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Phylogenetic relationships of *Antrodia* species and related taxa based on analyses of nuclear large subunit ribosomal DNA sequences

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ABSTRACT. This study aimed to evaluate relationships of *Antrodia* species and related taxa, including the taxonomic status of some *Antrodia* species that have been treated as separate genera (*Amyloporia*, *Fibroporia*, and *Taiwanofungus*). A comprehensive phylogenetic study of Homobasidiomycetes presented by Binder et al. in 2005, was consulted for sampling the taxa used for this analysis. The genera of the "residual" polyporoid clade and phlebioid clade of the Homobasidiomycetes were chosen as outgroups, and the genera belonging to the *Antrodia* clade and core polyporoid clade were selected as ingroup. Phylogenetic analyses of this study were based on sequence data derived from the nuclear large subunit ribosomal DNA (nuc-LSU rDNA). The analytical methods of maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI) were used. Results from these different analyses were generally consistent. Two main clades lacking high support were detected in the ingroup. Clade A consisted of taxa of the *Antrodia* clade, including all twelve studied *Antrodia* species and members of four other genera: *Daedalea*, *Fomitopsis*, *Neolentiporus,* and *Piptoporus*. The twelve *Antrodia* species were not clustered into a distinct subclade, indicating that *Antrodi*a is not a monophyletic group. Two species of *Fibroporia* (belonging to *Antrodia sensu lato*), characterized by having a fruiting body with a rhizomorphic margin, clustered together with very strong support. Five species with amyloid skeletal hyphae, diagnostic of *Amyloporia*, did not group together. The generic status of *Fibroporia*, but not *Amyloporia*, was supported in this study. Clade B consisted of the genera of the core polyporoid clade. Both species of the recently established genus *Taiwanofungus* formed a distinct subclade, supporting its generic status.

Keywords: *Amyloporia*; *Antrodia*; *Fibroporia*; *Fomitopsis*; Phylogeny; Polypore; *Taiwanofungus*.

INTRODUCTION

Antrodia P. Karst. is a polypore genus with more than 40 species causing brown rot of wood. The generic concept of *Antrodia* was amended by Gilbertson and Ryvarden (1986) and is summarized as follows: resupinate to effused-reflexed or effused-pileate basidiocarps; dimitic hyphal system with nodose-septate colorless generative hyphae, bearing mostly colorless skeletal hyphae that are inamyloid for most species and somewhat amyloid for a few species; without true cystidia; and with smooth, thin-walled and inamyloid basidiospores. Although earlier studies indicated that the genus *Antrodia sensu lato* is not monophyletic, some questions regarding the relationship remain unanswered.

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A number of genera have been segregated from *Antrodia sensu lato.* The genera *Amyloporia* Singer and *Fibroporia* Parmasto are regarded as congeneric with *Antrodia* by some authors (Gilbertson and Ryvarden, 1986, Ryvarden, 1991), but this hypothesis has not been tested with molecular analyses. Each genus only accommodates a few species. Amyloid skeletal hyphae are diagnostic for *Amyloporia*, and the fruiting body usually tastes bitter. Fruiting bodies of *Fibroporia* have a rhizomorphic margin. Ryvarden (1991) suggested that these characters might not be sufficient for supporting these two genera as separate from *Antrodia* if more convincing evidence can not be found. Two other species once placed in *Antrodia* (Wu et al., 1997; Chang and Chou, 2004) were recently referred to a new genus *Taiwanofungus* Sheng H. Wu et al. on the basis of morphological, ecological, and phylogenetic analyses derived from nuc-LSU rDNA (Wu et al., 2004).

Wu et al. (2004) included four species of *Antrodia*, and other more or less related genera of the polyporoid clade in their molecular analysis. The results indicated that *Taiwanofungu*s was distant to the two studied species of *Antrodia*, which included the generic type, and to other genera. The phylogenetic relationships of *Antrodia* and other studied genera were difficult to interpret from the results obtained due to a small sampling.

