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Molecular Identification of Asian Isolates of Medicinal Mushroom *Hericium erinaceum* by Phylogenetic Analysis of Nuclear ITS rDNA

PARK, HYUK-GU, HAN-GYU KO, SEONG-HWAN KIM[1], AND WON-MOK PARK*

School of Life Sciences and Biotechnology, Korea University, Seoul 136-701, Korea
[1]Department of Microbiology and Institute of Basic Science, Dankook University, Cheonan, Chungnam 330-714, Korea

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Abstract A reliable molecular phylogenetic method to identify *Hericium erinaceum*, the most industrially valuable species in the *Hericium* genus, was established. Sequencing and phylogenetic analyses of the PCR-amplified ITS and 5.8S rDNA from *Hericium* fungi, including 6 species and 23 isolates, showed that variation in nucleotide sequences and size exists in both ITS1 and ITS2 regions, but not in the 5.8S region. These two ITS regions provided different levels of information on the relationship of *H. erinaceum* to other *Hericium* species. Based on the ITS1 sequence, both the parsimony and neighbor joining trees clearly distinguished Asian *H. erinaceum* isolates from other *Hericium* species and isolates. The intraspecific divergence of the ITS2 region was suitable to dissect the Asian *H. erinaceum* isolates into a few groups.

Key words: *Hericium erinaceum*, medicinal mushroom, nuclear ribosomal DNA, ITS1-5.8S-ITS2, intraspecific variation, phylogenetic identification

Hericium are medium-sized to large, white, fleshy, edible basidiomycete fungi. These fungi grow mostly on dead or dying wood, but can be parasitic on some trees [1]. *Hericium* fungi are distributed throughout North America, Europe, and Asia, including China, Japan, Korea, and Malaysia. The species belonging to the *Hericium* genus are economically important, since they are valuable resources for agricultural and medicinal applications. In particular, *H. erinaceum*, commonly called "yamabushitake or lion's mane" is notable for its use in the treatment of diverse diseases such as gastritis, gastric ulcer, and tumors [8]. This mushroom has also been known to contain diverse pharmaceutically important compounds such as novel phenols and fatty acids that have possible chemotherapeutic effect on cancer and ameliorative effect in Alzheimer's dementia cases [10, 11]. Its medicinal application can be extended by the production of polysaccharides and protein complex that have hypolipidemic effect and biological response modifier characteristics [17, 19]. With increasing attention to the values of *H. erinaceum*, mushroom growers and industrial cultivators need to breed and improve *H. erinaceum* strains that can grow fast, produce good fruit body yields for food consumption, and generate pharmaceutically beneficial compounds. To accomplish their need, it is imperative to establish clear taxonomic and genetic relationships of *Hericium* fungi.

Mushroom growers and industrial cultivators of *H. erinaceum* have continuously been searching for new *Hericium* sources for strain improvements. These efforts have been done mainly by collecting field mushrooms at different geographic locations, especially in Southeast Asia regions, including China, Japan, Korea, etc. Morphology and chemical components of fruit body have been used for *Hericium* identification, however, these classical approaches are time-consuming and the results are often not conclusive. The scarcity of information on the genetic relationship between *H. erinaceum* from diverse origins also frequently generates confusion on the identification of the species.

The internal transcribed spacer (ITS) region of the nuclear rDNA cistron is one of the more frequently used regions for phylogenetic analyses at the genus and species levels [2, 7]. This ITS region conventionally includes entire ITS1, 5.8S gene, and ITS2 portion of the nuclear rDNA cistron. In fungi, the ITS region has commonly been used to study phylogenetic divergences, taxonomy, and species identification [4, 12, 13]. To facilitate *Hericium* identification, Lu *et al.* [9] designed PCR primers specific for the *Hericium* genus using the ITS sequences. Since the PCR probes can be used only at the genus level, the

*Corresponding author
Phone: 82-2-3290-3412; Fax: 82-2-3290-4374;
E-mail: wmpark@korea.ac.kr

method or criterion for identifying the collected isolates of *H. erinaceum* and for defining their genetic relationships are not yet currently available.

This study aimed to establish genetic criteria that help identification of *H. erinaceum*, the most industrially valuable species in Asia. We assessed the usefulness of each region of ITS1, ITS2, and 5.8S rDNA as a target site for molecular identification. Comparative analysis of the rDNA regions against all known *Hericium* species with extended numbers of *H. erinaceum* isolates from different geographic origins was performed, and the result on the exploration of sequence divergences and phylogenetic identification of *H. erinaceum* is described.

