ITS Sequence Comparison of Germplasm Resources 

Of Auricularia polytricha 

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Keywords: Auricularia polytricha, ITS, sequence, phylogeny

Abstract: The internal transcribed spacer (ITS) region in rRNA gene of Auricularia polytricha was cloned and sequenced, and the sequences were compared with those of some common species of Auricularia. The results showed that 6 strains among tested strains, except Teda with different origins, had the same length of ITS2. There were base variances in the ITS region of rRNA gene among the tested strains, and with 11 variant loci in ITS region. Compared with other 4 species from the Gene Bank, ITS1 and ITS2 sequences of A. polytricha varied in great extent. According to the analysis of distance matrix, the genetic relationship between strains and the related known species from systematic dendrogram, ITS sequence analysis supports the traditional classification of Auricularia based on morphology.

Introduction

rRNA is one of the most ancient molecules in cells. With the functional and evolutionary homology, rRNA is the living fossil of some research, including the origin of life, the early evolution and molecular phylogeny. The most conservative area of rDNA is 18S, 5.8S, 28S rRNA genes. Spacer in rRNA genes is the fastest evolution area[1], while ITS is a moderate conservative area, which is characterized by intraspecific relatively consistency and interspecific obviously difference [2].

In the study of molecular identification and phylogeny in ectomycorrhizal fungi, such as Tuber, Laccaria, Hebeloma, Tricholoma bakamatsutake, the ITS section have a common application [3].But the reports have been seldom in other higher fungus. In this paper, the ITS sequence of 7 strains A. polytricha rDNA were studied to reveal the genetic variations in different strains, which also could provide molecular evidence for A. polytricha identification and phylogeny.

Materials and Methods

Tested Materials. The fruiting bodies of seven A. polytricha strains with morphological differences were selected as the test material, including two wild isolates, five cultivated strains (Table 1).

Total DNA Extraction. The total DNA was extracted by CTAB (Cetyltrimethyl Ammonium Bromide)[1].
Table 1 Tested strains of *A. polytricha*

<table>
<thead>
<tr>
<th>No.</th>
<th>Abbreviation</th>
<th>Strain name</th>
<th>Source</th>
<th>Note</th>
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<td>AP7</td>
<td>Sichuan Academy of Agricultural Sciences</td>
<td>Wild poplar</td>
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<tr>
<td>2</td>
<td>A.pzj</td>
<td>Hybrid 1#</td>
<td>Edible Fungi Center of jinning Shangdong</td>
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<tr>
<td>3</td>
<td>A.pxs</td>
<td>Xiaoshan 3#</td>
<td>Edible Fungi Farm of Sichuan</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>A.ptd</td>
<td>Teda</td>
<td>Edible Fungi Farm of Sichuan</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>A.pte</td>
<td>Taier 319</td>
<td>Jiangsu Institute of Edible Fungi</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>A.pml</td>
<td>M 1</td>
<td>Hebei Institute of Microbiology</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>A.pel</td>
<td>AP059</td>
<td>Wolong Nature Reserve of Sichuan</td>
<td>Wild in Wolong</td>
</tr>
</tbody>
</table>

**ITS Fragments of PCR Amplification.** Fungi universal primers ITS1/ITS2 and ITS3/ITS4 were used to specifically amplify the segments of ITS region (ITS1, 5.8S rDNA and ITS2) in the PCR reaction (1 min initial denaturation at 94°C, and 36 cycles of 1 min denaturation at 94°C, 1 min at 54°C for annealing, 1.5 min at 72°C for extension, and a final extension at 72 °C for 10 min). The PCR products were examined by 1.4% agarose gel electrophoresis. After electrophoresis, the target fragments were purified by 3s Spin Agar Gel Purification Kit.

