Phylogeography of the rare and endangered fern *Isoetes yunguiensis*

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Background. *Isoetes yunguiensis* is a fern 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 study of the evolution of lithophytes in Yunnan–Guizhou Plateau. In recent years, human activities have caused the disappearance of many wild populations, and the number of plants in the existing population 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 non-coding 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.** In analysis with EST-SSR markers, *I. yunguiensis* showed high genetic diversity, and genetic variation between populations was significantly higher than that within 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* 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.

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Abstract

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Introduction

Isoetes, 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, lending great convenience in studies of the origins and evolution of pteridophytes (Zhang and

Taylor, 2013). Six species of *Isoetes* ~~are endemic to~~ China. Of these, *Isoetes orientalis* and *I. shangrilaensis* 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. Thus, the protection of the existing populations is urgent. *I. yunguiensis* is very similar in appearance to *I. japonica* 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 sp. nov. Subsequently, Xianchun Zhang synonymized this species as *I. chingiana* (unpublished) to honor Qin's work (Zhang, 2001). 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 of *I. yunguiensis* have mainly focused on morphology (Zhao et al., 2014), palynology (Liu et al., 2013), and molecular genetics (Ma et al., 2018; Dong et al., 2018). However, no studies of its evolution and biogeography have been published. Phylogeographic and genetic diversity studies of *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 current *I. yunguiensis* distribution patterns 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 the Biological Resources Laboratory of Guizhou

University. Figure 1 presents a map of sampling locations and geographical distribution of haplotypes. At the same time, 100 ml water samples were taken to the laboratory.

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

DNA was extracted from dried leaves using the TIANGEN Plant Genomic DNA Extraction Kit (DP305), and PCR analyses were performed using the EST-SSR primers SJSSR2, SJSSR6, SJSSR7, SJSSR8, and SJSSR11 and the cpDNA primers trnS-trnG, psbC-trnS, and psbD-trnT. PCR reactions were performed in 25-μL mixtures containing 12.5 μL 2× T5 Super PCR Mix, 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 pre-denaturation 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, and extension at 72°C for 1 min, and final extension at 72°C for 30 min. Reactions with cpDNA primers were performed with pre-denaturation 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, and 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. GeneMaker 2.2 was used to analyze 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 and sequenced using ABI 3730.

Data 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). Original peak images of non-coding cpDNA regions were manually checked using Chromas 2.6 and aligned using MEGA 7.0. DnaSP v5 (Librado & 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 group expansion events for *I. yunguiensis*, and Permut 2.0 (Pons & 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 (Excoffier et al., 2010) and IQ-TREE (Nguyen et al., 2014). A median-joining network map was constructed using Network 5.0 (Bandelt et al., 1999). 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). Data of 19 bioclimatic environmental variables were downloaded from the World Climate Data website (www.worldclim.org). 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, 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

Genetic diversities of EST-SSR loci in *I. yunguiensis* populations are shown in Table 2. Mean Na was 2.8343, ranging from 1.929 (SJSSR11) to 4.000 (SJSSR6). Mean Ne was 2.138, ranging from 1.521 (SJSSR11) to 2.981 (SJSSR6). Mean Shannon information index (I) was 0.782 ranging from 0.417 (SJSSR11) to 1.175 (SJSSR6). Ho ranged from 0 (SJSSR7) to 0.960 (SJSSR2), and He ranged from 0.273 (SJSSR11) to 0.642 (SJSSR6). Mean Ho and He were 0.475 and 0.463, respectively. PIC was high at a mean of 0.516, ranging from 0.313 (SJSSR11) to 0.641 (SJSSR6). Genetic diversity was the highest for SJSSR6 loci and the lowest for SJSSR11 loci.

Genetic diversity and structure of populations

Genetic diversity of different populations is shown in Table 3. Mean Na was 2.843, ranging from 2.4 (LS) to 3.4 (CX and XB). Mean Ne was 2.138, ranging from 1.777 (LS) to 2.675 (CX). 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. 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 4). The average interpopulation gene flow rate (Nm) was 1.534, which is considered high and indicates frequent gene exchange between populations. These observations are consistent with the lower genetic variation between populations than within populations.

