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Genetic Diversity and Genetic Relationship Analysis of *Platycladus orientalis* Germplasm Based on SSR Markers

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Abstract To investigate the genetic diversity and phylogenetic relationships among *Platycladus orientalis* germplasm resources in Zaozhuang City, simple sequence repeat (SSR) molecular markers were used to analyze genetic diversity and relatedness in 100 *P. orientalis* accessions collected from four regions. The results showed that a total of 32 allelic loci were detected using seven pairs of SSR primers, with an average of 6.571 alleles per primer pair. The mean number of alleles (Na) was 3.714, the mean effective number of alleles (Ne) was 1.900, the mean Shannon's information index (I) was 0.818, and the mean expected heterozygosity (He) was 0.440, indicating relatively high genetic diversity among the 100 sampled *P. orientalis* accessions. The Fst value was 0.0371, suggesting a high degree of similarity among populations, small genetic distances, and low genetic differentiation. Cluster analysis based on the estimation of the optimal K value showed that the maximum ΔK occurred at $K=3$, indicating that the 100 *P. orientalis* accessions could be divided into three groups rather than clustering strictly according to geographic origin, which implies the existence of gene flow among the sampled populations. Through preliminary screening and repeated validation, seven pairs of SSR primers with clear gel electrophoresis profiles were obtained, which showed stable amplification across all populations and yielded reliable, easily interpretable results. These microsatellite markers provide a useful reference for future studies on the origin and evolution of *P. orientalis* varieties, molecular identification and classification, hybrid breeding, and parental selection for genetic mapping.

Keywords *Platycladus orientalis*; SSR; Fingerprinting; Cluster analysis

1 Introduction

Platycladus orientalis, belonging to the family Cupressaceae, subfamily Cupressoideae, genus *Platycladus* (Fu, 1982), is the coniferous tree species with the widest natural distribution in China. It has a broad ecological amplitude, strong tolerance to drought and poor soils, vigorous vitality, and a long lifespan. It is one of the most commonly used pioneer tree species for afforestation of barren mountains in northern China, possessing extremely high economic and medicinal value, and it is also an important component of historical and cultural landscapes (Wu, 1986; Yang et al., 2014a). With global climate change, under the combined effects of natural disasters, deterioration of site conditions, pests and diseases, as well as subjective factors such as human disturbance and poor management, *P. orientalis* populations have shown varying degrees of decline, including weakened growth and even near death. The conservation and utilization of *P. orientalis* germplasm resources are facing great challenges, and its ecological value has not been effectively utilized (Su, 2003; Wang et al., 2004; Yang et al., 2014b).

Since the 1970s, extensive provenance trials have been carried out for *P. orientalis*, making it one of the earliest tree species in China to undergo such trials. In recent years, many scholars have conducted substantial research on provenance testing and patterns of genetic variation in *P. orientalis* (Wang, 2011). Numerous studies have also investigated and discussed the pharmacologically active components in different parts of *P. orientalis* (Miao Hui, 2018). During cultivation, a number of regional cultivars have been formed. Due to its wide distribution, wild populations of *P. orientalis* possess rich genetic resources. By studying the genetic variation and distribution patterns of *P. orientalis*, analyzing its genetic diversity and phylogenetic relationships, further revealing kinship and evolutionary relationships, and evaluating its genetic potential and value, a theoretical foundation can be established for the construction of core germplasm collections and the screening of superior genes. This is of great significance for the selection and utilization of elite germplasm.

With continuous advances in science and technology, methods for studying genetic diversity have also been constantly updated. Molecular marker techniques, owing to their advantages of being unaffected by environmental factors, developmental stage, or gene expression, have become important tools in genetic research (Liu et al., 2012). Geographic population variation in *P. orientalis* has shown significant effects on population selection and improvement (Jin, 2020). There is an urgent need to improve the genetic quality and adaptability of superior *P. orientalis* varieties through molecular marker technologies and to carry out research on the genetic basis of breeding populations. Among these methods, microsatellite markers (simple sequence repeats, SSRs), because of their high level of genetic information, good reproducibility and stability, and codominant inheritance (Maroof et al., 1994; Guichoux, 2011), have been widely used in studies of genetic diversity and phylogenetic relationships in forest trees (Reisch et al., 2007; Kalia, 2011; Lin et al., 2013; Huang et al., 2018). At present, sequenced genomes are mainly concentrated in cultivated plants and species with important economic value. Meanwhile, the development of new microsatellite primers is difficult and costly. However, species derived from a common ancestor often exhibit high sequence homology. Therefore, screening SSR primers required for the target species from closely related species with well-developed microsatellite primers has been widely adopted (Barbara, 2007).

