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ORIGINAL ARTICLE

Identification and Phylogenetic Analysis of *Antrodia camphorata* and Related Species Based on the Polymorphic D2 Region of LSU rDNA

Hua-Hsien Chiu[1,2*], Feng-Ping Lee[3], Jau-Kai Wang[1], Chih-Chung Chou[1,2]

[1]*School of Environmental and Life Sciences, Fooyin University, Kaohsiung, Taiwan*
[2]*Division of Genomic Medicine, Fooyin University Hospital, Ping Tung, Taiwan*
[3]*School of Nursing, Fooyin University, Kaohsiung, Taiwan*

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Mushroom polysaccharides are biologically active ingredients and potentially medicinally useful. In this study, we examined the medicinal fungus *Antrodia camphorata*. D2 Sequences of large subunit (LSU) ribosomal DNA (rDNA) were obtained from *A. camphorata* and related fungal taxa using the MicroSeq D2 LSU rDNA sequencing kit. This kit was used to reveal sequence similarities and phylogenetic relationships. A matrix analysis of sequence similarity for the LSU D2 region of six *A. camphorata* strains—B85, B86, B71 BCRC35396, BCRC35398, and BCRC35716—displayed 100% sequence identity. Sequence similarities of 91.1%, 86.4%, 82.4% and 83.1% were found when *Antrodia camphorata* TF971, *A. malicola*, *Antrodiella* spp., and *Trametes versicolor* were compared with *A. camphorata* B85, respectively. A phylogenetic tree with 12 strains, generated using a maximum parsimony method, did not show much difference compared with a neighbor-joining tree. According to the sequence data obtained, phylogenetic analysis allowed us to infer the phylogenetic relationships among *Antrodia*, *Antrodiella*, and *Trametes* species. Our sequence data establish a foundation for further expansion of MicroSeq D2 Fungal Database of the medically important fungus *A. camphorata*. The D2 LSU sequence polymorphisms, which contains unique alleles, can be used to provide a reliable method for characterizing wild unidentified ganodermataceae.

Key Words: *Antrodia camphorata*, LSU rDNA, medicinal fungus, phylogenetic analysis

Introduction

The medicinal fungus *Antrodia camphorata* is one of the most valuable mushrooms in Taiwan—it grows slowly on *Cinnamomum kanehirai*.[1] Traditionally, it has been used as a folk remedy in Taiwan for treating various disorders including food and drug intoxication, diarrhea, abdominal pain, hypertension, itchy skin, and liver cancer. Recent research has examined the biological activity of *A. camphorata*. The secondary metabolites of *A. camphorata* exhibit a wide range of biological activities, including antimicrobial, antiviral, antifungal, anticancer, procardiovascular, anti-inflammatory, antioxidant, and immunostimulating properties. Studies using CCl_4-intoxicated mice treated with *A. camphorata* extract have demonstrated that *A. camphorata* mycelia may have protective antioxidant effects.[2–4] *A. camphorata* mycelia has also been reported to have anticancer properties such as significant cytotoxicity against leukemia HL-60 cells.[4,5]

Mushroom polysaccharides are biologically active ingredients and potentially medicinally useful. Investigations of biologically active components of

*Corresponding author. Department of Biotechnology, Fooyin University, 151 Chin-Hsueh Road, Ta-Liao, Kaohsiung 831, Taiwan.
E-mail: sc055@mail.fy.edu.tw

A. camphorata have shown that their polysaccharides have an anti-hepatitis B virus effect.[6] In addition, extracts from cultured mycelia of *A. camphorata* display anti-inflammatory effects, by inhibiting reactive oxygen species production in human leukocytes at a pharmacologically applicable concentration.[7] Furthermore, biological studies have revealed that zhankuic acids, isolated from fruiting bodies of *A. camphorata,* exhibit anti-inflammatory activity.[8]

