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Identification of Medicinal Mushroom Species Based on Nuclear Large Subunit rDNA Sequences

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The purpose of this study was to develop molecular identification method for medical mushrooms and their preparations based on the nucleotide sequences of nuclear large subunit (LSU) rDNA. Four specimens were collected of each of the three representative medicinal mushrooms used in Korea: *Ganoderma lucidum, Coriolus versicolor*, and *Fomes fomentarius*. Fungal material used in these experiments included two different mycelial cultures and two different fruiting bodies from wild or cultivated mushrooms. The genomic DNA of mushrooms were extracted and 3 nuclear LSU rDNA fragments were amplified: set 1 for the 1.1-kb DNA fragment in the upstream region, set 2 for the 1.2-kb fragment in the middle, and set 3 for the 1.3-kb fragment downstream. The amplified gene products of nuclear large subunit rDNA from 3 different mushrooms were cloned into *E. coli* vector and subjected to nucleotide sequence determination. The sequence thus determined revealed that the gene sequences of the same medicinal mushroom species were more than 99.48% homologous, and the consensus sequences of 3 different medicinal mushrooms were more than 97.80% homologous. Restriction analysis revealed no useful restriction sites for 6-bp recognition enzymes for distinguishing the 3 sequences from one another, but some distinctive restriction patterns were recognized by the 4-bp recognition enzymes *AccII* and *HhaI*. This analysis was also confirmed by PCR-RFLP experiments on medicinal mushrooms.

Keywords: medicinal mushroom, *Ganoderma lucidum, Coriolus versicolor, Fomes fomentarius*, LSU rDNA, PCR-RFLP

For a long time in Asian countries, a variety of edible mushrooms have been taken as vitamin and mineral supplements. Recent progress in pharmacological studies on edible mushrooms has revealed some valuable activities related to biological response modification (Wasser, 2002; Wasser and Weis, 1999). In particular, chemopreventive, chemotherapeutic, immunomodulatory, hypoglycemic, and hypocholestemic effects have been observed in edible mushrooms, including: *Ganoderma lucidum* (Youngji), *Coriolus versicolor* (Unji), *Fomes fomentarius* (Malgup), *Inonotus obliquus* (Chaga), *Phellinus linteus* (Sanghwang), *Lentinus edodes* (Pyogo), *Tricholoma matsutake* (Song E), *Poria cocos* (Bokryung), and *Cordyceps sinensis* (Dongchunghacho). A variety of health-aid preparations from these mushrooms have been developed for medicinal and therapeutic purposes in Asian countries (Cui and Chisti, 2003).

Most mushrooms belong to the Basidiomycota, which produces a fruiting body composed of an umbrella -shaped cap, basidia-covered gills, and a club-shaped stalk. Up until now, taxonomic and phylogenetic studies of Basidiomycota have been based mainly on the analysis and comparison of morphological characters like the shape, size, and color of caps and gills.

During the last 10 years, a molecular approach for identification of several Basidiomycetes genera has been attempted by amplification and restriction of special DNA sequences by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP). This approach has been widely used for identification of eucaryotic specimens, including yeast and other fungi (Hopple and Vilgalys, 1994; Wesselink *et al.*, 2002).

In the case of bacteria, the diversity of ribosomal RNA (rRNA) gene (rDNA) sequences is useful for phylogenetic classification and taxonomy (Hur and Chun, 2004; Lim *et al.*, 2005). Similarly, rDNA genes have been successfully used as a target for species identification in taxonomic, evolutionary, and environmental studies of yeast and fungi (Borneman and

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Hartin, 2000; Schabereiter-Gurtner *et al.*, 2001; Smit *et al.*, 1999).

Basidiomycetes rRNAs, like other fungal rRNAs, are generally composed of 5S, 5.8S, and 18S small subunits (SSU) and a 25S~28S large subunit (LSU). Since rRNA structural genes have been known to be well conserved at the genus or species levels, most molecular phylogenetic studies have been focused on the internal transcribed spacer (ITS) region located between rRNA structural genes in the rDNA cistron (Chen *et al.*, 2004; Kim and Lee, 2000; Lee *et al.*, 2000; Park *et al.*, 2004). However, LSU rRNA gene has been revealed to have some variable regions with sequence divergency: D1, D2, and D3. These are enough to infer phylogenetic relationships between species (Hong *et al.*, 2000; Kurzman and Robnett, 1998; Wesselink *et al.*, 2002).