In this study, SSR molecular markers were used to analyze the genetic diversity and kinship relationships of 100 *P. orientalis* samples collected from four regions. Through preliminary screening and repeated validation, seven pairs of microsatellite primers were selected from all synthesized SSR primers. These primers produced clear gel electrophoresis patterns, could be stably amplified in each population, showed relatively ideal performance, and were easy to score and statistically analyze, and were thus used for subsequent analyses.

1 Results and Analysis

1.1 PCR amplification and primer polymorphism

Through primer screening and repeated validation, seven polymorphic microsatellite primers were successfully selected from 45 pairs of SSR primers. These primers produced clear gel electrophoresis profiles, could be stably amplified in all populations, showed relatively ideal performance, and were easy to score and statistically analyze. A total of 26 allelic loci were detected by the seven SSR primers, mainly distributed in the range of 125–309 bp (Table 1). On average, each SSR primer detected 3.714 alleles. The number of effective alleles (Ne) ranged from a minimum of 1.317 for primer 18 to a maximum of 2.819 for primer SF13, with a mean of 1.9. The observed heterozygosity (Ho) of *Platycladus orientalis* populations ranged from 0.140 to 0.610, with an average of 0.406. The expected heterozygosity (He) varied from 0.241 to 0.645, with a mean value of 0.440. The polymorphic information content (PIC) ranged from 0.212 to 0.579. Highly polymorphic primers (PIC>0.5) accounted for 28.6% of the total, primers with moderate polymorphism (0.25<PIC<0.5) accounted for 57.1%, and primers with low polymorphism (PIC<0.25) accounted for 14.3%, with an average PIC value of 0.398. The Shannon information index (I) ranged from 0.405~1.194, with an average of 0.818, indicating that the genetic diversity of the population of 100 *P. orientalis* accessions was relatively low.

Table 1 Genetic diversity characteristics of different SSR loci

Locus	Genotype No.	*Na*	*Ne*	*I*	*Ho*	*He*	u*He*	*PIC*
4	3	2	1.688	0.598	0.350	0.408	0.410	0.325
18	3	2	1.317	0.405	0.140	0.241	0.242	0.212
SF3	5	3	1.595	0.685	0.380	0.373	0.375	0.341
SF14	12	5	2.441	1.194	0.610	0.590	0.593	0.557
SF4	7	5	1.894	0.955	0.490	0.472	0.475	0.441
SF12	6	4	1.551	0.703	0.370	0.355	0.357	0.330
SF13	10	5	2.819	1.188	0.505	0.645	0.649	0.579
Mean	6.571	3.714	1.900	0.818	0.406	0.440	0.442	0.398

1.2 Genetic diversity and kinship analysis of *Platycladus orientalis*

Genetic variation among *Platycladus orientalis* populations was analyzed using seven SSR markers. Based on GenAlEx analysis, the results (Table 2) showed that at the population level, the observed number of alleles per population ranged from 2.571 to 3.571, with an average of 3.071. The effective number of alleles ranged from 1.865 to 1.987, with an average of 1.909. The Shannon information index ranged from 0.725 to 0.807, with a mean of 0.766. Observed heterozygosity ranged from 0.393 to 0.492, with an average of 0.423, while expected heterozygosity ranged from 0.431 to 0.452, with a mean of 0.439. The fixation index (Fst), which reflects the level of allelic heterozygosity among populations and is used to measure the degree of population differentiation, was 0.0371. This value falls within the range of 0–0.05, indicating a high degree of similarity among populations, small genetic distances, and very low genetic differentiation. Population A exhibited the highest values of polymorphism rate, Na, and I, indicating that this population had the highest genetic diversity. It is therefore inferred that population A represents the center of genetic diversity of *P. orientalis* among the four sampling regions.

Table 2 Genetic diversity of *Platycladus orientalis*

Population	N	*Na*	*Ne*	*I*	*Ho*	*He*	*uHe*
a	68	3.571	1.902	0.807	0.394	0.431	0.434
b	9	2.857	1.883	0.761	0.492	0.452	0.479
c	4	2.571	1.987	0.725	0.393	0.433	0.495
d	19	3.286	1.865	0.771	0.414	0.438	0.450
Mean	25	3.071	1.909	0.766	0.423	0.439	0.464

1.3 Genetic Differentiation Analysis of *Platycladus orientalis*

Analysis of variance (ANOVA) was used to assess genetic variation in *Platycladus orientalis*. The results (Table 3) showed that genetic variation in *P. orientalis* was mainly derived from within populations, accounting for 91% of the total variation, while genetic variation among populations accounted for 9%. This indicates that the genetic variation of *P. orientalis* is predominantly distributed within populations.

