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Genetic Consequence of Restricted Habitat and Population Decline in Endangered *Isoetes sinensis* (Isoetaceae)

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• *Background and Aims Isoetes sinensis* (Isoeteaceae) is a critically endangered aquatic quillwort in eastern China. Rapid decline of extant population size and local population extinction have occurred in recent years and have raised great concerns among conservationists.
• *Methods* Amplified fragment length polymorphisms (AFLPs) were used to investigate the genetic variation and population structure of seven extant populations of the species.
• *Key Results* Eight primer combinations produced a total of 343 unambiguous bands of which 210 (61·2 %) were polymorphic. *Isoetes sinensis* exhibited a high level of intra-population genetic diversity ($H_E = 0{\cdot}118$; $hs = 0{\cdot}147$; $I = 0{\cdot}192$; $P = 35{\cdot}2$ %). The genetic variation within each of the populations was not positively correlated with their size, suggesting recent population decline, which is well in accordance with field data of demographic surveys. Moreover, a high degree of genetic differentiation ($F_{ST} = 0{\cdot}535$; $G_{ST} = 0{\cdot}608$; $\theta^B = 0{\cdot}607$) was detected among populations and no correlation was found between geographical and genetic distance, suggesting that populations were in disequilibrium of migration-drift. Genetic drift played a more important role than gene flow in the current population genetic structure of *I. sinensis* because migration of *I. sinensis* is predominantly water-mediated and habitat range was highly influenced by environment changes.
• *Conclusions* Genetic information obtained in the present study provides useful baseline data for formulating conservation strategies. Conservation management, including both reinforcement for *in situ* populations and *ex situ* conservation programmes should be carefully designed to avoid the potential risk of outbreeding depression by admixture of individuals from different regions. However, translocation within the same regional population should be considered as a measure of genetic enhancement to rehabilitate local populations. An *ex situ* conservation strategy for conserving all extant populations to maximize genomic representation of the species is also recommended.

Key words: AFLP, genetic diversity, genetic differentiation, population structure, *Isoetes sinensis*, pteridophyte.

INTRODUCTION

Isoetes sinensis is a member of the Isoeteaceae, a family that consists of a single relict lycopsid genus with approx. 150 species occurring in lake, wetland and terrestrial habitats all over the world (Taylor and Hickey, 1992). Four species, *I. hypsophila*, *I. sinensis*, *I. taiwanensis* and *I. yunguiensis*, have been identified and documented in China (Wang *et al*., 2002). All Chinese *Isoetes* species are highly threatened with extinction due to habitat loss, agricultural land-use, and invasion by exotic species, and are therefore listed as endangered species (Fu and Jin, 1992; Ye and Li, 2003). *Isoetes sinensis* has four cytotypes, but only allotetraploid ($2n = 4x = 44$) plants occur in China (He *et al*., 2002), while hexaploid ($2n = 6x = 66$) and two aneuploids ($2n = 65$, $2n = 68$) of the species are found in Japan (Takamiya *et al*., 1994). A recent phylogenetic study suggested that *I. sinensis* was probably derived from hybridization between two diploid species: *I. yunguiensis* and *I. taiwanensis* endemic to China (Taylor *et al*., 2004). *Isoetes sinensis* is now a globally threatened taxon and is also listed as an endangered species in Japan (Takamiya 2001; Red List of Threatened Plants of Japan, http://www.biodic.go.jp/english/rdb/red_plants.csv). The species is discretely distributed in eastern China, mostly in limited areas in Anhui, Jiangshu, Jiangxi and Zhejiang provinces (Fu and Jin, 1992). A recent exhaustive survey of herbarium specimen and literature confirmed that the natural range of the species was very limited. *Isoetes sinensis* was previously documented in Dongxiang (DX) (Chen *et al*., 1998), Jiangxi province; Jiuxi (JX) (voucher nos HHBG 60934 and ZJFC 0188), Qinyuan (QY) (S. C. Zhu, pers. comm.), Tiantai (TT) (B. Y. Ding, pers. comm.) and Zhuji (ZJ) (voucher nos HZU 9754 and HZU 9810), Zhejiang province; and Xuanwuhu (XWH) (voucher no. NAS 00070205), Jiangshu province (Fig. 1). However, field surveys conducted in the past five years concluded that *I. sinensis* could be extinct in these six sites (Fig. 1) (Pang *et al*., 2003; Ye and Li, 2003). In addition, sizes of extant populations have decreased dramatically (Ye and Li, 2003). The rapid decline of the species and extinction of local populations have caused conservation concerns to save this endangered species in China (Pang *et al*., 2003; Wen *et al*., 2003; Ye and Li, 2003; Chen *et al*., 2004) and *I. sinensis* was recently categorized as a critically endangered species by IUCN (2004).

