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RESEARCH

SSR Marker–Based Genetic Diversity and Relationship Analyses of *Stephania tetrandra* S. Moore

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Abstract

Stephania tetrandra S. Moore (family: Menispermaceae), a dioecious herbaceous vine and the only species in the subgenus *Botryodiscia* of the genus *Stephania* of the family Menispermaceae, is mainly distributed in hilly areas south of the Huaihe River in China and found in many provinces of China, showing a high genetic diversity. This paper aimed to study genetic diversity of and genetic relationship among individuals of *S. tetrandra* within China to provide a basis for evaluation, exploitation, and utilization of *S. tetrandra* by using simple sequence repeat (SSR) molecular markers. Our results show that effective products were amplified from the 26 screened SSR gene loci, a total of 183 alleles amplified (2–16 alleles amplified by each pair of primers). Among the 26 loci, 16 had a *PIC* value higher than 0.5, indicating a high level of polymorphism. For most of the loci, the number of effective alleles was lower than that of the observed alleles, and the observed heterozygosity was lower than the expected heterozygosity. The genetic differentiation coefficient (0.021–0.547) was lower than 0.05 (low level of genetic differentiation) for 7 loci and higher than 0.25 (very high level of genetic differentiation) for 2 loci, and had a value representing a medium level of genetic differentiation for the remaining 17 loci. The intra-population inbreeding coefficient had a positive value for 21 loci, suggesting the presence of inbreeding and homozygous excess. The gene flow value was bigger than 1, indicating that genetic drift and natural selection played an unimportant role in population genetic differentiation of *S. tetrandra*. Based on discriminant analysis of principal components and Bayesian Information Criterion, *K*-means clustering was performed on 620 samples. These samples were divided into 9 genetic clusters, whose similarity coefficients and genetic distances were 0.755–0.918 and 0.067–0.280, respectively, indicating that these clusters were highly similar and short-distanced. The Bayesian clustering analysis was implemented in the STRUCTURE software to analyze the genetic structure of *S. tetrandra* and it was found that the 620 samples could be clustered into 5 ancestor groups; the 9 clusters and 40 natural populations inherited genes from the 5 groups to varying degrees, but the proportion of genes inherited from the 5 groups by each cluster and natural population differed. *S. tetrandra* was characterized by the presence of population structure and pronounced genetic subdivision, which, together with the presence of gene flow, may indicate a relatively stable recent state of these populations.

Keywords *Stephania tetrandra* · Genetic diversity · SSR markers · Population genetic relationship

Introduction

Stephaniae Tetrandrae Radix, the dried root of *S. tetrandra* in the family Menispermaceae, has been recorded in most editions of Chinese Pharmacopoeia (National Pharmacopoeia Committee 2015). It has the effect of inducing diuresis to alleviate edema, dispelling wind, and relieving pain, and is widely used clinically (Kang et al. 2014; Wang 2014; Wang et al. 2017). It showed good clinical effects on chronic hepatitis, early liver cirrhosis, allergic dermatosis, diabetes, prostatitis, seminal vesiculitis, and other diseases (Xue and Zhu 2012; Liu et al. 2017b; Wang et al.2017). Tetrandrine,

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its main active substance, has been demonstrated to have a good effect in anti-inflammation, analgesia, blood pressure lowering, anti-silicosis, blood sugar lowering, anti-free radical injury, anti-liver fibrosis, and anti-tumor (Kang et al. 2014; Wu et al. 2014; Qiu et al. 2014). *S. tetrandra* is mainly distributed in hilly areas south of the Huaihe River in China and found in many provinces of China, including Jiangxi, Anhui, Zhejiang, Fujian, Hunan, and Guangdong. *S. tetrandra* is a dioecious species (Chinese Academy of Sciences Institute of Botany 1972). According to our resource survey, the ratio of female and male individuals in wild populations of *S. tetrandra* is 1:3, resulting in a low level of seed production. We also found that dispersal of the fruit of this species by the wind was difficult because of its high mass, and it is slightly bitter in taste and maybe birds do not like eating it, restricting the habitat expansion of wild *S. tetrandra*. This species, however, possesses a strong ability to reproduce asexually, and in nature, it shows both seed propagation and vegetative propagation (Guo et al. 2007). In the process of sexual reproduction of plants, mutations such as deletion, duplication, inversion, translocation, and chromosome doubling can occur in the course of chromosome exchange, which can cause genetic variation (Li et al. 2023) and genetic diversity of species (Zhang et al. 2019). Asexual reproduction has less effect on genetic variation than sexual reproduction. At the same time, in the process of evolution, genetic variation and genetic drift in the germplasm may occur in plants in different distribution regions in response to environmental changes, resulting in differences in germplasm in different distribution regions (Peng and Tang, 2017; Lu et al. 2019). The study of genetic diversity can reveal the genetic structure and relationship of plant germplasm resources, and can be used for germplasm identification and high-quality resource evaluation (Xie and Yun 2000). So far, very few studies on the gender differentiation and genetic structure of *S. tetrandra* have been conducted. Whether there is any difference in the germplasm of *S. tetrandra* plant individuals from different distribution areas as well as the information on their genetic structure and evolutionary relationship remains to be disclosed.

Simple sequence repeats (SSRs) are widely distributed in the whole genome of eukaryotes and have highly polymorphic characteristics among individuals and populations (Chen et al. 2009). SSR sequences from different species showed a high allelic diversity in composition, length, mutation rate, and their distribution in chromosomes (Chen et al. 2009). Such sequences have relatively conservative flanking sequences, based on which SSR primers with high specificity can be designed and used for effective amplification of specific SSRs (Zhang 2014). This method has become a common approach for the development of SSR primers due to a large number of primers developed, simplicity, and low cost (Xie and Yun 2000; Chen et al. 2009; Ao et al. 2009; Zhang 2014). SSR molecular markers are codominant, in line with the Mendelian law, easy to operate, and highly repeatable, reliable, and polymorphic, so they have been widely employed in the study of the correlation between genomic differences and character variation, plant genetics, and breeding (Xie and Yun 2000; Liu et al. 2017a, b; Xie et al. 2017; Su et al. 2018). Therefore, we used SSR markers to analyze the genetic diversity and relationship of *S. tetrandra* in this paper.

S. tetrandra populations are present in different geographical locations. Their germplasms may differ, leading to the discrepancy in the quality of crude drug derived from them. The difference in germplasms is reflected by the difference in SSR markers. Therefore, in the present study, we tried to disclose the genetic diversity of the species *S. tetrandra* and the genetic relationship among different populations of *S. tetrandra* to provide a basis for resource conservation and utilization of that species.

Materials and Methods

Plant Materials and DNA Extraction

We gathered wild samples from 93 villages in 40 cities or counties in Guangdong, Anhui, Jiangxi, Zhejiang, Fujian, and Hunan provinces of China (Fig. 1). Sampling cities or counties (each one represents one population) were at least 50 km apart, and some (basically more than 5) plant individuals (Fig. 1) were chosen from populations in each city or county (depending on the distribution density of *S. tetrandra* populations). Fresh, healthy young leaves of 620 randomly chosen individuals of *S. tetrandra* (148 ones from Anhui, 53 from Fujian, 44 from Guangdong, 54 from Hunan, 279 from Jiangxi, and 42 from Zhejiang) were collected in the same period of time and quickly dried off for subsequent use. At the same time, specimens were collected from all sampling sites and confirmed by professor Songji Wei, a botanist from Guangxi University of Chinese Medicine, China, as genuine *S. tetrandra*, and grown at Xianhu Campus of Guangxi University of Chinese Medicine to establish a gene bank of *S. tetrandra*. The geographical and climatic information of the cities and counties where sampling sites were located are shown in Table 1. Genomic DNA was extracted using the magnetic bead–based kit for isolating genomic DNA from TIANGEN Biotech (Beijing), 2 to 3 μL of it was subjected to testing by 1.5% agarose gel electrophoresis, and the rest was stored at − 20 °C for PCR test.