**Cloning and Sequencing of ITS Fragment.** Connect the purified PCR products to Japanese Takara’s PMD-18T Vector, transform into E.coli competent cell JM109, screen by the AMP plate with X-gal IPTC, pick white colonies for culture, extract plasmid and test by PCR. After cloned strains successfully, two-way sequencing were done by Beijing Huadazhong Biological Technology Co. Limited of China, and the whole sequence was spliced by DNAMAN.

**Analysis of ITS Sequence.** The ITS sequence was automatic alignment by DNAMAN program. The published ITS sequence of *Tremell fuciformis* (*T.fuc*), *Auricularia auricular-judae* (*A.aur*), *Auricularia delicata* (*A.del*), *Auricularia fuscosuccinea* (*A.fus*) and *Auricularia mesenterica* (*A.mes*) were downloaded from NCBI Database. Analysis phylogenetic relationships of above mentioned strains. The range of ITS1 and IST2 were determined by the scope of *A. auricular-judae*. Data computing and phylogenetic trees constructing was carried out by DNAMAN software. *T. fuciformis* was the classified outgroup.

**Results**

**Variation Analysis of ITS Sequence.** The ITS sequences of 7 tested *A. polytricha* strains and other 4 species belonging to *Auricularia*, including *A. auricular-judae*, *A. delicata*, *A. fuscosuccinea* and *A. mesenterica*, and *T. fuciformis* were downloaded from Gene Bank (Figure 1).

The lengths of the ITS sequences were in the range of 502-599 bp for all of the strains tested. The shortest was that of *T. fuciformis* (502 bp), and the longest was that *A.polytricha* M1 (599 bp). Difference in the length of the ITS sequence was found in all strains. The length of the ITS sequence ranged from 532 to 599 bp for the *A.polytricha* strains. All the strains were the same in length of the 5.8 S rRNA genes (165 bp), except that *T. fuciformis* had a missing site. The lengths of the IST1 sequences were in the range of 163-230 bp. The shortest length of IST1 sequences was
found in Hybrid 1#, and the longest was found in M1. Taier 319 and Teda were the same in length of ITS1 (280 bp). The IST1 sequence of T. fuciformis was 182 bp in length. The lengths of ITS2 sequences were in the range of 163-210 bp. The shortest length of IST2 sequences was found in T. fuciformis, and the longest length of IST2 sequences was found in A. polytricha. All tested strains of A. polytricha were the same length of ITS2 (201 bp) except Teda (209 bp). Other 4 species of Auricularia were different length of IST2 sequences, namely A. auricular-judae (207 bp), A. delicata (183 bp), A. Fuscosuccinea (181 bp) and A. mesenterica (178 bp). Exclude insertions and deletions, the ITS region of all tested A. polytricha strains were 11 variant loci. 5 were found in IST1 region and 6 were found in ITS2 region. In figure 1, significant difference is found in three base sites. At the 168th base site, the base of Taier 319 and Teda are G, the other 5 are A. At the 532th base site, the base of Taier 319 and Teda are C, AP059 is missing, and the other 4 strains are T. At the 552th base site, the variation is the same as the 532th site.
<table>
<thead>
<tr>
<th>Strains</th>
<th>5.8S rDNA</th>
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<td>211</td>
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<table>
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<tr>
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<tr>
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<td>350</td>
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<tr>
<th>Strains</th>
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<th>421</th>
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<tr>
<th>Strains</th>
<th>650</th>
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</thead>
</table>
A.fuc - AAC\{-CGGCT\} - G\{-CTTG\}--- ---A----CT - GCG-TTTT- --GTG-TGGG
A.mes - AG\{-7\}GQCT - G\{-CTTG\}--- ---A----CT - G\{-G\}TTTT- --GTG-TGGG
A.pap - AAC\{-7\}QGCT - G\{-CTTG\}--- ---A----CT - GCG-TTTT- --GTG-TGGG
A.psj - AAC\{-CGGCT\} - G\{-CTTG\}--- ---A----CT - GCG-TTTT- --GTG-TGGG
A.pxs - AAC\{-CGGCT\} - G\{-CTTG\}--- ---A----CT - GCG-TTTT- --GTG-TGGG
A.ptd - AAC\{-CGGCT\} - G\{-CTTG\}--- ---A----CT - GCG-TTTT- --GTG-TGGG
A.pte - AAC\{-CGGCT\} - G\{-CTTG\}--- ---A----CT - GCG-TTTT- --GTG-TGGG
A.pml - AAC\{-CGGCT\} - G\{-CTTG\}--- ---A----CT - GCG-TTTT- --GTG-TGGG
A.pel - AAC\{-7\}GQCT - G\{-CTTG\}--- ---A----CT - GCG-TTTT- --GTG-TGGG