Small and isolated populations of endangered species are vulnerable to demographic, environmental and genetic stochasticity, and therefore face a higher risk of local extinction (Lande, 1999). Although the relative importance and consequence of these factors is still controversial (Lande, 1988; Spielman *et al*., 2004), genetic factors have been

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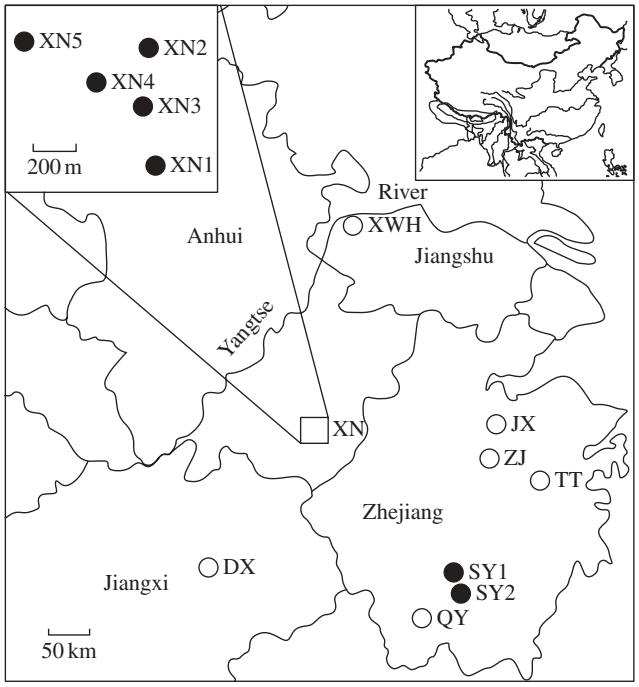

FIG. 1. Map of the historical and present distribution of *Isoetes sinensis* and sampling locations (open circles, historical sites where *I. sinensis* was previously documented but identified as extinct during field surveys in 2000–2003; closed circles, extant sites where samples were collected for the present study).

demonstrated to have direct and/or indirect impacts on the viability of endangered species (Frankham *et al*., 2002). Many endangered plant species with small population sizes in isolated or fragmented habitats suffer higher extinction risks due to the loss of genetic variation caused by genetic drift and inbreeding (Barrett and Kohn, 1991). Some critically endangered species may still go to extinction even when their natural habitats are restored because of severely depauperate genetic diversity and the lack of adaptive evolutionary potential in the face of an ever-changing environment (Frankham *et al*., 2002). It has been widely accepted by conservation geneticists that understanding the level of genetic diversity and population genetic structure is a prerequisite for formulating conservation programmes for endangered plant species. Such knowledge could provide critical information needed to understand the evolutionary history of populations and population genetics underlining potential risk in the long term, and also make available information for the genetic management of endangered species both *in situ* and *ex situ* (Frankham *et al*., 2002).

Of the available techniques in use to investigate population genetic diversity and genetic structure, the amplified fragment length polymorphism (AFLP) technique (Vos *et al*., 1995) has the combined advantages of RAPD and RFLP, with the promise of generating a large number of markers representative of the target genome. The technique is highly reproducible and cost-efficient, and has been used successfully in population genetic studies of several endangered plants (Travis *et al*., 1996; Gaudeul *et al*., 2000; Evans *et al*., 2001; Tero *et al*., 2003). However, few such studies have been attempted in pteridophyte species (Keiper and McConchie, 2000).

In this study, AFLP analysis was employed to address the following questions of conservation concern in *I. sinensis*: (*a*) What is the level of genetic diversity currently retained in the remnant populations? (*b*) How is the genetic variation distributed within and among populations? (*c*) Is population size associated with the level of population genetic variation, and geographic distance associated with genetic distance? These baseline data should be essential to understand the genetic consequences of population decline in remnant *I. sinensis* growing in isolated and fragile wetland habitats, so that adequate conservation measures could be taken without potential risk of admixture of genetically differentiated populations either for *in situ* conservation by translocation or for *ex situ* conservation.

MATERIALS AND METHODS

Study species

Isoetes sinensis Palmer is a small, aquatic and facultative evergreen quillwort with numerous spirally arranged leaves (sporophylls) arising from a globose, three-lobed corm. Pliant and hollow leaves are 2–4 mm wide and up to 30 cm long, depending on water depth in the wetland habitat. Sporangia are embedded in the broadened base of leaves. Megasporangia are usually borne on outer leaves, with a few white, granular and tetrahedric megaspores, while microsporangia are borne on inner leaves, with numerous grey-powdery and bilateral microspores (Fu and Jin, 1992). Both sexual and asexual reproduction has been observed in this species (Takamiya *et al*., 1994; Chen *et al*., 2004). *Isoetes sinensis* occurs in wetland habitats of rice paddy land, shallow ponds, swamps and creeks and appears to be a weak competitor with dominant companion species of *Eriocaulon decemflorum* Maxim, *Polygonum sagittatum* Linn., *Miscanthus sinensis* Anderss, *Prunella asiatica* Nakai and *Hydrangea* L. sp.

Field survey and sample collection

First an exhaustive literature search conducted on *I. sinensis* and visits made to the main Chinese herbaria (AAUB, ANU, ANUB, HHBG, HIB, HZU, NAS, PE and ZJFC) to look for specimen records resulted in a comprehensive compilation of historical and updated information of the geographic distribution and detailed locations of the species. Then, extensive field surveys across the entire range of *I. sinensis* were conducted during for four years (2000–2003), but this species was only found in two remnant sites (Fig. 1). Two populations (SY1 and SY2), approx. 1 km apart, were found in Songyang county, Zhejiang province (28°16′N, 119°16′E); while five small patches, 162–520 m apart, were observed in Xiuning county, Anhui province (29°30′N, 118°09′E). As this quillwort is a semi- and submerged aquatic the migration of spores or root-runners are restricted; therefore, each patch was presumed to be a population and was designated XN1, XN2, XN3, XN4 or XN5 (Fig. 1).

