The molecular diversity analysis of *Auricularia auricula-judae* in China by nuclear ribosomal DNA intergenic spacer

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**A R T I C L E   I N F O**

Article history:
Received 21 October 2013
Accepted 2 December 2013
Available online 28 December 2013

Keywords:
Germplasm resource
Genetic polymorphism
Strain identification
Ribosomal RNA genes

**A B S T R A C T**

Background: For the crossbreeding of *Auricularia auricula-judae*, selecting the appropriated parents in hybridization is very important. However, the classification and diversity analysis of *A. auricula-judae* has been equivocal, due to the similarity of the fruiting body morphology and its susceptibility to environmental influences. For this purpose, the molecular diversity of 32 *A. auricula-judae* commercial cultivars in China was analyzed by using the nuclear ribosomal DNA intergenic spacer.

Results: The complete nuclear rDNA gene complex of *A. auricula-judae* isolate is 11,210 bp long, and contains the 18S, 5.8S, and 28S rRNA gene as well as the ITS and IGS regions. Based on the sequence data, four more effective primer combinations for the IGS region of *A. auricula-judae* were designed. Nucleotide sequence variation in the IGS among 32 *A. auricula-judae* commercial cultivars in China were sorted into three strongly supported clades, which is correlated with geographical regions. Most strains originated from the same area were with a narrow genetic basis and could possibly be domesticated from the local wild-type strains.

Conclusion: The grouping information obtained in the present work provides significant information for further genetic improvement in *A. auricula-judae*, and suggested that the IGS region can be used as an excellent tool for identification of genetic variation.

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1. Introduction

*Auricularia auricula-judae* (Bull.) Quel. which has a global distribution in tropical, sub-tropical and temperate region, has been first cultivated in China more than one thousand years ago. It is an important edible and medical mushroom, and the annual production first cultivated in China more than one thousand years ago. It is an important edible and medical mushroom, and the annual production

To protect the rights of mushroom breeders, it is very important to discriminate among main cultivars of *A. auricula-judae*. However, the classification and diversity analysis of edible mushroom has been equivocal, due to the similarity of the fruiting body morphology and its susceptibility to environmental influences. Thus, problems frequently arise if the analysis is based entirely on morphological characteristics.

Fortunately, molecular biology techniques provide a useful methodology for systematic analysis of genetic polymorphism. The spacer regions of ribosomal DNA genes are useful for examination of close relationships between organisms, because of the divergence in their nucleotide sequences. The internal transcribed spacer (ITS) between the 18S and 25S rRNA genes is moderate and has been widely applied in phylogenetic studies of basidiomycetes [2]. The intergenic spacer (IGS) between the 25S and 18S rRNA genes is evolving fastest in the rDNA complex [3,4], and has been applied in the polymorphism analysis of edible fungi, such as *Agaricus bisporus* [5], *Laccaria bicolor* [6], *Hebeloma cylindrosporum* [7], *Lentinula edodes* [8], *Pleurotus eryngii* [9], *Tricholoma matsutake* [10], *Ferula sinkiangensis* [11], *Tuber borchii* [12] and *Rhodocollybia laulaha* [13]. However, sequence analysis of the IGS regions of *A. auricula-judae* has not been reported.

In this study, the complete rDNA repeat unit of *A. auricula-judae* was firstly sequenced and analyzed, particular emphasis was placed on the IGS region and the more effective primer combinations were designed. Based on the nucleotide sequence variation in the IGS, the genetic polymorphism of 32 *A. auricula-judae* commercial cultivars in China will be discussed.

2. Materials and methods

2.1. Mushroom strains

Thirty-two main cultivars of *A. auricula-judae* in China were used throughout this study. All cultivars were collected from local professional

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Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.
research institutes in different geographical regions of China. According to different floristic regions, tested strains were divided into six populations (Table 1).

Table 1

<table>
<thead>
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<th>No</th>
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<th>Source</th>
<th>Floristic region</th>
<th>GeneBank accession no</th>
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<td>JAU</td>
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<td>JF440703 JF440747</td>
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</table>

* MIHL, Heilongjiang Microbiological Institute; JAU, Jilin Agricultural University; DCH, Dongning County, Heilongjiang Province; HAU, Huazhong Agricultural University; BI, Biological Institute of Henan Scientific Academy; GEMC, Guangda Edible Mushroom Center, Jining; EMIC, Edible Mushroom Institute of Shouguang; MIHB, Microbiological Institute of Heibeii Province; EMIC, Edible Mushroom Institute of the Chinese Agricultural University; HIB, Hanzhong Institute of Botany, Shanxi Province; MIS, Microbiological Institute of Shaxi Province; XFH, Xingnan Edible Fungi Institute, Shaxi Province; SAAS, Shanghai Academy of Agricultural Sciences; HSCS, Haining Spawn Center of Suizhou; EMIK, Edible Mushroom Institute of Kunming.

