Flammispora gen. nov., a new freshwater ascomycete from decaying palm leaves

Umpava Pinruan[1*], Jariya Sakayaroj[1], E.B. Gareth Jones[1] and Kevin D. Hyde[2]

[1]*National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), 113 Phaholyothin Road, Khlong 1, Khlong Luang, Pathumthani, 12120, Thailand;* [2]*Centre for Research in Fungal Diversity, Department of Ecology & Biodiversity, The University of Hong Kong, Pokfulam Road, Hong Kong SAR, PR China*

**Correspondence*: Umpava Pinruan, umpava328@biotec.or.th

Abstract: *Flammispora bioteca* gen. et sp. nov., a freshwater ascomycete (*Ascomycota incertae sedis*) is characterised by black immersed ascomata, weakly persistent asci and 5-septate hyaline ascospores with a basal appendage. It is described from submerged decaying leaves of the peat swamp palm *Licuala longecalycata*. Although it is characteristic of the *Halosphaeriales*, sequencing data indicate it to be distantly related to this order. No genus can be found to accommodate this taxon and based on morphological and molecular evidence, a new genus is justified. The genus is, however, compared with the *Halosphaeriales* and its taxonomic position discussed.

Taxonomic novelties: *Flammispora* U. Pinruan, J. Sakayaroj, K.D. Hyde & E.B.G. Jones gen. nov., *Flammispora bioteca* U. Pinruan, J. Sakayaroj, K.D. Hyde & E.B.G. Jones sp. nov.
Key words: Freshwater ascomycetes, palm, heat swamp, systematics, tropical fungi.

INTRODUCTION

Submerged leaves of the peat swamp palm *Licuala longecalycata* Furt. have yielded a number of new fungal taxa (Hyde *et al*. 2002, McKenzie *et al*. 2002, Pinruan *et al*. 2002), including the freshwater ascomycete *Jahnula appendiculata* (*Jahnulales*) (Pang *et al*. 2002). Several freshwater ascomycetes from tropical locations have also been reported (Cai *et al*. 2003, Tsui *et al*. 2003, Lou *et al*. 2004). Those with appendaged ascospores are usually members of the *Annulatascaceae* (*Sordariomycetidae*) or *Halosphaeriales* while other taxa have sheaths (e.g. *Massarina velatospora* (Hyde & Borse 1986) or lack appendages (e.g. *Kirschsteiniothelia elaterascus*, Shearer 1995). Currently, 44 genera and 136 species are assigned to the *Halosphaeriales* (Pang 2002), while other taxa continue to be described (Hyde 2002, Pang *et al*. 2004). Most are marine, while a few are known from brackish and freshwater habitats e.g. *Aniptodera chesapeakensis*, *A. triseptata*, *Fluviatispora* spp., *Halosarpheia* spp., *Lignincola laevis, Nais inornata* (Hyde *et al*. 1999, 2000, Hyde 2002, Fryar *et al*. 2004, Tsui *et al*. 2004). Hyde (1994) described *Fluviatispora,* with two species from material collected in freshwater habitats in Ecuador, and Papua New Guinea, while a new species has recently been described from Brunei (Fryar & Hyde 2004). The *Annulatascaceae* comprise 10 genera and 38 species and are characterised by thin-walled cylindrical asci with a relatively massive refractive J^- apical ring and ascospores with polar appendages as gelatinous sheaths (Wong *et al*. 1998, Ranghoo *et al*. 1999). The purpose of this paper is to describe a new genus, which like the *Halosphaeriaceae*, has appendaged ascospores. We have used morphology and analysis of DNA sequences to establish whether the taxon should be included in the *Halosphaeriaceae*. The result and description of the new species are presented here.

