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RESEARCH ARTICLE

Genetic diversity in *Isoetes yunguiensis*, a rare and endangered endemic fern in China

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Abstract *Isoetes yunguiensis* is an endangered and endemic fern in China. Field survey indicated that only one population and no more than 50 individuals occur in the wild. The genetic variation of 46 individuals from the population remaining at Pingba (Guizhou Province, China) was assessed by Random Amplified Polymorphic DNA (RAPD) fingerprinting. Twelve primers were screened from sixty ten-bp arbitrary primers, and a total of 95 DNA fragments were scored. Of these, 62.1% were polymorphic loci, which indicated that high level genetic variation existed in the natural population. The accumulation of genetic variation in the history of the taxon and the apparent minimal reduction effect on genetic diversity following destruction of habitat might be responsible for the high level genetic diversity presently remaining in the *I. yunguiensis* population. However, with the continuing decrease of population size, the genetic diversity will gradually be lost. We suggest that the materials from the extant population should be used for re-establishment of the populations.

Keywords endemic fern, genetic diversity, *Isoetes*, random amplified polymorphic DNA (RAPD), rare and endangered

1 Introduction

Isoetes L. is a cosmopolitan genus of heterosporous Isoetaceae comprising about 200 species (Hoot and Taylor, 2001). Four species, *Isoetes yunguiensis*, *I. hypsophila*, *I. taiwanensis* and the East Asia endemic species *I. sinensis* have been reported in China.

Isoetes yunguiensis had been previously misidentified as *I. japonica*. *I. yunguiensis* is similar to *I. japonica* in external appearance and both species have megaspores with reticulate texture (Liu et al., 2002). Closer inspection of the specimens shows that *I. yunguiensis* is distinct from *I. japonica* in spore morphology (microspores of *I. yunguiensis* are smaller than those of *I. japonica*) and chromosome number (*I. yunguiensis* is a basic diploid with a chromosome number 2n = 22 while *I. japonica* 2n = 66, 67, 77, 87, 88, 89) (Liu et al., 2002). Based on these data, Wang et al., (2002) regarded the *Isoetes* plants distributed in the Yunnan- Guizhou Plateau in southwest China as a distinct, previously undescribed species of *Isoetes,* which they named *Isoetes yunguiensis* Wang Q. F. & W. C. Taylor. *I. yunguiensis* is a perennial distributed in ponds in riverside meadows and marshes at elevations of 1200–2200 m in Guizhou and Yunnan Provinces, China. *I. yunguiensis* in China has previously been reported in Kunming City and Xundian County of Yunnan Province and Pingba County and Guiyang City of Guizhou Province (Pang et al., 2003). In our recent field investigation, only one population, in Pingba County, Guizhou Province, was found in the whole China. The species is presently considered to be rare and threatened or endangered in China and is listed in the first category of the key protected wild plants.

Plant population genetic structures are determined by several factors, such as evolutionary history, distribution range and reproductive systems. A species without an appropriate amount of genetic diversity is thought to be unable to cope with changing environments or evolving competitors and parasites. Therefore, investigations of population genetic diversity and the population structure within a species may not only illustrate the evolutionary process and mechanism but also provide information useful for biological conservation (Ge and Hong, 1994; Ge, 1997; Meffe and Carroll, 1997). However, information on the genetic diversity of *I. yunguiensis* is still missing.

The Random Amplified Polymorphic DNA (RAPD) technique has several advantages over isozyme and other DNA marker methodologies, such as speed, low cost, and the use of small amounts of plant material. This technique has been

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widely used in investigating population genetic variation (Williams et al., 1990; Madeira et al., 1997; Qian et al., 2001; Angel, 2002). The principal goal of this study was to assess genetic variation among accessions of *I. yunguiensis* using RAPD markers, which may facilitate the conservation management for this species.

2 Materials and methods

2.1 Plant material

In January 2002, the extant population of *I. yunguiensis* in Pingba County, Guizhou Province, China was sampled. Field survey indicated that no more than 50 individuals were left in the wild. A total of 46 individuals were included in the study. About 5 g fresh leaves was harvested from each plant and immediately dried in a ziplock plastic bag containing about 70 g silica gels. The samples were stored at room temperature (25°C) until DNA was isolated in the laboratory.

