ISSR Analysis on Genetic Diversity of Endangered Plant *Parrotia subaequalis* in Dalonggou of Yixing, Jiangsu

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Abstract: In this study, 25 flower samples of *Parrotia subaequalis* individuals were collected in Dalonggou of Yixing, Jiangsu. ISSR markers were used to analyze the genetic diversity of the 25 individuals. Six primers were selected and used for PCR amplification. The result showed that 59 discernible DNA fragments were amplified, among them 47 are polymorphic loci and the percentage of polymorphic loci (PPB) is 79.7%. By analysis with POPGENE32, the effective number of alleles (Ne) is 1.4697, Nei's gene diversity (H) is 0.2792, Shannon's Information index (I) is 0.4195, the Ewens-Watterson Neutral value is between 0.02 to 1, and more than half of the neutral test values are above 0.5. Through cluster analysis with NTSYSpc-2.1, genetic similarity coefficients of 25 samples are between 0.25 and 1 with an average of 0.71. From the results of this study we can get a conclusion that the genetic differences among the wild populations of *P. subaequalis* in Dalonggou of Yixing, Jiangsu were large and rich in diversity. The aim of this study is to reveal the genetic diversity of wild population of *P. subaequalis* from molecular level and provide a theoretical basis for the protection of this rare and endangered plant.

1 Introduction

Parrotia subaequalis, belonging to the genus *Parrotia* of the Hamamelidaceae family, is one of the oldest angiosperms. It is the only "living fossil" species that has been rediscovered, is also endemic to China. And in the history of plant evolution, it has an important position of serving as a link between past (Gymnosperm) and future (Angiosperm). At the same time, the crown of the *P. subaequalis* blooms and the leaves are reddish purple in autumn. There are wisps of silvery silk in the flowers, and the shape of the flower is peculiar. *P. subaequalis* is also a rare and excellent tree for green ornamental with both flower viewing and autumn foliage. However, due to its narrow distribution area, it is scattered in the northern part of Mount Tianmu and the southeast of Mount Dabie. *P. subaequalis* has been extinct now because the number of its existing populations is extremely small. In 1999, it was listed in the national first-level protected wild plants list^[3,4] and listed by the International Union for Conservation of Nature (IUCN) as a Critically Endangered (CR) species^[5]. It has been proven by practice that endangered species, especially highly specialized single species, are the most unique and irreplaceable in classification or genetics. However, in recent years, the research work on *P. subaequalis* is active and most of the research is focused on morphological anatomy^[6,7], population structure^[8,9], biological characteristics^[10-12], breeding and siring^[13-15]

and other aspects, but the relevant research into the molecular level is very rare^[16].

In this study, we use ISSR (inter-simple sequence repeat) molecular marker technique to study the genetic diversity of the *P. subaequalis* population in Dalonggou of Yixing, Jiangsu. The aim of this study is to reveal the genetic diversity of wild population of *Parrotia subaequalis* from molecular level and provide a theoretical basis for the protection of this rare and endangered plant.

2 Materials and Methods

2.1 Materials

In this study, 25 individual samples of *P. subaequalis* were collected from Dalonggou, Yixing, Jiangsu, and the samples were all flower buds of adult plants. After field collection, the flower buds were quickly taken back into the laboratory in a ziplock bag containing the silica gel desiccant, and rapidly ground with liquid nitrogen and stored in a -80°C refrigerator for later use. The specific collection information of the sample is shown in Table 1.

Table 1 Sample messages of *Parrotia subaequalis*

Number	Longitude	Latitude	Height	Amount
1、2	119°44'53"E	31°15'33"N	300 m	2
3、4	119°44'51"E	31°15'29"N	260 m	2
5、6、7、8	119°44'55"E	31°21'41"N	258 m	4

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9、10、11、12	119°49'53"E	31°15'29"N	245 m	4
13、14、15、16、 17、18、19	119°44'51"E	31°15'15"N	235 m	7
20、21、22、23、 24、25	119°44'52"E	31°15'30"N	230 m	6

2.2 Methods

2.2.1 Genomic DNA Extraction

We took 0.1g of flower bud powder stored in the -80°C refrigerator and the total DNA was extracted by using TIANGEN Plant DNA isolation reagent (Plant DNA isolation Reagent). The concentration and quality of DNA were determined by UV spectrophotometry and 1.5% agarose gel electrophoresis.