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

Genetic relationships and cluster analyses

Unweighted pair group method with arithmetic mean (UPGMA) cluster analyses were performed based on 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 included only a unique population from the Yunnan Plateau, and the remaining 13 groups included populations from the Guizhou Plateau. With a genetic distance of 0.1, the second group was subdivided into groups IIa and IIb.

Group IIa predominantly included populations from the west of Guiyang City, and group IIb included 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* population. At K=2, ΔK reached the maximum value, and *I. yunguiensis* individuals were divided into two groups (Fig. 5). Group I (green; Fig. 6) mainly included individuals of the CX population, and group II included 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 the 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. 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 5.

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 tested 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 3) 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 within populations. Nm was 2.18, indicating a great gene flow among *I. yunguiensis* populations.

Population history dynamics based on cpDNA sequences

DnaSP v5 was used to test the combined cpDNA sequence of all 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 all significantly negative. The population growth index obtained by LAMARC simulation was 921.590 (G>200); all these results indicated 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. *I. flaccida* and *I. vallda* were used as outgroups. The 21 haplotypes were divided into two branches: the first branch including H1–H6 and H8–H21 and the second including H7 alone.

A mediation-links (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 the H1 and H2 diverged to form recent haplotypes.

Niche modeling

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

Discussion

Genetic diversity and structure

Compared with other rare, endemic, and endangered plants, such as *Ottelia acuminata jingxiensis* (He = 0.441; Li et al., 2019), *Dalbergia odorifera* (He = 0.37; Liu et al., 2018), and *Brasenia schreberi* (He = 0.256; Li et al., 2018), *I. yunguiensis* showed high genetic diversity. Most rare and endangered plants are seed plants that can achieve higher 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. In the present study, however, 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 within populations was higher than that between populations, which is consistent with the previously reported trends. 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 ($p>0.05$); these results indicate 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, 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 the results of UPGMA culture analysis, PCoA, and IQ-TREE based on Nei's genetic distances. The 2 groups are separated by the Wumeng Mountains, which follow the border between Yunnan and Guizhou Plateaus. Because water is the main medium through which *I. yunguiensis* spores are transmitted, the mountains between these provinces have isolated these *I. yunguiensis* populations, 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 difference in the CX population from Yunnan Plateau and the remaining 13 population 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 the 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 showed 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 *I. yunguiensis* habitat and dispersal. Few ancient haplotypes may have been randomly fixed in the population during migration, and young haplotypes could have then been fixed in the population due to 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, haplotypes polymorphism is gradually reduced. According to epigenetic theories, isolated populations 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 on the Guizhou Plateau. The CX population from the Yunnan Plateau contained 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 this study, and the specific location of the extinct population on the plateau remains unknown.