Strains 491 560

T.fuc CGCTCAAC CCCCCTAC

A.aur TAA-T-TCTG -G--AA--GC CCTGGGCCTC TTCAGCGGCG CTGCTTACAG TTCAGCGGCG CTGCTTACAG
A.del TAA-T-TCTG -G--AA--GC CCTGGGCCTC TTCAGCGGCG CTGCTTACAG TTCAGCGGCG CTGCTTACAG
A.fuc TAA-T-TCTG -G--AA--GC CCTGGGCCTC TTCAGCGGCG CTGCTTACAG TTCAGCGGCG CTGCTTACAG
A.mes TAA-T-TCTG -G--AA--GC CCTGGGCCTC TTCAGCGGCG CTGCTTACAG TTCAGCGGCG CTGCTTACAG
A.pap TAA-T-TCTG -G--AA--GC CCTGGGCCTC TTCAGCGGCG CTGCTTACAG TTCAGCGGCG CTGCTTACAG
A.pzj TAA-T-TCTG -G--AA--GC CCTGGGCCTC TTCAGCGGCG CTGCTTACAG TTCAGCGGCG CTGCTTACAG
A.pxs TAA-T-TCTG -G--AA--GC CCTGGGCCTC TTCAGCGGCG CTGCTTACAG TTCAGCGGCG CTGCTTACAG
A.ptd TAA-T-TCTG -G--AA--GC CCTGGGCCTC TTCAGCGGCG CTGCTTACAG TTCAGCGGCG CTGCTTACAG
A.pte TAA-T-TCTG -G--AA--GC CCTGGGCCTC TTCAGCGGCG CTGCTTACAG TTCAGCGGCG CTGCTTACAG
A.pml TAA-T-TCTG -G--AA--GC CCTGGGCCTC TTCAGCGGCG CTGCTTACAG TTCAGCGGCG CTGCTTACAG

Strains 561 630

A.fuc TCGTCCCTCT GCGGACAACT AATA–C-AAA G--T------ ----------
A.mes TCGTCCCCAG AGGGACAACT YTTT--C-AAA G--T------ ----------
A.pap TCGTCCCTCT GCGGACAACT YTTT--C-AAA G--T------ ----------
A.pzj TCGTCCCTCT GCGGACAACT YTTT--C-AAA G--T------ ----------
A.pxs TCGTCCCTCT GCGGACAACT YTTT--C-AAA G--T------ ----------
A.ptd TCGTCCCTCT GCGGACAACT YTTT--C-AAA G--T------ ----------
A.pte TCGTCCCTCT GCGGACAACT YTTT--C-AAA G--T------ ----------
A.pml TCGTCCCTCT GCGGACAACT YTTT--C-AAA G--T------ ----------

Strains 631 640

A.fuc AGCATATCAA T
A.mes - - - - - - - - - - - - - -
A.pap - - - - - - - - - - - - - -
A.pzj - - - - - - - - - - - - - -
A.pxs - - - - - - - - - - - - - -
**Genetic Distance Analysis.** The genetic distances of all strains based on ITS sequence were calculated by DNAMAN Software. In table 2, the genetic distances of all strains range from 0.002 to 0.351, and average is 0.110. The genetic distances of all species in *Auricularia* range from 0.002 to 0.301, and average is 0.0739. The genetic distances of all *A. polytricha* strains range from 0.002 to 0.301, and average is 0.0361. The genetic distances of family is greater than genus, and genus is greater than species, indicating uniformity between traditional classification and ITS classification, and also confirming the reasonableness of the traditional classification.