A. camphorata is a brown-rot fungus of the genus *Antrodia* (family: Polyporaceae, Aphyllophorales, Hymenomycetes, Basidiomycota). Chang and Chou placed this species in the *Antrodia* group for the following reasons: it has a dimitic hyphal system with generative hyphae (2.0–3.5 μm)/clamp connections, a hyaline and light brown skeletal hyphae, a width of up to 4.5 μm, and contains amyloid.[1] However, when this species was first published, it was incorrectly identified and classified.[1,9,10] In the original description, *A. camphorata* was recognized as a new *Ganoderma* species, *Ganoderma camphoratum*, due to its similar characteristics.[9] Recently, *A. camphorata* was suggested as a new name for *A. cinnamomea* since the fungus is associated with *C. kanehirai*.[11] In addition, not all *A. camphorata* strains show the same metabolic activity on the same medium. For example, the growth rates of five *A. camphorata* strains' mycelia were determined—the results showed BCRC35396 and 35398 grew faster than the others.[6] Polysaccharide profiles obtained from different strains of *A. camphorata* were found to exhibit polymorphisms.[6] Comparing the effects of mycelia extracts from five *A. camphorata* strains, the results suggest that *A. camphorata* B85 produced the strongest vasorelaxation in aortic preparations compared with five test strains.[12] Thus, accurate taxonomic identification and correct phylogenetic classification will give useful information for understanding useful genes and metabolites, and can be applied in future genetic engineering or cultivation of medicinal fungi. To date, only a few studies have focused on the molecular systematics of *Antrodia* fungi.[13] Therefore, it is necessary to further develop rapid and accurate molecular typing techniques for the identification of *A. camphorata* and related species.

Members of the genera *Antrodia, Antrodiella* and *Trametes* generally belong to the Polyporaceae family. These species show many variations in both macroscopic and microscopic characters, such as the shape of basidiocarp margins, spore morphology, the amyloidity of hyphae, sexuality, and substrate type.[14,15] Unfortunately, conventional identification methods may not accurately reflect the true fungal community in a sample. There is still no formal agreement of their nomenclature and taxonomy. In 1981, Julich placed *Antrodia*, *Antrodiella* and *Trametes* in the family Coriolaceae with genera such as *Datronia*, *Dichomitoporus*, and *Trichaptum*. However, his classification has been seriously criticized, since *Antrodia* was considered as a white rot fungus.[16] In 1989, Corner classified *Antrodia* and *Antrodiella* into a single *Tyromyces* group along with the genera *Anomoporia*, *Ceriporiopsis*, *Diplomitoporus*, *Flaviporus*, *Oligoporus* and *Postia*. Corner also grouped the *Trametes* group, with *Trametes* and 13 other genera.[17] This classification did not emphasize the rot type or pigments as much as hyphal systems. Observations suggest that transitions between hyphal states occur frequently in different phylogenetic lineages. Thus, the previously assembled *Tyromyces* or *Trametes* groups might not be a natural classification. Recently, Ryvarden classified *Antrodia* into the *Daedalea* group together with *Amylocystis*, *Daedalea*, *Auriporia*, *Fomitopsis*, *Gloeophyllum*, *Oligoporus*, *Piptoporus* and *Stiptophyllum*. This group is characterized by clamps, a di- to trimitic hyphal system, generative hyphae with a clam, spores that are oblong-ellipsoid to cylindrical, a hyaline, a thin-walled structure, a smooth surface, and non-amyloid activity. *Antrodiella* and *Trametes* were later classified into *Junghuhnia* and *Trametes* groups, respectively.[15] The evolutionary history and phylogenetic relationships of *Antrodia* species with related taxa, such as *Antrodiella* and *Trametes* fungi, remain controversial. To address these phylogenetic problems, correct taxonomy of many medicinal fungi needs to be further investigated.

Most identification of *A. camphorata* and related taxa were previously conducted on the basis of phenotypic characteristics. In recent years, the phenotypic descriptions are increasingly assessed and supplemented by molecular identification. Nucleic acid sequencing has already become an important molecular identification method applied to various taxonomic levels. The polymorphisms of the D2 variable domain of the large subunit (LSU) ribosomal DNA (rDNA) are useful for distinguishing closely related species within the genus. Recently, the MicroSeq D2 LSU rDNA Fungal Sequencing Kit (Applied Biosystems, Foster City, CA, USA) has been used to study fungal species.[18–20] After sequencing of the D2 region of LSU rDNA, a given fungus can be evaluated with the sequences on the MicroSeq D2 Fungal Database. Although this fungal database has more than 500 validated sequences from different fungal species, *Antrodia* species are not included. The goal of this study was to test MicroSeq D2 LSU rDNA Fungal Sequencing Kit for the identification of *A. camphorata* and related taxa. These data could be used to expand the MicroSeq D2 Fungal Database. Furthermore, we compared D2 LSU rDNA sequences

of *Antrodia*, *Antrodiella* and *Trametes* species and examined whether sufficient variability existed for identification at the species level. Therefore, D2 LSU sequences of *Antrodia* species were used to reveal their similarities. As well as discriminating among closely related *Antrodia* species, we sought to determine if the D2 alleles of the LSU rDNA could accurately infer the phylogenetic relationships among these related taxa.

