason for the low genetic differentiation among populations. Strategies for the conservation of the species in China are discussed. *Botanical Journal of the Linnean Society*, 2008, **157**, 657–671.

ADDITIONAL KEYWORDS: Ceratopteris thalictroides – conservation management – genetic variation – *ISSR – RAPD*.

INTRODUCTION

The genetic structure of populations of both seed plants and ferns reflects the interactions of many different processes, such as the long-term evolutionary history of the species (for example, shifts in distribution, habitat fragmentation, and/or population isolation), mutation, genetic drift, mating system, gene flow, and selection (Slatkin, 1987; Soltis & Soltis, 1990a).

In general, most species with wide distributions have high genetic diversity, both between and within populations (Hamrick & Godt, 1990). In contrast, many rare and endangered species may have reduced genetic variability because of their small population size, and are commonly differentiated into genetically unique populations adapted to local conditions for survival and growth (Ellstrand & Elam, 1993; Coates & Van Leeuwen, 1996; Krauss *et al*., 2000). However, some rare and endangered species have been known to exhibit high genetic diversity with either high or low population differentiation despite small numbers in the populations (Coates, 1988; Maguire & Sedgley,

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1997; Maki & Asada, 1998; Delgado *et al*., 1999; Zawko *et al*., 2001; Ge *et al*., 2003; Gao & Zhang, 2005).

Investigations of the population genetic diversity and structure of the populations within a species may not only illustrate the evolutionary process and mechanism, but also provide information useful for biological conservation (Schaal, Leverich & Rogstad, 1991). The results of such studies could lead to the adoption of the most appropriate population management strategy from the start of conservation efforts, when options may be the most flexible (Haig, 1998). The assessment of genetic variation and partitioning within and among populations is therefore an important prerequisite for the establishment of effective and efficient conservation management strategies for endangered species.

Ceratopteris thalictroides (L.) Brongn. is a polyploid, semi-aquatic, annual, homosporous fern with a wide geographical distribution in tropical and subtropical regions of the world (Watano & Masuyama, 1994); it commonly colonizes aquatic environments, such as paddy fields and marshes. In China, it mainly grows in agricultural fields, marshes, lakes, ditches, and ponds. Although *C. thalictroides* was widely distributed in China prior to the 1960s, it has since become severely vulnerable owing to the deterioration or loss of its primary habitats as a result of human disturbance. The species has declined rapidly in population quantity (number), and has even disappeared altogether from many locations (Hao *et al*., 2000; Dong *et al*., 2005). During our field investigations lasting from 2003 to 2004, only 13 extant populations of *C. thalictroides* were found from 25 tropical and subtropical sites surveyed in which *C. thalictroides* had been observed previously based on locality records on herbarium specimens. The species is now considered to be endangered in China and is listed amongst the second category of key protected wild plants (Yu, 1999). In several other countries, including neighbouring Vietnam and India, the species is also listed as being endangered (Raju, 1983).

Earlier studies of *C. thalictroides* have mainly dealt with its taxonomy, mating systems, sex determination, genetics, and molecular biology (Nishida & Kurita, 1963; Hickok, Warne & Fribourg, 1995). More recently, studies have been carried out focusing on the ecological factors causing the decline of *C. thalictroides* in China (Hao *et al*., 2000; Dong *et al*., 2005). However, the extent and patterns of genetic diversity within *C. thalictroides* populations are still largely unknown. Watano & Masuyama (1994) used allozymes to study the genetic variation among populations of *C. thalictroides* from Japan, and revealed 54% genetic differentiation. The results of their study indicated the existence of two allopatric types, referred to as the south type and north type. The study also indicated that the resolving power of allozymes in detecting the level of genetic diversity in the species was inadequate.

In recent years, a number of polymerase chain reaction (PCR)-based DNA markers have been widely used to investigate population genetic structure because they overcome the limitations of allozyme markers. The most popular markers are random amplification of polymorphic DNA (RAPD) and inter-simple sequence repeats (ISSRs), which have been used successfully in the study of plant systematics, evolutionary biology, and conservation genetics for the detection of genetic diversity in populations (for example, Schaal *et al*., 1991; Wolfe & Liston, 1998; Huang *et al*., 2001; Ge *et al*., 2003; Chen *et al*., 2005b).

The RAPD technique, which surveys the entire genome rather than selected fragments (Kingstona, Waldrenb & Smytha, 2004), is a good parameter to measure the pattern of genetic diversity of rare and endangered plants. However, RAPD has several limitations, including dominance, uncertain locus homology, and, in particular, sensitivity to the reaction conditions. In order to solve some of these problems, a relatively new technique, PCR amplification of ISSRs, represents one of the advantageous alternatives to assess genetic diversity (Zietkiewicz, Rafalske & Labuda, 1994). As a dominant marker, ISSR targets simple sequence repeats (microsatellites) that are abundant throughout the eukaryotic genome and evolve rapidly. As a consequence, ISSR amplification reveals a much larger number of polymorphic fragments per primer than does RAPD (Qian, Ge & Hong, 2001).

The principal goal of the present study was to provide information on genetic variation within and among populations of *C. thalictroides* in China, which may facilitate the conservation management of this species. A secondary aim was to compare the levels of genetic diversity detected by RAPD and ISSR as a way of assessing the suitability of these techniques for future investigations of genetic diversity in *Ceratopteris* populations.

MATERIAL AND METHODS

Plant material

The plant material used in this investigation was obtained from 13 extant populations, which represented a wide geographical distribution of the species in tropical and subtropical regions of China (Fig. 1). These populations were divided into five groups according to administrative regions, i.e. Central China (HN-1), East China (JS-1, ZJ-1, FJ-1), South China (GD-1, GD-2, GX-1, GX-2, GX-3), South-West

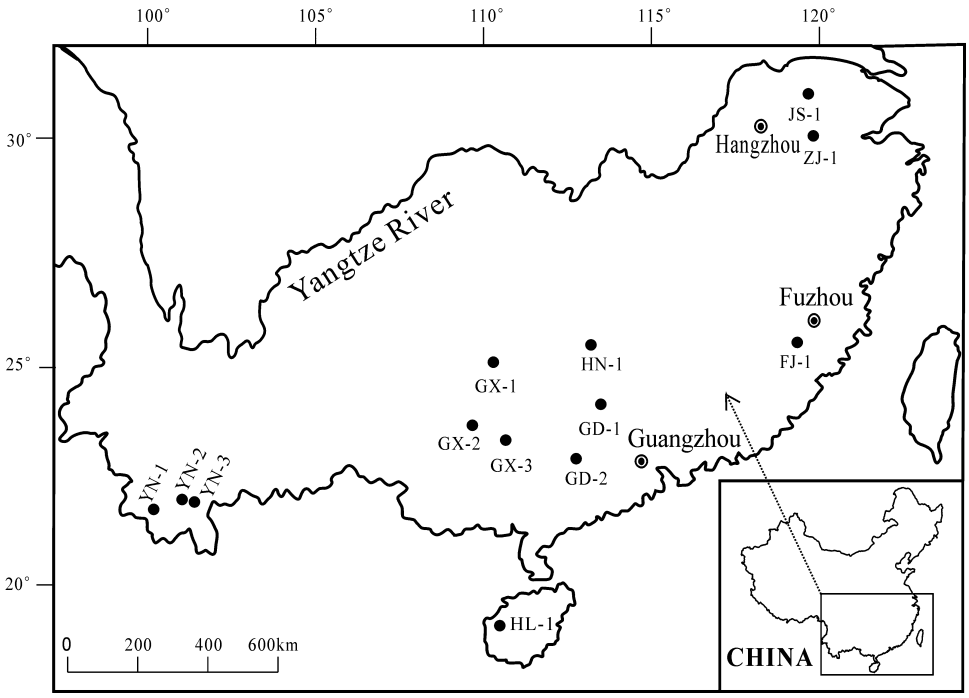

Figure 1. Map of *Ceratopteris thalictroides* populations, showing locations of the sample sites (see Table 1 for site names and descriptions).

China (YN-1, YN-2, YN-3), and Hainan Island (HL-1). Most of the populations are small and diminishing. In each of the 13 populations, a random sample consisting of 5–30 plants was chosen owing to the limited number of individuals. A total of 225 individuals from the 13 populations were included in the study using RAPD; 138 of the 225 individuals used for RAPD were analysed using ISSR markers. Details on collection sites and sample size are given in Table 1. About 5–10 g of fresh leaves per plant was collected and immediately dried in a sealed plastic bag containing about 50 g of silica gel. The samples were stored at room temperature until DNA was isolated in the laboratory at Wuhan University, Wuhan, China.

Total DNA extraction

Total genomic DNA was isolated using a modification of the cetyltrimethylammonium bromide (CTAB) extraction procedure of Doyle & Doyle (1987). About 0.5 g of silica-dried leaf tissue was ground in liquid nitrogen, mixed with 2 mL of extraction buffer [1.4 M NaCl, 100 mM Tris–HCl (pH 8.0), 20 mM ethylenediaminetetraacetic acid (EDTA), 2% CTAB, and 2% 2-mercaptoethanol] at 65 °C, and incubated at 65 °C for 30 min with gentle shaking every 5 min. DNA was extracted twice with 2 mL of chloroform–isoamylalcohol (CI; 24 : 1), and then centrifuged at 10 000 g for 2 min. RNase (10 mg mL^{-1}) was added to the supernatant and incubated for 2 h at 37 °C. The DNA was recovered as a pellet by centrifugation at 10 000 g for 2 min. The sediment was washed twice, each time with 400 μL of 70% ethanol, air-dried, resuspended in 100 μL of 0.1 × TE buffer (10 mM Tris–HCl, 0.1 mM EDTA), and then stored at –20 °C.

RAPD PCR amplification

A total of 60 primers from Genebase Co. Ltd. (Shanghai, China) was tested on four randomly selected individuals for PCR. The 11 primers that produced reproducible, clear, polymorphic electrophoretic bands were selected (Table 2). Amplification of genomic DNA was performed on a PTC-100 thermocycler (MJ Research, Inc.), and commenced with 4 min at 94 °C, followed by 45 cycles of 1 min at 94 °C, 1 min at 34 °C, 2 min at 72 °C, and ended with 7 min at 72 °C. Reactions were carried out in a volume of 25 mL containing 0.25 mM of each deoxynucleoside triphosphate (dNTP), 1.5 mM $MgCl_2$, 1 mM primer, 1 U Taq polymerase, and 50 ng of DNA template. Amplification products were resolved electrophoretically on 1.5% agarose gels run at 80 V in 0.5 × TBE buffer (Tris–boric acid–EDTA), visualized by staining with ethidium bromide, and photographed under ultraviolet light. The sizes of the amplification products were estimated using a 200-bp DNA ladder.

Table 1. Location, habitat, and population characteristics of the 13 *Ceratopteris thalictroides* populations studied

Population	Location	Elevation (m)	Coordinates	Habitat	Population size	Population area (m^2)	Sample size (RAPD/ISSR)
HN-1	Chenzhou, Hunan Province	176	25°48′N; 113°05′E	Rice field	20 000–25 000	300–400	28/12
FJ-1	Fuzhou, Fujian Province	11	25°54′N; 119°13′E	Rice field	200–300	80–100	28/12
ZJ-1	Hangzhou, Zhejiang Province	40	30°12′N; 119°43′E	Wasted rice field	150–200	35–40	14/11
JS-1	Wuxi, Jiangsu Province	3	31°31′N; 120°12′E	Pond	15–20	10–15	10/9
GD-1	Yingde, Guangdong Province	70	24°28′N; 113°37′E	Wasted rice field	50–80	180–200	20/13
GD-2	Zhaoqing, Guangdong Province	2	23°11′N; 112°23′E	Rice field	500–600	150–200	30/14
GX-1	Guilin, Guangxi Province	163	25°02′N; 110°19′E	Rice field	200–250	60–70	13/9
GX-2	Wuzhou, Guangxi Province	166	23°12′N; 110°22′E	Rice field	200–300	70–80	10/10
GX-3	Wuzhou, Guangxi Province	40	23°15′N; 111°19′E	Rice field	20–30	15–20	9/8
YN-1	Jinghong, Yunnan Province	640	21°32′N; 100°39′E	Wasted rice field	20–30	35–40	12/9
YN-2	Mengna,Yunnan Province	563	21°55′N; 101°15′E	Cistern	20–25	3–4	5/5
YN-3	Mengna, Yunnan Province	638	21°30′N; 101°34′E	Vegetable plot	50–60	60–70	20/13
HL1	Lingshui, Hainan Province	–	18°40′N; 109°50′E	Marsh	300–400	100–150	26/13

ISSR, intersimple sequence repeat; RAPD, random amplification of polymorphic DNA.