MATERIALS AND METHODS

Fungal Cultures

Sixteen isolates presumed to be *Hericium erinaceum* and six other *Hericium* species were included in this study. These isolates and species were selected to reflect the geographic diversity of *H. erinaceum* and potential taxonomic groups within it. Details of their collection and sources are provided in Table 1. For genomic DNA preparation, all fungal cultures used in this study were grown on 2% Oxoid malt extract agar (MEA) plates overlaid with sterile cellophane sheet and incubated for 5 to 7 days at 25°C in the dark.

Genomic DNA Preparation and PCR

Mycelia (about 250 mg) of each isolate grown on cellophane-layered MEA were harvested by scrapping with a scalpel, and genomic DNA was extracted using a specially designed drill bit according to the method described [5]. The extracted DNA was quantified with a GeneQuant II spectrophotometer (Amersham Pharmacia), and its final concentration was adjusted to 100 ng/μl. PCR amplification was carried out using ITS1 and ITS4 primers to amplify the ITS1, 5.8S, and ITS2 rDNA regions [18]. The PCR reaction mixture (a total volume of 50 μl) contained 200 ng of fungal genomic DNA, 40 pmol each of primer, 50 μM (each) of four deoxynucleotide triphosphates (dNTPs), 1×PCR buffer [10 mM Tris-Cl (pH 8.0), 1.5 mM $MgCl_2$, 50 mM KCl], and 1 unit of Thermostable DNA polymerase (Rose Scientific Co.). Amplification was done in a Hybaid Touch Down thermal cycler, and PCR conditions were programmed as follows: one cycle of denaturation at 94°C for 10 min, followed by 30 cycles of denaturation at 94°C for 50 s, annealing at 52°C for 50 s, and extension at 72°C for 50 s, and one final cycle of extension at 72°C for 10 min.

Table 1. *Hericium* species and isolates used in this study.

Fungal species	Culture code	Geographic origin	Size (bp) of ITS1/ITS2	GenBank accession no.
H. abietis	CBS 243.48	Canada	180/205	AY534579
H. alpestre	CBS 539.90	Austria	177/211	AY534580
H. americanum	CBS 493.63	U.S.A	180/204	AY534581
H. coralloides	IFO 7716	U.S.A	180/204	AY534582
H. erinaceus	CBS 485.95	U.S.A	180/204	AY534583
	KUMC e1	U.S.A	180/204	AY534596
H. laciniatum	ATCC 52480	Canada	189/215	AY534584
H. erinaceum	NFCF F01	Malaysia	180/204	AY534585
	NIAST 48001	Kwanreung, Korea	180/204	AY534586
	NIAST 48002	Kwanreung, Korea	180/204	AY534587
	NIAST 48006	Hongcheon, Korea	180/204	AY534588
	KUMC 1001	Pyeongchang, Korea	180/204	AY534589
	KUMC 1002	Pyeongchang, Korea	180/204	AY534590
	KUMC 1003	Pyeongchang, Korea	180/204	AY534591
	KUMC 1007	Pyeongchang, Korea	180/204	AY534592
	KUMC 1008	Pyeongchang, Korea	180/204	AY534593
	KUMC 1009	Pyeongchang, Korea	180/204	AY534594
	KUMC 1017	Cheonan, Korea	180/204	AY534595
	KUMC Y-2	Japan	180/204	AY534597
	KUMC e2	Saidama, Japan	180/204	AY534598
	KUMC e3	China	180/204	AY534599
	KUMC 1022	Yeongil, China	180/204	AY534600
	KUMC 1023	Yangpyong, Korea	180/204	AY534601

CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands. IFO: Institute for Fermentaion, Osaka, Japan. ATCC: American Type Culture Collection, USA. KUMC: Korea University Mycology Collection. NFCF: National Forestry Cooperatives Federation, Korea. NIAST: National Institute of Agricultural Science and Technology, Korea.

Cloning and Sequencing

PCR amplicons were separated on a 1% agarose gel, stained with ethidium bromide, and visualized under UV illumination. After identification, PCR amplicons were cut out and purified with the Qiaquick Gel Extraction kit (Qiagen). The gel-purified PCR products were cloned into the pCR®2.1-TOPO vector, using the TOPO™ TA cloning kit (Invitrogen), and transformed into One-shot competent *Escherichia coli* cells according to the manufacturer's protocols. Both strands of the PCR-amplified DNA fragments in the vector were sequenced, using M13R, F sequencing primers, and the PRISM Ready Reaction DyeDeoxy termination cycle sequencing kit. DNA sequences were determined in an Applied Biosystems ABI 373 DNA sequencer.

