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Genetic variation and gene flow in the endangered aquatic fern *Ceratopteris pteridoides* in China, and conservation implications

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Random Amplified Polymorphic DNA (RAPD) markers were used to measure the levels of genetic variation and patterns of the population structure within and among the five remaining populations of *Ceratopteris pteridoides*, an endangered aquatic fern in China. Fourteen RAPD primers amplified 101 reproducible bands, with 34 (33.66%) of them being polymorphic, indicating low levels of genetic diversity at the species level. The level of genetic diversity within the populations was considerably lower, with the percentage of polymorphic bands (PPB) ranging from 16.83% to 24.75%. AMOVA analysis revealed a low level of genetic variation (30.92%) among the populations. The UPGMA cluster of 72 samples detected that individuals from the same population did not form one distinct group, indicating high levels of gene flow between the populations. A Mantel test showed no significant relationship between genetic distance and geographic distance (r = 0.2166). Our results were similar to those obtained in an earlier ISSR analysis. Thus both RAPD and ISSR markers have comparable sensitivity, and could be employed to assess the partition of genetic diversity within and among populations. Several factors including clonal growth, inbreeding, high spore dispersal and the extensive hydrologic connectivity among populations which facilitate long-distance gene flow, might have played an important role in maintaining the genetic structure of the populations. In view of the genetic information currently available, we recommend establishing as many *in situ* conservation spots as possible and the cross transplanting of plants between populations in order to increase gene flow and preserve the genetic resources of the species.

Key words: *Ceratopteris pteridoides*, conservation, genetic variation, gene flow, RAPD

Introduction

The preservation of genetic diversity both within and among natural populations is a fundamental goal of conservation biology (Hamrick *et al*. 1991). Studies of the levels and distribution of genetic variation in rare plants not only enhance our understanding of population dynamics, adaptation and evolution, but also provide information useful for biological conservation (Schaal *et al*. 1991).

Genetic diversity of plant species reflects their breeding systems (Hamrick & Godt 1996). Earlier studies suggested that diploid species of homosporous pteridophytes have an inclination to gametophytic crossing, with higher levels of genetic variation within populations, whereas polyploid ferns are inbreeding and display high genetic variation among populations, but low levels of variation within populations (Masuyama & Watano 1990, Soltis & Soltis 1990a, Watano & Masuyama 1991, Maki & Asada 1998, Vogel *et al*. 1999). In general, outcrossing species commonly have higher levels of genetic diversity and lower differentiation among populations than selfing and clonal plants (Rossetto *et al*. 1995). Compared with widespread taxa, many rare and endangered species may become genetically depauperate because of their small population size (Bauert *et al*. 1998).

The extinction of species is closely correlated with genetic factors. Trends towards decline and possible extinction of a species are associated with genetic factors and population sizes as well as environmental factors (contingency events and environmental disasters) (Frankham *et al*. 2002). The loss of genetic diversity is a common occurrence in small populations and is a precursor of species extinction (Caughley 1994). It is critical that genetic factors including inbreeding depression, loss of genetic diversity, and genetic drift, which play an important role in species extinction, be studied especially for threatened species (Hong *et al*. 1995).

Ceratopteris is a genus of ferns that occurs in the New World and Old World tropics and subtropics. Species are either aquatic or subaquatic and are typically found in ponds, rivers, or other areas such as ditches, taro patches, or rice paddies (Hickok *et al*.1995). The genus contains six species (*C. cornuta*, *C. pteridoides*, *C. richardii*, *C. thalictroides*, *C. froesii*, and *C. siliquosa*), which exhibit one of the three mating systems: automixis, autogamy, or xenogamy (Hickok *et al*. 1995). Two species of *Ceratopteris* (*C. thalictroides* and *C. pteridoides*) occur in China (Diao 1990). *Ceratopteris* has been used as a model plant for many years in the study of genetics, biochemistry, cell biology, and molecular biology (Hickok *et al*. 1995, Chatterjee & Roux 2000).

