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SHORT COMMUNICATION

Homology search of genus *pleurotus* using an internal transcribed spacer region

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Abstract–In order to establish the phylogenetic relationships of genus *Pleurotus,* internal transcribed spacer (ITS) regions of nine strains of mushrooms were amplified and sequenced. Fungi-Seq-F1 primer and fungi-Seq-R1 were perfectly matched with 2007 and 2935 kinds of fungi, respectively. These results show that these primers can be used not only in the types of mushroom used in this study, but also in DNA sequencing analysis of the ITS region of any other mushroom. A BLAST search using about 500 bp of the 5' terminus of ITS region was carried out. The observed homology between some mushrooms and the ITS region was 98-100%. In order to investigate these results, we searched the GenBank databases. At the time of our search, the ITS region of the mushrooms was unknown and could not be found in the results of our database search in GenBank. Therefore, whole DNA sequencing of ITS region of the mushroom is considered to be of critical significance in view of future phylogenetic analyses. Additionally, the sequences of four mushrooms were aligned and showed 95-98% of homology.

Key words: *Pleurotus*, ITS Region, Homology

INTRODUCTION

Each mushroom characteristically contains many different bioactive compounds with diverse biological activities. Some edible mushrooms have various degrees of immunomodulatory, antitumor, and other beneficial or therapeutic health effects without any significant toxicity. Mushrooms have unique flavors and aromatic properties, and are considered to be rich in protein, fiber, carbohydrates, vitamins, and minerals [1,2]. Therefore, the artificial production of mushrooms is economically important in the world food industry, which has expanded over the past few years. Recently, the production of mushrooms and polysaccharides by various fungal strains has been extensively researched for potential applications using environmental parameters, as well as medium composition [3-5]. We previously investigated the possibility of artificial production of *Pleurotus ferulae* fruiting bodies in a solid-state culture. Specifically, when various ratios of garlic powder were used, yields of fruiting bodies were higher than those of a synthetic mixture without garlic powder in sawdust culture [6]. In order to investigate the viability of human cancer cell lines for the screening of antitumor substances contained in *Pleurotus ferulae*, the antitumor activities of these extracts were examined on three human solid carcinomas, one lung carcinoma, and two cervical carcinomas [7]. We also studied the batch cultures for effective polysaccharide production from *Pleurotus ferulae*. Additionally, a logistic model to describe the mycelial growth and a Leudecking-Piret model for polysaccharide production were proposed [8]. Using *Lentinus* sp., *Phloiota nameko*, and *Agrocybe aegerita*, the batch cultures employed for effective production of polysaccharide and mycelial growth from liquid cultures were also investigated [9-11]. For efficient mycelial growth and polysaccharide production from a liquid culture using *Pleurotus nebrodensis* Inzenga, the factors of the various environments affecting mycelial growth and production and biological effects of its extract were also investigated *in vitro* [12]. Additionally, in order to determine nutritional values, including crude fat, carbohydrate, protein, amino acid, vitamin, and mineral compositions, we studied the composition of fruit body extracts and mycelia of *Cordyceps militars* and *Morchella esculenta* and the composition of seasoning used [13-15].

Especially, the genus *Pleurotus* are promising as medicinal mushrooms, exhibiting hematological, antiviral, antitumor, antibiotic, antibacterial, hypocholesteroic and immunomodulation activities [16]. However, there are significant problems in classifying *Pleurotus* isolates using only morphological characteristics, which are often unreliable or inconclusive mainly due to the large influence exerted by environmental factors and compatibility experiments, which are based on the application of the controversial biological species concept. The genus *Pleurotus* shows great variation in morphology but due to a lack of appropriate applicable characteristics, few phylogenetic studies have been conducted to infer the relationships of taxa within the genus. Recently, molecular sequence data have been successfully applied for the study of evolutionary patterns and phylogenetic systematics in fungi. Nuclear small subunit ribosomal RNA gene regions are usually used as molecular tools to analyze fungal taxa at the family or order level, and ITS regions are commonly used to examine phylogenetic positions or relationships at the species or interspecies level. Therefore, in this study, ITS region sequences were used to infer phylogenetic relationships among the species of *Pleurotus* in Korea.