Table 3 Molecular variance analysis of *P. orientalis* germplasm

Sources of variation	df	SS	MS	Est. Var.	Percentage of variation
Among Pops	3	4.36	1.45	0	0%
Among Indiv	96	165.74	1.73	0.15	9%
Within Indiv	100	143	1.43	1.43	91%
Total	199	313.10		1.58	100%

1.4 Genetic structure analysis of *Platycladus orientalis*

Bayesian clustering analysis of 100 individuals from four populations was performed using STRUCTURE software. The number of subpopulations (K) was preset from 2 to 10, with 10 independent runs for each K value. The value of LnP(D) continuously decreased with increasing K. When $K=3$, ΔK reached its maximum peak, indicating that division of the experimental materials into three clusters was the most appropriate (Figure 1; Figure 2).

The distribution of individuals among the three clusters (Table 4) showed a relatively even composition, with mean Q values of 0.619, 0.476, and 0.461, respectively. When $Q \geq 0.6$, the genetic background of a sample is considered relatively pure, whereas when $Q < 0.6$, the genetic background is considered complex (Falush et al., 2003). In this study, the Q value of Subpopulation 1 was ≥ 0.6, indicating a relatively homogeneous genetic background. In contrast, Subpopulations 2 and 3 had Q values < 0.6, suggesting that these two subpopulations integrated genetic components from multiple clusters and exhibited evident gene flow.

The first cluster contained 39 individuals, including 27 from Shanting District, 2 from Yicheng District, 2 from Shizhong District, and 7 from Tengzhou City. The second cluster comprised 28 individuals, including 18 from Shanting District, 2 from Yicheng District, and 5 from Tengzhou City. The third cluster contained 23 individuals, including 68 from Shanting District, 9 from Yicheng District, 4 from Shizhong District, and 19 from Tengzhou City.

Using the method for estimating the optimal K value, Structure clustering analysis based on microsatellite data showed that ΔK reached its maximum at K=3; therefore, the optimal K value was 3. The studied populations were divided into three genetic clusters rather than being grouped according to sampling regions. This indicates the presence of gene flow among different regions, which is consistent with the results of the UPGMA clustering analysis.

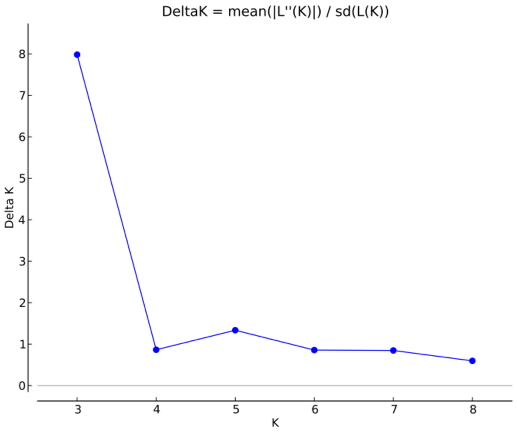

Figure 1 The deltaK (ΔK) values of structure output

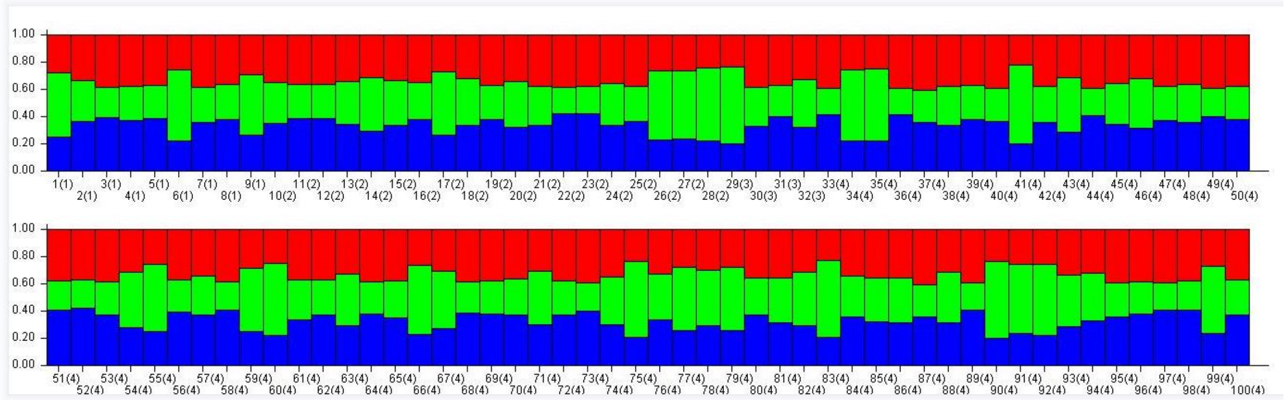

Figure 2 The structure output at K=3

Table 4 Distribution of *P. orientalis* germplasm subpopulations when K=3

Sub-population	Shanting	Yicheng	Shizhong	Tengzhou	Total	Q-Value
Sub-population 1	27	3	2	7	39	0.619
Sub-population 2	18	5	0	5	28	0.476
Sub-population 3	23	1	2	7	33	0.461
Total	68	9	4	19	100	0.5187

