Phylogenetic Study of *Ganoderma applanatum* and *Schizopora paradoxa* Based on 5S rRNA Sequences

Kim, Hak Hyun and Hack Sung Jung*

*Department of Natural Sciences, and Research
Center for Molecular Microbiology, Seoul National University, Seoul 151-742, Korea*

The sequences of the cytoplasmic 5S rRNAs (EMBL accession numbers X73589 and X73890) from two polypores, *Ganoderma applanatum* and *Schizopora paradoxa*, were determined by the direct chemical method for sequencing RNA and compared to the sequences of 9 reported mushrooms. 5S rRNAs of *Ganoderma applanatum* and *Schizopora paradoxa* consisted of 118 bases and fit the secondary structure model of the 5S rRNAs of basidiomycetes proposed by Huysmans *et al*. Based on Kimura's K_{nuc} values, the closest fungus to *Ganoderma applanatum* was *Ceratobasidium cornigerum* and the one to *Schizopora paradoxa* was *Bjerkandera adusta*. When the secondary structures of 5S rRNAs of 11 mushrooms were compared, the base substitution occurred at helix regions more than at loop regions. When a phylogenetic tree was constructed using the Neighbor program of the PHYLIP package, it partially discriminated and separated the mushrooms of the Hymenomycetes by the order.

KEY WORDS □ *Ganoderma applanatum*, *Schizopora paradoxa*, 5S rRNA sequences, phylogeny

The ribosomal RNAs (rRNAs) are involved in the structure of the ribosome as essential elements in protein synthesis. Due to their conserved function in cellular organisms, they are believed to have changed very little during the history of evolution and serve as ideal substances for assessing relationships between organisms (1). The rRNAs commonly used for systematic purposes are the 5S, 16S, and 23S molecules. Among them, 5S rRNAs of 118 to 120 nucleotides are easier to handle and interprete than the other larger ones even though there is an arguement that 5S rRNAs are too small for measuring relatedness between organisms (2). Recently, advanced techniques and sophisticated equipments have been widely introduced into biological sciences, especially in the study of nucleic acids, and 5S rRNAs of animals, plants, and prokaryotes have been commonly utilized in interpretation of systematics and evolution (1). Compared to other taxa, fungal 5S rRNA data used to be very poor but, during the last 10 years, a number of new sequences have been added to the EMBL Data Library, and accumulated data of 52 species in the Basidiomycotina are now available.

Wood-rotting fungi like *Ganoderma applanatum* and *Schizopora paradoxa* belong to the Aphyllophorales under the Hymenomycetes of the Basidiomycotina. They usually grow on dead trees or rotten wood and represent a cosmopolitan group consisting of characteristic species of a great diversity. Because wood-rotting fungi digest wood components enzymatically and utilize as a nutrient source, they are pathologically important in forest ecology (4). The fruitbodies are variable and grow as resupinate, reflexed, or pileate forms, or become sessile to imbricate forms. Futhermore, morphologically simple or similar species often turn out very complex or different microscopically, which has been an interesting subject in fungal systematics (6).

There have been just a few 5S rRNA data from wood-rotting fungi of the Aphyllophorales, so two common polypores of taxonomic importance were taken for a systematic consideration. *Schizopora paradoxa* (=*Poria versiporia*) is classified in the Polyporaceae but has some intermediate characteristics between the Corticiaceae and the Polyporaceae in its hyphal system. *Ganoderma applanatum* (=*Elfvingia applananta*) has double-walled spores whose ornamented inner layer penetrates into the outer layer and constitutes a distinct group representing the Ganodermataceae. The 5S rRNA data from these two species were compared to those of reported mushrooms of the Hymenomycetes and the Gasteromycetes in the Basidiomycotina and their phylogenetic positions and relationships were discussed in this study.

MATERIALS AND METHODS

Strains and culture

Ganoderma applanatum and *Schizopora paradoxa* strains used in this study werè FP-125024-T and FP-135563 stocked on MEA and donated by Dr. K.K. Nakasone (senior botanist, Center for Forest Mycology Research, USDA Forest Products Laboratory). They were grown in a shaking culture of PD broth at 25°C for 7 days and harvested when OD$_{600}$ arrived 10 by centrifugation at 3,000×g.