MATERIALS AND METHODS

1. Materials

The following nine species were used in this study and their sources: *Pleurotus ferulae*, *Pleurotus eryngii*, White *Pleurotus fer-*

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ulae, *Lentinus lepidens*, *Pleurotus ostreatus*, *Agrocybe aegerita*, *Phloiota namek*, *Sporassis crispa*, and *Macrolepiota procera*. Fungal cultures used in this experiment were maintained on media containing 24 g/L of potato dextrose broth with shaking at 24 °C in the dark for 7 days.

2. DNA Isolation of Fungal

Mycelia grown on 24-well plates were frozen, dried, and used for DNA isolation. DNA was extracted by the rapid method for nucleic acid extraction [17,18] with some modifications [19-21]. Genomic DNA of fungal was isolated as follows: the dried mycelia were ground into powder with liquid nitrogen. One ml of extraction buffer containing 10 mM Tris-HCl, 20 mM EDTA, 30 mM NaCl, and 1 μl of Protease K (20 mg/ml) was added, and the solution was gently shaken until the powder was thoroughly saturated at 50 °C. 100 μl of N-Lauroylsarcosine (10%) was added to the mixture, which was heated to 55 °C for 60 min and then centrifuged at 3,000 rpm (Union 32R Plus, Hanil, Republic of Korea) for 20 min at room temperature. The upper phase was transferred to a new tube, 60 μl of 3 M NaOAc (pH 5.2) was added, and the solution was centrifuged again. The supernatant was placed into a 1.5 ml tube, and a mixture of phenol and chloroform (1 : 1) solution was added, mixed, and centrifuged at 5,000 rpm for 15 min before a final extraction with chloroform only. The resulting volume of supernatant was measured, and a 600 μl volume of cold isopropanol (60%) was added to precipitate the genomic DNA. Genomic DNA was subsequently pelleted by centrifugation at 12,000 rpm for 10 min at 4 °C. 600 μl of isopropanol was decanted off the resulting aqueous phase, and 100 μl of ethanol (70%) was added. The DNA was pelleted by centrifugation at 12,000 rpm for 10 min. The ethanol was removed by speed vacuum and the DNA was dried at room temperature. The dried pellet was dissolved in 30 μl of dH_2O and stored at −20 °C.

3. DNA Application

Amplification was carried out in 50 μl of reaction mixture containing 50 ng of genomic DNA, 5 μl of buffer, 4 of dNTP mixture, 0.5 μl of ITS 4 primer (5'-TCCTCCGCTTATTGATATGC-3'), 0.5 μl of ITS 5 primer (5'-GGAAGTAAAAGTCGTAACAAGG-3'), 37.6 μl of H_2O, and 0.4 μl of Taq DNA polymerase.