Using PowerMarker software, UPGMA clustering based on Nei's genetic distance was performed on 100 samples from four populations. The clustering results showed both similarities and differences compared with the three clusters identified by STRUCTURE. The main difference was that the proportions of samples from each provenance differed among the clusters. The similarity was that germplasm from the Shanting provenance was distributed across all three clusters. Based on the clustering outcomes from both methods, further analyses of kinship relationships among germplasm accessions can be conducted. To some extent, the clustering results indicate that genetic relatedness among populations is associated with geographic distribution; however, most samples did not cluster strictly according to their sampling regions, suggesting that there is a certain level of gene flow among different regions.

The clustering analysis of *Platycladus orientalis* (Figure 3) showed that red represents Cluster I, blue represents Cluster II, and yellow represents Cluster III. Cluster I contained 13 individuals, including 7 from Shanting District, 4 from Tengzhou City, and 1 from Yicheng District. Cluster II contained 23 individuals, including 21 from Shanting District and 2 from Shizhong District. Cluster III contained 64 individuals, including 40 from Shanting District, 15 from Tengzhou City, 8 from Yicheng District, and 1 from Shizhong District.

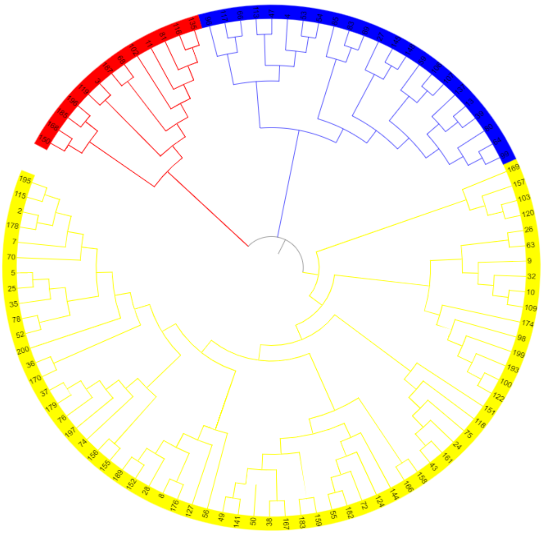

Figure 3 Phylogenetic tree of *Platycladus orientalis* based on SSR data

Using NTSYS software, UPGMA clustering analysis based on Nei's genetic distance was performed on 100 samples from four populations (Figure 4).

