Thailandiomyces bisetulosus gen. et sp. nov. (*Diaporthales, Sordariomycetidae, Sordariomycetes*) and its anamorph *Craspedodidymum*, is described based on nuclear SSU and LSU rDNA sequences

Pinruan, U.[1,2], Sakayaroj, J.[2], Hyde, K.D.[3,4] and Jones, E.B.G.[2*]

[1]Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand 50200
[2]National Center for Genetic Engineering and Biotechnology, 113 Thailand Science Park, Paholyothin Road, Khlong 1, Khlong Luang, Pathum Thani, Thailand 12120
[3]International Fungal Research & Development Centre, The Research Institute of Resource Insects, Chinese Academy of Forestry, Balonssi, Kunming 650224, PR China
[4]School of Science, Mae Fah Luang University, Tasud, Chiang Rai 57100, Thailand

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Thailandiomyces gen. nov. (*Diaporthales*) is described from senescent trunks of a palm (*Licuala longicalycata*) in a peat swamp. Phylogenetic analysis of *Thailandiomyces* was undertaken, with partial SSU and LSU rDNA sequences. Our morphological and molecular results show that this genus is well placed in the *Diaporthales*. However, it is not related to *Diaporthe* the genus it most closely resembles morphologically. It differs from *Diaporthe* species in number of characters: partially immersed to superficial ascomata, ascospore measurements, bipolar appendages, not surrounded by a mucilaginous sheath, and with *Craspedodidymum licualae* Pinruan as its anamorph. This is the first report of a teleomorph for the genus *Craspedodidymum*.

Key words: *Craspedodidymum* anamorph, freshwater ascomycete, molecular phylogeny, palm, peat swamp, rDNA

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*Corresponding author: E.B.G. Jones; e-mail: remispora@googlemail.com

Introduction

Between May 2002-October 2004 a detailed survey of the fungi associated with palms in Sirindhorn Peat Swamp Forest, Narathiwat Province, southern Thailand, was undertaken (Pinnoi *et al*., 2006; Pinruan *et al*., 2007). Species diversity on the selected palms was high with the discovery of 53 new taxa, 17 and 15 on *Licuala longicalycata* and *Eleiodoxa conferta,* respectively (Hyde *et al*., 2002; McKenzie *et al*., 2002; Pinruan *et al*., 2002; Pinnoi *et al*., 2003a,b; 2004; Pinruan *et al*., 2004a,b,c,d). New ascomycetes on senescent parts of the palm *Licuala longicalycata* included *Jahnula appendiculata* (*Jahnulales, Dothideomycetes incertae sedis*), *Phruensis brunneispora* (*Diaporthales, Sordariomycetidae, Sordariomycetes*), *Flammispora bioteca* (*Sordariomycetes incertae sedis*) and an unusual *Diaporthe*-like ascomycete with cylindrical bipolar appendaged ascospores and with *Craspedodidymum licualae* as its anamorph.

Material and methods

Fungal isolation

Senescent trunks of palm material were collected from Sirindhorn Peat Swamp Forest, Narathiwat, southern Thailand during May 2001. Palm samples were placed in plastic bags, returned to the laboratory, incubated in plastic boxes with moist tissue to allow fungal fruiting bodies to develop and examined within four weeks (Pinnoi *et al*., 2006; Pinruan *et al*., 2002, 2007). Type material has been deposited

in the BIOTEC Bangkok Herbarium (BBH) and cultures in the BIOTEC Culture Collection (BCC). Single ascospore isolations were made on corn meal agar (CMA) with added antibiotics to suppress bacterial growth. All observations, including photographic documenttation, and measurements were of material mounted in water, and examined in a differential interference microscope.

Growth of fungi, DNA extraction, amplification and sequencing

Stock cultures of the fungus were maintained on potato dextrose agar (PDA) at 25°C. The fungus was grown in potato dextrose broth (PDB) on a rotary shaker at 200 rpm at 25°C. Fungal biomass was harvested by vacuum filtration 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, Catalogue No. 740 590. 50). Nuclear SSU and LSU rDNA were amplified, using FINNZYMES, DyNAzyme^{TM} II DNA Polymerase Kit (MACHEREY-NAGEL, Product code F-551S), in a Perkin Elmer thermal cycler. The primer pairs and amplification cycles were performed following White *et al*. (1990), Bunyard *et al*. (1994), and Landvik (1996). The PCR products were purified using a NucleoSpin^{R} Plant DNA purification kit (MACHEREY-NAGEL, Catalogue No. 740 570. 50), then sequenced automatically by Macrogen Inc. (Korea) using primers NS1, NS3, NS4, NS5, NS6, LROR, JS5 and LR7 (White *et al*., 1990; Bunyard *et al*., 1994; Landvik, 1996).