Several phylogenetic analyses of *Antrodia* were previously presented by other researchers. An analysis of seven *Antrodia* species and related genera conducted by Kim et al. (2001) was based on sequences inferred from the internal transcribed spacer (ITS) region of nuclear ribosomal DNA. Kim et al. (2003) attempted to assess phylogenetic relationships of six *Antrodia* species and related taxa based on sequences of the mitochondrial small subunit ribosomal DNA (mt-SSU). However, the genera included in their analysis were highly diverse phylogenetically, and were distributed among almost all clades of Homobasidiomycetes. Nevertheless, both studies mentioned above showed that *Antrodia* species do not form a monophyletic clade.

Kim et al. (2005) evaluated the monophyly of *Fomitopsis*, based on sequence data derived from nuc-LSU rDNA. Their results showed that the four studied *Antrodia* species were clustered together with ten studied *Fomitopsis* species, and both of these genera were respectively shown to be non-monophyletic. Chiu (2007) conducted phylogenetic analysis of nine *Antrodia* spp. and eleven strains of *A. camphorata*, based on sequences inferred from ITS nrDNA. In Chiu's analysis, the ingroup consisted of only *Antrodia* spp., and hence his study chiefly revealed phylogenetic relationships among the studied *Antrodia* spp. and strains.

The aims of this study were to further evaluate the generic status of *Antrodia sensu stricto*, and of some taxa that have been treated as separate genera (*Amyloporia*, *Fibroporia*, and *Taiwanofungus*) by some mycologists, as well as their phylogenetic relationships with related polypore genera. The phylogenetic analyses were based on sequence data derived from nuc-LSU, a region widely adopted in analyzing phylogenetic relationships of the Homobasidiomycetes at and above the generic level.

MATERIALS AND METHODS

Taxon sampling

Twelve *Antrodia* species, including the generic type, *Ant. albida* (Fr.), and the members of 22 other genera with more or less close relationships to *Antrodia*, were chosen for this analysis. All belong to the polyporoid clade composed of a core polyporoid clade, an *Antrodia* clade, a phlebioid clade, and a "residual" polyporoid clade within Homobasidiomycetes according to the comprehensive study of Binder et al. (2005). In that study, the core polyporoid clade and *Antrodia* clade, and phlebioid clade and "residual" polyporoid clade clustered together; the former two as sister clades to the latter two. *Antrodiella semisupina* (Berk. & M.A. Curtis) Ryvarden and *Bjerkandera adusta* (Willd.) P. Karst. belonging to the "residual" polyporoid clade and phlebioid clade, respectively, were used as outgroup taxa. The ingroup consisted of genera belonging to the *Antrodia* clade and core polyporoid clade. Details of the studied taxa are presented in Table 1.

DNA extraction, PCR amplification, DNA cloning, and sequencing

Mycelia were transferred from agar cultures to 100 ml liquid medium (2% malt extract) and incubated on a rotary shaker (160 rpm) for 2~3 weeks at room temperature. DNA was isolated from freeze-dried or freshly cultured mycelia using the Plant Genomic DNA Extraction Miniprep System (Viogene, Taiwan) according to the manufacturer's instructions. The primer pair, LR0R/LR5 (Moncalvo et al., 2000), was used to amplify the nuc-LSU rDNAregion. PCR conditions were set according to the manufacturer's instructions (Viogene). The amplification products were purified with a PCR-M Clean Up kit (Viogene), and both strand sequences were produced using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit on an ABI 3730 DNA sequencer (Applied Biosystems, Foster City, Calif.). The primers, LR0R and LR5, were used for direct sequencing of the amplified fragments. For the strains with intragenomic heterogeneity, DNA cloning was performed using a yT&A cloning vector and competent ECOS^{TM} 9-5 cells (Yeastern Biotech, Taiwan). A single positive colony was picked for the following PCR amplification and DNA sequencing. The consensus data from the forward and reverse sequences were assembled using SeqWeb from the GCG Wisconsin Package (available at http://bioinfo.nhri.org.tw).

Sequence alignment and phylogenetic analyses

Fifty-six taxa were used, including 17 sequences newly derived for this study (Table 1). For the two *Taiwanofungus salmoneus* strains with intragenomic heterogeneity, only the representative clone sequences, EF036246 and EF036249, were chosen for analysis. Sequences were aligned using Clustal X 1.83 (Thompson et al., 1997) and were adjusted manually using BioEdit 7.0.4.1 (Hall, 1999). The optimized data matrix was deposited in TreeBase (Study accession number = S2416, Matrix accession number = M4581). Three analytical methods were used: maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI).

