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Original Article

Outcrossing Rates and Gene Flow in Natural Population of the Endangered Endemic Aquatic Lycophyte *Isoetes yunguiensis* as Revealed by ISSR Markers

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Abstract

In this study, mating system, genetic diversity, and genetic structure of the endangered endemic aquatic *Isoetes yunguiensis* in China was investigated using ISSR markers. The results of ISSR analyses showed that the estimate of multilocus outcrossing rate (t_m) was high at species level (t_m = 0.955), indicating that diploid *I. yunguiensis* is a predominant outcrossing species. Nine selected ISSR primers used in the study amplified 66 reproducible bands, 41 of which were polymorphic among 37 individuals. High level of genetic diversity was detected at the species level (PPB = 62.12%), whereas, relatively low genetic diversity existed within populations (PPB = 39.39%). Analysis of molecular variance (AMOVA) revealed that 31.99% of the genetic variation was attributable to differences between populations and the rest (68.01%) to variability within populationsof *I. yunguiensis.* Value of F_{st} (0.320) indicated that genetic differentiation between populations also was significant. These results showed that *I. yunguiensis* predominantly favors crossing, and has a high level of genetic diversity and highly significant genetic variation between and within populations. Gene flow (Nm) among populations is equal to 1.177. High outcrossing rates may be responsible for the high levels of genetic diversity observed in the *I. yunguiensis* population. To maintain the current level of genetic diversity for this species, we recommend increasing *in situ* conservation sites.

Keywords: endangered; genetic structure; ISSR; *Isoetes yunguiensis;* outcrossing rate

Introduction

Plant breeding systems determine gene flow, the genetic structure of populations, and the evolutionary potential of a species (Korpelainen, 1995). An accurate characterization of mating system is important for the conservation of the evolutionary potential of natural populations because it allows the outlining of strategies that optimize the sampling of genetic variability and the adoption of genetic-statistical models appropriate for the estimation of genetic parameters (Cánovas *et al*., 2015).

Isoetes L., the single remaining member of the family *Isoetaceae*, is a cosmopolitan genus of heterosporous lycopods comprising 200 or more species, and occupies a very important position in the evolutionary history of the pteridophytes (Hoot *et al*., 2001). Five species of *Isoetes* including *I. hypsophila*, *I. yunguiensis*, *I. taiwanensis*, *I. orientalis* and *I. sinensis* occur in China (Liu *et al*., 2005). *Isoetes yunguiensis* is an endangered and endemic aquatic fern in China, and is a basic diploid with a chromosome number $2n$ = 22 (Wang *et al*., 2002). The species is a perennial distributed in ponds in riverside meadows and marshes at elevations of 1200-2200 m in the Yunnan-Guizhou Plateau in southwest China. In recent years, *I. yunguiensis* has declined rapidly in the number and size of populations due to the impact of human activities. Pang *et al*. (2003) reported that five *I. yunguiensis* populations such as Heilongtan, Songhuaba, Xiaoshao, Shuangshao and Xuandian populations in Kunming City, Yunnan Province

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based on specimen records may become extinct during the field investigation (Fig.1, Table 1). In field investigation, two populations, in Pingba County and Hongfenghu Lake, Guizhou Province, were found in China (Dong *et al*., 2018a). The species is now considered to be rare and threatened or endangered and is listed among first category of the Key Protected Wild Plants in China (Yu, 1999). Most of the previous studies of *I. yunguiensis* have mainly focus on its morphology, taxonomy, cytology, ecology and reproductive development (Wang *et al*., 2002; Pang *et al*., 2003; Chen *et al*., 2007; Li *et al*., 2015). Using RAPD markers, Chen *et al*. (2005a) reported high level of genetic diversity (Percentage of polymorphic bands, PPB = 62.1%) from a remaining *I. yunguiensis* population in Pingba County, Guizhou Province, China. However, little is known about mating system, and population genetic structure of *I. yunguiensis*.

Mating systems of plants are commonly characterized by outcrossing rates. These estimates can be generated by examining allelic variation over many loci in progeny arrays from plants allowed to pollinate naturally, using a maximum-likelihood model to exclude progeny resulting from self-fertilization (Ritland and Jain, 1981). Outcrossing rates in plant populations have often performed using co-dominant markers such as SSR, allozyme (Chen *et al*., 2009; Suwarni *et al*., 2018). However, in the absence of sufficient allozyme polymorphism, several dominant markers such as RAPD (Gaiotto *et al*., 1997; Dong *et al*., 2006), ISSR (Han *et al*., 2009; Fernando *et al*., 2015; Saki *et al*., 2016), and AFLP (Gaiotto *et al*., 1997; Pometti *et al*., 2013., Zhao *et al*., 2014; Dong *et al*., 2018a; 2018b) have recently been used to estimate outcrossing rates in plant species.