Phylogenetic analyses

Two strains of *Thailandiomyces bisetulosus* (BCC00018, BCC00200) were sequenced to confirm their monophyly (Table 1). SSU and LSU rDNA sequences were aligned individually using BioEdit version 7.0.5 (Hall, 2005). The alignments were entered into PAUP* 4.0b10 (Swofford, 2002). SSU dataset comprised 28 taxa within the subclass *Sordariomycetidae* (the *Diaporthales, Magnaporthaceae, Ophiostomatales* and *Sordariales*) with the *Xylariales* as the outgroup. Sequences of LSU rDNA comprised 42 taxa from selected families and complexes in the *Diaporthales* (the *Gnomoniaceae, Melanconidaceae, Valsaceae, Diaporthaceae, Cryphonectriaceae, Togniniaceae*, *Schizoparme* complex and *Harknessia* complex) were chosen for the analysis, and the *Magnaporthaceae* served as the outgroup.

Phylogenetic trees for both datasets were generated using equally weighted maximum parsimony (heuristic searches with a stepwise starting tree, a random stepwise addition of 10 replicates and tree-bisection-reconnection (TBR) branch-swapping algorithm, with gaps treated as missing data). Bootstrap analysis (Felsenstein, 1985) based on equally weighted maximum parsimony was performed with full heuristic searches on 1,000 replicates, 10 replicates of random stepwise addition of taxa, TBR branch-swapping algorithm. Kishino-Hasegawa (K-H) maximum likelihood test (Kishino and Hasegawa, 1989) was performed in order to obtain the best phylogenetic hypothesis for the dataset.

Bayesian phylogenetic inference of SSU and LSU sequences was calculated using MrBayes 3.0b4 with general time reversible (GTR) model of DNA substitution and a gamma distribution rate variation across sites (Huelsenbeck and Ronquist, 2001). Four Markov chains were run from random starting trees for 2,000,000 generations and sampled every 100 generations. The first 100,000 generations were discarded as burn-in of the chain. A majority rule consensus tree of all remaining trees, as well as the posterior probabilities, was calculated. The alignments were deposited in TreeBase: matrix accession numbers = SN 3396, SN 3397. Accession numbers for taxa obtained from GenBank are indicated next to each taxon name in the trees.

Results

SSU phylogeny

The SSU dataset consists of 1,769 characters of which 1,500 are constant, 188 are parsimony informative and 81 are parsimony uninformative. This alignment consists of 402 and 14 inserted bases at the same position in two strains of *Thailandiomyces bisetulosus* BCC00018 and BCC00200, respectively. The maximum parsimony analysis resulted in eight

Table 1 Fungal isolates sequenced for this study.

Species name	GenBankaccession number	
	SSU	LSU
Thailandiomyces bisetulosus (BCC00018)	EF622229	EF622230
Thailandiomyces bisetulosus (BCC00200)	EF622228	EF622231

most parsimonious trees (MPTs) 469 steps long (CI = 0.673, RI = 0.776, RC = 0.523). The major branches are stable, and differ only in the position of *Lollipopaia minuta* AF301534 and *Phruensis brunneispora* AY581944 within the *Diaporthales*. The K-H test resulted in the best phylogenetic hypothesis for the dataset as shown in Fig. 19.

The two *Th. bisetulosus* isolates are monophyletic and positioned as a basal clade of the *Diaporthales* with *Togninia* species as a sister clade (50% bootstrap, 100% posterior probabilities). All taxa sampled in this analysis are well positioned in the *Diaporthales*, although with low bootstrap and posterior probabilities at node * (Fig. 19). *Thailandiomyces bisetulosus* forms an unsupported clade with *Lollipopaia minuta* AF301534 and is distantly placed to *Diaporthe phaseolorum* AY779278 and *D. eres* DQ471015 (the only two SSU sequences of *Diaporthe* available for the analysis in the GenBank), but without bootstrap and posterior probabilities support (Fig. 19).

LSU phylogeny

Further taxa from the *Diaporthales*, based on recent data by Gryzenhout *et al*. (2006) were added to the LSU analyses. The LSU dataset consists of 1,667 total characters of which 305 positions were excluded, due to the presence of an intron in the *Cryptodiaporthe corni* AF408343 sequence. Therefore 1,302 total characters, of which 1,020 were constant characters, 244 parsimony informative characters and 38 parsimony uninformative characters were included in the analyses. Maximum parsimony resulted in 24 MPTs with tree length, CI, RI and RC of 649 steps, 0.550, 0.803 and 0.442, respectively. The overall topology for all 24 MPTs are the same, and only differ in the minor swapping position within the *Diaporthaceae* and *Togniniaceae*. One of the 24 MPTs inferred with the best topology from K-H test is shown in Fig. 20.