The MP analysis was performed in PAUP* 4.0b10 (Swofford, 2002), using heuristic searches with 1000 random taxon stepwise addition sequences, TBR branch swapping, and the MAXTREES set to autoincrease. All transformations were considered unordered and equally weighted, with gaps treated as missing data. Bootstrap analysis (Hillis and Bull, 1993) was performed with 1000 replicates with random addition sequences for obtaining

Table 1. Taxa used in this study, along with their strain/specimen numbers and GenBank accession numbers.

Species	Strain/Specimen no.	GenBank accession no.
Amylocystis lapponica	HHB-13400-sp.	AF518598
Antrodia albida	FCUG 1396	AY333845
Antrodia albida	FCUG 1100	AY333846
Amyloporia (Antrodia) carbonica	DAOM197828	AF287844
Antrodia heteromorpha	FCUG 1244	AY333840
Antrodia juniperina	FP 97452-T	AY333839
Antrodia juniperina	WM 284T	AY333838
Antrodia malicola	MJL 1167SP	AY333835
Antrodia malicola	BCRC 35452	AY333837
Fibroporia (Antrodia) radiculosa	RLG 7629SP	AY333833
Fibroporia (Antrodia) radiculosa	L-9318SP	AY333834
Antroida serialis	GEL4465	AJ406519
Antrodia sinuosa	L-6192SP	AY333832
Antrodia sinuosa	RLG 1182R	AY333831
Amyloporia (Antrodia) sitchensis	HHB12513	AY333830
Fibroporia (Antrodia) vaillantii	P240	AJ583429
Antrodia variiformis	FP 90100SP	AY333827
Antrodia variiformis	FP 89848R	AY333828
Amyloporia (Antrodia) xantha	FCUG100	AY333826
Amyloporia (Antrodia) xantha	P289	AJ583430
Antrodiella semisupina	FCUG 960	AY333819
Auriporia aurea	FPL7026	AF287846
Bjerkandera adusta	DAOM215869	AF287848
Climacocystis sp.	KEW215	AF518609
Daedalea quercina	DAOM-142475	AF518613
Fomitopsis cajanderi	SFC 02040517	AY515337
Fomitopsis cupreorosea	CBS236.87	AY515325
Fomitopsis dochmia	CBS426.84	AY515326
Fomitopsis feei	CBS546.50	AY515327
Fomitopsis lilacinogilva	CBS422.84	AY515329
Fomitopsis (Laricifomes) officinalis	CBS164.30	AY515331
Fomitopsis (Laricifomes) officinalis	CBS565.83	AY515332
Fomitopsis palustris	CBS283.65	AY515333
Fomitopsis pinicola	CBS221.39	AY515334
Fomitopsis rosea	FP 104278-T	AY333809
Fomitopsis spraguei	CBS365.34	AY515335
Ganoderma australe	Wu 9302-56	AY333807
Grifola frondosa	zw-clarku005	AY218413
Ischnoderma benzoinum	GEL2914	AJ406543
Laetiporus sulphureus	DSH93-194	AF287870
Neolentiporus maculatissimus	Rajchenberg 158	AF518632
Oligoporus lacteus	KEW55	AY293205
Oligoporus rennyi	KEW57	AF287876
Osmoporus odoratus	Wu 0309-92	EF153195
Parmastomyces transmutans	L-14910-sp.	AF518635
Phaeolus schweinitzii	818-96	AF311050
Piptoporus betulinus	DSH93-186	AF287886
Polyporus alveolaris	FP-101937-Sp	AY826983
Pycnoporellus fulgens	T-325	AF518643
Sparassis spathulata	DSH93-184	AF287889
Taiwanofungus camphoratus	BCRC 35396	AY333844
Taiwanofungus camphoratus	CWN 01385	AY333841
Taiwanofungus salmoneus	BCRC 36937	EF036246
		EF036247
Taiwanofungus salmoneus	BCRC 36938	EF036248
		EF036249
		EF036250
Trametes suaveolens	DAOM-196328	AF518656
Tyromyces chioneus	KEW141	AF393080

[a]Taxa in bold indicate sequences from this study.

estimates of the reliability of the clades.