TABLE 1. *Polymorphic bands generated by each of eight pair primers in seven extant populations of* Isoetes sinensis

Primer pairs	Primer sequence (5′–3′)	Total bands	Polymorphic bands	Polymorphism (%)
E-AAC/M-CTG	GACTGCGTACCAATTCAACGATGAGTCCTGAGTAACTG	46	36	78·3
E-ACT/M-CTC	GACTGCGTACCAATTCACTGATGAGTCCTGAGTAACTC	25	18	72·0
E-ATG/M-CGT	GACTGCGTACCAATTCATGGATGAGTCCTGAGTAACGT	52	36	69·2
E-ACC/M-CGT	GACTGCGTACCAATTCACCGATGAGTCCTGAGTAACGT	42	29	69·0
E-ACG/M-CAT	GACTGCGTACCAATTCACGGATGAGTCCTGAGTAACAT	38	17	44·7
E-ACG/M-CTG	GACTGCGTACCAATTCACGGATGAGTCCTGAGTAACTG	47	24	51·1
E-AAC/M-CAT	GACTGCGTACCAATTCAACGATGAGTCCTGAGTAACAT	50	25	50·0
E-ACT/M-CTG	GACTGCGTACCAATTCACTGATGAGTCCTGAGTAACTG	43	25	58·1
Mean		42·9	26·3	61·6
Total		343	210	

Population area and population size were determined for each population by directly measuring the area and counting individuals within each population. Then, fresh leaves (sporophylls) were harvested from adult individuals, at least 2 m apart, to avoid sampling the same plant. Ten to 30 individuals were randomly sampled from each population, except population XN2, depending on sampling availability of adult plants in each of the seven populations. In population XN2, a total of 20 individuals were identified, but only five adult plants could be sampled keeping the minimum sampling distance (2 m). As a result, a total of 106 samples were collected from these seven populations at the two remnant sites (Fig. 1). Plant materials were immediately dried in silica gel before DNA isolation.

DNA isolation and AFLP analysis

Total genomic DNA was extracted from 0·3–0·5 g of dried leaves using a modified CTAB protocol outlined in Doyle and Doyle (1987). AFLP analysis was performed essentially as described previously by Vos *et al.* (1995) with a slight modification. Total genomic DNA (200 ng) was digested using 3 units of *Eco*RI and *Mse*I endonuclease mixture (Biolab) in a total volume of 25 μL for 2·5 h at 37 °C and digests were confirmed by electrophoresis on 1·5 % agarose gels. Then, 20-μL of ligation solution containing 4 pmol *Eco*RI adapter, 40 pmol *Mse*I adapter, and 1 unit of T_4 DNA ligase were added to the digests in a total volume of 40 μL. The ligation mixture was incubated overnight at 21 °C in a thermocycler. The resulting DNA (template DNA) was then diluted 1 : 5 in TE buffer. PCR pre-amplification was performed in a 20 μL solution containing 75 mM TRIS–HCl (pH 8·8), 20 mM $(NH_4)_2SO_4$, 1·5 mM $MgCl_2$, 0·1 ‰ Tween20, 0·20 mM each of dNTPs, 40 ng of primer *Eco*RI-A, 40 ng of primer *Mse*I-C, 0·5 units Taq polymerase (Fermentas), and 5 μL of template DNA for 20 cycles of the thermal profile: 94 °C for 30 s, 56 °C for 60 s and 72 °C for 60 s. The pre-amplification DNA was diluted 1 : 25 in TE buffer and used as template DNA for selective amplification. After pre-screening of 48 primer pairs, eight selective primer pairs were chosen for this study (Table 1). An aliquot of 2·5 μL diluted pre-amplification DNA was added to 7·5 μL of selective amplification cocktail (30 ng *Eco*RI primer, 30 ng *Mse*I primer, 0·20 mM dNTPs, 1× PCR buffer, 0·5 units Taq polymerase, 1·5 mM $MgCl_2$), and amplified with the thermal cycle profile: one cycle with 94 °C for 30 s, 60 °C for 1 min and 72 °C for 1 min, then 12 cycles of 94 °C for 30s, 65 °C (decreasing by 0·7 °C) for 1 min and 72 °C for 1 min, followed by 23 cycles of 94 °C for 30 s, 56 °C for 1 min and 72 °C for 1 min. The amplification products added with 7·5 μL loading buffer (98 % formamide, 10 mM EDTA (pH 8·0), 0·25 % bromophenol blue and 0·25 % xylene cyanol) were denatured at 94 °C for 5 min and electrophoresed on a 6 % denaturing polyacrylamide gel on a 40 × 45 cm EC160 DNA Sequencing System (Thermo). Silver staining was conducted according to protocol of the Silver Sequence™ (Promega) with slight modification.

Marker scoring and data analysis

Only intense and unambiguous bands (100–500 bp) were manually scored as present (1) or absent (0). After scoring all bands for all samples, the bands that were monomorphic across all individuals were then discarded for further analyses to reduce the statistical bias (Keiper and McConchie, 2000).

Due to the dominant nature of AFLPs, estimating heterozygosity and population differentiation could be problematic (Zhivotosky, 1999; Tero *et al.*, 2003). Traditional approaches to estimating heterozygosity and population differentiation using dominant markers assume that populations are in Hardy–Weinberg equilibrium, or require a known inbreeding coefficient, or treat multilocus DNA phenotypes as haplotypes (Zhivotovsky, 1999; Holsinger *et al.*, 2002). Earlier studies have suggested that such approaches may lead to severely biased estimates (Lynch and Milligan, 1994; Zhivotovsky, 1999). However, Krauss (2000) has demonstrated that accurate estimates of population genetic parameters can be obtained from dominant markers when a large number of polymorphic loci were used. More recently, Holsinger *et al.* (2002) introduced a Bayesian approach to estimating genetic diversity and population structure without prior information of the inbreeding coefficient (F_{IS}) and demonstrated that the Bayesian method could provide nearly unbiased estimates of heterozygosity, genetic distance and population differentiation.

