

Genetic variation in the endangered fern *Adiantum reniforme* var. *sinense* (Adiantaceae) in China

Xiu-Qun Liu^{1,2}, Robert Wahiti Gituru^{2,3} & Long-Qing Chen^{1,*}

¹ Key Laboratory of Horticultural Plant Biology (MOE), College of Horticulture and Forestry, Huazhong Agricultural University, Wuhan 430070, P.R. China (*corresponding author's e-mail: chenlq0206@163.com)

² Laboratory of Plant Systematics and Evolutionary Biology, College of Life Sciences, Wuhan University, Wuhan 430072, P.R. China

³ Botany Department, Jomo Kenyatta University of Agriculture and Technology, P.O. Box 62000-00200, Nairobi, Kenya

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Adiantum reniforme var. *sinense* (Adiantaceae) is a rare and endangered fern with only four known remaining populations restricted to a few sites in the Three-Gorges area of the Yangtze River in China. RAPD markers were used to investigate the level of genetic diversity in the four populations. Twenty of the 80 primers that were tested were selected and a total of 92 DNA fragments were scored. The low level of genetic diversity observed in the fern is attributed to its confinement to the east of Sichuan Province and west of Hubei Province, which constitute a refugium of plants from the Quaternary glaciation period. At the population level, the percentage of polymorphic bands (PPB), the effective number of alleles per locus (A_E), the expected heterozygosity (H_E), and Shannon's information measure (H) were 29.6%, 0.102, 1.114 and 0.1506, respectively; while at the species level, the corresponding values were 33.7%, 0.118, 1.133 and 0.1779, respectively. Nei's standard genetic distance (D) was low and ranged from 0.0136 to 0.0274. AMOVA analysis indicated that the most of the genetic variation (89.93%) resided within populations, and that only 10.03% of the variation resided among populations. In the UPGMA dendrogram based on Nei's genetic distance some individuals from different populations clustered together. The high gene flow detected in the populations ($N_m = 1.688$) may indicate that the four populations may originally have belonged to a single expansive population, which at present is fragmented into four discontinuous units due to disturbance by human activities. The endangered status of this taxon is attributed to the effect of low genetic diversity and habitat deterioration and disturbance.

Key words: *Adiantum reniforme* var. *sinense*, endangered fern, genetic variation, population genetics, RAPD

Introduction

Adiantum reniforme (Adiantaceae), the only unifoliate evergreen species of the genus, has an intraspecific disjunctive distribution, with *A. reniforme* distributed in the Azores, *A. reniforme* var. *assarifolium* in Madagascar, Mauritius and South Africa, and *A. reniforme* var. *sinense* in Asia. The latter being the only variety distributed in Asia makes it particularly suited for studying continental drift and flora evolution (Lin 1980). The taxon is also a domestic ornamental of considerable importance in China due to its lotus-like leaves.

Adiantum reniforme var. *sinense* was first reported from Wanzhou County of Chongqing City in 1978. Xu *et al.* (1987), Liu (2000) and Peng *et al.* (2000) reported that in China the species is distributed within a belt 100 km long and 3–5 km wide along both sides of the Yangtze River.

The dwarfish *A. reniforme* var. *sinense* has low reproductive ability and stringent habitat requirements, which causes it to be rare and endangered. It is of concern that the Three-Gorges Project is projected to cause the water level in the reservoir area to rise to 175 m a.s.l. and thereby submerge large belts of the low-lying area presently occupied by *A. reniforme* var. *sinense* between 80 and 430 m a.s.l.

In order to provide pertinent information and promote protection of *A. reniforme* var. *sinense*, studies on the taxon — including distribution studies (Xu *et al.* 1987), karyotype studies (Lin 1989), cultivation studies (Chen *et al.* 1990), spore propagation studies (Xu *et al.* 1998), soil studies (Shen *et al.* 1999), and isozyme analysis (Wang *et al.* 2000) — were conducted. However, up to date the extent of genetic diversity and the population genetic structure of *A. reniforme* var. *sinense* have not been reported.

RAPD markers have been used with success to detect population genetic structure and diversity in many different plant species (Jordano & Godoy 2000, Oiki *et al.* 2001, Lowe *et al.* 2003, Wang *et al.* 2003, Chapman *et al.* 2004). RAPD polymorphisms reflect the variation of the whole genomic DNA and are a good parameter to assess the patterns of genetic diversity of rare and endangered plants. RAPD parameters have

been used in many cases to provide information necessary for the conservation of endangered plant species (Etisham & Haq *et al.* 2001, Bekessy *et al.* 2002, Bouza *et al.* 2002, Kwon & Morden 2002, Schiller *et al.* 2003, Kingston *et al.* 2004, Moreira Reis & Grattapaglia 2004).