For the ML analysis, the best model of nucleotide substitution was determined using nested likelihood ratio tests calculated with Modeltest 3.7 (Posada and Crandall, 1998). Heuristic ML searches were conducted using PAUP* 4.0b10 with the appropriate model of evolution and the associated parameter estimates, ten random addition sequence replicates, and TBR branch swapping with the MULTrees option in effect.

The BI analysis was conducted using MrBayes v.3.1.2 (Ronquist and Huelsenbeck, 2003). Using the model identified by Modeltest and flat priors, four chains (three heated) were run for 8×10^6 generations, and trees were sampled every 1000 generations. Two thousand trees were discarded as part of the burn-in period. Posterior probabilities (PP) for the Bayesian approach were determined by calculating a 50% majority rule consensus tree from the remaining 6000 trees.

RESULTS

Analyses of nuc-LSU rDNA sequences

Amplification of the nuc-LSU rDNA region yielded fragments of approximately 980 base pairs long. The final alignment of 56 taxa included 3372 positions. After excluding ambiguous sites at both ends, 845 alignment sites were used for the phylogenetic analyses.

The MP analysis revealed ten most parsimonious trees (911 steps, consistency index (CI) = 0.425, retention index (RI) = 0.645). Of the 845 included sites, 541 were constant, 83 were variable but parsimoniously uninformative, and 221 (ca. 26.2%) sites were parsimony informative.

In the ML analysis, Modeltest selected the General Time Reversible model with a proportion of invariant sites and gamma-distributed site-to-site rate variations (GTR+I+G) as the best-fitting model for explaining evolutionary change within the selected taxa. The nucleotide frequencies were estimated (A = 0.2410, C = 0.2104, G = 0.3079, and T = 0.2407). A rate matrix of substitutions was created (A-C = 1.0543, A-G = 5.6405, A-T = 1.7413, C-G = 0.3591, C-T = 12.7360, and G-T = 1.0000). The gamma distribution shape parameter was 0.644. The optimal tree inferred under the ML criterion had a likelihood of -5618.80142.

For comparison, the likelihood values of the best states for the cold chain were 5685.94 and 5696.47 in the two parallel Bayesian runs, respectively. The average standard deviation of the split frequencies was 0.007214 at the end of the runs.

Phylogenetic relationships

The ten most parsimonious trees differed from each other mainly in whether the members of *Amyloporia* (*Amy. sitchensis* and *Amy. xantha*) were grouped together or not, and whether the relationship between *Amy. sitchensis* and the two *Amy. xantha* strains was resolved or not. One of these trees is presented (Figure 1). In this tree, the two main clades of the ingroup had weak bootstrap support (BS < 50%). Clade A was composed of all the *Antrodia* taxa and members of four other genera: *Daedalea*, *Neolentiporus*, *Fomitopsis* (excluding *Fom. officinalis*), and *Piptoporus*. Within this clade, *Antrodia* taxa did not cluster together. The following subclades were apparent: *Ant. juniperina* (BS = 63%), *Ant. variiformis-Ant. serialis* (BS = 98%), *Ant. malicola* (BS = 85%), *Ant. albida-Ant. heteromorpha* (BS = 100%), *Fib. radiculosa-Fib. vaillantii* (BS = 100%), *Ant. sinuosa* (BS = 99%), *Amy. sitchensis-Amy. xantha* (BS = 99%) with *Amy. sitchensis* and only one of the two *Amy. xantha* strains clustering together (BS = 60%). *Antrodia carbonica* was at the base of clade A. Clade B includes both brown-rot and white-rot genera belonging to the *Antrodia* clade and core polyporoid clade in Binder et al. (2005). Within this clade, the two species of *Taiwanofungus* formed a distinct subclade (BS = 100%), which did not cluster with any other genus with bootstrap support (BS) higher than 50%. Two strains of *Fom. officinalis* grouped together with 100% support in bootstrap analysis while other *Fomitopsis* species were placed in clade A.