2.2.2 ISSR-PCR Reaction System

The PCR reaction system used in this study is a PCR amplification system established and optimized by the research group in the previous stage as follows (25μL system): DNA template 2.5μL (20ng/μL), primer 1.00μL (10μmol/L), Taq enzyme 0.1μL (5U/μL), Mg²⁺ 3.00μL (25mmol/L), and dNTP 1.50μL (2.5mmol/L). The PCR reaction procedure was: pre-denaturation at 95°C for 5 min, then denaturation at 95°C for 30s per cycle, annealing at 54°C/56°C for 30s, extension at 72°C for 2 min, 38 cycles, and finally extension at 72°C for 10 min.

2.2.3 ISSR-PCR Reaction Primer Screening

The six primers used in this study with high reproducibility and polymorphism, these primers were selected by the research group in the previous stage. And the primers are 823, 824, 834, 835, 836, 890. The primer were synthesized by Shanghai Biotech Biotechnology according to the ninth set of ISSR primer sequences published on the University of Columbia (UBC) website. The primers information are shown in Table 2.

Table 2 Amplification results and polymorphism of ISSR amplification

Primer name	Primer sequences	Annealing temperature (°C)	Number of amplified bands	Number of polymorphic bands	Percentage of Polymorphism (%)
823	(TC) ₈ C	56	7	5	71.4
824	(TC) ₈ G	56	9	7	77.8
834	(AG) ₈ YT	56	12	11	91.7
835	(AG) ₈ YC	56	7	4	57.1
836	(AG) ₈ YA	54	12	10	83.3
890	VHV(GT) ₇	54	12	9	75

2.2.4 Data Analysis

In this study, the software POPGEN32 was used to analyze the genetic diversity parameters such as mean effective allele number (Ne), Nei's gene diversity index (H), Shannon information diversity index (I), polymorphic loci and index. And Genetic similarity coefficient calculation and cluster analysis were performed using software NTSYSpc2.1.

3 Results and Analysis

3.1 Polymorphism of ISSR Amplification Product

A total of 59 ISSR amplified bands were obtained by PCR amplification of 25 samples of *P. subaequalis* in Dalonggou, with good polymorphism, high stability and clear bands in the early stage of the research group. There were 47 polymorphic bands with a polymorphic ratio of 79.7%. On the average, there were about 10 amplified bands per primer, and the average number of polymorphic bands was about 7.7. Among them, the amplification effect of primer 834 was the best among the 6 primers. A total of 12 ISSR bands were amplified, and 11 polymorphic bands were obtained. The polymorphic ratio was as high as 91.7%. The amplification electropherogram is shown in Figure 1. And the primer amplification results and polymorphism are shown in Table 2.

Figure 1 ISSR amplification with primer 834
Note: 1~25: Sample 1~25 M: DL2000 Plus DNA Marker

3.2 ISSR Genetic Diversity Analysis

The POPGEN32 software was used to calculate the genetic diversity parameters of the population of *P. subaequalis* in Dalonggou. The results showed that the average effective allele number (Ne) of the 25 plants of *P. subaequalis* was 1.4697, Nei's gene diversity index (H) was 0.2792, Shannon information diversity index (I) was 0.4195, and there are 47 polymorphic loci and the polymorphism index was 79.66%. There is a large difference in each genetic locus. The maximum Nei's index is 0.4983, the minimum is 0, and the standard error (SD) is 0.1782. Shannon information diversity index (I) has a maximum value of 0.6914, a minimum of 0, and a standard error of 0.2492. These data indicate that although there are no geographical differences among individuals in the population of *P. subaequalis* in Dalonggou, there are different degrees of genetic variation between individuals under the conditions of long-term natural selection, and the genetic diversity is relatively rich. The specific genetic diversity indexes are shown in Table 3.