MATERIALS AND METHODS

Isolates

Submerged material of the palm *Licuala longecalycata* was collected from Sirindhorn Peat Swamp Forest, Narathiwat, southern Thailand on February 2002. The material was returned to the laboratory, incubated in plastic boxes on damp tissue paper and examined within 4 wks. Type material has been deposited in the BIOTEC Bangkok Herbarium (BBH) and cultures deposited in the BIOTEC Culture Collection (BCC) and Centraalbureau voor Schimmelcultures (CBS). Single-spore isolations were made on cornmeal agar (CMA) with added antibiotics to suppress bacterial growth following the method of Choi *et al*. (1999). All observations, including photographic documentation, were of material mounted in water and examined with a differential interference microscope.

Table 1. SSU rDNA sequences obtained from the GenBank.

Classification (Orders)	Taxon	GenBank accession no.
Halosphaeriales	*Nohea umiumi* Kohlm. & Volkm.-Kohlm.	U46878
	Halosphaeria appendiculata Linder	U46872
	Halosarpheia retorquens Shearer & J.L. Crane	AF352086
	Lignincola laevis Höhnk	U46873
	Nais inornata Kohlm.	AF050482
	Halosphaeriopsis mediosetigera (Cribb & J.W. Cribb) T.W. Johnson	U32420
	Nereiospora comata (Kohlm.) E.B.G. Jones, R.G. Johnson & S.T. Moss	AF050485
Microascales	*Pseudallescheria ellipsoidea* (Arx & Fassat.) McGinnis, A.A. Padhye & Ajello*Pseudallescheria boydii* (Shear) McGinnis, A.A. Padhye & Ajello	U43911 M89782
	Petriella setifera (J.C. Schmidt) Curzi	U43908
	Microascus cirrosus Curzi	M89994
Hypocreales	*Melanospora fallax* Zukal	U47842
	Kalichroma tethys (Kohlm. & E. Kohlm.) Kohlm. & Volkm.-Kohlm. BCC13048	AY722099
	Haematonectria haematococca (Berk. & Broome) Samuels & Nirenberg	AF141952
	Paecilomyces tenuipes (Peck) Samson	AB070372
	Sphaerostibella aureonitens (Tul. & C. Tul.) Seifert, Samuels & W. Gams	U32415
	Hypocrea lutea (Tode) Peck	AF543791
Phyllachorales	*Glomerella septospora* Sivan. & W.H. Hsieh	U78779
	Colletotrichum gloeosporioides (Penz.) Penz. & Sacc.	AY083798
	Plectosphaerella cucumerina (Lindf.) W. Gams	AF176951
	Polystigma ochraceum (Wahlenb.) Sacc.	AF276299
Sordariales	*Guanomyces polythrix* M.C. González, Hanlin & Ulloa	AF207683
	Chaetomium globosum Kunze	AB048285
	Sordaria fimicola (Roberge ex Desm.) Ces. & De Not.	X69851
	Ascovaginospora stellipala Fallah, Shearer & W.D. Chen	U85087
Ophiostomatales	*Ophiostoma piliferum* (Fr.) Syd. & P. Syd.	AY281094
	Ophiostoma ulmi (Buisman) Nannf.	M83261
Diaporthales	*Cryphonectria havanensis* (Bruner) M.E. Barr	L42440
	Endothia gyrosa (Schwein.) Fr.	L42443
Xylariales	*Xylaria* sp.	AB014042
	Xylaria carpophila (Pers.) Fr.*Monosporascus ibericus* J. Collado *et al.*	Z49785 AF340015
Dothideales	*Dothidea insculpta* Wallr.*Dothidea hippophaës* (Pass.) Fuckel	U42474 U42475
Ascomycota incertae sedis	*Flammispora bioteca* U. Pinruan *et al.*, sp. nov. BCC13367	AY722100
	Flammispora bioteca U. Pinruan *et al.*, sp. nov. BCC13368	AY722101

DNA extraction, amplification and sequencing

Stock cultures of *Flammispora bioteca* were maintained on potato-dextrose agar (PDA) at 25 ºC. Two strains of the fungus were grown in liquid GYP (Glucose-Yeast Extract-Peptone) (Abdel-Wahab *et al*. 2001) broth on a rotary shaker at 200 rpm at 25 ºC.