2.2. DNA extraction

Mycelia of all cultivars were separated from spawn and incubated on CYM (complete yeast media) agar plates at 25°C for 10 d. The agar plates with the grown mycelia were cut into small pieces, transferred into 100 ml CYM liquid medium, and cultured at 25°C for 14 d. Genomic DNA was extracted from freeze-dried mycelia of 32 A. auricula-judae cultivars by using the method of Tang et al. [14]. The DNA quality was confirmed by 1.0% (w/v) agarose gel electrophoresis, and concentrations were determined with a BioPhotometer 6131 (Eppendorf, Germany), then diluted to 50 ng/μl for PCR amplification.

2.3. PCR amplification

Polymerase chain reaction (PCR) amplifications for rDNA complete region of A. auricula-judae strain AU110 were done with combinations of universal primers (Table 2). On the basis of above sequence information, eight more effective primers for amplifying the IGS1 and IGS2 region of A. auricula-judae were designed by using the web-derived software “Primer 3” (http://frodo.wi.mit.edu/primer3/) (Table 2), and their relative position in the ribosomal repeat unit is indicated with arrows in Fig. 1. The main design parameters were as follows: primer optimum size, maximum size, and minimum size were all set to 22, 26 and 18 nucleotides respectively; primer optimum Tm, maximum Tm, and minimum Tm were set to 54°C, 60°C, and 50°C respectively. Their relative binding site in the A. auricula-judae ribosomal repeat unit is indicated in Table 2.

Using the new primers, we PCR-amplified the IGS1 and IGS2 domains from total DNA of 32 strains of Auricularia in a MyCycler thermal cycler (Bio-Rad, USA), which programmed as follows: initial denaturation 4 min at 94°C; 37 cycles of: denaturation 1 min at 94°C, annealing 1 min (at a temperature corresponding to the Tm of the primers used), extension 3 min at 72°C; and final extension 10 min at 72°C. Each reaction was performed in microfuge tubes 0.2 ml in a volume of 20 μl, including 0.25 mM dNTPs, 0.4 μM of each primer, 1.2 U Taq DNA polymerase (TaKaRa, Japan), 1 × PCR buffer, 2.0 mM MgCl₂, and 150 ng DNA template. The reaction products were analyzed on a 1% agarose gel in 1 × TAE buffer. A 2 kb DNA ladder (Gibco-BRL) was included as DNA size marker. PCR product purification and DNA sequencing were performed by Songon Biotechnology Company and Genecore Biotechnology Company in Shanghai. Sequences were deposited in GenBank (http://www.ncbi.nlm.nih.gov/).
2.4. Sequence analysis

The complete rDNA repeat analysis of strain AU110 was performed by Repeatmasker software (http://www.repeatmasker.org/). Alignments of IGS1 and IGS2 nucleotide sequences were made by using the MegAlign module of the Lasergene software system (DNASTAR, Inc.) implementing the Clustal W algorithm [15], with some manual adjustments.

Based on the IGS1, IGS2 and the combined sequence, phylogenetical tree contains 32 test cultivars were obtained with the Minimun Evolution and Maximum Parsimony methods. Kimura-2-Parameter and close-neighbor-interchange algorithm were used in ME and MP method respectively. Gaps were encoded as complete deletion and thus excluded from analyses; transition and transversion were set as the same weight. Tree topology was evaluated by performing bootstrap analysis of 1000 data sets using MEGA 3.1 [16]. Moreover, the IGS nucleotide sequences of L. edodes (GenBank accession numbers AB030581 and AB030582) were set as outgroup in the construction of the combined phylogenetical tree [8].

3. Results

3.1. Cloning and sequencing of the rDNA repeat unit

The complete rDNA region of A. auricula-judae cultivars AU110 was amplified, and then submitted to GenBank (GenBank accession number JN712676). The complete region was 11,210 bp long, and contained the 18S, 5.8S, and 28S rRNA genes as well as the ITS and IGS regions. Following DNA sequence alignment with the filamentous fungi [17], the exact size of each gene was estimated as follows: 1805 bp for the 18S rDNA, 513 bp for ITS1, 5.8S region and ITS2 sequence, 3135 bp for 28S, 2335 bp for IGS1, 118 bp for 5S and 3304 bp for the IGS2 region.