In the present study, the following parameters for revealing genetic diversity and population differentiation were estimated using different computer softwares: percentage

of polymorphic loci (P at the 0·99 level), Nei's unbiased expected heterozygosity (H_E) (Nei, 1978) and Wright' F-statistics (F_{ST}) (Weir and Cockerham, 1984) were estimated using the program TFGPA (Miller, 1997*a*). Shannon and Weaver's index (I) (Shannon and Weaver, 1949) and genetic differentiation (G_{ST}) (Nei, 1987) were calculated under the assumption of full selfing ($F_{IS} = 1$) within populations using the program POPGENE version 1.32 (Yeh *et al*., 1997). The Bayesian approach described by Holsinger *et al*. (2002) was also used to determine genetic diversity (hs, analogous to H_E) and population differentiation (θ^B, analogous to F_{ST}) using the program HICKORY 0.8 (Holsinger and Lewis, 2003), with five runs of each of three different models (full model, $f = 0$ model and f-free model) to ensure consistency of the results (burn-in = 50 000, no. of samples = 250 000, thinning = 50 in each run). The model chosen was based on the deviance information criterion (DIC) (Spiegelhalter *et al*., 2002). Models with smaller DIC are preferred, but a difference of >6 DIC units among different models is required to indicate that there is strong favouring of one model over another (Holsinger and Lewis, 2003).

Analysis of molecular variance (AMOVA) (Excoffier *et al*., 1992) was used to determine hierarchical genetic structure of the populations. The program Amova-prep (Miller, 1997*b*) was used to construct a Euclidean distance matrix. Then, AMOVA was carried out on the Euclidean distance matrix with the program WINAMOVA, Version 1.55 (Excoffier, 1995), with 1000 random bootstraps of the data set to partition the overall genetic variation into different hierarchical levels of population differentiation.

UPGMA cluster analysis was used to generate a dendrogram of all individual plants based on Nei's (1978) unbiased genetic distance. The analysis was performed using SAHN and TREE programs in NTSYS pc2.0 (Rohlf, 1997) and the resulting dendrogram was tested through 1000 bootstrap replicates. Linear regression was used to assess the relationship between population area (m^2) or population size (N) and the amount of genetic variation (H_E, hs and I) within populations. The analysis was performed using SPSS version 10.0 (SPSS, 1999). A Mantel test (Mantel, 1967) was used to determine if there was an association between genetic distances (pairwise Φ_{ST}) and geographical distances for the five Xiuning populations, and the significance of the test was also determined, using the program TFPGA with 5000 permutations (Miller, 1997*a*). The amount of gene flow (N_m) among the five Xiuning populations was estimated based on the mean value of pairwise genetic distances using the formula, $N_m = (1 - \Phi_{ST})/4\Phi_{ST}$.

RESULTS

AFLP polymorphism and genetic diversity within populations

The eight primer combinations generated a total of 343 reliable fragments ranging from 100 bp to 500 bp. Of these, 210 (61·6 %) were polymorphic across 106 individuals of *I. sinensis* (Table 1). Each individual sampled showed a unique AFLP phenotype. The number of fragments generated by different primer pairs varied from 25 by E-ACT/M-CTC to 52 by E-ATG/M-CGT with an average of 42·9 fragments. The percentage of polymorphic bands of the eight different primer pairs varied from 44·7 % to 78·3 %, with an average value of 61·6 % (Table 1).

TABLE 2. *Population area (P_A), population size (P_S), and within population genetic diversity in seven extant populations of* Isoetes sinensis*

Population	P_A	P_S	N	P (%)	H_E	hs	I
SY1	550	400	30	53·8	0·171	0·200	0·288
SY2	278	1000	25	34·8	0·115	0·147	0·198
XN1	50	60	11	34·3	0·117	0·141	0·186
XN2	18	20	5	25·2	0·083	0·132	0·146
XN3	70	60	10	38·6	0·125	0·135	0·172
XN4	60	30	15	29·0	0·104	0·148	0·206
XN5	53	50	10	31·0	0·109	0·125	0·147
Mean	154	231	15	35·2	0·118	0·147	0·192

* P_A, Population area (m^2); P_S, actual population size; N, sample size; P(%), percentage of polymorphism; H_E, Nei's (1978) unbiased expected heterozygosity; hs, genetic diversity using Bayesian approach; I, Shannon and Weaver's (1949) index.

The estimates of genetic diversity for each population are summarized in Table 2. The percentage of polymorphic loci ranged from 25·2 % in population XN2 to 53·8 % in population SY1, with a mean value of 35·2 %. The estimates of heterozygosity (H_E) based on the Lynch–Milligan procedure were generally lower than Bayesian estimates (hs). There was no significant correlation between estimates of H_E and hs ($R^2 = 0·429$, $P = 0·337$) and between estimates of H_E and I ($R^2 = 0·500$, $P = 0·253$), however, the correlation between hs and I was highly significant ($R^2 = 0·964$, $P = 0·000$). Population SY1 exhibited the highest heterozygosity in all three parameters ($H_E = 0·171$; $hs = 0·200$; $I = 0·288$) (Table 2). The mean values of H_E, hs and I across the seven populations were 0·118, 0·147 and 0·192, respectively.