The ML tree (Figure 2) is very similar in topology to the MP tree (Figure 1). It differs from the latter only in the placement of several clusters or taxa, e.g. the *Ant. variiformis-Ant. serialis* cluster, the *Fib. radiculosa-Fib. vaillantii* cluster, *Amy. carbonica*, and *Fom. officinalis*.

The consensus tree of the BI analysis (not shown) was identical in topology to the ML tree (Figure 2). The posterior probability derived from the BI is shown on the ML tree (Figure 2). The BI analysis found high posterior probabilities (PP > 95%) for all well-supported clusters (BS ≥ 90%) (excluding *Ant. sinuosa*) and several clusters with moderate support (BS > 70%) in the MP analysis (Figure 1).

DISCUSSION

In this study, the results derived from the three analyses (MP, ML, and BL) were generally consistent (Figures 1 and 2). Two main clades (clades A and B) were recognized with weak support. These two clades respectively correspond to the *Antrodia* clade and the core polyporoid clade of Binder et al. (2005). In clade A, *Antrodia* species were interspersed with species of other genera (Figures 1 and 2) although their relationships remain unclear due to low support in both MP and BL analyses. Similarly, the nine species of *Fomitopsis* in clade A grouped with *Antrodia* species and species assigned to other polypore genera including *Neolentiporus, Daedelea*, and *Piptoporus*. Our results, therefore, support those of previous studies that neither *Antrodia* nor *Fomitopsis* are monophyletic genera (Kim et al., 2001; 2003; 2005).

Fibroporia radiculosa and *Fib. vaillantii* formed a robustly supported subclade (BS, PP = 100%) (Figures