Fungal biomass was harvested and washed with sterile distilled water. The biomass was frozen in liquid nitrogen and ground with a mortar and pestle. DNA was extracted using a NucleoSpin^{R} Plant DNA extraction kit (Macherey-Nagel). Partial small subunit (SSU) ribosomal DNA (rDNA) was amplified using Finnzymes, DyNAzyme^{TM} II DNA Polymerase Kit (Macherey-Nagel, Product code F-551S), in a Perkin-Elmer thermal cycler. Primers NS1, NS4, NS5 and NS6 were used following White *et al*. (1990). The PCR product was purified using a NucleoSpin^{R} Plant DNA purification kit (Macherey-Nagel), then sequenced automatically by the Bio Service Unit (BSU) laboratory using the following primers: NS1, NS3, NS5 and NS6 (White *et al*. 1990).

Phylogenetic analysis

Partial SSU rDNA of *Flammispora bioteca* was analyzed along with other sequences obtained from the GenBank database (Table 1). Sequences were aligned in Clustal W 1.6 programme (Thompson *et al*. 1994) and refined visually in BioEdit version 6.0.7 (Hall 2004) and Se-Al v. 1.Oa1 (Rambaut 1999). Alignment was entered into PAUP v. 4.0b10 (Swofford 2002). Phylogenetic trees were generated using maximum parsimony, characters were equally weighted, followed by a heuristic search with a stepwise starting tree, a random stepwise addition of 100 replicates and tree-bisection-reconnection (TBR) branch-swapping algorithm, with gaps treated as missing data. Finally, bootstrap analysis (Felsenstein 1985) was performed using full heuristic searches on 1000 replicates, stepwise addition of sequence, 10 replicates of random addition of taxa and TBR branch-swapping algorithm.

RESULTS

Phylogenetic analysis

Initially, 1807 characters from the SSU sequences were included in the analysis (17.6 % parsimony-informative sites). Nine major orders were analysed (*Halosphaeriales, Microascales, Hypocreales, Phyllachorales, Sordariales, Ophiostomatales, Diaporthales* and *Xylariales*) including the *Dothideales* as outgroup. This yielded five most parsimonious trees (MPTs) with tree lengths, consistency indices (CI) and retention indices (RI) of 949 steps, 0.5933 and 0.7239, respectively. All five MPTs differ only with minor topological differences within the *Phyllachorales* (data not shown). The tree shown in Fig. 1 is the best hypothesis for our SSU dataset, resulting from the Kishino-Hasegawa (K-H) maximum likelihood test (Kishino & Hasegawa 1989).

The phylogenetic tree shows a number of major clades; A: *Halosphaeriales-Microascales* clade, B: *Hypocreales-Phyllachorales* clade, C: *Sordariales* clade and D: *Ophiostomatales-Diaporthales-Xylariales* clade (Fig. 1). The two strains of *Flammispora bioteca* sequenced form a distinct separate clade supported by 100% bootstrap value. In every analysis, *Flammispora bioteca* formed a distinct clade, with a long branch length, between clades A and B, although with low bootstrap support (Fig. 1).