Materials and Methods

Strains studied

A. camphorata strains BCRC35716, BCRC35396 and BCRC35398 were purchased from the Bioresource Collection and Research Center (BCRC, Taiwan). *A. camphorata* strains B85, B86, B71 and TF971 were provided by Dr. Tun-Tschu Chang from the Taiwan Forestry Institute. Four *A. camphorata* related species were obtained from BCRC, including *Antrodia malicola* BCRC35452, *Antrodiella* spp. BCRC35484, *Trametes versicolor* BCRC35683, and BCRC35644.

DNA extraction

Mycelium samples were collected from fresh fungal cells on plates of malt extract agar. Freeze-dried mycelium samples were ground in liquid nitrogen and 50 mg was transferred into a 1.5 mL microfuge tube. DNA was extracted by using plant genomic DNA extraction miniprep system (Viogene, Sunnyvale, CA, USA). Following the protocol provided by the manufacturer, DNA was purified and eluted with 100 μL of distilled water. One microliter of the DNA suspension was used for polymerase chain reaction (PCR) amplification.

PCR amplification and DNA sequencing

Amplification of the D2 region of the partial LSU rDNA was performed using the commercially available MicroSeq D2 LSU rDNA Fungal Sequencing Kit (Applied Biosystems). This kit provides all of the reagents necessary to amplify and sequence the D2 region. The D2 LSU rDNA fragment was amplified by adding 25 μL of diluted genomic DNA to 25 μL of master mix consisting of forward and reverse primers in the PCR module. PCR conditions used were as follows: 95°C for 10 minutes; 35 cycles at 95°C for 30 seconds; 53°C for 30 seconds; 72°C for 1 minute; and 72°C for 10 minutes. PCR-amplified D2 LSU segments of rRNA were purified from 2% agarose gels using the Wizard SV gel and PCR clean-up system (Promega, Madison, WI, USA) and eluted in 50 μL of nuclease-free water provided in the purification kit. Forty nanograms of purified DNA was used for direct sequencing with an automated ABI 3100 DNA sequencer in accordance with the protocol supplied by the manufacturer. These D2 LSU rDNA sequences were verified by direct sequencing with forward and reverse primers (Applied Biosystems). The raw data were processed using the Perkin-Elmer MicroSeq™ (version 1.4.3; Applied Biosystems).

Phylogenetic analysis

Multiple-sequence alignments of the D2 region of the partial LSU rDNA sequences were performed using the ClustalW software.[21] Phylogenetic and molecular evolutionary analyses were conducted using MEGA (version 2.1).[22] Phylogenetic trees were inferred from the alignments and analyzed by neighbor-joining (NJ) and maximum parsimony (MP) methods. One thousand bootstrap replicates were used to estimate the reliability of the nodes on phylogenetic trees. The distance matrix of neighbor-joining trees was calculated using Kimura's two-parameter model. The D2 LSU sequence of *Ganoderma applanatum* CBS250.61 was obtained from the MicroSeq D2 fungal library (version 1.4.2). *G. applanatum* CBS 250.61 was selected as out-group taxa.

Nucleotide sequence accession numbers

The D2 region of the partial LSU rDNA sequences of *Antrodia*, *Antrodiella* and *Trametes* species described in this report have been deposited in the GenBank database. The assigned sequence accession number are listed in Table 1 and described as follows: AY 873953, AY873954, AY873955, AY873956, AY873957, AY873958, and AY873959 (*A. camphorata* B85, B86, B71, TF971, BCRC35396, BCRC35398, and BCRC 35716, respectively); AY873962 (*Antrodiella* spp. BCRC35484), AY873963 (*A. malicola* BCRC 35452); and AY873960, AY873961 (*T. versicolor* BCRC35644 and BCRC35683, respectively).