In this study, the nucleotide sequences for nuclear LSU rDNA were determined and compared for three important medicinal mushrooms in Korea: *G. lucidum*, *C. versicolor*, and *F. fomentarius*. The possibility to distinguish between these mushroom species at the molecular level was investigated by PCR-RFLP technique.

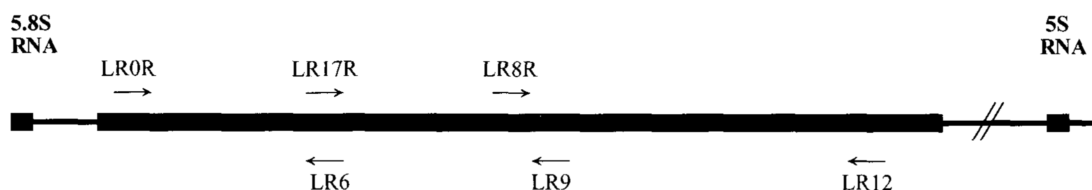
Materials and Methods

Sample collection

The mycelia of two different strains for each of the 3 mushroom species studied (*G. lucidum*, *C. versicolor*, and *F. fomentarius*) were kindly supplied by Prof. Tae Soo Lee of the Korean Wild Mushroom Bank, Incheon University (Incheon, Korea) and Prof. Hyung Tae Choi of Kangwon University (Chuncheon, Korea). Two different fruiting bodies from each medicinal mushroom species were also purchased from local natural herb collectors or from mushroom cultivators as shown in Table 1.

Table 1. Medicinal mushrooms used in this study and their GenBank accession numbers of nuclear LSU rDNA sequences.

Mushrooms	Source	Strain designation	Supplier	GenBank accession number
Ganoderma lucidum	Mycelium	IUM00298 (Isolated)	Korea Wild Mushroom Bank, Incheon University Incheon, Korea	DQ208410
		IUM01122 (Isolated)	Korea Wild Mushroom Bank, Incheon University Incheon, Korea	DQ208411
	Fruiting body	C-1 (Wild)	Mr. Dong-Myoung Jeon, Busan, Korea	DQ208412
		C-2 (Cultivated)	Mr. Young-Ho Bae, Andong, Gyongbuk, Korea	DQ208413
Coriolus versicolor	Mycelium	IUM00100 (Isolated)	Korea Wild Mushroom Bank, Incheon University Incheon, Korea	DQ208414
		951007-22 (Isolated)	Prof. Hyung Tae Choi, Kangwon University, Chuncheon, Korea	DQ208415
	Fruiting body	C-3 (Wild)	Mr. Dong-Myoung Jeon, Busan, Korea	DQ208416
		C-4 (Wild)	Mr. Kyoung-Je Jeon Jayeon-Cho, Inje, Gangwon, Korea	DQ208417
Fomes fomentarius	Mycelium	IUM00144 (Isolated)	Korea Wild Mushroom Bank, Incheon University Incheon, Korea	DQ208418
		IUM00214 (Isolated)	Korea Wild Mushroom Bank, Incheon University Incheon, Korea	DQ208419
	Fruiting Body	C-5 (Wild)	Mr. Dong-Myoung Jeon, Busan, Korea	DQ208420
		C-6 (Wild)	Mr. Il-Hwan Cha, Youngyang, Gyongbuk, Korea	DQ208421



Primer		Primer Sequence	Tm(°C)	Position	Estimated Size
Set 1	LR0R	5'- ACC CGC TGA ACT TAA TT - 3'	48	26-42	1,100 bp
	LR6	5'- CGC CAG TTC TGC TTA CC - 3'	52	1141-1125	
Set 2	LR17R	5'- TAA CCT ATT CTC AAA CTT - 3'	42	1033-1050	1,156 bp
	LR9	5'- AGA GAA CTG GGC AGA AA - 3'	49	2204-2188	
Set 3	LR8R	5'- AGC AGG TCT CCA AGG TG - 3'	44	1845-1865	1,262 bp
	LR12	5'- GAC TTA GAG GCG TTC AG - 3'	49	3124-3106	

Fig. 1. Three sets of primers used in this study and their location in nuclear LSU rDNA.