Conservation strategies for *I. yunguiensis*

Typically, the seed germination rates of endangered plants are thought to be low, leading to steady decline in populations, relatively narrow island-like geographical distributions, and disadvantages such as competitors in ecological communities. According to previous investigations, wild populations of *I. yunguiensis* have specific niche requirements and inhabit ditches, abandoned paddy fields, or lakesides. Thus, water quality in all sampled areas was measured; the pH of water was weakly alkaline (7.28–7.81), which is similar to the value reported by Liu et al. (2003) in Pingba (7.75). Other studies have identified few populations showing island-like narrow distributions, with some populations being restricted to very small areas, showing an obvious declining trend. In the present study, the XLC, XB, LS, GP, and PB populations included more than 100 individuals, while the remaining included less than 100, with some showing less than 50 individuals. *I. yunguiensis* is not dominant in the ecological community, as indicated by the pattern of origin of the PB population in this study. Previously, a wall was built in this region to protect *I. yunguiensis*; however, the survey indicated that only 10 *I. yunguiensis* inhabited areas within the walls, and these areas were dominated by companion species, which gradually reduced the number of *I. yunguiensis* individuals, forcing them to the verge of extinction. Outside the walls, however, *I. yunguiensis* formed islands of high plant density (~200 plants/m^2); numerous seedlings and no other companion species were noted in these communities. Some studies have shown poor growth of *I. yunguiensis* in mixed communities, indicating that companion species strongly influence its growth. Various conservation strategies have been proposed by many researchers. Liu (2005) suggested that a sustainable strategy be developed to conserve *I. yunguiensis* and other aquatic plants; controlling eutrophication and maintaining optimum water quality are few of the recommended means. This author also suggests using local and integrated conservation strategies and strengthening relevant biological research and public education. Based on the studies of *I. yunguiensis* community characteristics in Nayong Gongtong Nature Reserve, Gao et al. (2015) suggested that protective measures and artificial propagation techniques for *I. yunguiensis* should be strengthened. Likewise, Yuan et al. (2009) have suggested the establishment of protected areas for *I. yunguiensis* in the Pingba County. The present study showed that the BSH, CX, DJC, DPJ, GJS, SG, and PB populations showed high genetic diversity. Understanding of genetic diversity and population structure is important for establishing scientific and effective protection measures. Therefore, to protect the endangered *I. yunguiensis* plants, a provincial nature reserve has been established; the DPJ population in this study was sampled from that reserve. This population, however, has been difficult to protect. 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 the Longli Forest Farm; from this farm, seven populations were sampled, 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 the Longli Forest Farm and training of staff and residents of the surrounding areas in terms of the care of *I. yunguiensis* are warranted. In a previous study, populations with high genetic diversity were selected from similar environments for transplantation (Zhang, 2016). According to 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, Guizhou and Yunnan Plateaus could be divided into 2 regions to protect populations with high genetic diversity as well as 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 Guizhou and Yunnan Plateau 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 this study is similar to that reported previously. Genetic variation within populations was significantly greater than that 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 14 populations can be divided into two groups: Yunnan Plateau and Guizhou Plateau. This indicated that there are at least two refuges for *I. yunguiensis* during the glacial period. Because water flow is the key cause of the spread of *Isoetes*, 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 this study, only one population was found in the Yunnan Plateau. Further investigation is needed to identify the new population and discuss the area of refuge. At present, the cause of endangerment of *I. yunguiensis* remains unclear, and related research is urgently needed.

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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.

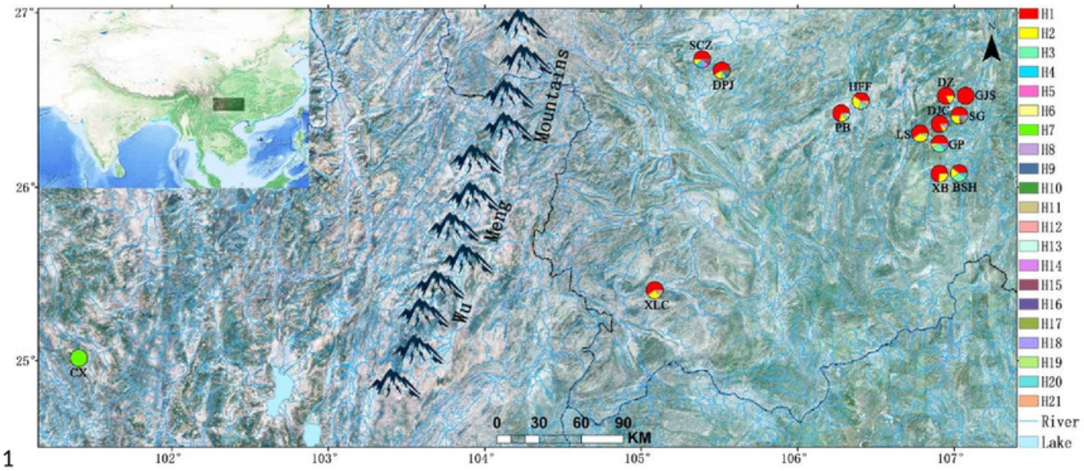

Figure 1: Sampling locations and geographical distributions of haplotypes.

