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Noble strain of *Sparassis latifolia* produces high content of β-glucan

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ABSTRACT

Objective: To classify four new *Sparassis* strains (CLM1, CKM1, CKM2, and KJM1) using the internal transcribed spacer sequence and to elucidate their β-glucan content and mycelial growth.

Methods: Two different microbiological media were used to determine growth rate. The β-glucan contents were analyzed using the Megazyme Mushroom and Yeast Beta-Glucan kit. To determine the genetic relationships, phylogenetic trees were constructed using ClustalX. Multiple sequence alignments were printed and shaded with the BOXSHADE 3.21 program.

Results: In this study, four new *Sparassis* strains were isolated from the southern region of the Korea Peninsula. They were all classified into the *Sparassis latifolia* clade as a monophyletic group based on the internal transcribed spacer sequence. Mycelial growth rate of the CLM1 strain was highest in potato dextrose agar and potato dextrose agar larch. The β-glucan content of the CLM1 strain was highest at 29.5% (w/w). A high degree of sequence divergence was detected in the RNA polymerase second largest subunit II gene (RPB2) within *Sparassis* spp. tested. The putative amino acid sequences of the RPB2 had a distinct sequence. The nucleotide sequences of the RPB2's intron were also divergent among *Sparassis* spp., even though their nucleotide length was well conserved within *Sparassis latifolia*.

Conclusions: These results indicate that the nucleotide sequences and the amino acid sequences of RPB2 can be used to identify individual *Sparassis* sp. The *Sparassis* strain CLM1 may be best for developing a remedy to prevent or treat cancer and other chronic diseases.

1. Introduction

Sparassis Fr. species are distributed in Europe, Eastern Asia, North America, and Australia. They were known as brown-rot producers with a bipolar mating system [1]. These species primarily grow on the stumps of coniferous trees such as pine (*Pinus densiflora*), larch (*Larix kaempferi*), and Korean pine (*Pinus koraiensis*). The edible mushroom, *Sparassis* spp., plays significant industrial and economic role as sources of pharmaceutics, therapeutics, health supplements and biotechnological products. Phylogenetic analyses of mushrooms using molecular-based methods have increased dramatically in the last decade. The phylogenetic relationships among *Sparassis* spp. have been studied using nucleotide sequence data from ribosomal DNAs (rDNA), mitochondrial rDNAs, and partial RNA polymerase subunit II gene (RPB2) [2–6]. These nucleotide markers as the primary fungal barcode genes have been used to correctly identify mushroom.

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At least eight clades were reported in *Sparassis* spp. [*Sparassis brevipes* (*S. brevipes*), *Sparassis crispa* (*S. crispa*), *Sparassis cystidiosa* (*S. cystidiosa*), *Sparassis latifolia* (*S. latifolia*), *Sparassis miniensis* (*S. miniensis*), *Sparassis radicata* (*S. radicata*), *Sparassis spathulata* (*S. spathulata*), and *Sparassis subalpina* (*S. subalpina*)]. *S. crispa* in Europe, *S. spathulata* in Eastern North America, and *S. radicata* Weir in Western North America are the main species found. Asian collections such as *S. crispa* are morphologically different from European collections. New *Sparassis* species such as *S. subalpina* in China was isolated [6], in which the flabellae are very broad, zonate and with only slightly contorted and thickened margins. This species originates from subalpine regions in Southwestern China [6].

S. latifolia was classified as separate species from *S. crispa* and *S. radicata* by Dai *et al*. [2]. *S. latifolia* is very widespread in Asia. The basidiocarps of *S. latifolia* are composed of numerous loosely arranged flabellae that are morphologically large, broad, dissected, and slightly contorted. The nuclear gene phylogeny, morphological differences, geographic distribution, and host shifts of *S. latifolia* are distinct from those of other *Sparassis* Fr. sp. Three clamp connection-producing species (*S. latifolia*, *S. crispa*, and *S. radicata*) form a clade, for which there is no sequence divergence in the *ATP6* gene. The nuclear gene data tree divides the three taxa into two highly divergent clades, one of which contains only *S. latifolia*. Korean collections also were redistributed into *S. latifolia* from *S. crispa* by molecular sequence analysis of the internal transcribed spacer (ITS) rDNA regions [4]. The 39 isolates collected from southern regions of Korea were grouped into subclade A of *S. latifolia*.

