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
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, Shanxi, 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 a 25 μL reaction volume containing 12.5 μL of 2× 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 Doc 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 (Na), effective number of alleles per locus (Ne), genetic differentiation among populations estimated by Nei’s gene diversity statistics 30) 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 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’ 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-
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 Jiling 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 Shanxi, 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 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

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

<table>
<thead>
<tr>
<th>No.</th>
<th>Primer number</th>
<th>Primer motif</th>
<th>No. of amplified loci</th>
<th>No. of polymorphic loci</th>
<th>Percentage polymorphism</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>UBC 808</td>
<td>(AG)(_G)</td>
<td>7</td>
<td>5</td>
<td>71.4</td>
</tr>
<tr>
<td>2</td>
<td>UBC 809</td>
<td>(GA)(_T)</td>
<td>9</td>
<td>5</td>
<td>60</td>
</tr>
<tr>
<td>3</td>
<td>UBC 817</td>
<td>(CA)(_A)</td>
<td>10</td>
<td>5</td>
<td>50</td>
</tr>
<tr>
<td>4</td>
<td>UBC 835</td>
<td>(AG)(_Y)C</td>
<td>8</td>
<td>5</td>
<td>62.5</td>
</tr>
<tr>
<td>5</td>
<td>UBC 840</td>
<td>(GA)(_Y)T</td>
<td>12</td>
<td>5</td>
<td>41.7</td>
</tr>
<tr>
<td>6</td>
<td>UBC 841</td>
<td>(GA)(_Y)C</td>
<td>11</td>
<td>5</td>
<td>45.5</td>
</tr>
<tr>
<td>7</td>
<td>UBC 842</td>
<td>(GA)(_Y)G</td>
<td>14</td>
<td>6</td>
<td>42.9</td>
</tr>
<tr>
<td>8</td>
<td>UBC 855</td>
<td>(AC)(_Y)T</td>
<td>10</td>
<td>5</td>
<td>50</td>
</tr>
<tr>
<td>9</td>
<td>UBC 864</td>
<td>(ATG)(_Y)</td>
<td>7</td>
<td>5</td>
<td>71.4</td>
</tr>
</tbody>
</table>

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**

<table>
<thead>
<tr>
<th>Population</th>
<th>(Na)</th>
<th>(Ne)</th>
<th>(He)</th>
<th>(I)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shanxi</td>
<td>1.93±0.24</td>
<td>1.61±0.27</td>
<td>0.35±0.12</td>
<td>0.52±0.17</td>
</tr>
<tr>
<td>Henan</td>
<td>1.72±0.45</td>
<td>1.57±0.36</td>
<td>0.32±0.20</td>
<td>0.45±0.28</td>
</tr>
<tr>
<td>Shanxi</td>
<td>1.91±0.28</td>
<td>1.66±0.31</td>
<td>0.37±0.14</td>
<td>0.54±0.19</td>
</tr>
<tr>
<td>Hubei</td>
<td>1.54±0.50</td>
<td>1.54±0.50</td>
<td>0.27±0.15</td>
<td>0.37±0.14</td>
</tr>
<tr>
<td>Gansu</td>
<td>1.39±0.49</td>
<td>1.31±0.39</td>
<td>0.17±0.21</td>
<td>0.24±0.31</td>
</tr>
<tr>
<td>Hebei</td>
<td>1.67±0.47</td>
<td>1.53±0.37</td>
<td>0.30±0.21</td>
<td>0.43±0.30</td>
</tr>
<tr>
<td>Yunnan</td>
<td>1.89±0.31</td>
<td>1.65±0.28</td>
<td>0.37±0.14</td>
<td>0.54±0.19</td>
</tr>
<tr>
<td>Sichuan</td>
<td>1.76±0.43</td>
<td>1.58±0.37</td>
<td>0.32±0.19</td>
<td>0.47±0.27</td>
</tr>
<tr>
<td>Tibet</td>
<td>1.47±0.50</td>
<td>1.47±0.50</td>
<td>0.23±0.25</td>
<td>0.33±0.35</td>
</tr>
<tr>
<td>Jilin</td>
<td>1.23±0.43</td>
<td>1.23±0.43</td>
<td>0.12±0.21</td>
<td>0.16±0.28</td>
</tr>
<tr>
<td>Heilongjiang</td>
<td>1.20±0.40</td>
<td>1.20±0.40</td>
<td>0.10±0.20</td>
<td>0.14±0.26</td>
</tr>
</tbody>
</table>

\(Na\), number of different alleles; \(Ne\), effective number of alleles; \(He\), Nei’s (1973) gene diversity; \(I\), Shannon’s information index.
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 Hebei, Jilin and Heilongjiang 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 known 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.25,38 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

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

<table>
<thead>
<tr>
<th>Source</th>
<th>Degrees of freedom</th>
<th>Sum of squares</th>
<th>Variance %</th>
<th>Variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Among populations</td>
<td>10</td>
<td>377.62</td>
<td>0.84</td>
<td>7.96</td>
</tr>
<tr>
<td>Within population</td>
<td>26</td>
<td>252.67</td>
<td>9.71</td>
<td>92.04</td>
</tr>
<tr>
<td>Total</td>
<td>36</td>
<td>630.29</td>
<td>10.55</td>
<td>100</td>
</tr>
</tbody>
</table>

Significance tests after 1000 permutations, p<0.001.
gation is still the main pathway adopted on Chinese farms owing to the absence of natural sclerotia (as “seeds”) and low production of artificial \textit{P. umbellatus} sclerotia.\textsuperscript{5} It is inevitable to lose genetic diversity during the asexual propagation of \textit{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 \textit{P. umbellatus}, owing to the unavoidable loss of genetic diversity during the long-term asexual propagation.\textsuperscript{41} Therefore, detailed studies of the reproductive biology of this species should be carried out to yield valuable domesticated \textit{P. umbellatus} sclerotia for conservation management of wild \textit{P. umbellatus}.

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Conflict of Interest The authors declare no conflict of interest.

REFERENCES

(2009).


29) Yeh FC, Yang RC, Boyle TB, Ye Z, Mao JX. POPGENE, the user-friendly shareware for population genetic analysis. Molecular Biology and Biotechnology Centre, University of Alberta, Canada, 10, (1997).