Results

LSU D2 region features

A. camphorata and related fungal taxa were used for the comparison of DNA sequences. D2 domains of LSU rDNA PCR product lengths were determined and ranged from 290 to 304 base pairs. Length variation was considerable among species with 14-bp difference between the shortest (*Antrodiella* spp. and *T. versicolor*) and longest (*A. camphorata* TF971). Among *Antrodia*, *Antrodiella,* and *Trametes* species

Table 1 Strains analyzed in this study, their D2 large subunit sequence lengths, GenBank accession numbers, source, and nucleotide frequencies

Species name	Length (bp)	GenBank accession number	Source	Nucleotide frequencies			
				A	C	G	T
Antrodia camphorata	302	AY873953	B85	0.2483	0.1656	0.2947	0.2914
	302	AY873954	B86	0.2483	0.1656	0.2947	0.2914
	302	AY873955	B71	0.2483	0.1656	0.2947	0.2914
	302	AY873957	BCRC35396	0.2483	0.1656	0.2947	0.2914
	302	AY873958	BCRC35398	0.2483	0.1656	0.2947	0.2914
	302	AY873959	BCRC35716	0.2483	0.1656	0.2947	0.2914
	304	AY873956	TF971	0.2303	0.1875	0.2993	0.2829
Antrodia malicola	293	AY873963	BCRC35452	0.2253	0.1980	0.3242	0.2526
Antrodiella spp.	290	AY873962	BCRC35484	0.2345	0.1966	0.3069	0.2621
Trametes versicolor	290	AY873960	BCRC35644	0.2379	0.2103	0.3172	0.2345
	290	AY873961	BCRC35683	0.2379	0.2103	0.3172	0.2345

BCRC: Bioresource Collection and Research Center; bp: base pair.

of this study, G+C content ranged from 46.03% to 52.76% in D2 sequences. For example, for *A. camphorata* B85, the nucleotide frequencies of A, C, G, and T were 0.2483, 0.1656, 0.2947, and 0.2914, respectively (Table 1). Multiple alignment of fungal D2 LSU sequences demonstrated nucleotide sequence diversity due to substitution, insertions or deletions among *Antrodia* species and related fungal taxa (Figure 1). The length and nucleotide base composition of D2 LSU sequences were unique and species specific for *A. camphorata* and related fungal taxa in this study.

Analysis of sequence similarity

To evaluate the utility of D2 LSU sequences to identify true *A. camphorata*, all *A. camphorata* strains except TF971 showed identical sequences and 100% similarity (Table 2). *A. camphorata* TF971 differed by 23 nucleotide substitutions and four insertions/deletions from the sequence with above-mentioned *A. camphorata* strains (Figure 1). Table 2 shows a matrix analysis of the sequence similarity of the LSU D2 region of *Antrodia* species and related fungal taxa. A sequence similarity of 91% was found between *A. camphorata* B85 and TF971. To further test LSU D2 sequences for the identification of *Antrodia* related species, *A. malicola* was selected and sequenced in this study. The result showed that *A. malicola* BCRC35452 had a more divergent sequence. It differed in 32 nucleotide substitutions and nine insertions/deletions from *A. camphorata* B85 (Figure 1). A sequence similarity of 86.4% was observed between *A. camphorata* B85 and *A. malicola* BCRC35452 (Table 2).

Table 2 shows sequence similarities among *Antrodia*, *Antrodiella*, and *Trametes* species. One hundred percent similarity was found between *T. versicolor* BCRC35644 and BCRC35683. The comparison of *T. versicolor* and *A. camphorata* B85 showed 83.1% similarity—the sequence was the same, aside for 38 nucleotide substitutions and 12 insertions/deletions (Figure 1). A sequence similarity of 89.7% was observed between *A. malicola* and *T. versicolor*.

Antrodiella are considered to be closely related to *Antrodia* in terms of phylogeny. In Figure 1, *Antrodiella* spp. BCRC35484 exhibited a sequence similarity of 82.4% and showed the greatest diversity we found—43 nucleotide substitutions and 11 insertions/deletions compared with *A. camphorata* B85. The comparison between *A. malicola*, *T. versicolor* and *Antrodiella* spp. showed 89.0% and 86.2% similarity, respectively (Table 2).