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 the EST-SSR markers.

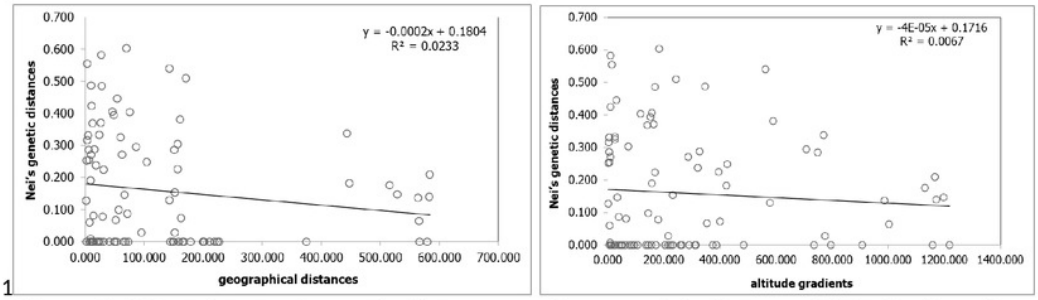

Figure 2: Mantel tests of genetic distances and geographic distances/altitudes based on the EST-SSR markers.

Figure 3

UPGMA cluster analysis of 14 *I. yunguiensis* populations based on the EST-SSR markers.

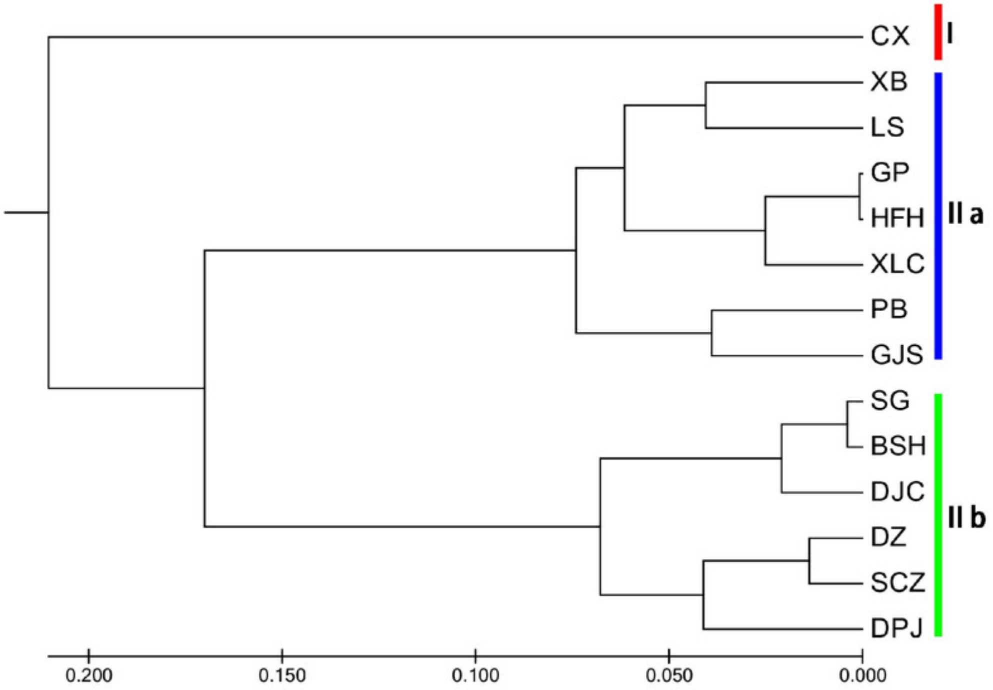

Figure 3 UPGMA cluster analysis of 14 *I. yunguiensis* populations based on the EST-SSR markers.

Figure 4

PCoA analysis of *I. yunguiensis* populations based on the EST-SSR markers.