Table 3 Genetic diversity of *Parrotia subaequalis*

Number of samples	Effective number of alleles (Ne)	Nei's gene diversity (H)	Shannon's information index (I)	Number of polymorphic sites	Percentage of polymorphic sites (%)
25	1.4068	0.2356	0.3512	34	68

3.3 Cluster Analysis

A total of 56 bands were generated from the ISSR amplification of 25 individual samples, and the genetic similarity coefficient was analyzed. The results showed that the genetic similarity coefficient of the individual plants of *P. subaequalis* in Dalonggou was between 0.25 and 1, with an average of 0.71. It shows that *P. subaequalis* in Dalonggou of Yixing, has a large genetic

difference at the molecular level and is rich in genetic diversity. Among them, the genetic similarity coefficient of individuals 19 and 20, 24 and 25 individuals is 1, indicating that the relationship between the two individuals is the closest, the genetic similarity coefficient between the 14 and 18 individuals is the smallest, is 0.25, indicating that the two individuals relationship is the farthest and the genetic difference is large (Table 4).

Table 4 The genetic similarity coefficient distances of *Parrotia subaequalis*

Sample number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1	1.00																								
2	0.67	1.00																							
3	0.67	0.67	1.00																						
4	0.58	0.42	0.75	1.00																					
5	0.67	0.50	0.83	0.92	1.00																				
6	0.67	0.67	0.67	0.75	0.83	1.00																			
7	0.83	0.50	0.67	0.58	0.67	0.67	1.00																		
8	0.67	0.67	0.50	0.42	0.50	0.50	0.67	1.00																	
9	0.67	0.83	0.83	0.58	0.67	0.83	0.67	0.50	1.00																
10	0.67	0.83	0.67	0.58	0.67	0.83	0.67	0.50	0.83	1.00															
11	0.75	0.42	0.75	0.67	0.75	0.58	0.75	0.75	0.58	0.42	1.00														
12	0.67	0.50	0.83	0.58	0.67	0.50	0.83	0.67	0.67	0.50	0.92	1.00													
13	0.83	0.67	0.83	0.75	0.83	0.83	0.67	0.67	0.83	0.67	0.75	0.67	1.00												
14	0.67	0.50	0.50	0.58	0.50	0.33	0.50	0.83	0.33	0.33	0.75	0.67	0.50	1.00											
15	0.50	0.50	0.50	0.42	0.33	0.50	0.67	0.50	0.67	0.50	0.58	0.67	0.50	0.50	1.00										
16	0.67	0.50	0.67	0.58	0.67	0.50	0.67	0.83	0.50	0.33	0.92	0.83	0.67	0.83	0.50	1.00									
17	0.83	0.67	0.83	0.75	0.83	0.83	0.67	0.67	0.83	0.67	0.75	0.67	1.00	0.50	0.50	0.67	1.00								
18	0.58	0.58	0.67	0.75	0.92	0.58	0.42	0.75	0.75	0.50	0.42	0.75	0.25	0.42	0.42	0.75	1.00								
19	0.67	0.50	0.83	0.75	0.83	0.67	0.50	0.50	0.67	0.50	0.75	0.67	0.83	0.50	0.33	0.67	0.83	0.75	1.00						
20	0.67	0.50	0.83	0.75	0.83	0.67	0.50	0.50	0.67	0.50	0.75	0.67	0.83	0.50	0.33	0.67	0.83	0.75	1.00	1.00					
21	0.67	0.50	0.67	0.58	0.67	0.67	0.67	0.67	0.67	0.50	0.75	0.67	0.83	0.50	0.67	0.83	0.67	0.58	0.67	0.67	1.00				
22	0.67	0.50	0.67	0.58	0.67	0.67	0.67	0.67	0.67	0.50	0.92	0.83	0.67	0.67	0.83	0.67	0.58	0.67	0.67	0.67	0.67	1.00			
23	0.67	0.50	0.83	0.58	0.67	0.50	0.83	0.67	0.67	0.50	1.00	0.67	0.67	0.67	0.83	0.67	0.42	0.67	0.67	0.67	0.83	1.00			
24	0.67	0.67	0.67	0.58	0.50	0.50	0.67	0.67	0.67	0.67	0.58	0.67	0.67	0.50	0.67	0.67	0.42	0.50	0.50	0.67	0.50	0.67	1.00		
25	0.67	0.67	0.67	0.58	0.50	0.50	0.67	0.67	0.67	0.67	0.58	0.67	0.67	0.50	0.67	0.67	0.42	0.50	0.50	0.67	0.50	0.67	1.00	1.00	