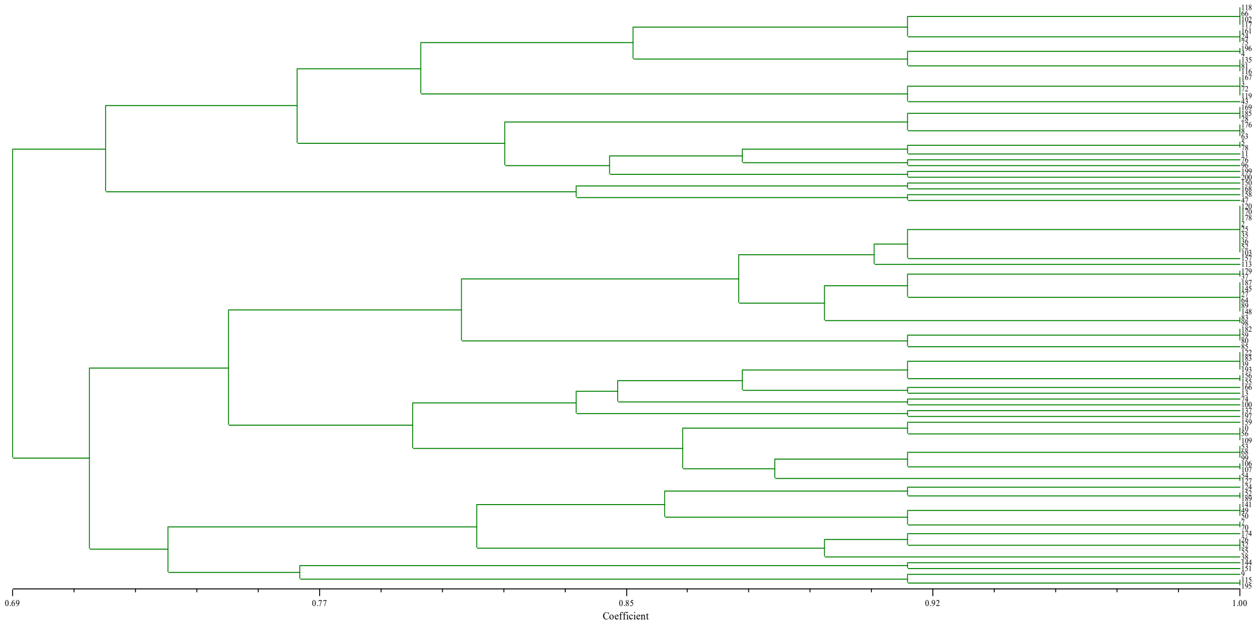

Figure 4 Dendrogram of *Platycladus orientalis* based on SSR data

At a similarity coefficient of approximately 0.69, the samples were divided into two clusters. Samples 135, 137, 141, and 145 collected from Shizhong District, which showed relatively close kinship, were grouped together with samples from other regions, while samples from the remaining regions were not strictly clustered according to their geographic origins. At a similarity coefficient of approximately 0.70, four clusters were identified, and the collected samples still did not cluster according to the four sampling regions. When the genetic similarity coefficient reached 0.77, the 100 *Platycladus orientalis* accessions were divided into eight clusters.

2 Discussion

In this study, seven polymorphic microsatellite primers with clear gel electrophoresis patterns and stable amplification across all populations were successfully selected, and corresponding DNA fingerprint profiles were established. These markers provide a valuable reference for future studies on the origin and evolution of *Platycladus orientalis* varieties, molecular-level identification and classification, hybrid breeding, and parental selection for genetic mapping. As an ideal type of molecular marker, microsatellites exhibit advantages such as good reproducibility, simplicity, efficiency, and high primer transferability in the identification of *P. orientalis* varieties. However, because the detection and application of SSR polymorphism largely depend on PCR amplification efficiency, different primers may require different reaction conditions. Therefore, it is essential to conduct preliminary optimization experiments for each primer and adopt appropriate strategies to maintain PCR reaction conditions at an optimal level.

In plant variety identification and classification studies, a more scientific approach is to integrate multiple methods and use them to complement and validate one another (Yuan et al., 2014). Identification results based on morphological traits and molecular markers are not always completely consistent. For example, some morphologically similar *P. orientalis* individuals were identified as hybrids in STRUCTURE clustering analyses. This phenomenon may be attributed to genetic variation caused by backcrossing and introgression, or to morphological variation resulting from convergent evolution and environmental selection (Rieseberg et al., 1999; Schwarzbach et al., 2001; Lexer et al., 2003). Similar patterns have also been observed in other populations exhibiting natural hybridization (Rieseberg, 1995). Any single method has inherent limitations, and relying on a single approach for species identification and classification makes it difficult to ensure the scientific rigor and reliability of the results.

Numerous studies have shown that factors such as genetic drift and gene flow have a substantial impact on population genetic structure. In recent years, parameters such as genetic differentiation coefficients have been widely used as important indicators for evaluating population genetic structure and kinship relationships among varieties (Song et al., 2011; Xu et al., 2014). Genetic variation analysis of *P. orientalis* populations using seven SSR markers showed that the average number of alleles (Na) among the 100 samples from four sampling regions was 3.714, the average effective number of alleles (Ne) was 1.900, the average Shannon index (I) was 0.818, and the average expected heterozygosity (He) was 0.440. These results indicate that the 100 sampled accessions possessed relatively rich genetic diversity. The fixation index (Fst), which reflects the level of allelic heterozygosity among populations and is used to measure the degree of population differentiation, was 0.0371. This value falls within the range of 0–0.05, indicating high similarity among populations, small genetic distances, and very low genetic differentiation.

Results from principal coordinate analysis (PCoA), UPGMA clustering, and STRUCTURE clustering based on microsatellite data consistently showed that the 100 *P. orientalis* accessions were not strictly clustered according to their geographic origins. This suggests that the genetic backgrounds of the germplasm resources are relatively similar and that varying degrees of natural hybridization may occur among *P. orientalis* germplasm from different sampling regions. Hybridization promotes gene flow among populations and contributes to genetic evolution, thereby influencing population genetic structure and altering its overall pattern.

The formation of this spatial genetic variation pattern may be the result of the combined effects of long-distance gene flow, natural climatic conditions, and geographic isolation. In theory, geographically proximate regions tend to have similar soil conditions and environmental climates, resulting in less pronounced differences in natural selection pressures and increased opportunities for interpopulation gene exchange. Consequently, populations located closer to each other tend to have smaller genetic distances and higher genetic similarity.