Sequence Alignment and Phylogenetic Analyses

The determined sequences of PCR amplicons were confirmed as fungal ITS rDNA through homology search of the GenBank nucleotide sequence database. The GenBank accession numbers of the ITS nucleotide sequences of all the *Hericium* isolates generated in this study are listed in Table 1. The nucleotide sequences were aligned with the multiple sequence alignment program CLUSTAL W [16] and rearranged manually. Regions of the sequences that could not be aligned with certainty were excluded from further analysis. Sequence identity was calculated by pairwise comparison. Phylogenetic analysis was performed using PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b10 [14]. Alignment gaps were treated as missing data. Heuristic searches, using maximum parsimony with 10 random addition sequence replicates, Tree Bisection and Reconstruction (TBR) branch swapping, MULPARS, and steepest descent options, were conducted. Distance analysis employed the F84 neighbor-joining method. Trees were rooted using *Hericium abietis* as the outgroup for both ITS1 and ITS2 analyses. Bootstrap analyses were performed with 1,000 replicates to evaluate confidence intervals at the branch points in the phylogenetic tree.

RESULTS AND DISCUSSION

PCR Amplification, Sequencing, and Sequence Analysis

The PCR amplification with ITS1 and ITS4 primers produced a single band of about 560 bp from all the *Hericium* isolates listed at Table 1. Sequencing and GenBank comparison of the PCR-amplified products showed that they are fungal rDNAs containing full length of ITS1, 5.8S, and ITS2. The real size of PCR amplicons ranged from 542 to 562 bp, indicating that size variation in the rDNA unit exists among *Hericium* species (Table 1). *H. laciniatum* had the longest ITS size, and all *H. erinaceum* isolates have the same size (542 bp). As for the ITS1 sequences, *H. laciniatum* was 189 bp, *H. alpestre* 177 bp, and other *Hericium* species 180 bp. All *Hericium* species had the same size of 5.8S (158 bp). Regarding the ITS2 sequence, its size was longer than that of ITS1 in all *Hericium* species, and ranged from 204 to 215 bp. The longest size of ITS2 was also found in *H. laciniatum*, and all *H. erinaceum* isolates also had the same 204 bp size of ITS2.

The results of pairwise comparison of *Hericium* ITS1 and ITS2 nucleotide sequences are depicted in Table 2. Nucleotide sequence identity levels among *Hericium* species were 89-99% in ITS1 and 78-99% in ITS2, showing that the ITS1 region is more conserved than ITS2 in *Hericium*. *H. erinaceum* isolates showed 88-99% identity in ITS1 and 76-99% in ITS2 with other *Hericium* species. The sequence identity among *H. erinaceum* isolates from Asian origin was 100% and 99% in ITS1 and ITS2, respectively, indicating that rDNA is quite well conserved in this species. The 5.8S region of 158 bp was very well conserved (99- 100%) among *Hericium* species (data not shown). Only one nucleotide variation was found in *H. alpestre*; This species had cytosine at position 145 of 158 bp 5.8S, replacing thymine that was contained in all other *Hericium* 5.8S.

Phylogeny of *Hericium* spp.

Due to its high sequence conservation, the 5.8S was excluded from phylogenetic analysis for *Hericium*, and the

Table 2. Nucleotide sequence identity of ITS1 and ITS2 between *Hericium* species.

ITS1/ITS2	*H. alpestre*	*H. americanum*	*H. coralloides*	*H. erinaceus*	*H. laciniatum*	*H. erinaceum*			
						China	Japan	Korea	Malaysia
H. abietis	88/77	98/97	98/97	98/97	95/94	97/98	97/97	97/97	97/97
H. alpestre		89/78	89/78	89/78	91/76	89/79	89/79	89/78	89/78
H. americanum			100/99	99/99	96/96	99/99	99/98	99/98	99/98
H. coralloides				99/98	96/95	99/98	99/98	99/98	99/98
H. erinaceus					95/96	98/99	98/98	98/98	98/98
H. laciniatum						96/97	96/96	96/96	96/96
H. erinaceum China							100/99	100/99	100/99
H. erinaceum Japan								100/99	100/99
H. erinaceum Korea									100/99