Phylogenetic analysis

Phylogenetic relationships were analyzed by NJ and MP methods (Figures 2A, 2B, respectively) based on the studies of 304 aligned positions of the partial LSU rDNA sequences of *Antrodia*, *Antrodiella*, and *Trametes* species shown in Table 1. Systematic studies—based on morphological characteristics—have shown that the *Ganoderma* genera belongs to the family Ganodermataceae, and had a clearly distinct phylogenetic lineage with the family Polyporaceae (e.g. genera *Antrodia*, *Antrodiella*, and *Trametes*). Thus, *G. applanatum* CBS250.61 was used as out-group taxa to root the tree. Phylogenetic trees were drawn using MEGA. The phylogenetic tree used in this study contained 12 strains. It was generated by MP, and did not show much difference compared with the NJ tree. In the phylogenetic trees, shown in Figure 2, all strains of the *A. camphorata*—except TF971—were clustered into a single clade. Their relationships were supported by the highest bootstrap

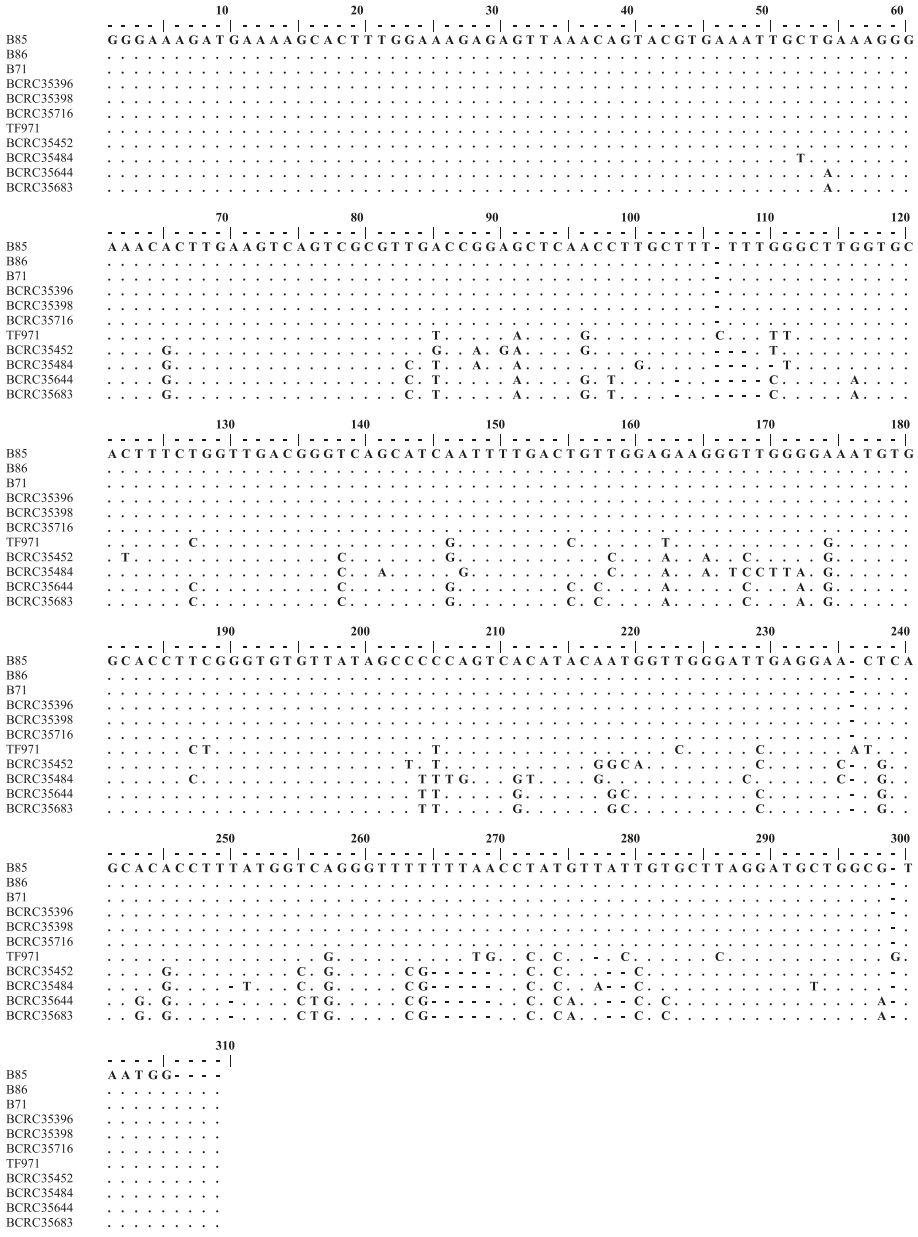

Figure 1 Multiple alignment of D2 large subunit sequences of *A. camphorata* (B85, B86, B71, BCRC35396, BCRC35398, BCRC35716, and TF971), *A. malicola* (BCRC35452), *Antrodiella* spp. (BCRC35484), and *T. versicolor* (BCRC35644 and BCRC35683). A dot designates the same base as the upper line. A dash designates an alignment gap.

