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Fragmented habitat drives significant genetic divergence in the Chinese endemic plant, *Urophysa henryi* (Ranunculaceae)

Deng-Feng Xie, Lin Zhang, Hao-Yu Hu, Xian-Lin Guo, Xing-Jin He*

Key Laboratory of Bio-Resources and Eco-Environment of Ministry of Education, College of Life Sciences, Sichuan University, Chengdu 610065, China

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ABSTRACT

Urophysa henryi (Oliv.) Ulbr., endemic to China with small populations, is known as a medicinal plant. In this study, ISSR markers were used to assess the genetic diversity and population structure throughout its entire distribution areas. Twelve primers revealed high genetic diversity at the species level ($PPB = 95.6\%$; $H = 0.3441$; $I = 0.5111$), as well as high level of genetic differentiation ($F_{ST} = 0.659$, $p < 0.001$; $G_{ST} = 0.677$) and restricted gene flow ($Nm = 0.239$) among populations. According to the UPGMA and PCoA analysis, the 9 populations were clustered into three main groups, which were roughly in accordance with their geographical regions. In addition, a significant correlation between the genetic difference and geographic distances among populations was detected from the IBD analysis ($r = 0.516$, $p = 0.003$). These results indicated that the habitat heterogeneity and physical barriers play important roles in the modern distribution pattern and population divergence of *U. henryi*. However, human activities have posed serious threat to its living environment and continued survival. It is necessary to adopt some measures to restrict anthropogenic disturbances and preserve the existing populations.

1. Introduction

The Guizhou Plateau and its adjacent regions are one of the most famous models of karst landform in the world with a wide area of 1.3×10^5 km^2 (Su, 2002). However, due to the vulnerability of these limestone areas characterized by the fluctuating ecological environments with an alternation between severe erosion in the rainy season and extreme drought in the long dry season that are stressful for plant growth (Huang et al., 2008), coupled with the irrational development and utilization of mankind, many species have become extinct in recent decades, such as *Tetrastigma dedavyi*, *Rotul aaquatica* and *Lepteodermis potaninii* (Zou, 2001). Some species have been extirpated from much of their historical ranges and now persist only in small, fragmented populations (Zou, 2001). Therefore, researching the genetic diversity and divergence of species in the Guizhou Plateau and adjacent areas can help people to understand the living status of the endemic species in these regions, and promote the protective measures.

Urophysa henryi (Oliv.) Ulbr. (Raunuculaceae) is an endemic perennial herb and known by people due to the medicinal value in treating contusion and bruise. It possesses unusual floral organs such as petaloid sepals, the staminodium, and petals with a saccate nectary, which allow the study of evolutionary novelties (Fig. 1) (Voelckel et al., 2010). Although the pollination

* Corresponding author.
E-mail address: xjhe@scu.edu.cn (X.-J. He).