The study species *Ceratopteris pteridoides* is an annual diploid (n = 39) floating fern found principally in Central and South America, southeastern Asia, eastern India, and China (Diao 1990, Hickok *et al*. 1995). The species is limited in aquatic habitat to ponds, lakes, rivers, and ditches (Lloyd 1974). In China, *C. pteridoides* is mainly distributed in the tropical and subtropical regions including Hubei, Hunan, Jiangsu, Anhui, Yunnan, and Guangxi provinces (Diao 1990). In recent decades, the number and size of *C. pteridoides* populations have rapidly declined because of human disturbance, contributing to the deterioration and loss of primary habitats. During our field investigations from 2003 to 2005, only five extant populations of *C. pteridoides* were found from thirteen tropical and subtropical sites from where the species had been reported earlier in published references and herbarium specimen labels. The species is now endangered and listed among the second category of the Key Protected Wild Plants in China (Yu 1999).

Earlier studies on *C. pteridoides* mainly dealt with its taxonomy and morphology (Hickok *et al*. 1995, Fan & Dai 1999, Carlquist & Schneider 2000). Knowledge of the molecular biology and genetics of *C. pteridoides* is limited in comparison to the other species of *Ceratopteris*.

In recent years, a number of PCR-based DNA markers have been widely used to investigate population genetic structure mainly because they overcome the limitations of allozyme markers. The most popular markers are RAPD and ISSR (e.g. Wolfe & Liston 1998, Huang *et al*. 2001, Ge *et al*. 2003, Liu *et al*. 2007). Dong *et al*. (2007), using ISSR markers, revealed a low level of genetic diversity (44.8%) among populations of *C. pteridoides* in China. There have been no studies of genetic diversity and genetic structure

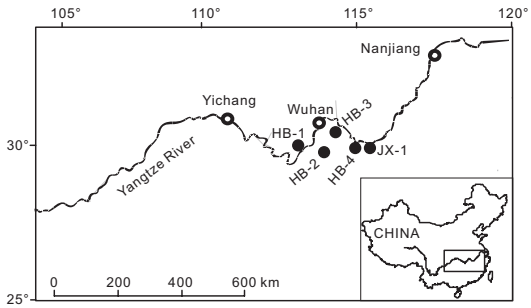

Fig. 1. Distribution map of the *Ceratopteris pteridoides* populations sampled. Population codes as in Table 1.

of *Ceratopteris* using RAPD techniques. RAPD markers survey the entire genome, rather than selected fragments (Kingston *et al*. 2004). In spite of the limitations of RAPD which include dominance, uncertain locus homology, and especially sensitivity to the reaction conditions, it has been used successfully in the study of plant systematics, evolutionary biology and conservation genetics for detection of genetic diversity in populations (Schaal *et al*. 1991, Wolfe & Liston 1998, Huang *et al*. 2001, Ge *et al*. 2003, Chen *et al*. 2004, Liu *et al*. 2007). RAPD data also are a good parameter to assess the patterns of genetic diversity and the genetic structure of rare and endangered plants and have been used to provide information necessary for the conservation of the endangered plants including lycophyte (*Isoëtes sinensis*) and fern (*Adiantum reniforme* var. *sinense*) species occurring in China (Chen *et al*. 2004, Liu *et al*. 2007).

The principal goal of the present study was to evaluate genetic variation within and among populations of *C. pteridoides* in China using RAPD, which may facilitate the conservation strategy for this endangered plant. A secondary aim was to compare the levels of genetic diversity revealed by RAPD and ISSR as a way of assessing the suitability of either technique for future investigations of genetic diversity in *Ceratopteris* populations.

