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Phylogenetic analysis of *Antrodia* species and *Antrodia camphorata* inferred from internal transcribed spacer region

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Abstract The species of *Antrodia* are one of the difficult-to-classify and obscure groups of poroid Aphyllophorales based on morphological appearance. However, it is becoming increasingly important to reliably identify the entire suite of *Antrodia camphorata* strains and *Antrodia* species due to the potential pharmaceutical value of their biologically active ingredients. In this study, the internal transcribed spacer (ITS) region of the ribosomal RNA gene (rDNA) was sequenced and phylogenetically analyzed in a number of *Antrodia* fungal species and strains. ITS amplicons from the *Antrodia* species tested ranged in size from 543 to 610 bp; the size of the ITS of *A. camphorata* strains ranged from 592 to 596 bp. The overall sizes of ITS2 and 5.8S ribosomal RNA gene of all *A. camphorata* strains tested in this study were shown to be 217 and 158 bp, respectively. A phylogenetic analysis of ITS data generated, which included sequences of 11 *A. camphorata* strains and nine other *Antrodia* species, showed three clearly distinct groups. Group 1 includes *A. camphorata*, *Antrodia salmonea*, and *Antrodia carbinca* strains. Within Group 2, *Antrodia sinuosa* and *Antrodia xantha* were clustered together. Group 3 contained *Antrodia albida*, *A. heteromorpha*, *A. serialis*, and *A. malicola*. The observed sequence diversity among ITS alleles provided an effective tool for differentiating strains of *A. camphorata*, *A. salmonea*, *A. xantha*, *A. sinuosa*, or *A. serialis*. Polymorphisms arising within the ITS1-5.8S-ITS2 region can provide practical markers for establishing a foundation for the further expansion of an ITS sequence database of medically important fungi.

Keywords *Antrodia* fungi · ITS · Polymorphisms · Ribosomal RNA gene

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Introduction

Antrodia is a relatively well-known genus among poroid wood-rotting fungi, with more than 40 described species (Dai and Niemela 2002). The genus is characterized by a dimitic hyphal system with clamped generative hyphae and a white or pale context, and by the production of a brown rot in its host plant (Dai and Niemela 2002; Ryvarden 1991). However, the nomenclature and taxonomy of its members is confusing at best and would still not appear to be taxonomically uniform (Ryvarden 1991). *Antrodia camphorata*, which has only been identified since 1994, typically grows slowly and, in Taiwan, only on the brown heartwood of the endemic aromatic tree *Cinnamomum kanehirai* (Hay) (Lauraceae). Its distinctive

morphological feature is the production of either red or red-orange to light-cinnamon basidiomata (Chang and Chou 1995; Wu et al. 1997). The existence of this species has brought into question the taxonomical designation of the species currently designated *Antrodia cinnamomea*, given that the host tree of this latter species is *Ci. kanehirai* rather than *Cinnamomum camphora* (L.) Presl. *A. cinnamomea* has been suggested to stand as the correct name for the fungus associated with *Ci. kanehirai* (Chang and Chou 2004). However, phylogenetic analysis based on sequence data derived from large ribosomal subunit (LSU) sequences of ribosomal RNA gene (rDNA) indicates that *A. camphorata* is distantly related to other species in *Antrodia* and, consequently, transferred to the new genus *Taiwanofungus* (Wu et al. 2004). Because this fungus is rich in polysaccharides and a special type of triterpenoid (Cherng et al. 1996), *A. camphorata* has been one of the most-valued medicinal fungi in Taiwan, particularly as a folk remedy for treating various human disorders. Recent studies have reported that the biologically active ingredients of *A. camphorata* mycelia may feature protective antioxidant (Song and Yen 2002) and anticancer properties (Hseu et al. 2002; Song and Yen 2003).

To the best of our knowledge, current studies pertaining to *A. camphorata* strains have focused on the strains' physiological, biochemical, and pharmacological properties (Cheng et al. 2005; Huang et al. 2005; Lee et al. 2002; Shen et al. 2004; Song et al. 2005; Wang et al. 2003). Compositional analysis of polysaccharides or lipopolysaccharide showed differences in the gel profiles and carbohydrate components among different *A. camphorata* strains (Cheng et al. 2005; Lee et al. 2002). An abundant number of different polysaccharide species from the fruiting bodies of such species have been identified in strains B85, B86, and BCRC35398. In addition, polysaccharides from *A. camphorata* isolates (B71, B85, B86, BCRC35396, and BCRC35398) appear to show varying levels of activity against the anti-hepatitis B virus (HBV) (Lee et al. 2002). In a study aimed at examining the effects of mycelial extracts from five different *A. camphorata* strains on vascular tension, Wang and his associates (2003) discovered that strain B85 produced the strongest vasorelaxation of the aorta among the five strains of *A. camphorata* tested.

Conventional identification of *A. camphorata* isolates is difficult because most show a basic uniformity in both microscopic and colonial appearance, although variations in colony morphology of cultured *A. camphorata* isolates do exist. These apparent strain differences are often not stable upon subculture, and they may simply be artifacts due to specific growth-condition requirements for the colony or may arise solely due to the presence of contaminating bacteria. It has been suggested that polymorphism analysis of internal transcribed spacer (ITS) regions of the ribosomal RNA gene is a valuable technique for both strain typing and species identification for a number of important fungi (Beltrame-Botelho et al. 2005; Healy et al. 2004; Tanabe et al. 2004). However, little use has been made of this technique in the study of phylogeny of genus *Antrodia*. The goal of this study was to compare the ITS1-5.8S–ITS2 region of the medically important *Antrodia* species and to determine whether there is sufficient variability in the sequence of this region to be used in phylogenetic analyses which would enable identification the members of this genus at the species or strain level. As such, any sequence polymorphism that was found within the ITS1-5.8S-ITS2 region was investigated with respect to whether it was able to provide practical markers which would facilitate the discovery of a potentially novel mushroom. A secondary aim was to establish the foundation for further expansion of an ITS sequence database for medically important fungi.

Material and methods

Source of fungal material

Four isolates of the *Antrodia* species were purchased from the Bioresource Collection and Research Center (BCRC, Taiwan) – *A. camphorata* BCRC35716, 35396, and 35398 and *Antrodia malicola* BCRC35452. Four isolates of *A. camphorata* – B85, B86, B71, and TF971 – were

kindly provided by Dr. Tun-Tschu Chang (Division of Forest Protection, Taiwan Forest Research Institute, Hsinchu, Taiwan). Two isolates of *A. camphorata* CH101 and CH102 were collected from the northern and southern regions of Taiwan, respectively.

DNA extraction

The mycelium sample was collected from fresh fungal cells on the culture plates of malt extract agar (MEA). The freeze-dried mycelium sample was ground in liquid nitrogen and the resultant powder (50 mg) transferred into a 1.5-ml microfuge tube. Total genomic DNA was extracted using a plant genomic DNA extraction mini prep system (Viogene, Sunnyvale, Calif.). The DNA was purified following the protocol provided by the manufacturer and eluted with 100 µl of distilled water. One microliter of the DNA suspension was used for PCR amplification.

PCR amplification and DNA sequencing

Amplification of the ribosomal ITS regions ITS1 and ITS2 flanking the 5.8S subunit was performed using a pair of primers –ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) – as described previously, with some modification (White et al. 1990). In addition, primers ITS1R (5′-GCTGCGTTCTTCATCGATGC-3′) and ITS4F (5′-GCATCGATGAAGAACGCAGC-3′) directed to the 5.8S sequence were used for separate amplification of each ITS spacer and also for sequencing. The amplifications were performed in a Gene Amp PCR System 9700 (Applied Biosystems, Foster City, Calif.) set at the following profile: an initial step at 95°C for 5 min; 35 cycles at 95°C for 30 s, 54°C for 30s, and 72°C for 45s; a final extension step at 72°C for 10 min. The PCR-amplified nuclear ITS ribosomal RNA gene was purified from 2% agarose gel using the Qiagen Gel Extraction kit (Qiagen, Valencia, Calif.) and sequenced directly. Sequence analysis was carried out using the BigDye Terminator Cycle Sequencing Reaction kit (Applied Biosystems) according to the manufacturer's protocol on an automated ABI Prism 3100 DNA Sequencer (Applied Biosystems).

Phylogenetic analysis

Relevant ITS sequences derived from the GenBank database and also from the results of this study (Table 1) were analyzed using CLUSTAL W software (Thompson et al. 1994) and implemented using BIOEDIT 5.0.9 (Hall 1999). The boundaries of the ITS1, 5.8S, and ITS2 sequences were determined by comparison with sequences of *A. camphorata* available in GenBank (accession no. AY37084; strain BRRC35396). Gaps were introduced into the sequences to increase their aligned similarity. Phylogenetic and molecular evolutionary analyses were conducted using MEGA software (ver. 3.1) (Kumar et al. 2001). Phylogenetic trees were inferred from the alignments and analyzed by the neighbor-joining (NJ) method. One thousand bootstrap replicates were used to estimate the reliabilities of the nodes on phylogenetic trees. The distance matrix of neighbor-joining tree was calculated using Kimura's two-parameter model (Kimura 1980). *Ganoderma applanatum* OE234 was selected as outgroup taxa.

Nucleotide sequence accession numbers

The ITS1-5.8S-ITS2 gene complex sequences of the various *Antrodia* species described in this report have been deposited in the GenBank database. The assigned sequence accession numbers are listed in Table 1 and described as follows: DQ013295, DQ013296, DQ013297, DQ455775 and DQ455776 (*A. camphorata* B71, CH101, CH102, B86 and BCRC35716, respectively); DQ013298 (*Antrodia salmonea* TF971); DQ013299 (*A. malicola* BCRC 35452).

Results

ITS length polymorphism analysis

Sequencing of the ITS region provided complete sequences for ITS 1, the 5.8S rRNA gene, and ITS 2 and provided partial sequences for the 18S (28 nucleotides) and 26S (32 nucleotides) rRNA genes. The 5.8S rRNA gene sequence of *Antrodia* species had minimal impact on the overall comparison since there is little interspecies variation

Table 1 Strains examined in this study, ITS length and GenBank accession number

Species of *Antrodia* studied	Strain no.	Length (base pair)				GenBank accession no.	Host
		Total	ITS1	5.8S	ITS2		
A. camphorata	B85	596	221	158	217	AJ496403	*Cinnamomum kanehirai*
	B86[a]	593	218	158	217	DQ455775	
	BRRC[b]35396	593	218	158	217	AY378094	
	BCRC35398	593	218	158	217	AY378095	
	BCRC35716[a]	593	218	158	217	DQ455776	
	B71[a]	593	218	158	217	DQ013295	
	CH101[a]	593	218	158	217	DQ013296	
	CH102[a]	593	218	158	217	DQ013297	
	P-1	593	218	158	217	AJ496398	
	A-1	593	218	158	217	AJ496295	
	#96	592	217	158	217	AJ496405	
A. salmonea	TF971[a,c]	609	226	158	225	DQ013298	*Cunninghamia konishii*
	U-1[d]	610	226	158	226	AJ496399	
A. malicola	BCRC35452[a]	577	203	158	216	DQ013299	*Pinus*
A. xantha	P209	566	191	158	210	AJ415569	*Pinus* sp.
	43	570	190	158	222	AXA6681	
A. carbinca	KHH-2001	572	190	158	224	AF423113	Not available
A. sinuosa	P115	570	190	158	221	AJ416068	*Tsuga canadensis*
	RLG1182R	577	190	158	229	AY966450	
A. serialis	P213	543	193	158	192	AJ344139	*Timber*
	P287	556	192	158	206	AJ345010	
A. vaillantii	4491	574	198	158	218	AY673075	*Salmon Bean*
A. albida	31	553	193	158	202	AJ006680	*Phoenix*
A. heteromorpha	M103	556	195	158	203	AF533964	*Picea abies*

[a] Strains newly sequenced in the study

[b] BCRC, Bioresource Collection and Research Center

[c] *A. camphorata* TF971 (accepted into GenBank as *A. salmonea*)

[d] Originally submitted to GenBank as *Antrodia camphorata*

in this subunit. The PCR product lengths of the ITS1-5.8S-ITS2 regions of *Antrodia* species were found to range from 543 to 610 bp (Table 1); consequently, there was a considerable variation in lengths, with a 67 bp difference between the shortest (*Antrodia serialis*) and the longest (*A. salmonea*). The overall length of the 5.8S region of all *Antrodia* species examined in this study was 158 bp. The size of the ITS1 spacer region for the *Antrodia* taxa ranged from 190 to 226 bp, and the corresponding size for the ITS2 spacer region ranged from 192 to 226 bp.

The size of the ITS of the *A. camphorata* strains ranged from 592 to 596 bp (Table 1). No variations in the lengths of the ITS2 and 5.8S regions were found among the different *A. camphorata* strains. The overall size of the ITS2 and 5.8S ribosomal RNA gene of all *A. camphorata* strains studied was, respectively, 217 and 158 bp. Sequence analysis of the ITS1 spacers among the different *A. camphorata* strains revealed sequence variability, with sizes ranging from 217 to 221 bp. *A. camphorata* #96 featured the shortest ITS1, namely 217 bp. The length of the sequence of the ITS1 regions of various *A. camphorata* strains from our newly sequenced isolates, including B71, CH101, CH102, BCRC35396, BCRC35398, BCRC35716 and B86, and from the GenBank database data, including P-1 and A-1, was always the same: 218 bp. Based upon our newly sequenced data and GenBank data, *A. camphorata* B85 featured the longest ITS1 spacer, 221 bp (Table 1).

ITS-based phylogeny of *Antrodia* species

The phylogenetic analysis, which included the sequences of 11 *A. camphorata* strains and nine other *Antrodia* species, showed three clearly distinguished groups (Fig. 1). Group 1 includes the *Antrodia camphorata*, *A. salmonea*, and

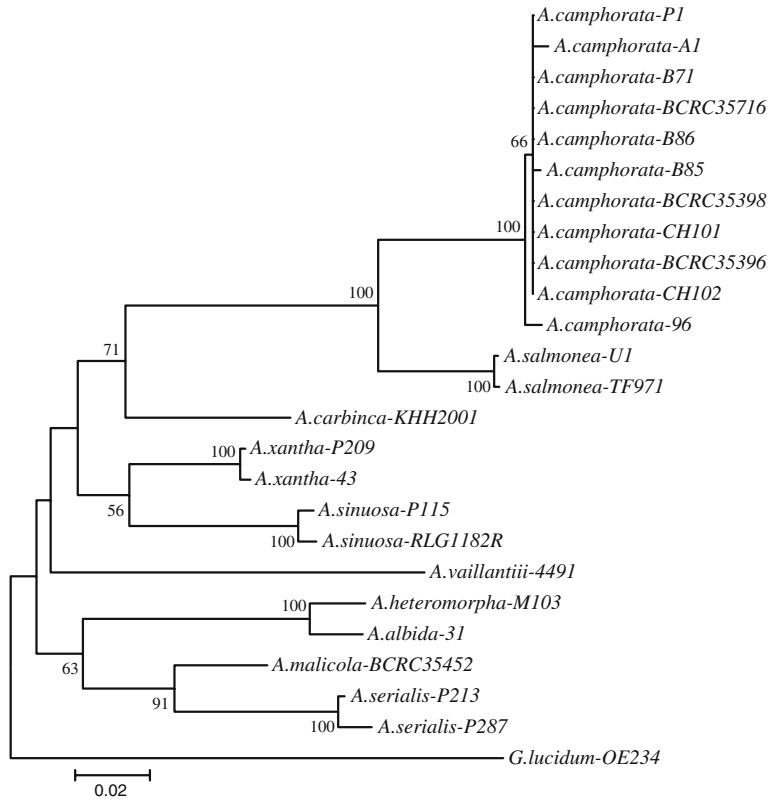

Fig. 1 Phylogenetic trees were inferred from the multiple-sequence alignments by the neighbor-joining (NJ) method. The distance matrix was calculated by use of Kimura's two-parameter model (Kimura 1980). The phylogenetic analysis which included the sequences of 11 *Antrodia camphorata* strains and nine other *Antrodia* species showed three clearly distinguished groups. *A. vaillantii* does not cluster together with *Antrodia* species of this study. *Ganoderma lucidum* was chosen as the outgroup. *Numbers* at the nodes indicate the bootstrap values. *Lower bars* indicate relative genetic distance

A. carbinca strains. This group is supported with a bootstrap value of 71% and contains the *A. camphorata* and *A. salmonea* subgroups supported by a bootstrap value of 100%. The former subgroup contains 11 *A. camphorata* strains. A comparison of the herein-described sequences of ITS sequences for other strains of *A. camphorata* with that of the *A. camphorata* B85 strain obtained from the GenBank database revealed nucleotide sequence polymorphisms for the ITS1-5.8S-ITS2 regions. This strain (B85) showed a 98.4–99.3% similarity with other strains of *A. camphorata* (BCRC35716, BCRC35398, P-1, B71, CH102, B86,CH101, BCRC35396, A-1, and #96) and a significant divergence in its ITS sequence for *A. salmonea*. The comparison of *A. camphorata* BCRC35716 with *A. camphorata* BCRC35398, P-1, B71, and CH102 revealed an identical sequence, with 100% similarity for the five strains; this was also the case for the comparison of B86 with CH101 and BCRC35396. The second subgroup of Group 1 includes the *A. salmonea* U-1 and TF971. Our results of phylogenetic analysis clearly demonstrate that *A. salmonea* and *A. camphorata* are two distinct species based on ITS sequence analysis. A comparison of the *A. camphorata* B85 strain with *A. salmonea* U-1 and TF971 revealed an 82.8 and 82.6% similarity, respectively. The comparison of *A. carbinca* with *A. camphorata* B85 revealed a 71.7% similarity.

Antrodia sinuosa and *Antrodia xantha* were clustered together within Group 2. This group contains two subgroups supported by a bootstrap value of 56%. One of these subgroups contains *A. sinuosa* P115 and RLG1182R strains, well supported by a bootstrap value of 100%. The other subgroup includes *A. xantha* P209 and 43 strains, also supported by a bootstrap value of 100%. The comparison of *A. xantha*, and *A. sinuosa* with *A. camphorata* B85 revealed 71.0 and 68.8% similarity, respectively. Group 3 includes two subgroups that are supported with a bootstrap value of 63%. One subgroup of group 3 consisting of *Antrodia albida* and *Antrodia heteromorpha* is well supported by a bootstrap value of 100%; the other subgroup contains *A. serialis* and

A. malicola with a bootstrap stability of 90%. The *A. serialis* P213 and P287 strains are well supported by a bootstrap value of 100%. The comparison of *A. malicola*, *A. albida*, *A. heteromopha*, and *A. serialis* with *A. camphorata* B85 revealed 69.3, 67.2, 66.9, and 66.3% similarity, respectively. *Antrodia vaillantii* could not be consistently determined using ITS-based phylogeny. A sequence similarity of 67.2% was found to exist between *A. camphorata* B85 and *A. vaillantii*.

Species identification and strain typing

A comparative analysis of the ITS sequence alignments for the herein studied *Antrodia* fungi was carried out using CLUSTAL W software (Thompson et al. 1994). This analysis revealed from four to six, from 67 to 104, and from 35 to 100 variable sites for the 5.8S region and the ITS1 and ITS2 spacers, respectively. An examination of Fig. 1 shows that *A. salmonea* is the most closely related strain to *A. camphorata*, with a total of 106 single nucleotide alterations in the ITS1-5.8S-ITS2 complex. These single nucleotide alterations included 30 nucleotide substitutions and 37 insertions/deletions for the ITS1 spacer, and 20 nucleotide substitutions and 15 insertions/deletions for the ITS2 spacer; there were four nucleotide substitutions and no insertions/deletions for the 5.8S region. A comparison of *A. serialis* and *A. camphorata* B85 revealed the greatest level of ITS1 sequence divergence – 60 nucleotide substitutions and 44 insertions/deletions. When the ITS2 sequence of *A. camphorata* B85 was compared with those of the different *Antrodia* species investigated in this study, *A. carbinca* (ITS2, 49 nucleotide substitutions and 51 insertions/deletions) and *A. vaillantii*, (ITS2, 63 nucleotide substitutions and 37 insertions/deletions) were revealed to have the greatest number of single nucleotide alterations in the ITS2 regions.

Interestingly, the B85 strain exhibited the greatest number of divergent ITS1 sequences of all *A. camphorata* strains examined. A comparison of the CTC bases located at position 64–66 within the ITS1 region of the B85 strain revealed that the deletions of these three-base sequences also occurred in the other strains of *A. camphorata* investigation. In addition to these differences within the ITS1 domain, the sequence comparison of the B85 strain with the #96 strain exhibited single nucleotide polymorphisms (SNPs), including a single base deletion at position 46 and T/C changes at position 166 within the ITS1 region. Although the size of the 5.8S ribosomal RNA gene for all *A. camphorata* strains was 158 bp, sequence analysis of the 5.8S region revealed SNPs characterized by base alterations: relative to the B85 strain, strain #96 exhibited an A/G change at positions 234 and 267 within the 5.8S region while strain A-1 featured a G/T change at position 263. For the ITS2 region, when compared to the B85 strain, only three strains of *A. camphorata*, namely BCRC35396, B86, and CH101, demonstrated a mononucleotide alternation featuring a T/C change at nucleotide position 446.

Discussion

It has often been reported that the ITS regions of the rRNA gene are often highly variable with respect to nucleotide composition and that this characteristic can be used to distinguish both morphologically distinct fungal species and strains of the same fungal species (Beltrame-Botelho et al. 2005; Healy et al. 2004; Tanabe et al. 2004). As such, the ITS region of the rRNA gene is generally believed to represent a convenient target for the molecular identification of specific species of fungi. The aim of the present investigation was to determine the lengths and phylogenetic relationships of the ITS1-5.8S-ITS2 of the rRNA gene of various members of the genus *Antrodia* and, subsequently, to determine the relative ''relatedness'' of various different strains of *A. camphorata*. In doing so, various *Antrodia* species were identified (Table 1). The results obtained herein on sequence polymorphisms for various *A. camphorata* strains (B86 and BCRC35716) represent – in the opinion of this author – the correct ITS lengths as compared to the corresponding data listed in the GenBank registry. The differences between the two data sets may be due to differences in the analytical methods used to determine ITS length.

On the basis of the data presented in Table 1, size polymorphism of the ITS1 and ITS2 spacers appears to indicate reasonably substantial levels of species-specific divergence for these regions in various *Antrodia* species, including *A. camphorata*. The comparative analysis of both ITS spacers of

the *A. camphorata* strains or *A. salmonea* strains included in this study indicates the presence, in these regions, of a strain-specific polymorphism. The results also suggest that a high degree of size conservation of the rRNA gene's ITS region is present within strains of the *A. camphorata* and *A. salmonea*, respectively. No variations in the length of the 5.8S region and the ITS2 spacer were detected for any of the strains of *A. camphorata* studied herein. In contrast,, the nucleotide length of the ITS1 spacer was found to vary considerably between different *A. camphorata* strains, especially the B85 strain, when compared to other strains. Consequently, length polymorphisms of the ITS1 and ITS2 regions were revealed to be an informative tool for differentiating among closely related *Antrodia* species (Table 1).

With respect to the determination of phylogenetic relationships, sequence polymorphisms of the ITS region enabled the identification of species-specific alleles for closely related *Antrodia* species and even of strain-specific alternations of *A. camphorata* strains which feature differences as slight as a single nucleotide. Therefore, sequence variability in the ITS1-5.8S–ITS2 region of the rRNA gene appears to provide a potential tool for discriminating interstrain differences at the subspecies level (Fig. 1). A double check of the sequences of *A. camphorata* strains B85, BCRC35396, and BCRC35398 described here confirmed that both the ITS1-5.8S-ITS2 regions obtained from the GenBank registry and our sequence data are coincident as regards both size (Table 1) and their phylogenetic relationships (Fig. 1). Moreover, the ITS regions of the rRNA gene sequences examined were verified by direct sequencing using forward and reverse primers designed for this study. This author is confident that he will be able to effectively use the results of the newly sequenced ITS regions for *A. camphorata* strains B86 and BCRC35716 for sequence analysis, as has been undertaken in this study, rather than simply using corresponding data derived from the GenBank registry (Table 1). According to Fig. 1, strain #96 of *A. camphorata* shows less similarity to the other strains of *A. camphorata* examined in this study (sequence similarity between strain B85 and strain #96 is approximately 98.4%). This result suggests that the details of the currently specified ITS sequence for strain #96 of *A. camphorata* featured in the GenBank database may need to be reviewed. If the current GenBank-specified description of *A. camphorata* strain #96 is, in fact, incorrect, a future investigation aimed at defining the compositions of the biologically active ingredients of strain #96 will be an interesting prospect.

Based upon the sequence length of ITS1 region,*A. camphorata* strain B85 was the most distant of all *A. camphorata* strains examined in this study (Table 1). The B85 strain has been reported to elicit the strongest vasorelaxation of the aorta among a group of five strains of *A. camphorata* tested (Wang et al. 2003). In addition, Lee et al. (2002) found that the relative anti-HBV activities of certain polysaccharides derived from various strains of *A. camphorata* (B71, B85, B86, BCRC35396, and BCRC35398) did vary somewhat, with the B86 strain exhibiting the greatest level of anti-hepatitis B surface-antigen effect of all the strains compared. Strain-specific variations in *A. camphorata* appear to be located in the ITS1-5.8S-ITS2 region, and the results of this study with respect to the presence of SNPs in the ITS regions reflect their phylogenetic relationships (Fig. 1) and well as provide the first molecular evidence of effective strain differentiation for *A. camphorata*. This is also the case for the *A. salmonea*, *A. xantha*, *A. sinuosa*, or *A. serialis* strains based on their phylogenetic relationships (Fig. 1). The presence of a molecular technique that is capable of determining such alterations, especially those between the different *A. camphorata* strains tested, is the first step in assessing the biologically active ingredients derived from the different strains of *A. camphorata*, and in being able to use such ingredients for future pharmaceutical purposes.

The ten species studied here could be easily differentiated on the basis of nucleotide base alternations by reference to either the ITS1 or ITS2 sequence. The observed sequence diversity among ITS alleles noted herein was useful in distinguishing between closely related *A. camphorata* species, particularly those that featured almost identical phenotypes. The ITS regions of the rRNA gene have also been used as targets for SNP

analysis in other organisms (Beltrame-Botelho et al. 2005; Healy et al. 2004; Hermosa et al. 2000; Lee et al. 1998): a certain level of sequence variation in the ITS sequence was typically found between species, although only minor variations were observed within strains of the same species (Henry et al. 2000). Based on these and the present results the ITS sequence would appear to provide a reliable means by which to rapidly identify known *Antrodia* species, facilitate the discovery of potentially novel *Antrodia* species, and establish a foundation for further expansion of an ITS sequence database of medically important fungi. For example, Shiang-Shan-Chih a fungus which is collected from the *Cu. konishii* tree, is very similar to *A. camphorata* (also called Niu-Chang-Chih). Recent studies of incompatible mating systems have suggested that Shiang-Shan-Chih be renamed *A. salmonea* and demonstrated that *A. salmonea* and *A. camphorata* are two distinct species of *Antrodia* fungus (Chang and Chou 2004). *A. camphorata* and *A. salmonea* are new combinations for two species of *Taiwanofungus* based on their morphological features for species identification (Wu et al. 2004). As illustrated by the data presented in Fig. 1, a sequence similarity of about 82% exists for these two species; consequently, our ITS results confirm that *A. camphorata* and *A. salmonea* are two closely related *Antrodia* species. This is the first time that such molecular typing techniques have been used to define the phylogenetic relationships between *A. camphorata* and *A. salmonea*. Based upon the results of this study, and although the sequence details of *A. salmonea* strain U-1, a ''Shiang-Shan-Chih'', was originally – and mistakenly – submitted to the GenBank database as the sequence for *A. camphorata*, this U-1 strain should be clearly recognized as *A. salmonea* and not confused with *A. camphorata*. Furthermore, our ITS sequence for TF971 proved to be virtually identical with the corresponding sequence for *A. salmonea* U-1, with the two exhibiting a sequence similarity of 99.6%.

The wood-rot system and the hyphal system, two important morphological characteristics, can be applied to a genera level for the identification of *Antrodia* species (Dai and Niemela 2002; Ryvarden 1991). Phylogenetic analysis based on sequence data derived from LSU sequences of the rRNA gene recently showed that *A. camphorata* is distantly related to *A. malicola*, *A. juniperina*, *A. variiformis*, and *A. albida* and transferred to the new genus *Taiwanofungus* (Wu et al. 2004). However, it is evident that genus *Antrodia* forms a heterogeneous group based on the phylogenetic analysis of ITS rDNA sequence (Fig. 1) and short (S)SU rDNA sequence (Kim et al. 2003). One monophyletic *Antrodia* group consists of *A. malicola*, *A. juniperina*, *A. variiformis*, and *A. albida*, which may be distantly related to another distinct monophyletic *Antrodia* group that includes *A. camphorata*, *A. carbinca*, *A. salmonea*, *A. xantha*, and *A. sinuosa*. In addition, information provided from the LSU sequences may be reduced due to the limited *Antrodia* species (Wu et al. 2004), and may influence the ability to decipher the phylogenetic relationship of the Polyporaceae family (Aphyllophorales) conclusively. Thus, it may be advisable in a comparative study to include a combination of more diverse sets of rDNA sequences from different monophyletic *Antrodia* groups in order to generate a more precise and comprehensive phylogenetic tree. The *Antrodia* genus is characterized by a di/trimitic hyphal system, and members of this genus have generative hyphae with clamps. Unfortunately, such conventional identification methods for species identification may not accurately reflect the true nomenclature and taxonomy of the *Antrodia* genus at the species level. According to our data and those of Kim et al. (2003), the identification of *Antrodia* species based on morphological characteristics is not necessarily fully concordant with their identification via ITS nucleotide alternations with respect to their phylogenetic relationships (Fig. 1). Many species featuring sexual reproduction in the genus *Antrodia* species are characterized by a heterothallic bipolar mating system, although *A. vaillantii* and *A. salmonea* exhibit a heterothallic tetrapolar mating system, and *A. malicola* is exceptional in that it features homothallic sexuality (Kim et al. 2003). Correspondingly, the analysis of sequence similarity in the ITS region between *A. vaillantii* and *A. salmonea* (65.7% sequence similarity) revealed a lower level of similarity than is the case for *A. vaillantii* and *A. malicola* (71.0% sequence similarity).

In conclusion, the analysis of the ITS region of the nuclear rRNA gene with respect to species identification can be considered to be a supplementary and effective tool to morphological analyses. Overall, our ITS sequence data provide a reliable means with which to clearly identify *Antrodia* species and even provide effective within-species comparison/identification. In addition, it is a potential tool for facilitating the discovery of potentially novel relevant mushroom species, thereby establishing a foundation for further expansion of an ITS sequence database for medically important fungal species.

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