Regular Article

Diversity Analysis of *Polyporus umbellatus* in China Using Inter-simple Sequence Repeat (ISSR) Markers

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Polyporus (P.) umbellatus, an endangered medicinal fungus in China, is distributed throughout most areas of the country. Thirty-seven natural *P. umbellatus* samples collected from 12 provinces in China were subjected to the inter-simple sequence repeat (ISSR) assay to investigate the genetic diversity within and among the 11 natural populations. Nine ISSR primers selected from 100 primers produced 88 discernible DNA bands, with 46 being polymorphic. The frequency of polymorphism varied from 19.57 to 93.48% with an average of 61.26% across all populations. At the population level, the within-population variance was much greater (92.04%) than the between-population variance (7.96%) as revealed by analysis of molecular variance. Eleven *P. umbellatus* populations were grouped into two major clusters, and the clustering pattern displayed four groups using the unweighted pair-group method with an arithmetic mean dendrogram. Principal coordinate analysis further indicated that the genetic diversity of *P. umbellatus* strains was unevenly distributed and displayed a clustered distribution pattern instead. Within these clusters, subgrouping (Henan and Hubei) and cluster II (Jilin and Heilongjiang) related to the geographic distribution were evident. The present study provides the first global overview of *P. umbellatus* diversity analysis in China, which may open up new opportunities in comparative genetic research on this medicinal fungus in other countries.

Key words genetic diversity; inter-simple sequence repeat (ISSR); *Polyporus umbellatus*; conservation

Polyporus (P.) umbellatus, a medicinal fungus belonging to Polyporaceae, Basidiomycetes, is widely distributed in China, Japan, Korea, and other temperate regions of the Northern Hemisphere.^{1,2)} *P. umbellatus* is widely distributed in most provinces around China.³⁾ The dried sclerotia of *P. umbellatus* have been used as a natural Chinese medicine in Asian countries especially in China for more than 2500 years.⁴⁾ It has been demonstrated that *P. umbellatus* sclerotia has significant pharmacological effects on treating edema, acute nephritis and diarrhea.⁵⁾ Many medical prescriptions containing *P. umbellatus*, such as Wulingsan (WLS), Choreito, CGX, Khz, Saireito and Yinchewuling San, have been used to treat various kidney diseases in China, Japan, India and other countries.⁶⁾ In addition, *P. umbellatus* has been verified with various pharmacological activities such as anti-tumor activity, diuretic activity and treatment of chronic kidney disease.^{7–14)} Moreover, the main chemical constituents of *P. umbellatus* sclerotia are steroids and polysaccharides,⁴⁾ which have been considered as important bioactive substances. The steroids compounds that isolated from *P. umbellatus* such as ergosterol and D-mannitol are both with diuretic effects, polyporosterones A–G are all with anticancer activity^{15–17)} and the *P. umbellatus* polysaccharides possess immuno-enhancing activity. Nowadays, the natural resource of *P. umbellatus* sclerotia has been decreased dramatically because of the lack of effective protection strategies, together with increasing commercial demand. In addition, the fungus is currently added to the endangered list in the China Red Book.¹⁾ Thus, effective measures should be adopted to protect the precious resource and guide the commercial production of *P. umbellatus* sclerotia.

It has been well documented that evaluating the extent and distribution of genetic variation within a species is a primary method for evolutionary comprehension, conservation of ge-

netic resources, and subsequent breeding strategies.¹⁸⁾ The loss of adaptive genetic variation makes wildlife populations at an increased risk of extinction.¹⁹⁾ Furthermore, establishing a population genetics framework makes it easy to evaluate the complex genetic structure within populations.²⁰⁾ Therefore, assessment of the genetic diversity and the genetic structure is necessary for fashioning conservation measures.

A number of polymerase chain reaction (PCR)-based techniques, such as Amplified Fragment Length Polymorphism (AFLP), Simple Sequence Repeat or microsatellites (SSR), sequence-related amplified polymorphism (SRAP) and inter-simple sequence repeat (ISSR) can be used to detect polymorphisms for studies of genetic diversity.^{21,22)} Markers such as sequence-related amplified polymorphism (SRAP),²³⁾ nuclear ribosomal DNA internal transcribed spacer (nrDNA ITS) and 28S ribosomal RNA (rRNA) (LSU, large subunit) sequences²⁴⁾ have been used to analysis the population structure of *P. umbellatus*. Nevertheless, each of these marker systems presents some drawbacks. SRAPs are dominant markers with poor consistency and low reproducibility.²⁵⁾ The heterozygosity of polymorphic loci for nrDNA ITS and 28S rRNA markers are limited because of single nucleotide mutations and insertion/deletion events.²⁶⁾ ISSR could be able to produce more reliable and reproducible bands because of the higher annealing temperature and longer sequence of ISSR primers. ISSR has been widely applied in genetic diversity analyses and molecular identification.^{27,28)} Therefore, ISSR markers have been proved to be a powerful tool for genetic studies in populations.