ITS1 and ITS2 sequences were used for the analysis. A heuristic maximum parsimony (MP) analysis of the ITS1 rDNA data, using 10 random stepwise addition sequences, yielded 4 equally parsimonious trees of 32 steps (consistency index [CI]=0.806, retention index [RI]=0.727, rescaled consistency index [RC]=0.659). A strict consensus tree rooted with the sequence of *H. alpestre* is shown in Fig. 1A. Within the *Hericium* genus, the separation of the *H. erinaceum* clade was clearly resolved with Asian isolates. No further separation was detected among the Asian *H. erinaceum* isolates. It is interesting to find that *H. erinaceus* is not grouped with *H. erinaceum*, but grouped with *H. abietis*. *H. erinaceus* has often been called *H. erinaceum*, because these two species have a common name, called "lion's mane." Thus, it would be possible that *H. erinaceum* has been misused as *H. erinaceus* or *vice versa*. So far, there has been no comparative study that clarifies the taxonomic relation between *H. erinaceus* and *H. erinaceum*. Our ITS1-based results on their relationship suggested that they are different species. At this point, it is hard to confirm the taxonomic status of the two species without fruit body. Thus, further works are necessary to clear its taxomonic position in the *Hericium* genus. A neighbor-joining tree, using ITS1 data (Fig. 1B), was also produced to compare with the phylogenetic tree results of Fig. 1A. This distance criterion-based analysis produced a tree topology similar to that of a character-based parsimony tree, supporting the separation of Asian *H. erinaceum* isolates from other *Hericium* species. Both trees also supported earlier observation that *H. coralloides* and *H. alpestre* are incompatible on mating test [3].

ITS2-based analysis has been successfully applied to the phylogenetic study of inky and ectomycorrhizal mushrooms [6, 13]. However, the medicinal mushroom *Hericium* showed no correlation between ITS2 sequence variation and morphology, because, compared to the ITS1 analysis (Figs. 1A and 1B), phylogenetic analyses of ITS2 sequences (Fig. 2) provided a different relationship between *H. erinaceum* and other *Hericium* species. Although we tried both the same search options as used in the ITS1 analysis and other options, many parsimony trees were constantly produced, and there was no relevant tree that could resolve the phylogeny of *Hericium* (data not shown). Thus, a distance-based neighbor-joining analysis was done, and a single tree was obtained using *H. alpestre* as the outgroup (Fig. 2). Similar to the ITS1 region analysis, *H. erinaceus*, *H. erinaceum* e1, *H. abietis*, *H. americanum*, and *H. coralloides* also formed their own distinct group with 53% bootstrap support. The outgroup *H. alpestre* formed its own lineage. However, some Asian isolates of *H. erinaceum* formed a separate lineage with 80% bootstrap support, and other Asian *erinaceum* isolates were split into