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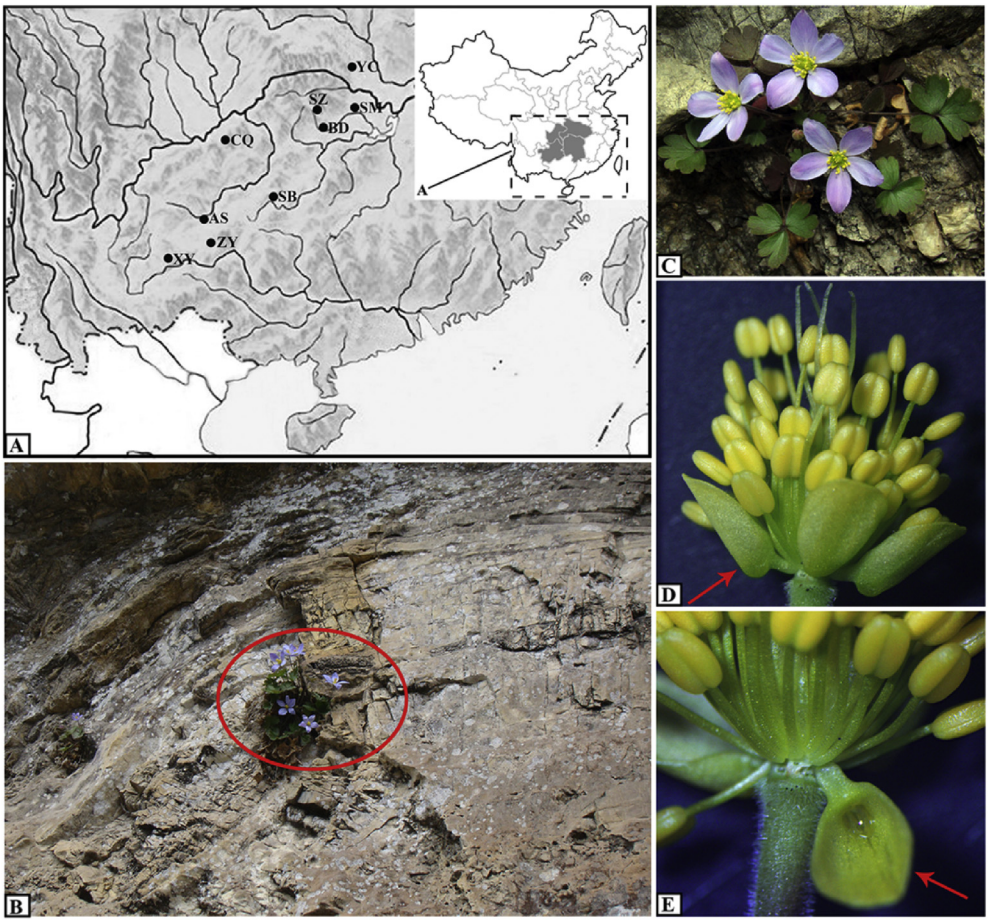

Fig. 1. Sampling localities and the morphological characters of *Urophysa henryi*. **A**: Sampling localities and geographical distribution; **B**: Habitat of *U. henryi* (circled in red line); **C**: Flowers; **D**: Spurred petal (pointed to with the red arrow); **E**: Nectar in the saccate petal (pointed to with the red arrow). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

biology of *U. henryi* has not been reported, the bisexual flowers of this species are possibly pollinated by bee and hawkmoth, as reported for its sister species *Urophysa rockii* (Oliv.) Ulbr. (Zhang et al., 2013). The fruit of *U. henryi* is dehiscing, five-carpel follicle, and exhibit gravity-dependent seed dispersal (similar to *U. rockii*). Populations of *U. henryi* are mainly distributed in the Guizhou Plateau and its adjacent regions (south Chongqing, north Hunan and west Hubei). Only very few populations were found so far and most of them appeared at steep and karstic cliffs separated by high mountains and deep valleys. In addition, human activities (tourism scenic spots and dam construction and excessive exploitations) have made serious damage to its living environment in recent decades. Unfortunately, less attention has been paid to *U. henryi*.

Most previous studies in the genus *Urophysa* were focusd on *U. rockii*, which is an endangered species. Zhang et al. (2013) found high genetic diversity at the species level but low genetic differentiation among *U. rockii* populations based on the ISSR markers. No genetic studies on *U. henryi* have been reported, although a few cases involving in such distribution pattern similar to *U. henryi* have been documented in these regions (Wang et al., 2009, 2014). Thus, *U. henryi* provides an excellent model to research the effects of karst habitat on organisms.

Microsatellite molecular markers such as ISSR fragments were developed by Zietkiewicz (Zietkiewicz et al., 1994). They have been widely used to investigate the genetic diversity and genetic structure of species (Qi et al., 2015; Tiwari et al., 2015; Zhao et al., 2016) due to their extensive polymorphisms and large numbers of highly informative and reproducible alleles relative to other markers (Hassanpour et al., 2013; Ferreira et al., 2015). The specific aims of our study are: (i) to evaluate the genetic diversity and differentiation of *U. henryi*. (ii) to investigate the effects of fragmented habitat on diversification and variation of *U. henryi*.

2. Materials and methods

2.1. Plant sampling

A total of 88 individuals from 9 wild populations covering almost the entire geographical ranges of *U. henryi* were collected (Table 1), and individuals were collected at least 10 m apart to avoid close relatives. Fresh leaves were dried in silica gel until total DNA was extracted. Voucher specimens were deposited in the Herbarium of Sichuan University (SZ).