The seven populations surveyed for population size and area varied in size from approx. 20 plants (XN2) to 1000 plants (SY2), and the population areas ranged from 18 m^2 (XN2) to 550 m^2 (SY1) (Table 2). Larger populations tended to harbour higher levels of genetic variation (P, H_E and hs), although no significant correlation was detected (Fig. 2A). However, significant correlations were found between population area and each of the three genetic diversity measures (Fig. 2B).

Population genetic differentiation

Using the Bayesian approach, the θ^B values obtained from the three different models are presented in Table 3. The smallest mean DIC 2858.8 was obtained under the f-free model, suggesting that the f-free model is more suitable than other models for this data set. Therefore, $\theta^B = 0·607$ was determined to be an unbiased estimate of population genetic differentiation under the f-free model, with an inbreeding coefficient $f = 0·504$. On the other hand, our estimate of F_{ST}, under the assumption of Hardy–Weinberg equilibrium was 0·535, which is smaller than θ^B obtained from the Bayesian approach, while the value of $G_{ST} = 0·608$ was obtained under the assumption of total

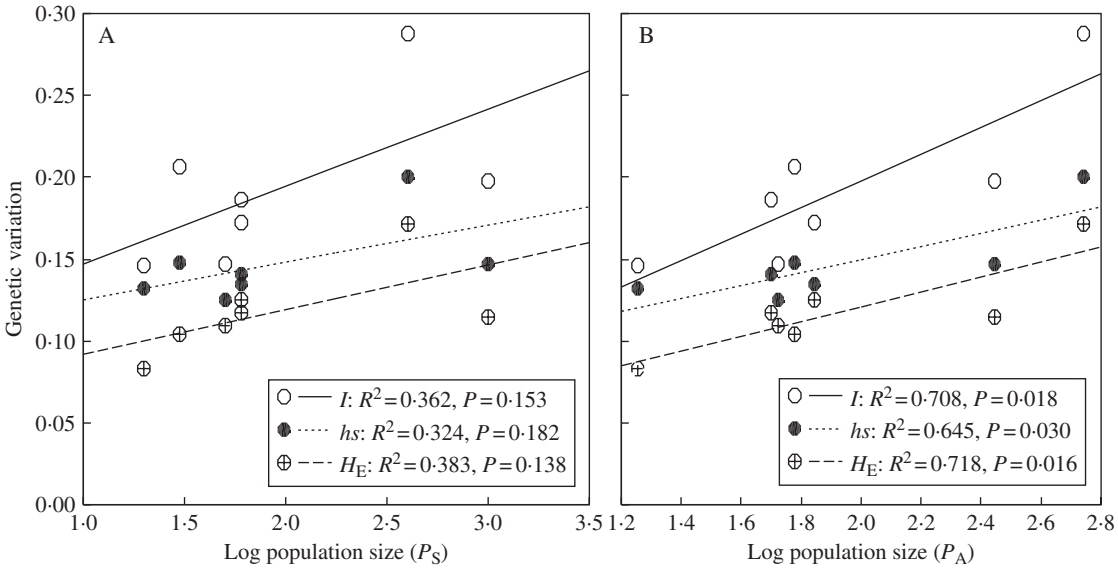

FIG. 2. Relationship of (A) population size and (B) population area *vs.* genetic variation measured as H_E, *hs* and *I*.

TABLE 3. *Wright's* F-*statistics* θ^B *calculated under three models of Bayesian approach (95 % confidence intervals are shown in parentheses)*

Model	*f*	θ^B	DIC
$f = 0$	0·000	0·565 (0·540–0·589)	2873·4
Full	0·006	0·566 (0·543–0·590)	2875·2
f-free	0·504	0·607 (0·577–0·635)	2858·8

DIC, Deviance information criterion; *f*, inbreeding index within populations.

selfing, which is almost equal to the $\theta^B = 0{\cdot}607$ derived from the *f*-free model. Thus, the results suggested that natural populations of *I. sinensis* should neither be completely outcrossed nor randomly mated. The values of $\theta^B = 0{\cdot}607$ and $G_{ST} = 0{\cdot}608$ would be more logical indexes to explain the population genetic differentiation for *I. sinensis*. Although there is a slight difference among the three measures, all of them indicate a strong genetic structure among *I. sinensis* populations.

AMOVA further revealed significant genetic differentiation across the sampled distribution of *I. sinensis* (Table 4). At the population level, 63·5 % of the total molecular variation was attributed to inter-population differentiation, and 36·5 % to individual differentiation within populations. However, when the total variance was partitioned into three hierarchical levels, the largest variance component (46·9 %) was found between the two regions sampled, while only 23·2 % and 29·9 % variance was found among populations within regions and among individuals within populations, respectively.

The mean pairwise genetic distance among populations (pairwise Φ_{ST}) was 0·530 ± 0·192, ranging from 0·093 between population XN2 and XN3 to 0·736 between population SY2 and XN5 (Table 5). All Φ_{ST} for each pairwise comparison was significant ($P < 0{\cdot}001$), except for Φ_{ST} between XN2 and XN3. In addition, the Mantel test revealed that no correlation between genetic distances (Φ_{ST}) and geographical distances existed among five local populations in Xiuning region (r = 0·267, P = 0·708), and the amount of gene flow estimated is N_m = 0·378.

TABLE 4. *Analysis of molecular variance (AMOVA) of 210 polymorphic AFLP markers for different hierarchical analyses of* Isoetes sinensis *populations*

Source of variation	d.f.	MSD	Variance component	Percentage of total	*P*-value*
Two hierarchical levels:					
Among populations	6	422·12	28·24	63·5	<0·001
Within populations	99	16·22	16·22	36·5	<0·001
Three hierarchical levels:					
Between regions	1	1606·42	25·49	46·9	<0·001
Among populations	5	185·26	12·60	23·2	<0·001
Within populations	99	16·22	16·22	29·9	<0·001

* Statistic significance is based on 1000 permutations.

TABLE 5. *Pairwise estimated values of* Φ_{ST} *among* Isoetes sinensis *populations**

Populations	SY1	SY2	XN1	XN2	XN3	XN4
SY2	0·495***					
XN1	0·664***	0·723***				
XN2	0·652***	0·728***	0·265***			
XN3	0·655***	0·724***	0·221***	0·093^{ns}		
XN4	0·657***	0·720***	0·398***	0·399***	0·396***	
XN5	0·668***	0·736***	0·503***	0·564***	0·552***	0·315***

* Statistic significance based on 1000 permutations was shown: ^{ns}, not significant; *** $P < 0{\cdot}001$.

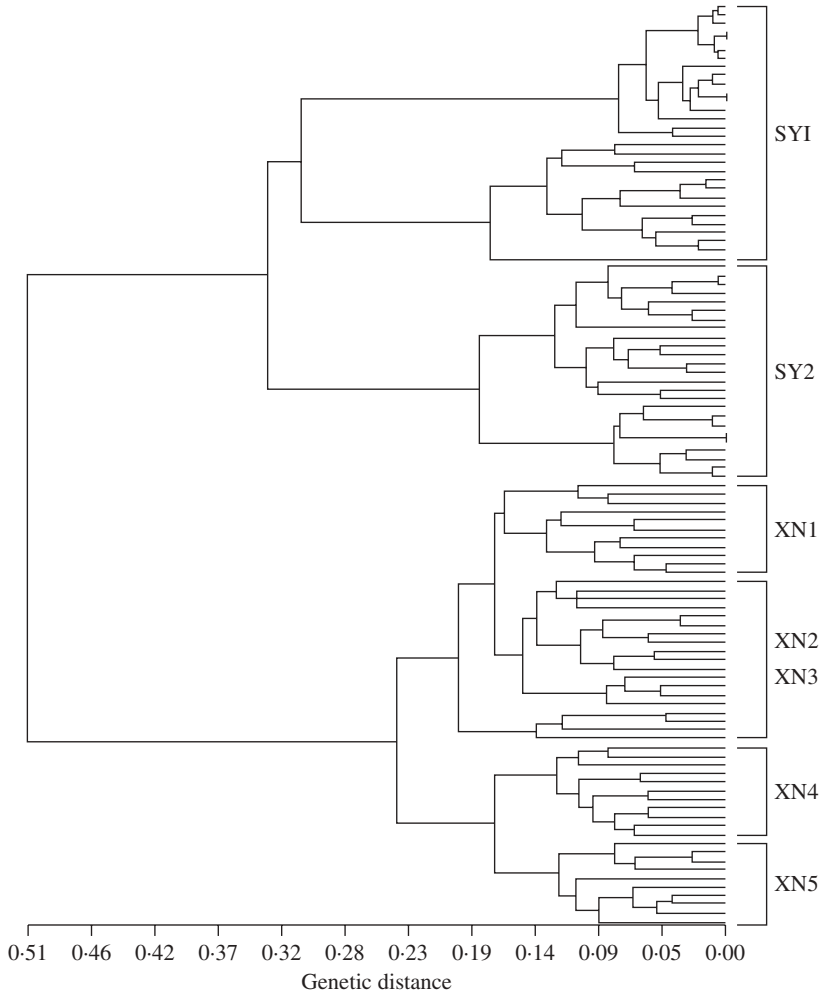

FIG. 3. UPGMA dendrogram of 106 individuals of seven extant *Isoetes sinensis* populations based on 210 polymorphic AFLP markers.

Cluster analysis

The UPGMA dendrogram (Fig. 3) was supported with high bootstrap values (>90 %), suggesting a strong population structure. The dendrogram of the 106 individual plants based on Nei's genetic distance coefficients revealed two well-defined clusters, which is well in accordance with their geographical distributions, namely the clusters SY and XN (Fig. 3). Furthermore, individuals from each population were mostly clustered into population-specific subclusters, except that individuals from population XN2 and XN3 grouped into a single subcluster and individuals in population XN4 were not clustered together. Individuals of SY1 and SY2 formed two distinct subclusters consistent with their habitats.

DISCUSSION

Genetic variation within populations

Most population genetic studies in pteridophyte species have used allozymes to assess the genetic diversity and population structures because the inbreeding coefficient (f) can be directly inferred from co-dominant allozyme markers. However, the advantage is largely compromised when the species is polyploid, such as the tetraploid *I. sinensis* (Chen *et al*., 2004), although there are recently developed protocols for analysing tetraploid populations (Thrall and Young, 2000). DNA-based genetic markers have been widely used in population studies of many seed plants and have proven to be a useful and effective tool for the investigation of genetic diversity and population genetic structures in natural populations of pteridophyte species (Schneller *et al*., 1998; Keiper and McConchie, 2000; Landergott *et al*., 2001; Pryor *et al*., 2001; Vitalis *et al*., 2002; Kingston *et al*., 2004). In the present study, heterozygosity within populations estimated by the Lynch–Milligan procedure was not consistent with that estimated by the Bayesian procedure recently suggested by Holsinger *et al*. (2002). For unbiased estimates of heterozygosity, the Bayesian procedure was believed to be more suitable over Lynch–Milligan procedure (Zhivotovsky, 1999), although Krauss (2000) found the Lynch–Milligan and Bayesian procedures could yield equivalent and accurate estimates of average heterozygosity in *Persoonia mollis*, which is a predominantly outbreeding species and exhibits high levels of