Primer Screening

Three samples were selected randomly for transcriptome sequencing using Illumina next-generation sequencing

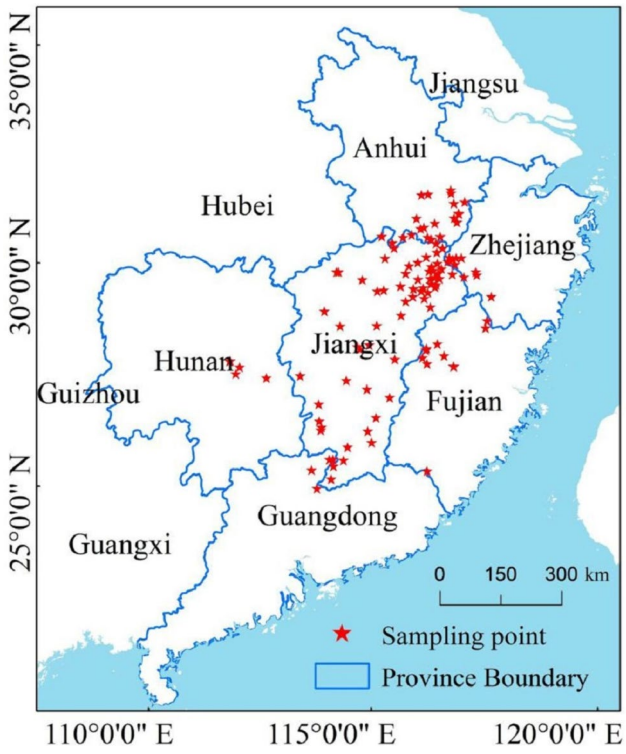

Fig. 1 A map showing the village-level sampling sites (all in China) selected for this study. The horizontal axis represents the longitude and the vertical axis represents the latitude. Each red five-pointed star is a village-level sampling site. The village-level sampling sites in each county or city are not too far apart and the cities and counties are at least 50 km apart

technology. A total of 45,024 SSR markers were mined from transcriptome sequences. Two hundred SSR markers were screened from marker loci which had 2 to 6 bases each and were present in more than 90% of samples and primers were designed according to conserved sequences on both ends of these SSR marker loci. Four individual plant samples from different populations were randomly selected and used to analyze the amplification effect and polymorphism of the 200 primers to select primers for polymorphic SSR markers for subsequent study (for each of the 4 plant samples, the amplified fragment size of the same SSR marker primer was different). The primers were synthesized by Tianyi Huiyuan Co., Ltd. in China.

Analysis of Population Genetic Diversity

The selected primers for polymorphic SSR markers were used to amplify DNA of 620 samples, in order to analyze SSR and population genetic diversity based on amplification results. The SSR fluorescent primer system used in PCR amplification (15μL in total) was as follows: mix, 7.5 μL; ddH_2O, 4.5 μL; forward primer, 1 μL (10 μmol/L); reverse primer, 1 μL(10 μmol/L); DNA template, 1 μL. The amplification procedure is shown in the Supplementary Materials. Post-amplification SSRs were detected by fluorescence-labeled capillary electrophoresis. Formamide and relative molecular weight internal standard were mixed at a ratio of 250:1 (v/v). A total of 9 μL of the resultant mixture was loaded into the microplate, to which 1 μL of PCR product diluted tenfold was added. The 3730XL sequencer was used for capillary electrophoresis, and the Fragment (Plant) fragment analysis software in GeneMarker was used to analyze the original data obtained by the sequencer. The position of the internal standard in each lane and the peak position of each sample were compared and analyzed to obtain the size of fragments.

Statistical Analysis

GenAlEx software was used to estimate various genetic parameters, including effective number of alleles per locus (*Ne*), observed number of alleles per locus (*Na*), Shannon information index (*I*), heterozygosity (*Ht*), observed heterozygosity (*Ho*), expected heterozygosity (*He*), fixation index (*F*), genetic differentiation coefficient (*Fst*), and gene flow value (*Nm*) (Xu et al. 2015). The polymorphism information content (*PIC*) of each locus was calculated using Cervus software (Slate et al. 2000). GenAlEx software was employed to calculate pairwise genetic differentiation coefficients (*Fst*) and perform molecular variance analysis of clusters. Using find.cluster in the R Package (adegenet), the *K*-means algorithm was run to generate a graph with different *K* values as the horizontal coordinate and the obtained BIC values as the vertical coordinate; the *K* value at the first inflection point was selected to determine the number of clusters (Jombart et al. 2010). The UPGMA (Unweighted Pair Group Method using Arithmetic Averages) clustering was performed, and tree maps were constructed using Phylip software based on Nei's genetic distances of clusters. The principal coordinate analysis (PCoA) was performed using GenAlEx software. Bayesian statistics and Markov chain Monte Carlo Simulation were used to estimate the assigned proportion of each individual in each group (membership coefficient, *Q*). Data on all *K* values (from 1 to 15) were input into STRUCTURE Harvester software v0.6.1 (available at http://taylor0.biology.ucla.edu/structureHarvester/) to estimate delta *K* and prepare the indfiles for the software CLUMPP (v1.1.2), which was used to perform alignments from the *Q* values of each *K* STRUCTURE group. distruct (v1.1) was used to visualize the resulting bar plots, and individuals were assigned to the cluster for which they showed the highest *Q* value. STRUCTURE software (v2.3.4) was used to infer the group structure with the following parameters: BURNIN 10000, NUMREPS 30000.

Table 1 Cities and counties where samples of *Stephania tetrandra* S. Moore were collected. Samples from each city/county represent a large natural population

