Phylogeography of the rare and endangered lycophyte *Isoetes yunguiensis* (#39715)

First revision

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Phylogeography of the rare and endangered lycophyte *Isoetes yunguiensis*

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Background. *Isoetes yunguiensis* Q. F. Wang et W. C. Taylor is a lycophyte of an ancient genus, and it is endemic to China. It is a first-class protected plant in China. This living fossil is used in paleoecology and studies on the evolution of Lycophytes in the Yunnan-Guizhou Plateau. In recent years, human activities have caused the disappearance of several wild populations, and the number of plants in the existing populations is low. Study of the genetic structure, distribution pattern, and historical dynamics of *I. yunguiensis* in all areas of its distribution is of guiding significance for its rational and effective protection. **Methods.** Expressed sequence tag-simple sequence repeat (EST-SSR) markers were used to study the genetic diversity and structure of *I. yunguiensis*, and noncoding chloroplast DNA (cpDNA) sequences were used to study the pedigree, population dynamics history, and glacial shelter of *I. yunguiensis*. A maximum entropy model was used to predict the past, present, and future distribution patterns of *I. yunguiensis*. **Results.** Analysis with EST-SSR markers revealed that *I. yunguiensis* showed high genetic diversity and that genetic variation was significantly higher within populations than between populations. Based on cpDNA data, it was concluded that there was no significant geographic pedigree in the whole area of *I. yunguiensis* distribution ($NST = 0.344 > GST = 0.183$, $p > 0.05$); 21 haplotypes were detected using DnaSP v5. Neutral test and LAMARC simulation showed that *I. yunguiensis* has experienced rapid expansion in recent years. The maximum entropy model predicted that the potential distribution area of *I. yunguiensis* in the last glacial maximum period has increased significantly compared with the present distribution area, but the future distribution area did not show substantial changes.

Phylogeography of the rare and endangered lycophyte *Isoetes yunguiensis*

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Abstract

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Methods. Expressed sequence tag-simple sequence repeat (EST-SSR) markers were used to study the genetic diversity and structure of *I. yunguiensis*, and noncoding chloroplast DNA (cpDNA) sequences were used to study the pedigree, population dynamics history, and glacial shelter of *I. yunguiensis*. A maximum entropy model was used to predict the past, present, and future distribution patterns of *I. yunguiensis*.

Results. Analysis with EST-SSR markers revealed that *I. yunguiensis* showed high genetic diversity and that genetic variation was significantly higher within populations than between populations. Based on cpDNA data, it was concluded that there was no significant geographic pedigree in the whole area of *I. yunguiensis* distribution ($NST = 0.344 > GST = 0.183$, $p > 0.05$); 21 haplotypes were detected using DnaSP v5. Neutral test and LAMARC simulation showed that *I. yunguiensis* has experienced rapid expansion in recent years. The maximum entropy model predicted that the potential distribution area of *I. yunguiensis* in the last glacial maximum period has increased significantly compared with the present distribution area, but the future distribution area did not show substantial changes.

Introduction

Phylogeography is first proposed by Avise et al (1987), which traces the evolutionary history of populations and explains the historical distribution of existing biota and the historical causes of population differentiation. The Yunnan–Guizhou Plateau is one of the tropics of biodiversity in the world (Myers et al., 2000). Located in the southwest of China, with an area of 3×105 km^2 and an elevation of 1000–3000 m, it is high in the west and low in the east. It is divided into the Yunnan and Guizhou Plateaus, with the Wumeng Mountains as its boundary. The Yunnan

Plateau is located in the higher elevation area in the west, and the Guizhou Plateau is located in the lower elevation area (Cheng et al., 2001). The Yunnan–Guizhou Plateau has played an important role in revealing the biological consequences of Cenozoic orogenic events. Since the Cenozoic, the Yunnan–Guizhou Plateau has been continuously uplifted, which has resulted in unique geomorphological structures and complex land conditions (Wu et al., 2008). In recent years, the phylogeography of some plants in the Yunnan–Guizhou Plateau are being revealed (Li et al., 2012; Wang et al., 2014). Therefore, it is necessary to study the pedigree history and distribution pattern of the endemic plant *Isoetes yunguiensis* Q. F. Wang et W. C. Taylor (Wang et al., 2002) in the Yunnan–Guizhou Plateau.

Isoetes L., the only genus of Isoetaceae, probably evolved from *Annalepis* (Meng, 1998). The evolutionary history of *Isoetes* almost spans the entire evolutionary history of vascular plants, and it is the only extant representative of plant groups that evolved with simplified plant bodies, thus lending great convenience in studies on the origins and evolution of pteridophytes (Zhang and Taylor, 2013). Six species of *Isoetes* are endemic to China(Li, 2014); among these, *Isoetes orien*talis H. Liu & Q. F. Wang and *Isoetes shangrilaensis* Xiang Li, Yuqian Huang, Xiaokang Dai & Xing Liu were described recently (Liu et al., 2005; Li et al., 2019) and were shown to have clear stepped distribution patterns.

I. yunguiensis is a perennial quillwort endemic to China (http://www.iplant.cn/rep/), and it is the first plant to receive national level I protection (Yu, 1999). In recent years, the species populations have declined greatly, and the plant has recently been listed as a critically endangered (CR) plant in China by Dong et al. (2017). *I. yunguiensis* was once sporadically distributed in the northern suburbs of Kunming (Yunnan Province) as well as in Xindan Tiansheng Bridge and Pingba (Guizhou Province). With climatic and environmental changes, wild *I. yunguiensis* populations are gradually dwindling, and several historical populations in Yunnan have already disappeared. Therefore, the protection of the existing populations is urgent.

I. yunguiensis is very similar in appearance to *Isoetes japonica* A. Braun and was treated as *I. japonica* for a long period. Renchang Qin, for the first time, distinguished *I. yunguiensis* from *I. japonica* and described it as *I. yunkweiensis* Ching. Due to lack of complete information, this finding ~~has not been publicly~~ published (Liu et al. 2002). Subsequently, Xianchun Zhang ~~synonymized~~ this species as *I. chingiana* to honor the work of Qin (Zhang, 2001) but ~~this findings has not been publicly published as well~~ (Liu et al. 2002). In a summary of previous studies, Wang et al. (2002) reported clear differences between the two species. In particular, *I. yunguiensis* megaspores are protuberant and numerous compared with *I. japonica* megaspores, and both species have distinct number of chromosomes (2n = 22 in *I. yunguiensis* vs. 2n = 66, 67, 77, 88, or 89 in *I. japonica*). ~~*I. chingiana* was later redescribed as the Chinese endemic species *I. yunguiensis* Q. F. Wang et W. C. Taylor~~. In recent years, studies on *I. yunguiensis* have mainly focused on its morphology (Zhao et al., 2015), palynology (Liu et al., 2013), and molecular genetics (Ma et al., 2018; Dong et al., 2018). However, to the best of our knowledge, no studies on its evolution and biogeography have been published. Phylogeographic and genetic diversity studies on *I. yunguiensis* provide the basic data necessary for determining relationships

across the evolutionary lineages of alpine plants in the Quaternary glacial period. Such relationships may provide a basis for the protection of endangered plants. Herein, expressed sequence tag-simple sequence repeat (EST-SSR) markers and chloroplast DNA (cpDNA) were used to determine the genetic diversity and structure of *I. yunguiensis* populations. The distribution and evolutionary patterns of genetically distinct *I. yunguiensis* groups were revealed, and the causes of the current distribution patterns of *I. yunguiensis* were discussed, which may provide scientific evidence for *I. yunguiensis* protection.

Materials & Methods

Materials

A total of 167 samples were collected from 14 populations in Yunnan and Guizhou Provinces from October 2018 to January 2019 (Table 1). Briefly, 8–15 samples that were distributed 10 m apart were randomly selected from each site (random sampling of minimal population). Healthy young leaves were collected in paper bags, dried immediately with silica, and stored at −20°C for further use. Voucher specimens were stored in Guizhou Agricultural College Plant Protection Department Herbarium(GACP). Fig. 1 presents a map of sampling locations and geographical distribution of haplotypes.

DNA extraction, polymerase chain reaction (PCR) amplification, and sequencing

DNA was extracted from dried leaves using the TIANGEN Plant Genomic DNA Extraction Kit (DP305, TIANGEN BIOTECH (BEIJING) CO., LTD.), and PCR analyses were performed using the EST-SSR primers SJSSR5, SJSSR12, SJSSR14, SJSSR45, and SJSSR53 and the cpDNA primers trnS-trnG, psbC-trnS, and psbD-trnT (Table 2). PCR reactions were conducted in 25 μL mixtures comprising 12.5 μL 2× T5 Super PCR Mix (Beijing TsingKe Biotech Co., Ltd.), 40 ng DNA template, and 1 μL each of (10 pmol/μL) forward and reverse primers, topped-up to final volume with double-distilled water. EST-SSR PCR was performed with a predenaturation step at 94°C for 5 min, followed by 28 cycles of denaturation at 94°C for 30s, annealing at respective temperatures for 30 s, extension at 72°C for 1 min, and final extension at 72°C for 30 min. Reactions with cpDNA primers were performed with predenaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at respective temperatures for 30 s, extension at 72°C for 1 min, and final extension at 72°C for 10 min. EST-SSR PCR products were visualized as clear bands using 1.5% agarose gel electrophoresis and detected by capillary electrophoresis with ABI 3730(Thermo Fisher Scientific). GeneMaker 2.2 was used to analyze the original peak patterns and to determine the sizes of EST-SSR marker fragments. Similarly, cpDNA PCR products were detected with clear bands using 1.5% agarose gel electrophoresis purified by electrophoretic cutting and magnetic beads and sequenced using ABI 3730(Thermo Fisher Scientific). All sequences are deposited in GenBank under accession numbers MN463102 - MN463268 for psbC-trnS, MN463269 - MN463435 for psbD-trnT, MN463436 - MN463602 for trnG-trnS.