The LSU data showed that the branches leading to the major families in the *Diaporthales* are reasonably stable, although with weak support in some of the terminal taxa. The two *Th. bisetulosus* strains are monophyletic and form a basal clade to the *Diaporthaceae* and *Diaporthales incertae sedis* with 100% bootstrap and 100% posterior probabilities, with the *Togniniaceae* as a sister clade (Fig. 20).

Taxonomy

Thailandiomyces Pinruan, Sakayaroj, Hyde & Jones, **gen. nov.**

MycoBank: 511583

Etymology: Thailand, in reference to the name of the host country.

Ascomata semi-immersa vel superficialia, globosa, nigra, coriacea, ostiolata, scattered. *Cello* longis cylindrical. *Peridium* cella crassitunicatum et textura angularis. *Paraphyses*. *Asci* cylindrica vel clavate, apedicellati, J-, apparatus subapicale praediti. *Ascosporae* rectae vel curvatae fusiformes, hyalinae, 1-septatae, guttulatae, appendiculae bipolaris.

Ascomata partially immersed to superficial, globose, black, coriaceous, ostiolate, scattered to gregarious, with long cylindrical necks, periphysate with short hyaline cells. *Peridium* composed of one stratum of compressed cells, (textura angularis), black to the outside, brown inwardly. *Paraphyses* present but deliquescent, irregular in width, rarely septate, tapering towards the apices, embedded in a mucilaginous matrix. *Asci* cylindrical to clavate, unitunicate, apedicellate, free-floating, apically truncate, with a J- subapical ring. *Ascospores* overlapping 2-seriate, fusoid, straight or curved, hyaline, 1-septate, smooth-walled, with bipolar appendages.

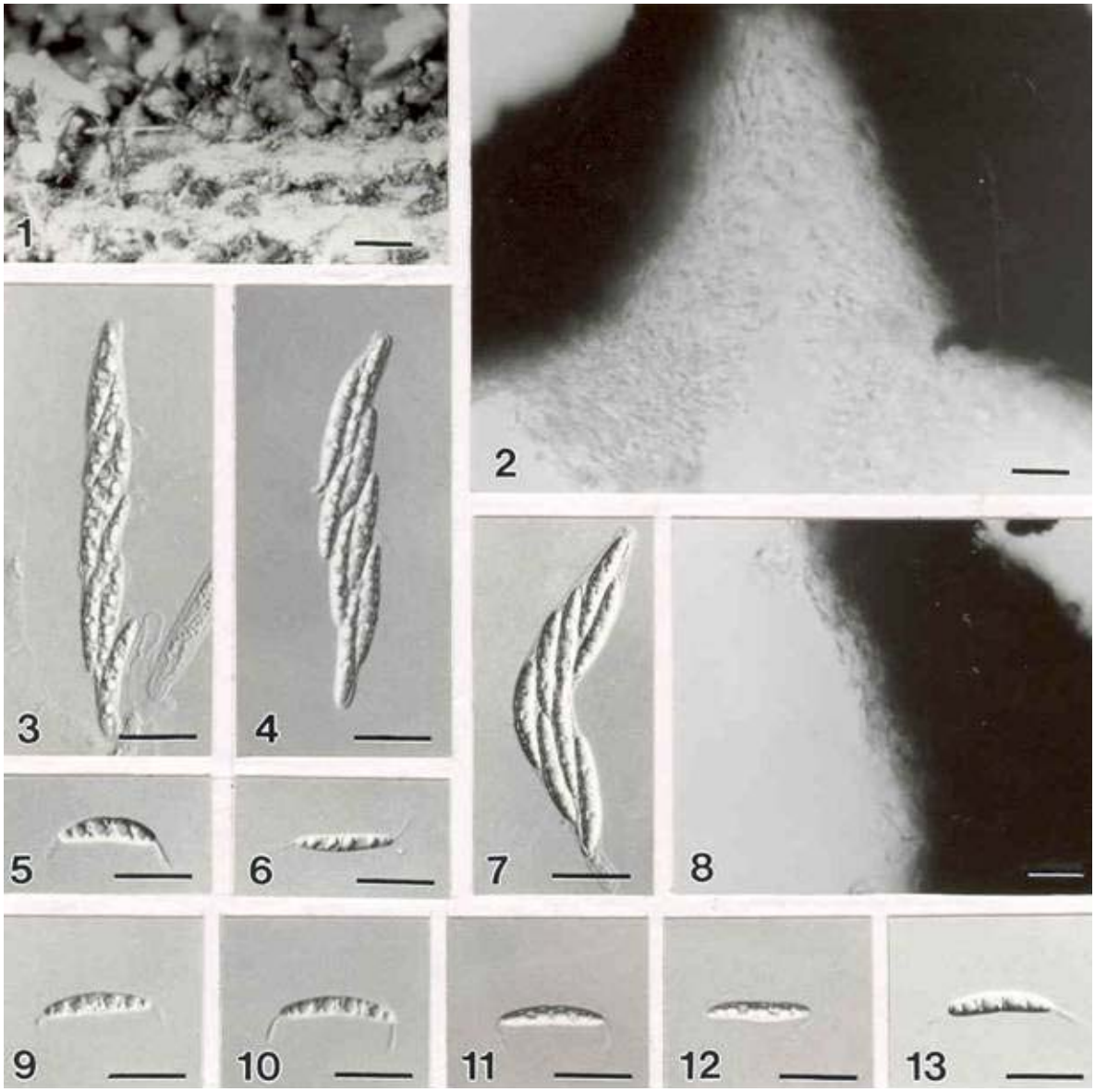
Anamorph: *Craspedodidymum* Hol.-Jech.