value of 100%. This clade sequentially clustered with *A. camphorata* TF971, which were strongly supported by bootstrap values of 99% and 98% in NJ and MP phylogenies. It was then placed into a sister group with *A. malicola* BCRC35452 and *Antrodiella* spp. BCRC35484, which was strongly supported by 94% in NJ phylogeny. *A. malicola* BCRC35452 and *Antrodiella* spp. BCRC35484 form a clade, and this branch was weakly supported by 43% in NJ phylogeny. Similar to the *A.camphorata* strains, *T. versicolor* BCRC35644 and BCRC35683 were clustered together and supported by 100% and 99% confidence levels in NJ and MP phylogenies, respectively (Figure 2).

Discussion

Recent studies of edible and medicinal fungal materials have focused on their physiological, biochemical, and pharmacological properties. Commercial fungi have become increasingly popular as a food source and have received increasing attention by the pharmaceutical industry. Moreover, there is a great deal of fake medicinal fungi in market. Thus, identification of high-quality species of economic fungi, such as *Antrodia camphorata*, *Ganoderma* spp., and *Agaricus blazei Murill*, is of great significance. The biologically active ingredients (such as polysaccharides)

Table 2 Pair wise sequence similarity (%) of the large subunit D2 region of taxa in this study

	B85	B86	B71	BCRC 35396	BCRC 35398	BCRC 35716	TF971	BCRC 35452	BCRC 35484	BCRC 35644	BCRC 35683
B85	100	100	100	100	100	100	91.1	86.4	82.4	83.1	83.1
B86	–	100	100	100	100	100	91.1	86.4	82.4	83.1	83.1
B71	–	–	100	100	100	100	91.1	86.4	82.4	83.1	83.1
BCRC35396	–	–	–	100	100	100	91.1	86.4	82.4	83.1	83.1
BCRC35398	–	–	–	–	100	100	91.1	86.4	82.4	83.1	83.1
BCRC35716	–	–	–	–	–	100	91.1	86.4	82.4	83.1	83.1
TF971	–	–	–	–	–	–	100	85.9	81.3	83.9	83.9
BCRC35452	–	–	–	–	–	–	–	100	89.0	89.7	89.7
BCRC35484	–	–	–	–	–	–	–	–	100	86.2	86.2
BCRC35644	–	–	–	–	–	–	–	–	–	100	100
BCRC35683	–	–	–	–	–	–	–	–	–	–	100

BCRC: Bioresource Collection and Research Center.

vary among different species of medicinal fungi. Currently, *A. camphorata* is very popular in Taiwan because it contains large amounts of polysaccharides and a special type of triterpenoids. However, *A. camphorata* can easily be confused with other related *Antrodia* species such as *A. salmonea*.[11] Analysis of biologically active ingredients have shown a difference in polysaccharides profile, anti-HBV activity, and vasorelaxation activity among five *A. camphorata* strains including B85, B86, B71, BCRC35396, and BCRC35398 tested in this study.[1,9,10] Accurate taxonomic identification and correct phylogenetic classification of *A. camphorata* is necessary and important to demonstrate its pharmaceutical application. The molecular identification system outlined in this study could be used for product quality control of *A. camphorata*. From a biotechnological point of view, molecular genetic markers have important applications, including product identification and quality control of *A. camphorata* and other medicinal fungi.

