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Phylogenetic Analysis of the Corticiaceae Based on Gene Sequences of Nuclear 18S Ribosomal DNAs

Seung-Shin Lee and Hack Sung Jung*

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

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The nuclear 18S ribosomal RNA genes of seven corticioid species were sequenced. These sequences were analyzed and compared with those of 24 other species of the order Aphyllophorales and phylogenetic trees were constructed using parsimonious methods. Phylogenetic analyses showed that two species among examined members of the Corticiaceae, *Resinicium bicolor* and *Thanatephorus praticola*, are located distantly from the remaining six species. The separation of *R. bicolor* seems to be phylogenetically significant because it has very unique cystidia. The independent lineage of *T. practicola* suggests that it is also phylogenetically distinct because it has unusual features like the homobasidium producing secondary spores and the septal ultrastructure of pore cap. Furthermore, *Auriscalpium vulgare, Bondarzewia berkeleyi,* and *Heterobasidion annosum* from different families of the Aphyllophorales proved to be closely related to the species of the Corticiaceae. They all have amyloid spores and grouped with *Aleurodiscus amorphus*, which is a member of the Corticiaceae. The amyloidity of spores seems to be an important character throughout the order of the Aphyllophorales.

Key words: 18S rDNA, Aphyllophorales, Corticiaceae, phylogeny

The Corticiaceae is one of the largest families in the order Aphyllophorales. It is considered to be an artificial group in that it is an assemblage of species with similar habitats (4). The Corticiaceae is often characterized by a resupinate fruitbody, which is one of its few common characters and by a rather smooth hymenium without tubes, gills, or spines.

At first, corticioid fungi were described as a tribe Resupinatus of the genus *Thelephora* (4). Patouillard considered the genus *Thelephora* as a taxon that has smooth or toothed hymenophores. Later, polyporoid and cantharelloid elements were added and the genus was raised to the family Thelephoraceae. After emphasis had been laid on microscopic structures, the traditional Thelephoraceae became split all over the Corticiaceae, the Cyphellaceae, the Stereaceae, and the Thelephoraceae (4, 12).

The Corticiaceae is considered an artificial group. This group is so chaotic that even its boundary is not clear. There is no clear cut difference between the Corticiaceae and the Stereaceae. In some respects, the Stereaceae and *Aleurodiscus* of the Corticiaceae have some common features: acanthocystidia or acanthohyphidia and amyloid spores. The main difference is that the Stereaceae can produce caps but the Corticiaceae has resupinate basidiocarps (4).

The Vuilleminiaceae is characterized by its remarkable basidia. But Donk thought that the family Vuilleminiaceae must be either expanded or sunk again in the Corticiaceae (4). The Meruliaceae, which has folded hymenium, appears to be very closely related to some genera like *Auriculariopsis, Cytidiella,* and *Phanerochaete* in which the hymenium does not become folded. Someone places the Meruliaceae within the Stereaceae but the monomitic hyphal system and the fruitbody structure of the Meruliaceae are not stereum-like (4).

Hericium, formerly placed in the Hydnaceae, was later transferred to the Corticiaceae due to common characters with *Gloeocystidiellum* which is treated as a subfamily of the Corticiaceae. But increasing hope that the boundaries of the Corticiaceae could be more sharply defined finally resulted in the Hericiaceae being separated from the Corticiaceae. However, the problem of where to draw the line between the remainder of the Corticiaceae and the Hericiaceae still remains (4).

Molecular techniques are becoming more important than ever as means for studying taxonomic and phylogenetic relationships among fungi. The ribosomal RNA gene is an ancient gene which all or-

* To whom correspondence should be addressed.

ganisms have. Ribosomal RNA genes form a mosaic pattern of conserved and variable regions which makes taxonomic analysis possible at many levels (18, 27). The region that is most appropriate for studying taxa at a particular level needs to be carefully selected. Because the nuclear small-subunit rRNA region has been appropriate for analysis at or above the level of orders, it was selected for phylogenetic study of the Corticiaceae.