Due to their dominant behaviour, these markers provide less information per locus than co-dominant markers. This is particularly relevant for applications that require genotype discrimination, as in the case of outcrossing-rate estimation. Through simulation studies, Ritland and Jain (1981) demonstrated, however, that this limitation could be readily overcome by multilocus estimation using a large number of dominant markers with intermediate gene frequencies.

In recent years, inter-simple sequence repeat (ISSR) have also been utilized as a tool in the study of conservation genetics and outcrossing rates (Han *et al*., 2009; Saki *et al*., 2016). ISSR is a technique that uses repeat-anchored primers to amplify DNA sequences between two-inverted SSR (Zietkiewicz *et al*., 1994). The technique does not require prior knowledge of the DNA sequence for primer design, and has advantages similar to those of RAPDs (Esselman *et al*., 1999). Furthermore, they are highly reproducible due to their primer length and to the high stringency achieved by the annealing temperature. Additionally, given the abundance of microsatellites sequences it is possible to analyze a large number of loci, giving high possibilities of finding polymorphisms, even in highly related genotypes (Carrasco *et al*., 2009). ISSR markers have been found to provide a much larger number of polymorphic fragments per primer than does RAPD and allozyme (Zietkiewicz *et al*., 1994; Han *et al*., 2009; Cheng *et al*., 2018). The objectives of our research was to use ISSR markers to evaluate outcrossing rates, and population genetic structure in natural populations of *I. yunguiensis* in China with the aim of providing baseline genetic information pertinent to the conservation and restoration of this endangered fern species.

Table 1. Geographic distribution, location, habitat, and sample size of *Isoetes yunguiensis* populations studied

Population code	Extant/ extinct population	Locality	Latitude/Longitude (N/E)	Altitude (m)	Habitats	Population size	Population area (m^2)	Sample size	Vouchers/ References
PB	Extant	Pingba, Guizhou	26°25′/106°17′	1365	Valley swamp	40-50	50-60	19	HCAS 75043/the present study
QHH	Extant	Hongfenghu, Guizhou	26°29′/106°24′	1247	Valley swamp	40-45	45-50	18	The present study
KM1	Extinct	Heilongtan, Kunming City,Yunnan	25°02′/102°42′	2000	Pond				KUN 0002883/ Pang *et al*., 2003
KM2	Extinct	Songhuaba, Kunming City, Yunnan	25°02′/102°42′	2000	Reservoir				KUN 0002885/ Pang *et al*., 2003
KM3	Extinct	Xiaoshao, Kunming City, Yunnan	25°02′/102°42′	2000	Rice-field stream				KUN 0002888/ Pang *et al*., 2003
KM4	Extinct	Shuangshao, Kunming City, Yunnan	25°02′/102°42′	2160	Rice-field stream				KUN 0002886/ Pang *et al*., 2003
XD1	Extinct	Xuandian, Kunming City, Yunnan	25°56′/103°25′	2080	Rice-field stream				KUN 65680/ Pang *et al*., 2003