No	Sampling cities/counties	I (°)	II (°)	III (℃)	IV (℃)	V (℃)	VI (mm)	VII (h)	Sample size
1	Dongzhi County, Anhui Province, China	117.06	29.77	16.9	28.0	1.0	1554.4	1288.0	13
2	Qingyang County, Anhui Province, China	117.86	30.64	16.1	40.6	−0.1	1600.0	1286.0	15
3	Shitai County, Anhui Province, China	117.47	30.15	16.0	40.0	−18.0	1624.4	1286.0	8
4	Qimen County, Anhui Province, China	117.53	29.91	15.6	41.0	−13.2	1781.4	1277.0	28
5	Shexian County, Anhui Province, China	118.42	30.02	16.4	40.8	−12.7	1477.0	1275.0	15
6	Xiuning County, Anhui Province, China	117.75	29.63	16.3	33.0	−3.0	1536.2	1277.0	17
7	Jixi County, Anhui Province, China	118.20	29.57	15.9	38.0	−7.4	1630.3	1643.4	16
8	Jing County, Anhui Province, China	117.91	30.21	15.0	42.7	−6.8	1500.0	2113.0	15
9	Jinde County, Anhui Province, China	118.15	30.70	16.0	41.5	−8.2	1429.6	1784.1	21
10	Shaowu City, Fujian Province, China	117.19	27.24	18.0	40.0	−5.0	1800.0	1668.0	19
11	Shunchang County, Fujian Province, China	117.79	26.79	18.5	31.0	8.0	1756.0	1740.7	8
12	Songhou County, Fujian Province, China	118.79	27.65	18.0	40.0	−5.0	1600.0	1900.0	11
13	Taining County, Fujian Province, China	117.07	27.08	17.1	38.7	−6.9	1775.0	1738.7	12
14	Dabu County, Guangdong Province, China	116.42	24.41	21.7	39.6	−2.4	1712.5	1916.5	6
15	Nanxiong City, Guangdong Province, China	114.60	25.16	19.9	40.4	−4.1	1495.2	1622.5	10
16	Shixing County, Guangdong Province, China	114.06	24.98	19.6	40.3	−4.3	1543.0	1582.7	9
17	Hengyang County, Hunan Province, China	112.59	27.38	18.7	40.0	−7.9	1470.9	1531.7	16
18	Shuangfeng County, Hunan Province, China	112.49	27.24	17.0	37.0	−8.1	1275.0	1550.0	4
19	Xiangxiang City, Hunan Province, China	112.38	27.53	17.1	39.6	−4.6	1326.8	1558.7	13
20	You County, Hunan Province, China	113.22	27.08	17.8	34.0	3.0	1410.0	1677.1	19
21	Dexing City, Jiangxi Province, China	117.57	28.78	18.0	40.9	−10.6	1869.6	1775.4	36
22	Wuzhou City, Jiangxi Province, China	116.15	27.65	17.5	42.1	−13.7	1750.0	1728.0	15
23	Quannan County, Jiangxi Province, China	114.50	24.74	18.6	39.0	−3.0	1695.0	1690.0	13
24	Jingdezhen City, Jiangxi Province, China	117.10	29.13	17.0	38.0	−2.0	1763.5	2009.8	14
25	Jiujiang City, Jiangxi Province, China	116.53	29.38	17.1	41.0	−4.0	1391.5	2076.3	18
26	Leping City, Jiangxi Province, China	117.33	29.18	18.3	39.0	−5.0	1672.3	1766.3	17
27	Nanchang City, Jiangxi Province, China	116.21	28.68	17.3	37.0	−2.0	1650.0	1771.5	17
28	Hengfeng County, Jiangxi Province, China	117.66	28.58	16.0	43.3	−14.3	1850.0	1940.0	21
29	Shangrao County, Jiangxi Province, China	117.80	28.75	17.8	39.0	−3.0	2066.1	1711.2	13
30	Wannian County, Jiangxi Province, China	117.12	28.62	17.4	41.2	−12.8	1808.0	1803.5	16
31	Wuyuan County, Jiangxi Province, China	117.85	29.33	16.8	41.0	−10.0	1962.3	1715.1	37
32	Yiyang County, Jiangxi Province, China	117.40	28.45	17.8	41.4	−11.2	2066.1	1711.2	19
33	Yugan County, Jiangxi Province, China	116.80	28.71	17.8	37.0	−2.0	1586.4	1872.0	11
34	Yushan County, Jiangxi Province, China	118.13	28.80	17.8	38.0	−3.0	2066.1	1711.2	11
35	Yifeng County, Jiangxi Province, China	114.30	28.17	17.2	39.0	−3.0	1737.1	1680.2	11
36	Guixi City, Jiangxi Province, China	117.29	28.53	17.9	38.0	−3.0	1350.0	1749.9	22
37	Yujiang County, Jiangxi Province, China	116.88	28.37	17.6	41.1	−15.4	1739.4	1788.8	7
38	Jiangshan City, Zhejiang Province, China	118.74	28.69	17.0	38.0	−3.0	2063.3	2000.0	14
39	Longquan City, Zhejiang Province, China	119.00	28.16	17.8	37.0	−4.0	1568.4	1676.6	7
40	Kaihua County, Zhejiang Province, China	118.08	29.09	16.4	37.0	−4.0	1814.0	1712.5	26

I longitude, *II* latitude, *III* annual average temperature, *IV* annual maximum temperature, *V* annual minimum temperature, *VI* annual rainfall, *VII* annual average sunshine

Results

Primer Screening

A total of 45,024 SSR markers were mined from the transcriptome sequences, of which 23,189 ones were single base repeats and 9272 ones were double bases. From the marker sites with 2–6 base repeats each which were present in more than 90% of the samples, 200 SSR markers were selected (see Supplementary Materials) and primers were designed. Twenty-six pairs of primers for the selected polymorphic SSR markers were randomly chosen and used to amplify the DNA of 620 samples of *S. tetrandra* (Table 2).

Genetic Diversity of SSR Loci

Statistical results of genetic diversity analysis of 26 SSR loci are shown in Table 3. The results show that effective products were amplified from all of the 26 SSR loci, including 183 alleles, 2–16 alleles per pair of primers. UG-29652-FAM had the highest *Na* (16 alleles), whereas UG-10578-HEX, UG-20112-FAM, and UG-33750-FAM had the lowest *Na* (only 2 for each). The *Ne* was 1.471–8.099 (3.162 on average). For most of the loci, the effective number of alleles was lower than the observed number of alleles, indicating the interaction between alleles. The *I* was 0.521–2.217 (1.202). The *Ho* was 0.128–0.804 (0.498).

Table 2 Characteristics of microsatellite loci developed for *Stephania tetrandra* S. Moore