Materials and Methods

Strains and cultures

The Corticiaceae strains used in this study are listed in Table 1. Cultures were grown on malt extract agar (MEA; malt extract 2%, peptone 0.5%, agar 1.5%) covered with a sterile cellophane disc at 24°C under dark condition for 7~14 days.

DNA isolation and PCR amplification

DNA extraction was done by the rapid method for nucleic acids extraction from petri dish-grown mycelia (15). Mycelium was harvested in an Eppendorf tube and 750 µl of extraction buffer [100 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 mM NaCl and 2% SDS] were added. After vortexing for 30 sec and then freezing for 30 sec in liquid nitrogen, the tube was incubated in a dry incubator for complete thawing at 70°C for 30 sec, and this process was repeated three times.

Extracted DNA was purified with classical phenol-chloroform extraction involving 3 steps: phenol extraction, phenol-chloroform extraction and chloroform extraction. Before the chloroform extraction, RNA was removed by RNase A by incubating at 37°C for 10 min. The purified DNA then precipitated with 1 volume of iso-propanol and centrifuged immediately at 12,000 rpm at room temperature. After removing the supernatant, the pellet was washed twice in 70% ethanol and allowed to air dry and resuspended in 40 µl of sterile TE (pH 8.0). Extracted DNA was stored at 4°C.

The region of small subunit ribosomal RNA gene was amplified using primers NS1 and NS8 designed by White *et al.* (26). PCR amplification of DNA was performed by Taq DNA polymerase (POSCO-CHEM) in 10 mM Tris-HCl (pH 9.0 at 25°C), 50 mM KCl, 0.1% Triton X-100, 2 mM $MgSO_4$, 0.2 mM of dATP, dGTP, dCTP, and dTTP, 0.5 µl of each primer with about 200 ng template DNA, and 3 unit of Taq DNA polymerase per PCR reaction. The total volume was adjusted to 100 µl and PCR was performed with the following parameters: an initial extension step at 94°C for 4 min, 1 min at 94°C for denaturation, 1 min at 50°C for annealing, and 2 min at 72°C for extension. After 32 cycles of denaturation, annealing and extension, there was a final extension step for 30 min at 72°C, and the reaction mixture was held at 4°C upon completion. Amplified PCR products were purified by chloroform extraction or by the purifying method using LMP agarose gel (20).

Cloning and DNA sequencing

The Promega pGEM-T vector system was used (17). 25 ng Promega pGEM-T vector and 100 ng PCR products were mixed with Promega T4 ligase 1× buffer and 1.5 Weiss units of Promega T4 DNA ligase. The mixture was incubated overnight at 16°C. *E. coli* was transformed with the ligation mixture by Sambrook's method (20). The automatic ALFexpress DNA sequencer (Pharmacia Biotech) was used for DNA sequencing using internal primers NS1, NS3, NS5, and NS7 designed by White *et al.* (26). For NS4, NS6, NS9 (26) and NS19SNU (14), the dideoxy chain termination method (21) was used.

DNA sequence analyses

The nuclear 18S rDNA sequences of 25 strains in GenBank were used (Table 2). Among them, *Tremella foliacea* was used as an outgroup. The first step of the analysis was to align complete sequences using a computerized alignment program CLUSTAL W. This alignment was verified manually. For phylogenetic reconstruction, the heuristic search option of parsimony algorithms in the

Table 1. List of corticioid species used for phylogenetic analyses of the study

Species	Family	Source
Aleurodiscus amorphus (Persoon: Fries) Schroeter	Corticiaceae	CBS 194.91
Botryohypochnus isabellinus (Fries) Donk	Corticiaceae	CBS 133.62
Hyphoderma setigerum (Fries : Fries) Donk	Corticiaceae	CBS 421.72
Peniophora quercina (Persoon : Fries) Cooke	Corticiaceae	CBS 838.72
Resinicium bicolor (Fries) Parmasto	Corticiaceae	CBS 253.73
Thanatephorus cucumeris (Frank) Donk	Corticiaceae	CBS 233.93
Trechispora mollusca (Persoon : Fries) Liberta	Corticiaceae	CBS 439.48