AFLP polymorphism. For *I. sinensis*, the observed relatively low proportion of polymorphisms (P = 35·2 %) (Table 2) may explain the bias. Recently, Tero *et al*. (2003) detected a high correlation between Lynch–Milligan and Bayesian estimates for *Silene tatarica* with the percentage of polymorphic loci ranging from 25·9 % to 54·9 %, which is comparable with *I. sinensis*, but they sampled a larger number of individuals per population (24–30 individuals). This might reduce the statistical bias. In the present study, sampling a larger number of individuals was prohibited due to the status of the critically endangered species with extremely small numbers of adult plants available for sampling in several populations (populations XN1–5). Therefore, a certain degree of statistical bias for estimating average heterozygosity might be expected when using the Lynch–Milligan procedure under the assumption of Hardy–Weinberg equilibrium. On the other hand, estimating population differentiation resulted in nearly identical values; this should provide reasonable confidence that Bayesian estimates of heterozygosity obtained for *I. sinensis* are adequately accurate.

Estimating the genetic diversity of *I. sinensis* at the population level revealed relatively high genetic diversity in *I. sinensis* (P = 35·2 %, H_E = 0·118, hs = 0·147 and I = 0·192) in comparison with other available data in pteridophytes. Caplen and Werth (2000) reported very low intra-population diversity for six diploid *Isoetes* species in North America based on isozymes, with P ranging from 0·0 to 22·9 % and H_E ranging from 0·000 to 0·093. Similarly, a low percentage of polymorphic loci (P = 23·7 %) and Nei's heterozygosity (H_E) = 0·073 were found in *Sticherus flabellatus* populations using AFLP (Keiper and McConchie, 2000). In a survey of allozyme data in fern species, the value of P and H_E at the population level for 32 diploid homosporous fern species was 31·5 % and 0·110, respectively (Maki and Asada, 1998). The relatively high genetic diversity observed in *I. sinensis* may be attributed to its allopolyploid origin (He *et al*., 2002). High genetic diversity was also detected in our previous allozyme analysis (H_E = 0·189; P = 43·3 %; Chen *et al*., 2004). As far as is known, most genetic diversity studies based on AFLP analysis so far conducted were for seed plants, particularly endangered seed plants. For example, the intra-population heterozygosity in a critically endangered *Astragalus cremnophylax* var. *cremnophylax* ranged from 0·037 to 0·134 (Travis *et al*., 1996). However, Evans *et al*. (2001) found very high genetic diversity within populations in the rare plant *Banksia saxicola*, with the value of H_E ranging from 0·19 to 0·26. Recently, Tero *et al*., (2003) reported genetic diversity for an endangered perennial plant *Silene talarica*, ranging from 0·075 to 0·176 (Nei's estimate) and from 0·131 to 0·190 (Bayesian estimate), which were similar to these values detected for *I. sinensis* (Table 2). Apparently, *I. sinensis* maintains high levels of genetic diversity in spite of small population sizes. It is likely that the reduction in population size was a relatively recent event in the natural range of *I. sinensis*. This is consistent with existing documentation that the species was more widely distributed in eastern China previously, especially in Zhejiang province (Fig. 1). Many populations became extinct very recently, such as the DX and TT populations (Fig. 1; field survey data). This recent decline of populations may not yet have had sufficient time to result in a decrease of the intra-population genetic diversity in *I. sinensis.*

Many empirical studies have confirmed the theoretical prediction that smaller populations harbour lower genetic variation than larger populations due to genetic erosion (Frankham, 1996; Travis *et al*., 1996; Gaudeul *et al*., 2000). However, in the present study, no significant correlation was detected between population size and the amount of genetic variation within populations (Fig. 2A). Similar results were also found in other rare and endangered species (Schmidt and Jensen, 2000; Tero *et al*., 2003). The lack of correlation between population size and genetic variation has been interpreted to be a consequence of recent population decrease or expansion (Tero *et al*., 2003). Interestingly, the genetic variation was significantly correlated to population area (Fig. 2B), suggesting that within-population migration of propagules was related to the spatial scale of isolated wetland habitat patches. A similar result was found in small natural populations of *Calystegia collina* (Wolf *et al*., 2000) in which genetic variation was not correlated to the number of individuals in the population, but significantly correlated to population area. Seemingly, the genetic diversity within each population of *I. sinensis* has been highly influenced by changes in the size of wetland habitats, possible reflecting fluctuations in annual rainfall and anthropogenic activities such as farmland irrigation management and fertilization methods with downstream effects on water quality. The complex dynamics of wetland habitats could be the greatest threat to *I. sinensis*. In addition, Wen *et al*. (2003) found that water quality had deteriorated highly in the wetland habitat of *I. sinensis*, which contained higher concentration of nitrates, dissolved carbon dioxide and heavy metal ions.