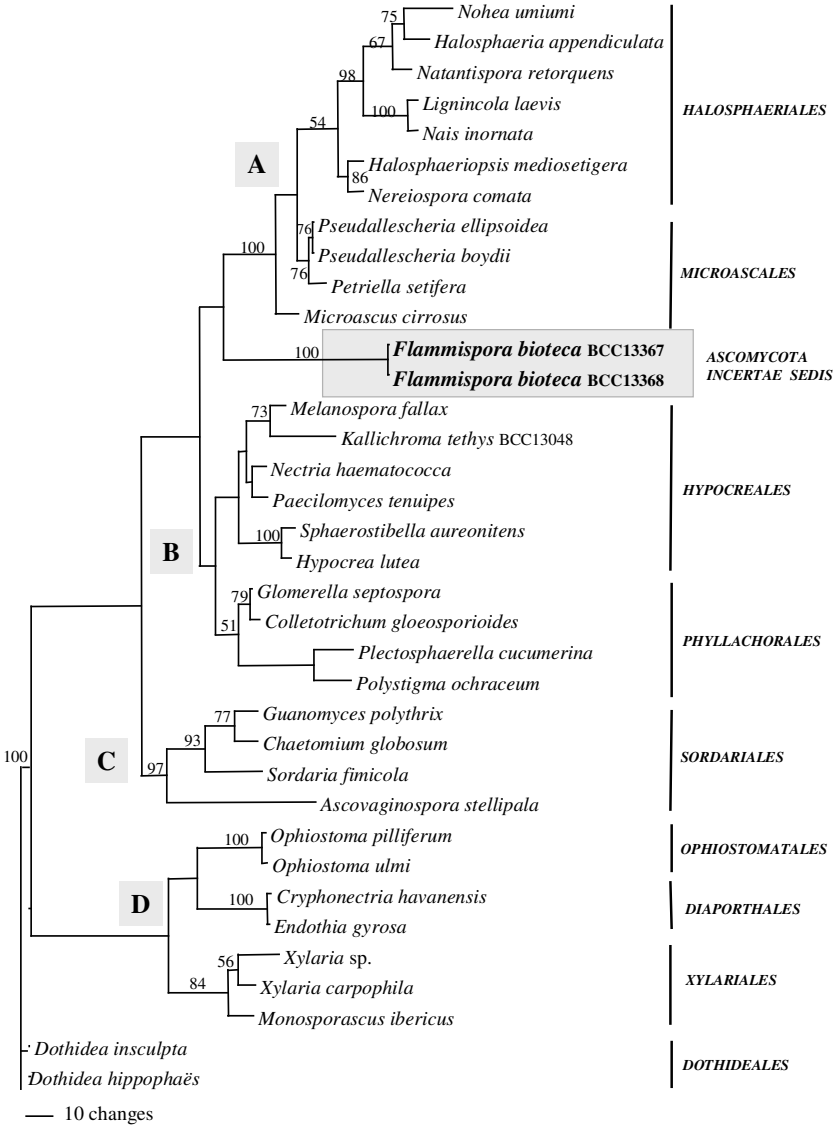


Fig. 1. One of the most parsimonious trees from partial SSU rDNA sequences. Bootstrap values higher than 50 % are given on the branches. Scale bar indicates ten character state changes.

Taxonomic descriptions

Flammispora U. Pinruan, J. Sakayaroj, K.D. Hyde & E.B.G. Jones, **gen. nov.** MycoBank MB500093. Figs 2–9.

Etymology: from Latin *flamma* – "flame" meaning in reference to the flame-like basal appendage.

Ascomata immersa vel semi-immersa, coriacea, ostiolata, solitaria. Asci octospori, unitunicati, clavati vel cylindro-clavati, pedicellati, deliquescens, sine paraphyses. Ascosporae biseriatae, fusiformes, hyalinae, septatae, appendiculatae.

Ascomata immersed, or semi-immersed, coriaceous, ostiolate and solitary. Asci 8-spored, unitunicate, clavate to cylindrical clavate, pedicellate and deliquescent. No paraphyses. Ascospores biseriate, fusiform, hyaline, septate, and appendaged.

