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Secondary Structure and Phylogenetic Implications of ITS2 in the Genus *Tricholoma*

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Abstract The internal transcribed spacer (ITS) region in the genus *Tricholoma* was analyzed, including for its primary nucleotide sequence and secondary structural characterization. The secondary structures of the ITS2 region in the genus *Tricholoma* were identified for use in bioinformatic processes to study molecular evolution and compare secondary structures. Ten newly sequenced ITS regions were added to the analysis and submitted to the GenBank database. The resulting structure from a minimum energy algorithm indicated the four-domain model, as previously suggested by others. The conserved secondary structure of the ITS2 sequences of the genus *Tricholoma* exhibited certain unique features, including pyrimidine tracts in the loops of domain A and a complete structure containing four domains, with motifs identified in other ITS2 secondary structures. A phylogenetic tree was derived from sequence alignment based on the secondary structures. From the resulting maximum parsimonious tree, it was found that the species in the genus *Tricholoma* had evolved monophyletically and were composed of four groups, as supported by the bootstrapping values and pileus color.

Key words: Internal transcribed spacer 2 (ITS2), genus *Tricholoma*, secondary structure, phylogenetics

Ribosomal DNA (rDNA) sequence data have been used to study the relationship between various taxa and to evaluate the classification of organisms within the ordinary structure of phylogenetic criteria [2, 5, 14]. Eukaryotic nuclear rDNA is tandemly repeated with units that consist of a nuclear small subunit (SSU), large subunit (LSU), and 5.8S rDNAs. The SSU and LSU are separated by internal transcribed spacers (ITS) and the 5.8S rDNA are located between these internal transcribed spacer regions (ITS1: between SSU - 5.8S rDNA spacer; ITS2: between 5.8S - LSU rDNA spacer). The spacer region seems to have a faster evolution rate than the rDNA coding region, because the lethal effect of mutations is smaller in the spacer region. Previously, SSU ribosomal RNA genes were introduced to molecular evolution research, because they contain highly conserved structures [13]. As such, it is possible to conduct long distance evolution analyses spanning genus to genus, family to family, and order to order. However, at the genus and species levels, the SSU ribosomal sequence character is not well adapted to other systematic classifications. Under these circumstances, many researchers have tried to apply the ITS sequences of ribosomal DNA from various sources to molecular systematics [3, 11].

Several researchers have found that the secondary structures of ITS are conserved across species and genera, although their primary structure is poorly conserved. van Nues *et al.* [27] found that the structural integrity of yeast ITS2 spacers is important for its processing and for the formation of pre-rRNA. The secondary structure predictions of ITS spacers have been studied using several techniques, including electron micrographs [4], chemical and enzymatic probing [29], and site directed mutagenesis [26, 27]. However, the most general method for predicting secondary structures is minimum energy modeling that relies on algorithms of the minimum free energy [30]. Despite poor primary sequence conservation and a much altered folding appearance, secondary structures including conserved motifs have been extensively studied in *Drosophila* [21], trematodes [6, 18, 19, 20], green algae, and flowering plants [3, 15]. Most previous reports have stated that the ITS2 region has four conserved domains (stem and loop structure) and is applicable to phylogenetic study. However, in fungal species, the secondary structures of ITS regions have still not been evaluated.

The current study proposes that the ectomycorrhizal mushroom has a certain phylogenetic constraint because of its dependent growth on a specific host. Accordingly, it is supposed that its mutational variation is more limited or

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peculiar than other nonectomycorrhizal basidiomycetes. Certain species in the genus *Tricholoma* have already been characterized as obligately ectomycorrhizal fungi [1]. One such species, *T. matsutake*, is known as the most delicious species in Asia and is involved in a variety of industrial applications. This mycorrhiza can form edible fruitbodies with an associated host. As such, it would be interesting to identify secondary structural information on ITS in species of the genus *Tricholoma*. In addition, many sequences of ITS regions in *Tricholoma* species have already been accumulated. Moreover, while analyzing the secondary structural modeling of the ITS2 in basidiomycetes, it was expected that the widespread ectomycorrhizal fungus *Tricholoma* has a typical structure and it can be applied to phylogenetic analysis in ordinary classifications.