According to the genetic similarity coefficient matrix, the phylogenetic relationship tree was constructed by software NTSYSpc2.1 with UPGMA method. The results showed that when the similarity coefficient was 0.42, the individual samples of No.4 were formed separately. When the similarity coefficient was 0.66, the remaining 24 individual samples clustered into two groups, in which individual samples 1, 17, and 22 are grouped, and the remaining 20 samples are grouped into the other. The similarity coefficients among the individuals in each major category also differed, indicating that the genetic differences among the individuals of the *P. subaequalis* were large, and the population of *P. subaequalis* is rich in genetic diversity(Figure 2).

Figure 2 Phylogenetic relationship of *Parrotia subaequalis* individuals

4 Conclusion and Discussion

The analysis of genetic diversity is useful for assessing the evolutionary potential of the population and predicting its future fate. It is also a prerequisite for the survival and evolution of organisms. It has important guiding significance in the protection and use of endangered wild plant resources^[18,19]. In this study, ISSR genetic diversity analysis was performed on the population of *P. subaequalis* in Dalonggou, Yixing, Jiangsu. The survival and resource status of the endangered plants were analyzed at the molecular level. In this study, the results have shown that there are large genetic differences among 25 individual samples within the population, and polymorphic alleles are abundant, with a polymorphism rate of 79.66%. In general, the abundance of alleles in a population indicates that a high genetic level is maintained in the population, which is conducive to the continuous development of species. Although the geographical distribution of witch *P. subaequalis* is narrow, it is shown at the molecular level that the genetic diversity of which is good, the population has not been completely reduced, and the mating among the relatives within the population is good. However, with the disturbance of human activities and changes in the natural environment, it is necessary to prevent the decline of genetic mating ability within the population and the break of genetic diversity in advance to prevent endangered species from being eliminated in natural selection, such as *P. subaequalis*.

P. subaequalis, as a rare and endangered plant endemic to China, the distribution only in Yixing of Jiangsu province, Jinzhai, Jixi, and Shucheng of Anhui province, Anji of Zhejiang province. Based on the genetic diversity of *P. subaequalis* populations in the Dalonggou of Yixing, this study proposes the following protection recommendations.

First, Delineate protected areas. Establish a nature reserve in the natural growth area of *P. subaequalis*, and correspondingly reduce human activities in the area. Regularly observe and record the flowering and breeding of wild witch hazel plants in the area, and statistically analyze the change law. Second, Strengthen survival and environmental protection. Aiming at the problems of weak photosynthetic capacity and low inter-species competition ability of the *P. subaequalis*, regular field observations of the plants can be carried out to clean up the interfering hybrids around the wild plants, to ensure that the wild *P. subaequalis* can obtain sufficient light, and to improve the utilization of light^[20]. Third, strengthen the intervention in the breeding season of plants. The breeding period of plants is susceptible to the external environment, especially the disturbance of climate change. The rainy weather often affects the spread of the pollen of *P. subaequalis*, the effects of self-propagation and external factors, which leads to the obvious phenomenon of large and small years of *P. subaequalis*. Intervention, artificial pollination of wild plants under necessary conditions to ensure the seed setting rate of *P. subaequalis*^[20]. Fourth, strengthen the study of asexual reproduction of plants. Asexual reproduction is an effective way to achieve rapid

proliferation and population expansion of some organisms in a suitable environment. Among them, cutting propagation and tissue culture are optional high-efficiency and low-cost asexual reproduction measures. Finally, increase the intensity of illegal digging. The plum bark of *P. subaequalis* is mottled, the tree is beautiful, and the autumn purple-red leaves are excellent garden ornamental trees. According to these reasons, the demand of the market has led some criminals to hack and dig wild adult plants, even ancient trees. Therefore, in order to combat theft and digging from the source, we must first ban the market for buying and selling *P. subaequalis* and increase the illegal costs of theft breakers. At the same time, the relevant law enforcement agencies should raise the people's awareness of the current survival situation and legislative protection of *P. subaequalis*, to create a good atmosphere for universal protection.

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