Isolation of 5S rRNA

Harvested cells were ground in a mortar in an equal volume of RNA zol B (Biotecx Lab) and centrifuged at 6,000×g for 20 minutes. The supernatant was repeatedly extracted three to four times with LETS-saturated phenol, and total nucleic acids were precipitated with ethanol. These were resuspended in a loading solution I (9) and electrophoresed on a 10% polyacrylamide-8 M urea gel (10) for 18 hours and then stained with EtBr. 5S rRNA was positioned using a UV illuminator and separated by eluting from the gel in an elution buffer for 12 hours, which was followed by ethanol precipitation.

RNA sequencing

The 3'-end of 5S rRNA was labelled with [5'-^{32}P]$_p$C$_p$ (Amersham) in a mixture of HEPES buffer solution, rATP, and T$_4$ RNA ligase (Pharmacia) for 4 hours (3). After reaction, the loading solution I was added and boiled for 1 minute and then cooled in ice for several minutes to avoid the reformation of secondary structure. Labelled 5S rRNA was positioned on electrophoresed gel by exposing to X-ray film and separated by eluting from the gel and then precipitated with ethanol. According to the direct chemical method of Peattie (9), 3'-labelled precipitates were divided in 4 tubes to modify bases through base-specific guanine, adenosine, cytosine, and uridine reactions and then digested with aniline. The partial digests were deveolped on 12 % polyacrylamide gel, and the sequence was analyzed by exposing to the X-ray film for 12 hours.

Data analysis

Primary sequences were deteremined and compared with those of 9 reported mushrooms of the Hymenomycetes and the Gasteromycetes. The number of different bases between each two compared sequences of 11 basidiomycetes was evaluated by calculating the corresponding K$_{nuc}$ values. Recent EMBL Data Library and Berlin RNA Databank of Wolters and Erdmann (12) were used to make a distance matrix calculated by Kimura's two parametér method (7). A phylogenetic tree was constructed using the Neighbor program of the Felsenstein PHYLIP package with the Neighbor-joining option.
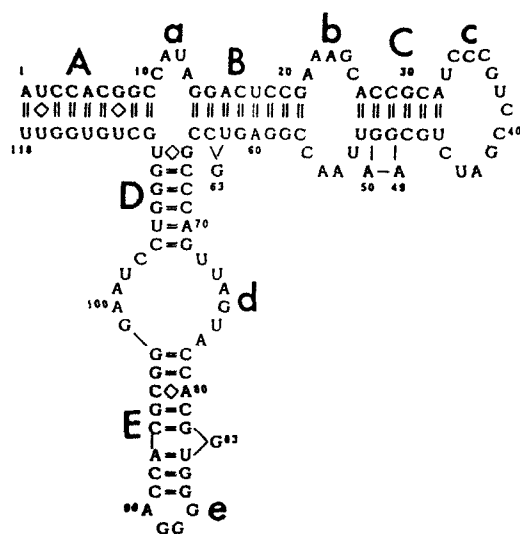

Fig. 1. *Secondary structure of Ganoderma applanatum 5S rRNA.*
The capital letters from A to E are the lettering of five helices and small ones from a to e the one of five loops. The double lines represent regular base-pairings and diamond symbols odd base-pairings. Bases of the numbers 49, 50, 63, and 83 are forming bulges on helices.

RESULTS AND DISCUSSION

The sequences (EMBL accession numbers X 73589 and X73890) of the cytoplasmic 5S rRNAs from *Ganoderma applanatum* and *Schizopora paradoxa* consisted of 118 bases each and belonged to the type B (2). They fit the secondary structure model of the 5S rRNAs of basidiomycetes, as shown in Fig. 1 for *Ganoderma applanatum*, proposed by Huysmans *et al.* (5) and belonged to the fifth sequence cluster proposed by Walker and Doolittle (11). Primary sequences of the two species and 9 known mushrooms are presented in an alignment and numbered serially in Fig. 2. Based on Kimura's K$_{nuc}$ values representing evolutionary distances in the distance matrix (Table 1), the closest fungus to *Ganoderma applanatum* of the Ganodermataceae was *Ceratobasidium cornigerum* of the Corticiaceae showing 3 nucleotide differences of 0.0259 1/2 K$_{nuc}$ value and the one to *Schizopora paradoxa* of the Polyporaceae was *Bjerkandera adusta* of the same family showing 2 nucleotide differences of 0.0171 1/2 K$_{nuc}$ value.