Data analysis

DNA analysis

Observed allele number (Na), effective allele number (Ne), observed heterozygosity (Ho), and expected heterozygosity (He) were calculated, and principal coordinate analyses (PCoA) and Mantel tests (Mantel, 1967) were performed using GenAlex 6.5 (Peakall and Smouse, 2012). Polymorphism information content (PIC) was calculated using CERVUS 3.0 (Kalinowski et al., 2007). Nei's genetic distances were computed by POPGene 32 (Yeh et al., 2000), and Bayesian clustering was performed using STRUCTURE 2.3.3 (Pritchard et al., 2009). The original peak images of noncoding cpDNA regions were manually evaluated using Chromas 2.6, and ClustalW program in the MEGA7.0 software was used to align and truncate the three gene fragments and examine whether poly structure existed or were deleted. Concatenate Sequence program in PhyloSuite v1.1.15 (Zhang et al., 2018) was used to connect the three gene fragments into one fragment. Insertion deletions are treated as mutation sites (Chen et al., 2008). DnaSP v5 (Librado and Rozas, 2009) was used to determine variation sites, haplotype diversity (Hd), and nucleotide diversity (π) of cpDNA sequences, and neutral tests and mismatch distribution analyses of cpDNA fragments were performed at the species level. LAMARC 2.1.8 was used to further verify the group expansion events for *I. yunguiensis*, and Permut 2.0 (Pons and Petit, 1996) was used to calculate total genetic diversity (Ht) and intrapopulation mean genetic diversity (Hs). To construct haplotype phylogenetic trees, analysis of molecular variance (AMOVA) was performed using Arlequian 3.5 (Excoffierand Lischer, 2010) and IQ-TREE (Nguyen et al., 2014). A median-joining network map was constructed using Network 5.0 (Bandelt et al., 1999).

Species distribution prediction

MaxEnt 3.4 (Phillips et al., 2006) was used to predict the distribution ranges of *I. yunguiensis* during the interglacial (LIG) period and last glacial maximum (LGM) as well as at the present and in the future. Maps were brought from National Catalogue Service for Geographic Information (http://www.webmap.cn). Climate data were downloaded from the World Climate Data website (www.worldclim.org; resolution choice is 30 s). Coordinates of *I. yunguiensis* distribution were determined using field records from the China Digital Herbarium (http://www.cvh.org.cn) as described previously (Pang et al., 2003; Yuan et al., 2012; Li et al., 2015). Briefly, environmental data and spatial coordinates were imported into MaxEnt 3.4, parameters were set to default values, and distribution ranges during various periods were predicted. Subsequently, ArcGis 10.2 was used to generate ASCII raster layers and simulated distribution maps.

Results

Genetic diversity of EST-SSR loci

The genetic diversity of EST-SSR loci in *I. yunguiensis* populations is presented in Table 3. The mean Na was 2.843, ranging from 1.929 (SJSSR53) to 4.000 (SJSSR12); mean Ne was 2.138, ranging from 1.521 (SJSSR53) to 2.981 (SJSSR12); and mean Shannon information index (I)

was 0.782, ranging from 0.417 (SJSSR53) to 1.175 (SJSSR12). Ho ranged from 0 (SJSSR14) to 0.960 (SJSSR5), and He ranged from 0.273 (SJSSR53) to 0.642 (SJSSR12). The mean Ho and He were 0.475 and 0.463, respectively. PIC was high at a mean of 0.516, ranging from 0.313 (SJSSR53) to 0.641 (SJSSR12). The genetic diversity was the highest for SJSSR12 loci and the lowest for SJSSR53 loci.

Genetic diversity and structure of populations

The genetic diversity of different populations is summarized in Table 4. The mean Na was 2.843, ranging from 2.400 (LS) to 3.400 (CX and XB); mean Ne was 2.138, ranging from 1.777 (LS) to 2.675 (CX); and mean I was 0.782, ranging from 0.595 (LS) to 1.016 (CX). Ho and He ranged from 0.251 (XB) to 0.673 (DJC) and from 0.335 (XLC) to 0.591 (CX), with mean values of 0.475 and 0.463, respectively. The genetic diversity was high in BSH, CX, DJC, DPJ, GJS, SG, and PB and low in LS, GP, and XB.

AMOVA using EST-SSR markers showed that 22.25% of the total genetic variation was present between *I. yunguiensis* populations and up to 77.75% was present within populations (Table 5). The average interpopulation gene flow rate (Nm) was 1.534, which is considered to be high, indicating frequent gene exchange between populations. These observations are consistent with the lower genetic variation between populations than within populations.

Mantel tests showed no significant associations of Nei's genetic distances with geographical distances ($p = 0.242 > 0.05$) (Fig. 2A) or altitude gradients ($p = 0.402 > 0.05$) (Fig. 2B).

Genetic relationships and cluster analyses

Unweighted pair group method with arithmetic mean (UPGMA) cluster analyses were performed on the basis of Nei's genetic distances using MEGA 7.0, and 14 UPGMA phylogenetic trees of *I. yunguiensis* populations were constructed (Fig. 3). With a genetic distance of 0.2, the 14 populations were divided into two groups. The first group CX comprised only one unique population from the Yunnan Plateau, and the second group comprised the remaining 13 populations from the Guizhou Plateau. With a genetic distance of 0.1, the second group was subdivided into groups II-a and II-b. Group II-a predominantly comprised populations from the west of Guiyang City, and group II-b comprised populations from to the east of Guiyang City; however, the difference between the two groups was not obvious. PCoA results (Fig. 4) were consistent with UPGMA results, in which the first and second components explained 51.50% and 18.76% of the variance, respectively.

STRUCTURE 2.3.3 was used to analyze the genetic structure of *I. yunguiensis* populations. At $K = 2$, ΔK reached the maximum value (Fig. 5), and *I. yunguiensis* individuals were divided into two groups (Fig. 6). Group I (blue; Fig. 6) mainly comprised individuals of the CX population, and group II comprised individuals from the remaining populations (red; Fig. 6). Overall, 30 of the 167 individuals showed differences of different degrees, indicating a variable degree gene exchange between populations.

cpDNA sequence characteristics and haplotype analyses

After aligning and connecting the three cpDNA sequences from *I. yunguiensis*, the total length was 1657 bp. Moreover, 36 mutation sites were detected, among which 11 were detected by trnS-trnG, including eight single-base mutations and three insertion/deletion sites. Only two mutation sites were detected by psbC-trnS, and both were single-base mutations. A total of 23 mutation sites were detected by spsbD-trnT, including 20 single-base mutations and three insertions/deletions.

A total of 21 haplotypes were detected using DnaSP v5, and haplotype H1 was the most widely distributed haplotype. In contrast to the CX population, the remaining 13 populations were of haplotype H1, followed by haplotype H2. Haplotype H3 was distributed in GP, BSH, DPJ, LS, and PB populations; haplotype H4 was distributed in GP and SCZ populations; and the unique haplotypes H5–H21 were distributed in only a single population but were evenly distributed across 10 populations, with only one CX population carrying the unique haplotype H7. Genetic diversity, nucleotide diversity, and haplotype compositions and frequencies of the sampled populations are shown in Table 6.

Population genetic structure based on cpDNA sequences

The total genetic diversity (Ht) was 0.631; mean genetic diversity (Hs) was 0.515; and the genetic differentiation coefficients between (GST) and within (NST) populations were 0.183 and 0.344, respectively. The haplotype variation structure of the 14 *I. yunguiensis* populations was examined using 1000 bootstrap replicates, which revealed a tendency of NST > GST, although the difference was not significant ($p > 0.05$); thus, *I. yunguiensis* exhibited no obvious phylogenetic structure in the studied region. Consistently, AMOVA (Table 7) showed that the genetic variation between populations was 34.00% and that within populations was 66.00% (Fst = 0.34, $p < 0.001$, 1000 bootstrap replicates). The genetic variation in *I. yunguiensis* is predominately higher within populations. The mean Nm was 2.18, which indicated a great gene flow between *I. yunguiensis* populations.

Population history dynamics based on cpDNA sequences

DnaSP v5 was used to examine the combined cpDNA sequence of all the individuals in the 14 sampled *I. yunguiensis* populations. Although the mismatch distribution was bimodal (Fig. 7), Tajima's D = −2.55856 ($p < 0.001$), Fu & Li's D = −8.03665 ($p < 0.02$), and Fu & Li's F = −6.94682 ($p < 0.02$) were significantly negative. The population growth index obtained by LAMARC simulation was 921.590 (G > 200). Taken together, these results indicated that the *I. yunguiensis* populations have experienced expansion events.