The level of genetic diversity and the way in which it is maintained among populations are of major concerns in conservation biology. In the present study, we investigate the level and partitioning of genetic diversity in *P. umbellatus* among the eleven populations throughout its known distribution area

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using ISSR markers. This preliminary study provides necessary genetic information for basic and applied research efforts related to *P. umbellatus* in future.

MATERIALS AND METHODS

Sclerotial Samples In total, 37 fresh sclerotial samples (Table 1, Figs. 1, 2) used for the present study were obtained from the following 12 provinces: Gansu, Shanxi, Shannxi, Henan, Hebei, Hubei, Sichuan, Yunnan, Tibet, Heilongjiang, Jilin and Beijing. For each sample, 3–5 individuals were selected randomly. In total, 165 individuals were included in the study. All the samples were taxonomically identified by Professor Shun-Xing Guo, Institute of Medicinal Plant Development, Chinese Academy of Medical Science and Peking Union Medical College. A voucher specimen (No. ALH-05-0618) was also deposited there for future reference.

DNA Extraction Fresh samples were washed with tap water and surface-sterilized in 75% ethanol for 1 min, followed by 3% NaClO solution for 3 min and then rinsed in sterile water three times. Then the sterilized sclerotia were cut into half and 50 mg inner tissue were frozen at -80°C for more than 30 min. Freeze-dried sclerotia were then ground to a fine powder with liquid nitrogen. Total genomic DNA was extracted using E.Z.N.A. Fungal DNA kit (Omega Bio-Tek, Doraville, GA, U.S.A.) following the manufacturer's instructions.

PCR Amplification PCR amplification was performed in 25 μL reaction volume containing 12.5 μL of 2 $\times$ Master PCR Mix (TIANGEN Biotech, Beijing, China), 1.0 μL template DNA (20 ng/ μL), 1 μL primer (10 μM) and 10.5 μL ddH₂O. The cycling parameters were as follows: initial denaturation at 94°C for 5 min, followed by 36 cycles: 94°C for 45 s, 45 s annealing at the annealing temperature of primers used and 2 min extension at 72°C with 10 min final extension at 72°C at the end. The amplification products were electrophoresed on a 2.0% agarose gel. The gels were stained with ethidium bromide and documented on Bio-Rad Gel DocTM XR⁺ system (Hercules, CA, U.S.A.).

Data Acquisition and Statistical Analyses The ISSR data was scored as “1” (presence of fragment) and “0” (absence of fragment) manually by visualizing electropherogram. The binary data matrix is input into POPGENE version 1.31.²⁹⁾ The following indices were used to quantify the amount of genetic diversity within each population examined: percentage of polymorphic loci, observation of number of alleles per locus (N_a), effective number of alleles per locus (N_e), genetic differentiation among populations estimated by Nei's gene diversity statistics³⁰⁾ and Shannon's information measure.³¹⁾

Analysis of molecular variance (AMOVA)³²⁾ was assessed to calculate the proportion of within and between geographic regions diversity using the GenAlex 6.5 software.³³⁾ As Beijing is geographically surrounded by the Hebei Province, the sample collected from Beijing was sorted into the population of Hebei for the following analyses.

The binary data for *P. umbellatus* Sclerotial were subjected to principal coordinate analysis (PCA)³⁴⁾ using the GenAlex 6.5.³³⁾ Ordination plots were drawn to indicate the multilateral genetic relationships among the *P. umbellatus* accessions. To examine the genetic relationship among populations, a dendrogram was also constructed based on Nei's genetic distance