RESULTS AND DISCUSSION

To amplify the ITS region from the collected strains, the primers of fungus were used. The locations on nuclear rDNAs of PCR primers are shown in Fig. 1. A Perkin-Elmer Cetus DNA thermal cycler (Model 9600) was used with the following program: denaturation for 5 min at 94 °C, 30 cycles for 1 min at 94 °C, 1 min at 48 °C, 1 min at 72 °C, and a final extension step for 10 min at 72 °C. Amplified DNA fragments were recovered from the gel and ligated into pGEM-T easy vector (Promega, Madison, WI) according to the protocol of the manufacturer. Plasmids were introduced into electrocompetent cells of *Escherichia coli* (DH10B) according to standard procedures. The transformants were selected on ampicillin (100 ug/ml) agar medium containing 5-bromo-4-chloro-3-indoyl-B-D-galactoside (80 ug/ml) and isopropylthio-B-D-galactosidase (120 ug/ml). Nucleotide sequences of amplified inserts were determined by using a BigDye DNA sequencing kit (BigDye Terminator Cycle Sequencing v 2.0, PE Biosystems, Foster City, CA 94404) on an ABI377 DNA sequencer (PE Applied Biosystems, Foster City, CA, USA). Sequencing was carried out in 10 μl of reaction mixture containing 400 ng of genomic DNA, 2 μl of Bigdye Terminator, 1 μl of primer (M13 reverse primer and ITS4,5 primer, 2 pmole), and 3 μl of SDW with the following program: denaturation for 2 min at 96 °C, 25 cycles for 10 sec at 96 °C, 5 sec at 50 °C, 4 min at 60 °C, and hold at 4 °C. Nucleotide analyses were performed by using a BLAST (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) algorithm in the GenBank database (National Center for Biotechnology Information: NCBI, http://www.ncbi.nlm.nih.gov/). Multiple sequence alignments and comparisons were performed with CLUSTALW [21,22]. For parsimony and distance analyses, the heuristic search option and the neighbor-joining method [23] were performed by using PAUP software (version 8.0 program) and were used for phylogenetic inference. Strengths of internal branches of resulting trees were statistically tested by bootstrap analysis with 1,000 replications [24]. 10 ng-2 ug of genomic DNA was isolated from other hyphae. This may be the result of different cell wall or cell membrane solubilities, as determined by guanidine isothiocyanate lysis buffer. The polymerase chain reaction (PCR) was carried out for amplification of ITS region from hypha with genomic DNA as a template. The results are shown in Fig. 2. A 0.5 kb of amplified segment was obtained. The amplified 16S rDNA gene segment was successfully ligated into the pEZ-T vector. 10 ug of recombinant plasmid DNA containing ITS region was extracted. The size of the ITS region of the mushrooms was known to be approximately 0.5 kb. Therefore, five primers were designed and synthesized for determining the DNA sequences. Sequencing of approximately 500-700 bp of 5' and 3' termini of the ITS region of the mushrooms was determined by using the T3 and T7 primer-binding sites in cloning vector (pEZ-T). The direction was analyzed by the MegAlign program. Based on

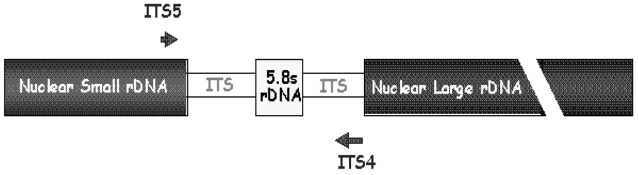

Fig. 1. Locations on nuclear rDNAs of PCR primers. The arrowheads represent the 3' end of each primer. The nuclear large rDNA is truncated in this figure.

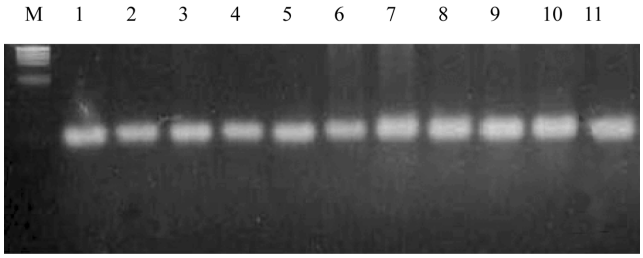

Fig. 2. Gel showing amplification of PCR products. M: Marker (0.5 kb ladder); Lane 1-3: *Pleurotus ferulae*; Lane 4: *Pleurotus eryngii*; Lane 5: *Pleurotus ostreatus*; Lane 6: *White Pleurotus ferulae*; Lane 7: *Sporassis crispa*; Lane 8: *Macrolepiot procera*; Lane 9: *Agrocybe aegerita*; Lane 10: *Lentinus lepideus*; Lane 11: *Pholiota Nameko.*