At present, only a portion of *P. orientalis* resources has been collected, and the limited sample size may introduce bias into the analyses. Therefore, a more comprehensive evaluation and utilization of *P. orientalis* germplasm resources will require further investigation and research. To adapt to diverse ecological conditions and geographic environments, wild plant resources have undergone prolonged evolutionary processes involving intense survival competition and extensive natural selection, resulting in extremely rich genetic diversity. These resources constitute complex natural gene pools that harbor many superior genes for disease resistance, pest resistance, and drought tolerance that are often absent in cultivated varieties, and thus can be used to improve the genetic basis of cultivated varieties (Tao et al., 2010; Jing et al., 2020). Influenced by artificial cultivation practices and breeding strategies, cultivated varieties have undergone substantial evolutionary changes; however, the patterns and directions of their evolution show considerable similarity to those of wild populations under natural conditions (Xu, 2005). The construction of genetic populations of *P. orientalis* and the exploration of their genetic diversity are therefore of great significance for the conservation and utilization of *P. orientalis* germplasm resources (Lei, 2018).

3 Materials and Methods

3.1 Overview of experimental material collection

In March 2021, a total of 100 *Platycladus orientalis* samples were collected from four regions in Zaozhuang City (Table 5): Shanting District (a68), Yicheng District (b9), Shizhong District (c4), and Tengzhou City (d19). A sampling strategy of collecting one mature individual at intervals of 10 m was adopted to avoid repeated sampling. Healthy upper leaves free of insect damage were collected as samples whenever possible. After collection, the samples were thoroughly dried using color-indicating silica gel, and DNA was subsequently extracted.

Table 5 Geographical location of *Platycladus orientalis* sampling sites

No.	Group	Region	Locality	Longitude	Latitude	Elevation (m)	Aspect
2	a	Shanting District	Huameizhuang	117°32′24″	35°01′05″	360	South
3	a	Shanting District	Shifosi	117°36′26″	35°01′38″	360	South
4	a	Shanting District	Shifosi	117°36′28″	35°01′39″	370	South
5	a	Shanting District	Shifosi	117°36′28″	35°01′40″	370	Southwest
7	a	Shanting District	Shifosi	117°36′28″	35°01′40″	384	Southeast
8	a	Shanting District	Shifosi	117°36′31″	35°01′39″	360	Southeast
9	a	Shanting District	Shifosi	117°36′22″	35°01′42″	410	East
10	a	Shanting District	Shifosi	117°36′37″	35°01′36″	330	South
11	a	Shanting District	Shifosi	117°36′22″	35°01′42″	390	South
13	a	Shanting District	Shifosi	117°36′29″	35°01′34″	380	South
24	a	Shanting District	Shifosi	117°36′32″	35°01′49″	326	Southwest
25	a	Shanting District	Glass Walkway South Mountain	117°35′27″	35°02′04″	340	Northeast
26	a	Shanting District	Glass Walkway South Mountain	117°35′25″	35°02′12″	330	North
27	a	Shanting District	Glass Walkway South Mountain	117°35′24″	35°02′01″	360	Northwest
28	a	Shanting District	Glass Walkway South Mountain	117°35′31″	35°02′01″	360	Northeast
32	a	Shanting District	Glass Walkway South Mountain	117°35′35″	35°01′54″	390	East
35	a	Shanting District	Mujia Cave West Mountain	117°35′54″	35°02′36″	270	Southwest
36	a	Shanting District	Mujia Cave West Mountain	117°35′55″	35°01′52″	280	West
37	a	Shanting District	Mujia Cave West Mountain	117°35′03″	35°02′19″	270	Southwest
38	a	Shanting District	Mujia Cave West Mountain	117°33′57″	35°02′02″	270	Northwest
39	a	Shanting District	Mujia Cave West Mountain	117°34′54″	35°02′36″	270	Southwest
43	a	Shanting District	Mujia Cave West Mountain	117°34′54″	35°02′36″	280	Southwest
47	a	Shanting District	East Dami Mountain	117°34′26″	35°03′00″	370	Southeast
49	a	Shanting District	East Dami Mountain	117°38′27″	35°02′59″	360	Southeast
50	a	Shanting District	East Dami Mountain	117°38′26″	35°03′04″	390	Northwest
52	a	Shanting District	Dajiao Mountain	117°38′27″	35°03′38″	390	West
53	a	Shanting District	East Dajiao Mountain	117°38′26″	35°02′51″	400	Southeast
54	a	Shanting District	Dajiao Mountain	117°38′27″	35°03′10″	246	Southwest
55	a	Shanting District	East Dajiao Mountain	117°38′25″	35°02′50″	400	East
56	a	Shanting District	Dajiao Mountain	117°38′15″	35°03′29″	400	West
59	a	Shanting District	East Dajiao Mountain	117°38′25″	35°02′48″	400	East
63	a	Shanting District	East Dajiao Mountain	117°38′25″	35°02′47″	410	Southeast
64	a	Shanting District	Northwest Mountain	117°36′31″	35°03′45″	280	East