In the current study we investigated genetic variation within four populations of *A. reniforme* var. *sinense* using RAPD polymorphisms, with the aim of identifying the causes of endangerment of this rare species and to provide information to assist conservationists and government planners in formulating appropriate conservation strategies.

Material and methods

Plant materials

The individuals of *A. reniforme* var. *sinense* used in this study were collected throughout their natural range in the Three-Gorges region of the Yangtze River in Chongqing, China. Only four remnant natural populations were found *viz.* populations A, B, C and D (Fig. 1). Owing to the small sizes of the populations no more than 15 individuals were taken from each population for the study, and individuals were sampled at a minimum distance of 50 m from each other. In total, 49 individuals (Nos. 1–49) were taken from the four populations. Population A, located in Tuche village, Wangchang Division, Xituo District, Shizu County of Chongqing City, yielded 15 individuals (Nos. 1–15); population B, located in Taiping Village, Zhougxing Division, Xituo District of Shizhu County, yielded 14 individuals (Nos. 16–29); population C, located in Shanshui Village, Xinxiang Division of Wanzhou District, yielded 13 individuals (Nos. 30–42), and population D, located in Jiangjun Village, Xinxiang Division of Wanzhou District, yielded five individuals (Nos. 43–47).

Total DNA extraction

Total genomic DNA was isolated from silica-dried leaf tissue using a modification of the CTAB extraction procedure (Doyle & Doyle

1987). Approximately 0.5 g leaf material was ground in liquid nitrogen, mixed with 800 μ l extraction buffer (0.1 M Tris-HCl pH 8.0, 0.05 M EDTA pH 8.0, 0.5 M NaCl, 1.5% SDS, 2% PVP, 0.5% β -mercaptoethanol), then placed into a 1.5 ml Eppendorf tube, and incubated at 65 °C for 30 min. The extracts were centrifuged at 12 000 rpm for 5 min and the supernatant was transferred into a new tube. Proteins were extracted three times with 2/3 volume of 2 ml of chloroform: isoamylalcohol (24:1), then centrifuged at 12 000 rpm for 2 min; DNA was precipitated in 1 volume -20 °C isopropanol, washed in 70% ethanol, air-dried and dissolved in 1 \times TE. In order to get further purification, we added RNase A (10 mg ml⁻¹) to the supernatant and incubated for 2 h at 37 °C. The mixture was centrifuged at 12 000 rpm for 2 min. The sediment was washed twice in 70% ethanol, air-dried, and suspended in 100 ml of 0.1 \times TE buffer (10 mM Tris-HCl, 0.1 mM EDTA), and then stored at -20 °C.

RAPD-PCR amplification

Twenty effective primers (Table 1), which produced clear and reproducible fragments, were selected for PCR from eighty obtained from SIGMA Co. Ltd. PCR was performed in volumes of 20 μ l consisting about 20 ng of DNA template, 3.0 μ l 10 \times buffer (500 mM KCl, 100 mM Tris-HCl pH 9.0 (25 °C), 1.0% Triton X-100, 15 mM MgCl₂), 0.3 Mm each dNTP, 5 pmol primer, 2U *Taq*-DNA polymerase. Ampli-

Table 1. Names of primers and sequences of 20 effective primers.

Primers	Sequences (5'-3')	Primers	Sequences (5'-3')
OPA07	GAAACGGGTG	OPG08	TCACGTCCAC
OPA10	GTGATCGCAG	OPG10	AGGGCCGTCT
OPA16	AGCCAGCGAA	OPG12	CAGCTCACGA
OPA17	GACCGCTTGT	OPG16	AGCGTCCTCC
OPC15	GACGGATCAG	OPG18	GGCTCATGTG
OPD03	GTCGCCGTCA	OPH04	GGAAGTCGCC
OPF12	ACGGTACCAG	OPK11	AATGCCCCAG
OPF13	GGCTGCAGAA	OPK18	CCTAGTCGAG
OPG04	AGCGTGTCTG	OPL03	CCAGCAGCTT
OPG05	CTGAGACGGA	OPM19	CCTTCAGGCA