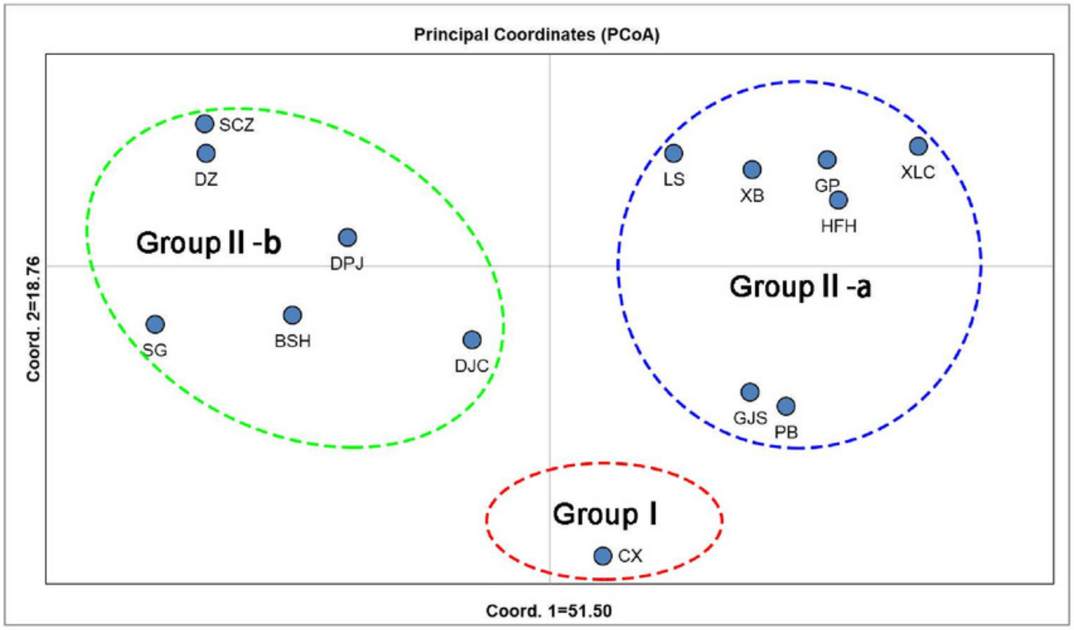

Figure 4: PCoA analysis of *I. yunguiensis* populations based on the EST-SSR markers.

Figure 5

Association between ΔK and K in the STRUCTURE analyses.

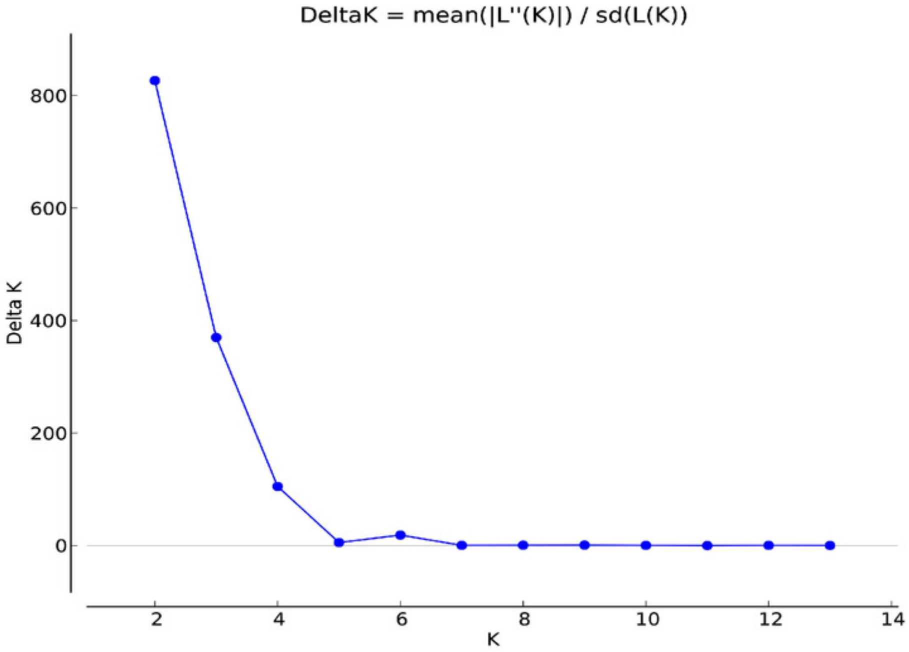

Figure 5: Association between ΔK and K in the STRUCTURE analyses.