Phylogenetic relationships based on cpDNA haplotypes

IQ-TREE was used to construct a phylogenetic tree of *I. yunguiensis* haplotypes (Fig. 8). The model with the best HKY+F value was selected. *Isoetes flaccida* and *I. vallda* were used as

outgroups. The 21 haplotypes were divided into two branches: the first branch included H1–H6 and H8–H21 and the second branch included H7 alone.
A mediation-link (median-joining) network diagram and phylogenetic tree based on the maximum likelihood method were constructed using Network 5.0 (Fig. 9). Because H1 and H2 appeared in the middle of the Network diagram, these were speculated to be relatively old haplotypes; moreover, there were few other haplotypes and variation coefficients were small, suggesting that recent haplotypes were derived from H1 and H2.

Niche modeling

MaxEnt 3.4 was used to predict distribution ranges during the LIG period (Fig. 10A) and LGM (Fig. 10B) as well as at present (Fig. 9C) and in the future (Fig. 10D). In all the models, the areas under the curve were >0.947, indicating that the predicted distribution ranges of *I. yunguiensis* are in the central Guizhou Plateau and northwestern and eastern Yunnan Plateau and that the suitable areas of distribution are mainly in the central Guizhou Plateau. The future distribution pattern is similar to the current distribution pattern. The range of *I. yunguiensis* distribution during the LIG period was significantly reduced compared with the present distribution range, and the distribution ranges in the Yunnan–Guizhou Plateau were clearly separated. The distribution range during LGM was slightly expanded in the Yunnan Plateau and reduced in the Guizhou Plateau compared with the future distribution range.

Discussion

Genetic diversity and structure

Compared with other rare, endemic, and endangered plants, such as *Isoetes sinensis* Palmer(He = 0.118; Kang et al., 2005), *Isoetes hypsophila* Hand. Mazz.(He = 0.039; Chen et al., 2010), *Isoetes malinverniana* Ces. & De Not.(H=0.1491 for ISSR data; H=0.2289 for AFLP data; Gentili et al., 2010), *Ottelia acuminata* var. *jingxiensis* H. Q. Wang & S. C. Sun J. (He = 0.441; Li et al., 2019), *Dalbergia odorifera* T. Chen (He = 0.37; Liu et al., 2019), and *Brasenia schreberi* J. F. Gmelin (He = 0.256; Li et al., 2018), *I. yunguiensis* showed high genetic diversity. Most rare and endangered plants are seed plants that can achieve a high gene flow through long-distance pollen and seed dispersal than ferns that rely solely on spores. Ma et al. (2018) investigated the genetic structure of *I. yunguiensis* using inter simple sequence repeat markers and showed inter- and intrapopulation genetic variations of 31.99% and 68.01%, respectively. Similarly, Dong et al. (2018) used amplified fragment length polymorphism markers and showed inter- and intrapopulation variations of 40.12% and 59.88%, respectively. However, in these previous studies, samples were collected from Pingba and Hongfeng Lake alone, which limited the representativeness of the genetic variation of *I. yunguiensis*. The 14 sampled populations covered all regions of *I. yunguiensis* distribution, and analyses of genetic structures and variations were conducted using EST-SSR and cpDNA sequences to reveal the genetic diversity of *I. yunguiensis*. Based on these markers, *I. yunguiensis* showed high genetic diversity. Moreover, AMOVA using these markers showed that the genetic diversity was higher

within populations than between populations, which is consistent with the previously reported studies. Reportedly, in the areas of *I. yunguiensis* distribution, the observed haplotypes accounted for 80.95% of the total haplotypes, and this species often did not show clear biogeographical patterns (Gao et al., 2012; Gao et al., 2016). Accordingly, in the present study, the population genetic differentiation index was higher than the gene differentiation index, although the difference was not significant ($p > 0.05$), indicating that *I. yunguiensis* shows no obvious biogeographical patterns. *I. yunguiensis* is mainly dispersed through spores released in flowing water (Yang et al., 2011), and it is distributed in the Yangtze and Pearl River water systems. In the present study, a high gene flow was observed between *I. yunguiensis* populations, which may explain the high genetic variation within populations (Huang et al., 2017).

Using STRUCTURE 2.3.3, the 14 *I. yunguiensis* populations were divided into two groups, and these results were consistent with those of UPGMA culture analysis, PCoA, and IQ-TREE based on Nei's genetic distances. The two groups are separated by the Wumeng Mountains, which follow the border between the Yunnan–Guizhou Plateaus. The mountains between these provinces have isolated these *I. yunguiensis* populations, thus explaining the genetic distances between the two main haplotype groups. However, based on the STRUCTURE analysis, the genetic structure of *I. yunguiensis* is relatively complex. Further studies are required to elucidate specific reasons underlying the difference between the CX population from the Yunnan Plateau and the remaining 13 populations from the Guizhou Plateau. However, Mantel tests showed no significant associations of Nei's genetic distances with geographical distances or altitude gradients.

Population structure and distribution ranges of *I. yunguiensis*

The genetic structures of extant plants can be traced back to the Quaternary glacial period. Based on this, Yu et al. (2013) have summarized the genetic structures of 36 species of alpine plants from the Qinghai–Tibet Plateau and surrounding areas. Some hardy plants did not migrate to lower altitudes during the glacial episodes; however, plants on the plateau surface experienced small-scale expansions of ranges following LGM. The genetic structures of species are often characterized by high variations or specific haplotypes, with population distributions in the highland mesa of one or more isolated areas. In addition, some species occupied narrow ranges during the glacial period, leading to small areas of expansion following LGM. These populations were characterized by high genetic diversity and unique haplotypes distributed evenly throughout the distribution range. The present study demonstrated that although *I. yunguiensis* shows high genetic variation, there is regional genetic variation that is not linked to longitude and latitude or elevation, presumably because during the Quaternary glacial period, the Yunnan–Guizhou Plateau remained free of ice. Thus, the influence of glacial episodes on *I. yunguiensis* was only characterized by the influences of changes in climate and availability of regional water, which affect the habitat and dispersal of *I. yunguiensis*. Few ancient haplotypes may have been randomly fixed in the population during migration, and young haplotypes could have

subsequently been fixed in the population because of genetic drift and the founder effect, thus forming the current population structure of *I. yunguiensis*.

Hewitt (2000) identified a small population that migrated to form populations with greater genetic variation and richer haploid types, potentially reflecting the duration of isolation and accumulation of genetic variants. When diffusion distances increase from outer diffusion migration groups that are prone to genetic drift or the founder effect, haplotype polymorphism is gradually reduced. According to coalescent theory, populations locaed in refuge show high genetic diversity and ancient haplotypes are often located in the center of the haplotype network maps. Thus, geographical locations of populations with high genetic diversity and ancient haplotypes can be used as bases for identifying potential habitats. In the present study, H1 and H2 were located in the center of the haplotype network map and may therefore be ancient haplotypes that are widely distributed in the Guizhou Plateau. The CX population from the Yunnan Plateau comprised the unique and stable haplotype H7 and is located far from the Guizhou Plateau. Perhaps, *I. yunguiensis* occupied specific areas of the Guizhou and Yunnan Plateaus during the glacial period. Because the ancestral population from the Yunnan Plateau is extinct, only one population was found in the Yunnan Plateau in the present study, and the specific location of the extinct population in this plateau remains unknown.

Conservation strategies for *I. yunguiensis*

The present study demonstrated that the BSH, CX, DJC, DPJ, GJS, SG, and PB populations showed high genetic diversity. It is important to understand genetic diversity and population structure to establish scientific and effective protection measures. Therefore, to protect the endangered *I. yunguiensis* plants, a provincial nature reserve has been established; the DPJ population used in the present study was sampled from this reserve. However, it has been difficult to protect this population. The SCZ population in the present study was sampled from a farm where sewage is directly discharged into the habitat. The DJC population was sampled from the middle of Longli Forest Farm; seven populations were sampled from this farm, accounting for half of the surveyed populations. However, although these populations were not under the threat of sewerage exposure, limited attention and protection have led to a state of low survival and even extinction. Therefore, establishment of a protected area dedicated to *I. yunguiensis* in Longli Forest Farm and training of staff and residents of the surrounding areas regarding protective measures for *I. yunguiensis* are warranted. In a previous study, populations with high genetic diversity were selected from similar environments for transplantation (Zhang, 2016). According to the principles of genetic diversity and conservation prioritization in special areas, the HFH populations, which showed the highest haplotype diversity (Hd = 0.8) should be given the highest priority, followed by the BSH, CX, DJC, DPJ, GJS, SG, and PB populations. As an overall conservation strategy based on the molecular genetic evidence provided in the present study, the Guizhou and Yunnan Plateaus can be divided into two regions to protect populations with high genetic diversity as well as those with high and stable haplotype diversity. The CX populations from the Yunnan Plateau and the BSH, HFH, DJC, SG, DPJ, GJS, and PB

populations from the Guizhou Plateau should be primarily protected. Both the Guizhou and Yunnan Plateaus should be protected, with special attention paid to the CX, BSH, and HFH populations. In addition, the water quality in *I. yunguiensis* habitats should be maintained to avoid contamination, and the growth of companion species should be controlled. Improved knowledge and awareness regarding the regions of *I. yunguiensis* distribution are paramount. To strengthen the conservation strategies for endangered species, further studies are required to identify threats. Moreover, given that the number of wild *I. yunguiensis* individuals in this survey was less than 10,000, which is declining further, the International Union for Conservation of Nature may soon list *I. yunguiensis* in the Critically Endangered category.