Table 1. Details of the Samples Used in the Present Investigation

Population (province)	Sample number	Origins	Collection data
Shannxi	1	Huanglong, Shannxi	April, 2014
	2	Ningshan, Shannxi	April, 2014
	3	Lueyang, Shannxi	April, 2014
	4	Fengxian, Shannxi	April, 2014
	5	Baoji, Shannxi	April, 2014
	6	Liuba, Shannxi	April, 2014
Henan	7	Xixia, Henan	March, 2014
	8	Luoyang, Henan	March, 2014
	9	Sanmenxia, Henan	March, 2014
Shanxi	10	Pingyao, Shanxi	May, 2014
	11	Guxian, Shanxi	May, 2014
	12	Lyvliang, Shanxi	July, 2014
	13	Huozhou, Shanxi	July, 2014
	14	Yuci, Shanxi	July, 2014
	15	Linfen, Shanxi	July, 2014
Hubei	16	Xinagyang, Hubei	May, 2014
Gansu	17	Shennongjia, Hubei	May, 2014
	18	Tianshui, Gansu	April, 2014
	19	Longnan, Gansu	April, 2014
Beijing	20	Gannan, Gansu	April, 2014
	21	Beijing	May, 2014
	22	Chengde, Hebei	May, 2014
Hebei	23	Laiyuan, Hebei	May, 2014
	24	Dali, Yunnan	June, 2014
	25	Lijiang, Yunnan	June, 2014
Sichuan	26	Zhaotong, Yunnan	June, 2014
	27	Lanping, Yunnan	June, 2014
	28	Guangyuan, Sichuan	May, 2014
Tibet	29	Aba, Sichuan	June, 2014
	30	Lushan, Sichuan	August, 2014
	31	Bomi, Tibet	April, 2014
Jilin	32	Milin, Tibet	August, 2014
	33	Linjiang, Jilin	August, 2014
	34	Ji'an, Jilin	August, 2014
Helongjiang	35	Tonghua, Jilin	August, 2014
	36	Wuchang, Helongjiang	August, 2014
	37	Hailin, Heilongjiang	August, 2014

using an unweighted paired group method of cluster analysis by arithmetic averages (UPGMA) of NTSYS-pc version 2.02c.

RESULTS

Genetic Analysis To evaluate and characterize the 37 samples, 100 UBC ISSR primers were initially screened with a subset of 12 samples from different provinces and finally 9 primers were proved to present clear and reproducible patterns. The nine ISSR primer combinations revealed 88 distinct bands among the 37 samples (Table 1), from which, 46 (52.2%) were polymorphic (Table 2). The number of amplified loci by the primers ranged from 7 (UBC 808) to 14 (UBC 842) with a size range of 180–2000 bp. The average number of amplified loci and polymorphic loci per primer were 9.78 and 5.11, respectively. At the population level, the percentage of polymorphic loci per population ranged from 19.57 to 93.48% with an average of 61.26%. The Shannon's information indices (I) ranged from 0.14 to 0.54, with an average of 0.38 at the population level. The samples of Yunnan and Shanxi pre-

Fig. 1. Locations of the 37 Sampled *P. umbellatus*

Blue triangle: Distribution records of specimens collected.

Fig. 2. The Aboveground Part (A) and the Sclerotia (Underground Part (B)) of *P. umbellatus*

sented the greatest level of genetic diversity (He : 0.37, I : 0.54, respectively) whereas the sample of Heilongjiang presented the lowest level of genetic diversity (He : 0.10, I : 0.14, respectively), as shown in Table 3.

Analysis of Intravarietal Genetic Variation Pairwise Nei's genetic distances ranged between 0.079 (Jilin/Heilongjiang) and 0.5534 (Gansu/Heilongjiang). The Nei's distances between Jilin and Heilongjiang (0.004) were not statistically significant (Table 4). The AMOVA based on Nei's values indicated that most of the genetic diversity occurred within populations (92.04%) while the variability among populations contributed 7.96% to the observed genetic diversity (Table 5).

ISSR-Based Genetic Relationships among *P. umbellatus* Population In order to represent the relationship among populations, cluster analysis (UPGMA) was used to generate a dendrogram based on Nei's genetic distance between the eleven populations studied (Fig. 3). The dendrogram obtained from UPGMA grouped eleven populations into two major clusters. Cluster I grouped 6 populations from Shannxi, Yunnan, Shanxi, Sichuan, Henan and Hubei provinces and Jilin and Heilongjiang province were included in clusters II. The populations, 'Tibet', 'Gansu' and 'Hebei' did not fall in any cluster but they were not distinct from others.