Table 2. Primers of random amplification of polymorphic DNA (RAPD) and intersimple sequence repeat (ISSR) analysis used in this study

Primer	Sequence (5′–3′)	Primer	Sequence (5′–3′)
RAPD		ISSR	
P-A-12	CCTGGGTCCA	SBS807	$(TC)_8A$
P-A-13	CCTGGGTGGA	SBS808	$(AG)_8C$
P-B-03	CTCCCTGAGC	SBS811	$(AC)_8C$
P-B-15	AGGGGCGGGA	SBS834	$(AG)_8$(C/G)C
P-B-17	GAGGGCGAGC	SBS835	$(AG)_8$(C/G)C
P-C-05	CTCGGGTGGG	SBS846	$(CA)_8$(A/T)T
P-C-15	TTCCGCGGGC	SBS847	$(CA)_8$(A/T)C
P-C-19	ATTGGGCGAT	SBS855	$(AC)_8$(C/G)T
P-D-01	GCTGTAGTGT	SBS856	$(AC)_8$(C/G)A
P-D-09	GAGCCCGTAG	SBS857	$(AC)_8$(C/G)G
P-D-10	CGATTCAGAG	SBS861	$(ACC)_5$
		SBS862	$(AGC)_5$

ISSR PCR amplification

Sixty-five primers from SBS Genetech Co. Ltd. (Shanghai, China) were screened on four randomly selected individuals for PCR; 12 primers (Table 2) that produced reproducible, clear, polymorphic electrophoretic bands were selected for the ISSR analysis of *C. thalictroides*. Amplification of genomic DNA was performed in the same Peltier thermal cycle as in the RAPD experiment, and programmed for an initial melting step at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 55 °C for 1 min, 72 °C for 1.5 min, and a final extension step at 72 °C for 7 min. Reactions were carried out in a volume of 25 mL containing 0.25 mM of each dNTP, 1.5 mM $MgCl_2$, 1.5 mM primer, 1 U Taq polymerase, and 50 ng of DNA template. PCR amplification products were analysed by electrophoresis on 1.5% agarose gels, visualized by staining with ethidium bromide, and photographed under ultraviolet light. The sizes of the amplification products were estimated using a 200-bp DNA ladder.

Data analysis

Amplified fragments of both RAPD and ISSR, with the same mobility according to the molecular weight (bp), were scored for the presence (1) or absence (0) of homologous bands. The two markers were used for the following statistical analyses. Genetic diversity was measured using the percentage of polymorphic bands (PPB), which was calculated by dividing the number of polymorphic bands at population, region and species levels by the total number of bands surveyed. At the species level, the coefficient of gene differentiation (G_{ST}) and the level of gene flow (N_m) were measured using Nei's (1973) gene diversity

Table 3. Analysis of genetic variations of 13 populations and five regions, generated by random amplification of polymorphic DNA (RAPD) and intersimple sequence repeat (ISSR) markers

		RAPD				ISSR			
Region	Population	Polymorphic bands	PPB (%)	*H*	*I*	Polymorphic bands	PPB (%)	*H*	*I*
Central China		35	34	0.12	0.17	39	29	0.09	0.14
	HN-1	35	34	0.12	0.17	39	29	0.09	0.14
East China		47	45	0.12	0.19	58	43	0.11	0.17
	FJ-1	36	35	0.10	0.16	39	29	0.08	0.13
	JS-1	31	30	0.10	0.15	35	26	0.09	0.14
	ZJ-1	24	34	0.09	0.13	29	22	0.06	0.10
South China		55	53	0.13	0.21	64	47	0.14	0.22
	GD-1	25	24	0.07	0.10	39	29	0.10	0.15
	GD-2	40	39	0.10	0.16	35	26	0.08	0.13
	GX-1	30	29	0.10	0.15	40	30	0.11	0.17
	GX-2	28	27	0.09	0.14	36	27	0.10	0.15
	GX-3	24	23	0.08	0.12	27	20	0.08	0.12
South-West China		38	37	0.13	0.19	63	47	0.15	0.23
	YN-1	34	33	0.11	0.17	45	33	0.12	0.18
	YN-2	16	15	0.06	0.09	31	23	0.09	0.13
	YN-3	31	30	0.11	0.17	53	39	0.12	0.19
Hainan Island		39	38	0.10	0.16	45	33	0.10	0.16
	HL-1	39	38	0.10	0.16	45	33	0.10	0.16
Species level		63	61	0.16	0.24	88	65	0.15	0.24

H, Nei's gene diversity; *I*, Shannon's information index; PPB, percentage of polymorphic bands.

statistics. Shannon's index of diversity (*I*) was also calculated (Lewontin, 1972). Nei's unbiased genetic identity (*H*) and genetic distance (*D*) between populations were computed (Nei, 1972). The PPB, *I*, *H*, and *D* values were estimated using POPGENE program 1.31 (Yeh *et al*., 1997).

In order to describe genetic structure and variability within populations, among populations, and among the five regions, the non-parametric analysis of molecular variance (AMOVA) was performed using squared Euclidean distances (Excoffier, Smouse & Quattro, 1992). Variation was apportioned to the following components: among individuals within populations, among populations within regions, and among regions. All genetic analyses were carried out using WINAMOVA program 1.55 (Excoffier, 1993). Input files for this program were generated using AMOVA-PREP (Miller, 1998). Significance tests were made after 1000 permutations.

To examine the genetic relationship among populations, a dendrogram was constructed for cluster analysis using an unweighted pair group method with arithmetic averaging (UPGMA) with NTSYSpc 2.02 (Rohlf, 1998). In addition, in order to test for a correlation between genetic distances (*D*) and geographical distances (in kilometres) among populations, and between the two types of marker, the Mantel matrix correspondence test was performed using the program TFPGA (Miller, 1997) (computing 3000 permutations).

RESULTS

RAPD POLYMORPHISM

A summary of the genetic data for the 13 populations and five regions is given in Table 3. The 11 selected primers generated a total of 104 bands (an average of 9.5 bands per primer) with fragments ranging in size from 100 to 1900 bp. Sixty-three bands were polymorphic amongst 225 individuals. The PPB for this species was 61%. The PPB for a single population ranged from 15% (YN-2) to 39% (GD-2), with an average of 29%. Nei's unbiased genetic identity (*H*) and Shannon's index of diversity (*I*) showed the same trends. Of the 13 populations, populations GD-2 and HL-1 exhibited the highest level of variability (PPB, 39% and 38%, respectively; *I*, 0.16 and 0.16, respectively), whereas population YN-2 exhibited the lowest level of variability (PPB, 15%; *I*, 0.09).

ISSR POLYMORPHISM

Table 3 summarizes the genetic data obtained using ISSR for each of the 13 populations of *C. thalictroides*

and each region. Of the 13 populations, 12 ISSR primers amplified 135 bands, 88 of which were polymorphic (PPB, 65%). There was a total of 135 bands, ranging in size from 100 to 2000 bp, with an average of 11.3 bands per primer. Genetic diversity varied among populations, with PPB ranging from 20% (GX-3) to 39% (YN-3). The *H* and *I* values also showed the same trends. Of the 13 populations, YN-3, YN-1, and HL-1 exhibited the highest level of variability (PPB, 39%, 33%, and 33%, respectively; *H*, 0.12, 0.12, and 0.10, respectively; *I*, 0.19, 0.18, and 0.16, respectively), whereas population GX-3 showed the lowest level of variability (PPB, 20%; *H*, 0.08; *I*, 0.12).

Genetic structure of the populations

The coefficient of genetic differentiation among populations (G_{ST}) was 0.39, as estimated by partitioning of the total gene diversity using RAPD. At the population level, the mean values of *H* and *I* were 0.16 and 0.24, respectively. Based on the G_{ST} values, the level of gene flow (N_m) was estimated to be 0.78 individuals per generation among populations.

The results of RAPD and ISSR analyses were similar. At the population level, G_{ST} and the mean values of *H* and *I* were 0.39, 0.16, and 0.24, respectively. The estimated number of migrants per generation (N_m) was 0.78 among populations.

The genetic distance based on Nei's unbiased measurements amongst 13 populations of *C. thalictroides* generated by RAPD markers showed a greater difference. The highest genetic variation noted was 0.13 between populations JS-1 and HL-1. The data obtained from ISSR markers showed that genetic variation between populations GX-3 and HL-1 was most significant (0.14). A cluster analysis (UPGMA) was used to generate two dendrograms based on Nei's genetic distance of RAPD and ISSR markers among the 13 studied populations (Fig. 2). The dendrogram indicated that the genetic similarity between populations ranged from 0.87 to 0.98. In each cluster, some individuals from the same population did not form a distinct group. Overall, UPGMA obtained from RAPD analysis was not inconsistent with that obtained from ISSR data among populations and among individuals. Clusters of both RAPD and ISSR were not related to the geographical distance between populations.

AMOVA of RAPD data revealed that there were highly significant ($P < 0.001$) genetic differences among the 13 populations of *C. thalictroides* (Table 4). Of the total genetic variation, 36% of the variation was attributable to that among populations and 64% was attributable to that among individuals within populations. The results of AMOVA are consistent with those of Nei's genetic statistics and Shannon's diversity estimation, indicating a low degree of population differentiation. An examination of the proportion of diversity among regions, among populations within regions, and within populations indicated that 12% of the total variation occurred among the five regions, whereas 25% and 63% occurred among populations within regions and within populations, respectively. AMOVA showed significant ($P = 0.005$) genetic differentiation among the five geographical regions.

AMOVA of ISSR bands showed highly significant ($P < 0.001$) genetic differences among the 13 populations of *C. thalictroides* (Table 4). A large proportion of genetic variation (66%) was found within populations, whereas only 34% was partitioned among populations. In addition, most of the variation (66% and 30%) existed among populations within regions and within populations, respectively, and only a small amount of the variation (4%) occurred among the five regions. AMOVA of ISSR bands also showed no significant ($P = 0.092$) genetic differentiation among the five geographical regions. In general, the results of AMOVA were in agreement with the *H* and *I* values obtained from ISSR. The results obtained from ISSR markers were similar to those obtained in the AMOVA of RAPD data.

In order to assess the level of relationship between RAPD and ISSR, a Mantel test was performed to compare Jaccard coefficients among the 13 populations. The correlation was $r = 0.24$ ($P = 1.00$). No significant correlation was found between genetic distance for both RAPD and ISSR markers and geographical distance (RAPD: $r = 0.53$, $P = 1.00$; ISSR: $r = 0.18$, $P = 0.87$) based on the Mantel test. This study reveals the lack of a clear geographical pattern in the distribution of the genetic variability in *C. thalictroides*.

DISCUSSION

Genetic diversity

By employing both RAPD and ISSR markers, this study has demonstrated that the level of genetic diversity among populations of *C. thalictroides* in China is higher than that within populations.

Compared with the results obtained for other fern species (Table 5), *C. thalictroides* from China presents low genetic diversity within populations and an intermediate level of genetic diversity at the species level using RAPD and ISSR, in spite of the small populations, contrary to an expected low genetic diversity among populations. The levels of genetic diversity (PPB: RAPD, 29.06%; ISSR, 28.09%) detected within populations of *C. thalictroides* were similar to those reported in previous studies on endangered pteridophytes, including *Dryopteris cristata*, *Isoëtes sinensis* (PPB, 5.04%), and *I. hypsophila* (PPB: RAPD, 15.9%;