Population structure

Population differentiation in *I. sinensis*, both at a regional scale and across its distribution range, was evident as measured by a range of different statistics (F_{ST}, G_{ST} and θ^B). The values of F_{ST} and G_{ST} differed slightly when different breeding systems were assumed (F_{ST} = 0·535 *vs*. G_{ST} = 0·608). Furthermore, the Bayesian estimate of θ^B = 0·607 is almost identical to Nei's G_{ST} = 0·608 when total selfing (F_{IS} = 1) was assumed for the species. The partitioning of genetic variation among populations has previously been reported in other *Isoetes* species. For example, the values of F_{ST} based on allozyme analysis for two diploid *Isoetes* species were 0·607 for *I. flaccida* and 0·947 for *I. engelmannii* (Caplen and Werth, 2000). Similar results have also been documented in other pteridophyte species (Schneller and Holderegger, 1996; Keiper and McConchie, 2000; Pryor *et al*., 2001). Using 469 AFLP markers, Keiper and McCondie (2000) reported a high mean F_{ST} estimate (0·783) among eight populations of the umbrella fern *Sticherus flabellatus*. High genetic differentiation was attributed to restricted gene flow, genetic drift and inbreeding (Loveless and Hamrick, 1984). Similar to other aquatic

Isoetes species, populations of *I. sinensis* are isolated from one another by their disjunct habitats, i.e. streams, hills and farmlands (Caplen and Werth, 2000). Dispersal of propagules can be achieved via transportation by water and occasionally by animals. Natural migration between the two populations in the Songyang region appears very limited since the populations are located on the opposing sides of the Anmin Mountain. During the 4-year field survey carried out for this study, no individuals were found in surrounding areas, which suggests current migration by human or animal activities is minimal. The populations in Xiuning region are much closer to each other, but they are also isolated by farmland and hills. The AMOVA analysis further suggested that a large proportion of genetic differentiation was attributed to geographic isolation (Table 4). Additional evidence from the UPGMA dendrogram revealed distinct clustering by geographic locations (Fig. 3), suggesting that gene flow was highly restricted between populations. As an aquatic quillwort, *I. sinensis* usually grows in damp sites that usually result in small clusters within each population. Therefore, the dispersal of this species is believed to be very limited and highly impacted by water flow regimes even at a microgeographical scale. This is in agreement with Taylor and Hickey (1992) who suggested that low levels of gene flow occur among *Isoetes* populations.

According to the theory of isolation by distance (Wright, 1943), when populations reach equilibrium between gene flow and genetic drift, a positive correlation should be detected between genetic distance and geographical distance. However, when populations are in disequilibrium, either gene flow or genetic drift can dominantly influence the population structure, and thus no correlation would be detected (Hutchison and Templeton, 1999). When the distance is not a limiting factor for the dispersal and gene flow is unlimited, populations would form a single uniform genetic unit, but when gene flow rate is greatly reduced, population genetic differentiation will increase due to genetic drift (Slatkin, 1977; Hutchison and Templeton, 1999). In the present study, the estimate of gene flow is very small ($N_m = 0{\cdot}378$), and no positive correlation between genetic distances (Φ_{ST}) and geographical distances was detected among the populations in Xiuning site, suggesting that genetic drift rather than gene flow played an important role in forming the present population structure. On the other hand, no significant genetic differentiation was found between population XN2 and XN3 ($\Phi_{ST} = 0{\cdot}093$) which might indicate adequate gene flow occurred between these two populations. This can be explained by their habitat situation: these two small populations were directly connected by a stream, although they were not the closest populations with respect to geographical distance (Fig. 1). This result confirms the assumption that hydrochory is the most important factor influencing gene flow among *I. sinensis* populations. A similar observation was also found in *Dryopteris cristata*, a fern species with high population differentiation and no significant correlation between genetic and geographical distances (Landergott *et al*., 2001).

Implications for conservation

It has been suggested that genetic variation is important for a species to maintain its evolutionary potential to cope with ever-changing environments. The information obtained in this study provides the first set of population genetic data to address conservation concerns for this critically endangered species. *Isoetes sinensis* populations appeared to maintain adequate genetic diversity in terms of neutral genetic variation. But in the absence of gene flow among populations and given the small population sizes, genetic drift might lead to a rapid genetic erosion and increase the extinction risk of local populations. An *in situ* conservation programme is urgently needed to mitigate further reduction of population size and deterioration of remnant habitats. Genetic reinforcement by translocation is an alternative measure to rehabilitate this species. However, considering the high differentiation among populations, the potential for inter-population outbreeding depression should be carefully investigated before such measures are implemented. If population differentiation was caused by different environmental adaptations, a mixture of individuals from genetically distinct populations may result in outbreeding depression (Hufford and Mazer, 2003). In the present study, since there are obvious ecological differences between Xiuning and Songyang regions, translocations between these two regions cannot currently be recommended. Alternatively, if population differentiation is caused by genetic drift, inter-population translocation will improve the performance of populations (Hufford and Mazer, 2003). Inter-population transplantations within regions would increase gene flow among populations and counteract the negative effects of genetic drift and inbreeding.

For *ex situ* conservation, the same caution should be taken to prevent mixture of individuals from different regions. Ideally, regional populations should be preserved separately in well-designed plots (ponds), while individuals from the same region need to be kept in the same plot (or pond) for genetic enhancement of outcrossing. A field sampling strategy should be developed based on the population genetic data so that sufficient genetic diversity of the extant populations could be maintained for the long-term survival of the species. Given the current situation of rapid decline of populations and natural habitats, it is strongly recommended that all extant populations are extensively sampled to maximize genomic representation of the species before further genetic erosion can occur.

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