No.	Group	Region	Locality	Longitude	Latitude	Elevation (m)	Aspect
66	a	Shanting District	Northwest Mountain	117°36′29″	35°03′40″	280	Southeast
68	a	Shanting District	Northwest Mountain	117°36′31″	35°03′43″	280	East
70	a	Shanting District	Northwest Mountain	117°36′38″	35°03′22″	280	South
72	a	Shanting District	Northwest Mountain	117°36′29″	35°03′40″	290	Southeast
74	a	Shanting District	Northwest Mountain	117°36′28″	35°03′40″	240	South
76	a	Shanting District	Northwest Mountain	117°36′27″	35°03′39″	280	Southwest
78	a	Shanting District	Northwest Mountain	117°36′40″	35°03′09″	280	South
80	a	Shanting District	Northwest Mountain	117°36′25″	35°03′38″	270	South
81	a	Shanting District	Yanggang Mountain	117°36′30″	35°02′55″	230	East
83	a	Shanting District	Yanggang Mountain	117°36′28″	35°02′57″	280	Southeast
85	a	Shanting District	Yanggang Mountain	117°36′10″	35°03′21″	300	Southwest
89	a	Shanting District	Yanggang Mountain	117°36′29″	35°02′56″	290	Southeast
96	a	Shanting District	Shengshan’an Pass	117°34′08″	35°00′19″	180	North
98	a	Shanting District	Shengshan’an Pass	117°34′10″	34°59′38″	290	Northwest
99	a	Shanting District	Yanggang Mountain	117°36′00″	35°03′22″	280	South
100	a	Shanting District	Shengshan’an Pass	117°34′10″	34°59′38″	216	Southwest
102	a	Shanting District	Shengshan’an Pass	117°34′10″	34°59′39″	213	Southwest
103	a	Shanting District	Jiguan Gu	117°36′57″	34°58′15″	270	Southwest
107	a	Shanting District	Jiguan Gu	117°37′09″	34°58′09″	260	Southwest
109	a	Shanting District	Jiguan Gu	117°36′46″	34°58′37″	300	Southwest
113	a	Shanting District	Baodu Gu	117°42′54″	34°59′11″	330	Northwest
115	a	Shanting District	Baodu Gu	117°42′55″	34°59′08″	340	Southwest
118	b	Yicheng District	Qingtan Temple	117°34′23″	34°57′39″	410	East
135	c	Shizhong District	Guishan Forest Farm	117°40′51″	34°46′43″	200	Northeast
151	d	Tengzhou City	East Mountain	117°23′13″	34°55′51″	120	East
155	d	Tengzhou City	Hutou Mountain	117°16′44″	34°53′28″	120	Northwest
166	d	Tengzhou City	Mushi Forest Farm	117°16′59″	34°58′08″	100	South
183	d	Tengzhou City	Hulutao	117°16′34″	34°52′58″	80	Northeast
200	a	Shanting District	Beiyu	117°25′53″	35°08′46″	360	Northeast

3.2 Experimental methods

3.2.1 Extraction and quality assessment of total plant DNA

Genomic DNA was extracted from the leaves of 100 *Platycladus orientalis* samples using a modified cetyltrimethylammonium bromide (CTAB) method. After the extracted DNA was completely dissolved, its quality was assessed by electrophoresis on 2% agarose gels.

3.2.2 Primer screening

In this study, SSR primers of *Platycladus orientalis* were developed based on the identification of SSR loci and primer screening from *P. orientalis* transcriptome sequences, and a total of 45 pairs of SSR primers were initially selected. Prior to large-scale PCR amplification and sequencing of all individuals, eight individuals were randomly selected for preliminary screening of the 45 microsatellite primers (Figure 5). Using the annealing temperature of 58 °C reported in the literature as a reference, PCR products were examined by 3% agarose gel electrophoresis and capillary sequencing. Primers with poor amplification efficiency or unclear banding patterns were discarded. Ultimately, seven pairs of SSR primers that produced clear gel electrophoresis profiles, could be stably amplified in all populations, showed satisfactory performance, and were easy to score and statistically analyze were selected for subsequent analyses.

3.2.3 SSR analysis

In this study, seven pairs of polymorphic primers with clear and well-resolved DNA bands were selected (Table 6). Detailed information on the PCR reaction system (Table 7) and amplification program is provided below.