Table 1. DNA sequences of the ITS region of nine strains

White Pleurotus ferulae	----ATGAATTCGCTATGGAGTTGTTGCTGG----CCTCTAGGGGCATGTGCACGCTTCA 52
Pleurotus ferulae	----ATGAATTCGCTATGGAGTTGTTGCTGG----CCTCTAGGGGTATGTGCACGCTTCA 52
Pleurotus eryngii	----ATGAATTCACTATGGAGTTGTTGCTGG----CCTCTAGGGGAATGTGCACGCTTCA 52
Pleurotus ostreatus	----ATGAATTCACTATGGAGTTGTTGCTGG----CCTCTAGGGGCATGTGCACGCTTCA 52
White Pleurotus ferulae	CTAGTCTTTCAA------CCACC-TGTGAACTTTTGGTAGATCTGCGAAGTCGT------ 99
Pleurotus ferulae	CTAGTCTTTCAA------CCACCCTGTGAACTTTTGG-AGATCTGCGAAGTCGT------ 99
Pleurotus eryngii	CTAGTCTTTCAA------CCACC-TGTGAACTTTTGATAGATCTGTGAAGTCGT------ 99
Pleurotus ostreatus	CTAGTCTTTCAA------CCACC-TGTGAACTTTTGATAGATCTGTGAAGTCGT------ 99
White Pleurotus ferulae	-------CTCTCAAGTCGTCAGACTTGGTTTGCTGGGATTTAAACATCTCGGT------- 145
Pleurotus ferulae	-------CTCTCAAGTCGTCAGACTTGGATTGCTGGGATTTAAACATCTCGGT------- 145
Pleurotus eryngii	-------CTCTCAAGTCGTTAGACTTGGTTTGCTGGGATGTAAACGTCTCGGT------- 145
Pleurotus ostreatus	-------CTCTCAAGTCGTCAGACTTGG-TTGCTGGGATTTAAACGTCTCGGT------- 144
White Pleurotus ferulae	------GTGACTACGCAGTCTAT--------TTACT-TATA-CACCCCAAATGTATGTCT 189
Pleurotus ferulae	------GTGACTACGCAGTCTAT--------TTACT-TATA-CACCCCAAATGTATGTCT 189
Pleurotus eryngii	------GTGACTACGCAGTCTAT--------TTACT-TATAACACCCCAAATGTATGTCT 190
Pleurotus ostreatus	------GTGACTACGCAGTCTAT--------TTACT-TACA-CACCCCAAATGTATGTCT 188
White Pleurotus ferulae	ACGAATGTCAT----TTAATGGGCCTTGTGCCTATAAACCATAATACAACTTTCAACAAC 245
Pleurotus ferulae	ACGAATGTCAT----TTAATGGGCCTTGTGCCTATAAACCATAATACAACTTTCAACAAC 245
Pleurotus eryngii	ACGAATGTCAT----TTAAAGGGCCTTGTGCCTATAAACCATAATACAACTTTCAACAAC 246
Pleurotus ostreatus	ACGAATGTCAT----TTAATGGGCCTTGTGCCTTTAAACCATAATACAACTTTCAACAAC 244
White Pleurotus ferulae	GGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGNTGAAT 305
Pleurotus ferulae	GGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAA---------- 293
Pleurotus eryngii	GGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAA---------- 294
Pleurotus ostreatus	GGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAA---------- 292