Isolation of total genomic DNA
The fruiting bodies of the collected or cultivated mushrooms were surface-sterilized with 70% ethanol and cut with a clean razor to obtain uncontaminated tissue. The tissue was frozen in liquid nitrogen and ground well into powder in a clean mortar. Mycelia of mushroom strains grown on potato dextrose agar plates were scratched out using a sterilized needle. Total genomic DNA from 40 mg of mushroom powder or mycelium was extracted for each species by using a DNeasy Plant Mini Kit (Qiagen, USA) according to the supplier's protocol.

DNA primers and DNA amplification
For amplification of nuclear LSU rDNA, three sets of primers were designed and supplied from Genotech (Daejon, Korea), as seen in Fig. 1. The amplification of nuclear LSU rDNA fragments was undertaken by using GeneAmp PCR System 2400 (Perkin Elmer, USA). The PCR mixture (50 μl) was composed of 4 μl of DNA solution (0.5 μg/ml), 5 μl of 10 × Taq buffer (100 mM Tris, pH 8.3, 500 mM KCl, 25 mM $MgCl_2$), 2 μl of 2.5 mM dNTP, 4 μl each of 25 μM forward primer and reverse primer, 0.4 μl of 2.5 unit/ml *Taq* DNA polymerase, and 30.6 μl sterilized water. The amplification cycle was set for 30 repetitions of DNA denaturation at 94°C for 30 sec, DNA annealing at 50~55°C for 45 sec, and DNA synthesis at 72°C for 1 min. However, initial denaturation was at 94°C for 5 min and the final extension delay was at 72°C for 10 min. The amplified PCR product was confirmed by electrophoresis on 1% agarose gel.

DNA sequencing and analysis
The amplified PCR products were purified with a QIAEX gel extraction kit (Qiagen, USA). This DNA fragment was inserted into pGEM-T easy vector (Promega, USA) by T4 DNA ligase, and the ligated mixture was transformed into *Escherichia coli* JM109 (*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi Δ(lac-proAB)*) (Sambrook *et al.*, 1989). Transformed bacteria formed white colonies on Luria-Bertani (LB) media (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.2) containing 50 μg/ml ampicillin, 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 0.04% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). The plasmid was isolated from these colonies and subjected to nucleotide sequencing. Sequence determination was performed using T7/SP6 universal primers by a custom service of Genotech (Daejon, Korea). The determined nucleotide sequence was deposited at GenBank (http://www.ncbi.nlm.nih.gov/BankIt/nph-bankit.cgi). The ClustalW program (http://www.ebi.ac.uk/clustalw/) was employed for base comparison, and Webcutter 2.0 program (http://rna.lundberg.gu.se/cutter2/) was used for the identification of restriction patterns.

PCR-RFLP analysis
The PCR fragments for nuclear LSU rDNA gene were subjected to restriction digestion by using either *Acc*II or *Hha*I as restriction enzymes. The length polymorphism of the restricted DNA fragments was identified by the procedure of Kim *et al.* (2001).

Results

PCR amplification and sequence determination of nuclear LSU rDNA from medicinal mushrooms

In order to develop an identification method for the biological origins of medicinal mushrooms, a PCR-RFLP approach was attempted using nuclear LSU rDNA. For this purpose, specimens each of *G.. lucidum*, *C. versicolor,* and *F. fomentarius*, were collected. Samples included two different mycelial cultures and two different fruiting bodies of wild or cultivated mushrooms (Table 1).

Using genomic DNA isolated from each specimen as a template, three divided fragments of nuclear LSU rRNA gene were amplified and subjected to determination of nucleotide sequences. All LSU rDNAs were determined to be composed of 3110~3111 base pairs after the sequences of the 3 divided fragments were assembled. Those sequences were deposited in GenBank as described in Table 1. In BLAST search, the most homologous sequences in GenBank databases were 28S rDNA sequences of two Hymenomycetes species; *Donkiporia* species showing 98.4% homology and *Antrodia* species showing 95.6% homology.