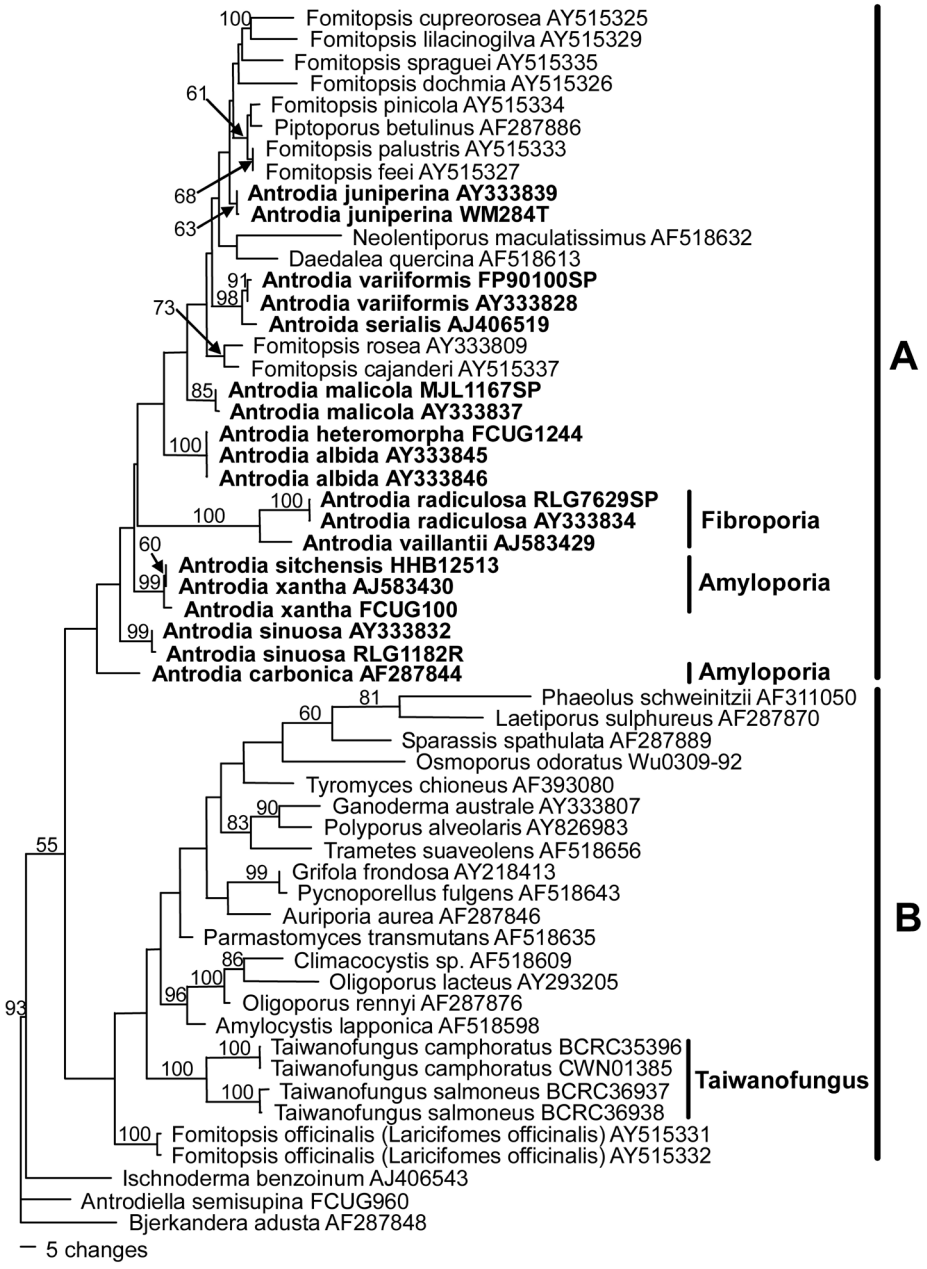

Figure 1. One of the ten most parsimonious trees derived from partial nuc-LSU rDNA sequence data. Bootstrap values are shown at nodes supported by no less than 50% from 1000 replicates. TL = 911, CI = 0.425, RI = 0.645.

1 and 2). These two species differ from other species of *Antrodia* by having a fruiting body with a rhizomorphic margin, and a tetrapolar mating system (Lombard, 1990) while most species of *Antrodia* possess a bipolar mating system. *Antrodia malicola* is an exception, with a homothallic mating system. Although *Fibroporia gossipina* was not included in our study, this species formed a well-supported clade with *Fibroporia vaillantii* in a previous study (Kim et al., 2001). It is, therefore, evident that the three members of *Fibroporia*, *Fib. radiculosa*, *Fib. gossipina,* and *Fib. vaillantii*, are closely related. Molecular results and sexuality along with morphological features support *Fibroporia* being a distinct genus.

Three species of *Amyloporia* with amyloid skeletal hyphae, i.e., *Amy. carbonica*, *Amy. sitchensis,* and *Amy. xantha*, nested within clade A. However, only two of them, *Amy. xantha* (the type of *Amyloporia*) and *Amy. sitchensis* formed a very strongly supported subclade in clade A (Figures 1 and 2). *Amyloporia carbonica* is separate from this subclade, but its position remains unresolved (Figures 1 and 2). In addition to molecular data indicating that *Amyloporia* is not a monophyletic genus, morphological delimitation from *Taiwanofungus* also appears problematic.

Both genera have amyloid skeletal hyphae, but the two species of *Taiwanofungus* endemic to Taiwan, *T. camphoratus* and *T. salmoneus,* formed a well-supported subclade within clade B (Figures 1 and 2), well separated from *Antrodia*, *Fibroporia*, and *Amyloporia*. This means that the generic status of *Amyloporia* cannot be recognized.