In fungi, the ribosomal RNA coding cistron (rDNA) has been widely utilized for molecular systematic studies. The sequence diversity among rDNA sequence alleles has been useful for distinguishing closely related species, particularly those that contain identical DNA sequences in the D1/D2 variable domain of the LSU rDNA. The LSU rDNA is a good candidate for the elucidation of phylogenetic characters because of its large size, slowly mosaic, rapidly evolving regions, and complex secondary structure variation. Divergence in the D2 domain of the LSU rDNA sequence is generally sufficient to identify individual species. Here, we present the first study to apply the MicroSeq D2 LSU rDNA Fungal Sequencing Kit to study *A. camphorata* and related species. Our results show that polymorphisms of the D2 LSU rDNA sequences can sufficiently and accurately determine individual species. *A. camphorata* B85, B86, B71, BCRC35396, BCRC-35398, and BCRC35716 were all clearly identified as the same species according to sequence data (Figure 1). Our data are consistent with polymorphisms of the large subunit ribosomal genetic region, which allow for better differentiation between genera and species of medical fungus. Furthermore, compared with the above-mentioned *A. camphorata* strains, TF971 had only very minor differences in morphological characteristics, except a white mycelium when cultured on potato dextrose agar. However, TF971 exhibited 91% sequence similarity. Most combination of strains in the same species exhibited 99% sequence similarity or higher, and one or fewer substitution per 100 nucleotides. Based on nucleotide divergence in the D2 LSU rDNA sequences, TF971 may be misidentified as *A. camphorata*. Phylogenetic analysis confirmed that TF971

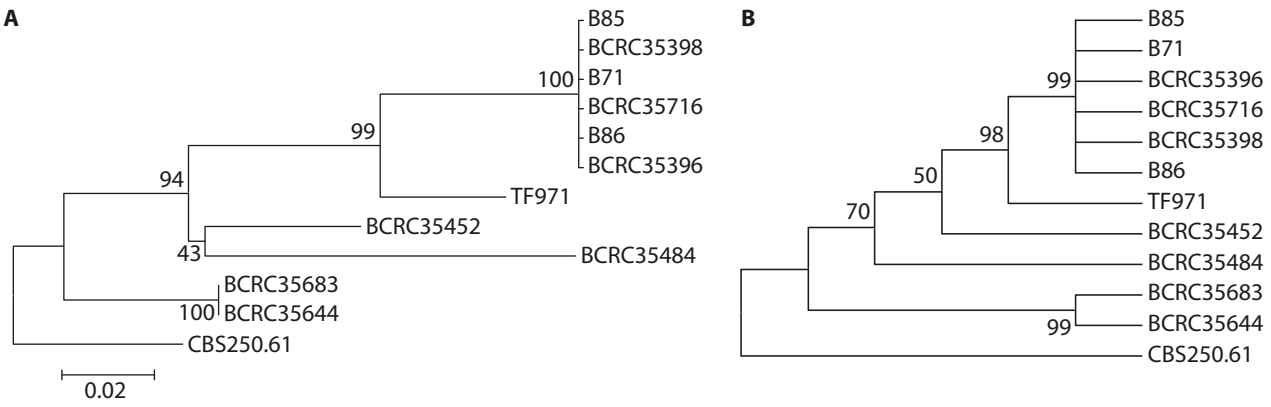

Figure 2 Phylogenetic trees were obtained from (A) neighbor-joining method, and (B) maximum parsimony method with 1000 bootstrap replicates based on studies of 304 aligned positions of the D2 region of partial large subunit (LSU) rDNA sequences of fungal strains. The D2 LSU sequences of *Antrodia camphorata* (B85, B86, B71, TF971, BCRC35396, BCRC35398, and BCRC35716), *Antrodia malicola* (BCRC35452), *Antrodiella* spp. (BCRC35484), *Trametes versicolor* (BCRC35644 and BCRC35683), and *Ganoderma applanatum* (CBS250.61) were used for tree building. The phylogenetic trees of neighbor-joining and maximum parsimony were drawn by using MEGA. GenBank accession numbers of sequences generated in this study are presented in Table 1. Numbers at the nodes indicate the bootstrap values. Lower bars indicate relative genetic distance.

exhibited a high bootstrap value and had relatively deep sublines branching from a position close to real *A. camphorata* strains. Therefore, we suggest that the TF971 strain belongs to a new single species of the genera *Antrodia*, a suggestion reported here for the first time. These findings again demonstrate that nucleic acid sequencing of the D2 LSU sequence is more accurate than morphological assessment in identification.