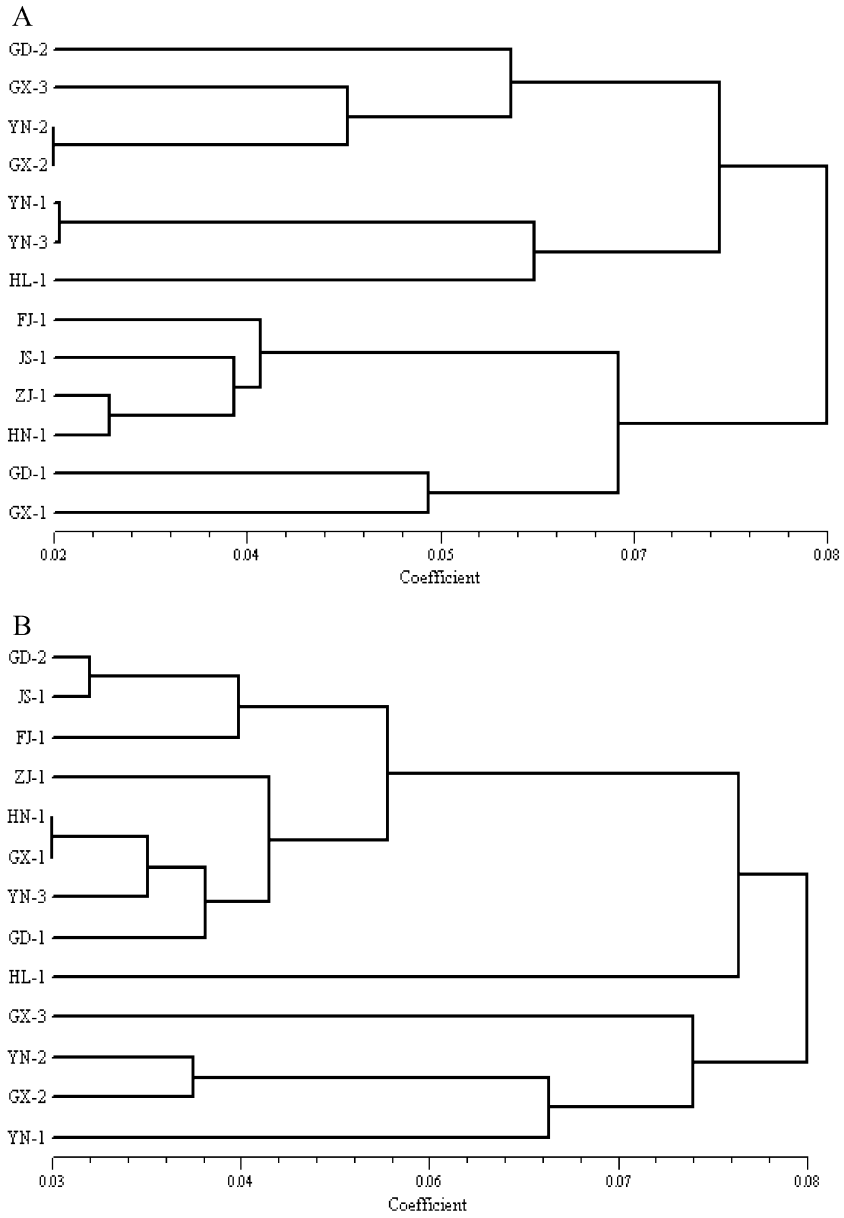

Figure 2. Dendrogram of 13 populations of *Ceratopteris thalictroides* based on Nei's genetic distance using random amplification of polymorphic DNA (RAPD) and intersimple sequence repeat (ISSR) markers. Cluster analysis using an unweighted pair group method with arithmetic averaging (UPGMA) with NTSYSpc 2.02. A, RAPD; B, ISSR. (See Table 1 for site names and descriptions.)

ISSR, 13%). In addition, the level of genetic diversity within populations of *C. thalictroides* was similar to that in the more widespread fern *Sticherus flabellatus* (PPB, 23.75%). However, the PPB within populations is lower than that found within populations of some other endangered or widespread ferns, such as *Angiopteris chauliodonta* (PPB, 57.1%) and *Polystichum otomasui* (PPB, 61.9%).

Among populations, the PPBs (RAPD, 61%; ISSR, 65%) detected by both RAPD and ISSR in *C. thalictroides* were low compared with those obtained for other fern species, including *I. hypsophila* using ISSR (PPB, 82%) and *P. otomasui* (PPB, 81.3%). The genetic diversity among populations in *C. thalictroides* was considerably higher than that reported in some other endangered ferns, including *D. cristata* (PPB, 1.9%), *I. sinensis* (PPB, 58.06%), and *I. hypsophila* using RAPD (PPB, 50%). The PPB value detected by RAPD and ISSR markers among populations of *C. thalictroides* in China was higher than that detected by

Table 4. Analyses of molecular variance (AMOVAs) of 13 populations (grouped into five regions) of *Ceratopteris thalictroides* using random amplification of polymorphic DNA (RAPD) and intersimple sequence repeat (ISSR) markers

Source of variation	d.f.	SSD	Variance component	Total variance (%)	*P*
RAPD					
Among regions	4	389.01	1.05	12	0.005
Among populations/regions	8	288.27	2.14	25	< 0.001
Within populations	21	1150.29	5.45	63	< 0.001
Among populations	12	677.27	3.01	36	< 0.001
Within populations	211	1150.30	5.45	64	< 0.001
Among regions	4	389.01	2.14	25	< 0.001
Within regions	219	1438.57	6.57	76	< 0.001
ISSR					
Among regions	4	222.47	0.44	4	0.092
Among populations/regions	8	317.76	3.24	30	< 0.001
Within populations	12	876.17	7.01	66	< 0.001
Among populations	12	540.23	3.60	34	< 0.001
Within populations	125	876.17	7.01	66	< 0.001
Among regions	4	222.47	1.83	17	< 0.001
Within regions	133	1193.93	8.98	83	< 0.001

Three AMOVAs, including nested analysis (among regions, among populations within regions and within populations), among-population analysis (among populations and within populations), and among-region analysis (among regions and within regions), were used. Significance tests after 1000 random permutations. Statistics include degrees of freedom (d.f.), sum of squared deviations (SSD), and probability (*P*).

Table 5. Genetic diversity of *Ceratopteris thalictroides* from China and other fern taxa used for comparison

Species	PPB within populations (mean %)	PPB at species level (%)	Methods	Reference
C. thalictroides	29.06	61	RAPD	This study
	28.09	65	ISSR	This study
	–	54	Allozyme	Watano & Masuyama (1994)
Isoëtes sinensis	5.04	58.06	RAPD	Chen *et al.* (2004)
I. hypsophila	15.9	50	RAPD	Chen *et al.* (2005a)
	13	82	ISSR	Chen *et al.* (2005b)
Angiopteris chauliodonta	57.1	42.9	RAPD	Kingston *et al.* (2004)
Polystichum otomasui	61.9	81.3	Allozyme	Maki & Asada (1998)
Dryopteris cristata	–	1.9	RAPD	Landergott *et al.* (2001)
Sticherus flabellatus	23.75	42	AFLP	Keiper & McConchie (2000)

AFLP, amplified fragment length polymorphism; ISSR, intersimple sequence repeat; PPB, percentage of polymorphic bands; RAPD, random amplification of polymorphic DNA.

allozymes between populations of *C. thalictroides* in Japan (PPB, 54%).

Certain factors, including inbreeding, small population, habitat fragmentation, and clonal growth, could have contributed to the low level of genetic diversity within populations of *C. thalictroides*.