Considering that two organisms are believed to be of same homology when the 1/2 K$_{nuc}$ value difference is less than 0.05 (8), pairs of *Ganoderma applanatum* and *Ceratobasidium cornigerum* were
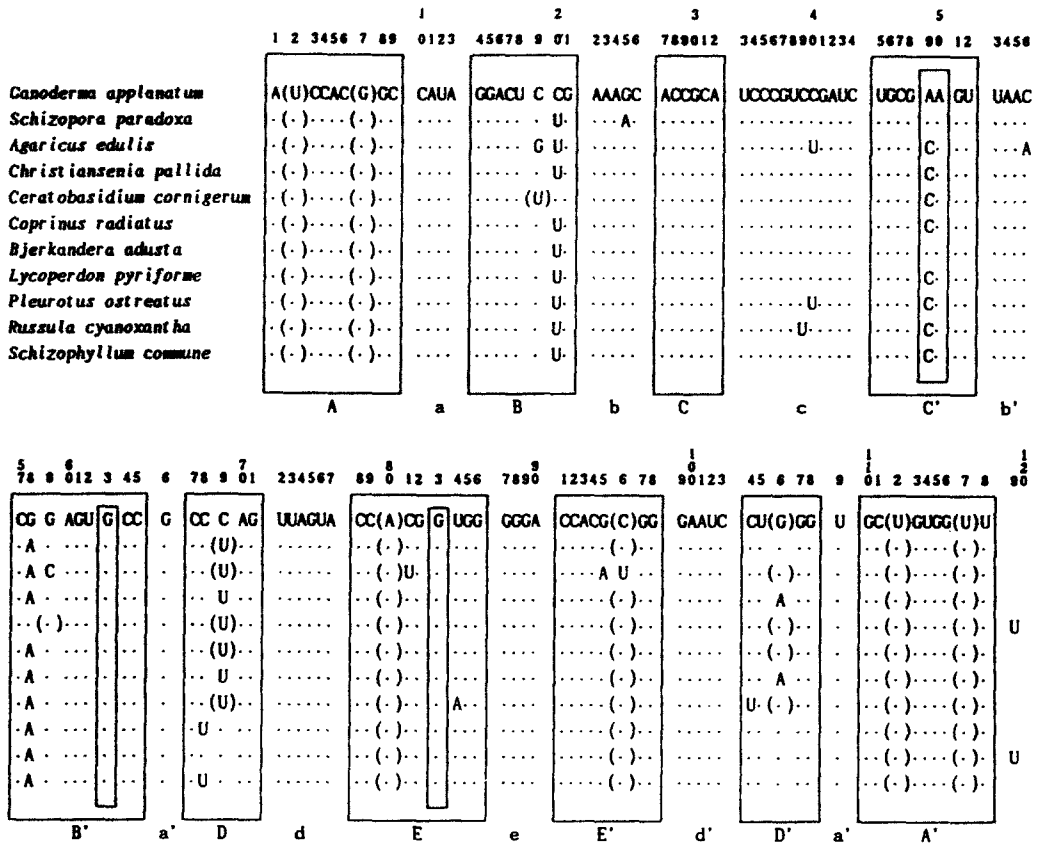
Fig. 2. Aligned primary sequences of 5S rRNAs from 11 mushrooms of the Hymenomycetes and the Gasteromycetes of the Basidiomycotina.

The 5S rRNAs studied here were composed of five helices, five loops, and three bulges on helices in their secondary structures. The boxed sequences indicate helix regions and open ones loop regions. The numbers of the top lines are serial numbers of the sequences and the letters of the bottom lines from A to E the symbols of helix regions and the ones from a to e those of loop regions. Primed letters are matching parts of corresponding regions. Dots are same bases as those of Ganoderma applanatum at given sequences and odd base-pairs are parenthesized. Bulges are indicated as narrow inside boxes below the 49th, 50th, 63rd and 83rd bases.

Table 1. Distance matrix of 11 mushrooms of the Hymenomycetes and the Gasteromycetes of the Basidiomycotina.