White Pleurotus ferulae	TGCAGAATTCAGTGAATCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATT 365
Pleurotus ferulae	----------AGTAATGTGAATT 306
Pleurotus eryngii	----------GTAATGTGAATT 306
Pleurotus ostreatus	----------GTAATGTGAATT 304
White Pleurotus ferulae	GCAGAATTCAGTGAATCATCGAATC--TTTGAACGCACC-TTGCGCCCCTTGGTATTCCG 422
Pleurotus ferulae	GCAGAATTCAGTGAATCATCGAATTCTTTTGAACGCACCCTTGCGCCCCTTGGTATTCCG 366
Pleurotus eryngii	GCAGAATTCAGTGAATCATCGAATC--TTTGAACGCACC-TTGCGCCCCTCGGTATTCCG 363
Pleurotus ostreatus	GCAGAATTCAGTGAATCATCGAATC--TTTGAACGCACC-TTGCGCCCCTTGGTATTCCG 361
White Pleurotus ferulae	A-GGGGCATGCCTGTTTGAGTGTCATTAAATTCTCAAACTCACTCTGGTTTTTTTCCAAT 481
Pleurotus ferulae	AAGGGGCATGCCTGTTTGAGTGTCATTAAATTCTCAAACTCACTCTGGTTTTTT-CCAAT 425
Pleurotus eryngii	A-GGGGCATGCCTGTTTGAGTGTCATTAAATTCTCAAACTCACTCTGGTTCTT--CCAAT 420
Pleurotus ostreatus	A-GGGGCATGCCTGTTTGAGTGTCATTAAATTCTCAAACTCACTTTGGTTTCTTTCCAAT 420
White Pleurotus ferulae	TG---TGATGTTTGGATTGTTGGGG--GCTGCTGGC----CTTGACAGGTCGGCTCCT-C 531
Pleurotus ferulae	TG---TGATGTTTGGATTGTTGGGG--GCTGCTGGC----CTTGACAGGTCGGCTCCTTC 476
Pleurotus eryngii	TG---TGATGTTTGGATTGTTGGGG--GCTGCTGGC----CTTGACAGGTCGGCTCCT-C 470
Pleurotus ostreatus	TG---TGATGTTTGGATTGTTGGGG--GCTGCTGGC----CTTGACAGGTCGGCTCCT-C 470
White Pleurotus ferulae	TTAAATGCATTAGCAGG--ACTTCTCATTGCCTCTGCGCATGATGTGA-TAATTATCA-- 586
Pleurotus ferulae	TTAAATGCATTAGCAGG--ACTTCTCATTGCCTCTGCGCATGATGTGAGTAATTATCA-- 532
Pleurotus eryngii	TTAAATGCATTAGCAGG--ACTTCTCATTGCCTCTGCGCATGATGTGA-TAACTATCA-- 525
Pleurotus ostreatus	TTAAATGCATTAGCAGG--ACTTCTCATTGCCTCTGCGCATGATGTGA-TAATTATCA-- 525
White Pleurotus ferulae	-CTCATCAATA-----GCACGCATGAAT-AGAGTCTAGCTCTCTAATCG-----TCCGCA 634
Pleurotus ferulae	-CTCATCAATA-----GCACGCA-GAAT-AGAGTCTAGCTCTCTAATCG-----TCCGCA 579
Pleurotus eryngii	-CTCATCAATA-----GCACGCATGAAT-AGAGTCTGGCTCTCTAACCG-----TCCGCA 573
Pleurotus ostreatus	-CTCATCAATA-----GCACGCATGAAT-AGAGTCCAGCTCTCTAATCG-----TCCGCA 573
White Pleurotus ferulae	AGGACAATTTGATAA---------- 649
Pleurotus ferulae	AGGACAA---------- 586
Pleurotus eryngii	AGGACAATTTGACAAT---------- 589
Pleurotus ostreatus	AGGACAATTTGACAAT---------- 589