A finding of interest is that strains of the same species exhibited a sequence similarity of 100%. For example, 100% similarity was found among *A. camphorata* strains B85, B86, B71, BCRC35396, BCRC35398, and BCRC35716. In addition, two *T. versicolor* strains, BCRC35644 and BCRC35683, exhibited a sequence similarity of 100% (Table 2). This study suggests the similarity should be 100% between the D2 LSU sequences of the same species. Evolutionary relationships elucidated could provide an important hint for further exploration of their active compounds.

According to partial mitochondrial SSU rDNA sequences of the *Antrodia* group, phylogenetic data suggested that *Antrodia* was not a monophyletic taxon, but a heterogeneous genus.[13] Based on phylogenetic analysis (Figure 2), the results also potentially indicate that the genus *Antrodia* does not form a monophyletic clade. Our analysis showed that *A. camphorata* strains form a clade separate from *A. malicola*. According to the similarity values shown in Table 2, *A. malicola* was more closely related to white rot fungi (*Antrodiella* spp. and *T. versicolor*) than to brown rot fungi (*A. camphorata*). Previous research has shown that members of the genus *Antrodia* may form a heterogeneous group and brown rot fungi have evolved convergently.[13] Our data for D2 LSU rDNA could support these finding.

The genera *Antrodia*, *Antrodiella* and *Trametes* belong to the same *Coriolaceae* family of the order Coriolales.[16] According to the wood rot system, *Antrodia*, *Antrodiella* and *Trametes* were classified into *Daedalea*, *Junghuhnia* and *Trametes* groups, respectively.[15] When the hyphal system was used in the identification and taxonomy of polyporoid fungi, *Antrodia* and *Antrodiella* were classified into the *Tyromyces* group, and *Trametes* was classified into the *Daedaleopsis* group.[17] According to this report, *Antrodia*, *Antrodiella* and *Trametes* species can be distinguished on the basis of multiple nucleotide polymorphic sites (Figure 1). *Antrodiella* spp. is more closely related to the *Antrodia* group than *T. versicolor*—a finding based on the phylogenetic analysis of their D2 LSU sequences. In Figure 2, the positions of *Antrodia* and related species in NJ and MP analysis trees confirm in part the traditional classification based on hyphal systems. Basidiocarps of these two genera—*Antrodia* and *Antrodiella*—are similar and they all have dimitic hyphal systems with clamped generative hyphae. The main difference is the type of wood rot. *Antrodiella* show white rot activity while members of the genus *Antrodia* show brown rot fungi.[14,17] Hyphal systems have been widely used in the identification and taxonomy of polyporoid fungi. Thus, *Antrodiella* was regarded as closely related to *Antrodia*. In addition, the relatively slow rate of molecular evolution makes the D2 region a good candidate for finding consensus-conserved sequences suitable for genus or higher taxonomic level detections.

On the other hand, the clades containing *Antrodiella* taxa did not show any particular relationships with the species of *Antrodia*—a finding based on the phylogenetic tree of partial mitochondrial small subunit rDNA sequences.[13] Most species relationships highlighted in this report were highly concordant between the traditional classification and molecular analysis—the research was also statistically well supported. However, the branch of *A. malicola* and *Antrodiella* spp. was poorly supported, with a 43% in NJ phylogeny. According to the similarity values shown in Table 2, *Antrodiella* spp. was positioned more closely to *T. versicolor* than *A. camphorata*. These results are in accordance with conventional classification. Based on morphological classification, the wood rot type of three genera are different. *Antrodiella* and *Trametes* groups show white rot activity, and members of the genus *Antrodia* show brown rot fungi.

The D2 region of LSU rDNA has been used as a target for phylogenetic analysis because it displays sequence variation between species.[18–20] Our sequence data establish a foundation for further expansion of the MicroSeq D2 Fungal Database. This can be used to identify medically important fungi. According to the sequence data from the D2 region of LSU rDNA, the phylogenetic analysis used in this study allowed us to determine species related to *Antrodia*, and to distinguish *Antrodia*, *Antrodiella*, and *Trametes* genera. Our D2 LSU sequence, which contains unique alleles, can be used to provide a reliable and efficient method for the characterization of previously unidentified organisms. Investigations are in progress to determine whether other medicinal fungus, including *Ganoderma* species, can be identified using the MicroSeq D2 LSU rDNA Fungal Sequencing Kit.

We conclude that the D2 domain of LSU rDNA can be exploited to development molecular markers for the rapid detection and identification of previously un-described species and pathogens isolated from the environment.

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