This result on the genetic variation of *C. thalictroides* in China indicates that the breeding systems and reproductive strategies of this species may not be consistent with those of other fern taxa used for the comparison of genetic diversity. Diploid *I. hypsophila* and tetraploid *I. sinensis* are two heterosporous ferns, producing mega- and microgametophytes from separate spores, with fertilization accomplished by sperm swimming through water. Limited gene flow has generally been assumed between *Isoëtes* populations (Duff & Evans, 1992). Owing to the long geographical distance (400 km) between the two regions (Sichuan and Yunnan) in China, Chen *et al*. (2005a, b) suggested that gene exchange may have been restricted,

hence increasing the interpopulation differentiation of *I. hypsophila*. Chen *et al*. (2004) suggested that inbreeding may have resulted in the low level of genetic diversity within populations in *I. sinensis*. The very low genetic diversity (PPB, 1.9%) and considerable interpopulation divergence in *D. cristata* indicate that this rare and endangered species is a predominantly selfing fern (Landergott *et al*., 2001). Outbreeding of *P. otomasui* may play an important role in the maintenance of high genetic diversity at the species level and low genetic differentiation among populations (Maki & Asada, 1998). Hsu, Moore & Chiang (2000) suggested that gene drift may lead to low-level genetic variation in *Archangiopteris itoi*, a rare endemic fern in Taiwan. Kingstona *et al*. (2004) suggested that the endangered homosporous fern *Angiopteris chauliodonta* may be an outcrossing fern. Low genetic diversity at the species level and high genetic variation ($F_{ST} = 0.783$) among populations, with average heterozygosity values ranging from 0.095 to 0.1175, were detected in *S. flabellatus*, a protected homosporous fern (Keiper & McConchie, 2000). These results indicate that the breeding system of *S. flabellatus* is predominantly inbreeding, with genetic diversity maintained in larger populations through variable rates of outcrossing.

Ceratopteris possesses three mating systems: outbreeding, intergametophytic selfing, and intragametophytic selfing (Watano & Masuyama, 1991; Hickok *et al*., 1995). Being a homosporous hermaphroditic fern gametophyte, *C. thalictroides* has the potential for self-fertilization (gametophytic selfing), although most of the diploid homosporous fern species are highly outcrossing (Soltis & Soltis, 1990b; Hickok *et al*., 1995). Recent reviews on mating systems of homosporous pteridophytes (Haufler, 1989; Masuyama & Watano, 1990; Soltis & Soltis, 1990b) have suggested that polyploid homosporous pteridophytes favour inbreeding. Watano & Masuyama (1991) demonstrated that there was predominant gametophytic selfing in *C. thalictroides*, a tetraploid species with $n = 77, 78$ (Nishida & Kurita, 1963; Löve, Löve & Pichi-Sermalli, 1977; Hickok, 1979). In addition, in studying the genetic diversity of 11 species of *Polygonella*, Lewis & Crawford (1995) found that the two most widespread species have a reduced within-population genetic diversity with respect to their narrowly endemic congeners. They hypothesized that the unexpected results resulted from high levels of self-fertilization for the widespread species. Moreover, a species can convert its mating system from outcrossing to selfing when a few closely related individuals mate and show clonal growth (Innes & Hermanutz, 1998). An example is *Solidago sempervirens*, which can change its mating system from outcrossing to selfing (Qian *et al*., 2001). Mating systems profoundly influence the effective population size in plants. Theoretical considerations have shown that selfing reduces the effective size of subpopulations (Maki & Asada, 1998). It is probable that high levels of gametophytic selfing may have played an important role in maintaining a low genetic diversity within populations of the endangered *C. thalictroides*.

Filed surveys have indicated that the geographical distribution of *C. thalictroides* in China has been declining rapidly, and that its populations have greatly reduced in size and become discontinuous. Presently, the number of individuals of *C. thalictroides* at 11 of the 13 existing natural populations is no more than 300. The main genetic consequences of a small population size are increased genetic drift and inbreeding (Ellstrand & Elam, 1993). Inbreeding and genetic drift caused by small extant populations will inevitably lead to decreasing genetic variability (Huang *et al*., 2001). Thus, small *C. thalictroides* populations derived from a few individuals may have accelerated the loss of genetic diversity within populations. The smaller the size of a population and the longer it remains small, the more genetic diversity it will lose. Earlier studies have shown that the degeneration of primary habitats, the decline in area of wetland coverage, and the deterioration of water quality as a result of contamination with domestic sewage and industrial effluents may have resulted in the degradation of *C. thalictroides* populations (Hao *et al*., 2000). Its habitats have been destroyed and have become fragmented (Dong *et al*., 2005). Studies of habitat fragmentation have shown that the process can lead to population extinction and the loss of genetic variation by not only minimizing suitable habitats, but also increasing the mating opportunity between genetically closely related individuals (Hunter, 1996; Qiu *et al*., 2004). Habitat fragmentation and deterioration caused by human disturbance are important factors leading to the low genetic diversity within populations of *C. thalictroides* (Hunter, 1996). The loss of genetic variation means a decreased ability to adapt to changing environments, hence placing *C. thalictroides* in an even more precarious situation with regard to habitat fragmentation caused by human disturbance.

Clonal growth and mating systems may also influence genetic structure (Qian *et al*., 2001). *Ceratopteris thalictroides* has a prolific capacity for vegetative reproduction (clonal growth) by means of numerous marginal leaf buds that rapidly develop into plantlets (Hickok, Warne & Slocum, 1987). Diao (1990) reported that the clonal growth of *C. thalictroides* was inferior to sexual reproduction in China. In the present study, the phenomenon was also observed at some natural populations of *C. thalictroides*, such as GD-2, YN-2, and YN-3. Because *C. thalictroides* is an

annual semi-aquatic fern, both its genets and ramets die down in winter. Extensive clonality can reduce the reproductive potential of outcrossing species and increase inbreeding rates in self-compatible plants (Frankham, Ballou & Briscoe, 2002). Extinction and re-colonization of patches by a few individuals could result in low genetic variation (Harrison & Hastions, 1996). Gilpin (1991) demonstrated that, even in a large population with a high turnover of extinction and colonization amongst its local populations, small effective population sizes can accelerate the loss of genetic diversity. It is therefore probable that clonal growth is partly responsible for the low diversity within populations of *C. thalictroides*. However, without detailed clonal growth data, the importance of clonal growth in the system is difficult to assess.

Genetic structure

Plant species differ markedly in the way in which genetic diversity is partitioned between populations. Analyses of ISSR markers using different approaches (Nei's genetic diversity analysis, Shannon's diversity index, and AMOVA) gave similar interpretations of the genetic structure of populations of *C. thalictroides*. The genetic variation in *C. thalictroides* detected using ISSR markers was similar to that obtained from RAPD analysis. AMOVA showed that over 60% of the genetic variation in both cases (64% by RAPD and 66% by ISSR) was partitioned within populations, and that populations were not significantly genetically differentiated from one another. Similar results have previously been obtained in a number of studies of rare and endangered species, including *Banksia cuneata* (Maguire & Sedgley, 1997), *Acacia anomala* (Coates, 1988), *P. otomasui* (Maki & Asada, 1998), *Leucopogon obtectus* (Zawko *et al*., 2001), and *Tetraena mongolica* (Ge *et al*., 2003). Other rare and endangered species have shown high genetic diversity at the species level and high interpopulation differentiation, such as *I. hypsophila* (Chen *et al*., 2005b) and *Pinus rzedowskii* (Delgado *et al*., 1999). Rare outbreeding diploid ferns can show high levels of genetic variation within populations, such as *Asplenium trichomanes* ssp. *inexpectans*, a rare calcicole (Vogel *et al*., 1999). Therefore, it is clear that rarity itself is not necessarily correlated with limited genetic variation within a population.