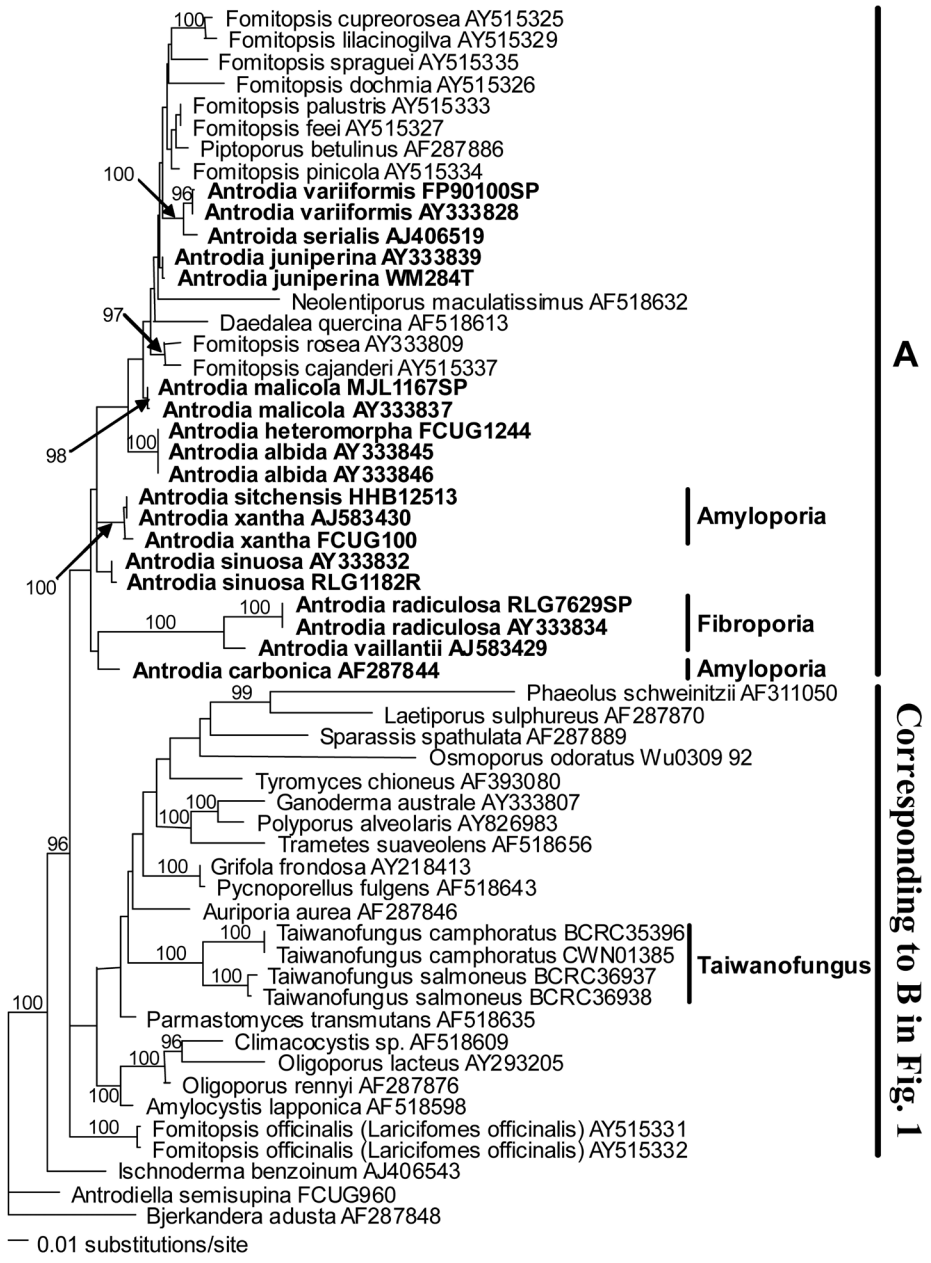

Figure 2. The maximum likelihood phylogram (-ln L = -5618.80142) based on partial nuc-LSU rDNA sequence data. Numerals associated with the nodes are posterior probabilities resulting from the Bayesian inference. Only posterior probabilities values > 95% are shown.

Kim et al. (2005) evaluated the monophyly of *Fomitopsis*, based on sequence data derived from nuc-LSU. Their results showed that the four studied *Antrodia* species were clustered together with ten studied *Fomitopsis* species, and both of these genera were respectively shown to be non-monophyletic.