Conclusions

I. yunguiensis was found to have higher genetic diversity than other rare and endangered plants, and its genetic structure demonstrated in the present study is similar to that reported previously. Genetic variation was significantly higher within populations than between populations. Based on the data of cpDNA, no obvious pedigree was found in the whole area of *I. yunguiensis* distribution, but there has been rapid expansion in recent years. The distribution pattern simulated using the MaxEnt model showed that the past and future distribution patterns of *I. yunguiensis* are not considerably different from its present distribution pattern. EST-SSR marker and cpDNA data support that the 14 populations examined in the present study can be divided into two groups: the Yunnan Plateau and Guizhou Plateau. This indicated that there were at least two refuges for *I. yunguiensis* during the glacial period. The Wumeng Mountains between the two plateaus have a great influence on gene exchange in *I. yunguiensis*. Based on the high genetic diversity and ancient haplotypes of the populations associated with the refuges, it can be concluded that the CX population from the Yunnan Plateau and the BSH, HFH, DJC, SG, DPJ, GJS, and PB populations from the Guizhou Plateau should be primarily protected. These are ice age refuges wherein these population need to be protected. In the present study, only one population was identified in the Yunnan Plateau. Further investigation is needed to identify the new population and discuss the area of refuge. To date, the cause of endangerment of *I. yunguiensis* remains unclear, and related research is urgently warranted.

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Figure 1

Sampling locations and geographical distributions of haplotypes

The pie charts show the proportions of different haplotypes in each population

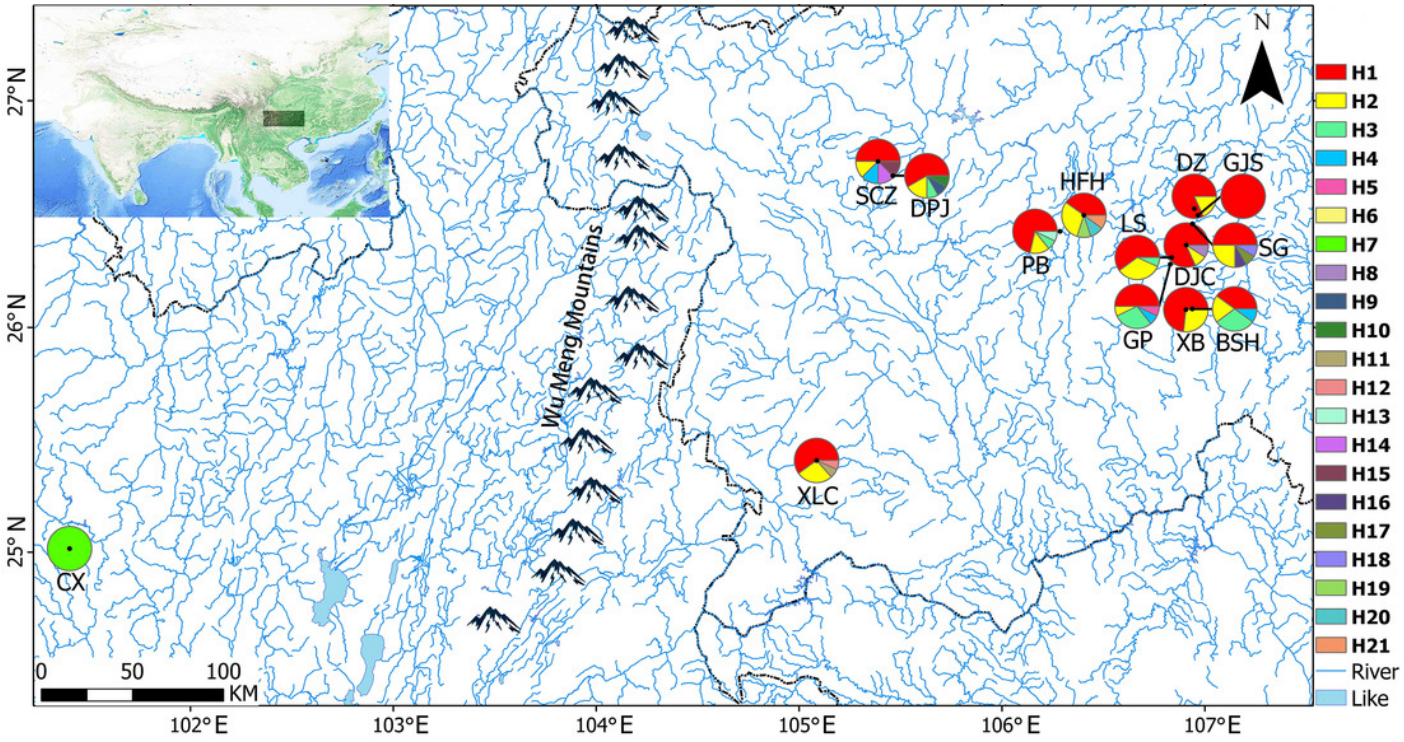
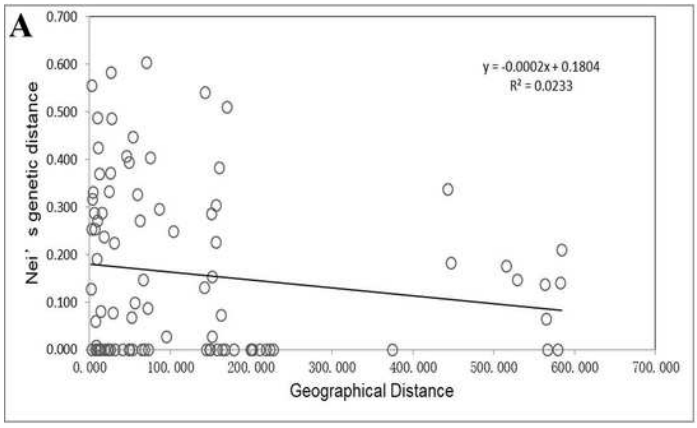


Figure 2

Mantel tests of genetic distances and geographic distances/altitudes based on EST-SSR markers



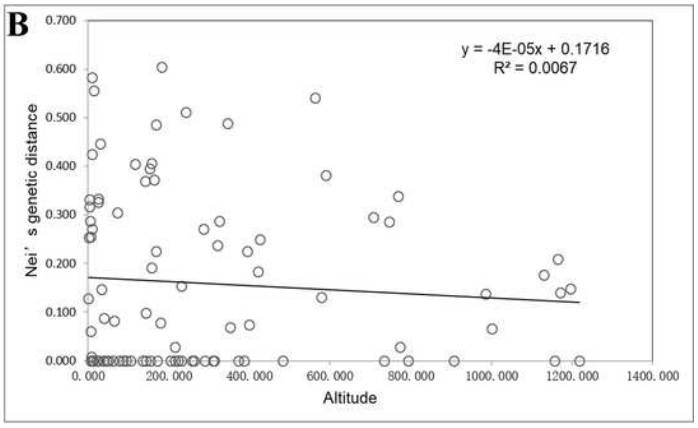


Figure 3

UPGMA cluster analysis of 14 *Isoetes yunguiensis* populations based on EST-SSR markers