Plant breeding systems determine gene flow, the genetic structure of populations, and the evolutionary potential of a species (Korpelainen, 1995). Polyploid ferns are inbreeding and display high genetic variation between populations, but low levels of variation within populations, whereas diploid species of homosporous pteridophytes have an inclination to gametophytic crossing, with higher levels of genetic variation within populations (for example, Masuyama & Watano, 1990; Soltis & Soltis, 1990a; Watano & Masuyama, 1991; Maki & Asada, 1998; Vogel *et al*., 1999). However, in this study, the tetraploid homosporous fern *C. thalictroides* in China showed an intermediate level of genetic diversity at the species level, and lower genetic differentiation among populations. Similarly, previous studies have indicated that other ferns do not always obey the law. A few diploid species are nearly exclusively inbreeding. For example, diploid species of *Botrychium dissectum* are characterized by high levels of inbreeding (intragametophytic selfing, 0.95) (see McCauley, Whittier & Reilly, 1985). The diploid fern *A. trichomanes* ssp. *trichomanes* is also predominantly an inbreeding taxon (Vogel *et al*., 1999). In only a few populations of *D. expansa* and *Heminonitis palmata* and a single population *of Blechnum spicant* are mixed mating systems found (Soltis & Soltis, 1990a; Ranker, 1992). Tetraploid species with high rates of intragametophytic selfing, such as *B. virginianum*, exhibit little interpopulation divergence ($F_{ST} = 0.080$) (Soltis & Soltis, 1990b). Therefore, Soltis & Soltis (1990b) suggested that the mating system may be of minor importance in determining the genetic structures of fern species.

High diversity and low population partitioning in rare plants have been attributed to a number of factors: insufficient length of time for genetic diversity to be reduced following a natural reduction in population size and isolation (Coates, 1988); adaptation of the genetic system to small population conditions (Coates, 1988; Zawko *et al*., 2001); recent fragmentation (via human disturbance) of a once continuous genetic system (Gao & Zhang, 2005); extensive gene flow and high outcrossing rates (Maguire & Sedgley, 1997; Maki & Asada, 1998; Zawko *et al*., 2001; Ge *et al*., 2003); and somatic mutation (Maki & Asada, 1998; Huang *et al*., 2001).

The distribution of genetic variability within a species reflects the patterns of dispersal, population establishment, and gene flow (Caplen & Werth, 2000). The G_{ST} value indicated high rates of gene flow among populations of *C. thalictroides* in China. Compared with the low gene flow of some ferns, including *I. engelmannii* ($N_m = 0.044$) (Caplen & Werth, 2000) and *P. lonchitis* ($N_m = 0.05$) (Soltis & Soltis, 1990a), and the high gene flow in some homosporous ferns, including *P. imbricans* ($N_m = 2.2$) (Soltis & Soltis, 1990a), an intermediate level of gene flow was observed among populations of *C. thalictroides* ($N_m = 0.78$). The considerable amount of gene flow (RAPD, 1.47; ISSR, 1.84) among the five regions of *C. thalictroides* from China also indicates that N_m is greater than one successful migrant per generation (Slatkin, 1987). A cluster analysis (UPGMA) of

C. thalictroides revealed that some individuals in a population did not fit into a distinct group, and that there was considerable gene flow among populations. In homosporous ferns, the ability to disperse spores may allow much greater interpopulation gene flow than in most seed plants because of the aerodynamic properties of the pteridophyte spore (Soltis & Soltis, 1990a). Previous studies have indicated that tetraploid *C. thalictroides* in Japan is an inbreeder (Masuyama & Watano, 1990; Watano & Masuyama, 1991, 1994). Watano & Masuyama (1991) suggested that an important factor promoting gametophytic selfing is the annual colonizing habit of this species. In Japan, this species grows in paddy fields every year. As the spores of this species are large and heavy and sink rapidly into the water, gene flow by spore dispersal between paddy fields should be negligible. Therefore, inbreeding may have a higher selective advantage than outcrossing, because it enables this annual to form many progeny in a short time, which are adapted for habitats similar to those of their parents. This species is widely distributed in the south of China, which has a well-developed river system. In this study, the samples were taken not only from agricultural fields, but also from marshes, lakes, ditches, and ponds. Because a considerable proportion of *C. thalictroides* populations from China grow in ditches and streams, spore dispersal by water flow within rivers and streams is probably more frequent and easier than spore dispersal between paddy fields (Watano & Masuyama, 1991). In addition, homosporous ferns possess a great potential for long-distance spore dispersal (Vogel *et al*., 1999; Landergott *et al*., 2001). The ease of spore dispersal may have facilitated extensive gene flow, increasing the rate of gametophytic crossing, and is probably responsible for the present-day structure of genetic variation and diversity. If gene flow is primarily between adjacent populations, it may be expected that populations in close geographical proximity would be more genetically similar than distant populations. However, in *C. thalictroides*, there was no significant relationship (RAPD, $r = 0.53$; ISSR, $r = 0.87$) between genetic distance and geographical distance amongst the 13 populations. This suggests that gene flow has not been limited to between nearest-neighbour populations, and that populations have received migrant genotypes from many sources (see Ge *et al*., 2003). In ferns, high spore dispersibility and high interpopulation gene flow may act as cohesive forces in the genetic structure of species, regardless of the mating system (Soltis & Soltis, 1990b). In addition, as *C. thalictroides* was widely distributed in China about 40–60 years ago, significant genetic differentiation between the populations is somewhat obscure, although population sizes have been reduced. Thus, the current high diversity and low level of genetic variation among populations may reflect the former large population sizes and wide geographical distribution of the species. Higher levels of gene flow among populations may have occurred before the rapid decline of the population as a result of human activities. In addition, gene flow via the dispersal of free-living bisexual gametophytes and other propagules, such as in clonal dispersal, is of considerable importance. It is evident that the high gene flow among populations of *C. thalictroides* is a probable reason for the low genetic variation among these populations.