	G.a.	S.p.	A.e.	C.p.	C.c.	C.r.	B.a.	L.p.	P.o.	R.c.	S.c.
Ganoderma applanatum	—	0.0345	0.1005	0.0436	0.0259	0.0347	0.0345	0.0529	0.0436	0.0347	0.0347
Schizopora paradoxa	0.0345	—	0.0184	0.0259	0.0436	0.0172	0.0171	0.0349	0.0436	0.0347	0.0347
Agaricus edulis	0.1005	0.0814	—	0.0716	0.0809	0.0623	0.0814	0.0184	0.0716	0.0809	0.0809
Christiansenia pallida	0.0436	0.0259	0.0176	—	0.0345	0.0085	0.0259	0.0345	0.0345	0.0258	0.0258
Ceratobasidium cornigerum	0.0259	0.0436	0.0809	0.0345	—	0.0258	0.0436	0.0436	0.0523	0.0430	0.0434
Coprinus radiatus	0.0347	0.0172	0.0623	0.0085	0.0258	—	0.0172	0.0172	0.0258	0.0171	0.0171
Bjerkandera adusta	0.0345	0.0171	0.0814	0.0086	0.0436	0.0172	—	0.0349	0.0436	0.0347	0.0347
Lycoperdon pyriforme	0.0529	0.0349	0.0814	0.0259	0.0436	0.0172	0.0349	—	0.0436	0.0347	0.0347
Pleurotus ostreatus	0.0436	0.0436	0.0716	0.0345	0.0523	0.0258	0.0436	0.0436	—	0.0258	0.0085
Russula cyanoxantha	0.0347	0.0347	0.0809	0.0258	0.0430	0.0171	0.0347	0.0347	0.0258	—	0.0171
Schizophyllum commune	0.0347	0.0347	0.0809	0.0258	0.0434	0.0171	0.0347	0.0347	0.0085	0.0171	—

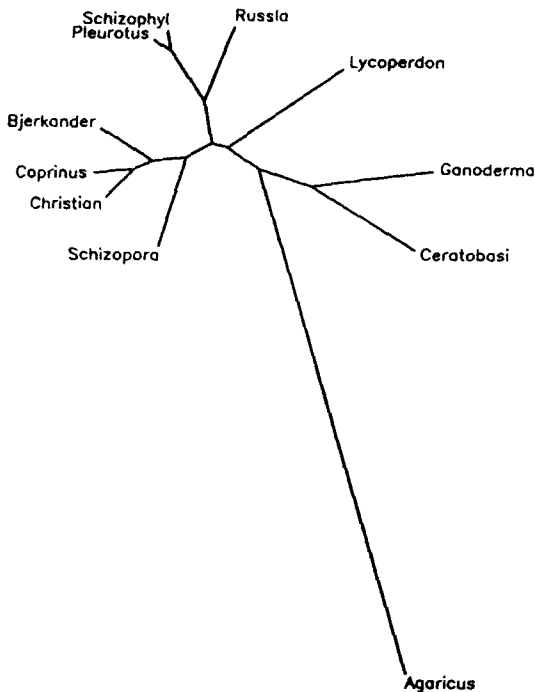Numbers are $1/2K_{nuc}$ values between 5S rRNA sequences of each two compared species.

Fig. 3. *Relationships between 11 mushrooms of the Hymenomycetes and the Gasteromycetes of the Basidiomycotina based on their 5S rRNA nucleotide sequences.*
The tree was constructed using the Neighbor program of the Felsenstein PHYLIP package with the Neighbor-joining option.

believed to have a same phylogenetic relationship even though they are presently placed under two different taxonomic families. *Schizopora paradoxa* was grouped with *Bjerkandera adusta* in a same family as was expected and proved to be a good member of the Polyporaceae rather than of the Corticiaceae. The present phylogeny based on 5S rRNA sequences using a technique of molecular biology suggests that the taxonomy of fungi based on morphology still holds good in the classification of mushrooms.

When the secondary structures of 5S rRNAs of 11 mushrooms were compared, the base substitution occurred at helix regions more rather than at loop regions (Fig. 2), suggesting a possibility that helix regions might have been less conserved and affected the formation of evolutionary branches more than loop regions within mushrooms. When a phylogenetic tree was constructed using the distance matrix calculated by Kimura's two parameter method (7) and the Neighbor program of the Felsenstein PHYLIP package with the Neighbor-joining option, the

present phylogeny partially discriminated and separated the mushrooms of the Hymenomycetes by the order as shown in Fig. 3. It also suggested that there were at least two lineages in the Aphyllophorales except the *Coprinus* and again two lineages in the Agaricales of the Hymenomycetes and that *Lycoperdon pyriforme* was forming an independent lineage for the Gasteromycetes.