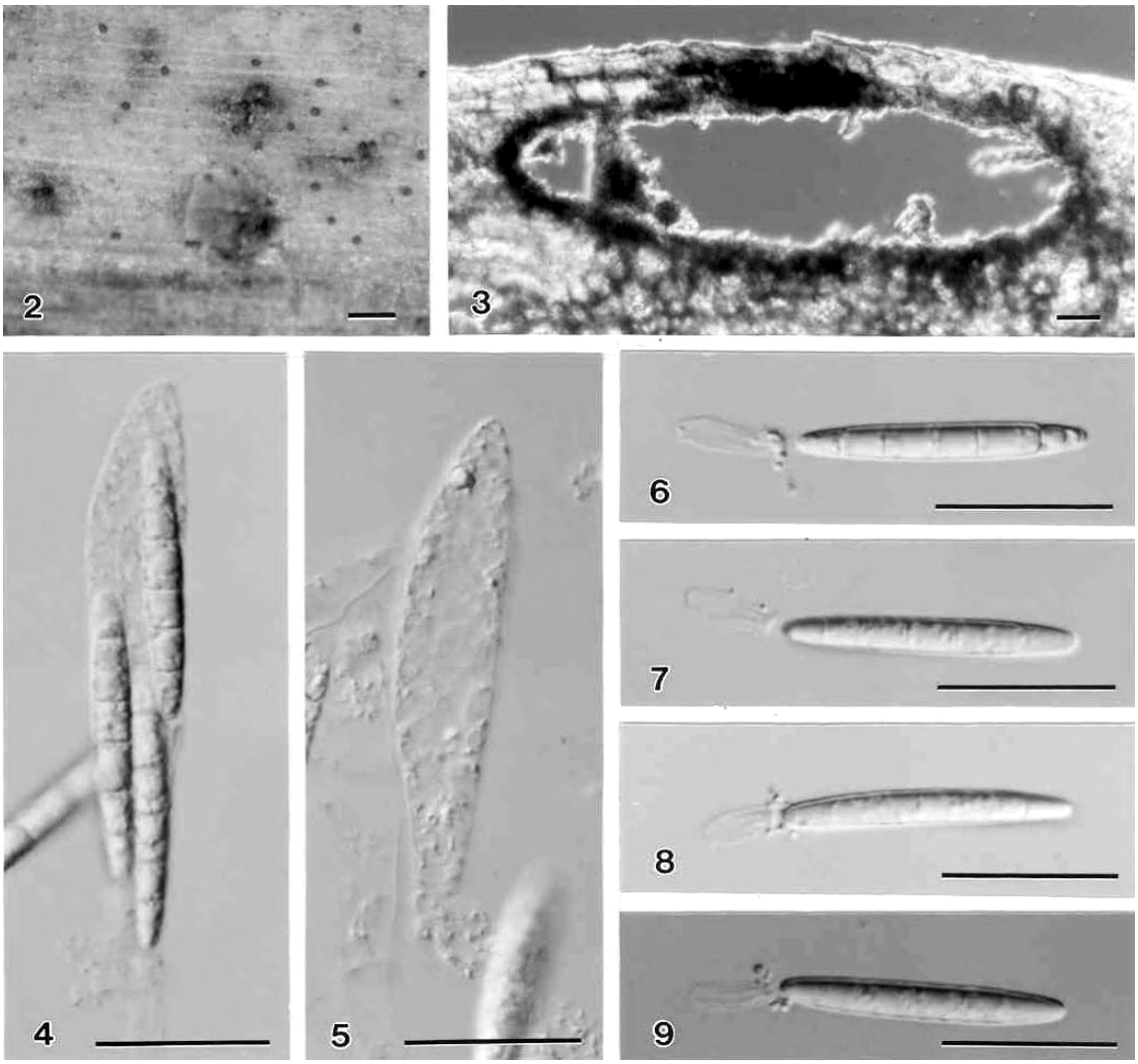
Typus generis: *Flammispora bioteca* U. Pinruan, J. Sakayaroj, E. B. G. Jones & K. D. Hyde, sp. nov.

Cultura ex-typus: BCC13367 (CBS 116642), BCC13368 (CBS 116643).

Flammispora bioteca U. Pinruan, J. Sakayaroj, K.D. Hyde & E.B.G. Jones, **sp. nov.** MycoBank MB500094. Figs 2–9.

Etymology: from Latin *bioteca* – named after a Thai Research Institute, BIOTEC.