The IGS1 region has no repeats. In the IGS2 region, the sequence from 8551 bp to 8638 bp in the 5′ end region was G-rich with (GGGGA)n repeat, and (TTAGG)n repeat existed in the sequence from 8768 bp to 8799 bp and from 8845 bp to 8880 bp, which increased the sequencing difficulty of complete IGS2 domain.

3.2. Species-specific primer development and PCR-amplification of IGS region

On the basis of the complete rDNA sequence of AU110, four species-specific primer combinations for amplified IGS region of A. auricula-judae were designed (Table 2, Fig. 1). The 5′ end and 3′ end of IGS1 region were amplified by using primer pairs IGS1-5F/IGS1-5R and IGS1-3F/IGS1-3R, and the product size were about 1200 bp and 1000 bp respectively (Fig. 2). The 5′ end of IGS2 region was amplified by using primer pairs IGS2-5F/IGS2-5R, and the product size was about 2500 bp, however, the region was G-rich and difficult for sequencing, thus excluded from further analyses. The product size of 3′ end of IGS2 region was about 800 bp (Fig. 3), which was amplified by using primer pairs IGS2-3F/IGS2-3R. The accession numbers of IGS1 and IGS2 3′ end sequence of all test cultivars are listed in Table 1.

The IGS1 fragment of 32 test isolates ranged from 2284 bp (JY-1, HME-1 and 186) to 2312 bp (ME-6), and the average size was 2299 bp; the 3′ end of IGS2 were nearly 800 bp. The detailed information of IGS region can be found in Table 3.
3.3. The phylogenetic analyses based on IGS1 complete sequence

The trees constructed by ME and MP methods based on the IGS1 alignment showed the same clustering patterns (Fig. 4). Five major clades were labeled; cluster I consisted of 8 strains cultivated in the East and Central regions; cluster II contained 6 strains, which were mainly cultivated in the Northeast, Northwest and Central regions; cluster III included 4 strains cultivated in North and Southwest regions; cluster IV merely consisted of the strains C21 and 8808, while cluster V consisted of 12 strains cultivated in the Northeast, North and Central regions.

3.4. The phylogenetic analyses based on IGS2 3′ end sequence

The 32 test strains were divided into 6 similar groups based on ME and MP methods (Fig. 5). Cluster I consisted of 10 strains mainly cultivated in the East and Central regions; clusters II and III contained 8 and 3 strains respectively, which were mainly cultivated in the

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Table 3
IGS sequence information of test A. auricula-judae cultivars.

<table>
<thead>
<tr>
<th>Sequence information</th>
<th>IGS1 entire region</th>
<th>IGS2 3′ end region</th>
<th>Complete IGS1 and IGS2 3′ end region</th>
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<tbody>
<tr>
<td>Alignment matrix length (bp)</td>
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<td>801</td>
<td>3114</td>
</tr>
<tr>
<td>Average region length (bp)</td>
<td>2299</td>
<td>800</td>
<td>3100</td>
</tr>
<tr>
<td>Nucleotide composition</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>A</td>
<td>20.4</td>
<td>22.2</td>
<td>20.9</td>
</tr>
<tr>
<td>T</td>
<td>27.6</td>
<td>25.9</td>
<td>27.2</td>
</tr>
<tr>
<td>C</td>
<td>23.1</td>
<td>21.7</td>
<td>22.8</td>
</tr>
<tr>
<td>G</td>
<td>28.8</td>
<td>30.2</td>
<td>29.2</td>
</tr>
<tr>
<td>transitional/transversional (si/sv)</td>
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<td>4.7</td>
<td>3.1</td>
</tr>
<tr>
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<tr>
<td>Variable sites, V</td>
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<tr>
<td>Parsimony-informative site, Pi</td>
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<tr>
<td>Singleton sites, S</td>
<td>77</td>
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<td>97</td>
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</tbody>
</table>

Fig. 4. The phylogenetic tree of A. auricula-judae test strains based on IGS1 sequence (a: ME tree; b: MP tree).
North, Southwest and Northwest regions; clusters IV, V and VI consisted of 5, 4 and 2 strains respectively, which were cultivated in the Northeast, Northwest and Central regions.

3.5. The phylogenetic analyses based on IGS1 and IGS2 3′ end combined sequence

The ME and MP analyzing result were nearly same based on the IGS1 with IGS2 3′ end combined sequence (Fig. 6), and one strain of L. edodes was set as the outgroup. All the test cultivars were divided into 3 main groups; group I consisted of 10 strains, DA-1, DA-2, DA-3, CBS-7, HEI-29, YM-1, ME-6, 139, AU110 and SN-A8, which were cultivated in the Northeast and Central regions; group II contained strains JY-1, XE-887, C21, 8808, ZJ-310, XE-987, DZ-1 and HME-1, which originated from the North and Southwest regions; while group III contained SHAN-1, ZHI-5, HE-3, 9809, 8129, HE-9, XK-1, DP-5, XP-10, YK-K3, 186, HEI-916, 97-1 and 173, cultivated in the Central, Northwest and East regions.