Principal Component Analysis The Principal Component Analysis (PCA) was performed, which reveals the first three most informative principal components with eigenvalues

Table 2. The ISSR Primers Used, Number of Amplified Loci, Polymorphic Loci and Percentage Polymorphism in Evaluation of Genetic Diversity

No.	Primer number	Primer motif	No. of amplified loci	No. of polymorphic loci	Percentage polymorphism
1	UBC 808	(AG) _n G	7	5	71.4
2	UBC 809	(GA) _n T	9	5	60
3	UBC 817	(CA) _n A	10	5	50
4	UBC 835	(AG) _n YC	8	5	62.5
5	UBC 840	(GA) _n YT	12	5	41.7
6	UBC 841	(GA) _n YC	11	5	45.5
7	UBC 842	(GA) _n YG	14	6	42.9
8	UBC 855	(AC) _n YT	10	5	50
9	UBC 864	(ATG) _n	7	5	71.4
Average			9.78	5.11	52.2

Table 3. Estimation of Different Genetic Diversity Based on 46 ISSR Loci of 11 Populations of *P. umbellatus*

Population	N_a	N_e	He	I
Shannxi	1.93±0.24	1.61±0.27	0.35±0.12	0.52±0.17
Henan	1.72±0.45	1.57±0.36	0.32±0.20	0.45±0.28
Shanxi	1.91±0.28	1.66±0.31	0.37±0.14	0.54±0.19
Hubei	1.54±0.50	1.54±0.50	0.27±0.15	0.37±0.14
Gansu	1.39±0.49	1.31±0.39	0.17±0.21	0.24±0.31
Hebei	1.67±0.47	1.53±0.37	0.30±0.21	0.43±0.30
Yunnan	1.89±0.31	1.65±0.28	0.37±0.14	0.54±0.19
Sichuan	1.76±0.43	1.58±0.37	0.32±0.19	0.47±0.27
Tibet	1.47±0.50	1.47±0.50	0.23±0.25	0.33±0.35
Jilin	1.23±0.43	1.23±0.43	0.12±0.21	0.16±0.28
Heilongjiang	1.20±0.40	1.20±0.40	0.10±0.20	0.14±0.26

N_a , number of different alleles; N_e , effective number of alleles; He , Nei's (1973) gene diversity; I , Shannon's information index.

of 32.74, 55.31, and 67.96%, respectively (Fig. 4). PCA is considered as a powerful tool for extracting a maximum of information from molecular marker data, if the first two or three

Table 4. Pairwise Nei's Genetic Distances between the Studied Populations

	Shannxi	Henan	Shanxi	Hubei	Gansu	Hebei	Yunnan	Sichuan	Tibet	Jilin	Heilongjiang
Shannxi	—	0.8007	0.8434	0.8573	0.8231	0.7572	0.8986	0.8664	0.8387	0.669	0.6418
Henan	0.2223	—	0.8088	0.8282	0.7921	0.6539	0.7906	0.8075	0.7549	0.7252	0.7441
Shanxi	0.1704	0.2122	—	0.7715	0.6673	0.8485	0.881	0.8716	0.7967	0.6968	0.6787
Hubei	0.154	0.1885	0.2594	—	0.7427	0.6848	0.8582	0.737	0.7447	0.6108	0.6571
Gansu	0.1946	0.233	0.4044	0.2974	—	0.5874	0.7481	0.7527	0.7312	0.6245	0.575
Hebei	0.2781	0.4248	0.1642	0.3786	0.532	—	0.7851	0.7383	0.7345	0.6228	0.6062
Yunnan	0.1069	0.2349	0.1267	0.1529	0.2903	0.2419	—	0.8421	0.769	0.693	0.6558
Sichuan	0.1434	0.2138	0.1374	0.3052	0.2841	0.3034	0.1718	—	0.7817	0.7691	0.7249
Tibet	0.1759	0.2811	0.2273	0.2948	0.3131	0.3085	0.2626	0.2462	—	0.7171	0.656
Jilin	0.402	0.3213	0.3613	0.4929	0.4708	0.4735	0.3667	0.2626	0.3325	—	0.9269
Heilongjiang	0.4434	0.2955	0.3876	0.4199	0.5534	0.5006	0.4219	0.3217	0.4216	0.0759	—

Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

Table 5. AMOVA Showing the Partitioning of Genetic Variation within and between Regions of *P. umbellatus*

Source	Degrees of freedom	Sum of squares	Variance	% Variation
Among populations	10	377.62	0.84	7.96
Within population	26	252.67	9.71	92.04
Total	36	630.29	10.55	100

Significance tests after 1000 permutations, $p < 0.001$.

principal coordinates explain more than 25% proportion of the total variation.³⁵⁾ As shown in Fig. 3, the results of the PCA are largely consistent with the known cluster analysis.