Table 2. Fungal species, family ranks, and GenBank accession numbers of small subunit rRNA sequences of compared taxa

Species	Family	GenBank Accession
Antrodia carbonica (Overholts) Ryvarden et Gilbertson	Polyporaceae	U59059
Auriscalpium vulgare S.F. Gray	Auriscalpiaceae	U59060
Bjerkandera adusta (Wildenow : Fries) Karsten	Polyporaceae	U59061
Bondarzewia berkeleyi (Fries) Bondartsev et Singer	Bondarzewiaceae	U59062
Ceriporia purpurea (Fries) Donk	Polyporaceae	U59065
Coltricia perennis (Linnaeus : Fries) Murrill	Hymenochaetaceae	U59064
Fomes fomentarius (Linnaeus) Fries	Polyporaceae	U59069
Fomitopsis pinicola (Swartz : Fries) Karsten	Polyporaceae	U59071
Heterobasidion annosum (Fries) Brefeld	Polyporaceae	U59072
Inonotus hispidus (Bulliard : Fries) Karsten	Hymenochaetaceae	U59074
Laetiporus sulphureus (Bulliard : Fries)	Polyporaceae	U59079
Lopharia spadicea (Persoon : Fries) Boidin[a]	Stereaceae	-
Meripilus giganteus (Persoon : Fries)	Polyporaceae	U59082
Panus rudis (Schweinitz) Fries	Pleurotaceae	U59086
Phanerochaete chrysosporium Burdsall	Corticiaceae (?)	U59084
Pleurotus tuberregium (Fries) Singer	Pleurotaceae	U59091
Polyporus squamosus (Hudson : Fries) Fries	Polyporaceae	U59089
Schizophyllum commune (Fries : Fries)	Schizophyllaceae	X54865
Sparassis spathulata (Schweinitz : Fries) Fries	Sparassidaceae	U59096
Spongipellis hirsutum (Willdenow : Fries) S.F. Gray	Polyporaceae	M59760
Thanatephorus praticola (Frank) Donk	Corticiaceae	M92990
Trametes suaveolens (Fries : Fries) Fries	Polyporaceae	U59098
Trichaptum abietinum (Dickson : Fries) Ryvarden	Polyporaceae	U59097
Tremella foliacea Fries[b]	Tremellaceae	L22262

[a] Sequence obtained through a personal communication with S.I. Yoon of this laboratory
[b] Outgroup sequence

computer program package PAUP 3.1.1. (24) was used with gaps treated as missing data. All characters were weighted equally. The confidences of internal branches from resulting trees were statistically estimated by the bootstrap analysis (9) of 500 replications using the stepwise addition option of the heuristic method of PAUP 3.1.1. (24).

Results and Discussion

Phylogenetic analyses

The size of the PCR products produced by primers NS1 and NS8 are as follows: 1732 bp for *Aleurodiscus amorphus*, 1735 bp for *Botryohypochnus isabellinus*, 1701 bp for *Hyphoderma setigerum*, 1718 bp for *Peniophora quercina*, 1733 bp for *Resinicium bicolor*, 1735 bp for *Thanatephorus cucumeris*, and 1708 bp for *Trechispora mollusca*.

The 18S rRNA gene sequences of 7 corticioid species and of 24 species from the Aphyllophorales were aligned with *Tremella foliacea* as an outgroup. Ambiguously aligned parts were removed and optimally aligned sequences were 1698 bp in length with 450 variable sites and 197 informative sites.

Parsimony method was applied to analyze aligned sequence data sets using PAUP 3.1.1. (24) and *Tremella foliacea* was used as an outgroup. When Swann and Taylor (23) analyzed higher taxa of the Basidiomycetes using 18S rRNA gene sequences, *Tremella foliacea* was found to be located clearly outside the Holobasidiomycetes including *Thanatephorus cucumeris* and proved to be a good species as an outgroup for the analyses of the Aphyllophorales which is an order of the Holobasidiomycetes.

