First record of *Lyophyllum shimeji* mashrooms in Dazhou

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Abstract. A new record of *Lyophyllum shimeji* was collected during field trip to masson pine at Dazhou (31.35° N, 107.72° E; 1100 m altitude) district in 16th of October 2017. It was identified using morphological and molecular techniques. Complete description was performed for the collected fresh fruiting bodies and isolated pure culture. Radial growth rate of culture was estimated on media1, media 2, media 3, media 4, media 5 and media 6 (0.196, 0.192, 0.122, 0.092, 0.200, 0.160 cm/ day, respectively). *Lyophyllum* are very close in morphological characters; hence, the identification was confirmed by DNA sequence analysis of the ribosomal 5.8S rRNA gene including the flanking internal transcribed spacers (ITS). The results showed that it was *Lyophyllum shimeji* and this is the first record in other parts of China. The suitable culture medium component was potato 200 g, MgSO₄·7H₂O 0.5 g, glucose 20 g, KH₂PO₄ 0.3 g, tryptone 0.5 g, VB 20 mg, agar 20 g, ddH₂O 1000 ml, PH5-6. These results could provide a new reference for its artificial domestication and cultivation studies.

1. Introduction

*Lyophyllum* is a world widespread genus, with many edible species such as *Lyophyllum decastes*, *Lyophyllum ulmarium*, *Lyophyllum shimeji* et al, and widely exist in North-temperate and arctic areas, and display broad ecological strategies, ranging from plant decayers to parasite species [1]. *Lyophyllum shimeji* has been recognized as a delicacy for centuries with known distribution in East Asia, especially in Japan and Korea but rare in China except in Yunnan. This species was reported by Peigui Liu from Jinggu, Yunnan in *Saprosma ternatum* woods and *pyrus pyrifolia* woods [2]. Hitherto, there is no any record for *Lyophyllum shimeji* in other parts of China.

Remarkable evolution has been made to affirm phylogenetic relationships in the largest order; Agaricales [3]. However, continued assessment of evolutionary relationships within this order is necessary. The species of *Lyophyllum* are generally regarded as a species complex and a great deal of taxonomic confusion, and are somewhat difficult to be differentiated by morphological descriptions. Thus, molecular techniques such as ribosomal RNA gene sequencing had been developed for their identification [4].

Though the *L. decastes* have traditionally been characterized as saprotrophic, several of these species are later seen to ectomycorrhiza and are difficult to cultivate artificially without host plant. However, *L. shimeji* example, in 1994, Ohta successfully obtained the fruit body through the artificial cultivation using barley grain without the host plant [5]. And Watanabe as well as Yoshida reported the same results at the same time, although the yields have been relatively low [6]. In recent years, a huge infections by the pine wood nematode caused a rapidly decline in the fructification of *L. shimeji* in Dazhou areas and the wild-grown *L. shimeji* is available only in high-class restaurants. Little research has been conducted for its artificial domestication and cultivation in China. Thus, it is necessary to explore its artificial
domestication and cultivation. In order to provide theoretical and applied references for propagation and artificial domestication and cultivation of *L. shimeji*, the purposes of this study were: (1) to morphology and molecular analysis of *Lyophyllum* mushroom that grows in Dazhou, China; (2) to screen the culture medium of *L. shimeji* for its artificial domestication and cultivation studying.

2. Material and method

2.1. Studied material

The fruit-bodies used in this experiment were found at Dazhou (31.35° N, 107.72° E; 780 m altitude) district in 16th of October 2017. Prior to the molecular analysis, the samples for the present study were fully described by expert field mycologists and assigned to taxa according to the literature.