4. Discussion

As the primary genetic material, DNA sequence can reflect the genetic relationship more directly, accurately and objectively than the morphological characteristics. To data, the DNA analyses of fungi focus on the ribosomal DNA sequence. Due to the different selective pressures of rDNA domains, the conservative degree of each rDNA domain was unequal. The 18S, 5.8S, 28S and 5S rDNA gene regions are highly conserved, and always used in phylogeny studies of the order and family [18,19]. ITS region is moderate variable, mainly for the identification of species and varieties [2]. The IGS domain, which evolved the fastest, was always applied for the analysis of the genetic diversity within species. In recent years, the IGS region has been successfully applied to study the genetic diversity of several edible mushrooms, such as Ferula sinkiangensis [11], Armillaria mellea [20], Pleurotus eryngii [21], Tuber borchii [12] and Rhodocollybia lousiha [13].

According to traditional theory [6], unequal exchange frequently occurred in mitosis, resulting in an amount of repetitive or sub-repetitive units existing in IGS region, whose structure and numbers were variable among different organisms, and represented as the IGS length differences. Saito et al. [8] have studied the IGS region of 16 L. edodes commercial cultivars in Japan, and found that there were significant differences within species. The IGS1 sequence number of different mushroom strains is 1–3 bands, and the sizes were between 0.9 kb and 1.5 kb; the IGS2 sequence number varied by 1–4 bands, and the sizes were between 2 kb and 3 kb; IGS1 and IGS2 domains of L. edodes contained intricate sub-repeat regions SR1 and SR2 respectively, while SR1 and SR2 have 6 kinds of short sub-repeats units, the structure and number in different strains were distinct, which caused the polymorphism of L. edodes IGS sequence. Similar phenomenon of Lentinula species has been observed in Nicholson et al. [22]. Huang et al. [9] analyzed the IGS2 region of P. eryngii and confirmed that the length and number of the IGS2 sequence amplified by different test cultivars demonstrated high polymorphism, and that the number of IGS2 fragment was among 1–4.

However, the opposite phenomenon also observed in other fungi, the IGS sequences of one certain strain amplified only a single band,
and there were no polymorphic bands among different materials. For example, Kim et al. [10] analyzed the IGS1 sequence of 29 North American Armillaria strains and found that the IGS1 fragment length of all test strains was 606 bp and contained 147 variable sites, and that the phylogenetic results based on ITS and IGS1 were nearly same; a similar phenomenon has also been found in Tilletia sp. [23] and Penicillium marneffei [24]. The research result demonstrated that there were obvious differences among different fungi, which means the IGS region required further in-depth analysis.

In this study, we firstly studied the complete rDNA sequence of A. auricula-judae, especially on the IGS region. Same as the amplified phenomenon of Armillaria [10], the amplification of IGS1 and IGS2 regions in A. auricula-judae obtained merely one specific band, and few length polymorphisms have been found among test cultivars. The IGS1 have no repeats, oppositely, the 5′ end of IGS2 region contained the rDNA transcription initiation site and terminator, have been found rich in GC and difficult for sequencing, which was same in the plant [25].

The phylogenetic analysis based on IGS1, IGS2 3′ end sequence and the combined sequence, as well as by ME or MP method, get the similar results, which demonstrated that the IGS sequence were highly effectively for analyzing the genetic variation among A. auricula-judae strains. What is more is that the combined IGS analysis result, which contains more nucleotide information, can divide all the strains more apparently; 32 test strains have divided into 3 groups, corresponding to the Northeast and Central regions; North and Southwest regions; as well as Central, Northwest and East regions respectively, which reflect the relationship between the genetic characteristics and geographical distribution. Most strains originated from the same area, were with a narrow genetic distance and could possibly be domesticated from the local wild-type strains. According to the grouping information, the genetic diversity of main A. auricula-judae cultivars in China was comparatively low, which indicates that the domestication of wild-type strains should attract more attention.

For the improvement of strains, selecting the appropriated parents in hybridization is very important. Hybridization program involving genetic diverse belonging to different distant clusters will facilitate the breeding program. Fortunately, the grouping information obtained in the present work provides significant information for further genetic improvement in A. auricula-judae, and is expected to be the reference for the similar studies in other countries.

Conflict of interest

No conflict of interest among the authors.

Acknowledgments

This work was supported by the Science Foundation of the Hubei Province, China (Grant No. 2013CFB421 and 2009CDA109).

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