Heuristic method with the stepwise addition option was used and 18 equally parsimonious trees (the shortest path length was 780) were obtained. A 50% majority consensus tree of 18 equal parsimony trees is presented in Fig. 1. Most of the bootstrap values are larger than 50%.

Polyphyly of the *Corticiaceae*

Fig. 1 shows that taxa of the *Corticiaceae* species used for the study are grouped together except *Resinicium bicolor, Thanatephorus praticola*, and *Phanerochaete chrysosporium*. However, *P. chrysosporium* has not yet been connected with a basidial state and produces a conidial state which has been referred to as the imperfect genus *Chrysosporium* (5). *P. chrysosporium* is not considered as a member of *Phanerochaete* in the Corticiaceae by European authors (5) and *P. chrysosporium* is ex-

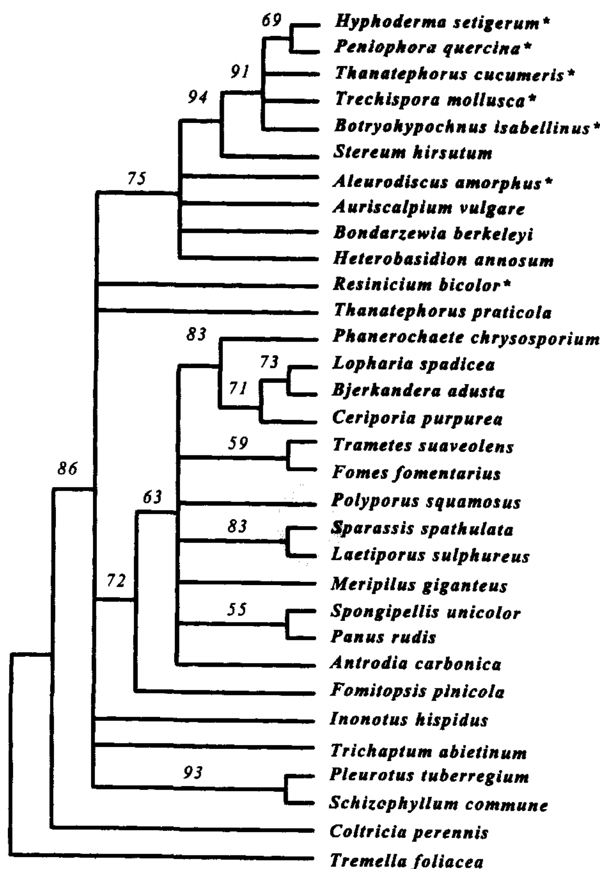

Fig. 1. Phylogenetic tree based on nuclear 18S rDNA sequences. Eighteen most parsimonious trees were constructed using the stepwise addition option of the heuristic method of PAUP 3.1.1. (24). *Tremella foliacea* was used as an outgroup. This is a bootstrap consensus tree of 18 parsimonious trees. Bootstrap values were obtained from 500 replications and are given above branches supported by more than 50%. Species followed by asterisks are corticioid species used for this study.

cluded from the discussion for the Corticiaceae and related families.

There are two species of *Thanatephorus*: *T. cucumeris* and *T. praticola*. As these names are taken to be synonyms, both species are known as a same species but the relationship appears very different judging from Fig. 1. It is predictable that one of two species, possibly *T. praticola* might have been misidentified or mislabeled. *Thanatephorus* is still a taxon of great taxonomic interest because it has been placed in the Tulasnellales or in the Ceratobasidiales or in the Corticiaceae of the Aphyllophorales depending on the varying views of different mycologists (11, 13, 19, 23).