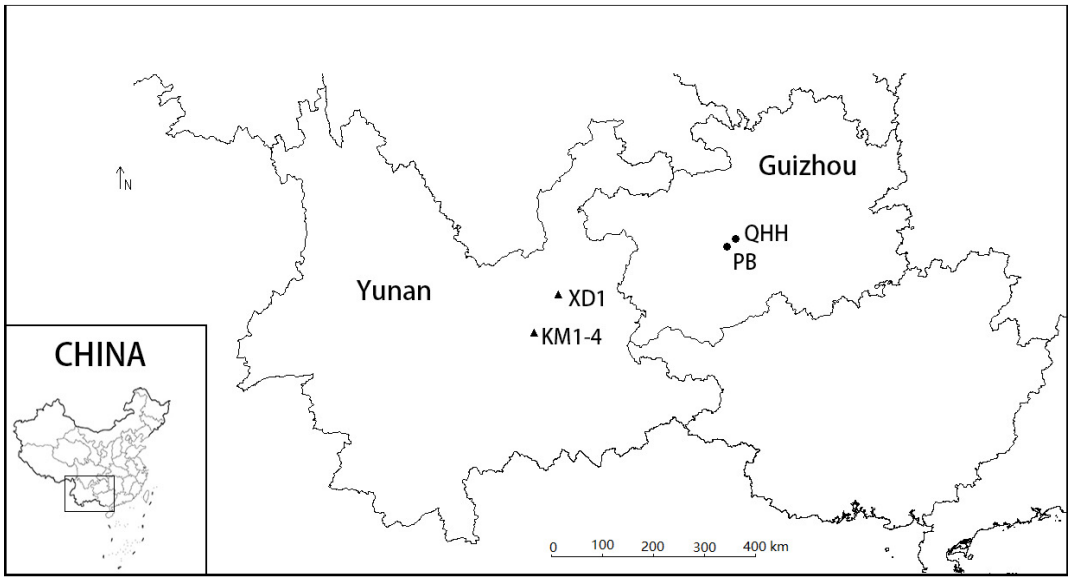

Fig. 1. Distribution map of *Isoetes yunguiensis* populations sampled in the present study. ● Sites of extant populations. ▲ Sites of extinct populations (see Pang *et al*., 2003). Codes correspond to populations in Table 1

Materials and Methods

Plant materials and total DNA extraction

From 2003 to 2015, the historic geographic distributions of *I. yunguiensis* were investigated in China based on specimen records and observations made. The two extant populations (designated as PB, QHH) be found and located in Guizhou Province in China during the field surveys (Fig. 1), and were small, containing fewer than 50 individuals (Table 1). At each sampling site, latitude, longitude, and elevation were measured by Global Positioning System (GPS). The habitat characteristics of *I. yunguiensis* populations were studied by collecting (Table 1). The population characteristics of the species including population numbers and population area, population sizes (numbers of individuals) were investigated (Table 1). Because of the two populations are small populations and in order to minimize effect on these populations, each population has been collected only 18-19 samples. Individuals in each study population were sampled at a minimum distance of 1 m from one another. A total of 37 individuals from the two remaining populations of *I. yunguiensi* in this study were collected. 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 0.3 to 0.5 g of silica-dried leaf tissue following the procedure described by Doyle and Doyle (1987). The DNA concentration of each sample was determined with an Eppendorf BioPhotometer plus (Eppendorf AG, Hamburg, Germany).

ISSR PCR amplification

A total of eighty primers from SBS Genetech Co. Ltd. (Shanghai, China) were tested for PCR. Those that produced reproducible, clear, polymorphic electrophoretic bands were selected. PCR reactions were performed in a PTC-100^{TM} thermocycler (MJ Research, Inc.) using the following temperature cycle profile: an initial melting step at 94 °C for 5 min, followed by 35 cycles 94 °C for 30 s, 55 °C for 45 s, 72 °C for 1.5 min, and a finally 7 min at 72 °C for final extension. Reactions were carried out in a volume of 25 μl, containing 0.25 mM of each dNTP, 25 mM $MgCl_2$, 1.5 mM primer, 2.5 U Taq polymerase and 4 μl (10 ng) of DNA template. PCR products were electrophoresed on 1.5% agarose gels stained with ethidium bromide, visualized under ultraviolet light, and photographed. Sizes of amplification products were estimated using a DL 2000 bp DNA ladder. Eighty ISSR primers were screened on four randomly selected individuals. Nine primers that could produce clear and reproducible fragments were chose for further analysis (Table 2).

Table 2. Primers used in the ISSR study

Primer	Sequence (5'-3')
SBS 816	$(CA)_8T$
SBS 834	$(AG)_8$ (C/G)T
SBS 836	$(AG)_8$ (C/G)A
SBS 840	$(GA)_8$(C/G)T
SBS 841	$(GA)_8$(C/G) C
SBS 842	$(GA)_8$(C/G) G
SBS 845	$(CT)_8$(A/T)G
SBS 858	$(TG)_8$(A/T)G
SBS 880	$(GGAGA)_3$