Figure 6

STRUCTURE analyses of 14 *I. yunguiensis* populations.

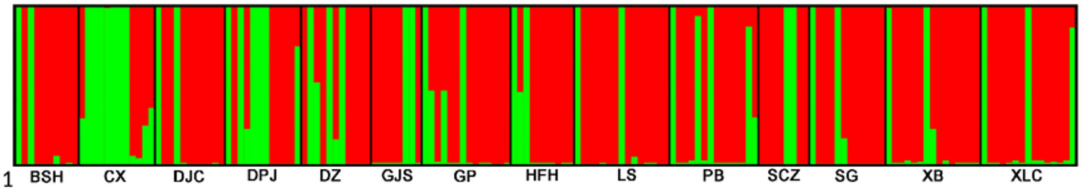

Figure 6: STRUCTURE analyses of 14 *I. yunguiensis* populations.

Figure 7

Mismatch distribution analysis among different inferred biogeographical groups.

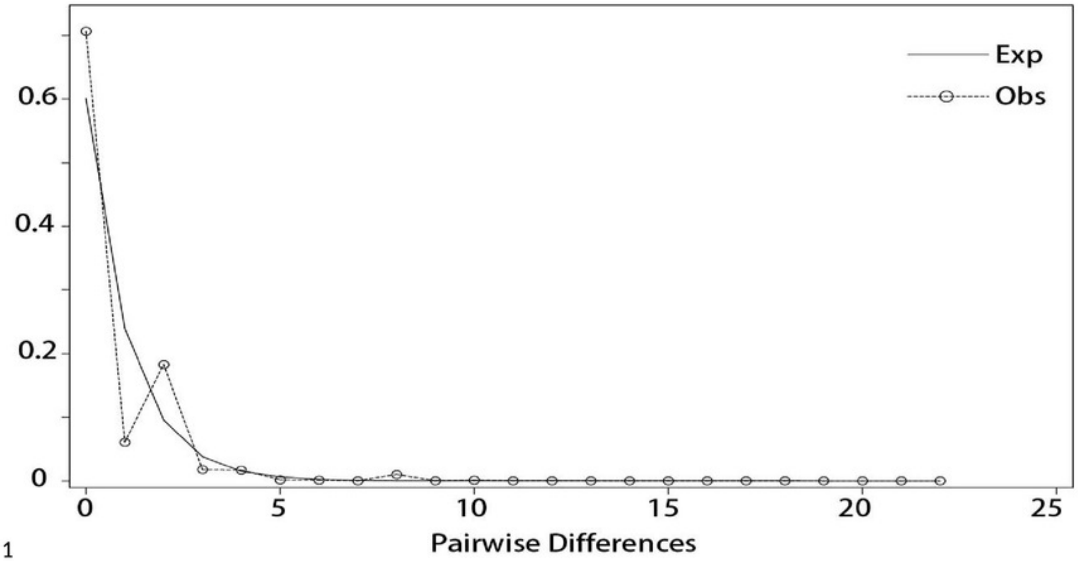

Figure 7: Mismatch distribution analysis among different inferred biogeographical groups.

Figure 8

Phylogenetic tree of *I. yunguiensis* haplotypes constructed using IQ-TREE.