2.2. DNA extraction and ISSR PCR

Total genomic DNA was extracted from dried leaves using plant genomic DNA kit (Tiangen Biotech, Beijing, China). ISSR primers were synthesized according to the primer set published by the University of British Columbia (UBC). Initially, a total of 73 primers were screened, of which, 12 primers that produced bright and discernible bands were employed for further analysis.

Polymerase chain reactions (PCRs) were performed in a 20 μL reaction volume containing 1.0 μL DNA (60 ng/μL), 2.0 μL reaction buffer (10 X), 1.2 μL $MgCl_2$ (25 mM), 0.4 μL dNTP mix (10 mM), 1.0 μL of each primer (10 pmol), 0.4 μL Taqpolymerase (2.5 U/μL) and 14 μL ddH_2O. The amplifications were performed with the following program: initial denaturation at 94°C for 5 min; 35 cycles of 94°C for 45 s, appropriate annealing temperature (see Table 2 for details) for 45 s, 72°C for 1 min; the last extension at 72°C for 10 min. Negative controls with no DNA added were employed in each PCR run to ensure quality.

The ISSR products were separated in 2% agarose gels (1 × TBE buffer), stained with ethidium bromide (0.5 mg/mL) and subjected to horizontal electrophoresis for two hours with a constant voltage (100 V). DL 2000 ladder (Shanghai Sangon Biological Engineering Technology and Service Co., Ltd., China) was used to estimate the sizes of bands. Ultraviolet light was applied to visualize the amplicons by using Bio-Rad Gel Documentation System (Bio-Rad Laboratories, UK).

2.3. Statistical analyses

A binary data matrix was constructed by scoring ISSR bands as 1/0 for the presence/absence of homologous bands for all samples. The program POPGENE version 1.32 (Yeh et al., 1999) was employed to calculate various genetic diversity parameters: the percentage of polymorphic bands (*PPB*), Shannon's information index (*I*), Nei's gene diversity (*H*), gene flow (N_m) and gene differentiation coefficient (G_{ST}). The analysis molecular variance (AMOVA) in Arlequin version 3.5 (Excoffier and Lischer, 2010) was used to measure the partitioning of genetic variability within and among populations.

NTSYS-pc v.2.21 (Rohlf, 2009) was used to perform the cluster analysis and construct the UPGMA (Unweighted Pair Group Method with Arithmetic mean) based on the obtained genetic distance matrix. The Principal Coordinates Analysis (PCoA) and Mantel test were carried out to group the populations and reveal the correlation between genetic and geographic distance matrices respectively using the GenALEx v.6.1 (Peakall and Smouse, 2006).

Table 1
Sample locations and genetic diversity of *Urophysa henryi*.

Population code	Location (all in China)	Sample size	Alt. (m)	Latitude	Longitude	Number of polymorphic loci	*PPB* (%)	*Na*	*Ne*	*H*	*I*
SZ	Sangzhi, Hunan province	10	426	29°40′	110°03′	46	29.1	1.2911	1.1736	0.1027	0.1540
BD	Sangzhi, Hunan province	10	434	29°39′	109°49′	49	31.0	1.3101	1.2142	0.1199	0.1754
SM	Shimen, Hunan province	10	267	29°56′	110°56′	51	32.3	1.3228	1.2238	0.1255	0.1836
YC	Yichang, Hubei province	10	245	30°42′	111°17′	38	24.1	1.2405	1.1498	0.0865	0.1289
CQ	Nanchuan, Chongqing municipality	8	523	30°04′	90°33′	39	24.7	1.2468	1.1722	0.0972	0.1424
SB	Shibing, Guizhou province	10	516	27°03′	108°19′	40	25.3	1.2532	1.1600	0.0924	0.1373
AS	Anshun, Guizhou province	10	627	26°13′	105°23′	53	33.5	1.3354	1.2481	0.1379	0.2001
ZY	Ziyun, Guizhou province	10	1082	25°41′	106°05′	53	33.5	1.3354	1.2287	0.1289	0.1890
XY	Xingyi, Guizhou province	10	961	25°08′	104°57′	43	27.2	1.2722	1.1960	0.1105	0.1610
Mean						45.8	29.0	1.2897	1.1963	0.1113	0.1635
Group I						111	70.3	1.7025	1.4281	0.2495	0.3722
Group II						87	55.1	1.5506	1.3735	0.2108	0.3097
GroupIII						127	80.4	1.8038	1.5049	0.2932	0.4358
Species level						151	95.6	1.9557	1.5990	0.3441	0.5111