Data analysis

Mating system

According to the molecular weight (bp), all individuals were scored for the presence (1) or absence (0) of the amplified ISSR fragments, and the data matrix of the ISSR phenotypes was constructed for further analysis. The programme MLTR 3.4 (Ritland, 2009) is based on the multilocus mixed-mating model and the estimation procedure of Ritland and Jain (1981) which assumes that progeny are derived from either random mating (outcrossing) or self-fertilization. Using the software MLTR 3.4 (Ritland, 2009), the following mating system parameters were calculated: the estimate of multilocus outcrossing rate (t_m, a maximum likelihood estimate of t_m=1.2) and single locus outcrossing rate (t_s), correlation of outcrossed paternity (rp), correlation of t_m among progeny arrays (rt), and fixation index of maternal parents (F). The biparental inbreeding rate was also estimated following Ritland (1990) as t_m - t_s. The standard errors for these parameters were calculated from 1000 bootstraps with resampling of individuals within families. Standard error was used to determine whether mating parameters were significantly lower than one or greater than zero.

Genetic diversity and gene flow

Genetic diversity was measured by the percentage of polymorphic bands (PPB), which was calculated by dividing the number of polymorphic bands at population, and species levels by the total number of bands surveyed. PPB, the Shannon index of diversity *(I)*, and the gene diversity index *(H)* were calculated to evaluate genetic diversity. All calculations were estimated using POPGENE program Version 1.32 (Yeh *et al*., 2000).

Genetic structure

Genetic variation within and among populations was further partitioned by analysis of molecular variance (AMOVA) using ARLEQUIN 3.5.2 (Excoffier and Lischer, 2015). The AMOVA-based estimate of population genetic differentiation between two populations (Fixation index, F_{ST}) was calculated. 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). Significance tests were made after 1000 permutations.

Results and Discussion

Mating system analysis

The estimate of multilocus outcrossing rates (t_m) and single locus outcrossing rates (t_s) were higher in species level (t_m= 0.955, t_s= 0.953, respectively) and all populations (PB population: t_m= 1.200, t_s= 1.164; QHH population: t_m= 1.200, t_s= 1.160, respectively), indicating that *I. yunguiensis* studied is mostly outcrosser (Table 3). The difference between t_m and t_s (0.002) in species level was insignificant, suggesting that biparental inbreeding was negligible (Ritland, 1990), indicating that there is a low tendency for mating between relatives (Table 3). The correlation of t_m within progeny arrays (rt) was low in all populations (PB population: -0.315 ± 0.185; QHH population: -0.999 ± 0.164, respectively), suggesting no differences in outcrossing rates among mother plants (Table 3). The low F –value (0.082) of the maternal parents suggested that there were an excess of heterozygotes and less inbreeding in the progeny population analyzed (Table 3).

The equivalent to crossing in higher plants involves crosses between gametophytes produced by spores from different sporophytes, termed intergametophytic crossing or xenogamy (Hickok *et al*., 1995). The reproductive structure and characteristics of ferns not only have a stable systematic significance, but also determine the reproductive mode and reproductive function (Wu and Qin, 1991). *I. yunguiensis* is a heterosporous fern, which can produce two kinds of different spore namely microspores and megaspores, and produce male gametophyte and female gametophyte respectively (Li *et al*., 2015). The reproductive structure and characteristics of *I. yunguiensis* determine that sexual reproduction model of *I. yunguiensis* is outcrossing by combining of sperm and egg cells from different sporophytes and gametophytes.

Genetic diversity and genetic structure

The nine selected primers generated a total of 66 bands (an average of 7.4 bands/primer). A total of 41 bands were polymorphic among 37 individuals (Table 4). The percentage of polymorphic bands (PPB) for this species was 62.12%, indicating high levels of genetic diversity at the species level. Nei's unbiased genetic identity (*H*) and the Shannon' information index (*I*) also showed a similar pattern of the genetic differentiation at the species level (*H* = 0.239; *I* = 0.354, respectively). Within populations, ISSR diversity (PPB= 39.39%) was lower than at the species level (Table 4). The results of ISSR diversity analysis among populations and within populations of *I. yunguiensis* in China obtained in the study were similar to those reported in previous studies on other fern species. For example, Chen *et al*. (2006) revealed 51.02% ISSR genetic diversity between populations and within populations (an average of PPB: ISSR, 16.32%) of the endangered aquatic fern *I. sinensis* in China based on ISSR data. Chen *et al*. (2005b) using RAPD and ISSR also reported high genetic diversity at the species level of the endangered aquatic fern *I. hypsophila* in China (PPB: RAPD 50.0%, ISSR 82%, respectively) and low genetic diversity among populations with PPB values ranging from 7.69 to 25.96% (RAPD), from 8 to 35% (ISSR), respectively. Using AFLP, Kang *et al*. (2005) revealed high genetic diversity (61.2%) among population levels of *I. sinensis*. Results of ISSR diversity analysis of *I. yunguiensis* in China obtained in this study were higher than those obtained for other rare and endangered fern species. For instance, Chen *et al. (2010a)* using AFLP data revealed low genetic diversity among population levels of *I. hypsophila*, an endangered alpine quillwort fern in China (PPB: 48.5%). Using AFLP, Kim *et al*. (2009) reported low genetic diversity (PPB) of six endangered *Isoetes* species from East Asia including *I. taiwanensis* (33.1-38.3%), *I. asiatica* (49.0%), *I. jejuensis*