Locus	Allele size range (bp)	Number of alleles*	Fluorescence labeling	Repeat motif	FPr1 (5′-3′)	RPr1 (5′-3′)
UG-00535	222–253	5	HEX	(CT)26	TGCTCTATACCCGTCAACCC	TCTTTGGAGAGAGAGCTCCG
UG-01284	290–292	2	HEX	(AG)6tgtgtgtgt(GC)6	GGCAAAACAAAGGCCAAATA	CAATGCCAGGCAAAAATCAT
UG-06879	174–189	3	FAM	(AGCT)7	CAGGAAACCGAAATTCAGGA	TTCACGAGAAACCGACCTTC
UG-08259	217–246	6	HEX	(TTG)16	TTAGTAGGGAAAGGCCGGTT	CGATCAACCAACTAAAGGCA
UG-10578	235–239	2	HEX	(GAAC)5	GATTGAGGAGTCTGCGAAGC	ATGTTGGACCAGTAGTCCGC
UG-15653	188–211	4	HEX	(CT)23	CCAGGGTGATCTTTGAGGAA	CCAGGAGTTTGCTTCTGAGG
UG-17146	116–137	5	FAM	(TC)26	CGAGGTTTGGTTATCTCGGA	GACGACTGATTGCCCAAGTT
UG-17278	256–281	3	FAM	(CAA)13	TTGAGATGGTCCTCCCTCAC	TCCTCTGGTCGCTTCTTTGT
UG-17625	285–293	5	HEX	(TGC)16	GGAGGCATTGAATTCTGCAT	GACCTTCACTTCAGCAAGGG
UG-18165	294–300	3	FAM	(TA)6(TG)14	AGGGAAACGCTTGGACTTCT	CCGCATTGGATAGGATGATT
UG-19736	229–232	2	FAM	(TTAT)5	AGATCGATCCAGCTTTCCCT	AGCACTTGCAGTTCACCCTT
UG-20112	212–218	2	FAM	(ACA)6	CCCTCACATTCTGTGGCTTT	CGACCCCAAGTAATTTGGAA
UG-20767	240–299	2	FAM	(TTC)7	CGCTCTGTCAGCACTCTGTC	GTTAATTTGCATTGGCATGG
UG-24783	117–130	2	FAM	(GA)23	TATTGTGGGCAATGAAGTCG	GGTAGTTTGGCCGTTAAGCA
UG-25537	234–251	3	FAM	(TCCT)6	GTTGTACTTGGAGCGCCATT	TACCGAGTGGCCGTTATCTC
UG-25587	268–283	3	HEX	(AATC)6	GCTCTTCGGAGTAGTCGGTG	CAGAACCTGTCGGGATTCAT
UG-26113	151–158	3	FAM	(TTG)12	ATCGAGCGCAACAGAAGAGT	GCACTGCAATGTCGAATGTC
UG-26775	174–188	2	FAM	(ATTT)6	AATGTTTGCCATCCTTCTGG	CCAAGCGGTAACTCAATGGT
UG-29088	153–166	4	FAM	(TTG)12	GCGCAATGGAAGAATTTGAT	TGTCCCAATCGACCTTAACA
UG-29652	176–205	4	FAM	(TG)27	TGTGCCTTTGTTTATCCGAA	TGGGATGAGACACCAAATGA
UG-30904	205–229	3	HEX	(TC)25	CAGCCATTGTTGCTTCTCTG	AATATCAGACGGCCCAGATG
UG-33750	180–188	2	FAM	(AAAT)6	CCTCATTTCCGCAACAGTTT	AAATTGTGGACCCTTGCTTG
UG-36824	217–224	3	FAM	(TTG)5	GGAATTGCCTTCCCTTTGTT	TGTCCATCAAGATTTGGGGT
UG-41999	248–264	3	HEX	(TCTT)5	GCTTTCGACTTTGAGATCCG	AAATTTCATGCACCCTTTGC
UG-53337	261–272	2	HEX	(ATCA)6	GGCTTCCAATTGCTGGACTA	TAAAGCTGAGAGGCAGAGGC
UG-57529	268–283	6	HEX	(CAA)11	GTCCGGACTCTGGTGTTCAT	GTTGCATTCAATTCTCGGGT

*The number of bands that each primer could amplify when the selected 26 primers were used for the amplification of 620 plant individuals

Table 3 Genetic parameters, *F*-statistics, and gene flow value of 26 SSR loci in *Stephania tetrandra* S. Moore

Locus	*N*	*Na*	*Ne*	*I*	*Ho*	*He*	*PIC*	*Fis*	*Fit*	*Fst*	*Nm*
UG-00535	581	4	2.572	1.067	0.503	0.611	0.541	0.115	0.175	0.068	3.420
UG-01284	615	7	1.735	0.897	0.211	0.424	0.400	0.472	0.506	0.065	3.575
UG-06879	602	5	2.322	1.036	0.510	0.569	0.515	0.081	0.108	0.029	8.250
UG-08259	584	9	2.653	1.380	0.520	0.623	0.597	0.120	0.168	0.054	4.407
UG-10578	570	2	1.784	0.631	0.451	0.439	0.343	−0.121	−0.027	0.084	2.741
UG-15653	586	15	7.196	2.171	0.616	0.861	0.847	0.256	0.289	0.044	5.371
UG-17146	595	14	6.864	2.118	0.804	0.854	0.838	0.010	0.061	0.052	4.519
UG-17278	566	10	3.612	1.456	0.555	0.723	0.677	0.135	0.239	0.121	1.815
UG-17625	599	10	3.879	1.594	0.564	0.742	0.708	0.200	0.247	0.058	4.067
UG-18165	578	6	1.685	0.760	0.372	0.407	0.363	0.022	0.080	0.060	3.911
UG-19736	614	4	1.471	0.521	0.269	0.320	0.272	0.073	0.175	0.110	2.017
UG-20112	616	2	1.695	0.600	0.377	0.410	0.326	−0.023	0.074	0.095	2.386
UG-20767	571	5	2.441	0.974	0.462	0.590	0.503	0.150	0.232	0.097	2.338
UG-24783	600	8	1.968	0.986	0.292	0.492	0.440	0.361	0.399	0.059	3.981
UG-25537	601	5	3.145	1.214	0.663	0.682	0.618	−0.073	0.033	0.099	2.275
UG-25587	613	5	2.810	1.213	0.626	0.644	0.583	0.009	0.030	0.0214	11.44
UG-26113	615	6	2.588	1.172	0.545	0.614	0.563	0.079	0.113	0.036	6.703
UG-26775	601	4	2.205	0.940	0.128	0.547	0.489	0.493	0.748	0.502	0.248
UG-29088	614	8	3.042	1.297	0.654	0.671	0.615	−0.084	0.037	0.112	1.990
UG-29652	570	16	5.835	2.008	0.799	0.829	0.809	0.015	0.045	0.030	8.107
UG-30904	610	13	8.099	2.217	0.714	0.877	0.864	0.122	0.186	0.073	3.165
UG-33750	587	2	1.886	0.663	0.161	0.470	0.359	0.205	0.640	0.547	0.207
UG-36824	598	3	1.556	0.664	0.313	0.358	0.329	0.066	0.135	0.074	3.128
UG-41999	599	7	2.845	1.244	0.616	0.649	0.595	0.018	0.055	0.038	6.338
UG-53337	562	3	2.024	0.726	0.511	0.506	0.384	−0.096	−0.008	0.08	2.876
UG-57529	574	10	4.293	1.707	0.722	0.767	0.737	0.025	0.060	0.035	6.852
Mean		7.038	3.162	1.202	0.498	0.603	0.551	0.101	0.185	0.102	4.082

Note: *N* samples size, *Na* observed number of alleles per locus, *Ne* effective number of alleles per locus, *I* Shannon information index, *Ho* observed heterozygosity, *He* expected heterozygosity, *PIC* polymorphism information content, *Fis* intra-population inbreeding coefficient, *Fit* inter-population inbreeding coefficient, *Fst* genetic differentiation coefficient, *Nm* gene flow

The *Ne* was 0.320–0.877 (0.603), UG-30904-HEX having the highest value and UG-19736-FAM having the lowest value. For all the 26 loci, except UG-10578-HEX and UG-53337-HEX, the *Ho* was lower than the *He*, indicating a departure from the Hardy–Weinberg equilibrium. The *PIC* was 0.272–0.864 (0.551); this parameter had a value bigger than 0.5 for 16 loci which were highly polymorphic and a value between 0.25 and 0.5 for 10 loci which were middle-level polymorphic.