MATERIALS AND METHODS

DNA Extraction and Manipulation

Chromosomal DNA was extracted according to the method used by Kim *et al.* [12] with certain modifications. The mycelia of the collected species were cultured in a PDA (potato dextrose agar, pH 5.6) medium. The mycelia were scraped and deposited in a microtube using a laboratory glass rod, then they were immediately frozen at −80°C, lyophilized, and crushed with micro tips. Two hundred μl of a lysis buffer was added, followed by SDS to a final concentration of 1% (w/v), then the mixture was incubated for one hour at 65°C. Centrifugation was carried out at 10,000 ×g for 10 min and the supernatant was removed. Next, NaCl was added to a final concentration of 0.7 M followed by the addition of one-tenth volume of 10% CTAB buffer (10% cetyl-trimethyl ammonium bromide, 100 mM EDTA, 50 mM Tris-HCl, 0.7 M NaCl, pH 8.0). After incubating for 10 min at 65°C, chloroform: isoamyl alcohol (24:1, v/v) extraction was repeated twice. Nucleic acid was concentrated by precipitation in two volumes of absolute ethanol and dissolved in 0.1× TE buffer (0.1 mM EDTA, 1 mM Tris-HCl, pH 8.0). RNase A was added to remove the RNA, and about 200 ng of nucleic acids were applied for the PCR reaction.

ITS Region Amplification and Sequencing

The ITS regions (ITS1, 5.8S, ITS2) were amplified with one set of PCR primers (#1 and #2). The SSU and LSU rDNA terminal sequence of *T. matsutkake* was used for the primer preparation [7, 8, 9]. The #1 primer was 5'-CAAG-GTTTCCGTAGGTGA-3' (positions 1769 to 1786), located at the 3'-terminal of the small subunit ribosomal RNA gene. The #2 primer was 5'-GTTAGTTTCTTTTCCTC-CGC-3' (positions 53 to 72), located at the 5'-terminal of the large subunit ribosomal RNA gene. The PCR reaction was performed for 32 cycles by denaturation at 95°C for 30 seconds, annealing at 55°C for 1 min, and polymerization at 72°C for 30 seconds, followed by a 10 min extension period. The PCR mixture was analyzed in 0.8% agarose gel by electrophoresis. The resulting DNA bands were excised and purified with GENE CLEAN II kit (BIO101 Co.) following the manufacturer's directions. The pGEM-T easy vector (Promega Co.) was used to clone the amplified DNA fragments and transformed into *Escherichia coli* strain DH5α. The selected *E. coli* containing the recombinant plasmid was cultivated and purified for the following sequencing reaction. An automated sequencing facility, ABI100 model 377 Perkin-Elmer, was used to analyze the nucleotide sequences. The resulting nucleotide sequences were submitted to the GenBank (Table 1), however, the AF241517 was found to be a false sequence and not used in the current study. The previously sequenced ITS2 region of *T. matsutake* (GenBank U62964) [9] and 24 retrieved sequences of ITS2 sequences were also used to obtain the phylogenetic relationships in the genus *Tricholoma*. The retrieved sequences were *T. caligatum* (GenBank AF309533), *T. ustale* (DDBJ AB036894), *T. imbricatum* (GenBank AF062608), *T. argyraceum* (GenBank AF062612), *T. saponaceum* (DDBJ AB036897), *T. ponderosum* (GenBank AF204811), *T. batschii* (GenBank AF062627), *T. nictitans* (GenBank AF062611), *T. equestre* (EMBL AJ236081), *T. japonicum* (DDBJ AB036900), *T. magnivelare* (DDBJ AB036893), *T. vaccinum* (GenBank AF062628), *T. terreum* (GenBank AF062618), *T. fulvocastaneum* (DDBJ AB036901), *T. flavovirens* (DDBJ AB036895), *T. populinum* (EMBL AJ272072), *T. scalpturatum* (GenBank AF062624), *T. orirubens* (GenBank AF062610), *T. portentosum* (DDBJ AB036896), *Collybia tuberosa* (GenBank AF065124), *C. cirrhata* (GenBank AF274382), and *C. cookei* (GenBank AF065123).