(9.3-29.3%), *I. hallasanensis* (22.3%), *I. coreana* (1.6-20.6%), and *I. japonica* (5.6-20.5%). A low level of gene diversity was also found at the population level in *C. pteridoides* based on ISSR, RAPD and AFLP markers (PPB: RAPD 33.6%, ISSR 44.8%, AFLP 17.4%, respectively) (Dong *et al*. 2007, 2010; Chen *et al*., 2010b).

Among several life-history traits, the breeding system is considered to be the most important to determine the level of genetic variability and its distribution in populations of plant species (Kim *et al*., 2009). In general, outcrossing species usually have higher levels of genetic diversity and lower differentiation between populations than selfing and clonal plants (Holsinger, 2000). Results of Peredo *et al*. (2013) also confirm the importance of reproduction system in the genetic diversity present in populations of some fern species such as *Blechnum spicant* and *Dryopteris affinis* ssp. *affini*. The results indicated that *I. yunguiensis* predominantly favour gametophytic crossing (t_m = 0.955) (Table 3). Therefore, it is probable that high outcrossing rate of this species may have played an important role in maintaining the generally high level of interpopulation genetic diversity in the endangered *I. yunguiensis*.

AMOVA of ISSR data revealed that 31.99% of the total genetic variation was attributable to among populations and 68.01% partitioned among individuals within populations of *I. yunguiensis*, indicating variation existed mainly within populations rather than among populations (Table 5). Generally, values of F_{ST} above 0.25 indicate very great genetic differentiation (Wright, 1978). In this study, the AMOVA-based estimate of population differentiation between two *I. yunguiensis* populations was F_{ST} (Fixation index) = 0.320, indicating genetic differentiation between populations (F_{ST}) was significant. Thus, the results indicated that there is almost always more variation explained among individuals within populations in all AMOVA analyses and this is not preclude there being strong and significant variation among/between populations. According to the Nei-Li genetic similarity of 0.80, a UPGMA cluster analysis of 37 individuals indicated that samples form two groups, one formed by the population QHH and other by the population PB, what the UPGMA tree has is only four QHH samples in the PB group (Fig. 2). The result of UPGMA cluster analysis further indicated that there was salient genetic differentiation between the two extant populations in China.

In the study, ISSR data showed that interpopulational gene flow (Nm) of *I. yunguiensis* was 1.177.

The observed level of gene flow was lower than that in the endangered aquatic *Isoetes* in China, such as *I. sinensis* (Allozyme analysis: Nm = 4.51; Chen *et al*., 2004). High gene flow in some diploid homosporous ferns, including *Polystichum munitum* (Nm = 24.00), *P. acrostichoides* (Nm = 12.69), *P. dudleyi* (Nm = 10.78), and *P. imbricans* (Nm = 2.2), was also observed (Soltis and Soltis, 1990). The results show that gene flow between populations of *I. yunguiensis* is quite restricted. In plants, gene flow is occasioned by movement of pollen, seeds, spores, and propagules (Orive and Asmussen, 2000). Dispersal of *Isoetes* spores is often accomplished via floating leaves (Small and Hickey, 1997). A survey indicates that young plants of *Isoetes* in China are swept away from the headstream on mountains to lower reaches of a river by floods (Liu *et al*., 2004). Habitat fragmentation and isolation of population may limit gene flow, the result are increased inbreeding and genetic drift, and leading to decreasing genetic diversity, resulting in population differentiation (Primack, 1993; Li and Jin, 2008). Field survey found that the distance of two *I. yunguiensis* populations is about 15 km, and have been isolated (Fig. 1). The isolation of population resulted in the limitation of spore dispersal, which reduced gene flow. Chen *et al*. (2005b, 2010a) also suggested that habitat isolation could have greatly limited gene flow between *I. hypsophila* populations from different geographical regions, hence increasing the interpopulation differentiation of *I. hypsophila*. Yang *et al*. (2011) revealed a high level of gene flow via spore dispersal (Nm = 16.66) in neighbor *ex-situ Isoetes* subpopulations alone main water flow and low genetic differentiation among conservation subpopulations (G_{st} = 0.07). Furthermore, Li *et al*. (2015) reported that the swimming speed of *I. yunguiensis* sperm is 79 μm /s and its life time is only 11 min, indicating long -distance movement of sperm among populations may be restricted. Therefore, spore dispersal and swimming sperm of *I. yunguiensis* is likely to be restricted between populations, and might have reduced gene flow. It is likely that the restricted gene flow between the populations in *I. yunguiensis* may have played an important role in determining the genetic differentiation of *I. yunguiensis* populations.

Because we are dealing with an endangered species and restricted population sizes at two sites, the sample sizes in the populations varied in the range 18-19; this may have led to bias in some statistical analyses of the data.

Table 3. Mating system parameter of *Isoetes yunguiensis* populations studied in China

Parameter	PB	QHH	Species level
t_m	1.200 (0.064)	1.200 (0.035)	0.955 (0.105)
t_s	1.164 (0.051)	1.160 (0.034)	0.953 (0.062)
$t_m - t_s$	0.036 (0.045)	0.040 (0.033)	0.002 (0.057)
rp(m)	-0.142 (0.300)	0.000 (0.350)	0.279 (0.034)
rp(s)	-0.250 (0.176)	-0.332 (0.127)	-0.047 (0.108)
rp(s)– rp(m)	-0.108 (0.317)	-0.332 (0.329)	-0.076 (0.078)
rt	-0.315 (0.185)	-0.999 (0.164)	-0.999 (0.000)
F	-0.200 (0.029)	-0.200 (0.019)	0.082 (0.141)

t_m, Multilocus outcrossing rate; t_s, Single-locus outcrossing rate; t_m-t_s, Difference of outcrossing rate or biparental inbreeding; rp(m), The multilocus correlation of paternity; rp(s), The single-locus correlation of paternity; rp(s)–rp(m), Parents correlation; rt, Correlation of t (or s) estimate; F, Inbreeding coefficient of the maternal parents; Numbers in parentheses are standard deviations (SD).

Table 4. Genetic diversity of two *Isoetes yunguiensis* populations studied in China

Population code	Number of polymorphic bands	PPB (%)	Na	Ne	*H*	*I*
PB	26	39.39	1.394 (0.492)	1.300 (0.405)	0.165 (0.216)	0.237 (0.308)
QHH	26	39.39	1.394 (0.492)	1.311 (0.407)	0.171 (0.218)	0.246 (0.311)
Species level	41	62.12	1.621 (0.488)	1.410 (0.372)	0.239 (0.200)	0.354 (0.289)

PPB, Percentage of polymorphic bands; Na, Observed number of alleles; Ne, Effective number of alleles; *H*, Nei's gene diversity index; *I*, Shannon's information index; Numbers in parentheses are standard deviations.

Table 5. Analysis of molecular variance (AMOVA) for ISSR data of 37 individuals from two populations of *Isoetes yunguiensis*

Source of variation	d.f.	SSD	Variance component	Percentage of variation (%)	*P* *
Among populations	1	43.742	2.122	31.99	<0.001
Within population	35	157.880	4.511	68.01	<0.001
Total level	36	201.622	6.633		

d.f., degree of freedom; SSD, sum of squared deviation; *Statistical significance is based on 1000 permutations.

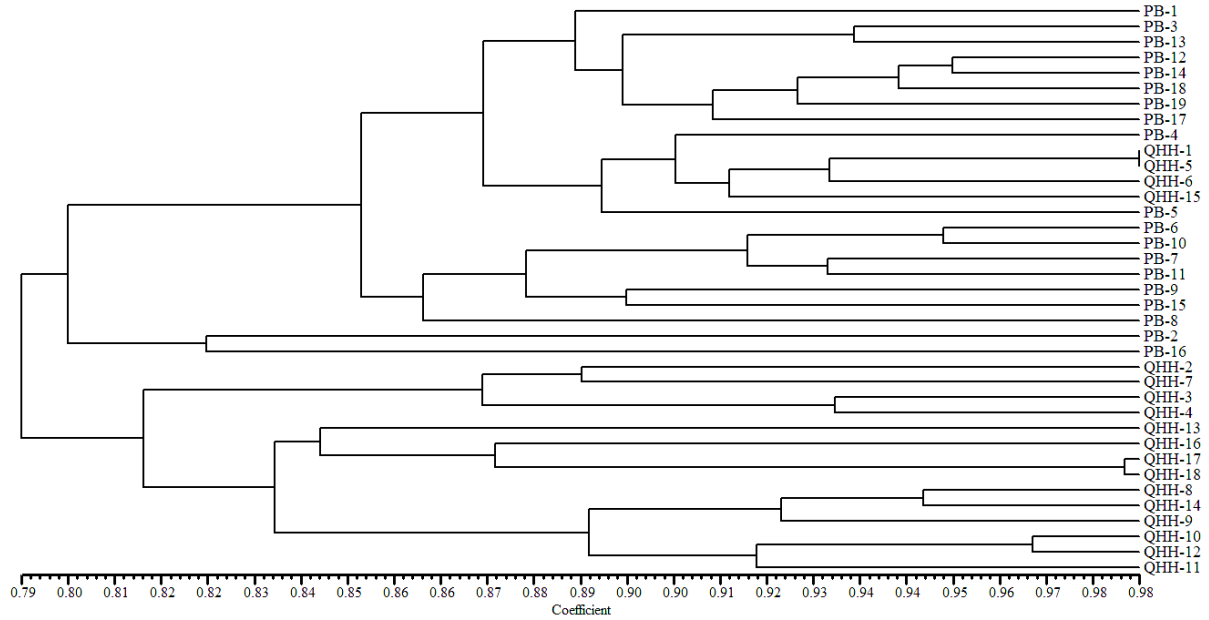

Fig. 2. UPGMA dendrogram of 37 individuals of *Isoetes yunguiensis* based on ISSR data. The first two or three letters represent the population code, and the number is the individual from each population

Implications for conservation

Habitat preservation is one of the most effective measures of conservation of species (Primack, 1993). The accelerated loss of habitat of *I. yunguiensis* in China puts the species at a risk of becoming extirpated (Pang *et al*., 2003; Chen *et al*., 2005a). The rapid reduction in numbers and sizes of *I. yunguiensis* populations in China is probably associated with human actives, contributing to the deterioration and loss of primary habitats (Pang *et al*., 2003). It is therefore, an important conservation strategy to protect more of the habitats of the remaining *I. yunguiensis* populations. It is worthy of notice that PB population have been protected by establishment of nature reserves at their locations. The *in situ* conservation was supported by grants from World Wide Fund for Nature or World Wildlife Fund (WWF) in 2007. In addition to habitat preservation, a key aim of conservation is to maintain a species' existing level of genetic variation in order to maximize its chances for persistence in the face of changing environments. Maintenance of genetic diversity is a major focus in conservation biology because it is important for a species to maintain its evolutionary potential to cope with an ever-changing environment (Chen *et al*., 2010a). Choice of *in situ* sites and the appropriate *ex situ* conservation strategies require adequate genetical data on the species to be conserved. The genetic information obtained in this study should help to provide a clear framework for developing a conservation program for the threatened species *I. yunguiensis*. The low level of genetic diversity within populations of *I. yunguiensis* indicated that efforts should try to preserve every existing population. However, both the extant PB population and QHH population was small population, with the continuing decrease of numbers and sizes of populations, the genetic diversity will gradually be lost. Therefore, we suggest that the materials from the extant population should be used for re-establishment of the populations.

A good knowledge of mating system of *I. yunguiensis* will provide critical base-line information for developing sustainable management strategies. Based on the results of

mating system of the species, we suggest that its conservation and restoration genetics should particularly also focus on the maintenance of historically significant processes such as high levels of outbreeding, and mixing more individuals from different populations in *ex situ* conservation, and minimizing inbreeding and enhancing gene flow in order to preserve the greatest extent of genetic resources within the species.

Conclusions

Our results revealed that *I. yunguiens*is predominantly favors crossing, and has a high level of genetic diversity and highly significant genetic variation between and within populations. Facts that may affect the genetic structure of the species include the high outcrossing rates, gene flow, spore dispersal and swimming sperms. The extant *I. yunguiensis* populations in China should be a priority for *in situ* conservation.

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