F-Statistics and Gene Flow

As shown in Table 3, the intra-population inbreeding coefficient (*Fis*) was − 0.121 to 0.493 (0.101 on average); this parameter had a positive value for 21 loci, suggesting the presence of inbreeding and homozygous excess. The inter-population inbreeding coefficient (*Fit*) was − 0.027 to 0.748

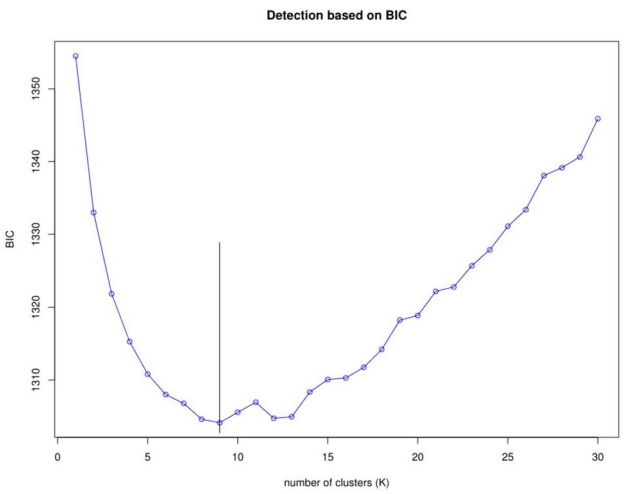

Fig. 2 Number of cluster (*K*) detection based on BIC

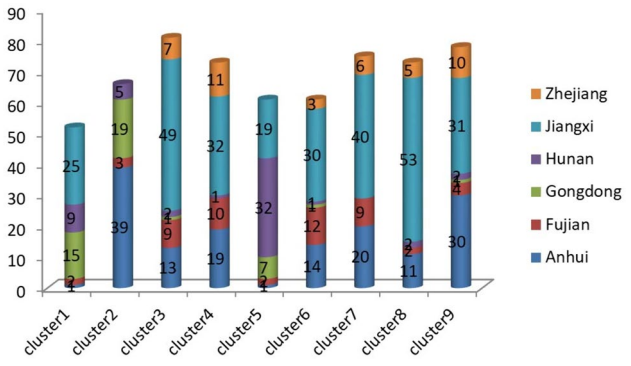

Fig. 3 Samples from different provinces contained in the nine clusters

(0.185). The genetic differentiation coefficient (*Fst*) was 0.021–0.547 (0.102); this parameter had a value lower than 0.05 (indicating a low level of genetic differentiation) for 7 loci, a value higher than 0.25 (indicating a very high level of genetic differentiation) for 2 loci, and a value indicating middle-level genetic differentiation for the remaining 17 loci. The gene flow (*Nm*) values varied from 0.207 to 11.440, with an average of 4.082; this parameter had a value higher than 1 for 24 loci, suggesting that inter-population genetic differentiation of these 24 loci was not a result of genetic drift.

Genetic Similarity, Distance, and Differentiation

K-means clustering was performed on amplified fragment length data of SSR primers for 620 plant individual samples collected from 40 cities or counties in 6 provinces of China. Bayesian Information Criterion (BIC) values were obtained by choosing different *K* values (the number of clusters). It was found that the value of *K* at the first inflection point was 9 (see Fig. 2); thus, it seemed most appropriate to divide the 620 samples into 9 clusters. The first cluster comprised 52 samples from Anhui, Fujian, Guangdong, Hunan, and Jiangxi; the second cluster consisted of 66 samples from Anhui, Hunan, Jiangxi, and Zhejiang; the 3rd, 6th, and 9th clusters had 81, 61, and 78 samples, respectively, which were collected from all the provinces; Clusters 4 and 8 each contained 73 samples from Anhui, Hunan, Jiangxi, Fujian, and Zhejiang. Cluster 5 had 61 samples, collected from Fujian, Guangdong, Hunan, and Jiangxi, whereas Cluster 7 had 75 samples from Anhui, Fujian, Jiangxi, and Zhejiang (see Fig. 3). It is clear that almost all clusters comprised samples from different geographical locations, indicating that geographical locations pose little impact on the genetic differentiation of *S. tetrandra* and grouping based on geographical regions is not reasonable.

Similarity test was performed on the 9 clusters to obtain their similarity coefficients and genetic distances (see Table 4). A UPGMA-based tree map was generated as shown in Fig. 4, in which the length of branch denotes Nei's genetic distance. As can be seen in Table 4, similarity coefficients of 9 clusters of *S. tetrandra* were 0.755–0.918, and genetic distances was 0.067–0.280, indicating that these clusters had a high level of similarity and short genetic distances.

Between-cluster genetic differentiation coefficients (*Fst*) were calculated for the 9 clusters, and a heat map (Fig. 5) was created. It can be seen in Fig. 5 that the genetic differentiation between Cluster 1 and Cluster 2 and between Cluster 1 and Cluster 4 was higher (circles having deeper color), Fst being above 0.095, whereas Clusters 3 and 4 showed the least genetic differentiation (the circle having the lightest color). *F*-statistics analysis results (Table 5) showed that *Fis*, *Fit*, *Fst*, and adjusted *Fst* were 0.172, 0.248, 0.092, and 0.219, respectively, indicating a moderate level of genetic differentiation. The gene flow value was 2.461, bigger than 1.

Principal Co-ordinates Analysis (PCoA) Result

PCoA can reveal the difference between two or two groups of samples by intuitively comparing the linear distance between the samples on coordinate axes. The shorter the

Table 4 Genetic distances and similarity coefficients of 9 clusters of *Stephania tetrandra* S. Moore

	Cluster1	Cluster2	Cluster3	Cluster4	Cluster5	Cluster6	Cluster7	Cluster8	Cluster9
Cluster1	****	**0.755**	**0.799**	**0.760**	**0.844**	**0.804**	**0.855**	**0.870**	**0.762**
Cluster2	*0.280*	****	**0.918**	**0.912**	**0.814**	**0.897**	**0.859**	**0.875**	**0.886**
Cluster3	*0.224*	*0.086*	****	**0.935**	**0.879**	**0.907**	**0.877**	**0.904**	**0.896**
Cluster4	*0.274*	*0.092*	*0.067*	****	**0.837**	**0.886**	**0.902**	**0.861**	**0.893**
Cluster5	*0.169*	*0.206*	*0.129*	*0.178*	****	**0.820**	**0.816**	**0.828**	**0.813**
Cluster6	*0.218*	*0.108*	*0.097*	*0.121*	*0.199*	****	**0.883**	**0.855**	**0.902**
Cluster7	*0.157*	*0.152*	*0.131*	*0.103*	*0.204*	*0.124*	****	**0.901**	**0.862**
Cluster8	*0.140*	*0.133*	*0.101*	*0.150*	*0.189*	*0.157*	*0.104*	****	**0.852**
Cluster9	*0.272*	*0.121*	*0.110*	*0.113*	*0.208*	*0.103*	*0.149*	*0.161*	****

Note: Figures in bold are similarity coefficients, for example, 0.755 in row 2 and column 3 is the genetic similarity coefficient between Clusters 1 and 2. Italic figures denote Nei's genetic distances, for example, the genetic distance between Clusters 1 and 2 is 0.280

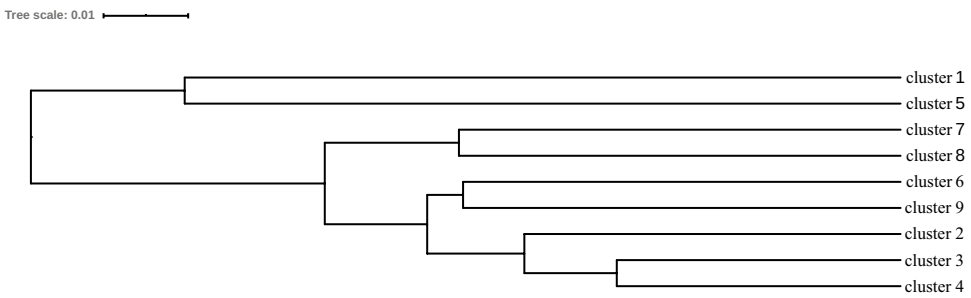

Fig. 4 Cluster analysis of Nei's genetic distances. The scale on the upper left corner denotes genetic distance which increases from right to left starting from zero. The lowermost branch point represents the genetic distance at which samples are divided into Clusters 3 and 4, and the branch point immediately to the left of it represents the distance where samples are divided into Clusters 2, 3, and 4, and other branch points can be interpreted in a similar fashion

linear distance between two or two groups of samples, the smaller the genetic difference. The PCoA result of the 9 clusters is shown in Fig. 6, from which we know that the PCoA result was basically consistent with that of the clustering analysis.