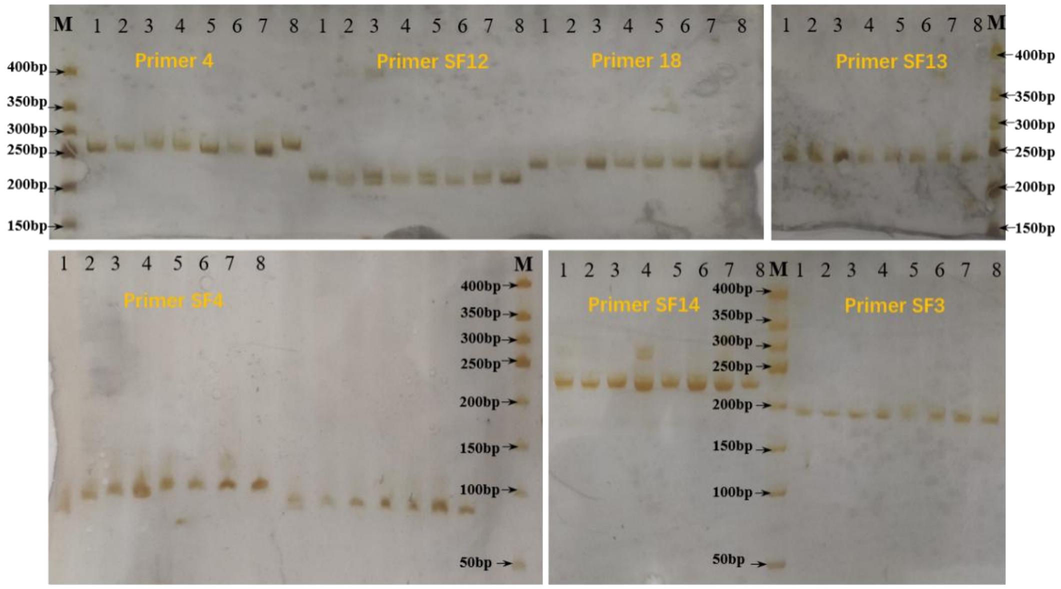

Figure 5 Amplification and detection of primers 4, 18, SF3, SF14, SF4, SF12 and SF13
Note: 1~8 The number of strains; M: 50bp DNA ladder

Table 6 Information of microsatellite primers used in this study

Locus name	Dye	Primer sequence (5´-3´)	Tm (°C)	Allele size (bp)	Repeated motif
SF12	5`6-FAM	F:AAACGAATGAGGCTGAATGG R:GGATGCACGCAATTTTCTTT	58	150-200	$(AT)_6$
SF3	5`6-FAM	F:GAGAGCTCTGCTGCCATCTT R:ATAACGTTCCCTGGCATCTG	58	150	$(TC)_6$
SF4	5`6-FAM	F:ATAAAAAGTCCCCGGAGCAT R:GCCAGTGAAATTGAGGTTGC	58	100-150	$(AG)_9$
18	5`6-FAM	F:ACATTGATTTGCATTGGGGT R:AGAGCACATTCCGGTACCAC	58	200-250	$(CA)_6$
SF13	5`HEX	F:ACGGCCTTTGTTTTCTCTCA R:AAACCGCCAACACAGGTAAT	58	250-300	$(GT)_7$
SF14	5`HEX	F:CTTCGTCCCCGATACAAGAG R:CATCATGCCCGATATCATCA	58	200-300	$(CAG)_6$
4	5`HEX	F:AGTGAGAGCACCTGCTGGAT R:AGCAGTGGGCTTTACCCTTT	58	300	$(TTC)_5/(GGGTAAA)_3$

Table 7 The PCR reaction system of the microsatellite markers

Component	Volume (μL)
(Vazyme)2×Taq Master mix	12.5
Forward primer	1.5
Reverse primer	1.5
ddH_2O	6
DNA template	1.5
Total	20

The PCR reaction program was as follows: 94 °C for 3 min; 30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min; followed by a final extension at 72 °C for 5 min, and then held at 4 °C. After completion of the PCR reactions, the products were examined by electrophoresis on 3% agarose gels. Qualified PCR amplification products were sent to an automated sequencer (Applied Biosystems) for allele genotyping. GeneMarker software was used to read allele sizes, and genotyping results were obtained for 100 *Platycladus orientalis* individuals.

PowerMarker V3.25 software was used to analyze the genotype data, including the number of alleles, number of genotypes, heterozygosity, and polymorphic information content (PIC) for different sample combinations. Genetic distances among varieties were also calculated, and clustering was performed using the unweighted pair-group method with arithmetic means (UPGMA) (Liu, 2005).

Author Contributions

Zhou Jilei and Li Jingtao were responsible for the experimental design and the execution of the experimental research. Zhou Jilei and Zhang Liudong carried out data analysis and prepared the first draft of the manuscript. Fu Yinyin completed the experimental design and analysis of the experimental results. Chen Yong participated in sample collection for the study. Li Jingtao conceived and led the project and provided guidance on experimental design, data analysis, and manuscript writing and revision. All authors read and approved the final manuscript.

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