Table 1. The collected strains from CBS and IFO cell cluture collections. The sequences of ITS2 were deposited in GenBank.

Strain	Culture collection	Genbank accession No.
Tricholoma lascivum	CBS100136	AF241513
Tricholoma unifactum	CBS296.64	AF241514
Tricholoma bakamatsutake	IFO30663	AF241515
Tricholoma album	CBS360.47	AF241516
Tricholoma sejunctum	CBS368.47	AF241518
Tricholoma squarrulosum	CBS371.47	AF241519
Tricholoma albo-brunneum	CBS572.96	AF241520
Tricholoma focal	CBS575.96	AF241521
Lepista personata	IFO7717	AF241522
Lepista sordida	IFO31013	AF241523

Secondary Structure and Phylogenetic Analysis

Most ITS2 nucleotide sequences were folded according to the RNA structure program, the parameters described by Mathews *et al.* [17]. The temperature dependent folding

was produced by an Internet MFOLD server. MFOLD is a RNA secondary structure calculation program with a minimum energy folding algorithm [30]. In the current study, every structure presented was calculated at 25°C. The predicted structures were viewed by the RnaViz program and imported to a graphic format.

The collected and retrieved sequences were treated in the multiple sequence alignment program, CLUSTALW [25], with options of gap opening penalty 5, and gap extension penalty 0.1, and rearranged manually with a focus on the secondary structures. The most parsimonious algorithm with a heuristic search option was adopted to obtain an optimum tree using the PAUP* program [23]. In addition, 100 bootstrapping was introduced to obtain confidence values. In order to evaluate the parsimony tree, a maximum likelihood tree with quartet puzzling was constructed.

RESULTS AND DISCUSSION

The average length of the ITS2 in the *Tricholoma* species was about 202 bases, with a range of 182 to 218 bases. The average GC content was about 43%. From the profile of the sequence alignment, it appeared that two-thirds of the region of the ITS2 sequences were more homologous than downstream of this conserved region. The identity of the sequences in the 5' two-third region had a mean of 61%, which dropped to 40% for the remaining region.

With the minimum-energy folding, despite a sequence variability in the 3' regions, the resulting secondary structures had similar folding structures. The ITS2 region sequences of 26 species were used to fold and predict a standard structure, which covered almost all the *Tricholoma* species previously published in the sequence databank. In a few cases, such as *T. vaccinum*, no optimum folding structure was found. Representative examples of the secondary structures are shown in Fig. 1(A). The conserved secondary structure of the ITS2 sequences in the *Tricholoma* species is shown in Fig. 1(B), which is hypothetical based on the conserved sequences. These sequences were elicited from the sequence-aligned profile based on each secondary structure. When the column sequence identity value was higher than 50%, the consensus sequence was employed to calculate the structure, therefore, it reflected the features of the major conserved sequences and mutational covariations to some extent. However, the hypothetical structure did not reflect certain loop regions that were less conserved and span sequences between domain to domain. The secondary structure of the *Tricholoma* ITS2 sequence had four domains (typical stem and loop structure in ITS2 region) as represented. Domain B had a typical loop consisting of pyrimidine tracts, as previously reported in the Volvocales cases. In addition, the genus *Tricholoma* had other pyrimidine tracts in domain A (Fig. 2). This was not the case in the Volvocales and Trematoda species [15, 20]. The only exception was *T. magnivelare*, which had one adenine nucleotide in the pyrimidine loop of domain A. Domain C, the largest stem and loop structure, consisted of more variable sequences, which resulted in a somewhat complicated stem and loop structure. Nonetheless, the apex of domain C was conserved. It has been previously reported that flowering plants have a UGGU motif that occurs in the end stems of domain C [15]. It also appeared that the genus *Tricholoma* had significant conservations at the tip of domain C in all species [Fig. 1(B)] (Table 2). In the sequence alignment and secondary structural folding or subfolding, the position of the conserved apex sequence subsisted in domain C of the secondary structure.

The phylogenetic tree of the *Tricholoma* species was determined on the basis of the secondary structures. Some of the species that failed to produce optimum structures were added to the pre-aligned sequences. Frequently, the primary sequence variation of the ITS region was too high to estimate the phylogenetic position of any species or taxa [19, 24, 28]. It was useful in differentiating between strains, however, the direct adaptation to a complicated phylogenetic estimation including higher taxomomic levels did not produce consistent results [10]. Therefore, the secondary structure of the *Tricholoma* species was determined and realigned following the conserved domain structures. The determined structure of the phylogenetic tree is shown in Fig. 3. The genus *Lepista* and *Collybia* were selected as outgroup taxa for the purpose of precise tree resolution. Accordingly, it would appear that the members of the genus *Tricholoma* were phylogenetically monophyly, supported by a high bootstrap value. In the tree, the *Tricholoma* species were grouped into 4 clusters (Fig. 3a-d), supported by a bootstrap percentage and maximum likelihood analysis.

In group a, the type species, such as *T. flavovirens*, *T. equestre*, and *T. portentosum*, formed a cluster, and *T. flavovirens* and *T. equestre* reflected yellow pileus. In group b, *T. terreum*, *T. argyraceum*, *T. scalpturatum*, and *T. squarrulosum* were characterized as gray pileus. However, *T. album* and *T. lascivum* exhibited a whitish cream pileus. In addition, *T. album*, *T. lascivum*, and *T. saponaceum* were characterized as a clamp connection present species, yet they were not differentiated from the species of clamp connection not present [22]. In the tree, *T. unifactum*, *T. sejunctum*, *T. japonicum*, *T. vaccinum*, and *T. imbricatum* were found not to be related to any groups. Nonetheless, *T. vaccinum* and *T. imbricatum* appeared to have some relationship, because they were originally characterized as squamulose and brown pileus. In addition, the maximum likelihood analysis using quartet puzzling indicated a 75% bootstrapping value between them and the other species. An interesting feature was found in group c, in which

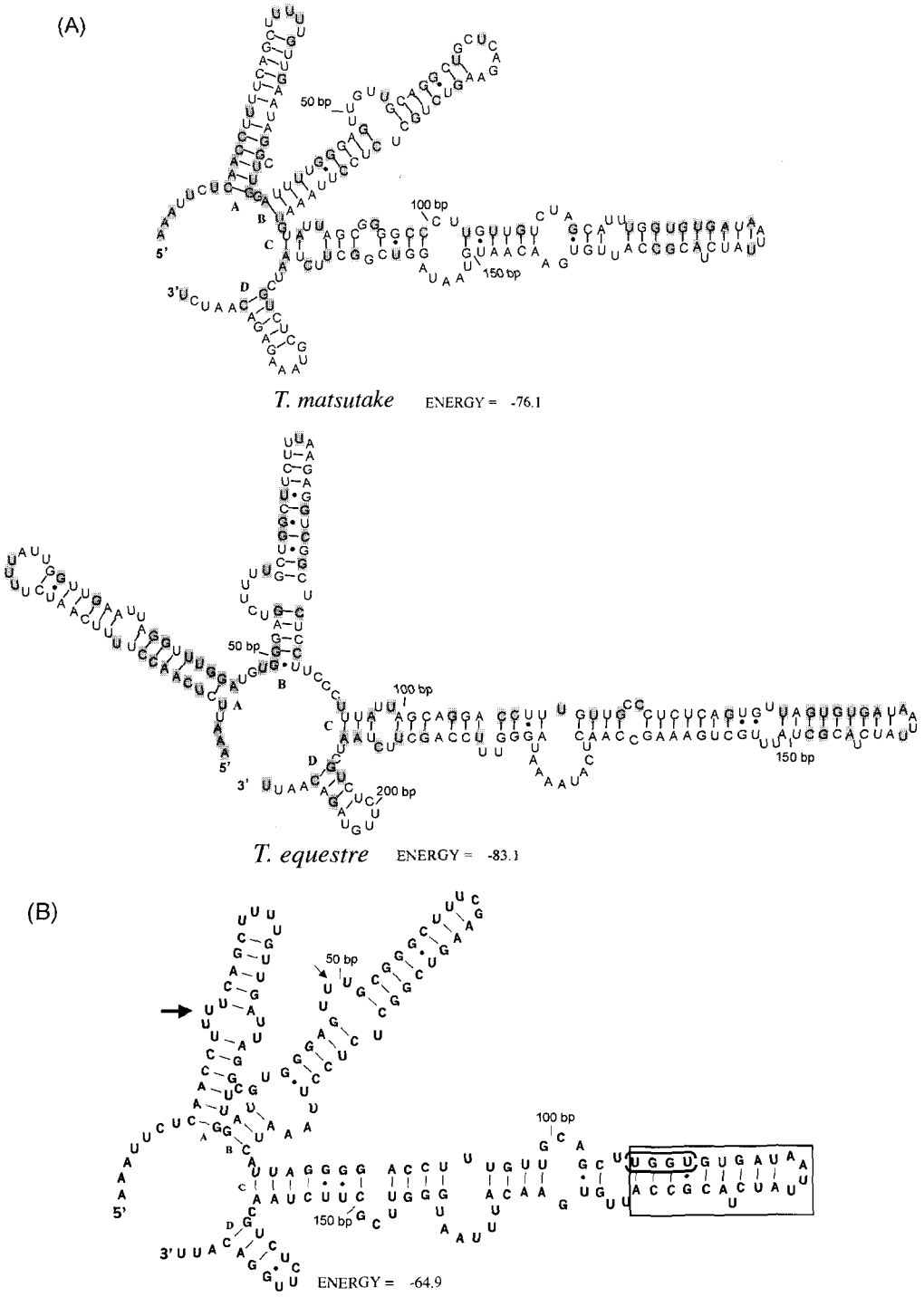

Fig. 1. Suggested ITS2 secondary structures of primary transcript rRNA in *Tricholoma* species.
The minimum energy folding methods were used to calculate the structures. A: Representative structure of *T. matsutake* and *T. equestre* species. B: Conserved structure from consensus alignment of ITS2 primary sequences. 171 base pair sequences with more than 50% column identity of aligned sequences were introduced to a standard structure prediction model. In the resulting structure, some single-strand regions were deleted. Each 50 bp span is indicated as a number bp –. The G, U pairing is marked as ●; the A, T and G, C pairing was marked as –. The four domains are represented as A, B, C, and D in the inner structure. In domain A, the large arrow indicates the pyrimidine tracts that are conserved in the genus *Tricholoma*. In domain B, the small arrow indicates the pyrimidine tracts that are also conserved in green algae and in the flowering plant. In domain C, the sequences surrounded by the upper and lower brackets, UGGU, are the motif sequences in the flowering plant. The nucleotides in the gray background indicate 100% conserved sequences in the 26 analyzed species. In the box, the apex structure with sequences was highly conserved over the entire set of *Tricholoma* species.

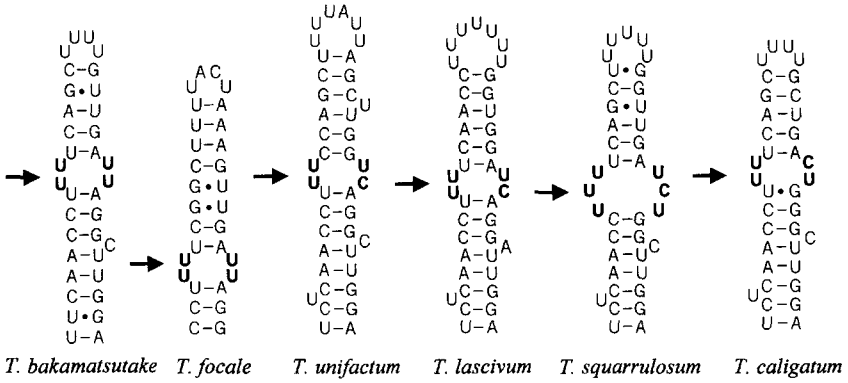

Fig. 2. Representative domain A structure from several *Tricholoma* species.
T. bakamatsutake, *T. focale*, *T. unifactum*, *T. lascivum*, and *T. squarrulosum* sequence information are shown in Table 1. The *T. caligatum* sequence was retrieved from the GenBank (AF309533). The arrows indicate the universally existing pyrimidine tracts in the genus *Tricholoma*.