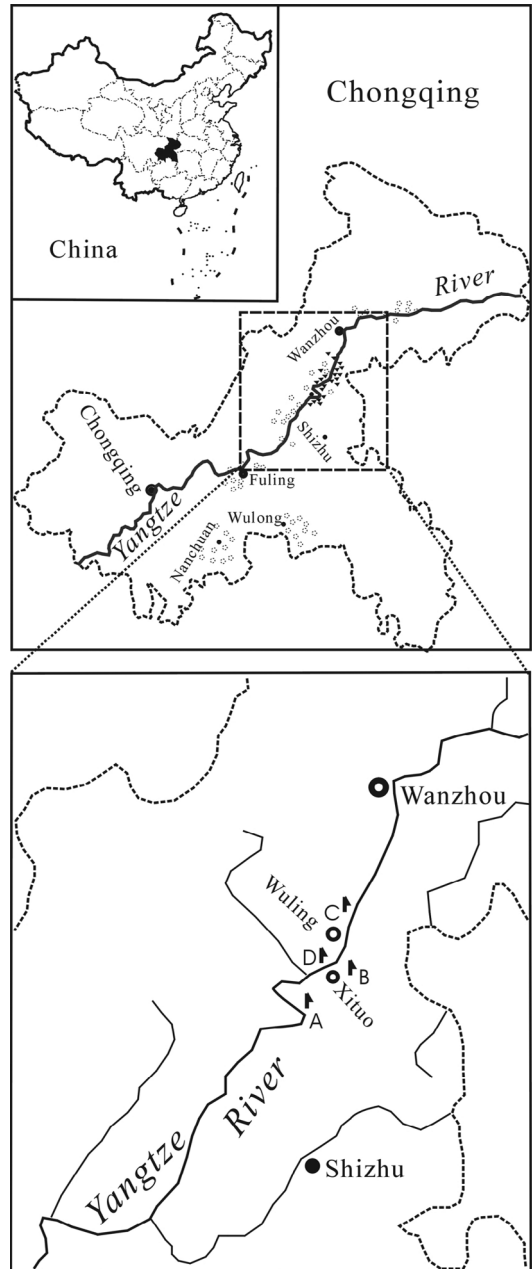


Fig. 1. Distribution of *Adiantum reniforme* var. *sinense*.

fication was performed in a 9700 Air Thermo-Cycler (GeneAmp® PCR System 9700, Japan) and commenced with 3 min at 94 °C, followed by 38 cycles of 1 min at 94 °C, 1 min at 36 °C, 2 min at 72 °C, and ended with 10 min at 72 °C (Chen *et al.* 1999). The amplified products were resolved electrophoretically on 1.4% agarose gel

run at 50 V for one hour and twenty minutes in $1 \times \text{TAE}$ (4 mM Tris-HCl, 1 mM EDTA) solutions, visualized by staining with $0.5 \mu\text{g } \mu\text{L}^{-1}$ ethidium bromide and photographed under ultraviolet light. Sizes of amplification products were estimated using a 200 bp DNA ladder.

Only those bands that showed consistent amplification were considered in final analysis. Smear and weak bands were excluded.

Data analysis

All individuals were scored for presence or absence of RAPD fragments, and the data were entered into a binary data matrix as discrete variables (1 for presence and 0 for absence). The matrix was assembled for the following analysis: the percentage of polymorphic bands (PPB), the effective number of alleles per locus (A_E), the expected heterozygosity (H_E). At the species level, genetic diversity measures (H_T : total population gene diversity; G_{ST} : coefficient of gene differentiation) and the level of gene flow (N_m) were measured using Nei's gene diversity statistics (Nei 1973). Nei's genetic distance (D) between populations was computed using the program (Nei 1972). Genetic diversity was estimated using Shannon's information measure (H) (Lewinton 1972):

$$H = -\sum_{i=1}^K p_i \ln p_i$$

where K is the number of RAPD bands produced with the respective primer and p_i is the frequency of the i th fragment. The H values were measured using POPGENE program 1.31 (Yeh *et al.* 1997).

Table 2. Expected heterozygosity (H_E), effective number of alleles per locus (A_E), percentage of polymorphic bands (PPB) and Shannon's information measure (H).