Ascomata 225–275 µm diam, immersa et semi-immersa, subglobosa, nigra, coriacea, ostiolata, solitaria, sine paraphysibus. Asci 82.5–87.5 × 16–21 µm, vel cylindricae-clavati vel clavati, unitunicati, pedicellati. Ascosporae 47.5–55 × 5–6.5 µm, 2–3-seriatae, fusiformes vel cylindrico, hyalinae, 5-septatae, guttulatae, appendicibus ad basim praeditae.



Figs 2–9. Light micrographs of *Flammispora bioteca* (from holotype). 2. Ascomata embedded in the substratum. 3. Vertical section of ascoma with dark brown to black perithecial wall. 4, 5. Asci cylindrical-clavate, thin-walled, ascospores 2–3-seriate, apical ring absent. 6–9. Ascospores with a single polar appendage, hyaline, and 5-septate. Scale bars: 2 = 100 µm, 3–9 = 20 µm.

Cultural characteristics: Colonies on PDA (BCC13367 and BCC13368 used for the molecular study) cottony, reaching 1 cm diam in 15 d at room temperature (22–24 ºC), with grey-brown mycelium, hyphae smooth-walled. No ascomata or anamorph formed in culture.

Holotype: Thailand, Narathiwat, Sirindhorn Peat Swamp Forest, on dead leaves of *Licuala longecalycata*, 13 Feb. 2002, U. Pinruan (WAH 134 in BBH **holotype**); cultures ex-type CBS 116642 and CBS 116643.

DISCUSSION

Flammispora bioteca cannot be assigned to a family or order at this time, although morphologically it shares a number of features with members of the *Halosphaeriales*. This includes simple ascomata, a thin-walled peridium, lack of paraphyses, thin-walled asci, which are weakly persistent and hyaline appendaged ascospores (Kohlmeyer & Kohlmeyer 1979, Jones 1995). *Flammispora* resembles aquatic genera with polar appendaged ascospores, especially some species with cylindrical to filiform ascospores and the taxa: *Ascosacculus aquaticus*, *Ascosalsum cincinnatulum, A. viscidulum* and *A. unicaudatum.* These species, however, differ from *Flammispora* in having hamate polar appendages, initially closely adpressed to the ascospore wall, then separating and eventually unfurling to form long, narrow appendages (Campbell *et al*. 2003). *Flammispora* can also be compared with *Halosphaeria cucullata*, which has cylindrical ascospores with or without a mucilaginous appendage (Kohlmeyer & Kohlmeyer 1979, Cai *et al*. 2002). *Flammispora* also superficially resembles species of *Fluviatispora*, the freshwater ascomycete described from the submerged rachides of the palm *Livistonia* in the Bensbach River, Papua New Guinea. *Fluviatispora tunicata* and *F. reticulats* differ from *Flammispora bioteca* in that the ascospores are unicellular and surrounded by a mucilaginous sheath.

Many freshwater ascomycetes in the *Annulatascaceae* have appendaged ascospores. However, all genera assigned to this family have cylindrical asci with a prominent large apical ring (Wong *et al*. 1998).

The appendages in *Flammispora bioteca* are interesting, as on release, ascospores appear as if the appendage forms as a sheath around the ascospore, which then evaginates to form the polar appendage. This occurs in several other ascomycetes with an exosporic sheath e.g. *Chaetosphaeria chaetosa* (Jones *et al*. 1983), and the secondary appendages of *Corollospora* species (Jones & Moss 1987). In ascospores observed within the asci, the appendages are apparent and this probably precludes this type of appendage formation. It therefore appears that the appendage is produced as an outgrowth of the cell wall at the basal pole. This is similar to several other ascomycete taxa with appendaged ascospores e.g. *Torpedospora radiata* Meyers.