Genetic Structure Analysis Result

For the purpose of further exploration of the genetic relationship between the 40 natural populations and between the 9 clusters, the STRUCTURE software was used to perform Bayesian clustering to compare their genetic structure. STRUCTURE is a calculation method based on the Bayesian model. A corresponding maximum likelihood is generated for the simulation of each K value. In STRUCTURE, the maximum likelihood is output as a ln-transformed value (ln likelihood, $L(K)$). The larger the $L(K)$ is, the more the K value is approximate to the true level. The $L(K)$ generally increases with the K value but will slowly plateau out. The goal of selecting the optimal

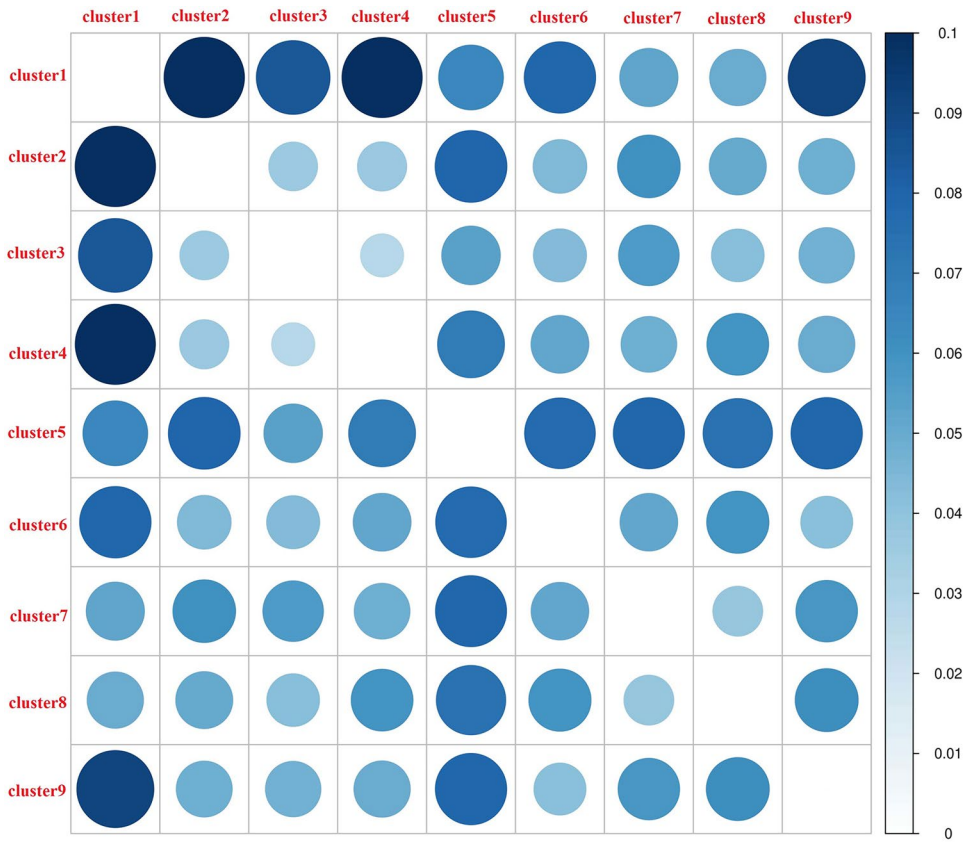

Fig. 5 Coefficients of genetic differentiation (Fst) among clusters. As indicated by the color scale on the right, Fst increases as the color becomes darker from bottom to top. This means that a darker circle denotes a higher Fst

Table 5 *F*-statistics for 9 clusters of *Stephania tetrandra* S. Moore

F-statistics	Value	*p*
Fst	0.092	0.001
Fis	0.172	0.001
Fit	0.248	0.001
Fst max	0.421	
F′st	0.219	
Nm	2.461	

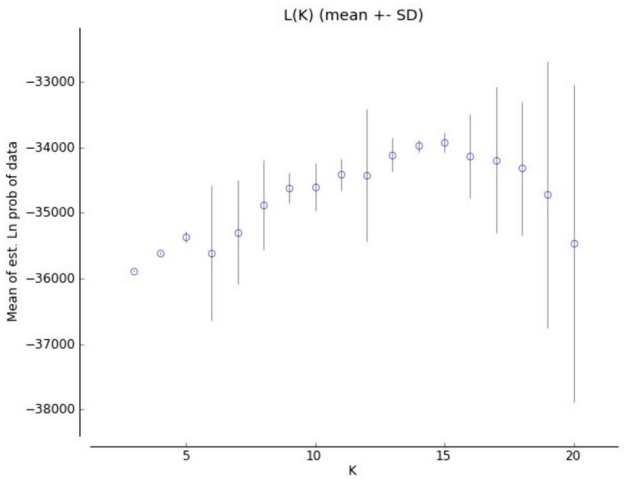

Fig. 7 Graph plotted with ln likelihood versus the number of clusters (*K*)

K value is to find the inflection point. Using this method, the change of *L*(*K*) with *K* and that of delta *K* with *K* were disclosed as shown in Figs. 7 and 8, respectively. In Fig. 8, delta *K* reached the maximum level when *K* was 5, suggesting that the 620 samples could be divided into 5 groups. Genetic maps of group structure were generated with $K = 5$ (see Fig. 9A–C). In Fig. 9A, all 620 plant samples from sampling sites in different cities or counties contained the genetic lineage of the 5 groups, and every group made contributions to the hereditary basis of the samples, resulting from crossing among the 5 groups. This indicates that the distribution of natural habitats had little influence upon the division of the five groups. In Fig. 9B, all the 9 clusters inherited genes from the 5 groups to varying degrees, but the proportion of genes inherited from the 5 groups by each cluster differed. Clusters 1, 2, and 8 inherited more genes from Group 1 than from other groups, and Cluster 3 was more influenced genetically by Group 5 than by other groups. In Fig. 9C, all natural populations inherited genes from the 5 groups to varying degrees; the proportions of genes inherited from the 5 groups were similar for all the natural populations except that GDNXS, GDSXX, HNXXX, and JXQNX inherited more genes from Group 2 than from other groups.