	H_E	A_E	PPB (%)	H
Pop-A	0.114	1.129	33.7	0.1817
Pop-B	0.121	1.137	33.7	0.1652
Pop-C	0.086	1.094	27.2	0.1264
Pop-D	0.089	1.097	23.9	0.1290
Avg. in population-level	0.102	1.114	29.6	0.1506
Avg. in species-level	0.118	1.133	33.7	0.1779

The analysis of molecular variance (AMOVA) was also used to partition the total phenotypic variance into within and among populations (Excoffier *et al.* 1992). AMOVA input files were generated with AMOVA-PREP 1.01 (Miller 1998). All analyses were carried out using AMOVA 1.5 provided by Laurent Excoffier (Genetics and Biometry Laboratory, University of Geneva, Switzerland). AMOVA variance components were used as estimates of the genetic diversity within and among populations. The dendrogram (UPGMA) was computed on the basis of the unweighted pair-group method using arithmetic means with the software TREECON (ver. 1.3b, Van de Peer 1997).

Results

RAPD polymorphism

Twenty effective RAPD primers generated a total of 92 bands (an average of 4.6 bands per primer), with fragments ranging in size from 200 to 2100 bp (mainly from 600 to 1800 bp). Thirty-one of the total 92 bands (33.7%) were polymorphic among 47 individuals, i.e., the percentage of polymorphic bands (PPB) for this species was 33.7%. The percentage of polymorphic bands (PPB) for a single population ranged from 23.9% to 33.7%. The average effective number of alleles per locus (A_E) was 1.133. Assuming Hardy-Weinberg equilibrium, the average gene diversity was estimated to be 0.102 within populations (H_E), and 0.118 at the species level (H_T). The Shannon's index (H) ranged from 0.1264 to 0.1817, with an average of 0.1506 at the population level and 0.1779 at the species level as shown in Table 2.

Genetic structure of populations

The coefficient of genetic differentiation between populations (G_{ST}) indicated that 12.9% genetic variability existed among populations and 88.1% of the genetic variation existed within populations. The level of gene flow (N_m) was estimated to be 1.688 individuals per generation among populations. Nei's standard genetic distance (D)

ranged from 0.0136 between populations B and C to 0.0274 between populations A and C (Table 3).

AMOVA analysis indicated that 10.07% of the total genetic diversity was attributable to among-populations diversity and the rest (89.93%) to within-populations diversity (Table 4).

The UPGMA tree of all individuals indicated that although individuals from a single population were always grouped together, they were just as likely to be grouped together with individuals from a different population (Fig. 2).

Discussion

In this study, we investigated the genetic diversity of four populations of *A. reniforme* var. *sinense* in China. The level of genetic diversity in this study (mean PPB value of 29.6%) is comparable to that reported in studies on other endangered pteridophytes (Hsu *et al.* 2000, Chen *et al.* 2004, 2005). The mean PPB value within populations of *Isoetes hypsophila* was reported by Chen *et al.* (2005) to be 13%. Working on *Isoetes sinensis*, Chen *et al.* (2004) found that the PPB values within populations ranged from 1% to 13%. Hsu *et al.* (2000) reported finding only five polymorphic RAPD bands after surveying 40 primers in *Archangiopteris itoi*, a rare endemic fern in Taiwan. The genetic diversity at population level in many rare and endangered fern species is considerably lower than that in more common and non-endangered fern species (Small & Hickey 1997, Maki & Asada 1998). Maki and Asada (1998) found 62% polymorphic allozyme loci at population level in *Polystichum otomasui*.

The low level of genetic diversity observed in *A. reniforme* var. *sinense* in China is attributed to its being restricted to the narrow area of the Three-Gorges region of the Yangtze River valley, which lies in the east of Sichuan Province and

west of Hubei Province. The area is a typical refugium of plants from the Quaternary glacial periods and the famous “living fossil” *Metasequoia glyptostroboides* was first reported from there (Hu & Cheng 1948).

The distribution of genetic variation in *A. reniforme* var. *sinense*, where 89.93% of the genetic variation is distributed within populations and 10.07% is partitioned among populations, is consistent with results from studies of some other fern species. Baker and Hauk (2003), working on *Sceptridium dissectum*, reported that 92% of the genetic variation was distributed within populations and 8.49% was distributed among populations.

Endemic and endangered plant species generally have low levels of gene flow ($N_m < 1$) (Hamrick & Godt 1996). However, this study revealed that *A. reniforme* var. *sinense* has a relatively high level of gene flow ($N_m = 1.688$). This indicates that a low level of gene flow is not the major causative factor of the endangered condition of this species.

Based on the low genetic diversity and high gene flow among populations observed in *A. reniforme* var. *sinense* in China, it is hypothesized that the four present-day populations historically belonged to a single continuous population that became fragmented into four discontinuous units largely due to human activities. What might appear to be high gene flow among populations might in actual fact represent gene flow

Table 3. Nei's unbiased genetic distance (D) among pairs of four populations.