Outcrossing has previously been recognized in the sexual reproduction of *C. thalictroides* (Watano & Masuyama, 1991). In composite cultures of gametophytes of *C. thalictroides*, two different types of gametophyte were observed (Schedlbauer & Klekowski, 1972), indicating that this species might undergo outcrossing in the populations. However, Watano & Masuyama (1991) have suggested that occasional gametophytic crossing in *C. thalictroides* may serve to compensate for the loss of genetic variability by inbreeding in cases in which the species is highly selfing. This phenomenon is similar to that in many inbreeders in seed plants, maintaining a morphological and/or genetic potential for occasional outcrossing (Stebbins, 1957). Although previous studies of gametophyte development have indicated that colonizing species may be inbreeders, both *Pteridium aquilinum* and *Equisetum arvense* are two colonizers with an outcrossing mating system (Soltis & Soltis, 1990b). The breeding system of the homosporous fern *S. flabellatus* is predominantly inbreeding, with genetic diversity maintained by occasional outcrossing in larger populations (Keiper & McConchie, 2000). In this study, the low genetic differentiation among populations and high gene flow indicate that *C. thalictroides* in China may possess a higher outcrossing rate. This is in common with many other rare taxa, which have high genetic diversity and low population differentiation (Coates, 1988; Maguire & Sedgley, 1997; Zawko *et al*., 2001; Ge *et al*., 2003). Outcrossing in *C. thalictroides* may occur mainly among populations by spore dispersal and in some larger populations. The outcrossing habit of *C. thalictroides* may also play an important role in the maintenance of the genetic variability of the species, in spite of its inclination towards inbreeding. Several factors, including high spore dispersal among populations by long-distance water flow, higher gene flow, and wide distribution of the species in China, with the occurrence of some large populations, may have resulted in a higher outcrossing rate in Chinese *C. thalictroides* than in Japanese *C. thalictroides*. In addition, it is worth noting that *C. thalictroides* in Japan has been

divided into two types (south and north types), and crossing tests have shown that the two types are almost completely cross-sterile in spite of occasional crossing (Masuyama *et al*., 2002).

Comparison of suitability of RAPD and ISSR markers

With the development and application of molecular techniques for the detection of the extent of genetic diversity and population genetic structure, it has become apparent that different markers have different properties and will reflect different aspects of genetic diversity. RAPD (Williams *et al*., 1990) and ISSR (Zietkiewicz *et al*., 1994) have been used extensively to estimate genetic diversity in population and species level studies. In the present study, both RAPD and ISSR surveys on the 13 study populations of *C. thalictroides* in China revealed high levels of polymorphism (PPB: RAPD, 61%; ISSR, 65%). Similarly, within populations, the two markers revealed roughly similar PPB values (RAPD, 15–39%; ISSR, 20–39%). This indicates that both RAPD and ISSR markers have similar discriminating power, and can be employed to accurately assess the partition of genetic diversity within and among populations of *C. thalictroides*. However, the Mantel test results between RAPD and ISSR markers ($r = 0.24$, $P = 1.00$) indicated a very poor fit. This is probably because the two techniques target different DNA segments. Furthermore, the two markers sample different genomic regions, which may have undergone different evolutionary processes under selection forces. Overall, our results indicate that both DNA markers may be effective in future work assessing the genetic variation in wild species of ferns.

Because we are dealing with an endangered species and restricted population sizes at some sites, the sample sizes in the populations varied in the range 5–30; this may have led to bias in some statistical analyses of the data.

Implications for conservation practice

The ultimate goal of conservation biology is to maintain the evolutionary potential of species by maintaining natural levels of genetic diversity (Kingstona *et al*., 2004). Molecular analysis is important in understanding the genetic systems governing endangered plants, and has the potential to contribute to the knowledge required for the conservation of genetic resources in *C. thalictroides* from China. An intermediate level of genetic diversity and weak genetic variation partitioned between populations indicate that a considerable amount of the overall genetic resources of the species could be adequately maintained in a few large and suitably protected populations.

Large extant populations with high levels of genetic variation, such as the HL-1 population which occurs on Hainan Island and the HN-1, GD-2, YN-1, and YN-3 populations which occur on mainland China, should be a priority for both *in situ* and *ex situ* conservation. Considering that *C. thalictroides* has a high extent of inbreeding as a result of its polyploid nature, and mostly occurs in habitats that are ephemeral, such as paddy fields, its conservation and restoration genetics should particularly focus on the maintenance of historically significant processes, such as high levels of outbreeding, gene flow, wide distribution, and large effective population sizes, by mixing more individuals from different populations in *ex situ* conservation.

In recent years, botanical gardens have played an important role in the *ex situ* conservation of rare and endangered plants (Maunder, 1994). Wuhan Botanical Garden and Xishuangbanna Tropical Botanical Garden, both of which are run under the auspices of the Chinese Academy of Sciences, currently play an important role in China in the conservation of species which once showed a wide distribution in tropical and subtropical regions of the country. The two botanical gardens have shown preliminary success in the conservation of species.

Habitat matching and the use of local propagules are considered to be the most successful factors in the long-term survival of a species (Krauss *et al*., 2000). The loss of genetic diversity associated with the extinction of populations is increasing, because of the small population sizes caused by human disturbance. Furthermore, genetic variation is inevitably being lost as a result of ongoing habitat destruction. From an evolutionary point of view, loss of genetic variation translates into a decreased ability to adapt to changing environments. Clearly, the situation of *C. thalictroides*, together with other rare and endangered Chinese ferns, is becoming increasingly precarious with habitat destruction caused by human disturbance. If the current situation is not remedied, loss of genetic elements in the populations will cause a decline in adaptive ability and could conceivably lead to an eventual extinction of the species. In attempts aimed at recovering populations of rare and endangered species, including *C. thalictroides*, and avoiding rapid genetic loss within populations, habitat conservation that allows a large number of individuals to survive will be of most importance. However, a uniform approach may not apply for all sites, and different sites should adopt different conservation methods as appropriate. Populations such as FJ-1, GD-2, and HL-1, located at the periphery of nature

reserves, should be placed under the administration of nature reserves. Other populations, such as GX-2, HN-1, and YN-3, which occur near forest, could be better conserved by implementing the government policy of a complete cessation of farming activities in the selected area to provide time for the forest to regenerate.

ACKNOWLEDGEMENTS

We acknowledge Dr Ren-Qing Wang, Dr Qing-Jun Li, Dr Xiao-Bao Deng, and Messrs Cui Wu, Yu-Hang Wang, and Zhe Shi for their help with fieldwork. We are also indebted to the team of Jing Xia and Wei-Guo Li for their assistance in data analysis. Dr Xiu-Qun Liu provided assistance with mapping. This study was supported by a grant from the Program for New Century Excellent Talents in University (from the Ministry of Education, People's Republic of China) granted to WQF (NCET-05-0619).

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