It was also revealed that all specimens belonging to the same species of medicinal mushrooms showed more than 99.48% nucleotide sequence homology (Table 2). However, the nucleotide sequence of nuclear LSU rDNA from the fruiting body of a wild specimen of *F. fomentarius* C-6 showed the lowest level of homology with others in the same species. This naturally collected sample (or specimen) could be a variant or subspecies of *F. fomentarius.*

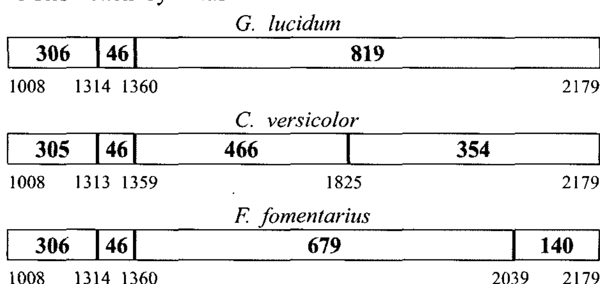
Table 2. Sequence homology (%) of nuclear LSU rDNA among the 3 mushroom species.

	G. lucidum	*C. versicolor*	*F. fomentarius*
G. lucidum	99.59 ± 0.29	97.94 ± 0.32	98.97 ± 0.43
C. versicolor	-	99.74 ± 0.16	97.80 ± 0.14
F. fomentarius	-	-	99.48 ± 0.48

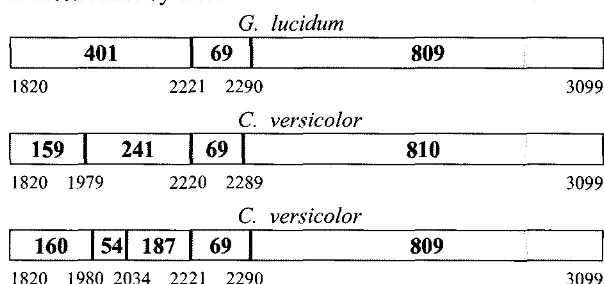
Restriction analysis of nuclear LSU rDNA from medicinal mushrooms

Consensus sequences were deduced through base alignment by ClustalW program. More than 97.80%

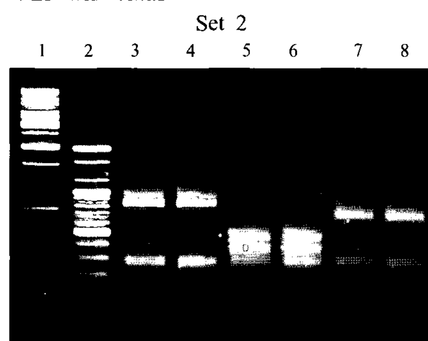
A Restriction by *Hha*I



B Restriction by *Acc*II



C PCR-RFLP with *Hha*I



D PCR-RFLP with *Acc*II

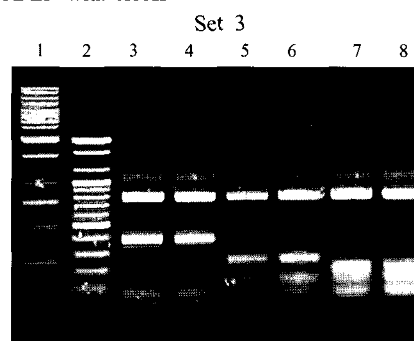


Fig. 2. Restriction analysis of nuclear LSU rDNA sequences from 3 different medicinal mushrooms and PCR-RFLP analysis based on restriction pattern. A, The middle fragments of nuclear LSU rDNA sequences showed different restriction pattern for *Hha*I. B, The downstream region of nuclear LSU rDNA sequences gave different restriction fragments by *Acc*II. C, PCR-RFLP analysis of the middle part fragment of nuclear LSU rDNA by *Hha*I. D, PCR-RFLP analysis of downstream fragment of nuclear LSU rDNA by *Acc*II. Samples in each lane are as follows: Lane 1, 1 kb DNA ladder, Lane 2, 100 bp ladder, Lane 3, *G. lucidum* IUM00298 mycelia, Lane 4, *G. lucidum* C-1 fruiting body, Lane 5, *C. versicolor* IUM00100 mycelia, Lane 6, *C. versicolor* C-3 fruiting body, Lane 7, *F. fomentarius* IUM00144 mycelia, Lane 8, *F. fomentarius* C-5 fruit body.

homology was found between the nucleotide sequences of the 3 different medicinal mushroom species (Table 2).