Genetic distance (D)	Pop-A	Pop-B	Pop-C	Pop-D
Pop-A	****			
Pop-B	0.0145	****		
Pop-C	0.0274	0.0136	****	
Pop-D	0.0237	0.0228	0.0266	****

Table 4. AMOVA analysis for 47 individuals from four populations of *Adiantum reniforme* var. *sinense*.

Source of variation	df	SSD	MSD	Variance component	% total	P^a
Among population	3	34.94	11.65	0.58	10.07	< 0.001
Within population	43	221.02	5.14	5.12	89.93	< 0.001

^a Significance tests after 5000 permutations.

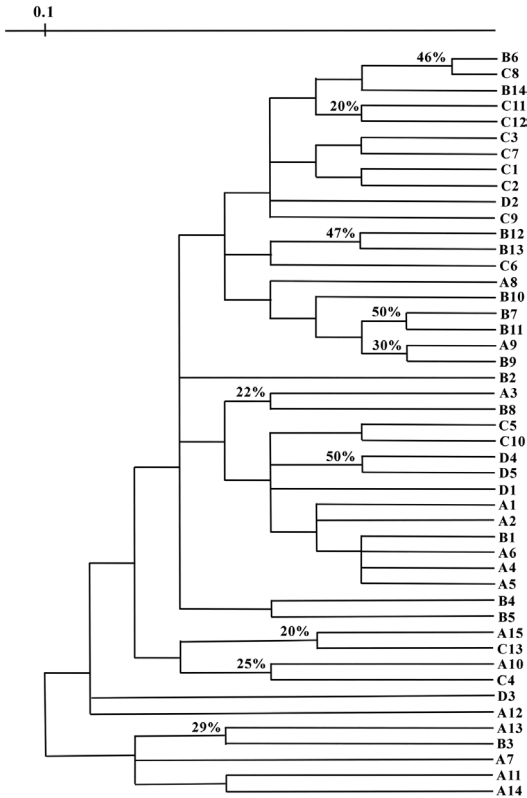


Fig. 2. The UPGMA dendrogram of *Adiantum reniforme* var. *sinense* individuals using the Nei and Li (1979) genetic distance matrix based on RAPD data. The percentages on the branches were the result of 2000 bootstrap replication. Only bootstrap values > 20% are indicated. The first letters (A–D) indicate the populations, the numbers following the letters the individuals.

within the same population. This hypothesis was supported by the dendrogram based on UPGMA method, which showed individuals from different populations clustering together (Fig. 2). The fern was previously distributed continuously in broad belts of more than 100 km along both banks of the Yangtze River in locations including Nanchuan (Peng *et al.* 2000), Wulong (Liu *et al.* 2000), Fuling (Xu *et al.* 2000), Shizhu and Wanzhou (Xu *et al.* 1987) (Fig. 1). Interviews with the local people at the study sites revealed that *A. reniforme* var. *sinense* is an ingredient of certain traditional Chinese medicines and has been collected for sale by the locals over the last twenty years. Human activities have extirpated several local populations and broken the previously continuous distribution of the species.

In addition to the effect of low genetic diversity and the impact of human activities on *A. reniforme* var. *sinense*, the endangered condition of this taxon is exacerbated by factors including habitat deterioration and the influence of the giant Three-Gorges hydroelectric power project. The species commonly grows in extreme habitats such as on cliff faces and in cracks on exposed rocks on cliff faces. Furthermore, the plentiful precipitation in the area (rainfall in the range of 1100–1300 mm annually) erodes the scant soil, on which the fern is dependent. The raining season in the area coincides with the period of maturation of the spores of *A. reniforme* var. *sinense*. Controlled experiments have indicated that excessive moisture is detrimental to successful spore germination in this species (Xu *et al.* 1998).

The projected water level expected to be achieved in the Three Gorges water reservoir is 175 m above sea level (Huang 2001). *Adiantum reniforme* var. *sinense* is distributed in the range of 80–430 (mainly 150–250) m a.s.l. It is unavoidable that the present habitat of *A. reniforme* var. *sinense* will be progressively submerged with the rise in the water level of the reservoir. An *ex-situ* conservation program is therefore of critical importance if extinction of this rare fern is to be avoided. This program should be complimented by *in-situ* conservation efforts in the areas of the habitat that lie above the ultimate projected water level of the reservoir.

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