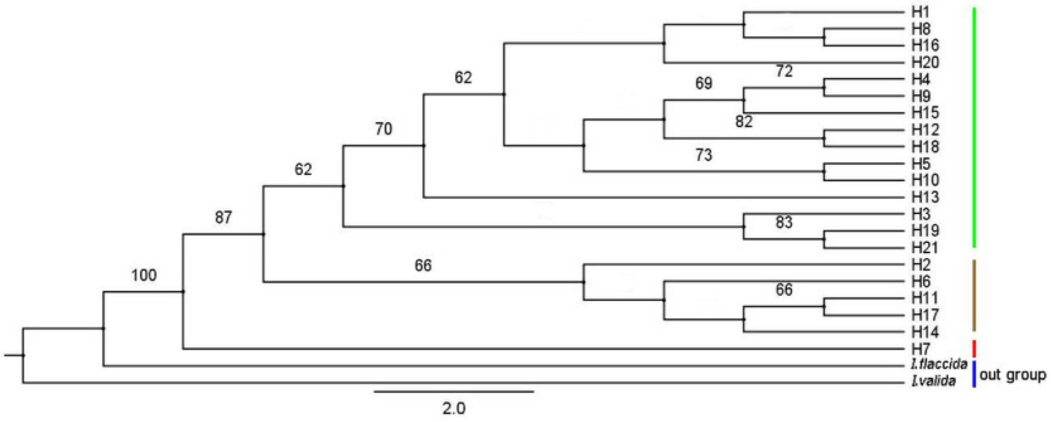

Figure 8: Phylogenetic tree of *I. yunguiensis* haplotypes constructed using IQ-TREE.

Figure 9

Median-joining network reflecting haplotype relationships.

Small open red circles represent potential intermediate haplotypes with other mutational steps between true haplotypes. Circle sizes are proportional to haplotype frequencies.

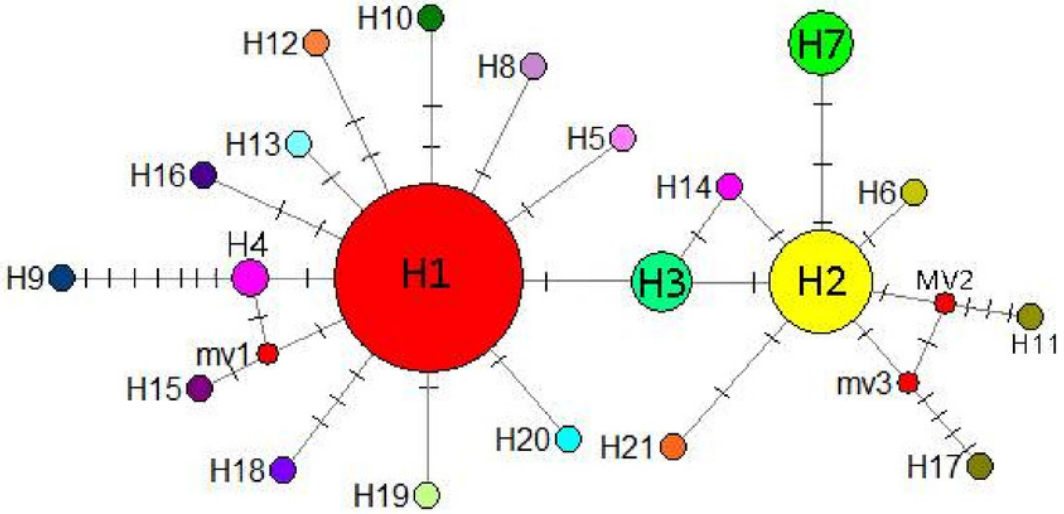

Figure 9: Median-joining network reflecting haplotype relationships.

Small open red circles represent potential intermediate haplotypes with other mutational steps between true haplotypes. Circle sizes are proportional to haplotype frequencies.

Table 1(on next page)

Details of sampling locations of the 14 *I. yunguiensis* populations.

Table 1:

Details of sampling locations of the 14 *I. yunguiensis* populations.