Material and methods

Plant material and total DNA extraction

A total of 72 individuals from the five extant populations were collected throughout the natural geographic distribution range of *C. pteridoides* in China during July and August 2005. The five remaining populations in China, four in Hubei Province (designated as HB-1, HB-2, HB-3 and HB-4) and one in Jiangxi Province (designated as JX-1), are in the area of the middle and lower reaches of the Yangtze River, which has thousands of shallow lakes most of which are interconnected to the main artery of the Yangtze River (Fig. 1). Individuals in each study population were sampled at a minimum distance of 5 m from each other (for collection sites, population sizes and sampling sizes *see* Table 1). Approximately 5–10 g of young leaves were harvested from each plant and immediately dried in a sealed ziplock plastic bag containing about 50 g of silica gel. Total DNA was extracted from 0.5 g of silica-dried leaf tissue following the procedure described by Fu *et al*. (2003).

RAPD PCR amplification

Eighty RAPD primers from SBS Genetech Co. Ltd. (Shanghai, China) were screened on four randomly selected individuals for PCR, and 14 primers that produced reproducible, clear, polymorphic electrophoretic bands were selected for further analysis (Table 2). The PCR reactions were performed in a PTC-100TM thermocycler (MJ Research, Inc.) using the following temperature-cycle profile: an initial melting step at 94 °C for 4 min, followed by 45 cycles of 94 °C for 1 min, 34 °C for 1 min, 72 °C for 2 min, and a final extension cycle of 10 min at 72 °C. Reactions were carried out in a volume of 25 μl, containing 0.25 mM of each dNTP, 1.5 mM $MgCl_2$, 1 mM primer, 1 U Taq polymerase and 3 μl (20 ng μl^{-1}) of DNA template. PCR products were electrophoresed on 1.5% agarose gels stained with ethidium bromide, and photographed under ultraviolet light. Sizes of amplification products were estimated using a 200 bp DNA ladder.

Data analysis

According to the molecular weight (bp), all individuals were scored for the presence (1) or

Table 1. Location, habitat, sample size, and genetic variability of the *Ceratopteris pteridoides* populations studied (PPB = percentage of polymorphic bands; *H* = Nei's gene diversity; *I* = Shannon's information index)

Population code	Location	Site coordinates	Altitude (m)	Habitats	Sample size	Number of polymorphic bands	PPB (%)	*H*	*I*
HB-1	Honghu, Hubei	29°55´N, 113°13´E	24	Ditch	18	22	21.78	0.0857(0.1724)	0.1250 (0.2476)
HB-2	Jiayu, Hubei	29°54´N, 114°01´E	19	lake	21	23	22.77	0.0980 (0.1862)	0.1407 (0.2649)
HB-3	Liangzihu, Hubei	30°15´N, 114°33´E	15	lake	14	25	24.75	0.0873 (0.1694)	0.1296 (0.2443)
HB-4	Yangxin, Hubei	29°55´N, 115°14´E	14	Fishpond	11	21	20.79	0.0739 (0.1578)	0.1100 (0.2285)
JX-1	Ruichang, Jiangxi	29°48´N, 115°43´E	21	Lake	8	17	16.83	0.0516 (0.1362)	0.0781 (0.1954)
At the species level					72	34	33.66	0.1177 (0.1842)	0.1757 (0.2665)

absence (0) of the amplified RAPD fragments. The percentage of polymorphic bands (PPB), the Shannon index of diversity (*I*) (Lewontin 1972), and the gene diversity index (*H*) were calculated to evaluate genetic diversity. At the species level, the coefficient of gene differentiation (G_{st}) and the level of gene flow (N_m) were measured using Nei's Index (1973); $N_m = 0.5(1 - G_{st})/G_{st}$ (McDermott & McDonald 1993). Nei's genetic distance (*D*) between populations was computed using Nei's program (Nei 1972). In order to examine correlation of the populations, a UPGMA tree (Nei 1972) was constructed. All calculations were carried out using POPGENE program ver. 1.31 (Yeh *et al*. 1997). Analysis of molecular variance (AMOVA) was also used to estimate genetic variability within and among populations using squared Euclidean distances (Excoffier *et al*. 1992). Genetic analyses were performed using the WINAMOVA program ver. 1.55 (Excoffier 1993). Input files for this program were generated using AMOVA-PREP (Miller 1998). Significance tests were made after 1000 permutations. The Nei and Li (1979) coefficient for measuring pairwise band similarities between individuals was calculated using NTSYSpc ver. 2.02 (Rohlf 1998). The dendrogram (UPGMA) of all individuals was computed using the unweighted pair-group method with an arithmetic average using NTSYSpc ver. 2.02 (Rohlf 1998). In addition, in order to assess a relationship between genetic and geographical distances (in km) among populations, a Mantel test was performed using the program TFPGA (Miller 1997). Significance tests were made after 3000 permutations.

Table 2. Name of RAPD primers, sequences of 14 effective primers.

Primer	Sequence 5´–3´	Primer	Sequence 5´–3´
P-A-17	CCTGGGCCTC	P-D-07	CGTGGGCAGG
P-B-15	AGGGGCGGGA	P-F-18	AGGCCGCTTA
P-C-06	CGTCTGCCCG	P-G-19	GTGGCCGCGC
P-D-01	GCTGTAGTGT	P-A-12	CCTGGGTCCA
P-D-02	CGCACCGCAC	P-A-13	CCTGGGTGGA
P-D-05	CTGGCGGCTG	P-B-17	GAGGGCGAGC5
P-D-06	GCCTGGTTGC	P-C-01	GCGGCTGGAG

Results

RAPD polymorphism

Fourteen effective RAPD primers generated a total of 101 bands (an average of 7.2 bands per primer) with fragments ranging in size from 200 to 2000 bp. Thirty-four of the 101 bands (33.66%) were polymorphic among the 72 individuals (Table 1). The percentage of polymorphic bands (PPB) was 33.66%. The percentage of polymorphic bands (PPB) for a single population ranged from 16.83% (JX-1) to 24.75% (HB-3), with a mean value of 21.38% ± 2.937%. The gene diversity (H) and the Shannon's information index (I) showed a similar pattern of the genetic differentiation between populations (Table 1). Among the five populations, the population HB-3 exhibited the highest level of variability (PPB, H and I values were 24.75%, 0.0873, and 0.1296, respectively), whereas the population JX-1 exhibited the lowest level of genetic variation (PPB, H and I values were 16.83%, 0.0516, and 0.0781, respectively) (Table 1).

Genetic structure of populations

The coefficient of genetic differentiation between populations (G_{st}) indicated 32.12% genetic variation existed among *C. pteridoides* populations, and 67.88% of the genetic variability existed within populations. The mean values of gene diversity (H) and the Shannon's information index (I) were 0.1177 and 0.1757, respectively. Based on the G_{st} values, the estimated number of migrants per generation (N_m) was 1.0565 among populations. AMOVA further revealed that 30.92% of the genetic variation was attributable to among-populations diversity and the rest (69.08%) to differences within populations (Table 3). Genetic distance (D) based on Nei's unbiased measures among the five study populations of *C. pteridoides* showed more differences. The highest genetic variance was 0.0768 between populations JX-1 and HB-4. The UPGMA cluster analyses of the five study populations showed that JX-1 population was clearly separated from the other populations (Fig. 2). A UPGMA cluster analysis of 72 individuals indicated that samples from the same population did not form a distinct group (Fig. 3). The Mantel test revealed that there is no significant correlation between genetic distance and geographic distance ($r = 0.2166$, $p = 0.7455$).

Discussion

Among populations, the PPB values detected by RAPD in *C. pteridoides* were low as compared with those obtained for other fern species. For instance, Watano and Masuyama (1994), using allozyme data, revealed 54% genetic diversity between populations of *C. thalictroides* from Japan. Based on allozyme analyses, Maki and Asada (1998) reported high genetic diversity both within and among populations in *Polystichum otomasui* (the PPB values were 61.9% and 81.3%, respectively). Chen *et al*. (2004, 2005) found very high (82%) ISSR diversity in the six extant populations of the critically endangered *Isoëtes hypsophila* and a high RAPD diversity (PPB: 58.06%) in *I. sinensis*. Results of RAPD diversity analysis among populations of *C. pteridoides* in China obtained in this study were similar to those obtained in an earlier ISSR diversity analysis (PPB = 44.8%) of the populations (Dong *et al*. 2007). Within populations, the two markers revealed a roughly similar range of PPB values (16.83% to 24.75% for RAPD and 14.4% to 32% for ISSR).

Table 3. Analyses of molecular variance (AMOVA) for RAPD data of 72 individuals in five populations of *C. pteridoides*.

Source of variation	d.f.	SSD	MSD	Variance component	Total percentage	p*
Among population	4	116.434	29.110	1.791	30.92	< 0.001
Within population	67	268.088	4.001	4.001	69.08	< 0.001

* Significance tests after 1000 random permutations.

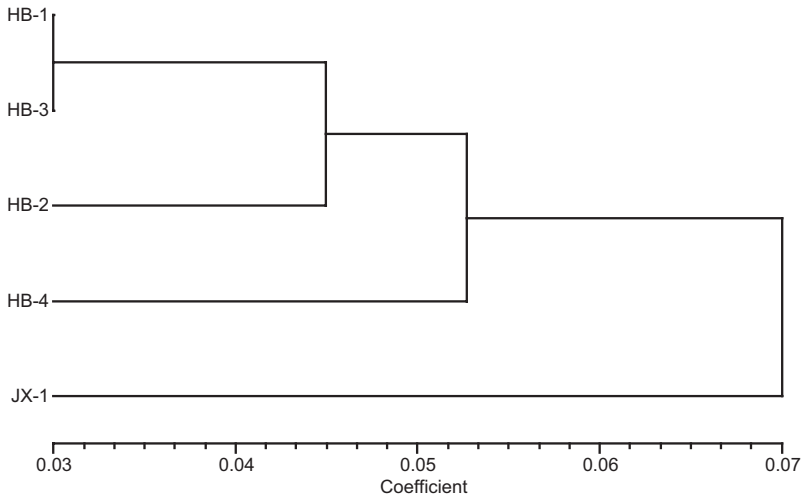

Fig. 2. Dendrogram of five populations of *Ceratopteris pteridoides* based on Nei's genetic distance using RAPD markers by UPGMA cluster analysis (NTSYS). Populations as in Table 1.

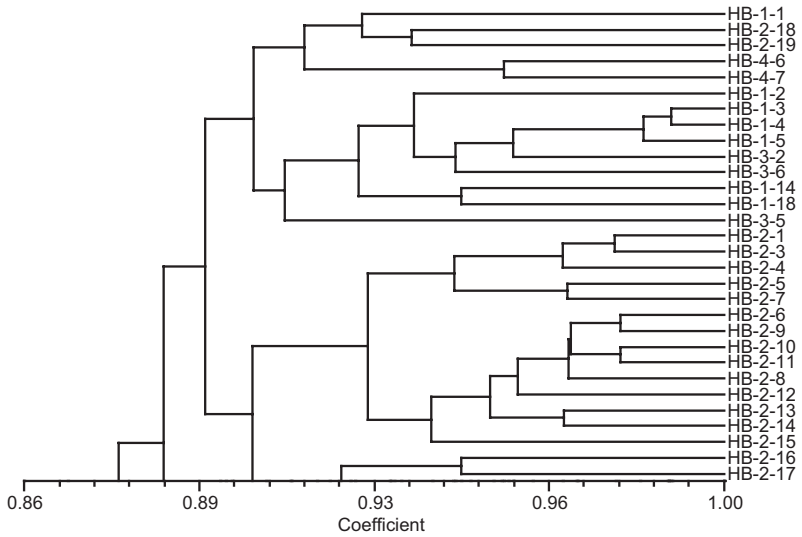

Fig. 3. UPGMA dendrogram based on RAPD data of 30 of 72 individuals of *Ceratopteris pteridoides*. The first two letters and one digit represent the population code, the last one or two digits represent number of individuals from each population.

AMOVA of RAPD bands showed highly significant ($p < 0.001$) genetic differences among the five populations of *C. pteridoides* (Table 3). A large proportion of genetic variation (69.08%) existed within populations, whereas only 30.92% was partitioned among populations, indicating a low level of genetic differentiation among the five populations. Results of AMOVA of RAPD are consistent with those of Nei's genetic statistics (0.1177) and Shannon's diversity index (0.1757), both of which indicated a low degree of population differentiation. Results of AMOVA of *H* and *I* from RAPD were also similar to those obtained from ISSR analysis (Dong *et al*. 2007). In addition, the Mantel test based on the RAPD data indicated no significant correlation between genetic distance and geographic distance ($r = 0.2166$, $p = 0.7455$). This finding was supported by results from ISSR markers ($r = 0.443$, $p = 0.912$) (Dong *et al*. 2007). Gene flow (N_m) among populations of *C. pteridoides* detected using RAPD markers ($N_m = 1.0565$) was similar to that obtained from ISSR analysis ($N_m = 1.19$) (Dong *et al*. 2007). On the whole, the results of both RAPD and ISSR analyses for *C. pteridoides* populations in China are similar. This suggests that for *C. pteridoides* both RAPD and ISSR markers have comparable sensitivity and can be used for assessing the patterns of genetic diversity and the genetic structure within populations

of the species. Both markers may be effective in future work on estimating genetic variation in wild species of ferns.

Several factors, including the long-term evolutionary history, mutation, genetic drift, mating system, gene flow, and selection, may determine the population genetic diversity and the genetic structure of populations of ferns (Soltis & Soltis 1990a). The breeding system of *C. pteridoides* may contribute to the low level of intra- and interpopulation genetic diversity within the populations. The potential for intragametophytic self-fertilization (automixis) makes homosporous pteridophytes such as *C. pteridoides* unique among vascular plants, owing to free-living gametophytes that are potentially bisexual (Masuyama & Watano 1990, Soltis & Soltis 1990b, Hickok *et al*. 1995, Haufler 2002). Inbreeding and genetic drift caused by small extant populations will inevitably lead to decreasing genetic variability (Huang *et al*. 2001). The effective population size in plants is profoundly affected by their mating systems. Theoretical studies have indicated that inbreeding reduces the effective size of subpopulations (Maki & Asada 1998). It is very probable that gametophytic selfing may have played an important role in maintaining the low level of genetic diversity within the endangered *C. pteridoides* in China.

Clonal growth is near universal among aquatic plants (Haynes 1988, Cook 1990). Clonal growth and mating system exert strong influence on the growth and genetic diversity of aquatic plant populations (Waycott 1995, Qian *et al*. 2001). The populations may be composed of a single genetic individual (genet) indicating a monoclonal structure, or they may be made up of several different clones and hence possess a multiclonal structure (Les 1991). Generally speaking, asexually reproducing species have low levels of genetic diversity, whereas sexually reproducing species exhibit high levels of population genetic variation (Loveless & Hamrick 1984). Clonal populations derived from a single individual (genet) are similar to small populations in that they both are likely to have increased genetic drift and inbreeding (Barrett & Kohn 1991, Ellstrand & Elam 1993). *Ceratopteris pteridoides* combines sexual reproduction with asexual reproduction through vegetative propagation by means of numerous marginal leaf buds that rapidly develop into plantlets (Hickok *et al*. 1987, Diao 1990). Both field observations and laboratory culture experiments have revealed that *C. pteridoides* has a prolific capacity for clonal growth. Field investigations indicated that HB-1 and HB-4 populations might have originated from a limited number of vegetative propagules transferred to the present site by human activities such as aquaculture. Consequences of extensive clonality include reduction in the sexual reproductive potential of outcrossing species and increased inbreeding rates in self-compatible plants (Frankham *et al*. 2002).

The strong colonizing ability of homosporous ferns results from the high dispersibility of their spores and the ability of a single, haploid propagule to become established and reproduce via intragametophytic selfing, which forms populations of genetically identical individuals (Soltis & Soltis 1990b). *Ceratopteris* lacks a perennial woody rhizome. Unlike many homosporous ferns *C. pteridoides* is an annual aquatic weed that forms new populations annually in lakes, ponds, and ditches. *Ceratopteris thalictroides* also has annual colonizing ability. Extinction and re-colonization of patches by a few individuals could result in low genetic variation (Harrison & Hastions 1996, Hedrick 1996). Thus, clonal growth, along with intragametophytic selfing may be responsible for the establishment of populations of *C. pteridoides* with genetically identical individuals, and may accelerate the loss of genetic diversity.

Gene flow is a common occurrence both within and among populations (Grant 1991) and is an important factor in determining the genetic structure of populations of plant species (Widen 1992). Gene flow could potentially prevent decline of genetic variation within populations and decrease inter-population differentiation (Slatkin1987). $N_m > 1$ is indicative of high levels of gene flow among populations (Soltis & Soltis 1990a). Endemic and endangered plant species generally have a low gene flow ($N_m < 1$; Hamrick & Godt 1996). However, the present RAPD analysis revealed a considerable level of gene flow among populations of *C. pteridoides* in China ($N_m = 1.0565$). The observed level of gene flow was also higher than the mean value in

aquatic plants (mean value of $N_m = 0.552$; Hamrick & Godt 1990). Liu *et al*. (2007) reported that the endangered fern *A. reniforme* var. *sinense* in China has a relatively high level of gene flow ($N_m = 1.688$). This finding together with the high level of gene flow detected in this study among populations of *C. pteridoides* indicates that for some fern species reduced gene flow is not a major causative factor of the endangered condition of the species. UPGMA cluster analyses revealed that in all five study populations individuals from the same population occasionally failed to cluster in one distinct group. The results further indicated extensive inter-population gene flow for *C. pteridoides* in China.

In plants, gene flow is occasioned by movement of pollen, seeds, spores, and propagules (Orive & Asmussen 2000). Soltis and Soltis (1990a) suggested that high levels of gene flow among populations via spore dispersal may be a much more important homogenizing force in species of ferns than interpopulational gene flow in most seed plants, suggesting that higher gene flow may be an important factor in maintaining genetic differentiation among populations. In view of the location of all the study populations in the middle and lower reaches of the Yangtze River, spore dispersal by water flow is likely to be a frequent occurrence. This efficient spore dispersal might have promoted gene flow in *C. pteridoides* and could be responsible for the present-day structure of genetic variation in the study populations.

The Mantel test showed no significant relationship ($r = 0.2166$) between genetic distance and geographic distance among the five study populations ($0.7 \leq r < 0.8$, poor fit; $r < 0.7$, very poor fit; Mantel 1967). This indicated that gene flow may have occurred not only among closely-neighboring populations, but that each population may have received migrant genotypes from sources far away from the recipient populations. Dispersal of free-living gametophytes and sporophytes may have played a part in the interpopulational gene flow. In addition, since *C. pteridoides* is a floating fern, individuals may have moved from one population to another by water flow.

Certain schools of thought, exemplified by Lande (1988), consider human activity to be the major factor responsible for extinction of species through the loss and destruction of primary habitats and over-exploitation. With the exception of the JX-1 population, all the studied populations of *C. pteridoides* in the central Chinese province of Hubei are in the area occupied in ancient times by a massive wetland known as the Yunmeng, which by 239 BC is reputed to have had a surface area spanning more than six million ha (Shi *et al*. 1989, Diao 1990). Over a period in excess of two thousands years, the surface coverage of the wetland has progressively declined albeit at different rates in different historical periods. The number of lakes in Hubei Province, known in Chinese as "The province of a thousand lakes", had decreased to 197 in 1988 with their total area reduced to 4909 km^2 in comparison with the condition in 1949 (Fang *et al*. 1995). Between 1958 and 1982 the total area of one lake (Honghu Lake) had been reduced by half (Lu & Jiang 2003). The rapid reduction in numbers and sizes of *C. pteridoides* populations is probably associated with sedimentation arising from soil erosion in the upper reaches of the Yangtze River, which is exacerbated by human activities including farming, excessive aquaculture, over-exploitation of water bodies, irrigation activities, reclamation of land from lakes, tourism, uncontrolled real estate development, and run-off water pollution (Shi *et al*. 1989, Gituru *et al*. 2002). For example, the demise of Dongliu Lake in the Yangtze River basin in Anhui province led to the extirpation of the *C. pteridoides* population in the lake. Excessive aquaculture and water pollution are the likely causes for the extinction of *C. pteridoides* from Haikou, Taibai, and Honghu Lakes in Hubei province (Jian *et al*. 2001, Lu & Jiang 2003). Field investigation established that fishermen removed *C. pteridoides* from HB-2 and HB-4 populations thus hampering re-establishment of populations and hastening the extirpation of *C. pteridoides* from these sites.