Type species: *Thailandiomyces bisetulosus* Pinruan.

Thailandiomyces bisetulosus Pinruan, Sakayaroj, Hyde & Jones, **sp. nov.** (Figs 1-13)

MycoBank: 511589

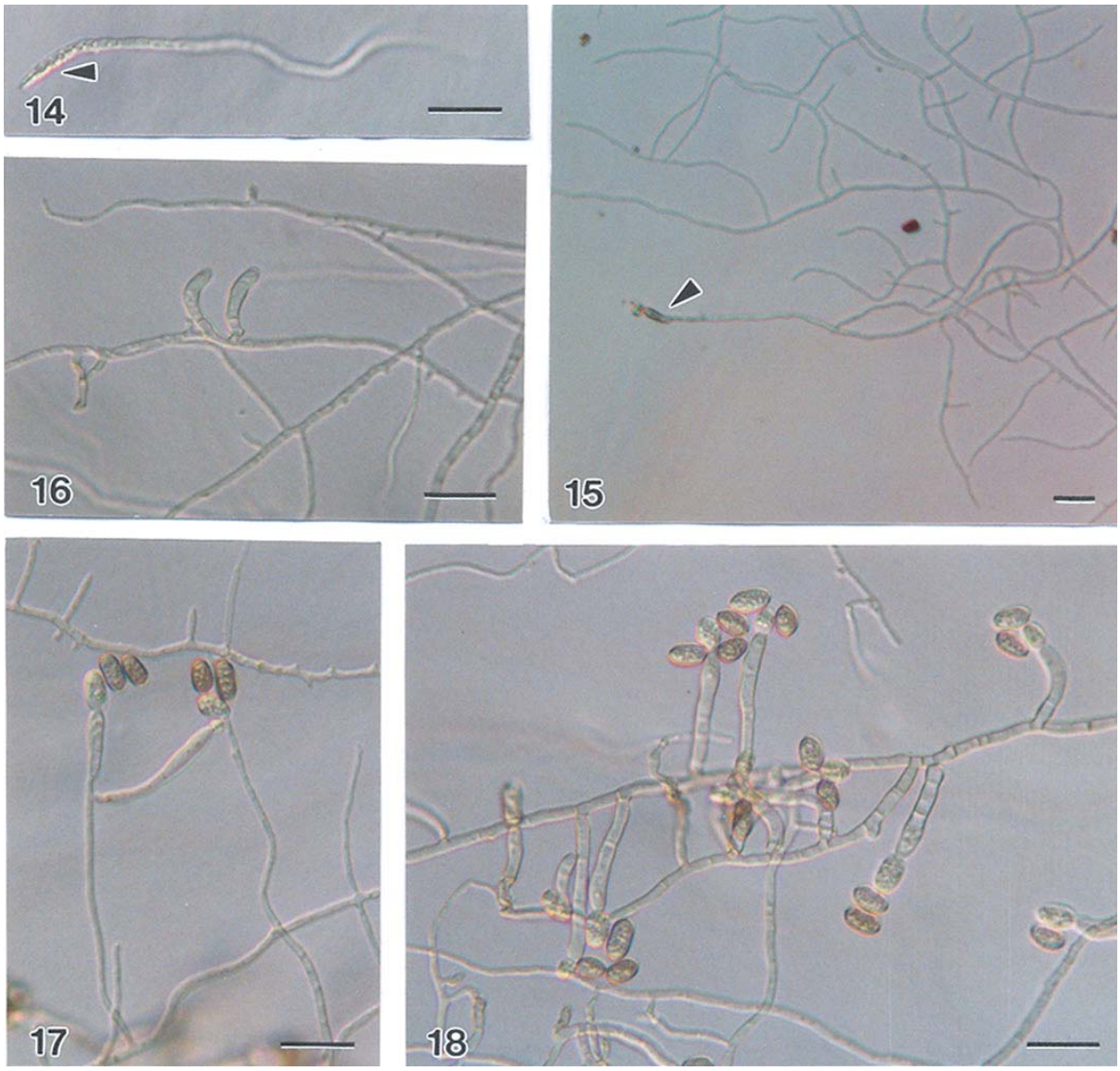
Etymology: from *bisetulosus*, in reference to the bipolar ascospore appendages.