Population code	Population geographic location	Latitude	longitude	Altitude (m)	Nind
GJS	Gengjia Mountain, Longli Forest Farm	106°58′E	26°30′N	1276	8
DZ	Dazhu Village, Longli County	106°57′E	26°31′N	1281	11
XB	Xinbai, Huishui County	106°54′E	26°04′N	1289	15
LS	Hongxing Village, Longli County	106°50′E	26°18′N	1445	15
GP	Huaxi, Guiyang City	106°49′E	26°17′N	1461	14
BSH	Baishuihe, Huishui County	106°56′E	26°05′N	134	10
CX	Zixi Mountain, Chuxiong City	101°24′E	25°01′N	2447	12
DJC	Luyuan Resort, Longli County	106°54′E	26°22′N	1603	11
DPJ	Dapingjing Wetland Park, Nayong County	105°27′E	26°40′N	2024	12
XLC	Xinglongchang, Xingren County	105°05′E	25°24′N	1540	15
PB	Pingba, Anshun City	106°17′E	26°25′N	1316	14
SCZ	Shuichanzhan, Nayong County	105°23′E	26°44′N	1677	8
SG	SG, Longli Forest Farm	106°56′E	26°27′N	1229	12
HFH	Hongfenghu, Qingzhen City	106°24′E	26°30′N	1250	10

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

Table 2(on next page)

Polymorphism analysis of the EST-SSR primers for 5 loci.

Table 2:

Polymorphism analysis of the EST-SSR primers for 5 loci.

Loci	Na	Ne	I	Ho	He	PIC
SJSSR2	2.929	2.361	0.899	0.960	0.565	0.552
SJSSR6	4.000	2.981	1.175	0.828	0.642	0.641
SJSSR7	2.857	2.191	0.869	0.000	0.520	0.553
SJSSR8	2.500	1.637	0.550	0.118	0.316	0.519
SJSSR11	1.929	1.521	0.417	0.468	0.273	0.313
Mean	2.843	2.138	0.782	0.475	0.463	0.516

Notes: 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.

Table 3(on next page)

Genetic diversity of 14 *I. yunguiensis* populations in analyses using EST-SSRs.

Table 3:

Genetic diversity of 14 *I. yunguiensis* populations in analyses using EST-SSRs.

Population	Na	Ne	I	Ho	He
BSH	3.200	2.407	0.956	0.578	0.569
CX	3.400	2.675	1.016	0.667	0.591
DJC	3.000	2.314	0.909	0.673	0.559
DPJ	2.800	2.546	0.913	0.503	0.550
DZ	2.800	2.245	0.803	0.400	0.466
GJS	2.600	2.116	0.746	0.600	0.455
GP	2.600	2.018	0.645	0.400	0.370
HFH	2.600	2.050	0.697	0.424	0.410
LS	2.400	1.777	0.595	0.386	0.362
PB	3.000	2.151	0.839	0.443	0.504
SCZ	2.600	2.013	0.729	0.425	0.442
SG	2.600	1.960	0.739	0.567	0.463
XB	3.400	1.843	0.749	0.251	0.407
XLC	2.800	1.818	0.611	0.333	0.335
Mean	2.843	2.138	0.782	0.475	0.463

Notes: 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.

Table 4(on next page)

AMOVA of 14 *I. yunguiensis* populations using EST-SSRs.

Table 4:

AMOVA of 14 *I. yunguiensis* populations using EST-SSRs.

Source of variation	d.f.	Sum of squares	Variance components	Percentage variation
Among populations	13.000	89.536	0.25247 Va	22.250
Within populations	320.000	282.234	0.88198 Vb	77.750
Total	333.000	371.769	1.134	FST=22.25% (p<0.01)

Note: d.f., degrees of freedom.

Table 5(on next page)

Haploidy and sequence characteristics of chloroplast DNA

Table 5:

Haploidy and sequence characteristics of chloroplast DNA

Population code	Chlorotype	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)	0.00000	0.00000
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

Note: Haplotypes and lineage distributions, and estimated haplotype diversity (Hd) and nucleotide diversity (π) are shown.

Table 6(on next page)

AMOVA of populations and geographical groups.

Table 6:

AMOVA of populations and geographical groups.

Source of variation	d.f.	Sum of squares	Variance components	Percentage variation
Among 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 (p<0.01)

Note: d.f., degrees of freedom.