PPB: Percentage of polymorphic bands; *Na*: Mean observed number of alleles; *Ne*: Mean effective number of alleles; *H*: Nei's genetic diversity; *I*: Shannon's information index.

Table 2
Amplified results of ISSR primers and genetic differentiation in this study.

Primers	Sequence (5′–3′)	T_A (°C)	Total amplified bands	*N*	(*PPB*/%)	(G_{ST})	(*N*m)
UBC815	$(CT)_8G$	47.5	15	15	100		
UBC823	$(TC)_8C$	50.5	8	8	100		
UBC834	$(AG)_8YT$	51.5	21	20	95.2		
UBC835	$(AC)_8CG$	50.5	14	14	100		
UBC836	$(AG)_8YA$	50.5	16	16	100		
UBC843	$(CY)_8RA$	47.0	11	11	100		
UBC844	$(CT)_8GC$	47.5	14	13	92.9		
UBC845	$(CT)_8GC$	53.0	11	10	90.9		
UBC850	$(GT)_8YC$	56.8	14	13	92.9		
UBC855	$(AC)_8YT$	47.5	7	7	100		
UBC873	$(GA)_8CA$	58.0	15	13	86.7		
UBC880	$GGA(GAG)_2$ AGGAGA	46.5	12	11	91.7		
Average			13.2	12.6	95.9	0.677	0.239
Group I						0.535	0.435
Group II						0.569	0.379
Group III						0.599	0.334

N: The number of polymorphic bands; *PPB*: Percentage of polymorphic bands; G_{ST}: Coefficient of gene differentiation; *N*m: Gene flow; Y = C/T, R = A/G.

3. Results

3.1. Polymorphism analysis

These 12 selected ISSR primers produced 158 discriminatory amplified molecular fragments, among which 151 (95.6%) were polymorphic bands. Each primer generated 7 (UBC855) to 21 (UBC834) obvious bands, with an average of 13.2 per primer. The size of bands ranged from 200 bp to 2500 bp. Information of bands obtained by each primer was showed in Table 2.

3.2. Genetic diversity

The mean effective number of alleles (*Ne*) ranged from 1.1498 to 1.2481 with 1.5990 at the species level. Nei's genetic diversity (*H*) was estimated to be 0.1113 at average level and 0.3441 at the species level. For the Shannon information index (*I*), the average level and species level were 0.1635 and 0.5111, respectively (Table 1). The percentage of polymorphic bands (*PPB*) changed from 86.7% (UBC873) to 100% with an average of 95.9% (Table 2). Additionally, we detected low level of genetic diversity in each population (Table 1). The genetic diversity of *U. henryi* from population AS ($Ne = 1.2481$, $H = 0.1379$, $I = 0.2001$) was the richest among nine populations and the lowest diversity was revealed in population YC. In terms of geographical regions, populations of group III possess the highest genetic diversity among three groups (Table 1).

3.3. Genetic differentiation among populations

The UPGMA dendrogram revealed the relationship among populations, which clustered the nine populations into three groups (Fig. 2A). The SZ, BD and SM were clustered into the first group, mainly distributed in north Hunan. YC and CQ formed the second group that sampled from west Hubei and Chongqing. The third group included SB, AS, ZY and XY, which were located in the Guizhou Plateau. Results of principal coordinate analysis (PCoA) also agreed with the relationship among individuals in these three groups (Fig. 2B). In addition, the Mantel test detected a significant positive correlation between genetic difference and geographic distances (Fig. 3; $r = 0.516$, $p = 0.003$).