2.2 Total DNA extraction and PCR amplification

Total genomic DNA was isolated from 0.5 g silica-dried leaf tissue using a modification of the CTAB extraction procedure (Doyle and Doyle, 1987). PCR reactions were carried out on a PTC-100 thermocycler (MJ Research). The PCR reaction systems and amplification procedure followed the methods described by Chen et al. (2004).

PCR amplification products were electrophoretically resolved on 1.5% agelose gels run at 100 V in 0.5 × TBE (Tris-boric acid-EDTA) buffer, visualized by staining with ethidium bromide, and photographed under ultraviolet light. Molecular weights were estimated using a 200 bp DNA ladder (Tian Yuan Biotech. Co. Ltd.).

2.3 Primer screening

Sixty ten-bp arbitrary RAPD primers were screened on eight samples of *I. yunguiensis* for unambiguous, clear and reproducible banding patterns. Twelve primers were selected for further analysis (Table 1).

2.4 Data analysis

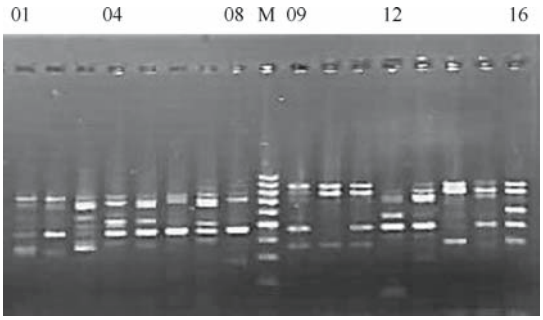
Amplified fragments were scored for the presence (1) or absence (0) of homologous bands. Genetic diversity was measured as the percentage of polymorphic loci (*PPL*), the Nei's gene diversity (*H*) (Nei, 1978) and as the Shannon index of diversity (*I*) (Lewontin, 1972) using POPGENE program 1.31 (Yeh et al. 1997).

3 Results and discussion

Sixty RAPD primers were screened on eight randomly selected individuals. Twelve primers that produced clear and reproducible fragments were selected for further analysis (Table 1). These primers generated a total of 95 bands (an average of 7.9 bands per primer) with fragments ranging in size from 200 to 2000 bp (Fig. 1). A total of 95 bands were polymorphic among 46 individuals, i.e., the percentage of *PPL* for this species was 62.1%. The *H* was 0.2021 and the (*I*) was 0.3048.

Table 1 Name of primers, sequences and amplifications of 12 effective primers

Primers	Sequences (5′–3′)	Number of loci scored	Number of polymorphic loci
P-A-12	CCTGGGTCCA	7	5
P-A-13	CCTGGGTGGA	6	6
P-A-14	CCTGGGTTTC	8	7
P-A-19	GCCCGGTTTA	8	6
P-B-03	CTCCCTGAGC	9	7
P-B-08	TTCCCGGAGC	9	5
P-B-09	TTCCGGGTGC	9	1
P-B-17	GAGGGCGAGC	6	2
P-C-01	GCGGCTGGAG	11	8
P-C-10	TAGCCCGCTT	7	2
P-C-15	TTCCGCGGGC	9	4
P-C-19	ATTGGGCGAT	6	6



M: 200 bp DNA Ladder (Tian Yuan Biotech. Co., Ltd.)

Fig. 1 RAPD bands of 16 individuals of *I. yunguiensis* amplified with primer P-B-03 (Lane 01−08 and 09−16)

The level of genetic diversity detected in *I. yunguiensis* in the present study (*PPL* = 62.1%) was higher than that in the other endangered species in the same genus. By using RAPD markers on four populations of *I. sinensis* from China, Chen et al. (2004) found that the *PPL* values within populations ranged from 0.81% to 12.9%. Studies on the genetic diversity within six populations of *I. hypsophila* from China using RAPD and ISSR markers indicated that the *PPL* values within populations were all lower than that within the *I. yunguiensis* population (for RAPD markers the *PPL* values ranged from 8% to 26%, Chen et al., 2005; and for ISSR markers from 8% to 35%, Chen and Wang, unpublished data). The level of genetic diversity within population detected in *I. yunguiensis* was higher than in some other unendangered species in the same genus. For example, Small and Hickey (1997) reported a mean of 33% polymorphic allozyme loci within populations of *I. karstenii*. Compared with the values reported on other pteridophytes, *I. yunguiensis* showed relatively high within-population genetic diversity. For example,