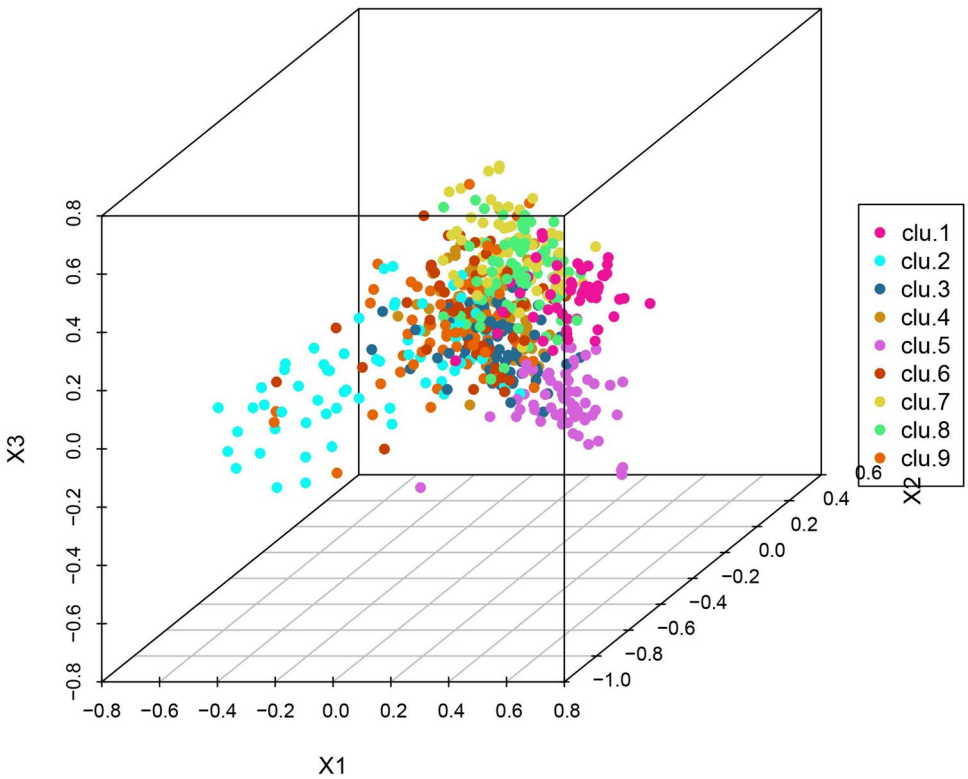

Fig. 6 Principal Co-ordinates Analysis result

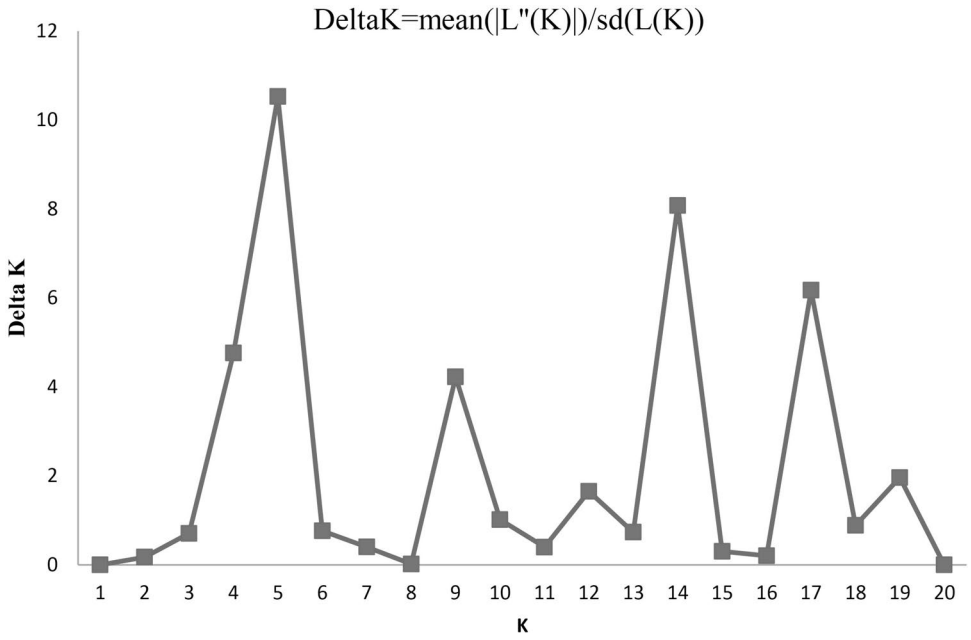

Fig. 8 Graphic plotted with delta *K* versus *K*

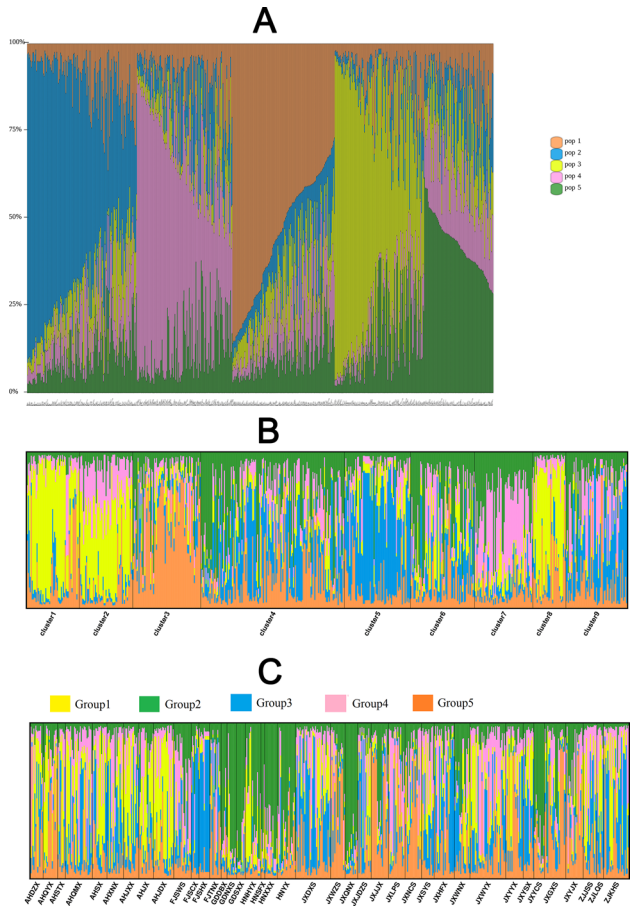

Fig. 9 Population structure analysis results. **A** Assigned proportion of each individual in the groups when $K=5$. **B** Assigned proportion of each cluster in the groups when $K=5$. **C** Assigned proportion of each natural population in the groups when $K=5$. In **C**, the abbreviations stand for sampling cities and counties, the first two letters representing the name of a province, and those followed being the name of a city or a county (X means a county and S means a city; e.g., AHDZX is "Dongzhi County, Anhui Province" (see Table 1))