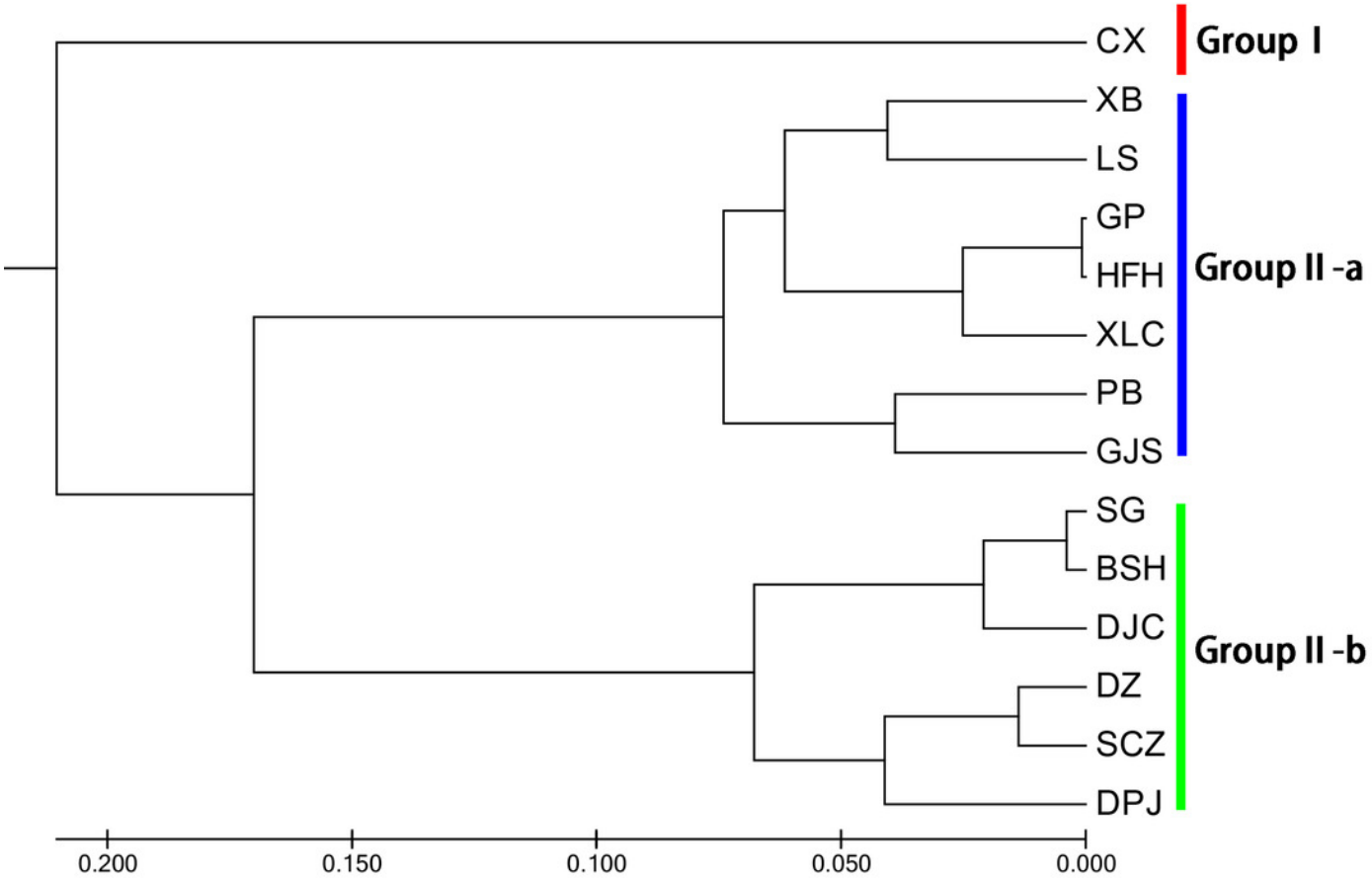


Figure 4

PCoA analysis of *Isoetes yunguiensis* populations based on EST-SSR markers

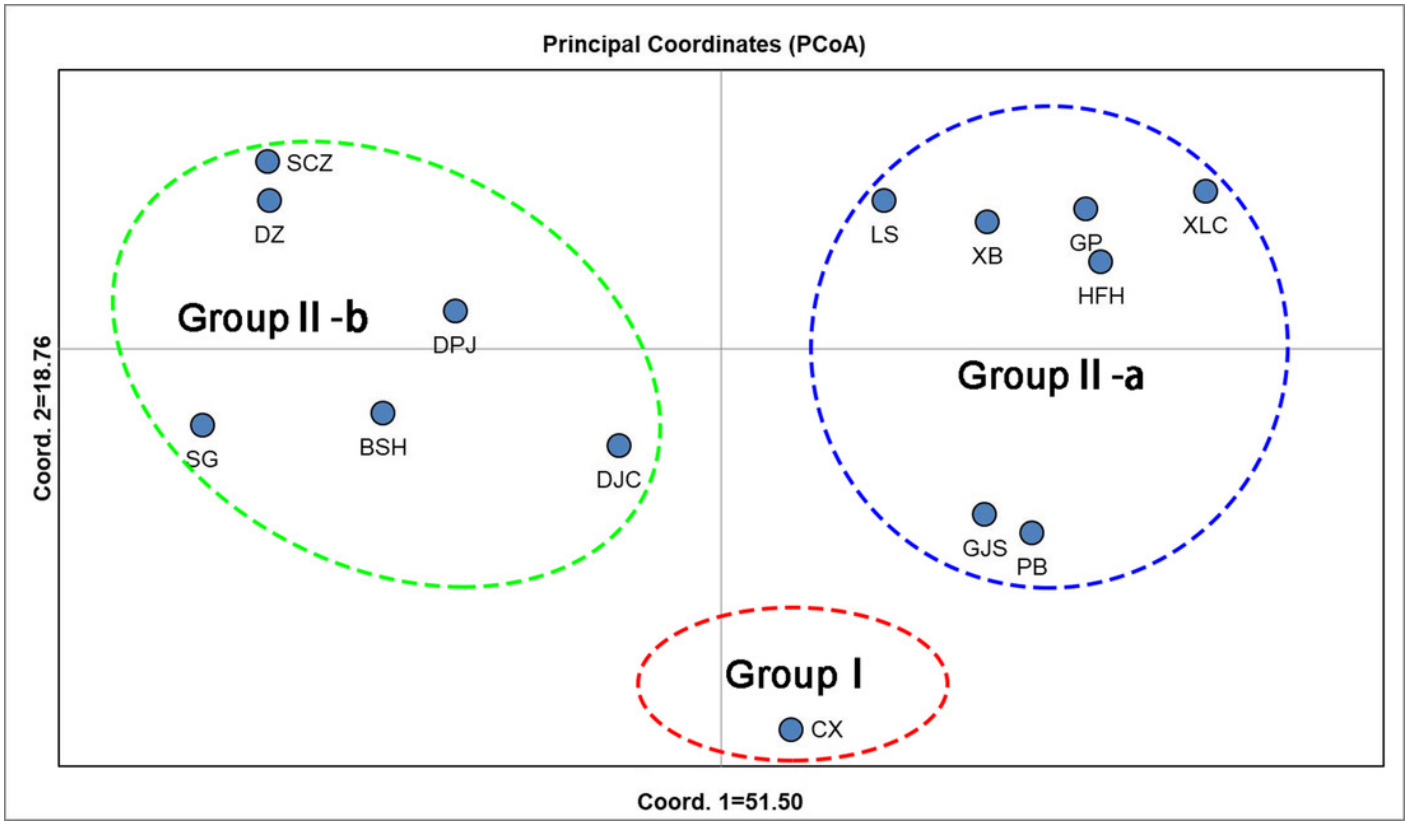


Figure 5

Association between ΔK and K in the STRUCTURE analyses

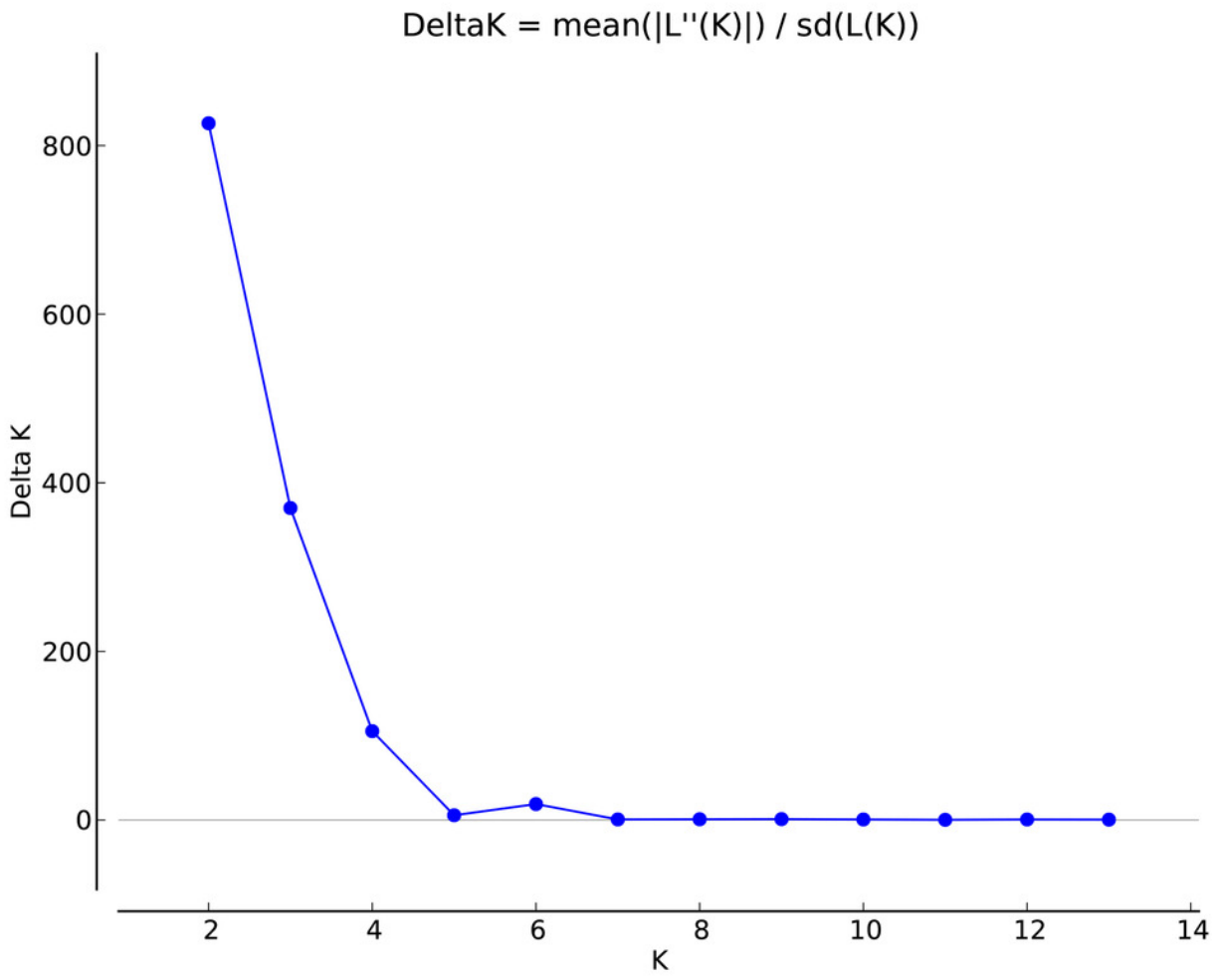


Figure 6

STRUCTURE analyses of 14 *Isoetes yunguiensis* populations based on EST-SSR markers