**Table 2 Genetic distance matrix based on ITS sequence**

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<tbody>
<tr>
<td>A. pte</td>
<td>0.013</td>
<td></td>
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<td></td>
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<tr>
<td>A. ptd</td>
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<td>0.002</td>
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<tr>
<td>A. pxs</td>
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<td>0.007</td>
<td>0.005</td>
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<tr>
<td>A. pzj</td>
<td>0.011</td>
<td>0.008</td>
<td>0.006</td>
<td>0.004</td>
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<td></td>
<td></td>
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<tr>
<td>A. pap</td>
<td>0.014</td>
<td>0.010</td>
<td>0.009</td>
<td>0.007</td>
<td>0.006</td>
<td></td>
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</tr>
<tr>
<td>A. aur</td>
<td>0.129</td>
<td>0.121</td>
<td>0.121</td>
<td>0.112</td>
<td>0.122</td>
<td></td>
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<tr>
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<td>0.080</td>
<td>0.082</td>
<td>0.082</td>
<td>0.061</td>
<td>0.086</td>
<td>0.132</td>
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<tr>
<td>A. fus</td>
<td>0.074</td>
<td>0.067</td>
<td>0.069</td>
<td>0.050</td>
<td>0.073</td>
<td>0.138</td>
<td>0.063</td>
<td></td>
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<tr>
<td>A. mes</td>
<td>0.156</td>
<td>0.146</td>
<td>0.146</td>
<td>0.150</td>
<td>0.134</td>
<td>0.148</td>
<td>0.097</td>
<td>0.143</td>
<td>0.138</td>
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<tr>
<td>T. fuc</td>
<td>0.298</td>
<td>0.293</td>
<td>0.294</td>
<td>0.302</td>
<td>0.310</td>
<td>0.300</td>
<td>0.312</td>
<td>0.333</td>
<td>0.338</td>
<td>0.351</td>
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<tr>
<td>A. pel</td>
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<td>0.014</td>
<td>0.012</td>
<td>0.009</td>
<td>0.007</td>
<td>0.126</td>
<td>0.076</td>
<td>0.076</td>
<td>0.156</td>
<td>0.301</td>
</tr>
</tbody>
</table>

**Phylogenetic Analyses.** Figure 2 is the phylogenetic tree of *Auricularia* based on ITS sequence, described by DNAMAN and *T.fuciformis* was classified outgroup. From the figure, at less than 94% supporting strength, 11 strains in ingroup are divided into five groups. At 99% supporting strength, a separate branch is formed in the 7 strains of *A. polytricha* in the A group, with 12 bp base differences of ITS sequences, distinguishing from other species.

At 86% supporting strength, all species of *Auricularia* are gotten together, showing their phylogenetic relationships. With 95% supporting strength for the border, each species is divided into single branch, namely group A~E for *A.polytricha, A. delicate, A. fuscosuccinea, A. auricular-judae and A. mesenterica*, indicating that ITS sequence analysis supports species of *Auricularia* divided by morphological characteristics.

All *A. Polytricha* strains including two wild strains are subsumed into group A, showing their close genetic relationship, and matching their morphological characteristics. Teda and Taier 319, Hybrid 1# and Xiaoshang 3# are clustered together. The reasons may be a strain with two names. Variations is found in two wild strains (AP7 and AP49) and AP57 *A.auricular-judae, A. delicate, A. fuscosuccinea* and *A. mesenterica* have been reported that their ITS sequence exist some interspecific variability.[1]
Discussion

Ultrastructure plays an important role in the phylogenetic classification of Basidiomycetes. For a long time, the main classified indicators based on the type of basidium covered the authentic Auricularia phylogeny status \(^4\). Basidium type was considered to be an important indicator to define higher taxa. On the basis of basidium type, Introductory Mycology divided basidiomycetes into three subclasses that are Holobasidiomycetidae, Phragmobasidiomycetidae and Teliomycetidae \(^5\). Along the classification system, fungi, with heterobasidium divided into four cells by diaphragm or mediastinum, compose Phragmobasidiomycetidae. In this subclass, Tremellales and Auriculariales are made up of saprophytic bacteria. Tremellales is characterized by heterobasidium divided into four cells by mediastinum. Phlogiotis spp, some species of Exidia spp. and Tremella spp. are part of Tremellales. The members of Auriculariales are characterized by heterobasidium and divided into four cells by diaphragm. However, according to The Macrofungi in China and some traditional classification, Phlogiotis spp. Exidia spp. and Auricularia are filed under Auriculariales. Further, P. helvelloides, E. glandulosa and E. recisa are filed under Auricularia \(^6\). The classification is based on the idea that fungal basidiocarp is glia to the deme. Dacrymycetales, Tremellales and Auriculariales are included in gelatinous fungus. But the Dacrymycetales’s basidiocarp has no diaphragm, and some species of Eichlerilla incarnate are classified in Tremellaceae.

Bandoni, combining microscopic morphology with ultrastructure, ecology and embryology data, come up with a selective classification opinion for Tremellales and Auriculariales’ division \(^7\). He put the Auriculariales’ species with simple septal pore out, so Auriculariales include basidiocarp with diaphragm or mediastinum, hyphae haploid phase, continuous bunghole overlap. This classification is supported by rRNA and rDNA sequence analysis. So Auriculariales, except Auricularia, also include some species under Tremellales classified by Martin, and Tremellales just
include species with septal pore complex and hyphae haploid phase\cite{8}.

*Exidia* and *Auricularia* have different basidium type. *Auricularia* has diaphragm. Some species of *Exidia* and *Exidia glandulosa* Fr. have mediastinum. *Auricularia* Fr and *Exidia* Fr. are similar in morphology, fruiting body anatomy, pigments accumulation and the stage of non-morphological. Wei M, who took advantage of ITS sequence analysis to study the phylogenetic relationship of *A.auricula* and related taxa of it. The result support that *Exidia, Auricularia* and some species in *E. deglugens* are coexist in *Auriculariales*, distinguishing tremellales, which include *Tremella, Sirobasdium magnun*, and *Filobasidiella neoforman*\cite{4}.

Mycologist put forward many classification systems\cite{3}, but no one is the academic consensus. McGuires pointed out: because of lacking connected information on inconspicuous characteristics, we may not be making sure the real relationship in *Auriculariales*. Taking advantage of ITS sequencing and other molecular biology techniques, combining with morphology, microstructure and ultrastructure can make phylogenetic status of *Auriculariales* more objective and make the relationship with related taxa *Auriculariales* closer to the actual\cite{9}.

Fungal rDNA is conservative, but IST on rDNA evolves quickly. In same genus, different strains may have variation. Interspecific variation of ITS sequence in *A.auricula-judae, A. delicata, A. fuscosuccinea* and *A. mesenterica* have been reported\cite{11}.For the first time, PCR amplification, cloning and sequencing to the ITS region gene segments of *A.auricula-judae* are successfully carried out. The results showed: variation is found in the ITS sequences of seven strains, and nucleotide variation range from 1-4 bp. Combining sequence from Gene Bank, comparative analysis is carried out, the finding *A.polytricha, A.auriculajudae, A. delicata, A. fuscosuccinea* and *A. mesenterica*’s ITS sequence variation is greater than the intraspecific variation of *A.polytricha*.

Finally, the results of ITS sequence analysis support the traditional classification of *Auricularia* and *Tremella*, so do the taxonomy of species in *Auricularia* and the separation of strains in species.

References