Hsu et al. (2000) found only five polymorphic RAPD bands after surveying 40 primers in *Archangiopteris itoi*, a rare and endemic fern in Taiwan Province, China. The genetic diversity at population level in *I. yunguiensis* is comparable to that in certain other fern species, which are not endangered. For example, Miki and Asada (1998) found 62% polymorphic allozyme loci at population level in *Polystichum otomasui*. The Nei's gene diversity (0.2021) and Shannon index (0.3048) obtained for *I. yunguiensis* are compared to those recorded for other species in the same genus. The levels of Nei's gene diversity and Shannon index within the extant *I. yunguiensis* population were higher than those in other quillworts at both population and species level. For example, studies on genetic diversity in *I. hypsophila* using RAPD molecular markers indicated that the Nei's gene diversity ranged from 0.028 to 0.093 and the Shannon index ranged from 0.042 to 0.139 at population level. At the species level Nei's gene diversity and the Shannon index were 0.152 and 0.237, respectively (Chen et al., 2005).

By using RAPD primers, we demonstrated that there was a high level genetic variation within the extant population of *I. yunguiensis*. The present results that *I. yunguiensis* display high level genetic variation are most likely attributed to multiple causes rather than to a single event. *I. yunguiensis* is an ancient species distributed in the Yunnan-Guizhou Plateau in southwest China. Several probable events might have happened during the history of the population, including sexual recombination, the accumulation of somatic mutation in history and founder effect. All of these factors might have played an important role in increasing or maintaining the high level genetic diversity in *I. yunguiensis* population. Another possible explanation for the high level genetic diversity found in *I. yunguiensis* population is that the genetic diversity has been less affected by the current destruction of habitat in spite that in the present-day, *I. yunguiensis* population has declined in the number of individuals due to increasing intensity of human activity in the area. It however appears that the remaining population of *I. yunguiensis* has not suffered heavy losses of genetic diversity that may have resulted from inbreeding and genetic drift. Erosion of genetic diversity in the species may, however, have not been readily discernible due to initial high level diversity in the species.

The final aim of biodiversity conservation programs is to ensure continuity of a species or population and to maintain the evolutionary potential of species (Hamrick and Godt, 1996). Genetic diversity is a precondition for a species to increase its adaptive ability, thereby to reduce its susceptibility in the changing biotic and abiotic environments. Therefore, the level of genetic diversity within a species is of critical importance to its long-term survival (Ellstrand and Elam, 1993).

The present-day *I. yunguiensis* population has, rather unexpectedly, maintained high levels of genetic diversity. This indicates that the species may be capable of adapting to environment variation. Due to human activity and intense competition from other hydrophyte species, the extant *I. yunguiensis* population has faced decline in the number of individuals during recent years (Pang et al., 2003). In our recent field investigation, no more than 50 individuals of *I. yunguiensis* were found in the Pingba population, Guizhou Province, China. There is real danger that if the trend of declining numbers is left unchecked, genetic consequences associated with small isolated populations including genetic drift and inbreeding will diminish genetic diversity in *I. yunguiensis*. Thus, there is an urgent need to pay more attention to the diminishing population size and to adopt appropriate conservation strategies for the long-time survival of this species.

On-going efforts have to be made to produce plants of *I. yunguiensis* by tissue culture, and artificially germinating spores in the laboratory have met considerable success (Chen and Wang, unpublished data). These will provide young sporophyles for replanting to natural populations of *I. yunguiensis*. Nevertheless, RAPD data from the present study indicate that extensive genetic variation still exists within the single remaining population of *I. yunguiensis*. Thus, this population can provide materials for re-establishing other populations.

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