Present-day low levels of genetic variation among populations of *C. pteridoides* may be a reflection of earlier periods when the populations were much more expansive. It is likely that high levels of interpopulational gene flow might have occurred before the onset of the rapid decline of the populations as a result of the combined

effects of geographic events and human disturbance.

Previous studies have suggested that diploid species of homosporous pteridophytes have an inclination to gametophytic crossing, with some exceptions favoring gametophytic selfing as a derived condition (Haufler 2002), whereas polyploid homosporous pteridophytes predominantly favor gametophytic selfing (Masuyama & Watano 1990). Soltis and Soltis (1990a) reported that six diploid species of homosporous pteridophytes in *Polystichum* showed both outcrossing and low interpopulational divergence. The presence of herbarium specimens with both 16-and 32-spored sporangia and exhibiting mixed features of *C. pteridoides* indicates that hybridization occurs naturally in this genus (Lloyd 1993). The results of both RAPD and ISSR analyses (Dong *et al.* 2007) showed high gene flow among populations of *C. pteridoides* in China, indicating the species may possess a higher outcrossing rate. Outcrossing and higher gene flow may have had a founder effect on the diploid *C. pteridoides*, thereby maintaining a low level of genetic differentiation in the species.

Both *in situ* and *ex situ* conservation approaches are required for protecting the remaining *C. pteridoides* populations in China. However the most appropriate conservation strategy would be to protect more habitats of the species. In recent years, the policy promulgated by the local administrative authorities in China of "protecting natural species and renewing the lake habitat" has started to register success as indicated by the reduction in introduced aquatic species observed at Honghu Lake (HB-1) and Liangzihu Lake (HB-3) in Hubei province. The policy appears to be a good approach towards improving the lacustrine ecosystem and protecting threatened aquatic life forms such as *C. pteridoides*. A key aim of conservation, in addition to habitat preservation, is to maintain a species' existing level of genetic variation in order to maximize its chances for persistence in the face of changing environments (Keiper & McConchie 2003). When decisions need to be made about choosing populations for protection, those with the highest genetic diversity should have higher priority to maintain the species' potential for evolutionary change (Hamrick & Godt 1996, Zawko *et al.* 2001).

Choice of sites and the appropriate *ex situ* conservation strategies require adequate genetical data on the species to be conserved (Hogbin & Peakall 1999). The genetic information presented in our study is intended to guide policy makers in formulating the appropriate strategies for the conservation of natural populations of *C. pteridoides*. The low level of genetic diversity at the species level and the low level of genetic variation among populations of *C. pteridoides* requires more focus on the conservation of the genetic resources of the species by protecting the existing small populations and encouraging increase in the number of individuals in the populations. In this regard, populations HB-1, HB-2, and HB-3 should be a priority for both *in situ* and *ex situ* conservation. Considering that only a few extant populations remain in China, and that outbreeding and high levels of interpopulational gene flow are a feature of the reproductive biology of the species, it would be advisable to establish as many *in situ* conservation sites as possible with transplanting of plants among populations to minimize inbreeding and enhance gene flow in order to preserve the greatest extent of genetic resources within the species.

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