However, *Thanatephorus* has at least two special characters which confuse mycologists. The first is the holobasidium producing secondary spores (11), which is a general phenomenon of the Heterobasidiomycetes while there is only direct germination in the Homobasidiomycetes (1, 4). Lowy made a new taxon Metabasidiomycetidae which included taxa with incompletely divided or aseptate basidia having notably enlarged or swollen sterigmata and with basidiospores germinating by repetition or conidia (16).

The second special character of *Thanatephorus* is the ultrastructure of septa. Most of the Holobasidiomycetes have a complex dolipore septum with multiperforated pore caps. On the other hand, the Phragmobasidiomycetes have simple non-perforated pore caps. The pore cap structure is considered to be useful in distinguishing the latter group from the former group (13). *Thanatephorus* has a septum which is different from that of the general Holobasidiomycetes and similar to that of the Phragmobasidiomycetes. *Thanatephorus* has only three pores whose average diameter is significantly greater than that of other multiperforated caps (25).

Because of such exceptional characters as these, *Thanatephorus* is placed in the Holobasidiomycetes or in the Heterobasidiomycetes or even in the Metabasidiomycetes (4, 13, 25). Considering these characters, it seems to be natural that *Thanatephorus* has a distant relationship to the Corticiaceae and can be located within the boundary of the Holobasidiomycetes. *Thanatephorus praticola* in Fig. 1 is in accordance with this view.

On the other hand, there are other views. Septa are likely to be conserved structures and apparently have phylogenetic significance. But septal characters alone cannot be used to establish natural relationships without regard to basidial characters (13). It seems unnatural to place the *Thanatephorus* in Heterobasidiomycetes but seems reasonable that major emphasis must be placed on the basidial morphology. *Thanatephorus* has an unusual septal structure. The septum has fewer pores with larger diameter than other septa and a perforated septal pore cap even though poorly developed. According to this view, it is not strange that the relationship between *T. cucumeris* and other species of the Corticiaceae is close as appears in Fig. 1. If we can get more data from species with similar characters as those of *Thanatephorus* (e.g. *Uthatobasidium*, *Ypsilonidium*, *Ceratobasidium* and *Oliveonia*), we should be able to get more reliable conclusions in the future.

Resinicium bicolor has a distant relationship with the rest of the Corticiaceae used in this study. The distinguishing characteristic of the genus *Resinicium* is the presence of halocystidia. *R. bicolor* has asterocystidia in addition to halocystidia.

The fruitbody of *R. bicolor* gets greenish tints because its basal layer is usually associated with living cells of green algae. If there is a symbiotic connection between *R. bicolor* and green algae, it is unclear yet and the phylogenetic significance of green algal association is still questionable. Having both halocystidia and asterocystidia might be a distinguishing character which separates the *R. bicolor* from the other species of the family. Except for *R. bicolor*, the remaining seven species of the Corticiaceae do not have such cystidial characters.

Hyphoderma setigerum and *Peniophora quercina* have the closest relationship among the eight species of the Corticiaceae. Their remarkable common character is cystidia encrusted with crystalline matter (5, 8). *Thanatephorus cucumeris, T. praticola, Trechispora mollusca* and *Botryohypochnus isabellinus* have no cystidia, but *Aleurodiscus amorphus* has moniliform cystidia (11). *H. setigerum* and *P. quercina* have many common characters: monomitic hyphae, clamps at all septa, basidia with four sterigmata, basidia with a basal clamp, spore size, allantoid and smooth spore of no amyloidity.

Fig. 1 shows that *Thanatephorus cucumeris, Trechispora mollusca*, and *B. isabellinus* are closely related. However, there are no primary characters which connect them. Possible common characters are non-amyloid spores, basidia with four sterigmata and monomitic hyphal systems (11). Non-amyloid spores and monomitic hyphal systems may be considered as the main characters which separate these three species from the Group 2 species in Fig. 2. In addition to these two characters, a third character is that they all have no cystidia. In the cases of *R. bicolor* and *H. setigerum-P. quercina*, cystidia were a prominent character. It is interesting that cystidial taxa *R. bicolor* and *H. setigerum-P. quercina* are distantly related but *H. setigerum-P. quercina* is closely located to the non-cystidial taxa *T. cucumeris-T. mollusca-B. isabellinus*.