DISCUSSION

Genetic Diversity In recent years, limited studies have found that high levels of genetic variability in *P. umbellatus* using SRAP, nrDNA ITS, and 28S rRNA (LSU, large subunit) sequences.^{23,24)} However, the materials in this research using SRAP did not cover all geographic regions (only 7 samples) and the nrDNA ITS and 28S rRNA markers are limited because of single nucleotide mutations and insertion/deletion events compared to ISSR although 42 samples included in the study. In present study, the analysis of the 12 populations using 9 polymorphic ISSR revealed high values of genetic diversity within populations. This is the first study of the *P. umbellatus* using ISSR molecular marker, which is useful in detecting genetic polymorphism in large portion of the genome simultaneously. Nine primers are screened out that are useful for *P. umbellatus*. Our results show a relatively high level of genetic diversity, with 52.2% of loci being polymorphic. This relatively high genetic diversity may own to several characteristics as follows. First, geographic isolation has played an important role during the formation of genetic diversity. Second, changes in distribution or habitat may be the other important factor leading to high genetic diversity within populations of *P. umbellatus* as it has been used as a natural medicine in China for more than 2500 years.⁴⁾ The estimators of genetic diversity are higher in populations of Yunnan, Shanxi and Shannxi, which may be attributed to the wide distribution of *P. umbellatus* in these three provinces. So the changes in distribution, together with the wide distribution of *P. umbellatus*, seriously contributed to the high level of genetic diversity at

the population level.

Genetic Structure Analysis of the ISSR markers using different approaches such as Nei's gene diversity statistics, Shannon's information measure and AMOVA demonstrated similar interpretations of the genetic structure of the populations of *P. umbellatus*. AMOVA analysis showed that 92.04% of the total variation resulted from differentiation within populations while between populations variation only accounted for 7.96% of the total genetic diversity. The high values of within population genetic diversity in *P. umbellatus* are in accordance with other studies in conifers.^{23,24)}

The ISSR data after UPGMA analysis has revealed some interesting trends. Our results show that two of clusters in the dendrogram displayed some strict relationship with geographical distribution as Henan and Hubei, Jilin and Helongjiang are respectively geographical contiguity (Fig. 3), whereas the result carried out by Zhang *et al.* using SRAP demonstrated no strict geographic relationship among *P. umbellatus*.²³⁾ The lack of geographic relationship in previous study between populations might be due to the small subset of *P. umbellatus* samples were used in their study. It is well know that the *P. umbellatus* sclerotia's growth depend on a symbiotic relationship with the forest pathogenic fungus *Armillaria* species.³⁶⁾ Conversely, *Armillaria* species are widely distributed throughout the world and the fungal species comprise several biological species in North America, Europe, Australia and China.³⁷⁾ Additional studies will be conducted to clarify whether the genetic structure of *P. umbellatus* is affected by the composition of *Armillaria* species population.

Conservation Implications The ultimate goals of Chinese Herb resource conservation are to ensure sustainable survival of populations and preserve their good quality as well. Loss of genetic diversity could make the degradation of a species' quality.^{23,38)} Examining the chemical constituents is important to control the quality of natural *P. umbellatus*. Therefore, knowledge of species distribution and the levels of genetic diversity are important for the conservation of *P. umbellatus*.³⁹⁾ Although the wild resource is widely distributed around China,⁴⁰⁾ the genetic diversity of *P. umbellatus* is endangered by the decline of population size because of the overexploitation. As the extinction of any one population would reduce total genetic variability considerably, we should preserve every population for the conservation of genetic variability. Even the *P. umbellatus* sclerotia can be produced using artificial infection with *Armillaria* species, asexual propa-

Fig. 3. The Dendrogram Tree of 11 Natural *P. umbellatus* Populations in China Based on Nei's Genetic Distance Using Unweighted Pair Group Method with Arithmetic Mean Method from ISSR Markers

Fig. 4. Scatter Plot of the *P. umbellatus* Using Principal Component Analysis

gation is still the main pathway adopted on Chinese farms owing to the absence of natural sclerotia (as “seeds”) and low production of artificial *P. umbellatus* sclerotia.⁵⁾ It is inevitable to lose genetic diversity during the asexual propagation of *P. umbellatus* sclerotial production in nature. The extensive gene flow obtained through sexual hybridization between different types could be best implications for the genetic diversity conservation in *P. umbellatus*, owing to the unavoidable loss of genetic diversity during the long-term asexual propagation.⁴¹⁾ Therefore, detailed studies of the reproductive biology of this species should be carried out to yield valuable domesticated *P. umbellatus* sclerotia for conservation management of wild *P. umbellatus*.

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Conflict of Interest The authors declare no conflict of interest.

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