AKNOWLEDGEMENTS

REFERENCES

1. **Austin, B. and F. Priest,** 1986. Modern bacterial taxonomy. van Nostrand Reinhold, Berkshire.
2. **Blanz, P.A. and M. Unseld,** 1987. Ribosomal RNA as a taxonomic tool in mycology, p. 247-258. *In* G.S. de Hoog, M. Th. Smith, and A. C. M. Weijman (ed.), The expanding realm of yeast-like fungi. Elsevier Science Publishers, Amsterdam.
3. **England T.E. and O.C. Uhlenbeck,** 1987. 3'-Terminal labelling of RNA with T4 RNA ligase. *Nature* **275,** 560-561.
4. **Gilbertson, R.L.,** 1980. Wood-rotting fungi of North America. *Mycologia* **72,** 1-49.
5. **Huysmans, E., E. Dams, A. Vandenberghe, and R. de Wachter,** 1983. The nucleotide sequences of the 5S rRNAs of four mushrooms and their use in studying the phylogenetic position of basidiomycetes among the eukaryotes. *Nucl. Acids Res.* **11,** 2871-2880.
6. **Jung, H.S.,** 1987. Wood-rotting Aphyllophorales of the southern Appalachian spruce-fir forest. Bibliotheca Mycologica Band **119,** J. Cramer, Stuttgart.
7. **Kimura, M.,** 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* **16,** 111-120.
8. **Kumazaki, T., H. Hori, and S. Osawa,** 1983. Phylogeny of protozoa deduced from 5S rRNA sequences. *J. Mol. Evol.* **19,** 411-419.
9. **Peattie, D.A.,** 1979. Direct chemical method for sequencing RNA. *Proc. Natl. Acad. Sci. USA* **76,** 1760-1764.
10. **Vournakis, J.N., J. Celantano, M. Finn, R.E. Lockard, T. Mitra, G.Pavlakis, A. Troutt, M. van den Berg, and R.M. Wurst,** 1981. Sequence and structure analysis of end-labeled RNA with

nucleases, p. 267-298. *In C.* Papas (ed.), Gene amplification and analysis, Vol. 2. Elsevier Science Publishers, Amsterdam.

11. **Walker, W.F. and W.F. Doolittle**, 1983. 5S rRNA sequences from eight basidiomycetes and fungi imperfecti. *Nucl. Acids Res.* **11**, 7625-7630.

12. **Wolters, J. and V.A. Erdmann**, 1988. Compilation of 5S rRNA and 5S rRNA gene sequences. *Nucl. Acids Res.* **16**, r1-r69.

초 록: 5S rRNA 염기서열에 의한 잔나비걸상과 좀구멍버섯의 계통학적 연구

김학현 · 정학성*(서울대학교 자연과학대학 미생물학과, 서울대학교 분자미생물학연구센터)

담자균류 균심류의 영지과에 속하는 잔나비걸상(*Ganoderma applanatum*)과 구멍장이버섯과에 속하는 좀구멍버섯(*Schizopora paradoxa*) 두 종의 5S rRNA 염기서열들을 (EMBL accession numbers X73589 and X73890) direct chemical method로 분석 결정하고 담자균류 균심류와 복균류의 기존에 밝혀진 9종 버섯의 염기서열과 비교하였다. 잔나비걸상과 좀구멍버섯의 5S rRNA는 각각 118개의 염기로 구성되어 있으며 B형 5S rRNA에 해당하였고 Huysmans 등이 제시한 2차구조의 모델에 들어 맞으며 Walker와 Doolittle이 제시한 제5 염기서열군에 속하였다. 진화거리를 나타내는 Kimura의 염기치환상수 K$_{nuc}$ 값에 의하면 잔나비걸상과 가장 가까운 종은 고약버섯과의 *Ceratobasidium cornigerum*으로서 염기 3개의 차이를 보였으며 좀구멍버섯과 가장 가까운 종은 구멍장이버섯과의 줄버섯(*Bjerkandera adusta*)으로서 염기 두개의 차이를 보였다. 11개 5S rRNA의 이차구조를 비교하였을때 염기의 치환은 loop 부분보다는 helix 부분에서 많이 일어났으며, 이는 helix 부분이 loop 부분보다는 진화적으로 덜 보존되어 있고 진화 분지를 형성하는데 보다 많은 영향을 주었음을 시사하였다. Kimura의 two parameter method로 계산된 distance matrix를 사용하고 Felsenstein PHYLIP package의 Neighbor program에서 Neighbor-joining option을 이용하여 계통수를 그렸을 때 균심류의 버섯들은 부분적으로 목별로 구분되었다. 균심류의 민주름버섯목에는 적어도 먹물버섯류(*Coprinus radiatus*)를 제외한 2개의 계통분지가 있고 주름버섯목에도 2개의 계통 분지가 있으며, 복균류에는 말불버섯(*Lycoperdon pyriforme*)이 독립된 계통분지를 형성하고 있음을 시사하였다.