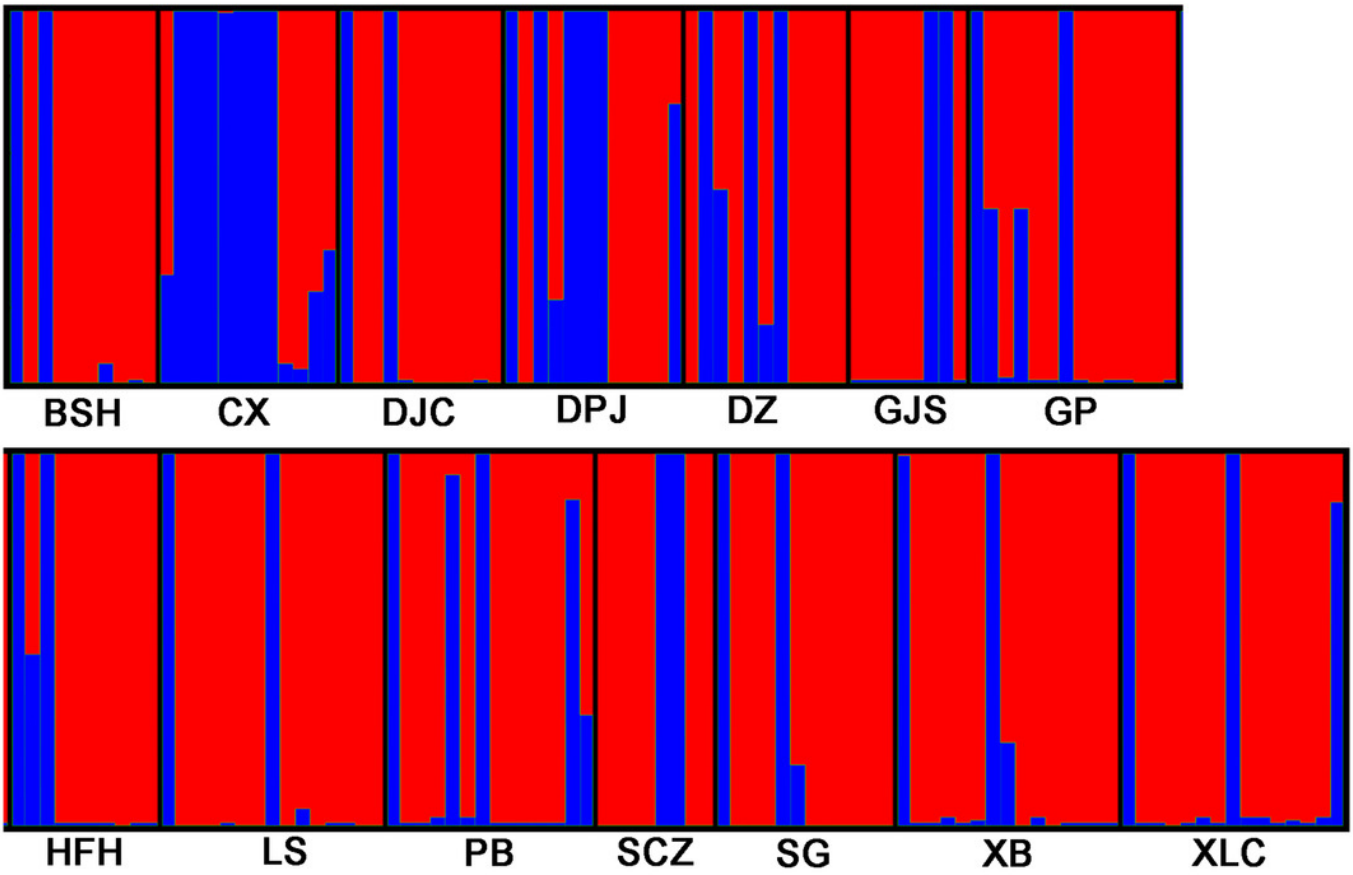


Figure 7

Mismatch distribution analysis among different inferred biogeographical groups

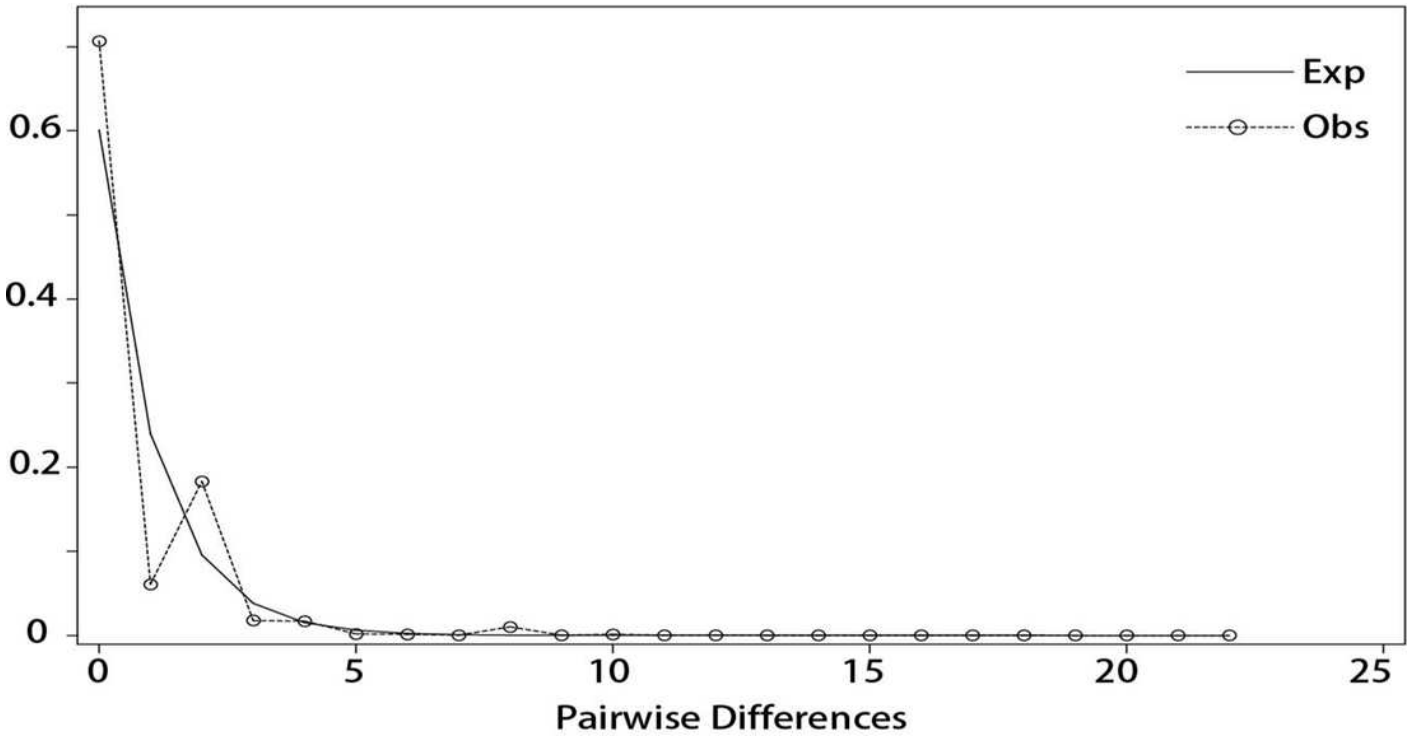


Figure 8

A phylogenetic tree of *Isoetes yunguiensis* haplotype constructed using IQ-TREE based on cpDNA

The number above the node represents the bootstrap support rate (showing a value >60)