Our molecular data using two strains of *Flammispora bioteca* (isolated from the holotype on the same occasion) confirms they are monophyletic. *Flammispora bioteca* is distantly related to the *Halosphaeriales*, although it shares some morphological characteristics. It forms a distinct clade to other ascomycetes with unitunicate asci, between the *Microascales* and *Hypocreales* clades, although with weak support (Fig. 1). However, a long branch length was observed in the sequence of *F. bioteca*. This could indicate a high number of autapomorphic molecular characters, and a sequence difference with neighbouring clades, or may be due to the lack of other closely related taxa in the analyses. At the moment, no family or order is appropriate to accommodate this fungus, therefore a new genus with uncertain taxonomic position is proposed.

ACKNOWLEDGEMENTS

This project is supported by research grants BRT R_145008 and BRT R_245002. We are grateful to Graduate School Chiang Mai University, Saisamorn Lumyong, Ruud Valyasevi, and Morakot Tanticharoen for continued support, to Ittichai Chatmala and Aom Pinnoi for field assistance and to Manetr Boonyanant, and his staff, for research facilities at the Sirindhorn Field and Nature Study Center, Narathiwat.

REFERENCES

Abdel-Wahab MA, Pang K-L, El-Sharouny HM, Jones EBG (2001). *Halosarpheia unicellularis* sp. nov. (*Halosphaeriales*, *Ascomycota*) based on morphological and molecular evidence. *Mycoscience* **42**: 255–260.

Cai L, Tsui CKM, Zhang K, Hyde KD (2002). Aquatic fungi from Lake Fuxian, Yunnan, China. *Fungal Diversity* **9**: 57–70.

Cai L, Zhang KQ, McKenzie EHC, Hyde KD (2003). Freshwater fungi from bamboo and wood submerged in the Liput River in the Philippines. *Fungal Diversity* **13**: 1–12.

Campbell J, Anderson JL, Shearer CA (2003). Systematics of *Halosarpheia* based on morphological and molecular data. *Mycologia* **95**: 530–552.

Choi YW, Hyde KD, Ho WH (1999). Single spore isolation of fungi. *Fungal Diversity* **3**: 29–38.

Felsenstein J (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**: 783–791.

Fryar SC, Booth W, Davies J, Hodgkiss IJ, Hyde KD (2004). Distribution of fungi on wood in the Tutong River, Brunei. *Fungal Diversity* **17**: In press.

Fryar SC, Hyde KD (2004). New species and genera of ascomycetes from fresh and brackish water in Brunei: *Ayria appendiculata* and *Sungaiicola bactrodesmiella* gen. et spp. nov., *Fluviatispora boothii, Torrentispora*

crassiparietis and *T. fusiformis* spp. nov. *Cryptogamie Mycologie*: In press.

Hall T (2004). *BioEdit version 6.0.7*. Department of Microbiology, North Carolina State University. *http://www.mbio.ncsu.edu/BioEdit/bioedit.html*

Hyde KD (1994). Aquatic fungi on rachides of *Livistonia* in the Western Province of Papua New Guinea. *Mycological Research* **98**: 719–725.

Hyde KD (2002). *Aniptodera triseptata* sp. nov. (*Halosphaeriales*) from submerged wood in freshwater. *Cryptogamie Mycologie* **23**: 5–7.

Hyde KD, Borse BD (1986). Marine fungi from Seychelles. IV. *Massarina velatospora*, a new marine *Ascomycotina* from mangrove wood. *Mycotaxon* **27**: 161–167.

Hyde KD, Ho WH, Tsai KM (1999). The genera *Aniptodera, Halosarpheia, Nais* and *Phaeonectriella* from freshwater habitats. *Mycoscience* **40**: 165–183.

Hyde KD, Sarma VV, Jones EBG (2000). Morphology and taxonomy of higher marine fungi. In: *Marine mycology-a practical approach.* Fungal Diversity Research Series 1, (Hyde KD, Pointing SB, eds). Fungal Diversity Press, Hong Kong: 172–204.

Hyde KD, Yanna, Pinnoi A, Jones EBG (2002). *Goidanichiella fusiforma* sp. nov. from palm fronds in Brunei and Thailand. *Fungal Diversity* **11**: 119–122.