The Nei's coefficient of genetic differentiation (G_{ST}) across the entire populations was 0.677 (Table 2), which means that 67.7% of the total genetic variability was attributed to inter-population and 32.3% to intra-populations. AMOVA analysis also revealed similar results that 65.9% and 34.1% of the total genetic variation was partitioned inter-populations and intra-populations ($p < 0.001$), respectively. The level of gene flow (N_m) was estimated to be only 0.239 among populations. When populations of *U. henryi* were devided into three groups, most genetic diversity occurred among populations (52.3% of the total variance) ($p < 0.001$). The Nei's coefficient of genetic differentiation (G_{ST}) in group III was most obvious among the three groups and gene flow (N_m) in group I was most prominent.

4. Discussion

4.1. Genetic diversity

Our data revealed a high genetic diversity at the species level ($PPB = 95.6\%$; $H = 0.3441$; $I = 0.5111$) and a low genetic variation at the population level (Table 1), these results are similar to previous research on its sister species *U. rockii* (Zhang et al., 2013). Many factors were found to influence genetic diversity, such as living environment, mating system and geographical distribution range (Nybom, 2004). *U. henryi* mainly distributes in the Guizhou Plateau and adjacent regions, and

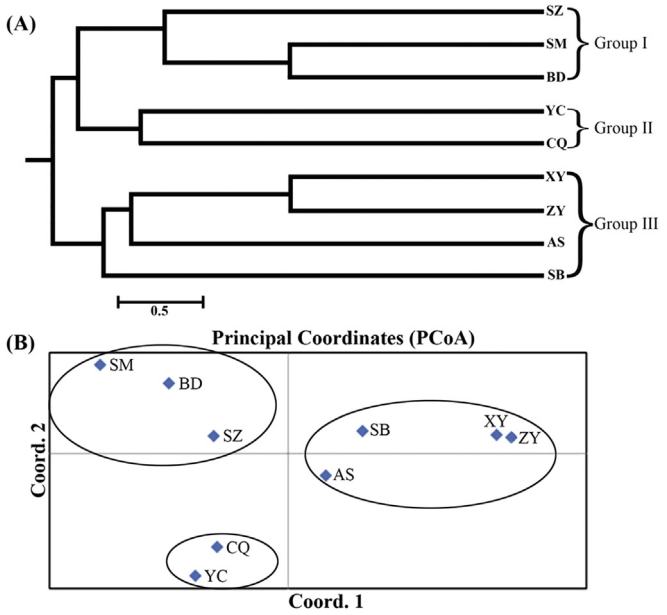

Fig. 2. UPGMA dendrogram **(A)** and Principal Coordinates analysis (PCoA) **(B)** of nine *Urophysa henryi* populations.

its populations grow on cliffs or fissures of rocks (Fu and Orbelia, 2001) (Fig. 1). Such special environment led to the continued ancient populations with high genetic variation separating into different small populations and provided ample opportunities for isolation, drift and mutation to keep a high level of genetic variability (Wang et al., 2009). Propagation method of plant species is also regarded as one of the most important factors determining the levels of genetic diversity (Hamrick and Godt, 1996). Self-compatibility is a wide-spread phenomenon in species of Aquilegiinae Tamura and their variation in spur color and length has been demonstrated to be adaptative for different pollinators (Fior et al., 2013; Li et al., 2014). Thus, as a member of Aquilegiinae Tamura, inbreeding depression is much more likely to occur and ultimately result in low level of genetic variability in populations of *U. henryi*. Moreover, populations of *U. henryi* isolate from each other far away and each population produces a great number of seeds every year, but only a few seeds can germinate successfully, about less than 2% (Zhang et al., 2013). Such extremely low level of germination rate, coupled with limited number of individuals in each population would have caused genetic drift that could later result in a decrease in genetic diversity and an increase in inbreeding.

4.2. Population genetic structure and differentiation

Significant genetic variation was detected among populations (G_{ST} = 0.677; F_{ST} = 65.9%, $p < 0.001$). The distribution pattern of genetic variability may be ascribed to the following reasons: firstly, as an entomophilous plant, flowers of *U. henryi* deposit nectar into its saccate petals (Fig. 1), which could attract pollinators. However, it blooms in winter when animals' activities are infrequent and most of insects can not spread pollen over a long distance, which may lead to weak pollen flow (Li et al., 2011). In addition, the same to its sister species, seeds of *U. henryi* are small and dispersed to rock fissures based on the

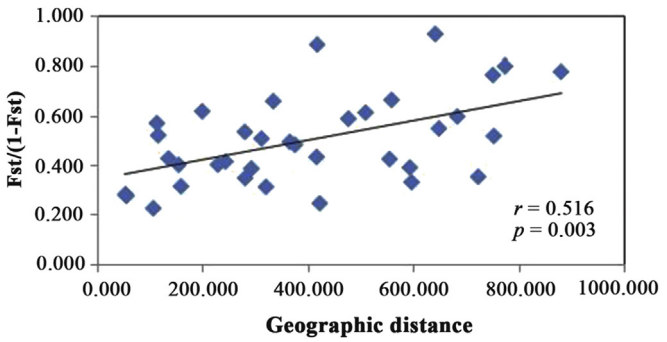

Fig. 3. Scatterplots representing relationships between Plots of genetic difference [Fst/(1 − Fst)] and geographic distance (Km), $r = 0.516$, $p = 0.003$.

mechanical strain of follicles crack spontaneously when seeds matured (Zhang et al., 2013). But most of seeds disperse near the mother plants, which would reduce seed-mediated gene flow among populations and increase differentiation. The restricted gene flow among populations is also corroborated in our result ($Nm = 0.239$). Secondly, the habitats of *U. henryi* are highly fragmented and only a few, relatively small and isolated populations distribute in steep and karstic cliffs of the Guizhou Plateau and adjacent regions, where many high mountains are situated in. These natural barriers separated populations of *U. henryi* into different geographical areas with increasing geographic isolation from each other, reduced or disrupted meta-population dynamics, and probably further aroused the differentiation among populations. This prediction was also supported by the Mantel test, which revealed a significant correlation between genetic difference and geographic distance (isolation by distance) ($r = 0.516$, $p = 0.003$).

Generally, populations sampled from neighboring geographical regions were inclined to cluster together, for instance, the populations in group I (SZ, BD and SM) and group III (XY, ZY, AS and SB), which suggested that populations within each group have a closer genetic relationship. High levels of gene flow among populations could reduce population differentiation and increase genetic similarity (Whitlock and Mccauley, 1999). However, it is noteworthy to mention the populations YC and CQ, which are geographically distributed far away from each other but are genetically closer populations. This observation may be attributed to that individuals of the two populations retained vast ancestral polymorphisms and possessed the similar environmental conditions. We presumed that the ancient populations of *U. henryi* may be widely distributed in the Guizhou Plateau and adjacent areas, and later their habitats fragmented by some geological events or environment changes such as orogenesis and glacial movements. Fortunately, the carbonate substrate of karst landform provide suitable living conditions for *U. henryi*, its populations can evolve independently in different geographical regions and finally lead to such pattern of population genetic structure.

4.3. Considerations for the conservation of U. henryi

Maintenance of genetic diversity and habitat integrity is important for long-term survival of a species or population (Frankel and Soulé, 1981). For *U. henryi*, genetic diversity within population was very low, along with the number of population is limited and individuals in each population are very small, which may be vulnerable to genetic drift or completeness loss and finally lead to species more susceptible to habitat deterioration. Additionally, small fragmented populations are sensitive to inbreeding, which may reduce heterozygosity and fitness-related genetic variation (Reed and Frankham, 2003). Worse, as a traditional Chinese medicinal plant, the habitats of *U. henryi* have been severely damaged by the human activities. In the past decades, many dams and tourism scenic spots have been built in areas where the wild populations of *U. henryi* are located in, which have caused serious damage to its natural habitats and inevitably increased the risk of extinction. Therefore, some relevant protective measures should be carried out. On the one hand, the maintenance of the existing populations and the avoidance of human disturbance are priority requisites. On the other hand, more energy should be focused on artificial cultivation (such as seed germination, plant tissue culture) to keep different genotypes and to increase molecular variance and fitness of individuals in the future.

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