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 populations of *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).

*Ioletes* L., the single remaining member of the family *Isoetaceae*, is a cosmopolitan genus of heterosporous lycophods 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. hypophila*, *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...
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 (m²) | 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                   | Heilongan, 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                   | Shuanghao, Kunming City, Yunnan | 25°02'/102°42" | 2160         | Rice-field stream |                |                    |             | KUN 0002886/ Pang et al., 2003 |
| XD1            | Extinct                   | Xuanzhan, Kunming City, Yunnan | 25°56'/103°25" | 2080         | Rice-field stream |                |                    |             | KUN 056880/ Pang et al., 2003 |

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.

Ma M et al / Not Bot Horti Agrobo, 2019, 47(2):339-346
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) were 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. yunguiensis* 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 silicadried 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™ 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 final 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)$_8$T |
| 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)$_3$ |

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.
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 \( t_o \) (a maximum likelihood estimate of \( t_o = 1.2 \)) and single locus outcrossing rate \( t_s \), correlation of outcrossed progeny arrays \( r_p \), correlation of \( t_o \) among progeny arrays \( r_t \), and fixation index of maternal parents \( F_s \). The biparental inbreeding rate was also estimated following Ritland (1990) as \( F_s = t_o - t_s \). 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 NTSSYSpC ver. 2.02 (Rohlf, 1998). The dendrogram (UPGMA) of all individuals was computed using the unweighted pair-group method with an arithmetic average using NTSSYSpC ver. 2.02 (Rohlf, 1998). Significance tests were made after 1000 permutations.

Results and Discussion

Mating system analysis

The estimate of multilocus outcrossing rates \( t_o \) and single locus outcrossing rates \( t_s \) were higher in species level \( t_o = 0.955, t_s = 0.953 \), respectively, and all populations (PB population: \( t_o = 1.200, t_s = 1.164 \); QHH population: \( t_o = 1.200, t_s = 1.160 \), respectively), indicating that \( I. yunguensis \) studied is mostly outcrosser (Table 3). The difference between \( t_o \) 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 \( t_o \) within progeny arrays \( r_t \) was low in all populations (PB population: \( -0.315 \pm 0.185 \); QHH population: \( 0.999 \pm 0.164 \), respectively), suggesting no differences in outcrossing rates among mother plants (Table 3). The low \( F_s \)-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). \( I. yunguensis \) 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 \( I. yunguensis \) determine that sexual reproduction model of \( I. yunguensis \) 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 \( (H = 0.239; I = 0.354) \). 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 \( I. yunguensis \) 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 \( I. hypophila \) in China (PPB: RAPD 50.0%, ISSR 82%, respectively) and low genetic diversity among populations with PPB values ranging from 7.69 to 25.95% (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 \( I. yunguensis \) 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 \( I. hypophila \), 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%), \( I. jejuensis \) (33.1-38.3%), and \( I. issatiae \) (49.0%).
(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. affinis. 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 (\( N_m \)) 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: \( N_m = 4.51 \); Chen et al., 2004). High gene flow in some diploid homosporous ferns, including Polystichum minutum (\( N_m = 24.00 \)), P. acrostichoides (\( N_m = 12.69 \)), P. dudleyi (\( N_m = 10.78 \)), and P. imbricata (\( N_m = 2.2 \)), was also observed (Solís and Solís, 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. hypophila populations from different geographical regions, hence increasing the interpopulation differentiation of I. hypophila. Yang et al. (2011) revealed a high level of gene flow via spore dispersal (\( N_m = 16.66 \)) in neighbor ex-situ Isoetes subpopulations alone main water flow and low genetic differentiation among conservation subpopulations (\( G_a = 0.07 \)). 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 |
|-----------------|-------------|-------------|---------------|
| \( t_a \)       | 1.200 (0.064) | 1.200 (0.035) | 0.955 (0.105) |
| \( t \)         | 1.160 (0.054) | 1.160 (0.034) | 0.953 (0.062) |
| \( t_a-t \)     | 0.056 (0.045) | 0.040 (0.035) | 0.002 (0.057) |
| \( r_p(s) \)    | -0.142 (0.300) | 0.000 (0.350) | 0.279 (0.034) |
| \( r_p(s) \)    | -0.250 (0.176) | -0.332 (0.127) | -0.047 (0.108) |
| \( r_p(m) \)    | -0.108 (0.317) | -0.332 (0.329) | -0.075 (0.078) |
| \( r_t \)       | -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) |

Note: \( t_a \): Multilocus outcrossing rate; \( t \): Single-locus outcrossing rate; \( t_a-t \): Difference of outcrossing rate or biparental inbreeding; \( r_p(s) \): The multilocus correlation of paternity; \( r_p(m) \): The single-locus correlation of paternity; \( r_p(s)-r_p(m) \): Parents correlation; \( r_t \): Correlation of \( t \) (or \( s \)) estimate; \( F \): Inbreeding coefficient of the maternal parents. Numbers in parentheses are standard deviations (SD).
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. yunguiensis* 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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