Jones EBG (1995). Ultrastructure and taxonomy of the aquatic ascomycetous order Halosphaeriales. *Canadian Journal of Botany* **73**: 790–801.

Jones EBG, Johnson RG, Moss ST (1983). Spore appendage development in the lignicolous marine pyrenomycetes *Chaetosphaeria chaetosa* and *Halosphaeria trullifera*. *Transactions of the British Mycological Society* **80**: 193–200.

Jones EBG, Moss ST (1987). Key and notes on genera of the Halosphaeriaceae examined at the ultrastructure level. *Systema Ascomycetum* **6**: 179–200.

Kishino H, Hasegawa M (1989). Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order of Hominoidea. *Journal of Molecular Evolution* **29**: 170–179.

Kohlmeyer J, Kohlmeyer E (1979). *Marine mycology: the higher fungi.* Academic Press. New York.

Luo J, Yin JF, Cai L, Zhang K, Hyde KD (2004). Freshwater fungi in Lake Dianchi, a heavily polluted lake in Yunnan, China. *Fungal Diversity* **16**: 93–112.

McKenzie EHC, Pinnoi A, Wong MKM, Jones EBG, Hyde KD (2002). Two new hyaline *Chalara* species and key to species described since 1975. *Fungal Diversity* **11**: 129–139.

Pang K-L (2002). Systematics of the *Halosphaeriales*: Which morphological characters are important? In: *Fungi in Marine Environments*. (Hyde KD, ed). *Fungal Diversity Research Series* **7**: 35–57.

Pang K-L, Abdel-Wahab MA, Sivichai S, El-Sharouney HM, Jones EBG (2002). *Jahnulales (Dothideomycetes, Ascomycota)*: a new order of lignicolous freshwater ascomycetes. *Mycological Research* **106**: 1031–1042.

Pang KL, Jones EBG, Vrijmoed LLP (2004)**.** Two new marine fungi from China and Singapore, with the description of a new genus, *Sablecola***.** *Canadian Journal of Botany* **82**: 485–490.

Pinruan U, Jones EBG, Hyde KD (2002). Aquatic fungi from peat swamp palms: *Jahnula appendiculata* sp. nov. *Sydowia* **54**: 242–247.

Rambaut A (1999). *Se-Al. v1.Oa1.* Department of Zoology, University of Oxford, Oxford OX1 4JD, UK. *http://evolve.zoo.ox.ac.uk/Se-Al/Se-Al.html*

Ranghoo VM, Hyde KD, Liew ECY, Spatafora JW (1999). Family placement of *Ascotaiwania* and *Ascolacicola* based on DNA sequences from the large subunit rRNA gene. *Fungal Diversity* **2**: 159–168.

Shearer CA (1995). A new species of *Kirschsteiniothelia* (*Pleosporales*) with an unusual fissitunicate ascus. *Mycologia* **85**: 963–969.

Swofford DL (2002). *PAUP*: *Phylogenetic analysis using parsimony.* Version 4.0b10. Illinois Natural History Survey, Champion, Illinois.

Thompson JD, Higgins DG, Gibson TJ (1994). CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**: 4673–4680.

Tsui CKM, Hyde KD (2003). *Freshwater Mycology*. *Fungal Diversity Research Series* **10**.

Tsui CKM, Hyde KD (2004). Biodiversity of fungi on submerged wood in a stream and estuaries in the Tai Ho Bay, Hong Kong. *Fungal Diversity* **15**: 171–186.

White TJ, Bruns T, Lee S, Taylor J (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocol: a guide to methods and applications* (Innis MA, Gelfand DH, Sninsky DH, White TJ, eds.): Academic Press, San Diego, USA: 315–322.

Wong SW, Hyde KD, Jones EBG (1998). *Annulatascaceae*, a new ascomycete family from the tropics. *Systema Ascomycetum* **16**: 17–25.