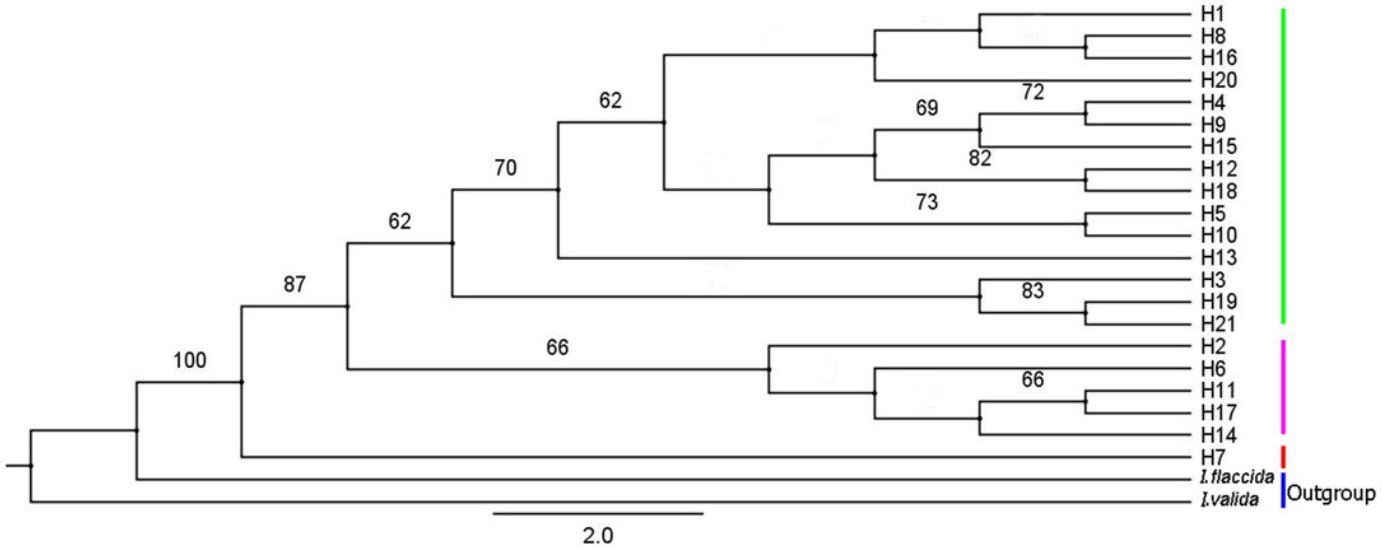


Figure 9

Median-joining network reflecting haplotype relationships

Small red circles represent potential intermediate haplotypes, The sizes of circles are proportional to individual number of haplotype, The solid line indicates a base mutation step

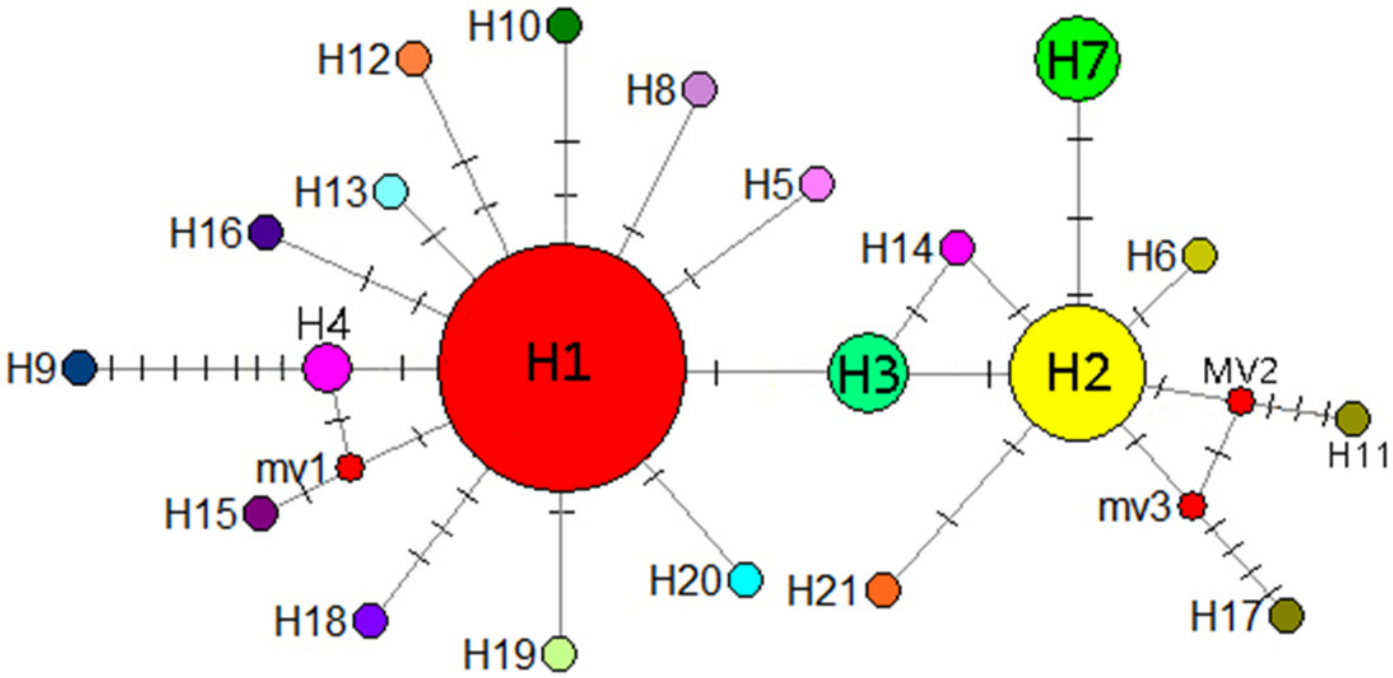


Figure 10

Potential distribution modeling of *Isoetes yunguiensis* based on scenarios of the MIROC model

A. the last interglacial (LIG, ca. 130 Kya), B. the last glacial maximum (LGM, ca. 22 Kya BP), C. present day (average for 1970–2000), and D. future in 2070 (average for 2061–2080)

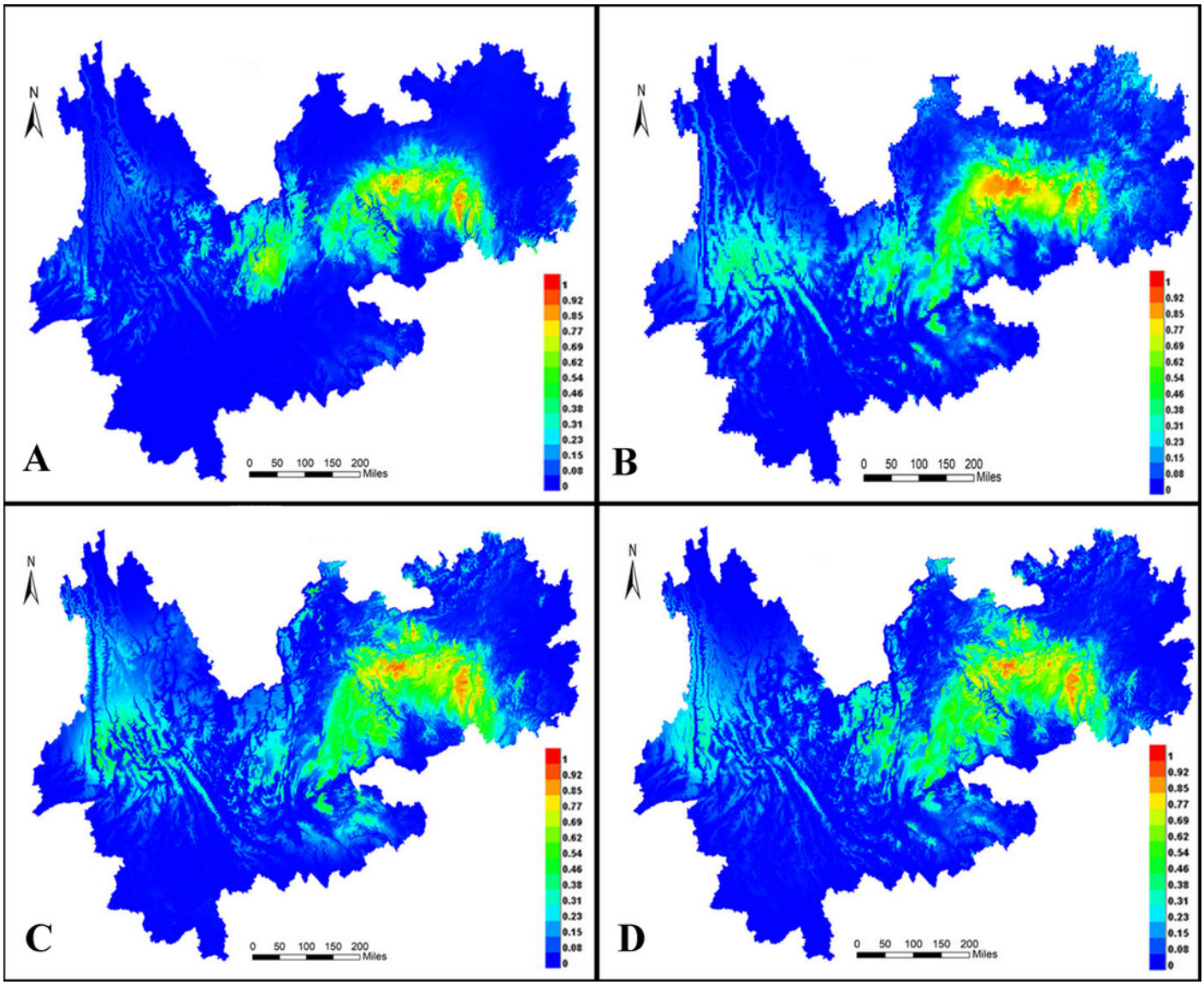


Table 1(on next page)

Details of sampling locations of the 14 *Isoetes yunguiensis* populations

Coordinates and number of individuals sampled (Nind) are shown for each population