Medicinal mushrooms have a long history of frequent use in traditional Asian therapy. The extracted materials from various mushrooms have been used as a remedy for treatment of cancers or other diseases [7–9]. Polysaccharides represent the major class of bioactive compounds found in mushrooms. *Sparassis*, an edible mushroom, is popular worldwide because it shows potentially great pharmaceutical properties. The purified β-glucan from *Sparassis* sp. is a polysaccharide, which exhibits various biological activities, such as immune stimulation, enhancement of the hematopoietic response, and anticancer effects (antiangiogenic and antimetastatic) [7,9,10]. Kwon *et al*. reported that oral administration of *S. crispa* can improve the impaired healing of diabetic wounds by increasing the migration of macrophages and fibroblasts, and β-glucan from *S. crispa* directly increases the synthesis of type I collagen [11]. Thus, *Sparassis* sp. extracts have been applied as health supplements in food, drinks, and drugs.

Four new cauliflower mushrooms were collected in the southern region of the Republic of Korea, and their mycelia were induced and cultured. They were all identified as a *S. latifolia* strain by molecular sequence analyses. We wanted to look for and identify *Sparassis* strains that produce high amount of β-glucan. This study was carried out to assess which strain of *S. latifolia* can grow faster and produce more amount of β-glucan.

2. Materials and methods

2.1. Fungus growth

Fruiting body parts of four *Sparassis* strains were sterilized, isolated, and incubated on potato dextrose agar medium (PDA) including 100 mg/L ampicillin and kanamycin. The induced mycelia were cultured on Petri-dish plates (150 mm in diameter) to compare growth. Two different microbiological media [PDA and potato dextrose agar larch (PDAL)] were used to determine growth rate. Mycelia of the *S. latifolia* strains were cultured on medium PDA and PDAL. PDA was composed of 4 g potato starch, 20 g dextrose, 15 g agar, and 1 L distilled water. PDAL was prepared as PDA plus 1 L larch extract. The larch extract was prepared by extracting 100 g larch sawdust in 1 L distilled water for 2 h at 100 °C. After autoclaving the larch extract, the sawdust mixture was removed by filtering with a membrane. Petri dishes (150 mm in diameter) containing 100 mL of culture medium were sterilized at 121 °C for 20 min. The optimal pH for all isolates was 6.0 in PDA (data not shown). Mycelium that had been cultured for 4 weeks on PDA media was placed on medium surface. Small plugs of mycelium were used as inoculum to measure the growth rate of the isolate in each treatment and were placed on the center of the plate. The diameter of the growing mycelium was measured every 7 days to represent growth. The mycelium growth was measured in 150 mm Petri-dish plate at 28 days of inoculation (DOI). All replicates were grown together in a controlled temperature chamber at 25 °C in the dark during the whole period. All plates were sealed with double layers of wrap, which permitted gas exchange. Linear growth rate was estimated for each replicate by measuring the colony diameter weekly. Both PDA and PDAL were used to measure growth. All analyses were conducted in triplicate.

2.2. Determination of β-glucan content

Each strain of mycelia was cultivated in potato dextrose broth or potato dextrose larch broth for 30 days under the same culture conditions (25 °C in the darkness at 150 r/min). The cultured mycelium was harvested, rinsed to delete the growth medium, and frozen-dried with a speed vacuum. The β-glucan contents of lyophilized mycelium samples were analyzed using the Megazyme Mushroom and Yeast Beta-Glucan kit (K-YBGL, Megazyme, Wicklow, Ireland). All analyses were done in triplicate and reported on a dry matter basis.

2.3. Molecular techniques

Genomic DNAs were isolated from the mycelium. The materials were ground to a fine powder in liquid nitrogen. The DNA was extracted with GeneAll Exgene Plant SV mini kit (GeneAll, Seoul, Korea). *S. latifolia* sequence data were generated based on a previous study: (1) mitochondrial large subunit ribosomal DNA genes (mls rDNA), (2) nuclear small subunit of ribosomal DNA (nss rDNA), (3) ITS, and (4) RPB2 [5]. Wang *et al*. showed all of the primary primer sets we used in this study [5]. Another primer set was used for the *RPB2* genes (RPB2LP853; CTTAAATACTCCCTTGCCAC and RPB2RP2509; GTACGTGATACCGATAGTACC) to amplify a longer fragment (1656 bp) [5]. PCR reaction mixes contained 5 μL of 10 × buffer, 2.5 mmol/L deoxynucleotide triphosphates Mix, and 5 unit of *Taq* polymerase (Genotech, Daejion, Korea). The amplification program included 30–35 cycles of 95 °C for 20 s, 50 °C for 30 s, and 72 °C for 90 s. The PCR product was purified and sequenced using ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA).