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Project Title: Relationship of the genus *Savoryella* (teleomorph ascomycete) and its anamorph *Canalisporium*, as inferred by multiple gene phylogenies

(BRT R_252057)

Submitted by: Mr. Nattawut Boonyuen*, Miss Charuwan Chuaseeharonnachai and Professor E.B. Gareth Jones

BIOTEC-Mycology, Bioresources Technology Unit, National Center for Genetic Engineering and Biotechnology, 113 Paholyothin Rd., Klong 1, Klong Luang, Pathumthani 12120 THAILAND

***E-mail: Nattawut@biotec.or.th**

Tel: 66 2564 6700 ext. 3204

Fax: 66 2564 6707

SUMMARY

The taxonomic placement of freshwater and marine *Savoryella* species has been widely debated and no their anamorphs have been reported. This study incorporates individual phylogenetic datasets and a combined dataset, based on the small subunit rDNA (SSU), large subunit rDNA (LSU), to determine the ordinal position of the genera *Ascotaiwania*, *Canalisporium* and *Savoryella*, all based on strains isolated from Thai substrata. Other genes sequenced include ITS region, the partial RPB1 (DNA dependent RNA polymerase II largest subunit), the partial RPB2 (DNA dependent RNA polymerase II largest subunit) and translation elongation factor (EF1-α) genes to further elucidate the phylogeny of the genera *Ascotaiwania*, *Canalisporium* and *Savoryella* (ACS clade). These data provide a comprehensive view of their phylogenies and reveal a new lineage of aquatic Ascomycota.

In this study, the ascomycete *Ascotaiwania* which is morphologically similar to *Savoryella*, was included in the study. *Ascotaiwania* is characterized by ascospores that are generally more than 3-septate with hyaline end cells, asci with a relatively massive, non-amyloid apical ring. The ordinal status of these two genera (*Ascotaiwania* & *Savoryella*) is unknown and consequently classified as Ascomycota *incertae sedis*. We also describe *Ascothailandia* gen. nov. from submerged wood at Hala Bala Wildlife Sanctuary, Thailand. The new genus (teleomorph) is characterized by perithecoid, globose, dark-brown, ostiolate ascomata, paraphysate, asci cylindrical, unitunicate with a J-refractive apical ring and versicolourous, 3-euseptate ascospores. Ascospores germinated producing a *Canalisporium* (*C. grenadoidia* sp. nov.) anamorph. This genus is morphologically similar to *Ascotaiwania* and *Savoryella* species, but it differs in the morphology of the acomata, asci with apical ring and spores (shape, dimension and colour). Our phylogenetic results show that this taxon is well placed in the Hypocreomycetidae and bears close phylogenetic affinities to the anamorphic genus *Canalisporium*.

Phylogenetic analyses indicate that the genera *Savoryella, Ascotaiwania, Canalisporium* and *Ascothailandia* share a common ancestor and are closely related. In the SSU rDNA, LSU rDNA, RPB1, RPB2 and EF1-α dataset, *Savoryella* shows no affinities with the Hypocreales, Halosphaeriales, Sordariales and Xylariales (subclass

Hypocreomycetidae, Sordariomycetes). The finding presented here suggests a new lineage of aquatic ascomycetes that have invaded both the marine and freshwater environments. Although these genera are related, tree topologies between the different datasets vary as they contain differenet taxa. However, they form a distinct group similar to the unclassified group of marine ascomycetes comprising *Swampomyces, Torpedospora* and *Juncigera* (The TMB clade).

A number of trends can be discerned:

1. *Savoryella* species form a monophyletic clade, although the marine and freshwater species are placed in different sister groups.
2. *Savoryella* species do not show any affinities with the Hypocreales, Halosphaeriales, Sordariales and Xylariales as previously suggested by other authors.
3. *Ascotaiwania* is not monophyletic with the different species grouping with different anamorphs.
4. *Canalisporium* species form a monophyletic clade in all analyses, with a new ascomycete (*Ascothailandia* gen. et sp. nov., *C. grenadoidia* sp. nov.) and is well placed in Hypocreomycetidae. It shares phylogenetic affinities with the genus *Savoryella.* They form a unique group (ACS clade) with in the sub class Hypocreomycetidae.
5. *Canalisporium* species morphologically and phylogenetically form 2 groups: a) those with more than 1 row of cells, and b) those with only 1 row of cells.
6. Despite our original project title, *Canalisporium* is not the anamorph of *Savoryella.*

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GENERAL INFORMATION

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Project Title: Relationship of the genus *Savoryella* (teleomorph ascomycete) and its anamorph *Canalisporium,* as inferred by multiple gene phylogenies

Principal Project: Mr. Nattawut Boonyuen, M.sc
Position: Researcher Assistant, BIOTEC
Address: BIOTEC-Mycology, Bioresources and Technology Unit, 113 Paholyothin Rd., Klong 1, Klong Luang, Pathumthani 12120, Thailand, Tel: 02-564-6700 ext. 3242/3204
Fax: 02-564-6707 E-mail: Nattawut@biotec.or.th

Institution/Organization Head: Dr. Lily Eurwilaichitr
Position: Director of Bioresources Technology Unit
Address: 113 Paholyothin Rd., Klong 1, Klong Luang, Pathumthani 12120, Thailand, Tel: 02-564- 6700 Fax: 02-564- 6707 ext. 3464
E-mail: Lily@biotec.or.th

Investigation team:

Principal Investigator: Mr. Nattawut Boonyuen, M.Sc.
Position: Researcher Assistant, BIOTEC
Address: BIOTEC-Mycology, Bioresources and Technology Unit, 113 Paholyothin Rd., Klong 1, Klong Luang, Pathumthani 12120, Thailand, Tel: 02-564-6700 ext. 3242/3204 Fax: 02-564-6707
E-mail: Nattawut@biotec.or.th
Project responsibility: Molecular taxonomy, data analyses, manuscript

Research assistant: Miss Charuwan Chuaseeharonnachai, B.Sc
Position: Researcher Assistant
Address: BIOTEC-Mycology, Bioresources and Technology Unit, 113

Paholyothin Rd., Klong 1, Klong Luang, Pathumthani 12120,

Thailand, Tel: 02-564-6700 ext. 3532 Fax: 02-564-6707

E-mail: Charuwan.chu@biotec.or.th

Project responsibility: Cultivation, DNA extraction and PCR

Collaborators/External experts:

Professor E.B. Gareth Jones, Ph.D., D.Sc.

Position: Specialist, BIOTEC

Address: BIOTEC-Mycology, Bioresources and Technology Unit, 113

Paholyothin Rd., Klong 1, Klong Luang, Pathumthani 12120,

Thailand, Tel: 02-564-6700 ext. 3204 Fax: 02-564-6707

E-mail: Remispora@gmail.com

Project consulting: Discussion and manuscript preparation

PART 1
BACKROUND AND RATIONAL

An investigation of the fungal diversity of Thailand known as "*lignicolous freshwater fungi*" has been in progress for 8 years with over 400 species from various locations and wood species documented (Sivichai, 1999; Sivichai et al., 2002; Sivichai et al., 2003 a,b; Sivichai and Boonyene, 2004). The majority of the water-dwelling fungi recorded in this study were mitosporic fungi, with few Ascomycota and only one Basidiomycota (Sivichai, 1999; Sivichai and Jones, 2004).

Savoryella is one of the most commonly reported unitunicate ascomycete genus from submerged wood in rivers or streams (Sivichai et al., 2002, 2003a). The phylogenetic assignment of the genus is unresolved and it has been referred to a number of orders and families in the Sordariomycetes, Sordariomycetidae (Zhang et al., 2006) as shown in the following Table 1.

Table 1. Taxonomic assignment of the genus *Savoryella*

Authors & References	**Order**	**Family**	**Comments**
Jones & Eaton 1969	_	_	Authors did not assign to any family
Kohlmeyer & Kohlmeyer,1979	Sphaeriales *incertae sedis*	_	_
Kohlmeyer, 1986; Eriksson & Hawksworth, 1986	Ascomcyetes *incertae sedis*	_	_
Eriksson & Hawksworth, 1987	Xylariales	Amphisphaeriaceae	_
Barr, 1990	Halosphaeriales	_	Presence of catenophyses-like paraphyeses
Jones & Hyde,	Sordariales	Tripterosporaceae,	Presence of brown

1992		Lasiosphaeriaceae	ascospores , asci with a refractive apical ring
Read et al. 1992	_	_	It is difficult to assign *Savoryella* to any existing group and suggested it was congeneric with *Ascotaiwania* and has shared features with *Aniptodera*, which belongs to the Halosphaeriales
Ho et al., 1997	Sordariales	_	-
Vijaykrishna, 2006	Hypocreales	_	Based on molecular analysis (SSU data)

Jones and Eaton (1969) first described this genus with black perithecial ascomata, cylindrical asci with a comparatively flattened apex and brown ascospores with hyaline end-cells. It was collected on wooden slats in a water cooling tower run with brackish water at Connah's Quay, North Wales. Currently 11 species are recognized of which three are marine, one occurs on wood associated with sand, while the remainders are found in freshwater habitats (Table 2).

Table 2. Current name, author and year of the *Savoryella* species

No.	Fungal name	Authors and (Year)
1.	*Savoryella appendiculata*	K.D. Hyde & E.B.G. Jones (1992)
2.	*Savoryella aquatica*	K.D. Hyde (1993)
3.	*Savoryella curvispora*	W.H. Ho, K.D. Hyde & Hodgkiss (1997)
4.	*Savoryella fusiformis*	W.H. Ho, K.D. Hyde & Hodgkiss (1997)
5.	*Savoryella grandispora*	K.D. Hyde (1994)
6.	*Savoryella lignicola*	E.B.G. Jones & R.A. Eaton (1969)
7.	*Savoryella limnetica*	H.S. Chang & S.Y. Hsieh (1998)

8.	*Savoryella longispora*	E.B.G. Jones & K.D. Hyde (1992)
9.	*Savoryella melanospora*	Abdel-Wahab & E.B.G. Jones (2000)
10.	*Savoryella paucispora*	(Cribb & J.W. Cribb) J. Koch (1982)
11.	*Savoryella verrucosa*	Minoura & T. Muroi (1978)

No anamorph has been reported for *Savoryella* (Tsui and Hyde, 2003). The establishment of the anamorph-teleomorph link between taxa can be phylogenetically informative. Of the 400 freshwater species, only 56 links have been established between ascomycetes and their anamorphic fungi (Sivichai and Jones, 2003a). Of 22 anamorph/teleomorph connections reported by Sivichai (1999), most of these were detected by observing the anamorph/teleomorphs growing together on the same substratum and then verifying the connections by cultural studies.

The anamorphic genus *Canalisporium* is characterized by possession of a dolipore-like septum at the transmission electron microscope level, but no teleomorph is known for the genus (Ho, 1999; Goh et al., 1989; Nawawi and Kuthubutheen, 1989). Currently nine *Canalisporium* species have been described (Table 3).

Table 3. Current name, author and year of the *Canalisporium* species

No.	Fungal name	Authors and (Year)
1.	*Canalisporium caribense*	(Hol.-Jech. & Mercado) Nawawi & Kuthub. (1989)
2.	*Canalisporium elegans*	Nawawi & Kuthub. (1989)
3.	*Canalisporium exiguum*	Goh & K.D. Hyde (1998)
4.	*Canalisporium jinghongensis*	L. Cai, K.D. Hyde & McKenzie (2003)
5.	*Canalisporium kenyense*	Goh, W.H. Ho & K.D. Hyde (1998)
6.	*Canalisporium pallidum*	Goh, W.H. Ho & K.D. Hyde (1998)
7.	*Canalisporium panamense*	A. Ferrer & Shearer (2005)
8.	*Canalisporium pulchrum*	(Hol.-Jech. & Mercado) Nawawi & Kuthub. (1989)
9.	*Canalisporium variabile*	Goh & K.D. Hyde (2000)

Ascotaiwania is a unitunicate ascomycete characterized by ascospores that are generally more than 3-septate with hyaline end cells, asci with a relatively massive, non-amyloid apical ring. The genus *Ascotaiwania* is reported from freshwater habitat and from terrestrial palms with 12 species (Table 4).

Table 4. Current name, author and year of the *Ascotaiwania* species

No.	Fungal name	Authors and (Year)
1.	*Ascotaiwania hsilio*	H.S. Chang & S.Y. Hsieh (1998)
2.	*Ascotaiwania hughesii*	Fallah, J.L. Crane & Shearer (1999)
3.	*Ascotaiwania licualae*	J. Fröhl. & K.D. Hyde (2000)
4.	*Ascotaiwania lignicola*	Sivan. & H.S. Chang (1992)
5.	*Ascotaiwania mauritiana*	Dulym., P.F. Cannon, K.D. Hyde & Peerally (2001)
6.	*Ascotaiwania mitriformis*	Ranghoo & K.D. Hyde (1998)
7.	*Ascotaiwania pallida*	K.D. Hyde & Goh (1999)
8.	*Ascotaiwania palmicola*	K.D. Hyde (1995)
9.	*Ascotaiwania pennisetorum*	M.K.M. Wong & K.D. Hyde (2001)
10.	*Ascotaiwania persoonii*	Fallah, J.L. Crane & Shearer (1999)
11.	*Ascotaiwania sawadae*	H.S. Chang & S.Y. Hsieh (1998)
12.	*Ascotaiwania wulai*	H.S. Chang & S.Y. Hsieh (1998)

Presently, a one gene approach has been used for studying the relationships between fungi, but it may not infer the whole evolution of fungal taxa as different genes evolve at different rates (Li and Graur, 1991; Geiser et al., 2000). Molecular phylogeny techniques on nuclear ribosomal genes (LSU, SSU, 5.8S rDNA) offer the chance to investigate the taxonomic placement and sexual/asexual relationships of a wide range of fungal taxa. Other genes that can also enhance our knowledge of fungal evolution include RNA polymerase II subunit (Kurtzman and Robnett, 2003), and translation elongation factor EF1-1α (O'Donnell, 2000; Kurtzman and Robnett, 2003).

OBJECTIVES

1. To determine the taxonomic placement of *Savoryella* by multiple gene phylogeny

2 To elucidate the phylogeny of *Savoryella* with other phenotypically similar genera

3. To examine the interrelationships of the genera *Savoryella* and *Ascotawania* with the anamophic genus *Canalisporium* from different habitats (freshwater and marine environments) based on morphological and molecular data

MATERIAL AND METHODS

1. Specimen collection and fungal growth

Fungi were isolated from various substrata from freshwater and marine locations in Thailand (Sivichai and Boonyene, 2004; Sakayaroj et al., 2005; Pinruan et al., 2002) and maintained on Corn Meal Agar (CMA) or Potato Dextrose Agar (PDA) media with seawater or freshwater (Table 5). All cultures were grown on PDA at room temperature of 25°C for 4-16 weeks (depending on the growth rate of each species).

2. Genomic DNA extraction

Actively growing mycelia were scraped off the surface of a culture and transferred to micro-centrifuge tubes and the biomass lyophilized at -80°C for 2 days before DNA extraction which followed a modified protocol of Tigano-Milani et al. (1995). The lyophilized-mycelia were ground with a sterile pipette tip in 2 ml microcentrifuge tube. The resulting powder was transferred to a 1.5-mL pre-warmed (65°C) microcentrifuge tube with 700 µl extraction buffer (0.7 M NaCl; 50 mM Tris-

HCl, pH 8; 10 mM EDTA, pH 8; 1% CTAB) and incubated at 65°C for 1 hour. In the CTAB-based method, DNA was extracted once with 500 µl (24:1) chloroform-isoamyl alcohol (CIAA) and centrifuged at 12.000 rpm for 20 minutes. The supernatant was transferred to a 1.5-mL new microcentrifuge tube containing 1/10 volume of 10% CTAB, added with 700 µl CIAA and centrifuged for 20 minutes at 12.000 rpm. The 1000 µl precipitation buffer (50 mM Tris-HCl, pH 8.0; 10 mM EDTA, pH 8.0; 1% CTAB) was added to the aqueous phase of supernatant for 30 minutes at room temperature. The 300 µl Tris-EDTA High Salt (1 M NaCl; 10 mM EDTA, pH 8.0; 1 mM EDTA, pH 8) buffer was added to the pellet, washed with 400 µl ethanol 70%, and resuspended in 30 µL sterilized deionized water containing 5 µ RNase A (100 µg/mL). The DNA pellet after centrifugation (20 minutes, 12.000 rpm, 4 °C) was washed in 400 µl 70% ethanol and air-dried. Finally, the DNA re-suspended in 50 µl TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA pH 8.0).

3. PCR amplification

DNA was amplified with Taq DNA polymerase. Different regions of the partial SSU, LSU ribosomal DNA, ITS region (Figure 1), partial RPB2 (Figure 2), partial RPB1 (Figure 3) and translation elongation factor EF1-α (Figure 4) were amplified using primers (Table 6) NS1, NS3, NS4, NS5, NS6, JS1, JS8, LROR, LR5, LR7, ITS1, ITS4, ITS5, RPB2-5F2, RPB2-7CR, RPB2-5F1, RPB2-7R, 983F, 2218R, CEFF2, CEFR2, CRPB1, CRPB1A and RPB1Cr (White et al., 1990; Bunyard et al., 1994; Landvik, 1996; Liu et al., 1999; Rehner, 2001) using PCR Model MJ Research DYAD ALD and PCR reaction (Table 7) were carried out in total volume of 50 µl containing 10-50 ng DNA template. The 10X PCR buffer, 1.5 mM $MgCl_2$, 2 mM dNTPs, 0.2 µM each primer and 0.5 U of Taq Polymerase (DNA Polymerase Kit, Vivantis Technologies). Amplification cycles were performed following the procedure of Tang et al. (2007) composed of 95°C for 5 min, followed by denaturation step at 35 cycles, 52°C for 1 min (for SSU or LSU rDNA), 55°C for 1 minute (ITS region), 52°C for 1.5 minute (for RPB1, RPB2 & EF1-α) at annealing step, 72°C for 1.5-2.5 minutes (elongation step) and the final step of 72°C for 10 minutes (Table 8). The size of each amplified fragment was verified by gel electrophoresis with ethidium bromide staining of a 2 mL product sample and visualized over an ultraviolet transilluminator. PCR products were purified using NucleoSpin^{R} Extract Kit (Macherey-Nagel, Germany), following the manufacturer's instructions. Then checking for the quantity and quality in a 1% agarose gel electrophoresis was applied. Finally, the purified PCR product was used directly for DNA sequencing.

Table 5. Fungal isolates sequenced for this study

Species	Isolates numbers	Sources	Substrate origins/Habitats	Collection sites	Fungal references
Ascotaiwania sawadae	SS00051	BCC03343	Submerged Hard wood/Freshwater	Khao Yai National Park, Nakhon Nayok, Thailand	H.S. Chang & S.Y. Hsieh (1998)
Ascothailandia grenadoidia gen. et sp. nov	SS03615	BCC20507	Submerged *Wrightia tomentosa*/Freshwater	Khlong I-Gading stream, Hala-Bala Wildlife Sanctuary, Narathiwat, Thailand	N/A
Canalisporium sp.(*caribense*)	SS03732	BCC21424	Submerged wood/Freshwater	Stream at Ban Krang, Kaeng Krachan National Park, Phetchaburi, Thailand	(Hol.-Jech. & Mercado) Nawawi & Kuthub. (1989)
Canalisporium caribense	SS03683	BCC21022	Submerged wood/Freshwater	Wang Kar Leung Waterfall, Wang Kan Lueng Arboretum, Lop Buri, Thailand	(Hol.-Jech. & Mercado) Nawawi & Kuthub. (1989)
Canalisporium caribense	SS03839	BCC24239	Submerged wood/Freshwater	Khlong I-Gading stream, Hala-Bala Wildlife	(Hol.-Jech. & Mercado) Nawawi & Kuthub. (1989)

				Sanctuary, Narathiwat, Thailand	
Canalisporium elegans	SS00523	BCC03625	Submerged *Xylia dolabriformis*/Freshwater	Stream at road marker at km 29.2, Khao Yai National Park, Nakhon Ratchasima, Thailand	Nawawi & Kuthub. (1989)
Canalisporium elegans	SS00877	BCC09963	Submerged wood/Freshwater	Stream at road marker at km 18, Kaeng Krachan National Park, Phetchaburi, Thailand	Nawawi & Kuthub. (1989)
Canalisporium elegans	SS00895	BCC12772	Submerged *Stereospermum neuranthum* /Freshwater	Stream at road marker at km 29.2, Khao Yai National Park, Nakhon Ratchasima, Thailand	Nawawi & Kuthub. (1989)
Canalisporium elegans	SS03483	BCC26225	Submerged wood/Freshwater	Bor Kleng Hot Spring, Ratchaburi, Thailand	Nawawi & Kuthub. (1989)
Canalisporium elegans	SS03491	BCC18364	Submerged wood/Freshwater	Kaeng Krachan National Park, Phetchaburi, Thailand	Nawawi & Kuthub. (1989)

Canalisporium exiguum	SS00809	BCC12770	Submerged wood/Freshwater	Khao Soi Dao Wildlife Sanctuary, Chanthaburi, Thailand	Goh & K.D. Hyde (1998)
Canalisporium pallidium	SS00091	BCC03350	Submerged *Alstonia scholaris*/Freshwater	Streams at road marker at km 29.2, Khao Yai National Park, Nakhon Ratchasima, Thailand	Goh, W.H. Ho & K.D. Hyde (1998)
Canalisporium palladium	SS00498	BCC03608	Submerged *Xylia dolabriformis*/Freshwater	Stream at road marker at km 29.2, Khao Yai National Park, Nakhon Ratchasima, Thailand	Goh, W.H. Ho & K.D. Hyde (1998)
Canalisporium pulchrum	SS00170	BCC03406	Submerged *Alstonia scholaris*/Freshwater	Stream at road marker at km 29.2, Khao Yai National Park, Nakhon Ratchasima, Thailand	(Hol.-Jech. & Mercado) Nawawi & Kuthub. (1989)
Canalisporium pulchrum	SS03773	BCC21030	Submerged Leaf/Freshwater	Khlong I-Gading Stream, Hala-Bala Wildlife Sanctuary, Narathiwat, Thailand	(Hol.-Jech. & Mercado) Nawawi & Kuthub. (1989)

Canalisporium pulchrum	SS03788	BCC22507	Submerged wood/Freshwater	Khao Pra - Bang Khram Wildlife Sanctuary, Krabi, Thailand	(Hol.-Jech. & Mercado) Nawawi & Kuthub. (1989)
Canalisporium pulchrum	SS03819	BCC21221	Submerged wood/Freshwater	Khao Pra-Bang Khram Wildlife Sanctuary, Krabi, Thailand	(Hol.-Jech. & Mercado) Nawawi & Kuthub. (1989)
Canalisporium pulchrum	SS03823	BCC21428	Submerged wood/Freshwater	Khao Pra-Bang Khram Wildlife Sanctuary, Krabi, Thailand	(Hol.-Jech. & Mercado) Nawawi & Kuthub. (1989)
Canalisporium pulchrum	SS03982	BCC23549	Submerged wood/Freshwater	Haew Narok waterfall, Khao Yai National Park, Nakhon Nayok, Thailand	(Hol.-Jech. & Mercado) Nawawi & Kuthub. (1989)
Savoryealla aquatica	SS00096	BCC03345	Submerged *Anisoptera oblongal*/Freshwater	Streams at road marker at km 29.2, Khao Yai National Park, Nakhon Ratchasima, Thailand	K.D. Hyde (1993)
Savoryella aquatica	SS00359	BCC03521	Submerged *Alstonia scholaris*/Freshwater	Streams at Tad Tha Phu, Khao Yai National Park, Nakhon Ratchasima,	K.D. Hyde (1993)

				Thailand	
Savoryella aquatica	SS00583	BCC03641	Submerged *Xylia dolabriformis*/Freshwater	Streams at Tad Tha Phu, Khao Yai National Park, Nakhon Ratchasima, Thailand	K.D. Hyde (1993)
Savoryella aquatica	SS03801	BCC22509	Submerged wood/Freshwater	Khao Pra - Bang Khram Wildlife Sanctuary, Krabi, Thailand	K.D. Hyde (1993)
Savoryella lignicola	SAT00908	-	-	Tammarang Pier, Saton, Thailand	E.B.G. Jones & R.A. Eaton (1969)
Savoryella longispora	SAT00320	BCC23612	Mangrove wood/Marine	Tammarang Pier, Satun, Thailand	E.B.G. Jones & K.D. Hyde (1992)
Savoryella longispora	SAT00322	BCC23592	Mangrove wood/Marine	Tammarang Pier, Satun, Thailand	E.B.G. Jones & K.D. Hyde (1992)
Savoryella paucispora	SAT00866	BCC28374	Mangrove wood/Marine	Laem TaLum Phuk, Nakhonsithammarat, Thailand	(Cribb & J.W. Cribb) J. Koch (1982)
Savoryella paucispora	SAT00867	BCC28375	Mangrove wood/Marine	Laem TaLum Phuk, Nakhonsithammarat,	(Cribb & J.W. Cribb) J. Koch (1982)

				Thailand	
Savoryella verrucosa	SS00042	BCC03342	Submerged Elephant grass/Freshwater	Khao Yai National Park, Nakhon Ratchasima, Thailand	Minoura & T. Muroi (1978)
Savoryella verrucosa	SS00052	BCC03344	Submerged Twig/Freshwater	Khao Yai National Park, Nakhon Ratchasima, Thailand	Minoura & T. Muroi (1978)
Savoryella verrucosa	SS00582	BCC03642	Submerged *Xylia dolabriformis*/Freshwater	Streams at Tad Tha Phu, Khao Yai National Park, Nakhon Ratchasima, Thailand	Minoura & T. Muroi (1978)
Savoryella verrucosa	SS03331	BCC24236	Submerged *Stereospermum neuranthum*/Freshwater	Streams at Tad Tha Phu, Khao Yai National Park, Nakhon Ratchasima, Thailand	Minoura & T. Muroi (1978)

a Isolates with the prefix SS and SAT are from the BIOTEC Culture Collection (BCC);

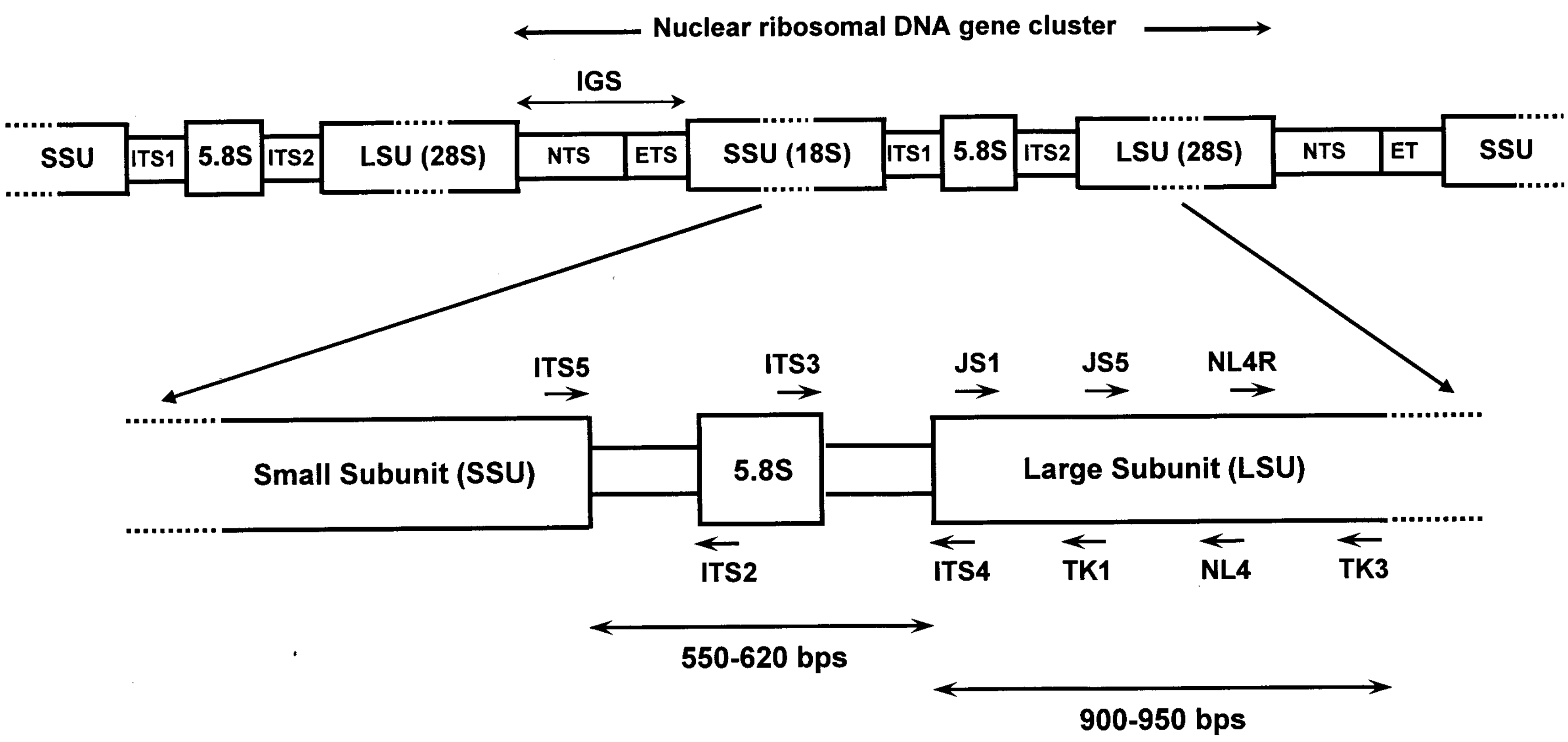

Figure 1. Diagrammatic representation of the nuclear ribosomal DNA gene cluster showing the primer positions for the PCR and DNA sequencing. The gene is divided into coding (SSU, 5.8S and LSU genes) and non-coding (IGS and ITS) regions. Position and direction of replication of each primer are shown. Picture from Kwong, 2003

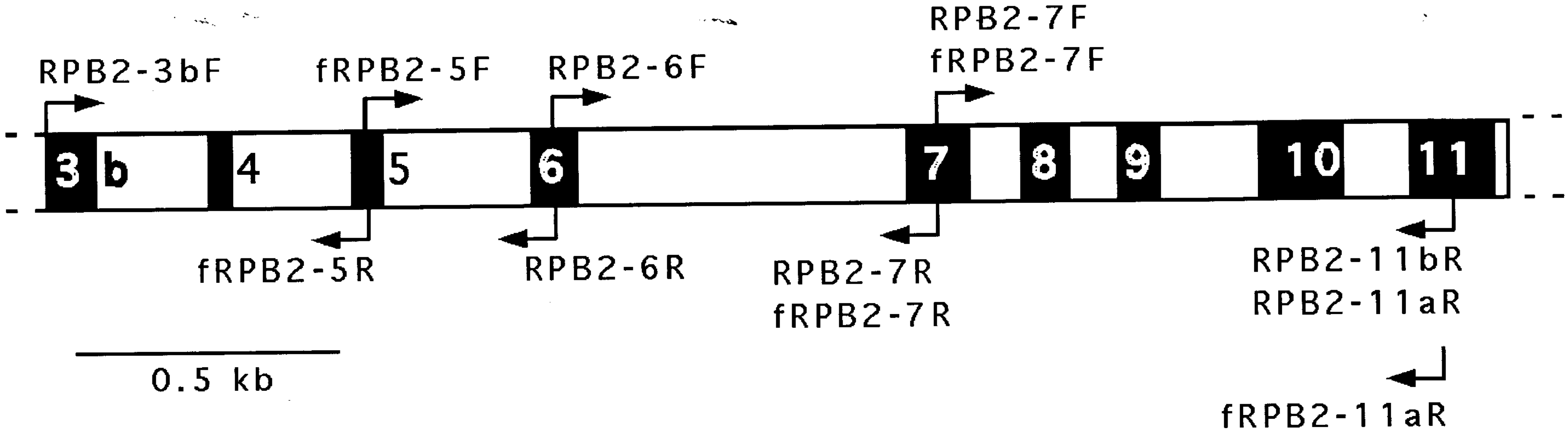

Figure 2. Diagrammatic representation of the RNA polymerase II gene (RPB2) encoding the second largest protein subunit showing the primer positions for the PCR and DNA sequencing. Blocks with shading are exons (coding regions) and blocks without shading are introns (non-coding regions). Picture from http://www.clarku.edu/faculty/dhibbett/Protocols_Folder/Primers/Primers.htm

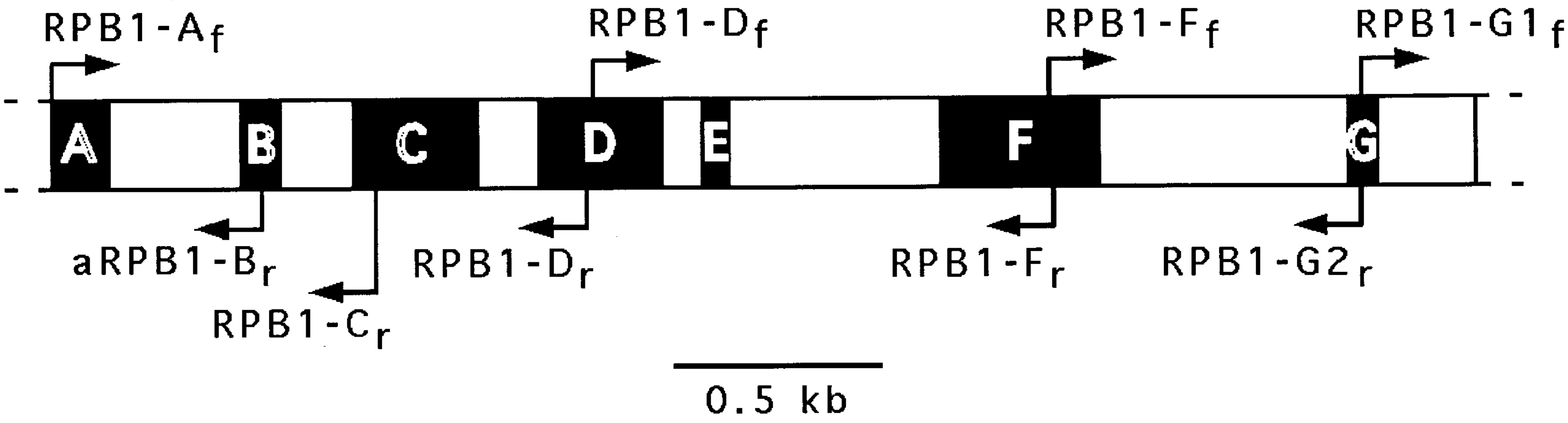

Figure 3. Diagrammatic representation of the RNA polymerase II gene (RPB1) encoding the second largest protein subunit showing the primer positions for the PCR and DNA sequencing. Blocks with shading are exons (coding regions) and blocks without shading are introns (non-coding regions). Picture from http://www.clarku.edu/faculty/dhibbett/Protocols_Folder/Primers/Primers.htm

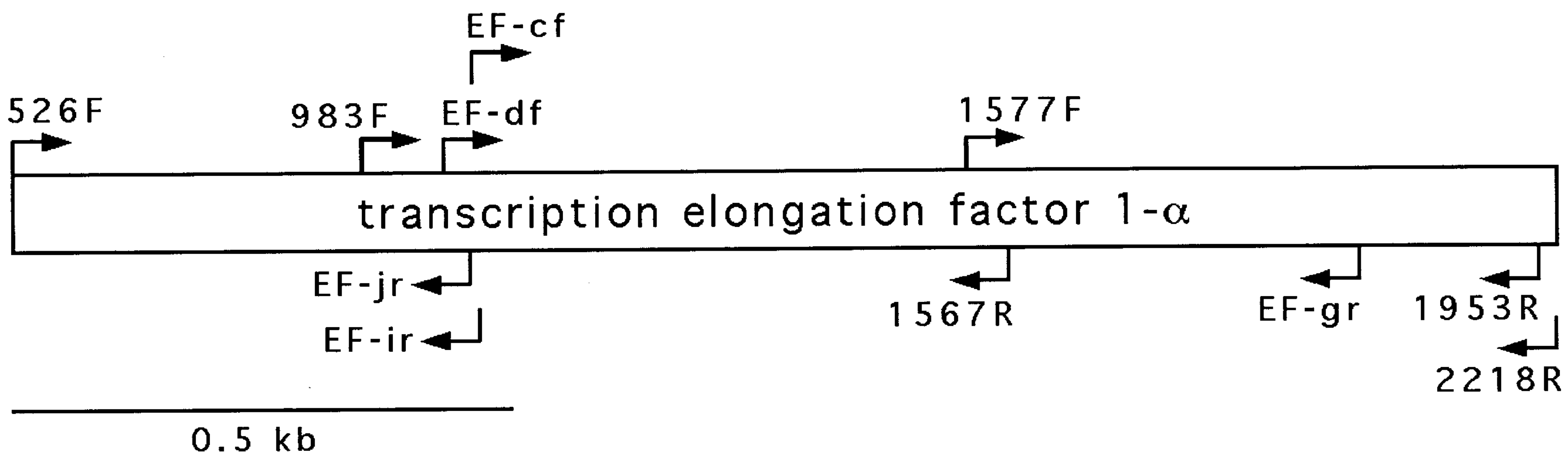

Figure 4. Diagrammatic representation of the Translation elongation factor 1a, EF-1a showing the primer positions for the PCR and DNA sequencing. Picture from http://www.clarku.edu/faculty/dhibbett/Protocols_Folder/Primers/Primers.htm

Table 6. Primers used for PCR and DNA sequencing

Small subunit (18S)	**Sequence (5' – 3')**
NS1	GTA GTC ATA TGC TTG TCT C
NS3	GCA AGT CTG GTG CCA GCA GCC
NS4	CTT CCG TCA ATT CCT TTA AG
NS5	AAC TTA AAG GAA TTG ACG GAA G
NS6	GCA TCA CAG ACC TGT TAT TGC CTC
Large subunit (28S)	**Sequence (5' – 3')**
JS1	CGC TGA ACT TAA GCA TAT
JS8	CAT CCA TTT TCA GGG CTA
LR7	TAC TAC CAC CAA GAT CT
LROR	ACC CGC TGA ACT TAA GC
Internal Transcribed Spacers (ITS)	**Sequence (5' – 3')**
ITS1	TCC GTA GGT GAA CCT GCG G
ITS4	TCC TCC GCT TAT TGA TAT GC
ITS5	GGA AGT AAA AGT CGT AAC AAG G
Polymerase II second largest subunit regions 5-7 (RPB2)	**Sequence (5' – 3')**
RPB2-5F1	GAY GAY MGW GAT CAY TTY GG
RPB2-5F2	GGG GWG AYC AGA AGA AGG C
RPB2-7cR	CCC ATR GCT TGT YYR CCC AT
RPB2-7R	CCC ATW GCY TGC TTM CCC AT
Translation elongation factor EF1(alpha), TEF1-α	**Sequence (5' – 3')**
EF1-983F	GCY CCY GGH CAY CGT GAY TTY AT
EF1-2218R	ATG ACA ACC RAC RGC RAC RG TYT G
EF1-CEFF2	GGC TTC AAC GTG AAG AAC G
EF1-CEFR2	GRG GGT CGT TCT TGG WGT C
RNA polymerase II largest subunit, RPB1	**Sequence (5' – 3')**
RPB1-CRPB1	CCW GGY TTY ATC AAG AAR GT
RPB1-CRPB1A	CAY CCW GGY TTY ATC AAG AA
RPB1-RPB1Cr	CCN GCD ATN TCR TTR TCC ATR TA

RPB2-7R, RPB2-5F2/RPB2-7CR, CRPB1/CRPB1A, RPB1Cr, EF1-983/EF1-2218R,		95 °C	5 minutes
		52 °C	1.5 minutes
		72 °C	1.5 minutes
		72 °C	10 minutes

4. PCR product purification

The PCR product was purified directly following the manufacturer's instructions of NucleoSpin^{R} Extract (MACHEREY-NAGEL). Then checking for the quantity and quality in a 1% agarose gel electrophoresis was applied. Finally, the purified PCR product was used directly for DNA sequencing.

5. DNA Sequencing

PCR products were directly sequenced by Macrogen., Inc in Korea using primers NS1, NS3, NS4, NS5, NS6, JS1, JS8, LROR, LR5, LR7, ITS1, ITS4, ITS5, RPB2-5F1, RPB2-5F2, RPB2-7cR, RPB2-7R, EF1-983F, EF1-2218R, EF1-CEFF2, EF1-CEFR2, RPB1-CRPB1, RPB1-CRPB1A and RPB1-RPB1Cr (White et al., 1990; Bunyard et al., 1994; Landvik, 1996; Liu et al., 1999; Rehner, 2001).

6. Phylogenetic analyses

Fungal lists with various taxa were analyzed along with other sequences obtained from the GenBank Database, with a suitable outgroup taxa and aligned initially with the computer program Bioedit (Hall, 2006) and Clustal W (Thompson et al., 1997) with default parameter settings, and alignments were manually edited by inserting gaps for optimization using Se-Al (Rambaut, 2002). Phylogenetic analyses of SSU rDNA, LSU rDNA, ITS region, RPB1, RPB2 and EF1(alpha) gene were performed with maximum parsimony employing a heuristic search (1000 random replicates) in PAUP* v 4.0b10 (Swofford, 2002). Ambiguously aligned regions also were excluded from the phylogenetic analyses. Maximum parsimony trees were found using 1,000 heuristic searches and including parsimony-informative characters in stepwise (random) addition and tree bisection and reconstruction (TBR) as branch swapping algorithm. Branch support for all parsimony analyses was estimated by performing 1,000 bootstrap replicates (Felsenstein, 1985) with a heuristic search of 10 random-addition replicates for each bootstrap replicate. Descriptive tree statistics tree length (TL), consistency index (CI), retention index (RI), rescaled consistency index (RC) and homoplasy index (HI) were calculated for all trees generated under different optimality criteria.

RESULTS

The phylogenetic relationship of the genera *Ascotaiwania*, *Canalisporium*, *Savoryella* & *Ascothailandia* inferred by multiple gene data

In the nuclear small subunit ribosomal DNA (nSSU rDNA/18S rDNA), nuclear large subunit ribosomal DNA (nLSU rDNA/28S rDNA) and Internal Transcribed Spacer (ITS) datasets, the phylogenetic analyses indicate that the genera *Ascotaiwania* (A), *Canalisporium* (C) and *Savoryella* (S) are closly related within the subclass Hypocreomycetidae, Sordariomycetes, Ascomycota sharing a common ancestor (Part 3, Page 54). The genus *Savoryella* shows no affinities with the Hypocreales, Halosphaeriales, Sordariales and Xylariales (subclass Hypocreomycetidae, Sordariomycetes, Ascomycota, Fungi). Our findings suggest a new lineage (ACS clade = *Ascotaiwania, Canalisporium, Savoryella*) of aquatic ascomycetes that have invaded both the marine and freshwater habitats. Although these genera are related, they are not monophyletic. However, they form a distinct group similar to the unclassified group of marine ascomycetes comprising the genera *Swampomyces, Torpedospora* and *Juncigera* (TBM clade). In the second year of work, we present the new datasets that includes an analysis of the partial RPB1 (DNA dependent RNA polymerase II largest subunit), the partial RPB2 (DNA dependent RNA polymerase II largest subunit) and TEF-la (translation elongation factor 1 alpha) genes to further elucidate the phylogeny of the genera *Ascotaiwania*, *Canalisporium* and *Savoryella* (ACS clade). These data provide a comprehensive view of these phylogenies and define new lineages of these genera (Part 3, Page 54). The results of separate analyses from RPB1, RPB2 and TEF-1a are in agreement with their combined analyses of that resolve a monophyletic Hypocreomycetidae, Sordariomycetes comprising three well-supported clades {Draft Manuscript entitled:

A new lineage of aquatic ascomycetes inferred by multiple gene phylogenies of the genera *Ascotaiwania*, *Ascothailandia,* *Canalisporium*, and *Savoryella*}.We also describe *Ascothailandia* gen. nov. (*Canalisporium grenadoidia* sp. nov.) from submerged wood at Hala Bala Wildlife Sanctuary, Thailand (Part 2, Page 36). This genus is morphologically similar to the genera *Ascotaiwania* and *Savoryella*, but it differs in the acomata, asci, apical ring and spores (shape, dimension and colour) from these genera. Our phylogenetic results (18S rDNA+28S rDNA and ITS region) show that *Ascothailandia* gen. nov. is well placed in the Hypocreomycetidae and bears close phylogenetic affinities to *Canalisporium* species {Manucript entitled: Wood inhabiting freshwater fungi from Thailand: *Ascothailandia grenadoidia* gen. sp. Nov *Canalisporium grenadoidia* sp. with a key to *Canalisporium* species (Sordariomycetes, Ascomycota)}.

FUTURE WORK

Further work on publication in draft will be submitted to appropriate journals.

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PART 2

Manuscript entitled: Wood inhabiting freshwater fungi from Thailand: *Ascothailandia grenadoidia* gen. et sp. nov. *Canalisporium grenadoidia* sp. nov., with a key to *Canalisporium* species (Sordariomycetes, Ascomycota)

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Wood inhabiting freshwater fungi from Thailand: *Ascothailandia grenadoidia* gen. et sp. nov., *Canalisporium grenadoidia* sp. nov. with a key to *Canalisporium* species (Sordariomycetes, Ascomycota)

Veera Sri-indrasutdhi[1], Nattawut Boonyuen[1], Satinee Suetrong[1,2], Charuwan Chuaseeharonnachai[1], Somsak Sivichai[1] and E.B. Gareth Jones[1]

V. Sri-indrasutdhi
N. Boonyuen
S. Suetrong
C. Chuaseeharonnachai
S. Sivichai
E.B. Gareth Jones

[1]Bioresources Technology Unit, National Center for Genetic Engineering and Biotechnology (BIOTEC),113 Thailand Science Park, Phaholyothin Road., Khlong 1, Khlong Luang, Pathumthani 12120, Thailand

Corresponding author: V. Sri-indrasutdhi
Tel: +66-2564-6700 ext. 3531
Fax: +66-2564-6707
E-mail: veera@biotec.or.th

Text 20 pages; 3 Tables; 17 Figures

Abstract *Ascothailandia grenadoidia* gen. et sp. nov. is described and illustrated from submerged wood (*Wrightia tomentosa*) in a stream at Hala Bala Wildlife Sanctuary, southern Thailand. The new genus (teleomorph) is characterized by perithecoid, globose, dark-brown, ostiolate ascomata, paraphysate, asci cylindrical, unitunicate with a prominent J-refractive apical ring and versicolourous, 3-euseptate ascospores. Ascospores germinated producing a *Canalisporium* (*C. grenadoidia* sp. nov.) anamorph. The morphological characterization of this new fungus is reported and compared with the genera *Ascotaiwania* and *Savoryella*. Phylogenetic analyses of the combined partial 18S, 28S ribosomal DNA and internal transcribed spacer (ITS), including 5.8S regions, of *Ascothailandia grenadoidia* and 10 *Canalisporium* species were undertaken and analyzed with maximum parsimony and Bayesian methods. The molecular data indicate that *A. grenadoidia* is closely related to *Canalisporium elegans* in the Sordariomycetes, Hypocreomycetidae, order *incertae sedis*. Both

morphological and molecular characterization provides sufficient evidence to support the description of a new genus. A key to *Canalisporium* species is provided.

Key words anamorphic fungi, combined 18S and 28S rDNA, Hypocreomycetidae, ITS

Introduction

A long term study (3 years) of the fungal colonization of nine tropical timbers (*Azadirachta indica* A. Juss var. *siamensis* Valeton, *Erythrophleum teysmannii* Craib, *Melaleuca cajuputi* Powell, *Shorea obtusa* Wall., *Shorea roxburghii* G. Don, *Shorea siamensis* Miq., *Wrightia tomentosa* Roem. & Schult., *Xylia xylocarpa* (Roxb.) W. Taub, *Zollingeria dongnaiensis* Pierre) submerged in a stream at Hala Bala Wildlife Sanctuary, Narathiwat province, Thailand, has been in progress. During this study a fungus with morphological features (especially the asci and ascospores) similar to *Ascotaiwania* Sivan. & H.S. Chang and *Savoryella* E.B.G. Jones & R.A. Eaton was found on the test blocks of *Wrightia tomentosa*. *Ascotaiwania* and *Savoryella* species are frequently collected on trapped wood in streams and rivers, in both tropical and temperate zones (Jones and Eaton 1969; Sivanesan and Chang 1992; Jones and Hyde 1992; Chang et al. 1998; Sivichai et al. 2000a, b). The taxonomic position of *Ascotaiwania* and *Savoryella* has not been resolved, and are referred to the Sordariales *incertae sedis* and Hypocreales *incertae sedis*, respectively (Cai et al. 2006). A phylogenetic study of these two genera was undertaken with the view of resolving the identification of the new ascomycete. Nine species of *Canalisporium* Nawawi & Kuthub., saprobic on wood and plant debris, often in freshwater, have been described but have no known teleomorph (Nawawi and Kuthubutheen 1989; Goh et al. 1998; Goh and Hyde 2000; Cai et al. 2002; Ho and Hyde 2004). However, Cai et al. (2006) suggested a connection with the Tubeufiaceae but this was not supported by a molecular study. In this study, a new genus and species of *Ascothailandia grenadoidea* (anam. *Canalisporium grenadoidia*), is described and illustrated. In addition, the partial small subunit (SSU), large subunit (LSU) of rDNA and the internal transcribed spacer region (ITS) sequences of six *Canalisporium* species were compared to resolve their phylogenetic relationships.

Materials and methods

Morphological studies

Submerged wood test blocks (15 × 2.5 × 2.5 cm) of *Wrightia tomentosa* were collected from a stream at Hala Bala Wildlife Sanctuary, Narathiwat province, southern Thailand, washed of surface debris and incubated in a plastic box with damp tissue paper, then returned to the laboratory. Samples were periodically examined for two weeks. Single ascospore isolations were made on Corn Meal Agar (CMA) with added antibiotics (penicillin G 0.5 g/l and streptomycin 0.5 g/l) and germinating spores transferred to Potato Dextrose Agar (PDA). Axenic cultures were deposited at BIOTEC Culture Collection (BCC). Collections of *Canalisporium* species were made from various sites in Thailand, and deposited in BCC. Dried specimens were deposited at BIOTEC Bangkok Herbarium (BBH).

Phylogenetic studies

The taxonomic placement of the selected freshwater lignicolous fungi was undertaken by comparing the partial 18S, 28S ribosomal DNA and ITS regions.

Culture selection

Selected fungal cultures used in this study are listed in Table 1 along with collection and isolation data. Fungi were grown on PDA for 4–16 weeks at 25°C. Actively growing mycelium was scraped off the surface of a culture and transferred to 2 ml of microcentrifuge tubes and the biomass lyophilized at -80°C for 24 hours.

DNA extraction

Extraction buffer (1% CTAB, 0.7 M NaCl, 50 mM Tris-HCl, 10 mM EDTA, pH 8) was added to fungal samples. The samples were ground in a 2 ml microcentrifuge tube and the volume adjusted by adding 700 µl extraction buffer and mixed by inverting the tubes and incubated at 65°C for 1 hour. Samples were centrifuged at 12,000×*g* for 10 min at 25°C. The aqueous supernatant was transferred into a new microcentrifuge tube with phenol-chloroform-isoamyl alcohol by mixing gently and by centrifugation at 12,000×*g* for 10 min at 25°C. The upper liquid phase was transferred to a new microcentrifuge tube containing 7.5 M of ammonium acetate.

The DNA was precipitated by ethanol (–20°C overnight) by centrifugation at 12,000×*g* for 10 min at 15°C. The DNA-pellet was washed with ice-cold 70% ethanol and dried at 25°C. The pellet was redissolved in 50 μl of TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA pH 8.0).

PCR amplification

Primers used for PCR amplification and for sequencing of SSU, LSU rDNA and ITS were NS1, NS3, NS4, NS6, JS1, JS8, ITS1, ITS4 and ITS5 (White et al. 1990; Bunyard et al. 1994; Landvik 1996). Amplification was performed in a 50 μl reaction mix: 10 mM of each dNTP (1 μl), 10 μM of each primer (1 μl), 10% of dilution buffer (5 μl), 25 mM of Mg (5 μl), 4 M of enhancer (5 μl) and 60–62% of sterile distilled water (30.8 μl), 0.2 μl of *Taq* DNA polymerase kit from FERMENTAS and 10–50 ng of genomic DNA template (1 μl) carried out using a PCR Model MJ Research DYAD ALD in 200 μl reaction tubes. (95°C, 0.5 min; 52°C, 1 min; 72°C, 1.5 min; 35 cycles). PCR products (7 μl aliquots) were checked by electrophoresis in 1% agarose gels with 0.003% ethidium bromide in 0.5×TBE buffer (0.044 M Boric acid, 1.1 mM EDTA, 0.045 M Tris, pH 8) for purity.

DNA purification and sequencing

PCR products were purified using NucleoSpin® Extract Kit (Macherey-Nagel, Germany). The PCR products were sequenced by Macrogen Inc. in Korea with the same primers as in the PCR amplification. The sequences obtained were deposited in the National Center for Biotechnology Information (NCBI) and the accession numbers are listed in Table 1.

Phylogenetic analyses

Each sequence was checked for ambiguous bases and refined visually, assembled using BioEdit 6.0.7 (Hall 1999). The consensus sequences for each DNA region were multiple aligned by Clustal W 1.6 (Thompson et al. 1994) with all sequences derived from the GenBank database and the accession numbers that are included in the phylogenetic trees.

The alignment (P.I.N. 3801) included the most similar sequence identified through BLAST search. *Daldinia concentrica* (Bolton) Ces. & De Not. and *Xylaria hypoxylon* (L.) Grev. were chosen as the outgroup to root the phylogenetic tree for all analyses.

The analyses of combined nuclear ribosomal DNA dataset (18S and 28S rDNA) and ITS dataset were performed using PAUP*4.0b10 (Swofford 2002). Gaps were treated as missing data. The most parsimonious trees (MPTs) were searched using maximum parsimony (MP) in PAUP*4.0b10 with heuristic searches algorithm with tree bisection–reconnection (TBR) branch swapping. One hundred replicates of random stepwise sequence addition were performed and the shortest trees over all replicates kept and assumed to be the most parsimonious reconstructions, to increase the chance of finding the best tree(s). The tree length (TL), Consistency indices (CI), and Retention indices (RI), Rescaled consistency index (RC) and homoplasy index (HI) were calculated for each tree generated. Trees were visualized with TreeView (Page 1996).

Tree topologies from different parsimony analyses were tested with the Kishino-Hasegawa (K-H) maximum likelihood test (Kishino and Hasegawa 1989) to find the most likelihood tree. Bootstrap support (BS) for the branches was based on 1000 replicated with 10 replicates of random stepwise addition of sequence.

The model of substitution used for Bayesian analysis was chosen with Mrmodeltest 2.2 (Nylander 2004). Independent Bayesian phylogenetic analysis was performed in MrBayes3.0.b4 (Huelsenbeck and Ronquist 2001) using a uniform GTR+I+G (combined 18S+28S rDNA dataset) and HKY+G model (ITS dataset), with general time reversible (GTR) model for DNA distribution and gamma distribution rate variation across sites. The Metropolis-coupled Markov chain Monte Carlo (MCMC) sampling approach was used to calculate posterior probabilities. Four simultaneous Markov chains were run from a random starting tree for 2000000 generations and sampled every 100 generations. The first 2000 generations were discarded as burn in of the chain. A 50% majority rule consensus tree of all remaining trees, as well as the posterior probabilities (PP), was calculated. Parsimony bootstrap value greater than 50% and Bayesian Posterior Probabilities greater than 0.95 are given above and below each clade, respectively.

Results

Species descriptions

Ascothailandia Sri-indrasutdhi, Boonyuen, Sivichai & E.B.G. Jones, gen. nov.

Ascomata immersa, semi-immersa vel superficialia, perithecoidia, globosa vel subglobosa, brunneus versus ater, solitarius, dispersus, longus collum, ostiolata. Ostiolum maximam pariem centralis aut si ascomatis superjectus horizontalis versus hospes pagina, nunc ad uni-extremum et curvisursum, longus vel brevis, prerumque brunneus vel ater, periphyses. Paraphyses hypha similus, numerosus, contractus distal, non in gelatina matricis inclusus. Asci 8-spora, longus collum, pedunculus, unitunica, apicalis truncate, with a relative massive (ca. 4–8 µm diam.), J-, refractivus, collum, apicalis annulus, persistens. Ascospora uniseriatus vel imbricatus uniseriatus, fusiformis rectus vel curvamen, 3-euseptenatus et versicolorus.

MycoBank no.: MB 515145

Type species: *Ascothailandia grenadoidia* Sri-indrasutdhi, Boonyuen, Sivichai & E.B.G. Jones

Ascomata immersed, semi-immersed or superficial, perithecioid, globose or subglobose, dark-brown to black, solitary, scattered, ostiolate. Ostiole mostly central but if ascomata are lying horizontal to the host surface, then at one end and curving upwards, long or short, usually brown or black, periphysate. Paraphyses hypha-like, numerous, tapering distally, not embedded in a gelatinous matrix. Asci 8 spored, long cylindrical, pedunculate, unitunicate, apically truncate, with a relative massive (ca. 4–8 µm diam.), with an apical ring J-, refractive, cylindrical, persistent. Ascospores uniseriate or overlapping uniseriate, fusiform, straight or curved, 3-euseptate and versicolourous.

Etymology: from *asco* in reference to Ascomycota and *Thailandia* in reference to Thailand-country of origin.

Ascothailandia grenadoidia Sri-indrasutdhi, Boonyuen, Sivichai & E.B.G. Jones, sp. nov. Figs. 1–9

Ascomata immersa vel semi-immersa, dispersa, pyriformia, brunnea versus ater, 110–200 µm ata ($\overline{x}$ = 160, n = 6), 100–150 µm in diam ($\overline{x}$ = 123, n = 6). coriacea, ostiolata, papilata. Paraphyses sparsae, hyalinae, numerosae, contractus distal, non in gelatina matricis inclusus. Asci 70–86 × 10–12 µm ($\overline{x}$ = 11.5×78.6 µm, n = 6), 8-spora, collum, unitunica, brevis pedicellati, apicibus truncatus, apicalis annulus J-refractivus, Ascospora 16–22 × 4–8 µm ($\overline{x}$ = 19.7×5.9, n = 25), uniseriatus, ovoideae vel fusiformes, rectae vel curvae, hyalinae, 3-euseptatae, leves, guttula in quoque cellula, muco temri curcumcinato.

Holotypus: In lignum submerses emortuisque *Wrightia tomentosa* 26/08/2005 S. Sivichai, BBH26383.

Ascomata immersed or semi-immersed, scattered, pyriform, dark-brown to black, 110–200 μm high ($\overline{x}$ = 160, n = 6), 100–150 μm in diam ($\overline{x}$ = 123, n = 6). coriaceous, ostiolate, papilate. Paraphyses sparse, hyaline, numerous, tapering distally, not embedded in a gelatinous matrix. Asci 10–12 × 70–86 μm ($\overline{x}$ = 11.5 × 78.6 μm, n = 6), 8-spored, cylindrical, unitunicate, short pedicellate, apically truncate, with a refractive, J-apical ring. Ascospores 16–22 × 4–8 μm ($\overline{x}$ = 19.7 × 5.9, n = 25), uniseriated ovoid to fusoid, straight to curved, hyaline, 3-euseptate, smooth-walled, with a large guttule in each cell, surrounded by a thin layer of mucilage (Figs. 1–9).

Holotype: Thailand, Narathiwat, Hala Bala Wildlife Sanctuary, on submerged wood of *Wrightia tomentosa* Roem & Schult., August 26, 2005, collected and isolated by S. Sivichai, BBH26383 (single ascospore isolate ex holotype SS03615 = BCC20507).

MycoBank no.: MB 515146

Etymology: from *grenade* and *–oidia*, in reference to the similarity of the conidia of the anamorph in culture to a grenade outline.

rDNA sequence ex holotype: GQ390252 (18S), GQ390267 (28S), GQ390282 (ITS)

Anamorph: *Canalisporium grenadoidia.*

Canalisporium grenadoidia Sri-indrasutdhi, Boonyuen, Sivichai & E.B.G. Jones, sp. nov. Figs. 10-15

Colonia on CMA, crescens tardus, palus brunnea versus obscurus brunnea, effusio vel punctiformis, densus, cum parvus aerius mycelia, hypha alba versus obscurus brunnea, 2–2.5 μm latus, septa, germino, conidia obscurus olea versus brunnea. Conidia solitarius, acrogena, globosa versus sub-globosa vel ovatus, holoeverto. Densus paries, levis curvamen, 3–6 longitudinalis septa, et 4–6 transversa septa, atris, crassis praedita, brunnea versus nigrobrunnea, subsidium ad palus brunnea parvus cellula basali, Conidiorum 17–26, apicalis series cum 3–4 cella, nigro lumen canalis 1–2 µm.

Holotypus: On CMA 18/10/2005 by S. Sivichai, BBH26384.

Colonies on CMA, slow growing, pale-brown to dark-brown, effuse or punctiform, compact, with little aerial mycelium; hyphae white to pale-brown, 2–2.5 µm wide, septate, branched; conidia are pale-olive to brown. Conidia solitary, acrogenous, globose to sub-globose or oval, holoblastic, thick-walled, slightly curved, with 3–6 longitudinal septa, and 4–6 transverse septa, some constricted at the septa, brown to dark-brown at the septa, supported by a pale-brown small basal cell, the number of cells per conidium varies from 17–26, apical row with 3–4 cells, cell lumen connected by canals obscured by dark pigment, 1–2 µm.

Holotype: In culture of CMA, October 18, 2005 by S. Sivichai, BBH26384

Etymology: from *grenade* and *–oidia*, in reference to the similarity of the conidia of the anamorph in culture to a grenade outline.

MycoBank no.: MB 515143

We also compare the *Canalisporium grenadoidia* with currently known species of *Canalisporium* in shape and size of the conidia (Table 3).

Molecular phylogeny of combined 18S and 28S rDNA dataset

The combined 18S rDNA and 28S rDNA dataset including the new genus (One taxon) and *Canalisporium* strains (14 taxa) from the BIOTEC Culture Collection (BCC) were aligned along with 37 representative taxa from the Class Sordariomycetes and three Subclasses: Hypocreomycetidae (Microascales and Hypocreales), Sordariomycetidae (Diaporthales, Ophiostomatales and Sordariales) and the Lulworthiales with their accession numbers (Fig. 16). Two representative taxa of the order Xylariales (*Daldinia concentrica* and *Xylaria hypoxylon*) were used as an outgroup. The maximum parsimony analysis, with gaps treated as missing data, yielded eight trees (TL = 2316, CI = 0.506; RI = 0.494; RC = 0.422; HI = 0.494). The final aligned dataset comprised 2301 characters, out of which 592 were parsimony informative, 233 parsimony uninformative and 1476 constant characters. The difference between the eight trees is in the branch swapping pattern in the clade Lulworthiales and Diaporthales (results not shown). Bootstrap values, greater than 50%, are shown on the upper nodes, whereas Bayesian posterior probabilities greater than 0.95 are indicated on lower nodes. Based on Kishino-Hasegawa (K-H) maximum likelihood test, one of the eight trees (the best likelihood tree) is shown in Fig. 16. Phylogenies based on the same dataset sequences calculated on K-H maximum likelihood test under different loci of small ribosomal DNA and large ribosomal DNA were almost identical to those obtained from individual datasets, except for the position of the Lulworthiales in the 18S rDNA and 28S rDNA dataset with minor branch swapping (results not shown).

The new ascomycete is well placed in the *Canalisporium* clade with strong support (1.00 PP and 100% BS). This clade comprised two subclades: Subclade A composed of *Ascothailandia*, *C. elegans* Nawawi & Kuthub., *C. jinghongensis* L. Cai, K.D. Hyde & McKenzie and *C. pulchrum* (Hol.-Jech. & Mercado) A. Nawawi & A. J. Kuthubutheen with strong support (1.00 PP and 99% BS), Subclade B consisted of *C. caribense* (Hol.-Jech. & Mercado) A. Nawawi & A. J. Kuthubutheen (type species) and *C. pallidum* T. K. Goh, W. H. Ho & K. D. Hyde with low support, while another species, *C. exiguum* T. K. Goh & K. D. Hyde, grouped as a sister taxon to Subclade A and Subclade B.

The relationship between *A. grenadoidia* (teleomorph of *C. grenadoidia*) and the other *Canalisporium* species showed that this new taxon has close phylogenetic affinities with *C. elegans* (SS00523, SS00895) with a bootstrap support of 77% and posterior probabilities of 1.00 while *C. exiguum* formed a basal clade to the other *Canalisporium* species. Therefore, *A. grenadoidia* is proposed as a new ascomycete with its anamorph (*C. grenadoidia*) and supported by both morphological and molecular data.

Molecular phylogeny of internal transcribed spacer (ITS) dataset

A dataset consisting of 17 taxa including the new ascomycete, *Canalisporium* species and outgroup taxa (*D. concentrica* and *X. hypoxylon*) is presented in Fig. 17. This dataset contained 748 characters, 218 parsimony-informative, 415 constant and 115 parsimony-uninformative. The maximum parsimony analysis yielded three most parsimonious trees (MPTs) 443 steps long (with CI = 0.905, RI = 0.915, RC = 0.828, HI = 0.095). The overall topologies for all three MPTs are the same, and only differ in the minor swapping position of *C. caribense* (SS003839). One of the three MPTs inferred with the best topology from K-H test is shown in Fig. 17.

Maximum parsimonious phylogenies from the ITS dataset showed that the branches leading to the *Canalisporium* species are reasonably stable with respect to the position of *A. grenadoidia* (teleomorph of *C. grenadoidia*) and essentially similar to those derived from the combined 18S+28S rDNA and each individual datasets (18S and 28S rDNA). An exception was *C. exiguum* swapping position with the *C. pallidum* and *C. caribense* subclade with low support (less than 50% BS and 0.95 PP). The new taxon grouped with *C. elegans* with high statistical bootstrap support (92%) and Bayesian values (1.00).

Discussion

The genera *Ascotaiwania*, *Savoryella* and *Ascothailandia* share many common morphological features (Table 2), especially the nature of ascomata, paraphyses and versicolourous ascospores. However morphologically they can be separated by two characters: *Ascotaiwania* has a non-amyloid apical ring and ascospores with more than 3 septa, *Savoryella* lacks an apical ring and ascospores are 3-septate, while *Ascothailandia* has an apical ring and 3-septate ascospores. Further more, molecular data show they are not monophyletic (Boonyuen et al., personal communication).

However this paper focuses on the new freshwater ascomycete (*Ascothailandia*) and its *Canalisporium* anamorph, the first time the genus has been linked to a teleomorph. Molecular sequences show that the *Canalisporium* spp. and the new ascomycete form a well supported monophyletic clade in the Hypocreomycetidae, with the Hypocreales and Microascales as sister clades. The long branches of the *Canalisporium* clade and weak bootstrap support suggests little affinity with the Hypocreales and Microascales. *Ascothailandia* and *Canalisporium* share few morphological characters with the Microascales and Hypocreales, especially the versicolorous 3-septate ascospores and multicellular, pigmented conidia.

Goh et al. (1998) emended the generic concept of *Canalisporium* from that of Nawawi and Kuthubutheen (1989), both in the habitat of the taxa, conidial septation, order and arrangement of septa in the conidial body and the presence of septal canals, basal cells, conidial secession, conidiogenesis, morphology of the mycelium in pure culture and fossilized conidia features.

Canalisporium grenadoidia differs from all other *Canalisporium* species in shape and size of the conidia (Table 3). Currently there are nine *Canalisporium*: *C. caribense* (type species), *C. elegans*, *C. pulchrum* (Nawawi and Kuthubutheen 1989); *C. exiguum*, *C. kenyense* T. K.Goh, W. H. Ho & K. D. Hyde, *C. pallidum* (Goh et al.

1998); *C. variabile* T. K. Goh & K. D. Hyde (Goh and Hyde 2000); *C. jinghongensis* (Cai et al. 2003) and *C. panamense* A. Ferrer & C. A. Shearer (Ferrer and Shearer 2005). *Canalisporium grenadoidia* is described by as the anamorph of *Ascothailandia grenadoidia.*

Within the *Canalisporium* clade, two subclades are observed: subclade A. *Canalisporium* spp. with many rows of cells and subclade B. these forming only two rows, whether these differences are sufficient to designate them as separate genera remains to be resolved. No previous sequences have been available for *Canalisporium*, although Cai et al. (2003) drew attention to the similarities of the conidia to those of *Dictyosporium* Corda. Clearly, *Canalisporium* species are not the anamorphs of members of the Tubeufiaceae as suggested by Cai et al. (2006).

Key to species of *Canalisporium* (after Goh et al. 1998)

1a. Conidiophore absent..*C. panamense*

1b. Conidiophore present...2

2a. Conidia with the three smaller cells at the base and have a single cell at the apex..*C. kenyense*

2b. Conidia with a single cell at the base and one (rarely), two or more cells at the apex...3

3a. Conidia with a single column of longitudinal septa, scattered, pale olivaceous with clearly visible septa and canals, septa thin and not banded.................*C. pallidum*

3b. Conidia with a single, double, or 4–5 column(s) of longitudinal septa, pale-brown to dark-brown, septa usually thick and darkly banded, canals obscured or not readily visible...4

4a. Conidia with a single column of longitudinal septa...................................5

4b. Conidia with two or more columns of longitudinal septa...........................6

5a. Conidia 24–51 × 15–29 × (8–)10–16 μm, with 3–6(–7) rows of transverse septa...*C. caribense*

5b. Conidia 18–25 × 13–15 × 5–8 μm, with 2–3(–4) rows of transverse septa...*C. exiguum*

6a. Conidia regularly with 2 columns of longitudinal septa...............................7

6b. Conidia irregularly with 4–5 columns of longitudinal septa..........................8

7a. Conidia with 2–4 rows, 1 cell at the apex, 22–35 × 15–23 × 10–10.5 µm, 2.5–5 wide..*C. variabile*

7b. Conidia with 2–4 rows, 1–4 cells at the apex, 25–33 × 20–28 × 7.5–11.5 µm, up to 25 µm long and 1.5–2 µm wide ....*C. jinghongenses*

8a. Conidia with 3–9 rows, 1–3 cells at the apex, 25–63 × (16–)20–32 × 12–17 µm ....*C. pulchrum*

8b. Conidia with 5–8 rows, 1–5 cells at the apex, 32–58 × 25–38 × 10–13 µm ....*C. elegans*

8c. Conidia with 4–6 rows, 3–4 cells at the apex, 27.5–37.5 × 24–27.5 × 17.5–22.5 µm..*C. grenadoidia*

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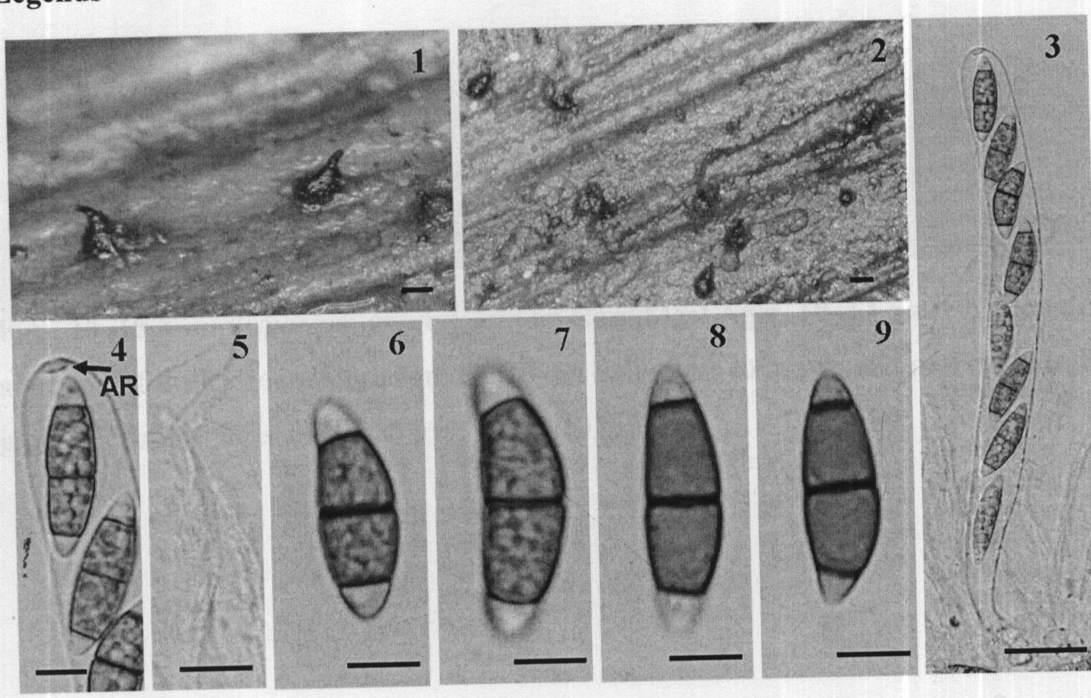
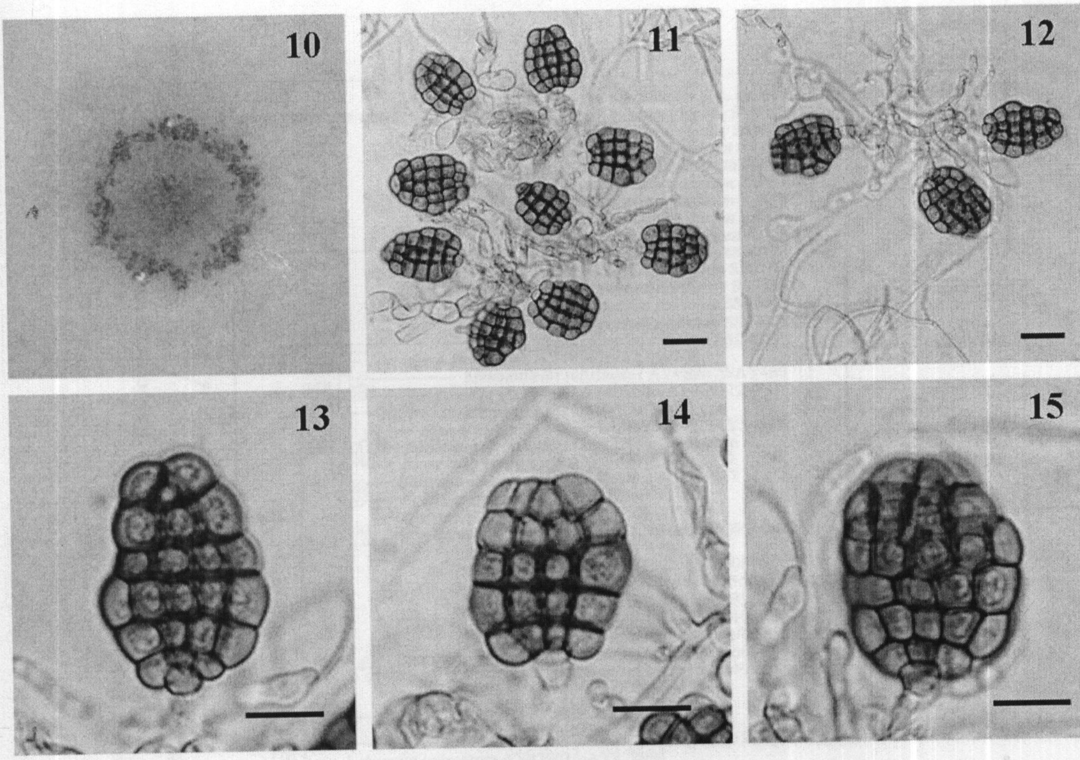
Legends



Figs. 1–9 Morphological characteristics of *Ascothailandia grenadoidia*. **1, 2** Light microscope micrographs of ascomata with long neck on the test blocks. **3** Ascus and hyaline paraphyses. **4** Apical ring (*AR*). **5** Hyaline paraphyses. **6–9** Ascospores uniseriate to overlapped biseriate squeezed from ascus. *Bars* 1, 2 100 μm; 3–5 20 μm; 6–9 5 μm



Figs. 10–15. *Canalisporium grenadoidia* on Corn Meal Agar. **10–12** Squash mount of a portion of the sporodochium conidia. **13–15** Pale-olive to brown and globose to sub-globose conidia. *Bars* 11, 12 20 µm; 13–15 10 µm

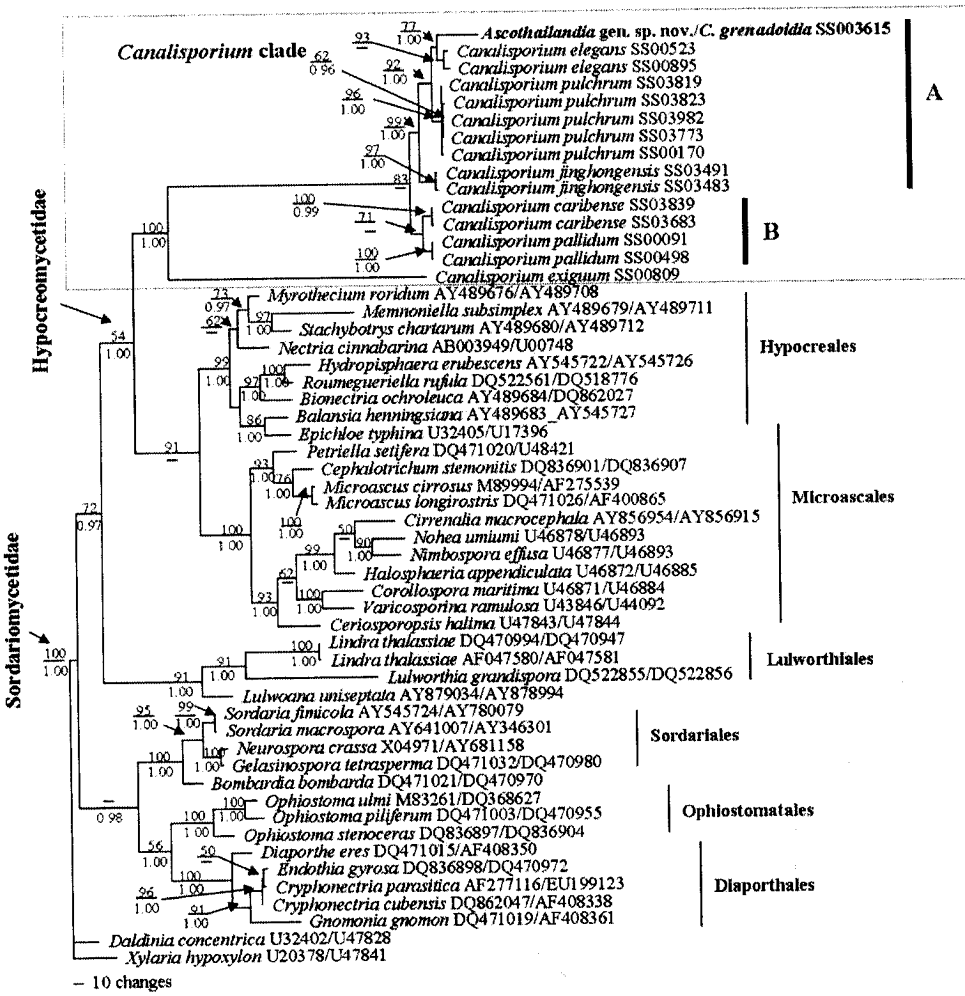


Fig. 16. Most parsimonious tree obtained from combined (18S+28S) rDNA dataset. The tree rooted with *Xylaria hypoxylon* and *Daldinia concentrica* from the Order Xylariales. Bootstrap values higher than 50% from maximum parsimony analysis are given above nodes and Bayesian posterior probabilities more than 0.95 are indicated as below nodes.

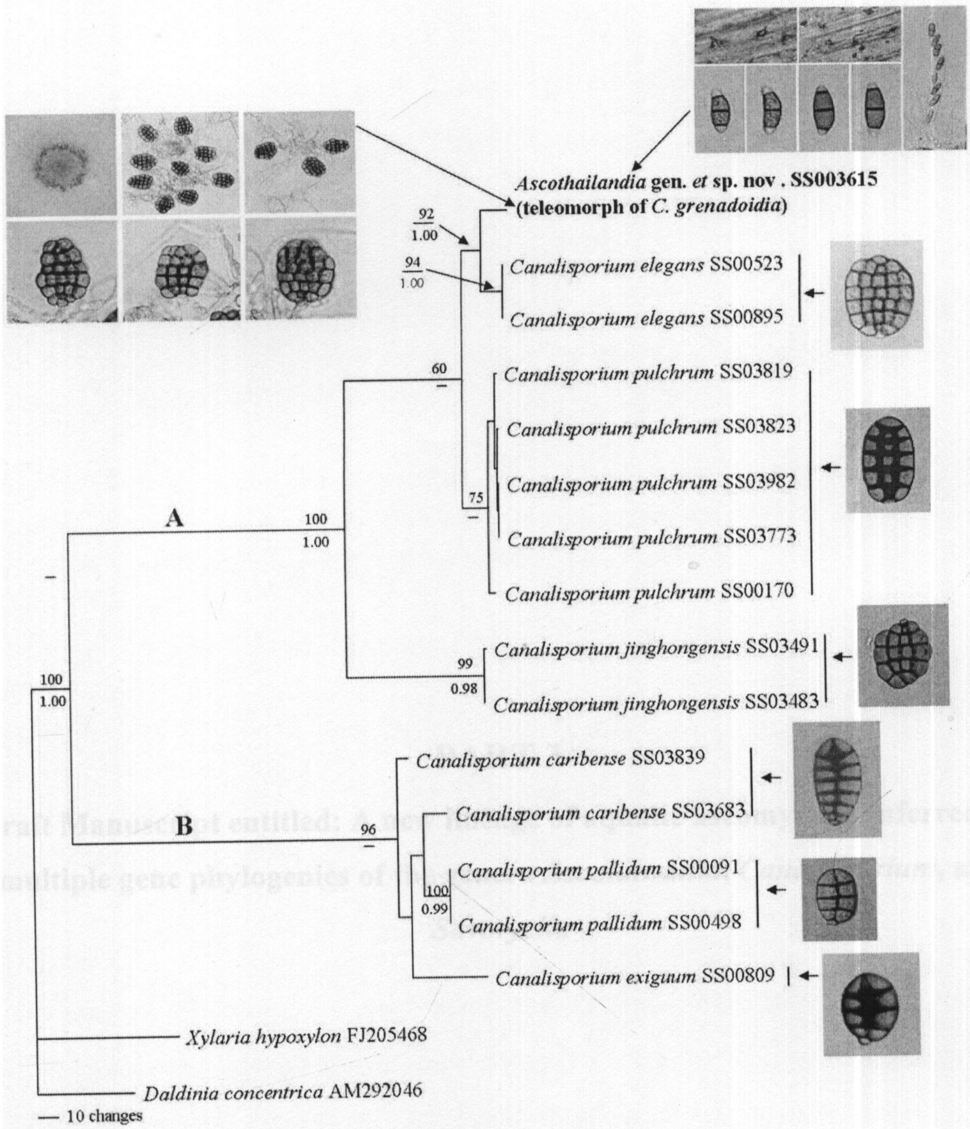


Fig. 17. Phylogeny obtained from ITS rDNA dataset. Bootstrap values more than 50% are shown above the branches and Bayesian posterior probabilities more than 0.95 are indicated as below nodes.

PART 3

Draft Manuscript entitled: A new lineage of aquatic ascomycetes inferred by multiple gene phylogenies of the genera *Ascotaiwania*, *Canalisporium*, and *Savoryella*

Draft Manuscript entitled: A new lineage of aquatic ascomycetes inferred from multiple-gene phylogenies of the genera *Ascotaiwania, Canalisporium*, and *Savoryella*

Nattawut Boonyuen[1*], Charuwan Chuaseeharonnachai[1], Satinee Suetrong [1,2], Veera Sri-indrasutdhi[1], Ka-Lai Pang[3], Somsak Sivichai[1] and E.B. Gareth Jones[1]

[1]Bioresources Technology Unit, National Center for Genetic Engineering and Biotechnology (BIOTEC), 113 Thailand Science Park, Phaholyothin Road., Khlong 1, Khlong Luang, Pathumthani 12120, Thailand
[2]Department of Microbiology, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla, 90112, Thailand
[3]Institute of Marine Biology, National Taiwan Ocean University, Keelung, Taiwan

*Corresponding author: Nattawut Boonyuen
Tel: +66-2564-6700 ext. 3242
Fax: +66-2564-6707
E-mail: nattawut@biotec.or.th

ABSTRACT

The taxonomic placement of freshwater and marine *Savoryella* species has been widely debated, and the genus has been tentatively assigned to various orders in the Sordariomycetes. We performed phylogenetic analyses of the partial small subunit rRNA (SSU), large subunit rRNA (LSU), internal transcribed spacer rDNA (ITS), DNA-dependent RNA polymerase II largest subunit (partial RPB1), DNA-dependent RNA polymerase II largest subunit (partial RPB2), translation elongation factor 1-alpha (TEF-l) and combined SSU and LSU sequences. Our results indicate that *Savoryella* species form a monophyletic group. Furthermore, *Savoryella*, *Ascotaiwania*, *Ascothailandia* and its anamorphic relatives (*Canalisporium*) form a clade, indicating that these genera share a common ancestor and are closely related. *Savoryella* shows no affinity with the Hypocreales, Halosphaeriales, Sordariales and Xylariales, despite earlier assignments to those orders. Because the genus shows no clear relationship with any named order or family, it is best referred to the subclass Hypocreomycetidae *incertae sedis* within Sordariomycetes. Our findings support the

recognition of a new lineage (ACS clade = *Ascotaiwania, Canalisporium, Savoryella*) of aquatic ascomycetes that have invaded both marine and freshwater habitats.

Keywords: Ascomycota *incertae sedis*, Hypocreomycetidae, Fungal systematics, unitunicate ascomycetes.

Introduction

Savoryella is one of the most commonly reported unitunicate ascomycete genera from submerged wood in rivers and streams (Sivichai et al., 2002, 2003) and in the marine environment (Jones and Hyde, 1992). The species *S. appendiculata* and *S. melanospora* have been recovered from wood in contact with sand (Jones and Hyde, 1992; Abdel-Wahab and Jones, 2000). The phylogenetic position of the genus is unresolved, and it has been referred to a number of orders and families in the Sordariomycetes, Sordariomycetidae (Zhang et al., 2006). Eleven species have been recognized: *Savoryella appendiculata, S. aquatica, S. curvispora, S. fusiformis, S. grandispora, S. lignicola, S. limnetica, S. longispora, S. melanospora, S. paucispora* and *S. verrucosa*. Of these species, five are found in marine habitats, and six are found in freshwater (Cai et al., 2006).

Jones and Eaton (1969) established the genus *Savoryella* with *S. lignicola*, which was isolated from wooden slats in a water-cooling tower run on brackish water, as the type species. *Savoryella* species are characterized by dark brown to black ascomata, clavate to cylindrical asci with a comparatively flattened apical ring and versicolorous septate ascospores, brown central cells and hyaline end cells. No anamorph has been reported for *Savoryella* (Tsui and Hyde, 2003). The establishment of the anamorph-teleomorph link between taxa can be phylogenetically informative. The genus has been variously referred to Sphaeriales *incertae sedis* (Kohlmeyer and Kohlmeyer, 1979), Ascomycetes *incertae sedis* (Kohlmeyer, 1986; Eriksson and Hawksworth, 1986), Amphisphaeriaceae (Eriksson and Hawksworth, 1987) and Sordariales (Jones and Hyde, 1992). Barr (1990) and Read et al. (1993) considered *Savoryella* to be best referred to Halosphaeriales based on morphological features (catenophyses-like paraphyses) and ultrastructural observations, respectively. Based on large subunit (LSU) rDNA data, Vijaykrishna (2006) and Cai et al. (2006) recently placed two *Savoryella* species (*S. elongata* and *S. longispora*) in the order Hypocreales within the subclass Hypocreomycetidae, but the relationship of Hypocreales to other orders could not be elucidated with good statistical support.

The genus *Ascotaiwania* has been isolated from freshwater habitats and from terrestrial palms and includes 12 species: *Ascotaiwania hsilio*, *A. hughesii*, *A. licualae*, *A. lignicola*, *A. mauritiana*, *A. mitriformis*, *A. pallida*, *A. palmicola*, *A. pennisetorum*, *A. persoonii*, *A. sawadae* and *A. wulai* (Sivanesan and Chang, 1992).

Ascotaiwania (Sivanesan and Chang, 1992; Chang et al., 1997; Ho et al., 1998) morphologically resembles *Savoryella* in having asci with versicolorous ascospores and long necks that are mostly lighter in color. However, *Ascotaiwania* differs in having cylindrical asci with a relatively massive, non-amyloid apical ring and ascospores that are three- to seven-septate. Additionally, *Ascotaiwania* anamorphs have been reported in *Monotosporella* (*A. sawadae, A. mitriformis*) and *Helicoon farinosum* (*A. hughesii*) (Sivichai et al., 1998; Cai et al., 2006).

Previous molecular studies have failed to resolve the taxonomic position of *Ascotaiwania* (Ranghoo et al., 1999; Campbell and Shearer, 2004). Cai et al. (2006) refer *Ascotaiwania* to Sordariales *incertae sedis*.

In our ongoing research on Thai freshwater fungi (Sivichai et al., 2002, 2003; Pang et al., 2002; Pinruan et al., 2002, 2004a, 2004b; Pinoi et al., 2003), we have recovered several *Canalisporium* species from submerged or trapped wood (Sivichai and Boonyene, 2004). Recently, a new species of *Canalisporium* has been reported in association with a newly described teleomorph genus, *Ascothailandida*, which morphologically resembles *Savoryella* and *Ascotaiwania* (Sri-indrasutdhi et al., in press). However, phylogenetic and morphological data indicate that the new *Canalisporium* species shows affinity with the Hypocreales (subclass Hypocreomycetidae, Sordariomycetes). Consequently, the new species and genus have been classified as Ascomycota *incertae sedis*.

To better understand the relationships and ordinal placements of *Savoryella*, *Ascotaiwania*, *Ascothailandia* and *Canalisporium* species, we conducted phylogenetic analyses using multiple genes. The objectives of this study were 1) to determine the taxonomic placement of *Savoryella* using a multiple-gene approach, 2) to elucidate the phylogeny of *Savoryella* and morphologically similar genera, including *Ascotaiwania* and *Ascothailandia* and 3) to use molecular data to examine the interrelationships of *Savoryella* and *Ascotaiwania* with the anamorphic genus *Canalisporium* from various habitats (freshwater and marine environments).

Materials and methods

Specimen collection

Fungi were isolated from various substrata collected from freshwater and marine locations in Thailand (Sivichai and Boonyene, 2004; Sakayaroj et al., 2004; Pinruan et al., 2002). Isolates were maintained on cornmeal agar (CMA) or potato dextrose agar (PDA) media prepared with seawater or freshwater.

Fungal isolates and growth

Fungal cultures were deposited and maintained in the BIOTEC Culture Collection (BCC). Taxa used in this study are listed in Table 1. All cultures were grown on PDA at 25°C for 4-16 weeks, depending on the growth rate of each species.

Genomic extraction and PCR amplification

Actively growing mycelia were scraped off from the surface of a culture and transferred to micro-centrifuge tubes. The biomass was lyophilized at -80°C for two days before DNA extraction, which followed a protocol modified from Tigano-Milani et al. (1995). The lyophilized mycelia were ground with a sterile pipette tip in a 2-ml microcentrifuge tube. The resulting powder was transferred to a 1.5-ml pre-warmed (65°C) microcentrifuge tube containing 700 µl of extraction buffer (0.7 M NaCl; 50 mM Tris-HCl, pH 8; and 10 mM EDTA, pH 8; 1% CTAB) and incubated at 65°C for one hour. In the CTAB-based method, DNA was extracted once with 500 µl of 24:1 chloroform-isoamyl alcohol (CIAA) and centrifuged at 12000 rpm for 20 minutes. The supernatant was transferred to a new 1.5-mL microcentrifuge tube containing 1/10 volume of 10% CTAB and 700 µl of CIAA. This mixture was then centrifuged for 20 minutes at 12000 rpm. Next, 1000 µl of precipitation buffer (50 mM Tris-HCl, pH 8.0; 10 mM EDTA, pH 8.0; 1% CTAB) was added to the aqueous phase of the supernatant, and the mixture was incubated for 30 minutes at room temperature. To purify the precipitated DNA, 300 µl of high-salt Tris-EDTA buffer (1 M NaCl; 10

mM EDTA, pH 8.0; and 1 mM EDTA, pH 8) was added, and the pellet was washed with 400 µl of 70% ethanol and resuspended in 30 µL of sterile deionized water containing 5 U RNase A (100 µg/mL). After centrifugation (20 minutes, 12000 rpm, 4°C), the DNA pellet was washed in 400 µl of 70% ethanol and air-dried. Finally, the DNA was resuspended in 50 µl of TE buffer (10 mM Tris-HCl, pH 8.0; and 1 mM EDTA, pH 8.0).

The partial SSU and LSU ribosomal DNA, ITS, partial RPB1 and RPB2 and TEF1-α regions were amplified using primers NS1, NS3, NS4, NS5, NS6, JS1, JS8, LROR, LR5, LR7, ITS1, ITS4, ITS5, RPB2-5F1, RPB2-5F2, RPB2-7CR, RPB2-7R, RPB1-CRPB1, RPB1-CR, EF1-983F, EF1-2218R, EF1-CEFF2 and EF1-CEFR2 (White et al. 1990; Bunyard et al., 1994; Landvik, 1996; Liu et al., 1999; Carbone et al., 1999; Rehner, 2001). PCR reactions were carried out in a total volume of 50 µl containing 10-50 ng of DNA template, 5 µl of 10X PCR buffer, 1.5 mM $MgCl_2$, 2 mM dNTPs, 0.2 µM each primer and 0.5 U of Taq Polymerase (DNA Polymerase Kit, Vivantis Technologies). Amplification cycles were performed according to the procedure of Tang et al. (2007) and consisted of an initial denaturation step at 95°C for 5 minutes followed by 35 cycles of denaturation (95°C), annealing (52°C for 1 min for SSU and LSU rDNA or 55°C for 1.5 minute for ITS, RPB1, RPB2 and TEF-l) and elongation (72°C for 1.5 minutes) and a final elongation step at 72°C for 10 minutes. In order to verify the size of each fragment, a 2-µl sample of the product was run on an agarose gel and visualized with ethidium bromide via an ultraviolet transilluminator. PCR products were purified using the NucleoSpin[R] Extract Kit (Macherey-Nagel, Germany), following the manufacturer's instructions. The quantity and quality of each purified product was verified by electrophoresis on a 1% agarose gel. Finally, purified PCR products were used directly for DNA sequencing.

DNA sequencing

PCR products were sequenced by Macrogen, Inc. (Korea) using the same forward and reverse primers used for amplification (White et al., 1990; Bunyard et al., 1994; Landvik, 1996; Liu et al., 1999; Carbone et al., 1999; Rehner, 2001). Each sequence was checked for ambiguous bases and assembled using Bioedit 7.5.03 (Hall, 2006).

Sequence alignment and phylogenetic analyses

A BLAST search was employed to obtain the closest-matching sequences in the GenBank database (Altschul et al., 1990). The SSU rDNA, LSU rDNA, ITS rDNA, RPB2, RPB1 and TEF-1 sequences were aligned with related sequences obtained from GenBank (Zhang et al., 2006; Tang et al., 2007; Schoch et al., 2007; Spatafora et al., 2006; Hibbett et al., 2007) using ClustalW 1.6 (Thompson et al., 1994). Alignments further adjusted manually using BioEdit 7.5.0.3 (Hall, 2006). Gaps were coded as missing data. Regions in which the alignment was ambiguous due to a large number of gaps were deleted from the analysis. Representatives of the order Pezizales were chosen as the outgroup taxa for all analyses.

Aligned datasets were subsequently analyzed using maximum parsimony (MP) in PAUP 4.0b10 (Swofford, 2002). To obtain the most parsimonious trees (MPTs), we performed heuristic searches with tree bisection–reconnection (TBR) branch swapping and 1000 replicates of random stepwise sequence addition. Gaps were treated as missing data and were given equal weight. The Kishino–Hasegawa (K–H) test was used to estimate the best tree topology (Kishino and Hasegawa, 1989).

Bootstrapping analyses (Felsenstein 1985) were performed with 1000 replicates of a full heuristic search (each consisting of 10 replicates of the random-swapping algorithm). The tree length (TL), consistency index (CI), rescaled consistency index (RC), retention index (RI) and homoplasy index (HI) were calculated for each tree. Representative sequences for each order within the class Sordariomycetes were retrieved from Genbank and added to the alignment.

Results

SSU phylogeny

To investigate the monophyly of the genera *Ascotaiwania, Canalisporium* and *Savoryella* and determine their phylogenetic position at the ordinal level, we included the type species of *Savoryella* (*S. lignicola*) and *Canalisporium* (*C. caribense*) in the 18S rDNA dataset. Sequences from 33 taxa of *Ascotaiwania, Canalisporium* and *Savoryella* obtained from the BIOTEC Culture Collection (BCC) were aligned with sequences from representatives of the two main subclasses of Sordariomycetes, Hypocreomycetidae and Sordariomycetidae. Representatives of subclass Hypocreomycetidae included various taxa from three orders (Microascales, Hypocreales and Melanosporales) and Hypocreomycetidae *incertae sedis*. These taxa formed the TMB clade (see below). Representatives of six major orders within subclass Sordariomycetidae (Diaporthales, Coniochaetales, Chaetosphaeriales, Ophiostomatales, Sordariales and Boliniales) and five representatives of Lulworthiales (Spathulosporomycetidae) were also included. Members of the orders Xylariales and Pezizales were chosen as outgroup taxa.

The aligned SSU dataset comprised a total of 1189 characters, of which 532 were parsimony informative, 497 were constant, and 160 were variable but parsimony uninformative. Maximum parsimony analysis resulted in 18 most parsimonious trees (MPTs) with a tree length (TL) of 2309 steps (CI=0.472, RI=0.846, RC= 0.400, HI=0.528) is shown in Figure 1.

The genera *Savoryella* (S), *Canalisporium* (C) and *Ascotaiwania* (A) formed a well-supported clade [100% bootstrap percentage (BP)]. This clade, hereafter referred to as the ACS clade, was clearly distinct from Halosphaeriales, Hypocreales, Melanosporales, Microascales (Hypocreomycetidae) and Sordariales (Sordariomycetidae) within Sordariomycetes. The seven *Canalisporium* species (*C. caribense*, *C. elegans*, *C. exigum*, *C. grenadoidia*, *C. jinghongensis*, *C. pallidum* and *C. pulchrum*) that composed subclade C and the six *Savoryella* species (*S. aquatica*, *S. fusiformis*, *S. lignicola*, *S. longispora*, *S. paucispora* & *S. verrucosa*) that composed subclade S together formed a moderately well-supported monophyletic clade (85% BP), while *Ascotaiwania sawadae* (SS00051) was grouped as a sister taxon to Subclade A plus Subclade B.

LSU phylogeny

This 28S rDNA dataset was used to investigate the phylogenetic relationships of *Savoryella* (five Thai marine isolates and eight Thai aquatic isolates), *Canalisporium* (eighteen strains) and *Ascotaiwania sawadae* (one strain, SS00051). The aligned dataset comprised a total of 1241 characters, of which 289 were parsimony informative and 812 were constant. The number of most parsimonious trees (MPT), tree length (TL), consistency index (CI), retention index (RI), rescaled consistency index (RC) and homoplasy index (HI) are listed in Figure 2.

The Kishino–Hasegawa (K–H) test was used to identify the best tree topology shown in Figure 2. The tree obtained from unweighted parsimony analysis yielded the best KH-likelihood scores, as shown in Figure 2. All topologies were similar to the phylogeny generated from the SSU dataset (data not shown). According to our analyses, the LSU rDNA sequences were divided into at least three major clades. Clades with bootstrap support values (BP) greater than 50% are described below.

Clade S (the *Savoryella* clade) consisted of *S. lignicola* (SAT00908), *S. longispora* (SAT00320 and SAT00322), *S. paucispora* (SAT00866 and SAT00867), *S. veruscosa* (SS00042, SS00052, SS00582 and SS03331), *S. aquatica* (SS00096, SS00359, SS03801 and SS00583) and *S. fusiformis* (SS00783). This clade included two distinct groups of species characterized by their habitat of origin (marine or freshwater). Most internal nodes within each clade had moderate to high bootstrap support (data not shown). Within clade S, the first sub-group comprised three marine species, *S. lignicola* (Alai), *S. longispora* (SAT00320 and SAT00322) and *S. paucispora* (SAT00866 & SAT00867), while the second sub-group comprised three species (*S. aquatica*, *S. fusiformis* and *S. verrucosa*) that were isolated from submerged wood collected in a freshwater environment.

Clade C (the *Canalisporium* clade) consisted of *C. grenadoidia* (SS03615), *C. elegans* (SS00523, SS00877 and SS00895), *C. pulchrum* (SS00170, SS03773, SS03788, SS03819, SS03823 and SS03982), *Canalisporium* sp. (SS03732), *C. exiguum* (SS00809), *C. caribense* (SS03839 and SS03683), *C. jinghongensis* (SS3483 and SS03491) and *C. palladium* (SS00091 and SS00498).

Clade A (the *Ascotaiwania* clade) comprised *A. sawadae* (SS00051) and several sequences derived from GenBank. *A. sawadae* (AF132323) plus *A. mitriformis* (AF132324) formed a subclade that was sister to clade C. *M. setosa* (AF132334) plus *A. hughesii* (AY316357) formed a sister subclade to all other ACS taxa except *A. persoonii* (AY590295), which was basal to all other ACS taxa. However, this relationship lacked support. The two *A. sawadae* sequences (SS00051 and AF132323) were grouped with 100% BP, while *A. mitriformis* (AF132324) formed a basal sister group with *M. setosa* (AF132334) and *A. hughesii* (AY316357) with weak support (data not shown). *M. setosa* (AF132334) and *A. hughesii* (AY316357) were closely related with high bootstrap support (data not shown).

Canalisporium and *Savoryella* appeared to be monophyletic, but the *Ascotaiwania* species did not form a monophyletic clade. *A. hughesii* and its anamorph (*M. setosa*) formed a sister group to clade S plus clade C, while *A. sawadae* (SS00051) and *A. mitriformis* formed a clade separate from the *Savoryella* plus *Canalisporium* clade.

Combined SSU and LSU phylogeny

We compared tree topologies obtained by combining the SSU and LSU datasets to those obtained by separately analyzing the LSU dataset, the ITS dataset, the RPB2 dataset, the combined ITS+RPB2 dataset and the ITS+LSU rDNA dataset.

The combined SSU and LSU dataset consisted of 3369 characters, of which 1053 were parsimony informative, 390 were variable but parsimony uninformative and 1926 were constant. An initial analysis of this dataset yielded eight trees with a tree length of 3528 (CI= 0.613, RI= 0.803, RC= 0.492, HI=0.387), shown in Figure 3.

The results of the combined SSU+LSU analysis were consistent with those of the separate SSU and LSU analyses. The MP trees resolved a monophyletic Hypocreomycetidae comprising three well-supported clades (A, C and S).

Ascotaiwania was polyphyletic, with *A. sawadae* (SS00051 and AF132323) and *A. mitriformis* (AF132324) forming a weakly supported sister group to the *Canalisporium* plus *Savoryella* clade and *A. hughesii* (AY316357), *A. persoonii* (AY590295) and *M. setosa* (AF132334) forming clades separate from *Canalisporium* plus *Savoryella. Canalisporium* formed a monophyletic group with *A. sawadae* as its sister clade. Five *Savoryella* species (*S. aquatica, S. lignicola, S. longispora, S. paucispora* and *S. verrucosa*) formed a monophyletic subclade with high bootstrap support (data not shown).

RPB2 phylogeny

To further elucidate ordinal relationships of *Ascotaiwania* (A), *Canalisporium* (C) and *Savoryella* (S), we sequenced part of the RPB2 gene (DNA-dependent RNA polymerase II largest subunit) for comparison with the SSU, LSU and combined SSU+LSU datasets.

The partial RPB2 dataset contained 32 taxa (14 *Savoryella*, 17 *Canalisporium*, 1 *Ascotaiwania*) from the BIOTEC Culture Collection (BCC). We also included the type species of *Savoryella* (*S. lignicola* AlaiPang) and *Canalisporium* (*C. carebense* SS03683 and SS03839). Eighty-eight representative taxa from the two subclasses of Sordariomycetes, Hypocreomycetidae and Sordariomycetidae (Zhang et al., 2006), were aligned with the *Ascotaiwania* (A), *Canalisporium* (C) and *Savoryella* (S) sequences. From subclass Hypocreomycetidae, various representative taxa from two orders and one new clade consisting of Microascales, Hypocreales and the TMB clade (Hypocreomycetidae *incertae sedis*) were included in the analysis. From subclass

Sordariomycetidae, representatives of six major orders (Diaporthales, Coniochaetales, Chaetosphaeriales, Ophiostomatales, Sordariales and Boliniales) were included in the RPB2 dataset. Members of the order Xylariales (*Daldinia concentrica* DQ368651 and *X. hypoxylon* DQ470878) were chosen as outgroup taxa. The aligned dataset comprised 940 characters, of which 626 were parsimony informative, 261 were constant, and 53 were variable but parsimony uninformative. Figure 4 shows the strict consensus of six MPTs with a tree length of 2309 (CI=0.140, RI=0.607, RC= 0.085, HI=0.860).

The genera *Savoryella* (S), *Canalisporium* (C) and *Ascotaiwania* (A) formed a well-supported clade (ACS clade) with 97% bootstrap support. The ACS clade was clearly distinct from Hypocreales, Microascales (subclass Hypocreomycetidae) and Sordariales (Subclass Sordariomycetidae).

The ACS clade comprised three subclades. The *Savoryella* clade (S), composed of five species (*S. aquatica, S. lignicola, S. longispora, S. paucispora* and *S. verrucosa*), formed a monophyletic subclade with 98% bootstrap support. The *Canalisporium* clade (C), consisting of seven *Canalisporium* species (*C. caribense, C. elegans, C. pallidum, C. pulchrum, C. exiguum, C. jinghongensis* and *C. grenadoidia*/*Ascothailandia* gen. et sp. nov.) formed a well-supported monophyletic subclade (100% BP). *Ascotaiwania* (A) was the sister group of the *Canalisporium* clade; however, this relationship was unsupported. These results are consistent with those of the SSU and LSU rDNA analyses and the combined SSU plus LSU analysis with respect to the phylogenetic placement of taxa within Hypocreomycetidae *incertae sedis*, although some of the members of each clade or subclade (i.e., the species identity of sequences obtained from GenBank) differed among datasets.

RPB1 phylogeny

We further investigated the phylogeny of the ACS clade using DNA sequences from the largest subunit of RNA polymerase II (RPB1). We compared the results of this analysis to those of the previous analyses of the SSU, LSU, SSU+LSU and RPB2 datasets for the same set of taxa within the ACS genera. The RPB1 dataset comprised 69 taxa, including selected species and reference species from GenBank representing the class Sordariomycetes (Spatafora et al., 2006). *Xylaria hypoxylon* and *X. acuta* (order Xylariales) were selected as outgroup taxa. Taxa were selected based on the current ordinal classification of the Hypocreomycetidae *incertae sedis* group, consisting of Microascales, Hypocreales, the TMB clade (Hypocreomycetidae *incertae sedis*), Diaporthales, Ophiostomatales and Sordariales. Two equally parsimonious trees (TL=5246, CI=0.261, RC=0.154, RI =0.590 and HI=0.734) were obtained, and the consensus tree is shown in Figure 5. Clades with bootstrap support values (BS) above 50% were designated as follows.

Clade A (the *Ascotaiwania* clade) consisted of *A. sawadae* (SS00051). Clade C (the *Canalisporium* clade) included *C. elegans* (SS00877, SS00523 and SS00895), *C. pulchrum* (SS03819, SS03823, SS03982, SS00170, SS03788 and SS03773), *C. jinghomgensis* (SS03491 and SS03483), *C. exiguum* (SS00809), *C. palladium* (SS00498 and SS00091), *Canalisporium* sp. (SS03732) and *C. caribense* (SS03839 and SS03683). Clade S (the *Savoryella* clade) consisted of *S. aquatica* (SS00096, SS00583, SS00359 and SS03801), *S. veruscosa* (SS00042, SS03331, SS00582 and SS00052), *S. lignicola* (LaiPang), *S. longispora* (SAT00320 and SAT00322), *S. paucispora* (SAT00866) and *S. paucispora* (SAT00867).

Maximum parsimony analysis of RPB1 produced the same topology as that obtained from the LSU, SSU, combined SSU+LSU and RPB2 analyses. The individual RPB2 and RPB1 datasets, though not significantly incongruent, conflicted in the placement of some taxa that exhibited long branches in the RPB1 data set (data not shown). In contrast, the RPB2 terminal branch lengths were rather uniform. Bootstrap support was greater for clades recovered in the RPB1 analysis (data not shown). Combining the two datasets (RPB2+RPB1) increased the degree of confidence for several relationships (data not shown). Overall, the RPB1 data did not yield a robust phylogeny when RPB1 sequences were used alone. This multi-gene study indicates that the ACS clade is a monophyletic group composed of three distinct lineages: part of *Ascotaiwania*, *Canalisporium* and *Savoryella*. Within the *Savoryella* lineage, two subclades were recovered in the RPB1 and combined datasets: one composed of species originating from marine habitats and one composed of species from freshwater habitats.

Translation elongation factor 1-alpha (TEF1-α) phylogeny

The genera *Savoryella* and *Canalisporium* have traditionally been differentiated by the morphologies of their ascospores, asci and conidiogenous cells. We assessed their phylogenetic relationships using protein sequences from a portion of the tef1 gene region. Sixteen isolates from the three genera were closely related within subclass

Hypocreomycetidae, Sordariomycetes. Maximum parsimony analysis (with gaps treated as missing data) yielded a single tree (TL=2214, CI=0.293, RI=0.520, RC=0.152, HI=0.707). The final aligned dataset comprised 919 characters, of which 339 were parsimony informative, 75 were parsimony uninformative and 505 were constant (Figure 4). When GenBank sequences for related species were excluded, maximum parsimony analysis yielded 15 most parsimonious trees (MPTs) that were 919 steps long (CI=0.729, RI=0.841, RC=0.613, HI=0.271). The overall topologies for all 15 MPTs were similar, differing only in the position of *C. grenadoidia* (SS003615). Most species were placed in one of two well-supported basal clades (*Savoryella* clade, 94% BP; *Canalisporium* clade, 98% BP) (Figure 6). However, no representative of *Ascotaiwania* was included in this phylogenetic tree. The ten isolates of *Savoryella* formed multiple well-supported lineages within a monophyletic clade. The *Canalisporium* species also appeared in a single clade. Most clades that were supported by sequence data appeared to be predictive of generic relationships.

ITS phylogeny

The ribosomal (ITS1, 5.8S, ITS2) sequence dataset was analyzed using maximum parsimony. The aligned dataset comprised 35 sequences, with *Xylaria hypoxylon* (FJ205468) and *Daldinia concentrica* as outgroup taxa. There were a total of 758 characters, of which 491 were parsimony informative and 196 were constant. An initial analysis of this dataset yielded 46 MPTs with a tree length (TL) of 1693 (CI= 0.615, RI= 0.808, RC=0.497, HI=0.385), shown in Figure 7.

In the ITS analysis, the two *A. sawadae* strains were monophyletic with 85% bootstrap support. *Canalisporium* and *Savoryella* formed a clade with 67% bootstrap support. Fifteen *Canalisporium* taxa formed a well-supported monophyletic clade (100% BP). Twelve *Savoryella* species constituted a well-supported monophyletic clade (97% BP) and appeared to be phylogenetically distinct from other genera, such as *Canalisporium*, *Monotosporella*, *Ascotaiwania* and *Helicoon*. Within the *Savoryella* clade, most internal subclades did not have reliable branch support. The Thai marine strains *Savoryella* cf. *longispora* (SAT00320) and *S. paucispora* (SAT00866 and SAT00866) were grouped together with weak statistical confidence. However, *S. lignicola* (SAT00908), the type species and a marine isolate, did not cluster with other *Savoryella* derived from marine habitats. Instead, *S. lignicola* was sister to a group of Thai freshwater *Savoryella* species. In the Thai freshwater

Savoryella subclade, four *S. aquatica* isolates grouped consistently with 85% bootstrap support, while *S. verrucosa* isolates formed a separate cluster with 86% bootstrap support. The *Monotosporella* strains and the two *Helicoon* strains did not group with the *Ascotaiwania* strains.

The congruence of the ITS- and LSU-derived phylogenies was tested by analyzing the respective datasets independently with both Bayesian (data not shown) and parsimony analyses. Separate parsimony analyses of the ITS and LSU datasets resulted in similar topologies, and both provided better resolution of the deeper nodes.

DISCUSSION

The ACS clade, a newly inferred lineage

Hibbett et al. (2007) recognized three subclasses within Sordariomycetes: Hypocreomycetidae (including the orders Coronophorales, Hypocreales, Melanosporales, and Microascales), Sordariomycetidae (including the orders Boliniales, Chaetosphaeriales, Coniochaetales, Diaporthales, Ophiostomatales, and Sordariales) and Xylariomycetidae (including the order Xylariales). The orders Lulworthiales, Meliolales, Phyllachorales and Trichosphaeriales were represented as Sordariomycetes *incertae sedis.*

The genera studied here (*Ascotaiwania*, *Canalisporium* and *Savoryella*) form a clade (referred to as the ACS clade) within the Hypocreomycetidae. Members of the Coronophorales and the TBM clade form sister clades to the ACS clade. The ACS clade is distinct from the orders Halosphaeriales, Microacales and Hypocreales, and genera grouped in the TBM clade are morphologically diverse compared to those in the ACS clade. Members of the ACS clade have a number of shared features, including ascomata that are generally swan-like in shape (rarely with a central neck); unitunicate asci that are persistent, clavate to cylindrical, short pedunculate, with or without paraphyses and generally with an apical pore; ascospores, asci cells, hyphae-like cells, and brown central cells. Most ascospore appendages are lacking, except in the marine species *S. appendiculata.*

All ACS species are saprobes; most are aquatic and grow well on lignocellulosic materials, such as decayed wood (Sivichai et al., 2002, 2003). However, few ACS species actively degrade lignocellulose (Jones & Eaton 1969). The ACS clade represents a newly inferred lineage of the Hypocreomycetidae. It is interesting to note that both the TMB and ACS clades occur in aquatic habitats that are transitional from terrestrial to freshwater to brackish and fully saline habitats.

Although the ACS clade represents a newly recognized lineage of ascomycetes, it is premature to erect a new order to accommodate this group of taxa.

No anamorphs have been reported for *Savoryella*, but several dematiaceous hyphomycetes have been reported in the genus *Ascotaiwania*, including *Monotosporella* sp. (*A. sawadae*; Sivichai et al., 1998), *M. setosa* (*A. sawadae*; Ranghoo et al., 1999) and *Helicoon* (*A. hughesii*; Fallah et al., 1999; Tsui and Berbee, 2006). Our analyses suggest that *Ascotaiwania* is not monophyletic, although these species form a distinct group (Ranghoo et al., 1999; Cambell and Shearer, 2004).

Ordinal placement of **Savoryella** *and* **Canalisporium** *species*

This study expands the dataset of Vijaykrishna et al. (2006) with additional sequences representing a broader taxonomic and phylogenetic sampling of Sordariomycetes. Therefore, we use the tree derived from the 18S rDNA dataset as a basis for discussing the ordinal position of the genera.

The phylogenetic positions of *Savoryella* and *Canalisporium* inferred by MP methods are similar for different genes (LSU, RPB2 and combined gene datasets), and the branching patterns with respect to the placement of ingroup taxa are similar to those obtained from a phylogenetic analysis of SSU nu-rDNA (Zhang et al., 2006; Schoch et al., 2007; Tang et al., 2007). However, some of the clades and subclades differed in composition, with different taxa obtained from GenBank for each gene.

Our results clearly show that *Savoryella*, which is morphologically similar to *Ascotaiwania* (Sordariales *incertae sedis*, Sordariomycetidae), is not closely related to the order Hypocreales within Hypocreomycetidae (Vijaykrishna, 2005; Cai et al., 2006). Therefore, *Savoryella* and its relatives should be assigned to another order. The ACS clade is best referred to Hypocreomycetidae *incertae sedis*, Sordariomycetes. These findings suggest that a newly recognized lineage of aquatic ascomycetes has invaded both marine and freshwater habitats. Although *Savoryella*, *Ascotaiwania* and *Canalisporium* are closely related, the exact topologies of the different datasets vary because they contain different taxa. The three genera form a distinctive group similar to the unclassified group of marine ascomycetes comprising *Swampomyces, Torpedospora* and *Juncigera* (Sakayaroj et al., 2005; Schoch et al., 2007).

The monophyly of the genera Savoryella* and *Canalisporium

All of our analyses support the monophyly of *Savoryella* and *Canalisporium*, but the taxonomic assignment of these genera is unresolved because they have been referred to several orders and families within Sordariomycetes, Sordariomycetidae.

Morphologically, the genus *Savoryella* resembles *Ascotaiwania* Sivan. & H. S. Chang. *Savoryella* shares several traits in common with *Ascotaiwania*, such as versicolorous ascospores, but differs in having cylindrical asci with a relatively massive, non-amyloid apical ring and four- to eight-septate ascospores (Chang et al., 1998). No anamorph is known for any of the described species of *Savoryella*. However, our analyses show that *Savoryella* and the anamorphic genus *Canalisporium* are related to *Ascotaiwania*. In our results, *Ascotaiwania* is polyphyletic, with different species grouping with different anamorphs (*Monotosporella, Helicoon* and *Canalisporium*) and is more distantly related to *Savoryella* and *Canalisporium* species than the latter groups are to each other.

The genus *Savoryella* clusters with *Canalisporium* (18S rDNA phylogenies); however, *Ascotaiwania* occupies a basal position relative to other members of both genera. Due to the limited availability of sequences from public databases, the phylogenetic relationships among *Savoryella*, *Canalisporium* and *Ascotaiwania* species cannot be ascertained. Additionally, we lacked the type species of *Ascotaiwania* for comparison in this study.

In the ITS data, most internal nodes were supported by the bootstrap analysis. Both freshwater and marine *Savoryella* species formed reciprocally monophyletic groups based on their habitats of origin. In the parsimony analysis, most taxa of *Savoryella*, including *S. lignicola* (the type species of the genus), *S. aquatica*, *S. lignicola*, *S. longispora, S. paucispora* and *S. verrucosa*,- were sorted into a large cluster, forming a monophyletic clade. *S. aquatica* and *S. verrucosa* formed a branch that was strongly supported by statistics and concordance. *S. lignicola*, *S. longispora* and *S. paucispora* formed a well-supported lineage of marine origin, and this group was well supported as the sister group of *Savoryella aquatica* plus *S. verrucosa*, which were collected from freshwater streams.

The topology derived from this analysis is consistent with independent analyses based on the position of the *Savoryella* and *Canalisporium* clades, showing non-polyphyletic genera. In contrast to the independent RPB2 dataset and the combined RPB2 dataset, the ITS dataset produced ambiguous results for the placement of *Ascotaiwania* spp.

The ITS dataset supported the monophyly and phylogenetic distinctiveness of *Savoryella*. Other genera (*Canalisporium*, *Ascotaiwania*, *Monotosporella* and *Helicoon*) were split across subclades in this analysis. *Savoryella aquatica* and *Savoryella verrucosa* were grouped together with high bootstrap and Bayesian support values, consistent with their shared freshwater habitat.

Our data shows that ITS sequence data can be used confidently to distinguish *Canalisporium* species. Further phylogenetic analyses of ITS sequences should include a greater number of species having a broader representation of the morphological variation in the genus. Likewise, additional species that are more restricted in their ecological habitats should be included. To confidently infer the phylogenetic relationships among members of *Canalisporium* and related genera, some species groupings that we have observed on the ITS tree will need to be verified by including more type strains in the dataset. Taxon sampling can have a strong effect on phylogenetic inference.

ACKNOWLEDGEMENTS

This research was financially supported by the Biodiversity Research and Training Program of Thailand (BRT R_252057 and R_251009). We thank Professor Morakot Tanticharoen, Dr. Kanyawim Kirtikara and Dr. Lily Eurwilaichitr at BIOTEC for their continual interest and constant support. We also thank Dr. Sayanh Somrithipol for providing comments and suggestions on the manuscript.

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Legends

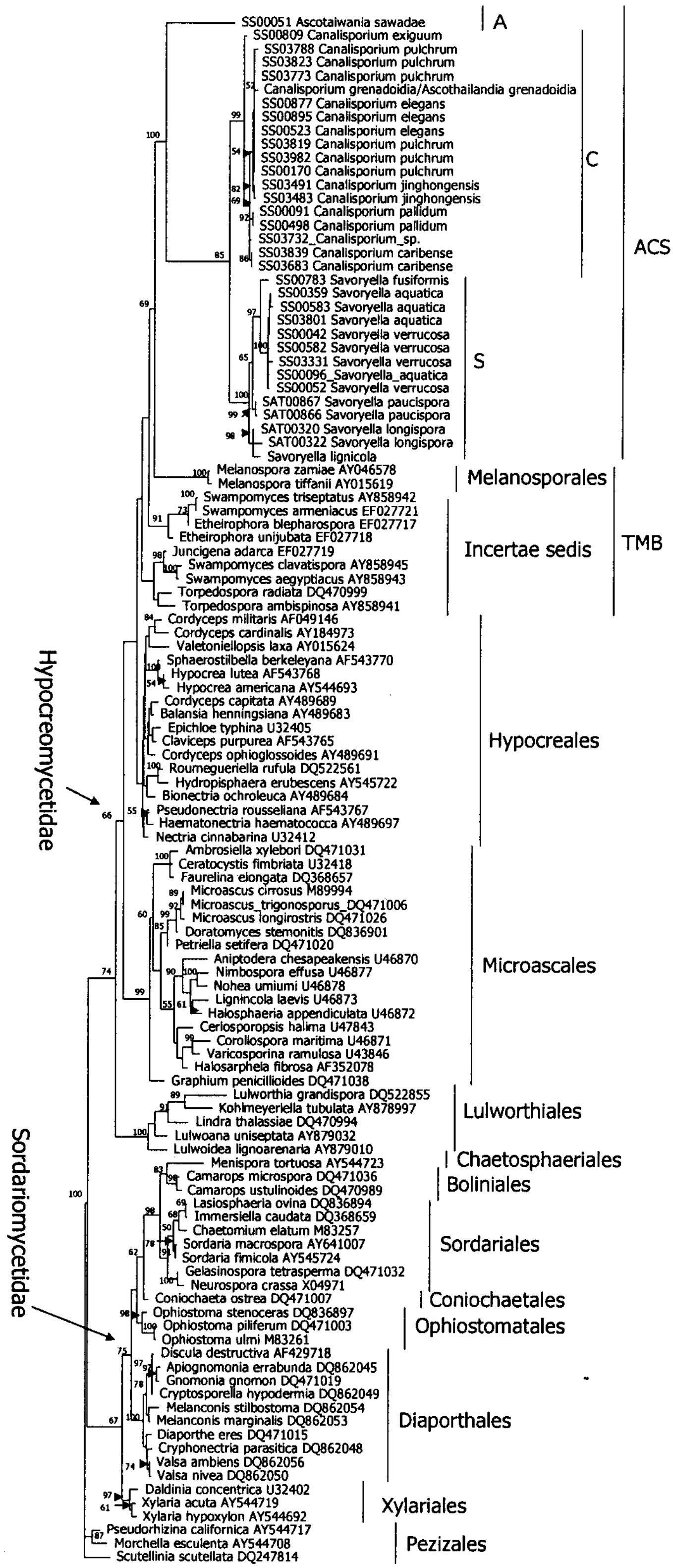

Fig 1 One of 18 most parsimonious trees for the Sordariomycetes inferred from SSU rDNA sequences. Bootstrap values greater than 50% (1,000 replicates) are given above the corresponding branches. NCBI GenBank accession numbers and names of taxonomic orders are provided to the right of the species names.

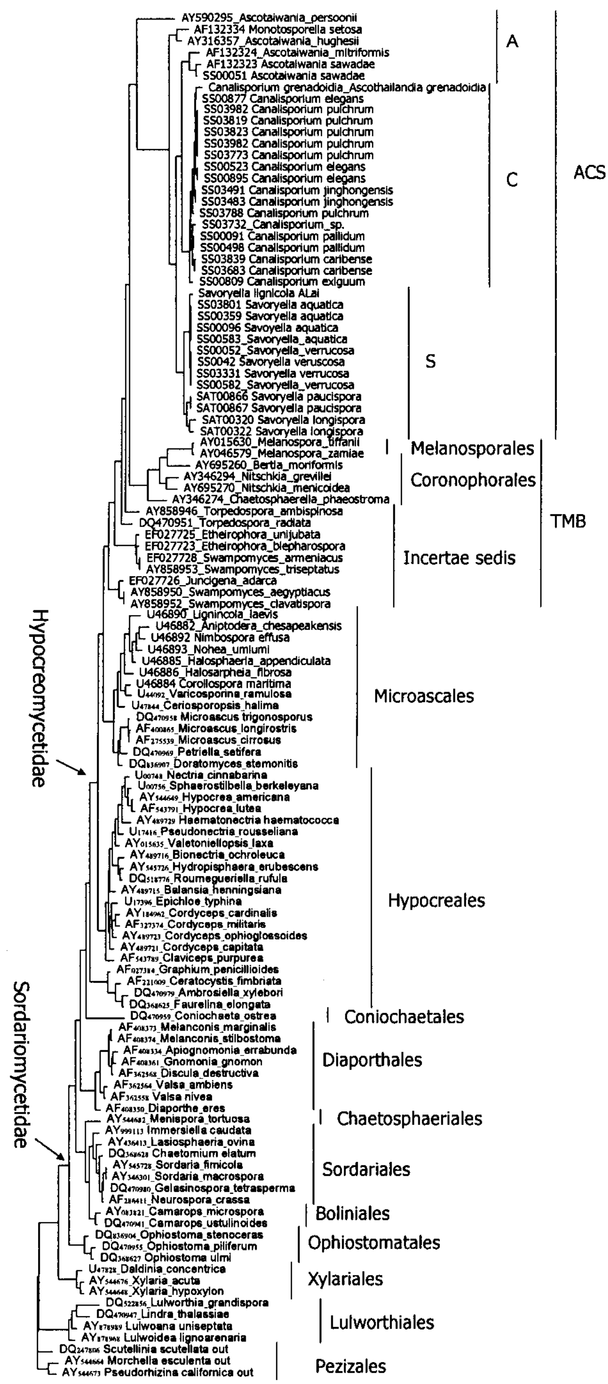

Fig 2 The most parsimonious tree for the Sordariomycetes inferred from partial LSU rDNA sequences. NCBI GenBank accession numbers and names of taxonomic orders are provided to the right of the species names. All *Savoryella, Canalisporium* and *Ascotaiwania* species collectively form a monophyletic group.

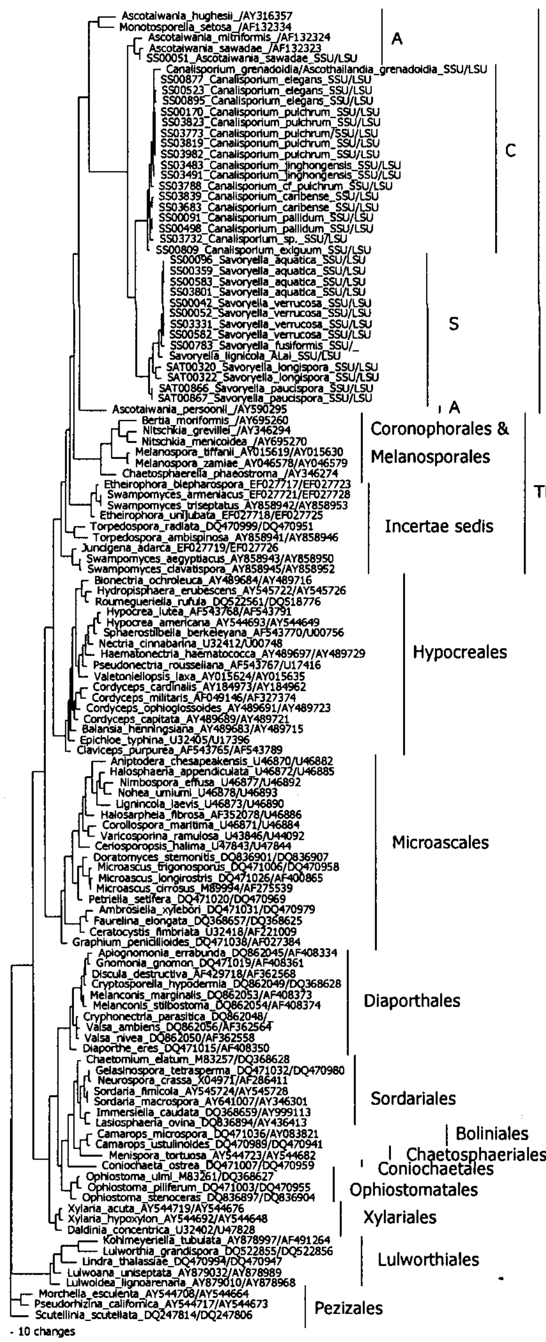

Fig 3 One of eight most parsimonious trees inferred from combined SSU rDNA + LSU rDNA sequences. The tree is rooted with members of the order Pezizales as outgroups. Bootstrap values greater than 50% (1,000 replicates) are given above the corresponding branches. Related anamorphic taxa are denoted by GenBank accession numbers. The type species of *Savoryella* and *Ascotaiwaniana* are printed in bold.

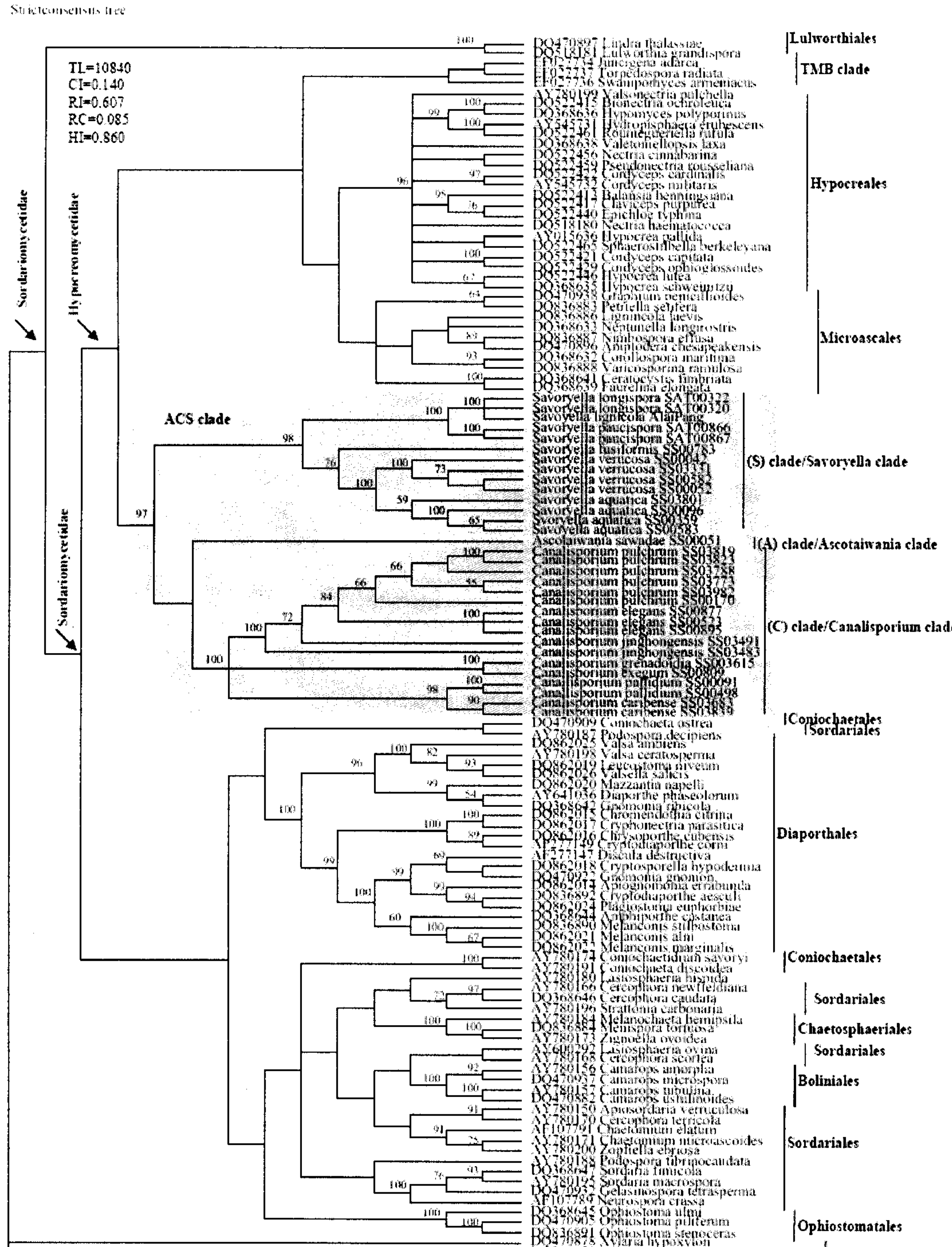

Fig 4 Strict consensus phylogram of the six most parsimonious trees inferred from RPB2 gene sequences, showing phylogenetic relationships between species of *Savoryella*, *Canalisporium* and *Ascotaiwania* and related taxa obtained from GenBank. Bootstrap values greater than 50% (1,000 replicates) are given above the corresponding branches. RPB2 sequences are indicated by their GenBank accession numbers. The phylogram is rooted with members of the order Xylariales as outgroups.

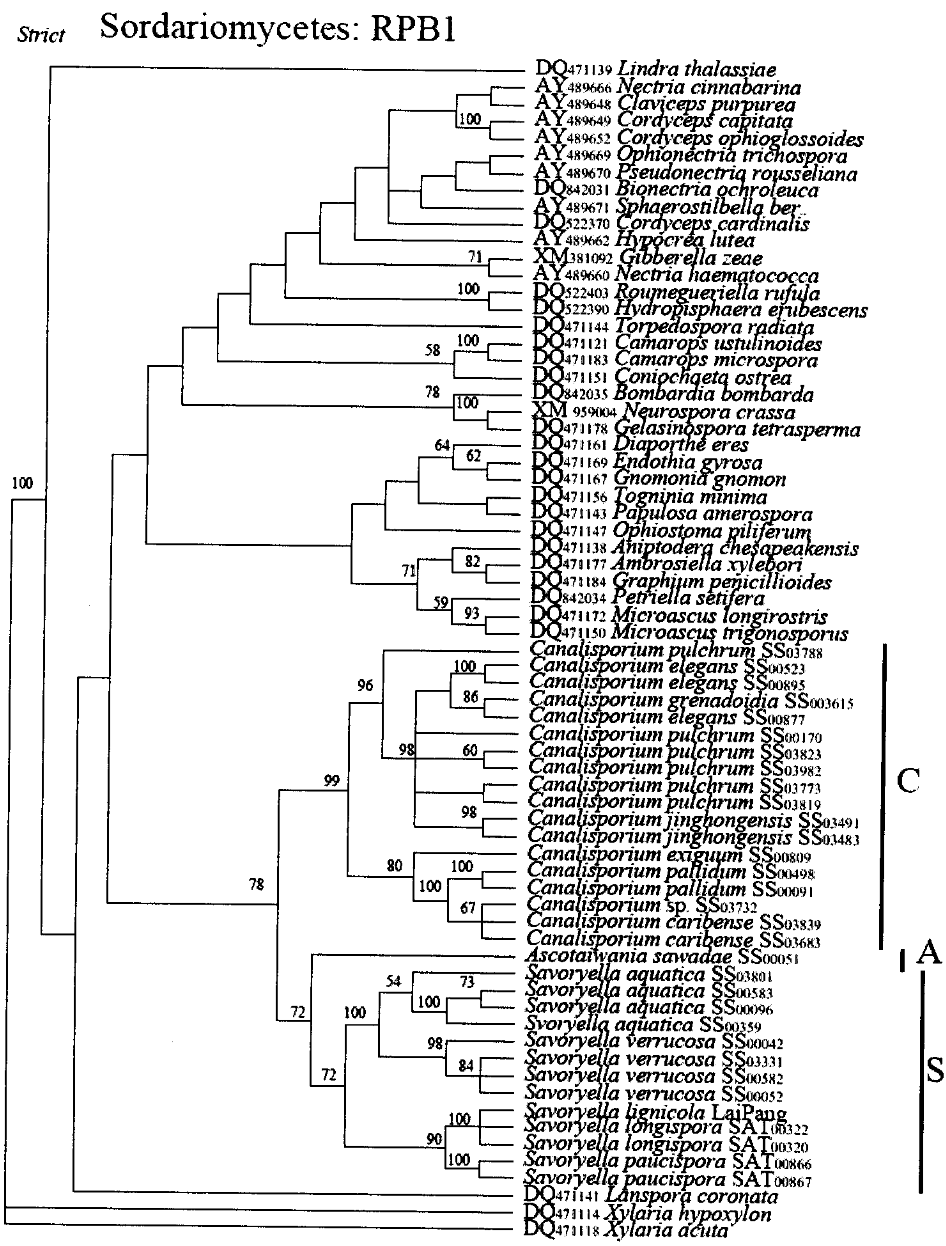

Fig 5 Strict consensus phylogram of two equally parsimonious trees inferred from partial RPB1 gene sequences. Bootstrap values greater than 50% (1,000 replicates) are given above the corresponding branches. The phylogram is rooted with members of the order Xylariales as outgroups.

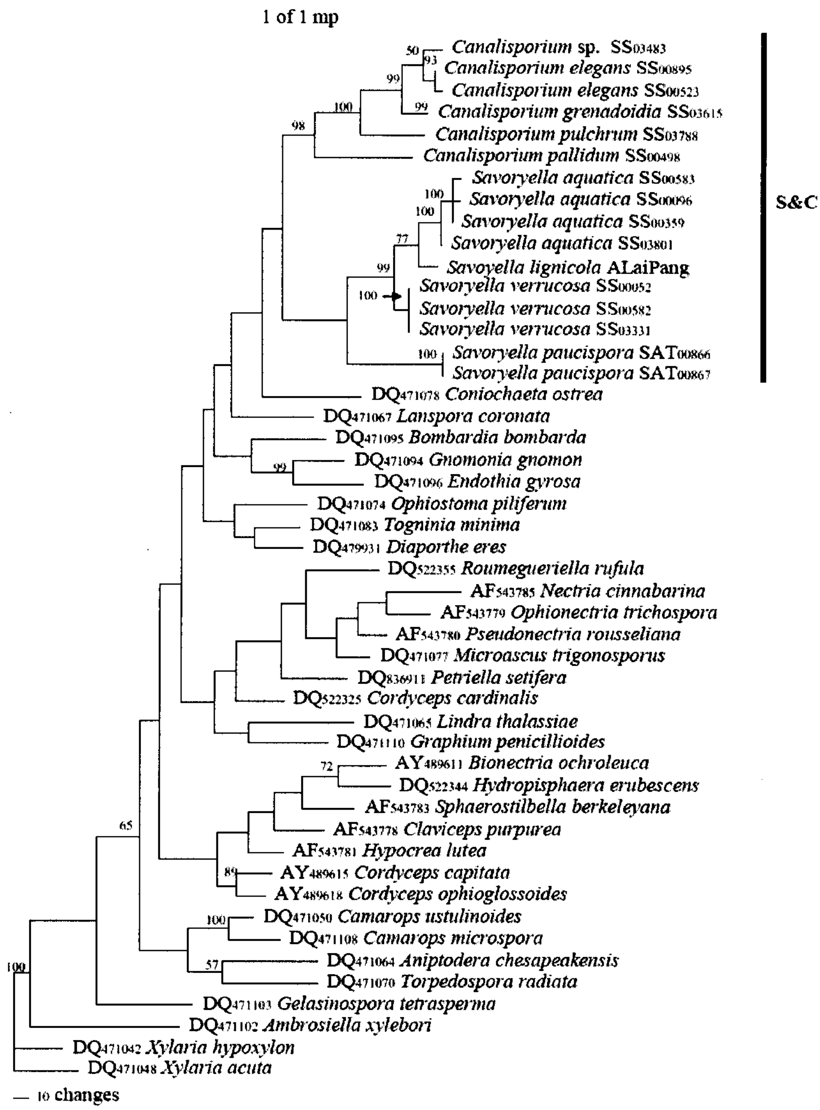

Fig 6 The single most parsimonious tree inferred from partial translation elongation factor 1-alpha (tef-1) gene sequences. Bootstrap values greater than 50% (1,000 replicates) are given above the corresponding branches. The phylogram is rooted with members of the order Xylariales as outgroups.

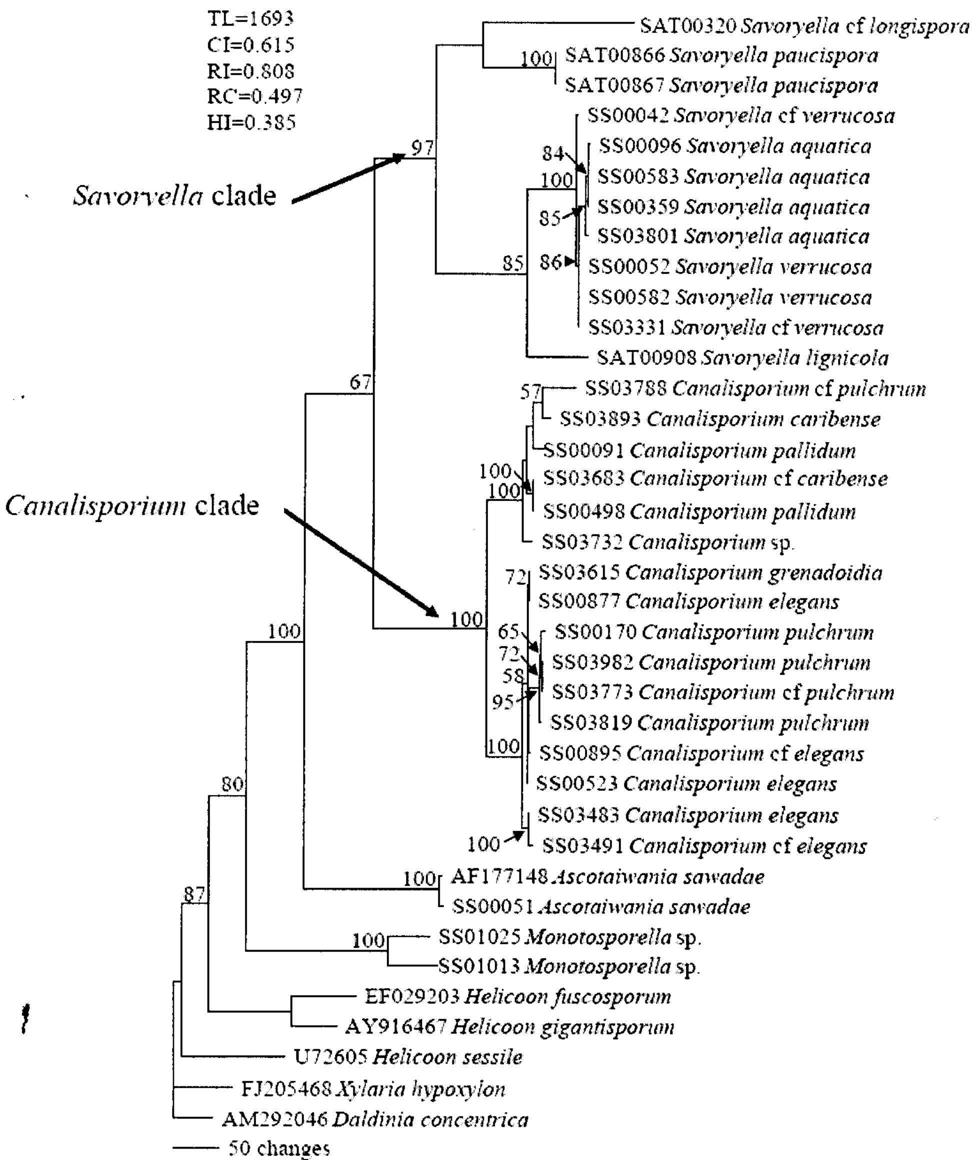

Fig 7 One of 46 most parsimonious trees inferred from nuclear ribosomal ITS sequences of *Savoryella*, *Canalisporium*, *Ascotaiwania* and anamorphs of *Ascotaiwania* (*Monotosporell' setosa* and *Helicoon* spp.), rooted with *Xylaria* and *Daldinia*. Bootstrap values greater than 50% (1,000 replicates), are given above the corresponding branches.

PART 4

POSTER BRT AND ABSTRACT OUTPUT 2008

The 12th BRT Annual Conference, Daimon Hotel Plaza, Surathani, Thailand, October, 10-13, 2008 (Abstract and Poster). Charuwan Chuaseeharonnachai, Nattawut Boonyuen, Somsak Sivichai and E.B. Gareth Jones (2008). ***Relationship of the genus Savoryella (teleomorph ascomycete) and its anamorph Canalisporium, as inferred by SSU and LSU data***

Relationship of the genus *Savoryella* (teleomorph ascomycete) and its anamorph *Canalisporium* as inferred by multiple gene phylogenies

Nattawut Boonyuen, Charuwan Chuaseeharonnachai, Somsak Sivichai and E.B. Gareth Jones*
*National Center for Genetic Engineering and Biotechnology, Pathum Thani, Thailand, *e-mail: nattawut@biotec.or.th*

The taxonomic placement of selected freshwater *Savoryella* species and some marine *Savoryella* species as well as putative *Canalisporium* species that originated from submerged woods in aquatic habitats have not been classified into any family or order with certainty. Results based on individual molecular data analyses of the partial small sequence (SSU data), indicate that *Savoryella* form a monophyletic clade and group within the subclass Hypocreomycetidae, Sordariomycetes. The genus *Savoryella* shows no affinities with the Hypocreales despite earlier assignment to that order. In addition, we can confirm using the large subunit rRNA gene (28S rDNA) the taxonomic position within Hypocreomycetidae, which is in good agreement with the 18S rDNA gene. Further analyses will be conducted including more strains of these taxa, and combining molecular analyses, such as ITS, RPB1, RPB2 and EF1-α, for determining the precise taxonomic placement of these genera.

RELATIONSHIP OF THE GENUS *SAVORYELLA* (TELEOMORPH ASCOMYCETE) AND THE ANAMORPH GENUS *CANALISPORIUM*, AS INFERRED BY SSU AND LSU DATA

Charuwan Chuaseeharonnachai, Nattawut Boonyuen, Somsak Sivichai and E.B. Gareth Jones

National Center for Genetic Engineering and Biotechnology (BIOTEC), 113 Thailand Science Park, Phahonyothin Road, Klong 1, Klong Luang, Pathumthani 12120, Thailand

ABSTRACT

The taxonomic placement of selected freshwater and marine *Savoryella* species, as well as *Canalisporium* species originating from submerged wood from aquatic habitats have not been classified into any family or order with certainty. Results based on individual molecular data, analyses of the partial small sequence (SSU data), indicate that *Savoryella* form a monophyletic clade and groups within the subclass Hypocreomycetidae, Sordariomycetes. The genus *Savoryella* shows no affinities with the Hypocreales despite earlier assignment to that order. These findings suggest a new lineage of ascomycetes that have invaded both the sea and freshwater environments. Interestingly, both *Savoryella* and *Ascotaiwania* share few morphological features in common, although clustering in the same clade. Based on morphology and sequence evidence, we suggest their referral to the Hypocreomycetidae incertae sedis, Sordariomycetes, until further information is available.

OBJECTIVES

1. To determine the taxonomic placement of *Savoryella* and *Canalisporium* species based on SSU and LSU sequences.2.To examine the interrelationships of the genera *Savoryella* with the anamophic genus *Canalisporium*.
2. To examine the interrelationships of the genera *Savoryella* with the anamophic genus *Canalisporium*

MATERIAL AND METHODS

The SSU and LSU were amplified and sequenced by using primer NS1, NS3, NS5, NS6, JS1, JS8, LROR, LR7, ITS1, ITS4 and ITS5 (White et al. 1990). Phylogenetic trees were generated from Maximum Parsimony incorporated in PAUP*4.0b10 (Swofford 2002).

CONCLUSION

1. *Savoryella* species form a monophyletic clade with the anamophic *Canalisporium* species in a sister group (SSU data).
2. *Ascotaiwania* is the teleomorph of *Canalisporium* species (large subunit rRNA gene).
3. The ascomycetes *Savoryella* and *Ascotaiwania* share a common ancestor and differ in some morphological features (28S rDNA gene).
4. The freshwater ascomycetes, previously identified as a *Savoryella* (SS3615) can be assigned to *Ascotaiwania* with the confidence, family a sister group with *Canalisporium* (LSU sequence).

Future work

Further analysis will be focused on wide taxa sampling and sequence of futher genes (ITS, RPB1, RPB2 and EF1-a).

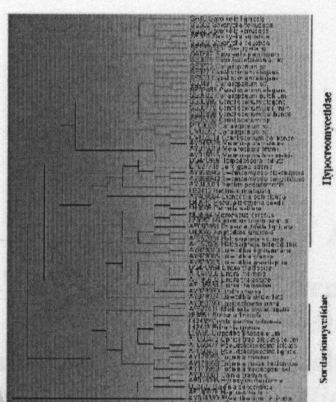

Fig 2 One of 52 parsimonious tree (SSU data)

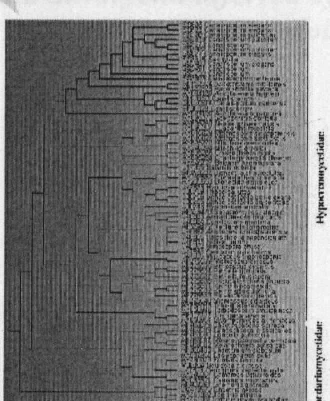

Fig 3 One of 47 parsimonious tree (LSU data)

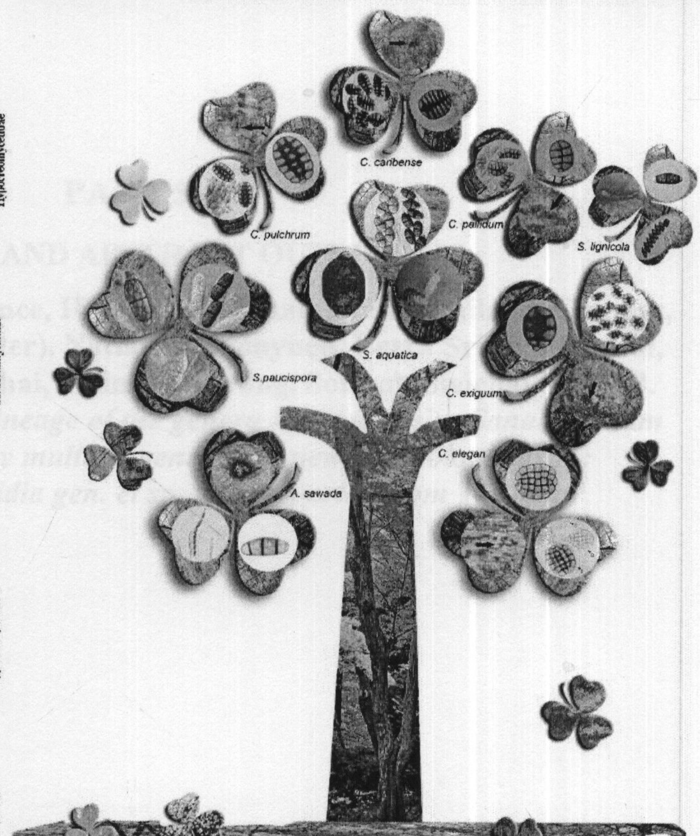

Fig 1 Tree pictures of various spore in this study

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PART 5

POSTER BRT AND ABSTRACT OUTPUT 2009

The 13th BRT Annual Conference, Holiday Inn, ChaingMai, Thailand, October, 12-14, 2009 (Abstract and Poster). Nattawut Boonyuen, Veera Sri-indrasutdhi, Charuwan Chuaseeharonnachai, Satinee Suetrong, Somsak Sivichai and E.B. Gareth Jones. (2009). ***A new lineage of the genera Ascotaiwania, Canalisporium and Savoryella inferred by multiple gene and a new lignicolous taxon: Ascothailandia grenadoidia gen. et sp. nov., reported from Thailand***

A new lineage of the genera *Ascotaiwania*, *Canalisporium* and *Savoryella* inferred by multiple gene and a new lignicolous taxon: *Ascothailandia grenadoidia* gen. *et* sp. nov., reported from Thailand

Nattawut Boonyuen, Veera Sri-indrasutdhi, Charuwan Chuaseeharonnachai, Satinee Suetrong, Somsak Sivichai and E. B. Gareth Jones

Bioresources Technology Unit, National Centre for Genetic Engineering and Biotechnology, Pathumthani, Thailand, e-mail: nattawut@biotec.or.th

The taxonomic placement of freshwater and marine *Savoryella* species has been widely debated and assigned to various orders. This study incorporates individual phylogenetic datasets and a combined datasets, based on the small subunit rDNA (SSU), large subunit rDNA (LSU) and RNA polymerase II the second largest subunit (RPB2) and ITS region to determine the ordinal position of the genera *Ascotaiwania* (A), *Canalisporium* (C) and *Savoryella* (S), all based on strains isolated from Thai substrata and including their type species. The ordinal status of (A) and (S) is unknown and consequently classified as Ascomycota *incertae sedis*. The anamorphic genera: *Monotosporella, Helicoon* (anamorphs of *A. sawadae, A. mitriformis* and *A. hughesii*), and *Canalisporium* species have also been studied. Phylogenetic analyses indicate that the genera (S), (A), and (C) share a common ancestor and are closely related. In the SSU, LSU, RPB2 and ITS dataset, *Savoryella* shows no affinities with the Hypocreales, Halosphaeriales, Sordariales and Xylariales despite earlier assignment to the orders Sordariales and Hyprocreles. Our findings suggest a new lineage (ACS clade= *Ascotaiwania, Canalisporium, Savoryella*) of aquatic ascomycetes that have invaded both the marine and freshwater habitats. We also describe *Ascothailandia* gen. nov. from submerged wood at Hala Bala Wildlife Sanctuary, Thailand. This genus is morphologically similar to the genera (A) and (S), but it differs in the acomata, asci, apical ring and spores (shape, dimension and colour) from these genera. Our phylogenetic results show that this taxon is well placed in the Hypocreomycetidae and bears close phylogenetic affinities to *Canalisporium* species.

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Wood inhabiting freshwater fungi from Thailand: *Ascothailandia* gen. et sp. nov., *Canalisporium grenadoidia* sp. nov.

Charuwan Chuaseeharonnachai[1], Veera Sri-indrasutdhi[1], Nattawut Boonyuen[1], Satinee Suetrong [1,2], Somsak Sivichai[1] and E.B. Gareth Jones[1]

[1]Bioresources Technology Center, Mycology Laboratory, National Center for Genetic Engineering and Biotechnology, 113 Paholyothin Road, Khlong Luang, Khlong 1, Pathum Thani 12120, Thailand.

[2]Department of Microbiology, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla, 90112, Thailand

Summary

Ascothailandia grenadoidia gen. et sp. nov. is described an illustrated from submerged wood (*Wrightia tomentosa*) in a stream at Hala Bala Wildlife Sanctuary, southern Thailand. Phylogenetic analysis of the combined partial 18S, 28S ribosomal DNA and internal transcribed spacer (ITS), including 5.8S regions, of *A. grenadoidia* and ten *Canalisporium* species was undertaken and analyzed with maximum parsimony and Bayesian methods. The molecular data indicate that *A. grenadoidia* is closely related to *C. elegans* in the Sordariomycetes, Hypocreomycetidae, order *incertae sedis*. Therefore, *A. grenadoidia* is proposed as a new genus with its anamorph (*C. grenadoidia*) and supported by both morphological and molecular data.

Objectives

1. To describe a new genus and species of *Ascothailandia* (*Canalisporium grenadoidia*) based on morphological data
2. To determine the taxonomic placement and relationship of a new fungus and its anamorph by molecular data (ITS data, the combined 18S and 28S ribosomal DNA)

Materials and Methods

1. Submerged wood test blocks (15×2.5×2.5 cm) of *Wrightia tomentosa* were collected from a stream at Hala Bala Wildlife Sanctuary, Narathiwat province to examine fungal presence.
2. A new taxon was examined Under microscope and described based on morphological traits.
3. Phylogenetic trees of combined rDNA (18S and 28S) and ITS dataset by using primer NS1, NS3, NS4, NS6, JS1, JS8, ITS1, ITS4 and ITS5 (White et al. 1990 and Bunyard et al. 1994) with statistical values were generated by Maximum Parsimony (MP) in PAUP*4.0b10 (Swofford 2002).

Acknowledgments

This research was supported by the Biodiversity and Training Program (BRT R_252057 and R_251009) of Thailand. We would like to thank Prof. Morakot Tanticharoen, Dr. Kanyawim Kirtikara, Dr. Lily Eurwilaichitr at BIOTEC for their continual interest and support.

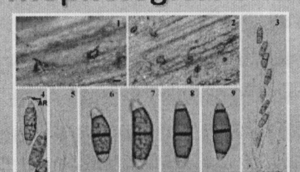
References

Bunyard BA, Nicholson MS, Royse DJ (1994) A systematic assessment of *Morchella* using RFLP analysis of the 28S ribosomal RNA gene. Mycologia 86:762-772

Swofford DL (2002) PAUP*4.0b10: Phylogenetic analysis using parsimony (*and other methods). Sinauer, Sunderland, Massachusetts, USA

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sininsky JJ, White TJ (eds) PCR protocols. San Diego, California, Academic Press, 315-322

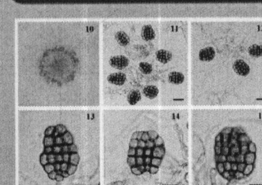
Mophological and Molecular Results



Figs. 1-9 Morphological characteristics of *A. grenadoidia*.

1. The new genus (teleomorph) is characterized by perithecoid, globose, dark-brown, ostiolate ascomata, paraphysate, asci cylindrical, unitunicate with a J-refractive apical ring and versicolourous, 3 - euseptate ascospores. Ascospores germinated producing a *Canalisporium* (*C. grenadoidia* sp. nov.) anamorph.

2. The new species (*C. grenadoidia* sp. nov.) is characterized by conidia solitary, acrogenous, sub-globose or oval, holoblastic, Thick-walled, 3-6 longitudinal septa, and 4-6 transverse septa, some constricted at the septa, brown to dark-brown at the septa, supported by a pale-brown basal cell, the number of cells per conidium 17-26, apical row with 3-4 cells, cell lumen connected by canals obscured by dark pigment, 1-2 µm.



Figs. 10-15 *Canalisporium grenadoidia* sp. nov., on Corn Meal Agar (CMA).

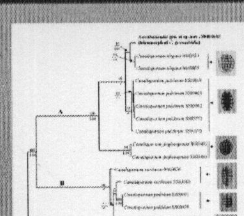


Fig. 16 Combined ribosomal (18S/28S rDNA) of the most parsimonious trees.

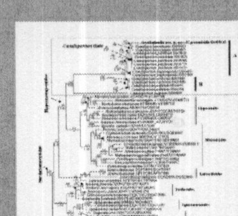


Fig. 17 Phylogeny obtained from ITS rDNA dataset.

3. The new ascomycete is well placed in the *Canalisporium* clade with strong support in Subclass Hypocreomycetidae (subclade A). This new taxon of *A. grenadoidia* (teleomorph of *C. grenadoidia*) has close phylogenetic affinities with *C. elegans* based on combined 18S and 28S rDNA dataset.
4. The new taxon grouped with *C. elegans* with high statistical bootstrap support (92%) and Bayesian values (1.00) calculated on ITS dataset.

PART 6

Oral presentation entitled: A new lineage of aquatic ascomycetes inferred by multiple gene phylogenies of the genera *Ascotaiwania*, *Canalisporium* and *Savoryella*, in Asian Mycological Congress 2009 & 11th International Marine and Freshwater Mycology Symposium, November 15-19, 2009, Taichung, Taiwan

Tuesday , 17/11/2009

Symposium B: Bioactive Compounds from Aquatic Fungi

Chair: Dr. Rainer Ebel and Dr. Thomas dela Cruz

10:00-10:25	SB-1: Natural product diversity of marine-derived fungi *Dr. Rainer Ebel*	O-149
10:25-10:50	SB-2: Mining our seas: strategies for isolating biologically Active Marine Fungi *Dr. Thomas Edison dela Cruz*	O-150
10:50-11:10	SB-3: Biological activities of marine fungi isolated from Philippine seagrasses *Ms. Dianne L. Dizon*	O-151
11:10-11:30	Coffee Break/Poster Session	

Symposium C: Ecology, Taxonomy and Phylogeny of Freshwater Fungi

Chair: Prof. Kevin Hyde and Dr. Somsak Sivichai

11:30-11:50	SC-1: A new lineage of aquatic ascomycetes inferred by multiple gene phylogenies of the genera *Ascotaiwania*, *Canalisporium* and *Savoryella* *Mr. Nattawut Boonyuen*	O-153
11:50-12:20	SC-2: Variation of fungal communities between lentic and lotic habitats, a case study from Thailand *Prof. Kevin D. Hyde*	O-154
12:20-12:50	SC-3: The dynamics of wood decay in tropical freshwater habitats *Prof. E. B. Gareth Jones*	O-155
12:50-14:10	Lunch/poster session	

Symposium D: Phylogeny and Ecology of Lower Fungi

Chair: Prof. Chiu-Yuan Chien and Dr. Daiske Honda

14:10-14:35	SD-1: Phylogeny and taxonomy of the genus *Pythium* *Dr. Shihomi Uzuhashi*	O-157
14:35-15:00	SD-2:A survey of *Phytophthora* species on Hainan Island of South China *Prof. Hon H. Ho*	O-158
15:00-15:20	SD-3: Isolation and identification of anaerobic chytrids from ruminants *Dr. Yo-Chia Chen*	O-160
15:20-15:40	SD-4: Diversity and ecology of chytrids in Taiwan *Assoc. Prof. Shu-Fen Chen*	O-161
15:40-14:10	Coffee Break/Poster Session	

A new lineage of aquatic ascomycetes inferred by multiple gene phylogenies of the genera *Ascotaiwania*, *Canalisporium* and *Savoryella*

Nattawut Boonyuen, Charuwan Chuaseeharonnachai, Satinee Suetrong, Somsak Sivichai and E. B. Gareth Jones
Mycology Laboratory, BIOTEC Central Research Unit, National Centre for Genetic Engineering and Biotechnology, Thailand Science Park, Pathumthani

The taxonomic placement of freshwater and marine *Savoryella* species has been widely debated and assigned to various orders: e.g. Hypocreales, Sordariales. The genus is characterized as processing paraphyses that deliquesce early, elongate, clavate to cylindrical asci with a poorly developed apical ring and versicoloured, 3-septate ascospores. This study incorporates individual phylogenetic datasets and a combined datasets, based on the small subunit rDNA (SSU), large subunit rDNA (LSU), to determine the ordinal position of the genera *Ascotaiwania*, *Canalisporium* and *Savoryella*, all based on strains isolated from Thai substrata and including their type species. Other genes sequenced include ITS region and RNA polymerase II the second largest subunit (RPB2). *Ascotaiwania* is characterized by ascospores that are generally more than 3-septate with hyaline end cells, asci with a relatively massive, and a non-amyloid apical ring. The ordinal status of *Ascotaiwania* and *Savoryella* is unknown and consequently classified as Ascomycota *incertae sedis*. The anamorphic genera: *Monotosporella, Helicoon* (anamorphs of *Ascotaiwania sawadae, A. mitriformis* and *A. hughesii*), and *Canalisporium* species have also been studied. Phylogenetic analyses indicate that the genera *Savoryella, Ascotaiwania* and *Canalisporium* share a common ancestor and are closely related. In the SSU rDNA, LSU rDNA and RPB2 dataset, *Savoryella* shows no affinities with the Hypocreales, Halosphaeriales, Sordariales and Xylariales (subclass Hypocreomycetidae, Sordariomycetes) despite earlier assignment to the orders Sordariales and Hyprocreles. Our findings suggest a new lineage (ACS clade= *Ascotaiwania, Canalisporium, Savoryella*) of aquatic ascomycetes that have invaded both the marine and freshwater habitats. Although these genera are related, they are not monophyletic. However, they form a distinct group similar to the unclassified group of marine ascomycetes comprising the genera *Swampomyces, Torpedospora* and *Juncigera* (TBM clade).

Keywords: Ascotaiwania, Canalisporium, Monotosporella, Savoryella, *phylogeny, systematics*

PART 7

LIST OF SPECIMENS COLLECTED IN THIS STUDY

PART 7

LIST OF SPECIMENS AND CULTURES COLLECTED IN THIS STUDY

List of *Savoryella* strains used in this study

Original Code	BCC Code	BBH Code	Order	Family	Genus	Epithet	Collection Date	IsolationDate	Substrate	SubSite	Site	District	Province
SS00042	3342	-	Sordariales	*Incertae sedis*	*Savoryella*	*verrucosa*	03-Sep-96	17-Oct-96	Elephant grass	-	Khao Yai National Park	-	Nakhon Ratchasima
SS00052	3344	12667	Sordariales	*Incertae sedis*	*Savoryella*	*verrucosa*	25-Sep-96	29-Oct-96	Twig	-	Khao Yai National Park	-	Nakhon Ratchasima
SS00582	3642	-	Sordariales	*Incertae sedis*	*Savoryella*	*verrucosa*	10-Jun-98	15-Jul-98	*Xylia dolabriformis*	Tad Tha Phu	Sakaerat Environmental Research Station	-	Nakhon Ratchasima
SAT00320	23612	-	Sordariales	*Incertae sedis*	*Savoryella*	*longispora*	-	-	Mangrove wood	-	Tammarang Pier	-	Satun
SAT00322	23612	-	Sordariales	*Incertae sedis*	*Savoryella*	*longispora*	-	-	Mangrove wood	-	Tammarang Pier	-	Satun
SAT00908	-	-	Sordariales	*Incertae sedis*	*Savoryella*	*lignicola*	-	-	Mangrove wood	-	Tammarang Pier	-	Satun
Alai	-	-	Sordariales	*Incertae sedis*	*Savoryella*	*lignicola*	-	-	-	-	-	-	-
SAT00866	28374	-	Sordariales	*Incertae sedis*	*Savoryella*	*paucispora*	-	-	Mangrove wood	-	Laem TaLum Phuk	-	Nakhonsithammarat
SAT00867	28375	-	Sordariales	*Incertae sedis*	*Savoryella*	*paucispora*	-	-	Mangrove wood	-	Laem TaLum Phuk	-	Nakhonsithammarat
SS03331	24236	-	Sordariales	*Incertae sedis*	*Savoryella*	sp.	11-Apr-05	26-Apr-05	*Stereospermum neuranthum*	Tad Tha Phu	Sakaerat Environmental Research Station		Nakhon Ratchasima
SS00096	3345	12702	Sordariales	*Incertae sedis*	*Savoryella*	*aquatica*	14-Nov-96	29-Dec-96	*Anisoptera oblonga*	Road marker at km 29.2	Sakaerat Environmental Research Station	-	Nakhon Ratchasima
SS00359	3521	-	Sordariales	*Incertae sedis*	*Savoryella*	*aquatica*	11-Jul-97	06-Aug-97	*Alstonia scholaris*	Tad Tha Phu	Sakaerat Environmental Research Station	-	Nakhon Ratchasima
SS00583	3641	-	Sordariales	*Incertae sedis*	*Savoryella*	*aquatica*	10-Jun-98	15-Jul-98	*Xylia dolabriformis*	Tad Tha Phu	Sakaerat Environmental Research Station	-	Nakhon Ratchasima
SS03801	22509	-	Sordariales	*Incertae sedis*	*Savoryella*	*aquatica*	26-Jan-06	15-Mar-06	Wood		Khao Pra - Bang Khram Wildlife Sanctuary		Krabi

List of *Ascotaiwania* strains used in this study

Original Code	BCC Code	BBH Code	Order	Family	Genus	Epithet	Collection Date	Isolation Date	Substrate	SubSite	Site	District	Province
SS00051	3343	-	Sordariales	*Incertae sedis*	*Ascotaiwania*	*sawadae*	25-Sep-96	29-Oct-96	Hard wood	-	Khao Yai National Park	-	Nakhon Nayok

List of *Ascothailandia grenadoidia* gen. *et* sp. nov., used in this study

Original Code	BCC Code	BBH Code	Order	Family	Genus	Epithet	Collection Date	Isolation Date	Substrate	SubSite	Site	District	Province
SS03615	20507	-	Sordariales	*Incertae sedis*	*Ascothailandia*	*grenadoid ia*	-	-	Submerged *Wrightia*	Khlong I-Gading stream	Hala-Bala Wildlife Sanctuary	-	Narathiwat

List of *Canalisporium* strains used in this study

Original Code	BCC Code	BBH Code	Order	Family	Genus	Epithet	Collection Date	Isolation Date	Substrate	SubSite	Site	District	Province
SS00091	3350	12699	*Incertae sedis*	*Incertae sedis*	*Canalisporium*	*pallidium*	14-Nov-96	27-Dec-96	*Alstonia scholaris*	Road marker at km 29.2	Sakaerat Environmental Research Station	-	Nakhon Ratchasima
SS00170	3406	12764	*Incertae sedis*	*Incertae sedis*	*Canalisporium*	*pulchrum*	02-ธ.ค.-96	11-ก.พ.-97	*Alstonia scholaris*	Road marker at km 29.2	Sakaerat Environmental Research Station	-	Nakhon Ratchasima
SS00498	3608	-	*Incertae sedis*	*Incertae sedis*	*Canalisporium*	*pallidium*	06-Nov-97	09-Mar-98	*Xylia dolabriformis*	Road marker at km 29.2	Sakaerat Environmental Research Station	-	Nakhon Ratchasima
SS00523	3625	-	*Incertae sedis*	*Incertae sedis*	*Canalisporium*	*elegans*	14-Apr-98	24-Apr-98	*Xylia dolabriformis*	Road marker at km 29.2	Sakaerat Environmental Research Station	-	Nakhon Ratchasima
SS00809	12770	-	*Incertae sedis*	*Incertae sedis*	*Canalisporium*	*exiguum*	20-Sep-00	29-Sep-00	Wood	-	Khao Soi Dao Wildlife Sanctuary	-	Chanthaburi
SS00877	9963	-	*Incertae sedis*	*Incertae sedis*	*Canalisporium*	*elegans*	26-Sep-00	10-Nov-00	Wood	Road marker at km 18	Kaeng Krachan National Park	-	Prachuap Khiri Khan
SS00895	12772	-	*Incertae sedis*	*Incertae sedis*	*Canalisporium*	*elegans*	13-Nov-00	29-Nov-00	Stereospermum neuranthum	Road marker at km 29.2	Sakaerat Environmental Research Station	-	Nakhon Ratchasima

SS03483	26225	-	*Incertae sedis*	*Incertae sedis*	*Canalisporium*	*elegans*	08-Apr-05	17-Jul-05	Wood	-	Bor Kleng Hot Spring	-	Ratchaburi
SS03491	18364	-	*Incertae sedis*	*Incertae sedis*	*Canalisporium*	sp.	08-Apr-05	19-Jul-05	Wood	-	Kaeng Krachan National Park		Prachuap Khiri Khan
SS03683	21022	-	*Incertae sedis*	*Incertae sedis*	*Canalisporium*	sp.	13-Jul-05	07-Feb-06	Wood	Wang Kar Leung Waterfall	Wang Kan Lueng Arboretum		Lop Buri
SS03732	21424	-	*Incertae sedis*	*Incertae sedis*	*Canalisporium*	sp.	17-May-04	21-Feb-06	Wood	Ban Krang	Kaeng Krachan National Park		Phetchaburi
SS03773	21030	-	*Incertae sedis*	*Incertae sedis*	*Canalisporium*	sp.	28-Jan-06	10-Mar-06	Leaf	Khlong I-Gading	Hala-Bala Wildlife Sanctuary		Narathiwat
SS03788	22507	-	*Incertae sedis*	*Incertae sedis*	*Canalisporium*	sp.	26-Jan-06	15-Mar-2006	Wood	-	Khao Pra - Bang Khram Wildlife Sanctuary		Krabi
SS03819	21221	-	*Incertae sedis*	*Incertae sedis*	*Canalisporium*	*pulchrum*	26-Jan-06	15-Mar-06	Wood	-	Khao Pra - Bang Khram Wildlife Sanctuary		Krabi
SS03823	21428	-	*Incertae sedis*	*Incertae sedis*	*Canalisporium*	*pulchrum*	26-Jan-06	15-Mar-06	Wood	-	Khao Pra - Bang Khram Wildlife Sanctuary		Krabi
SS03839	24239	-	*Incertae sedis*	*Incertae sedis*	*Canalisporium*	*caribense*	25-Feb-06	17-May-06	Wood	Khlong I-Gading	Hala-Bala Wildlife Sanctuary		Narathiwat

PART 8

THAI ARTICLE SENT TO BRT MAGAZINE

การจัดหมวดหมู่ ราสกุล *Savoryella* โดยใช้เทคนิคทางชีวโมเลกุลของยีนหลายชนิดร่วม

นัฐวุฒิ บุญยืน จารุวรรณ เชื้อสีหะรณชัย สาทินี ชื่อตรง วีระ ศรีอินทร์สุทธิ์ สมศักดิ์ ศิวิชัย และ ศ.อีวาน เบนจามิน กาเร็ธ โจนส์
ศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ สำนักงานพัฒนาวิทยาศาสตร์และเทคโนโลยีแห่งชาติ อุทยานวิทยาศาสตร์
113 ถนนพหลโยธิน ตำบลคลองหนึ่ง อำเภอคลองหลวง จังหวัดปทุมธานี 12120

การศึกษาค้นคว้าวิจัยงานทางด้านอนุกรมวิธานทางด้านราวิทยามีความสำคัญอย่างยิ่งในการต่อยอดองค์ความรู้ในงานทางด้านราวิทยาต่างๆเพื่อนำไปใช้ประโยชน์ทางเทคโนโลยีชีวภาพและพันธุวิศวกรรมและสาขาอื่นๆที่เกี่ยวข้อง ราจัดเป็นจุลินทรีย์ในอาณาจักรราซึ่งมีบทบาทและหน้าที่ในการย่อยสลายอินทรีย์วัตถุในระบบนิเวศน์เพื่อนำแร่ธาตุต่างๆกลับคืนสู่ธรรมชาติ ราหลายชนิดถูกนำมาใช้ประโยชน์ต่างๆอย่างมากมายในชีวิตประจำวัน ทั้งด้านเกษตรกรรม อุตสาหกรรมอาหาร อุตสาหกรรมเกษตร และทางด้านการแพทย์ เนื่องจากรามีความหลากหลายทางชีวภาพ ทั้งในแง่สกุล ชนิด และสายพันธุ์ ดังนั้นการนำรากลุ่มใดก็ตามไปใช้ประโยชน์ในด้านต่างๆจึงจำเป็นต้องทราบข้อมูลทางพื้นฐานเบื้องต้นก่อนในไปใช้ประโยชน์ หรือ นำไปใช้ประกอบการศึกษาวิจัยในด้านต่างๆที่เกี่ยวข้อง ซึ่งทางด้านราวิทยาถือว่าเป็นงานวิจัยทางด้านพื้นฐานช่วยสนับสนุนงานวิจัยทางด้านการใช้ประโยชน์ให้มีประสิทธิภาพยิ่งขึ้น

ในการจัดกลุ่มและการจัดจำแนกราต้องอาศัยการศึกษาทางด้านเทคนิคทางลักษณะทางสัณฐานวิทยาและเทคนิคเชิงลึกในระดับโมเลกุลของราควบคู่กันไป เพื่อทำให้ทราบระดับอนุกรมวิธานที่ชัดเจนก่อน โดยหลักการจำแนกกลุ่มรานักราวิทยาจะพิจารณาภาพรวมจากกลุ่มใหญ่ไปยังกลุ่มย่อย ซึ่งเรียกว่า แทกโซโนมี มีข้อมูลพื้นฐานประกอบด้วย ไฟลัม คลาส ออเดอร์หรือระดับ แฟมิลี่หรือวงศ์ จีนัสหรือสกุล สปีชีส์หรือชนิด และ สายพันธุ์หรือสเทนส์

การจัดจำแนกโดยอาศัยข้อมูลลำดับเบสของ ดีเอ็นเอ ถือว่าเป็นเทคนิคที่มีความแม่นยำสูงมากเมื่อเปรียบเทียบกับการจัดจำแนกโดยอาศัยลักษณะทางสัณฐานวิทยาของราเพียงอย่างเดียวซึ่งใช้โครงสร้างต่างๆและส่วนสืบพันธุ์ของราในการจัดจำแนกเท่านั้น เนื่องจากอาจมีข้อจำกัดบางประการในการจัดจำแนกอาศัยลักษณะทางสัณฐานวิทยาของราแต่ละชนิดซึ่งอาจมีความความผันแปรเกิดขึ้นได้ ตามสภาพแวดล้อมที่เปลี่ยนไป ดังนั้นจึงทำให้ยากในการการจัดหมวดหมู่กลุ่มราต่างๆถึงแม้นักราวิทยาจะมีความเชี่ยวชาญในรากลุ่มดังกล่าวก็ตาม

ราสกุล *Savoryella* มีรูปร่างของสปอร์ที่เกิดจากการสืบพันธุ์แบบอาศัยเพศ พบได้ทั้งน้ำจืดและน้ำทะเล พบได้บนซากอินทรีวัตถุ ทั้งใบไม้ และ ไม้ที่ย่อยสลาย มีการเจริญเติบโตค่อนข้างช้าบนอาหารเลี้ยงเชื้อราในสกุลดังกล่าว มีการค้นพบทั้งหมด 11 สปีชีส์ ดังนี้ *Savoryella appendiculata, S. aquatica, S. curvispora, S. fusiformis, S. grandispora, S. lignicola, S. limnetica, S. longispora, S. melanospora, S. paucispora* และ *S. verrucosa* ซึ่งข้อมูลจากอดีต ยังไม่เคยมีการรายงานรูปร่างของสปอร์ที่เกิดจากการสืบพันธุ์แบบไม่อาศัยเพศ อีกทั้งการศึกษาเบื้องต้นพบว่าข้อมูลทางด้านสัณฐานวิทยาของราดังกล่าวมีความคลุมเครือในการจัดจำแนกในระดับ ออเดอร์ ซึ่งเคยถูกรายงานตามการจำแนกโดยเพียงลักษณะทางสัณฐานวิทยาเท่านั้น ผลการศึกษาดังกล่าวยังมีความไม่ชัดเจนในการจัดกลุ่ม โดยการศึกษาครั้งนี้ใช้การหาความสัมพันธ์เชิงวิวัฒนาการในระดับโมเลกุลมาช่วยตอบคำถาม และช่วยหาตำแหน่งของอนุกรมวิธานระดับโมเลกุลของราสกุล *Savoryella* โดยใช้ ข้อมูลในลำดับเบสบริเวณของไรโบโซมอลอาร์เอ็นเอชนิด 18S rDNA และ 28S rDNA ซึ่งเป็นยีนที่ช่วยจัดกลุ่มและจำแนกในระดับ คลาส ออเดอร์ และแฟมิลี่ หรือ วงศ์ได้อย่างกว้างๆได้ดีในขณะที่ ดีเอนเอของลำดับเบสในบริเวณไอทีเอส 1 และ 2 รวมกับ ไรโบโซมอลอาร์เอ็นเอ ชนิด 5.8rDNA สามารถบ่งชี้ใน ระดับสกุลและสปีชีสหรือชนิดได้ดีที่สุด ในขณะที่ยีนอื่นๆเช่น อาร์พีบีวัน (RPB1) อาร์พีบีทู (RPB2) และอีเอฟวันแอลฟ่า (EF1α) ถูกนำมาใช้เป็นข้อมูลเสริมเพื่อยืนยันผลของ 18S rDNA และ 28S rDNA ให้มีการจัดกลุ่มและการจัดจำแนกราดังกล่าวมีความน่าเชื่อถือมากขึ้น ผลการศึกษาการจำแนกโดยใช้เทคนิคในระดับโมเลกุลพบว่า กาจัดจำแนกราสกุล *Savoryella* ในระดับโมเลกุลในยีนหลายชนิดให้ผลไปในทิศทางเดียวกัน คือ ราสกุล *Savoryella* ไม่สามารถจัดในออเดอร์ ที่มีการรายงานโดยอาศัยลักษณะทางสัณฐานวิทยาของรา ดังกล่าว อีกทั้งข้อมูลของ ราสกุล *Savoryella* ต่างสปีชีส์ มีความใกล้เคียงกันอย่างมากและมีบรรพบุรุษร่วมกัน ต้นไม้ความสัมพันธ์เชิงวิวัฒนาการในยีนที่ต่างกันยังแสดงให้เห็นว่าราสกุล *Savoryella* ที่แยกมาจากน้ำจืดจับกลุ่มด้วยกันเองซึ่งแยกมากจากกันราสกุล *Savoryella*ที่แยกมาจากน้ำทะเล ซึ่งแสดงให้เห็นว่าความสัมพันธ์วิวัฒนาการมีผลจากแหล่งที่พบด้วย ดังนั้นการศึกษาความสัมพันธ์ทางวิวัฒนาการระดับโมเลกุลของยีนที่ต่างกันหลายชนิด สามารถนำมาเป็นเครื่องมือในการแก้ปัญหาการจัดหมู่และการจัดจำแนกราสกุล *Savoryella* ได้

การศึกษาครั้งนี้ใช้เป็นส่วนหนึ่งในวารสาร Mycosciene เรื่อง Wood inhabiting freshwater fungi from Thailand: *Ascothailandia grenadoidia* gen. sp. nov. *Canalisporium grenadoidia* sp. with a key to *Canalisporium* species (Sordariomycetes, Ascomycota) และอยู่ระหว่างการเตรียมการตีพิมพ์ในหัวข้อเรื่อง A new lineage of aquatic ascomycetes inferred by multiple gene phylogenies of the genera *Ascotaiwania, Ascothailandia, Canalisporium,* and *Savoryella*