T. matsutake (S. Ito et Imai) Singer [22] and other similar mushrooms with economic interest, such as *T. magnivelare*, *T. bakamatsutake*, and *T. caligatum*, were clustered. Although *T. matsutake*, *T. caligatum*, *T. focale*, and *T. ponderosum* were preliminarily classified based on annulate stipe and brown pileus, *T. focale* was found to belong to group d in the current study. *T. nictitance*, *T. batchii*, *T. albo-brunneum*, *T. ustale*, *T. saponaceum*, and *T. populinum* were grouped in group d, and reflected mostly brown pileus.

Theoretically, collaboration of molecular analysis and morphological characteristics should lead to accurate systematics. In addition, integrated studies of morphological and molecular characteristics in the genus *Tricholoma* are important due to its gradually enlarged database, and some researchers have even attempted reclassification [16]. In the current study, it was found that the genus *Tricholoma* exhibited a significant correlation between its ITS2 sequence variation and morphological characterization, such as gray

Table 2. The represented sequences in the conserved apex in domain C. The gap is showed by - and the dot is identical character.

T. matsutake	UGGU - GUGAUAA - UU-A - UCU-ACGCC - A
T. focal	. . . . - . . . . . - . . - . - . . . - . . . . . - .
T. populinum	. . . . - . . . . . - . . - . - . . . - . . . . . - .
T. ustale	. . . . - . . . . . - . . - . - . . . - . . . . . - .
T. saponaceum	. . . . - . . . . . - . . - . - . . . - . . . . . - .
T. albo-brunneum	. . . . - . . . . . - . . - . - . . . - . . . . . - .
T. batschii	. . . . - . . . . . - . . - . - . . . C . . . . . - .
T. equestre	. A . . - . . . . . - . . - . - . . . - . . . . U - .
T. argyraceum	. . . . - . . . . . - . . - . - . . . - . . . . . - .
T. scalpturatum	. . . . - . . . . A . . A . - - . A - . . - . . . . . - .
T. squarrulosum	. . . . - . . . . . - . . - . - . . . - . . . . . - .
T. fulvocastaneum	. . . . - . . . . . - . . - . - . . . - . . . . . - .
T. terreum	. . . . - . . . . . A . . C . - . U . - . U . . . C .
T. lascivum	. . . . - . . . . . - . . - . - . . . - . U . . . - .
T. magnivelare	. . . . - . . . . . - . . - . - . . . - . . . . . - .
T. ponderosum	. . . . - . . . . . - . . - . - . . . - . . . . . - .
T. bakamatsutake	. . . . - . . . . . G - . . - . - . . . - . . . . . - .
T. caligatum	. . . . - . . . . . - . . - . - . . . - . . . . . - .
T. unifactum	. . . . - . . . . . - . . - . - . . . - . . . . . - .
T. sejunctum	. . . . - . . . . . - . . - . - . . . - . . . . . - G
T. japonicum	. . . . - . . . . . - . . - . - . . . - . . . . . - .
T. imbricatum	. . . . - . . . . . G - . . - - - . UC - . . . . . - .
T. nictitans	. . . . G . . . . . - . . - . U . . . - . . . . . - .
T. orirubens	. . . . - . . . . . - . . - - . U . - . . . . . - .
T. vaccinum	. . . . - . . . . . G - . . - . - . . . - . . . . . - .
T. portentosum	. . . . - . . . . . - . . - . - . . . - . . . . UU .

Fig. 3. Maximum parsimonious tree based on ITS2 sequences from *Tricholoma* species.
176 characters out of a total of 341 were parsimony-informative. The CI value was 0.522 and RI value was 0.616. The gaps were treated as the fifth base [23]. Branches supported by bootstrap percentages higher than 50% are indicated as bold lines. Bootstrap percentages above 75% are indicated as double bold lines.

and brown pileus. However, the characteristic of the clamp connection did not have any influence on the phylogenetic relationship, and further study is needed.

As for ectomycorrhizal characteristic species, the genus *Tricholoma* exhibited some interesting secondary structures, as previously described. Typically, the secondary structure was well conserved with an entirely shared structural motif and indicated the four-domain model. A phylogenetic tree based on the conserved secondary structures and its application are presented in this study, however, the adaptation of higher-level taxa based on secondary structure information remains unclear and further studies are in need with expanded sequence data.

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