Based on the consensus sequences for these 3 mushroom species, restriction patterns of LSU rDNA were compared. Although their restriction patterns were almost the same, some differences in restriction sites for 4-base recognition enzymes, especially *Acc*II and *Hha*I, were found between the 3 mushroom species (Fig. 2A and 2B). In particular, the middle parts of nuclear LSU rDNA showed different restriction patterns for *Hha*I, and the downstream region of nuclear LSU rDNA had different restriction sites for *Acc*II.

Molecular discrimination of medicinal mushrooms by PCR-RFLP of nuclear LSU rDNA

In order to verify the results obtained from restriction analysis, PCR-RFLP analysis was performed on the amplified nuclear LSU rDNA fragments. As expected, the middle part of nuclear LSU rDNAs, which were amplified using set 2 primers (LR17R and LR9), showed species-specific differences in restriction patterns for *Hha*I, as shown in Fig. 2C. When amplified using set 3 primers (LR8R and LR12), the downstream sections of the nuclear LSU rDNAs from different mushrooms also gave different restriction fragments when digested with *Acc*II, as shown in Fig. 2D.

Discussion

The identification of natural herbs used for medicinal purposes is very important in order to obtain the desired species. For a long time, natural herbs were examined with the naked eye based on phenotypic characters, but it was impossible to distinguish between genetically related species by this method.

Recently a molecular approach has been introduced to detect the biological source or origin of natural herbs. This procedure is convenient, rapid, accurate and requires only a small amount of sample. Furthermore this method can be employed even with samples in the form of powders or extracts in mixed drug formulas. However, this procedure does not directly reflect the pharmacological activity of the specimen in question.

The identification procedure of medicinal mushrooms is also required for quality control of functional health-aid preparations as well as nutritional supplements. In our study, PCR-RFLP analysis of nuclear rDNA was attempted to identify the biological identity of these medicinal mushrooms widely used in Korea: *G. lucidum*, *C. versicolor,* and *F. fomentarius*.

At first, the molecular identification of these medicinal mushrooms was based on nuclear SSU rDNA sequences. However, no useful restriction site to distinguish between these three mushrooms was found in SSU rDNA sequences. In fact, the sequences of 3 different medicinal mushroom species were over 98% homologous (data not shown).

Because nuclear LSU rDNA is known to have more diversity than nuclear SSU rDNA (Hong *et al.*, 2000; Kurzman and Robnett, 1998; Wesselink *et al.*, 2002), nuclear LSU rDNA sequences were used for the identification of medicinal mushroom species. Nuclear LSU rRNA genes were amplified and their nucleotide sequences were determined and deposited in GenBank (Table 1). The BLAST search in GenBank databases suggested that 28S rDNA sequences of *Donkiporia* and *Antrodia* species are the most homologous with those ones sequenced from medicinal mushrooms.

Based on the determined nuclear LSU sequences, the possibility to distinguish between these three medicinal mushroom species was examined through restriction analysis. Among the 4-base recognition enzymes, *Acc*II and *Hha*I were found to show varying restriction patterns of LSU rDNAs between different medicinal mushroom species.

This difference in restriction patterns was confirmed by PCR-RFLP analysis of nuclear LSU rDNAs; *Hha*I-digested PCR product in the middle part and *Acc*II-digested PCR product in the downstream region of LSU rDNAs. This analysis could be employed for the identification and classification of these medicinal mushrooms commonly used in health-aid preparations or drug formulas: *G. lucidum*, *C. versicolor,* and *F. fomentarius*. Additional taxonomic information for other medicinal mushrooms could be also established based on nuclear LSU rDNA sequences.

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