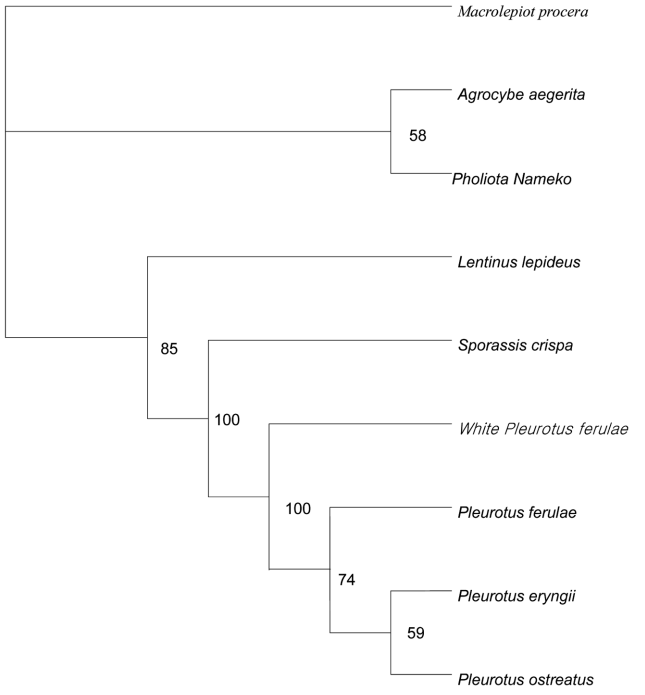

Fig. 3. Phylogenetic tree base on ITS region sequences of nine strains.

the results of this analysis, the phylogenetic tree of the DNA sequence was analyzed by the MegAlign program. In addition, primers for sequencing of other ITS were designed by using the Primer Select program. (Fungi-Seq-F1, 5'-ACG GGG AGG TAG TGA CAA TA-3' & Fungi-Seq-R1, 5'-GAA CAT CTA AGG GCA TCA CAG-3'). These primers were aligned using Probe Check (version 2.1r3; http://rdp.cme.msu.edu/cgis/probe_match.cgi). As a result, fungi-Seq-F1 primer and fungi-Seq-R1 were in perfect agreement with 2007 and 2935 kinds of fungi, respectively. These results showed that fungi-Seq-F1 and fungi-Seq-R1 primer can be used not only in the mushrooms used in this study, but also in DNA sequencing analysis of the ITS region of any other mushroom. DNA sequence results of the ITS region of nine strains are shown in Table 1. A BLAST search using about 500 bp of 5' termini of ITS region was carried out and is shown in Fig. 3. The homology between some mushrooms and ITS region was 98-100%, In order to investigate our results the databases in GenBank were searched. At the time of our search, the ITS region sequence of the mushrooms was unknown, which could be proved from the results of the GenBank database search. Therefore, whole DNA sequencing of the ITS region of mushrooms in the future is considered to be of critical significance for future phylogenetic analysis. Additionally, sequences between four mushrooms were aligned and showed 95-98% homology. Unusually, the homology was higher in similar mushrooms than in mushrooms that differed greatly.

We are in the process of determining the entire DNA sequences and constructing the 16S rDNA genes of some strains classified into *Agrocybe aegerita*. If we clarify the DNA sequences of the ITS region of the mushrooms used this study and carry out homology searches between these and the 16S rDNA genes of other mushrooms, re-classification according to the DNA sequence of ITS region may be performed for future phylogenetic analysis.

CONCLUSIONS

The identification of genus *Pleurotus* is very imoprtant in order to obtain the desired species. For a long time, genus *Pleurotus* were examined with the naked eye based on phenotypic characters, but it was impossible to distinguish between genetically related species by this method. Therefore, in our study, ITS region sequences were used to infer phylogenetic relationships among the genus *Pleurotus* in Korea. Fungi-Seq-F1 primer and fungi-Seq-R1 show that these primers can be used not only in the types of mushroom used in this study, but also in DNA sequencing analysis of the ITS region of any other mushroom. A BLAST search using about 500 bp of 5' termini of ITS region was carried out. The observed homology between some mushrooms and the ITS region was 98-100%. Additionally, sequences between four mushrooms were aligned and showed 95-98% homology. Unusually, the homology was higher in similar mushrooms than in mushrooms that differed greatly.

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