Population code	Population geographic location	Latitude	Longitude	Altitude (m)	Nind
GJS	Gengjia Mountain, Longli Forest Farm	106°57′57″E	26°29′45″N	1276	8
DZ	Dazhu Village, Longli County	106°56′50″E	26°31′29″N	1281	11
XB	Xinbai, Huishui County	106°54′20″E	26°04′40″N	1289	15
LS	Hongxing Village, Longli County	106°50′10″E	26°18′31″N	1445	15
GP	Huaxi, Guiyang City	106°49′41″E	26°16′43″N	1461	14
BSH	Baishuihe, Huishui County	106°56′13″E	26°04′51″N	1434	10
CX	Zixi Mountain, Chuxiong City	101°24′14″E	25°00′55″N	2447	12
DJC	Luyuan Resort, Longli County	106°54′25″E	26°21′48″N	1603	11
DPJ	Dapingjing Wetland Park, Nayong County	105°27′41″E	26°40′10″N	2024	12
XLC	Xinglongchang, Xingren County	105°05′03″E	25°24′35″N	1540	15
PB	Pingba, Anshun City	106°17′07″E	26°25′29″N	1316	14
SCZ	Shuichanzhan, Nayong County	105°23′17″E	26°44′05″N	1677	8
SG	SG, Longli Forest Farm	106°56′15″E	26°27′25″N	1229	12
HFH	Hongfenghu, Qingzhen City	106°24′13″E	26°30′01″N	1250	10

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Table 2(on next page)

Information of primer pairs used to amplify by PCR

Primer	Sequence (5'-3')	Tm (°C)	Reference
SJSSR5	**F:** CACACCCAACATACATACGCA, **R:** ATGGGGTGAAACAACAGGAG	53	Gichira et al., 2016
SJSSR12	**F:** GTGGTTGATTTGGGGTCATC, **R:** CCCTCTTTGCCAACAGTGAT	53	Gichira et al., 2016
SJSSR14	**F:** GGCCAGAGAACAGGAGAAAG, **R:** CCAAGTGGAAATTATGTCGCT	55	Gichira et al., 2016
SJSSR45	**F:** AAGGCCAACACAAAAACTGG, **R:** CGCCCACTAATCAGGACACT	52	Gichira et al., 2016
SJSSR53	**F:** AGGGATTGCTAGCGCTGTTA, **R:** GGCAAAACAAAAGCATCCAT	54	Gichira et al., 2016
psbC-trnS	**F:** TGAACCTGTTCTTTCCATGA, **R:** GAACTATCGAGGGTTCGAAT	52	Nishizawa & Watano, 2000
psbD-trnT	**F:** CTCCGTARCCAGTCATCCATA, **R:** CCCTTTTAACTCAGTGGTAG	55	Shaw et al., 2007
trnS-trnG	**F:** AGATAGGGATTCGAACCCTCGGT, **R:** GTAGCGGGAATCGAACCCGCATC	52	Shaw et al., 2007

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Table 3(on next page)

Polymorphism analysis of the EST-SSR primers

Na, Number of alleles observed; Ne, Effective number of alleles; I, Shannon's information index; Ho, Observed heterozygosity; He, Expected heterozygosity; PIC, Polymorphic information content; SD, standard deviation

Loci	Na (mean ± SD)	Ne (mean ± SD)	I (mean ± SD)	Ho (mean ± SD)	He (mean ± SD)	PIC
SJSSR5	2.929 ± 0.917	2.361 ± 0.437	0.899 ± 0.211	0.960 ± 0.042	0.565 ± 0.068	0.552
SJSSR12	4.000 ± 0.555	2.981 ± 0.678	1.175 ± 0.228	0.828 ± 0.321	0.642 ± 0.113	0.641
SJSSR14	2.857 ± 0.363	2.191 ± 0.467	0.869 ± 0.197	0	0.520 ± 0.122	0.553
SJSSR45	2.500 ± 1.225	1.637 ± 0.576	0.550 ± 0.414	0.118 ± 0.158	0.316 ± 0.234	0.519
SJSSR53	1.929 ± 0.616	1.521 ± 0.510	0.417 ± 0.319	0.468 ± 0.446	0.273 ± 0.228	0.313
Mean	2.843 ± 0.461	2.138 ± 0.334	0.782 ± 0.174	0.475 ± 0.203	0.463 ± 0.097	0.516

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Table 4(on next page)

Genetic diversity of 14 *Isoetes yunguiensis* populations in analyses using EST-SSR markers

Na, Number of alleles observed; Ne, Effective number of alleles; I, Shannon's information index; Ho, Observed heterozygosity; He, Expected heterozygosity; SD, standard deviation

Population	Na (mean ± SD)	Ne (mean ± SD)	I (mean ± SD)	Ho (mean ± SD)	He (mean ± SD)
GJS	2.600 ± 1.140	2.116 ± 0.783	0.746 ± 0.464	0.600 ± 0.548	0.455 ± 0.264
DZ	2.800 ± 1.095	2.245 ± 0.943	0.803 ± 0.497	0.400 ± 0.509	0.466 ± 0.282
XB	3.400 ± 0.548	1.843 ± 0.610	0.749 ± 0.310	0.251 ± 0.380	0.407 ± 0.195
LS	2.400 ± 1.140	1.777 ± 0.685	0.595 ± 0.432	0.386 ± 0.529	0.362 ± 0.254
GP	2.600 ± 1.673	2.018 ± 1.025	0.645 ± 0.619	0.400 ± 0.548	0.370 ± 0.343
BSH	3.200 ± 1.304	2.407 ± 0.554	0.956 ± 0.275	0.578 ± 0.485	0.569 ± 0.087
CX	3.400 ± 1.342	2.675 ± 0.926	1.016 ± 0.352	0.667 ± 0.445	0.591 ± 0.127
DJC	3.000 ± 1.000	2.314 ± 0.388	0.909 ± 0.218	0.673 ± 0.466	0.559 ± 0.069
DPJ	2.800 ± 0.837	2.546 ± 0.973	0.913 ± 0.376	0.503 ± 0.432	0.550 ± 0.197
XLC	2.800 ± 1.304	1.818 ± 0.882	0.611 ± 0.537	0.333 ± 0.437	0.335 ± 0.303
PB	3.000 ± 0	2.151 ± 0.546	0.839 ± 0.223	0.443 ± 0.456	0.504 ± 0.155
SCZ	2.600 ± 0.894	2.013 ± 0.770	0.729 ± 0.381	0.425 ± 0.527	0.442 ± 0.213
SG	2.600 ± 0.894	1.960 ± 0.517	0.739 ± 0.251	0.567 ± 0.518	0.463 ± 0.133
HFH	2.600 ± 1.140	2.050 ± 0.918	0.697 ± 0.512	0.424 ± 0.490	0.410 ± 0.296
Mean	2.843 ± 0.275	2.138 ± 0.200	0.782 ± 0.104	0.475 ± 0.121	0.463 ± 0.058

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Table 5(on next page)

AMOVA of 14 *Isoetes yunguiensis* populations using EST-SSR markers

d.f., degrees of freedom.*, $p < 0.001$, Fst, degree of population differentiation

Source of variation	d.f.	Sum of squares	Variance components	Percentage variation
Among populations	13	89.536	0.25247 Va	22.25
Within populations	320	282.234	0.88198 Vb	77.75
Total	333	371.769	1.134	Fst = 0.223*

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Table 6(on next page)

Haploidy and sequence characteristics of chloroplast DNA

Hd: Haplotype diversity; π: Nucleotide diversity

Population code	Haplotype (number)	Hd	π
GJS	H1 (8)	0.00000	0.00000
DZ	H1 (9), H2 (2)	0.32727	0.00040
XB	H1 (11), H2 (4)	0.41905	0.00051
LS	H1 (9), H2 (5), H3 (1)	0.56190	0.00060
GP	H1 (7), H2 (1), H3 (4), H4 (1), H5 (1)	0.70330	0.00017
BSH	H1 (4), H2 (2), H3 (3), H6 (1)	0.77778	0.00012
CX	H7 (12)	0	0
DJC	H1 (9), H2 (1), H8 (1)	0.34545	0.00011
DPJ	H1 (7), H2 (2), H3 (1), H9 (1), H10 (1)	0.66667	0.00101
XLC	H1 (9), H2 (4), H11 (1), H12 (1)	0.60000	0.00048
PB	H1 (10), H2(2), H3 (1), H13 (1)	0.49451	0.00046
SCZ	H1 (4), H2 (1), H4 (1), H14 (1), H15 (1)	0.78571	0.00056
SG	H1 (6), H2 (3), H16 (1), H17 (1), H18 (1)	0.72727	0.00071
HFH	H1 (4), H2 (3), H19 (1), H20 (1), H21 (1)	0.80000	0.00034

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Table 7 (on next page)

AMOVA of 14 *Isoetes yunguiensis* populations using cpDNA markers

d.f., degrees of freedom. *, $p < 0.001$, Fst, degree of population differentiation

Source of variation	d.f.	Sum of squares	Variance components	Percentage variation
Between populations	13	53.826	0.299 Va	34.00
Within populations	153	88.875	0.581 Vb	66.00
Total	166	142.701	0.880	Fst = 0.34*

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