The status of *Taiwanofungus* as a genus separate from *Antrodia* was supported in this analysis. Several characteristics delimit this genus from *Antrodia*. First, fruiting bodies have amyloid skeletal hyphae and a bitter taste. These characters are shared with *Amyloporia*. Second, basidiospores are small [< 5 µm long and < 2 µm wide, according to Chang and Chou (1995; 2004)] while the type species of *Antrodia* (*Ant. albida*) and the species that clusters with it (*Ant. herteromorpha*) have distinctly larger spores [> 10 µm long and > 3.5 µm wide, according to Gilbertson and Ryvarden (1986)]. Third, two species known in this genus are capable of producing both arthroconidia and chlamydospores in culture while *Antrodia* species do not. Fourth, both species of *Taiwanofungus* are tetrapolar in sexuality (Chang and Chou, 2004). *Fibroporia* is another genus with a tetrapolar mating system, but this genus has morphological characters similar to other species of *Antrodia* rather than *Taiwanofungus*. Fifth, both species of *Taiwanofungus* are specific to their tree hosts, at species level. *Taiwanofungus camphoratus* occurs only on trunks of *Cinnamomum kanehirai*, and *T. salmoneus* occurs strictly on *Cunninghamia konishii*. Specific relationships between fungi and their plant hosts have not been reported for *Antrodia* species.

The ten taxa studied of *Fomitopsis* were clearly divided

into several clusters (Figures 1 and 2). Nine of them were included in analyses grouped in clade A. Only one, *Fom. officinalis,* was placed in clade B, i.e., as a subclade of clade B (Figure 1) or as a separate clade (Figure 2). Obviously, *Fom. officinalis* has a distinct taxonomic status from the other species of *Fomitopsis* used in this study. A similar conclusion was also obtained in the analysis of Kim et al. (2005). Kotlaba and Pouzar (1957) established the genus *Laricifomes* Kotl. & Pouzar based on *Boletus officinalis* Vill. The present authors consider that *Laricifomes officinalis* (Vill.) Kotlaba & Pouzar is the correct valid name for the famous medicinal fungus *Fom. officinalis*.

As also indicated in our phylogenetic analyses of nuc-LSU rDNA sequences, only terminal clades were strongly or moderately supported, and the majority of relationships below this level are still not clearly resolved (Figures 1 and 2). More characters (preferably from unlinked loci) may be required to resolve the relationships in the future.

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分析核糖體大亞基核酸序列研究薄孔菌屬 (*Antrodia*) 種類與相關分類群的系統關係

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本研究旨在評估薄孔菌屬 (*Antrodia*) 種類與相關分類群的關係，包含一些曾被處理為其他屬（粉孔菌屬 (*Amyloporia*)，絲孔菌屬 (*Fibroporia*)，台芝屬 (*Taiwanofungus*)）的薄孔菌屬種類的分類地位探討。Binder 等人在 2005 年提出的同擔子菌綱 (Homobasidiomycetes）系統學的一項廣泛性研究，為本研究選取分析類群之參考。外群取“residual”polyporoid clade 以及 phlebioid clade 的屬，內群則選取 *Antrodia* clade 以及 core polyporoid clade 的屬。藉由分析核糖大亞基核酸序列進行系統發生學研究。分析方法為「最大簡約法」(maximum parsimony)、「最大似然法」(maximum likelihoood) 以及「貝葉氏導出式分析」(Bayesian inference)，這些分析所得結果基本一致。內群包含兩個未具有高支持度的支序群，支序群 A 由 12 個薄孔菌屬的種以及迷孔菌屬 (*Daedalea*)，擬層孔菌屬 (*Fomitopsis*)，新鏡孔菌屬 (*Neolentiporus*)，滴孔菌屬 (*Piptoporus*) 等屬的種類組成，這些都隸屬於薄孔菌支序群。這 12 個薄孔菌屬的種並未聚成一次支序群，顯示它們非為單系群。12 個薄孔菌屬種類中兩種屬於絲孔菌，其識別特徵為子實體具有菌索狀邊緣，它們聚成高支持度的一群。本研究中具有粉孔菌屬類澱粉質的骨骼菌絲特徵的五種並未形成單系群。絲孔菌屬的屬級地位在本研究中得到支持，但粉孔菌屬的屬級地位則未得到支持。支序群 B 由一些屬於 *Antrodia* clade 和 core polyporoid clade 的屬組成。台芝屬的兩種在本研究中聚成一明顯的次支序群，其屬級地位得到支持。

關鍵詞： 粉孔菌屬 ； 薄孔菌屬 ； 絲孔菌屬 ； 擬層孔菌屬 ； 系統發育 ； 多孔菌 ； 台芝屬。