2.2. Medium compositions and culture conditions

Isolation into pure culture was carried out directly after collection from the field sites according to the method of El-Gharabawy et al [7]. The pure culture mycelium, obtained by separation and purification from wild *Lyophyllum* fruiting bodies were cultured on different culture mediums (medium1: CaCl$_2$ 0.05 g, MgSO$_4$ 0.15 g, NaCl 0.025 g, FeCl$_3$ (1%) 0.001, KH$_2$PO$_4$ 0.5 g, VB,100 ug, (NH$_4$)$_2$HPO$_4$ 0.25 g, malt extract 10 g, glucose 10 g, citric acid 0.2 g, ager 20 g, ddH$_2$O 1000 ml; medium2: potato 200 g, MgSO$_4$·7H$_2$O 0.5 g, glucose 20 g, KH$_2$PO$_4$ 0.3 g, tryptone 0.5g, VB 20 mg, ager 20 g, ddH$_2$O 1000 ml, PH5-6; medium3: KH$_2$PO$_4$ 1.0 g, Na$_2$MO$_4$·2H$_2$O 0.0027 g, MgSO$_4$·7H$_2$O 0.5 g, CaCl·2H$_2$O 0.05 g, H$_3$BO$_3$ 0.028 g, VB,0.0001 g, MnCl$_2$·2H$_2$O 0.003 g, ZnSO$_4$·7H$_2$O 0.0023 g, EDTA 0.02 g, CuCl$_2$·2H$_2$O 0.0063 g, glucose 20.0 g, ager 15 g, ddH$_2$O 1000 ml, pH5.4±0.2 (25°C); medium4: malt extract 20 g, soy tryptone 1 g, glucose 20 g, ager 15 g, ddH$_2$O 1000 ml, medium5: yeast extract 3 g, glucose 20 g, KH$_2$PO$_4$ 3 g, MgSO$_4$·7H$_2$O 5 g, tryptone 5 g, VB, 20 mg, ager 20 g, potato 200 g+pine 80 g were soaked in distilled water at constant temperature 100°C for 30 min, filter and let stand for 1000 ml, PH 5.5-6.0; medium6: potato 200 g, ager 20 g, glucose 20 g, ddH$_2$O 1000 ml) under sterile conditions.

2.3. Determination of Radial growth rate (RGR)

Isolation plates were incubated at 25°C and pure cultures were maintained on potato dextrose agar (PDA) slopes at 4°C. Radial growth rate (RGR) was quantified on 9 cm petridishes of six different culture mediums using 1cm discs of actively growing cultures at 25°C.

2.4. DNA extraction and polymerase chain reaction amplification parameters

Genomic DNA was extracted from the fresh fruiting body by the cetyltrimethylammonium bromide (CTAB) method [8]. The oligonucleotide primers (ITS1:5’-TCCGTAGGTGAACCTGCGG-3’ and ITS4: 5’-TCTTCCGCTTATTGATATGC-3’) used for amplification and the PCR amplifications were performed in ABI 9700 thermal cyclers. The PCR mixture with a total volumes of 25 μl and contained 0.5 μl of each primer, 0.5μl template (20-50 ng), 2.5 μL 10 × Taq buffer (Mg$^{2+}$ plus), 0.5 μl dNTP (2.5 mM), and 0.5 μl Taq DNA polymerase (Tiangen Biotech, Beijing Co., Ltd.). Thermal condition was performed with initial denaturation at 94°C for 4 min, followed by 35 cycles at 94°C for 45s, 55°C for 45s and 72°C for 1 min, then final extension at 72°C for 10 min. PCR products were analyzed and separated on 1.0–1.5% (w/v) agarose gels, stained with ethidium bromide, and visualized using UV light. The expected PCR products were purified using the SanPrep Column DNA Gel Extraction Kit (Shanghai Biological Technology Co., Ltd.). Obtained ITS nucleotide sequences were subjected to a BLAST search against the NCBI database (http://www.ncbi.nlm.nih.gov/) to match the best similarities with other related ITSs on database.

3. Results

3.1. Morphological characteristics
The taxonomic position: Fungi – Basidiomycota – Agaricomycetes – Agaricales – Tricholomataceae – Lyophyllum.

The samples were collected during field trip to masson pine at Dazhou (31.35° N, 107.72° E; 1100 m altitude) district in 16th of October 2017. The fruit bodies were gregarious with the base of a bulged waist and usually in areas where rotten branches and leaf litter collects; cap is 2 to 6 cm across; cap surface is smooth and the middle part slightly convex or concave; Gills sinuate; Stem is 2 to 8 cm long and 1.5 to 3.5 mm in diameter and white at base with no ring (figure 1).

![Figure 1. Morphology of Lyophyllum shimeji.](image1)

3.2. Molecular characterization of L. shimeji

![Figure 2. PCR of the electrophoresis pattern.](image2)
The best DNA sequence similarities with our ITS nucleotide sequence (664 bp) (figure 2) were obtained from NCBI GenBank and aligned results (figure 3) showed that the sample which collected at Dazhou (31.35° N, 107.72° E; 1100 m altitude) district in 16th of October 2017 was *L. shimeji*.

#### Figure 3. target ITS nucleotide sequence alignment results.

3.3. **The mycelium morphology and culture characteristics analysis**

#### Figure 4. Mycelium morphology of 3 days later after inoculation in different mediums. 1 stands for medium 1, 2 stands for medium 2, 3 stands for medium 3, 4 stands for medium 4, 5 stands for medium 5, 6stands for medium 6.
The mycelium morphology of different culture mediums were shown in figures 4 and 5, and the culture characteristics analysis were shown in table 1. The mycelia began to germinate 2 days later on medium 1, medium 5 and medium 6 while the mycelia began to germinate 3 days later on medium 2, medium 3 and medium 4. The mycelia are all white except on medium 3, in which the mycelium is canary. The mycelia are denser on medium 2, medium 3 and medium 5, dense on medium 1 and medium 6, sparsely on medium 4. Culture showed more aggressive appearance on medium 2 with thick growing tenacious mycelium.

![Mycelium morphology of 7 days later after inoculation in different mediums](image)

**Figure 5.** Mycelium morphology of 7 days later after inoculation in different mediums. 1 stands for medium 1, 2 stands for medium 2, 3 stands for medium 3, 4 stands for medium 4, 5 stands for medium 5, 6 stands for medium 6.

| mediums | mycelium germination time(d) | mycelium dense | mycelium color | colony marginal morphology |
|---------|------------------------------|----------------|----------------|--------------------------|
| 1       | 2                            | ++             | white          | neatly                   |
| 2       | 3                            | +++            | white          | neatly                   |
| 3       | 3                            | +++            | canary         | neatly                   |
| 4       | 3                            | +              | white          | irregular                |
| 5       | 2                            | +++            | white          | neatly                   |
| 6       | 2                            | ++             | white          | irregular                |

Note: +++ denser, ++ dense, + sparsely.

### 3.4. Radial growth rate (RGR) of mycelium

The radial growth rate (RGR) of mycelium were shown in table 2. Although the RGR were not significant different at 0.05 level on medium 2 and medium 5, the RGR on medium 2 and medium 5 were significantly different on medium 3. According to the RGR and the mycelium morphology and culture characteristics analysis, medium 2 is the best culture medium for *Lyophyllum shimeji*. 

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*Note: This text is a natural representation of the document content.*
Table 2. Radial growth rate (RGR) of mycelium on different mediums.

| mediums | Radial growth rate (cm⁻¹d) |
|---------|----------------------------|
| 1       | 0.196±0.001a               |
| 2       | 0.192±0.004a               |
| 3       | 0.122±0.002c               |
| 4       | 0.092±0.002d               |
| 5       | 0.200±0.005a               |
| 6       | 0.160±0.003b               |

Note: the data are the mean ± standard error of 10 replicates, and the different lowercase letters indicated significant differences at 0.05 level.

4. Discussion

The ectomycorrhizal fungus *Lyophyllum shimeji* (Kawam.) Hongo is one of the most valuable edible mushrooms in East Asia, especially in Japan but rare in China [9]. Hitherto, there is no any record for *Lyophyllum shimeji* in other parts of China except in Yunnan which reported by Peigui Liu in 1993. In this study, morphological characteristics and molecular techniques results showed that the samples fund at Dazhou (31.35°N, 107.72°E; 780 m altitude) district in 16th of October 2017 was *Lyophyllum shimeji*. The existence of *Lyophyllum shimeji* in other parts of China is reported for the first time.

The ectomycorrhizal fungi are difficult to cultivate artificially without the host plant. However, in 1994, Ohta successfully obtained the fruit body through the artificial cultivation using barley grain without the host plant, although the yields have been relatively low. In recent years, a huge infections by the pine wood nematode caused a rapidly decline in the fructification of *L. shimeji* in Dazhou areas and the wild-grown *L. shimeji* is available only in high-class restaurants. Little research has been conducted for its artificial domestication and cultivation in China. Therefore, in order to explore its artificial domestication and cultivation without the host plant, we screen the suitable medium for this fungus in this report. Results showed that the suitable medium component was potato 200g, MgSO₄·7H₂O 0.5g, glucose 20 g, KH₂PO₄ 0.3 g, tryptone 0.5g, VB₁ 20 mg, ager 20 g, ddH₂O 1000 ml, PH5-6. These results could provide a new reference for its artificial domestication and cultivation studies.

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