Though there were only eight species of the Corticiaceae used for this study, at least two species, *R. bicolor* and *T. praticola*, were found to be quite distantly located from the remaining species, which suggests that the Corticiaceae can be a good example of polyphyletic groups.

The Corticiaceae within the Aphyllophorales

Before drawing phylogenetic trees, discussing the relationship between species of the Corticiaceae and other families of the Aphyllophorales proved to be unpredictable and unreliable. Some pileate species like *Auriscalpium vulgare, Bondarzewia berkeleyi, Heterobasidion annosum*, and *Stereum hirsutum* are closely related to members of the Corticiaceae. Fig. 2 summarizes that the fundamental relationship between corticioid species-related taxa seems to be based on the non-amyloidity of spores. Among 31 species of the Aphyllophorales, only *S. hirsutum, A. amorphus, A. vulgare* and *B. berkeleyi* have ameyloid spores and are grouped together. Group 2 is composed of morphologically and ecologically heterogeneous members but these members have amyloid spores in common except *Heterobasidion annosum*. However, the spores of *H. annosum* are asperulate and their ornamentation is positive in amyloid reaction (22).

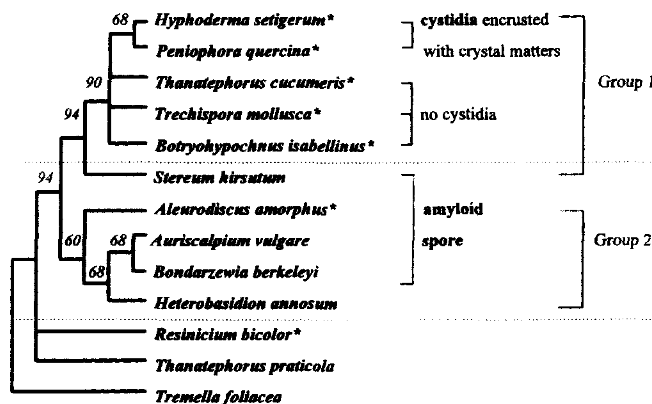

Fig. 2. Phylogenetic tree based on nuclear 18S rDNA sequences of the corticioid species and their neighbors in Fig. 1. *Tremella foliacea* was used as an outgroup. This is a strict consensus tree of 2 equally parsimonious trees. Bootstrap values were obtained from 500 replications and are given above branches supported by more than 50%. On the right, taxonomically important characters of taxa are indicated. Groups 1 and 2 represent two major clusters of taxa except *Resinicium bicolor* and *Thanatephorus praticola*. Species followed by asterisks are corticioid species used for this study.

Amyloid spores seem to be a special character in the Aphyllophorales. Species which have amyloid spores fall into several groups that do not appear to be mutually closely related (4). But Donk proposed that they seem to be mutually connected by a central body or central core of the Aphyllophorales (4). Phylogenetic classification of the Polyporaceae using mitochondrial rRNA sequences by Hibbett and Donoghue (10) and the present results support the central core concept, suggesting that *H. annosum* is closely related to the central core. Both mitochondrial rRNA analysis (10) and the present nuclear 18S rRNA analysis support this conclusion.

Spore amyloidity seems to be a very important character of the Aphyllophorales but it is believed that amyloidity alone is not a strong boundary character to separate genera of the family. As shown in Fig. 2, *S. hirsutum*, which has amyloid

spores, is included in Group 1 but not in Group 2. The major limitation in the present conclusions is that a relatively small number of species were analyzed for the study compared with the large number of species both in the Corticiaceae and in the Aphyllophorales. Furthermore, only one species is used for each genus or each family. Because of the question if each species is represent given genus or family of the study, the present results should be restricted to this study and considered as temporary evidences for the relationships between compared taxa. To improve the present taxonomic interpretations and conclusions, more molecular data must come out in the future.

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