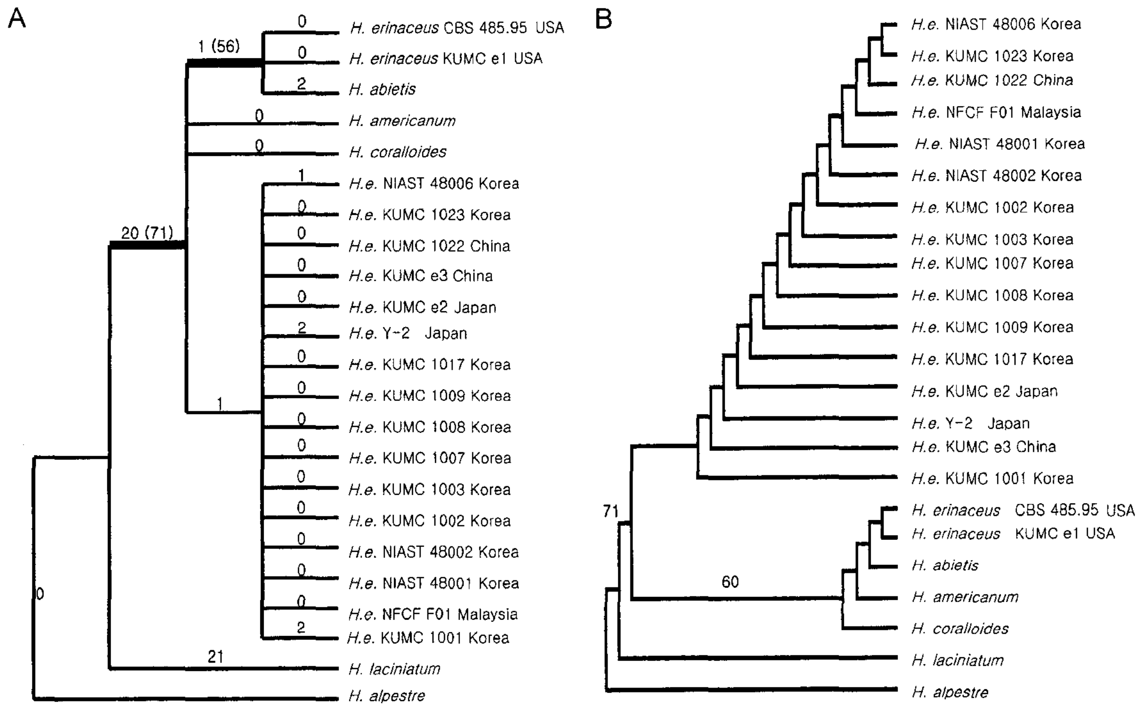

Fig. 1. Phylogenetic analyses of *Hericium* species, based on the ITS1 sequence data.
A: One of the four most equally parsimonious trees. Plain figures indicate branch lengths and figures in brackets show the bootstrap support value >50%, calculated from 1,000 replications. Branches with 100% support in a consensus tree are indicated in bold. B: Neighbor-joining tree. Numbers above nodes are bootstrap intervals >50%, calculated from 1,000 replications. *H. alpestre* was used as an outgroup taxon in both trees.

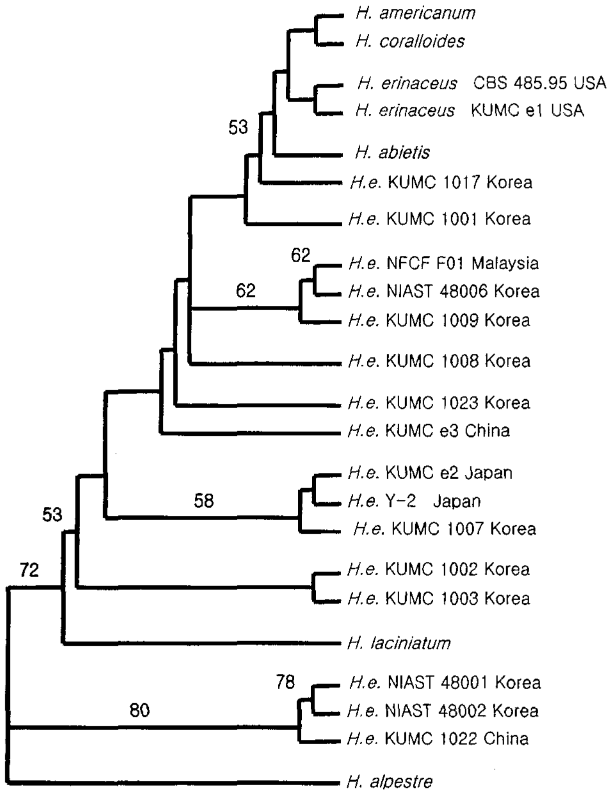

Fig. 2. Phylogenetic tree recovered from the neighbor-joining analysis of *Hericium* ITS2 data.
Numbers above nodes are bootstrap intervals. *H. alpestre* was used as an outgroup taxon.

a few subclades that contained different geographic origins and other species. This result indicates that the ITS2 sequence is quite diverged in *Hericium*, but the divergence is not congruent with morphology-based species differentiation. Thus, the ITS2-based phylogenetic tree is not suitable to separate each *Hericium* species.

Lu *et al.* [9] used combined sequences of ITS1, ITS2, and 5.8S regions for the phylogenetic analysis of homobasidiomycetes, including a few species of *Hericium*. They resolved *Hericiaceae* from other homobasidiomycete families. However, the separation tree node had only 30% bootstrap support. In addition, this low confidence level (<50%) was present in tree nodes that formed five other different clades of family separation. Based on the results of the current work that difference existing between the ITS1 and ITS2 trees prevented their combinative analysis, we wonder whether the combined sequence of ITS regions is suitable for the phylogeny of homobasidiomycetes. For solid conclusion on the relationships of *Hericiaceae* to other fungal families, our work suggests that independent analysis with ITS1 or ITS2 should be done, and homoplasy analysis should be performed to check if the ITS1- and ITS2-based tree produces similar tree topology with enough statistical support [15].

In conclusion, this study identified that the ITS1 region is a useful site for distinguishing Asian *H. erinaceum* isolates (which are a major source for medicinal *Hericium* supply) from other *Hericium* species. The ITS1-based phylogenetic method will surely provide a genetic clue to solve frequently encountered species uncertainties in Korea and other Asian countries. In addition, this method should help identify field-isolated *Hericium* that could be used for breeding *H. erinaceum* strains with good commercial values as medicinal and edible mushroom. Based on the results of the present work, we are currently in a process to develop an ITS1-derived quick and easy genotyping method to identify *H. erinaceum* and to speed up screening of many field-isolated *Hericium* for commercial application.

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