Discussion

SSR Genetic Diversity Analysis

Genetic diversity refers to the sum of genetic variations among individuals within a species or within a population. Intraspecific diversity is not only an important source of species genetic diversity, but also a decisive factor for a species to respond successfully to external disturbance, and an important index used for the evaluation of germplasm resources (Nafees 2015; Zhen 2017). *S. tetrandra* is the only species in the subgenus *Botryodiscia* of the genus *Stephania* of the family Menispermaceae. Retrieval in TIMETREE (http://timetree.org/) using terms "Menispermaceae" and "Stephania" showed that the evolution time of plants in the genus *Stephania* was about 36.4 MYA, and the evolution time of *S. tetrandra*, about 4.96MYA, was shorter than that of other species in the genus *Stephania*. This short evolution time may have a certain influence on the degree of species differentiation of *S. tetrandra*. In our experiment, the genetic diversity of SSRs was analyzed on 620 samples from Anhui, Fujian, Guangdong, Hunan, Jiangxi, and Zhejiang of China. The amplification results of 26 SSR loci showed that the values of *Na* varied from 2 to 16. For all loci, there were great differences in *PIC*, *Ne*, *I*, *Ho*, and *He* (Table 3), consistent with findings of other species (Huang 2019; Ismail et al. 2019). These differences may be due to the fact that the EST-SSRs used in our study are transcribed regions of the genomes; therefore, they can be either neutral markers or included into genome regions associated with adaptively important traits (i.e., being subject to selection) and thus can determine the ability of *S. tetrandra* to adapt to any

environmental stress factors; meanwhile, they are of great significance to the evaluation of germplasm resources (An et al. 2009). *PIC* values of 16 loci among the 26 loci were bigger than 0.5, indicative of a high level of polymorphism; and the values of *PIC* of the remaining 10 loci were greater than 0.25 (a medium level of polymorphism). According to Purvis and Franklin (2005), loci with $PIC > 0.7$ are the best genetic markers. In the present work, 6 loci had a *PIC* value bigger than 0.7, suggesting that it was possible to select SSR loci for efficiently investigating the genetic diversity of *S. tetrandra* from the loci tested. From the results of our experiment, it was also found that *Ho* for all loci except for UG-10578-HEX and UG-53337-HEX was lower than *He*, suggesting that most loci in daughter populations violated the Hardy–Weinberg equilibrium, maybe due to the fact that parental populations have been affected by non-random factors in the process of gene transmission. *Fst* can be used to assess the population differentiation degree and genetic structure; a value between 0 and 0.05 indicates no or low-level differentiation, a value between 0.05 and 0.15 indicates a medium level of differentiation, a value between 0.15 and 0.25 indicates a high level of differentiation, and a value greater than 0.25 means differentiation is at a very high level (Wright 1977). In our study, the value of *Fst* was bigger than 0.05 for most loci, suggesting that there was a medium level of genetic differentiation. The gene flow *Nm* were 0.102 and 4.082 (individuals per generation), greater than 1 for most loci, meaning that the current genetic structure has been maintained and genetic differentiation due to genetic drift has been overcome (Ellstrand and Elam 1993). Moreover, the results of *Fst* and *Nm* suggested that there was a moderate level of subdivision of populations (Wright 1965). A high heterozygosity across the whole species may make it more resilient and enable its sustainable development, since individuals with greater heterozygosity within a population have increased developmental stability (Leary et al. 1983; Novicic et al. 2011). From the point of view of breeding, however, heterozygosity may bring some difficulties to the selection and breeding of good germplasms (Liu 2005); that is to say, a low level of heterozygosity may be beneficial to the selection and breeding of high-quality germplasms.

Genetic Variation and Structure Analyses

All samples collected in the present study came from wild populations of *S. tetrandra*. Prior to the analysis of genetic differentiation and genetic structure, DAPC (discriminant analysis of principal components)–based genotyping on the 620 samples was performed. We found that the resulting genetic clusters were not closely associated with natural populations (refer to the Supplementary Materials for the genotyping results: detection based on BIC and cluster. Neis_distance). In the present study, we aimed to analyze the genetic diversity and genetic structure of the species *S. tetrandra*, and since there were great differences among individuals from the same natural population, we carried out further analyses using genetic clusters obtained from the genotyping instead of natural populations. According to the genotyping results, optimally the 620 samples should be divided into 9 clusters, each one of which contained individuals from different geographical locations (i.e., from different natural populations), suggesting that genetic clustering based on natural populations was suboptimal. In our study, the similarity coefficients and genetic distances of the 9 genetic clusters were 0.755–0.918 and 0.067–0.280, respectively, genetically highly similar and short-distanced. Furthermore, as demonstrated by *F*-statistics analysis, there was a medium level of differentiation between the 9 clusters, and a value of gene flow greater than 1 indicated that gene exchange occurred frequently between the clusters, so the clusters were highly similar (the similarity coefficients being 0.755–0.918), suggesting that genetic drift and natural selection may play an unimportant role in *S. tetrandra*; germplasm differentiation of *S. tetrandra* may have mainly resulted from individual variation. For the purpose of further exploration of the genetic relationships between populations of *S. tetrandra*, the Bayesian clustering analysis was implemented in the STRUCTURE software to compare the genetic structure of 620 samples of *S. tetrandra* (Porras-Hurtado et al. 2013; Rosenberg 2004). Results of the STRUCTURE analysis showed that the 620 samples could be divided into 5 groups. Due to the influence from gene flow, the 9 clusters and 40 natural populations inherited genes from the 5 groups to varying degrees.

Factors Associated with the Genetic Differentiation of *S. tetrandra*

S. tetrandra is a dioecious species with unisexual flowers and reproductive characteristics typical of the family Menispermaceae (Cheng 2022). Self-crossing is not possible since male and female reproductive organs are located in different plant individuals; thus, the homozygous excess should be mainly due to inbreeding between closely related individuals. We conducted a comprehensive field survey on the distribution, habitat, and individual biological characteristics of wild *S. tetrandra* in China, and found that the ratio of female and male individuals in wild populations of *S. tetrandra* was 1:3, severe gender bias existing. Moreover, dispersal of the fruit of this species by the wind is difficult because of its high mass (about 0.789 g per fruit) and that it is not easy to shed from the plant, and it is slightly bitter in taste and birds do not like eating it, resulting in difficult dispersal of its seeds. Therefore, the geographical distribution of *S. tetrandra* is limited to a certain range. From the distribution map of the sampling sites as shown in Fig. 1 of

our study, Jiangxi had the highest distribution density of *S. tetrandra* populations, and the distribution density in other provinces decreased with the distance between them and Jiangxi. The roots and rhizomes of *S. tetrandra*, however, possess a strong ability to reproduce, and in nature, this species shows both seed propagation and vegetative propagation (Guo et al. 2007). In the present study, all plant individuals instead of natural populations from different geographical locations were used in cluster analysis and genetic structure analysis because individuals from the same natural population could not be grouped into the same genetic cluster, indicating the presence of population structure and pronounced genetic subdivision.

S. tetrandra is mainly distributed in Guangdong, Hunan, Jiangxi, Anhui, Zhejiang, and Fujian in China. Affected by various factors, germplasms and phenotypic characteristics of *S. tetrandra* from different habitats differ (Yang et al. 2020). The difference in medicinal quality of traditional Chinese drugs has an adverse effect on the development of their resources and their clinical use, and the quality difference is mainly affected by germplasms (Meng et al. 2013). The analysis of genetic differences of germplasms from different geographical locations can provide a basis for the selection of excellent germplasms (Zawedde et al. 2015) and the selection of suitable environmental conditions of producing areas as well as the development and utilization of medicinal resources (Ma and Xiao 1998).

Conclusion

Altogether, the species studied is characterized by the presence of population structure and pronounced genetic subdivision, which, together with the presence of gene flow, may indicate a relatively stable recent state of these populations. However, the overexploitation of *S. tetrandra* resources, as well as other anthropogenic factors, can disturb the existing equilibrium. In this regard, the obtained results can be used for monitoring, rational using, and restoration of these populations. Moreover, developed markers will also be useful for the design of plantation cultivation, and since they mark the transcribed genome, they can be applied to research on increasing the productivity of *S. tetrandra* and developing alternative sources of target biologically active substances (for example, via in vitro cultivation).

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Availability of Data and Materials All relevant data are within the manuscript and its Supporting Information files.

Declarations

Ethical Approval Not applicable.

Competing Interests The authors declare no competing interests.

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