Figs 1-13. Light micrographs of *Thailiomyces setulis* sp. nov. (**from holotype**). **1.** Ascoma on substratum. **2.** Periphyses. **3-4, 7.** Asci. **8.** Peridium. **5-6, 9-13.** Ascospores. Bars: 1 = 400 µm; 2-13 = 10 µm.

Ascomata 275-325 µm diam, semi-immersa vel superficialia, globosa, nigra, coriacea, ostiolata, scattered. *Neck* ad 1 mm longa, 100 µm diam, cylindrica. *Peridium* ad 45 µm crassum, cella crassitunicatum et textura angularis. *Paraphyses*. *Asci* 65-75 × 6-7.5 µm, cylindrica vel clavate, apedicellati, sup-apicale J- praediti, 2 × 2 µm. *Ascosporae* 20-25 × 3.5-5 µm, rectae vel curvatae fusiformes, hyalinae, 1-septatae, guttulatae, appendiculae bipolaris.

Ascomata 275-325 µm diameter, partially immersed to superficial, globose, black, coriaceous, ostiolate, scattered to gregarious (Fig. 1). *Neck* up to 1 mm long, 100 µm diam., periphyses with short hyaline cells, central, cylindrical, black (Fig. 2). *Peridium* up to 45 µm thick, composed of one stratum of compressed cells, of *textura angularis*, black to the outside, brown inwardly (Fig. 8). *Paraphyses* present but deliquescent, irregular in width, up to 5-6.5 µm wide, rarely septate, tapering towards apices, embedded in a mucilaginous matrix. *Asci* 65-75 × 6-7.5 µm ($\bar{x}$ = 67.5 × 6.8 µm, n = 20), 8-spored, cylindrical to clavate, unitunicate, apedicellate, free-floating, apically truncate, with a J- subapical ring, 2 × 2 µm (Figs 3-4, 7). *Ascospores* 20-25 × 3.5-5 µm ($\bar{x}$ = 22.9 × 4 µm, n = 30), overlapping 2-seriate, fusoid, straight or curved, hyaline, 1-septate, smooth-walled, with 4-5 large guttules, with bipolar



Figs 14-18. Light micrographs of *Craspedodidymum licualae* anamorph in culture. **14-15.** Single ascospore germination (arrowed). **16-18.** Conidiophores and conidia. Bars: 14-18 = 10 µm.

spine-like appendages, usually bent laterally, 5-7.5 µm long, 1-1.5 µm diam (Figs 5-6, 9-13).

Anamorph: *Craspedodidymum licualae* Pinruan (Figs 14-18).

Colonies on natural substratum effuse, black. *Mycelium* superficial. *Conidiophores* macronematous, mononemataus, erect, brown, paler toward the apex, straight or flexuous, smooth, but rough at the apex. *Conidiogenous cells* integrated, terminal, 20-27.5 × 6.2-7.5 µm, enteroblastic and monophialidic. *Conidia* 13.7-17.5 × 7.5-10 µm, obovoid or ellipsoid, broadly rounded at both ends, brown, papillate at the basal end, O-septate.

Holotype: THAILAND, Narathiwat, Sirindhorn Peat Swamp Forest, on submerged trunk of *Licuala longicalycata*, 12 May 2001, U. Pinruan (Wah 110) in BBH.

Cultural characteristics: Colonies on PDA (BCC00018 and BCC00200 used for the molecular study) cottony, reaching 1 cm diam in 3 days at room temperature (22-24ºC), with dark grey mycelium, hyphae smooth-walled. Anamorph and teleomorph sporulate on the same plate in culture, developing after 2 weeks,

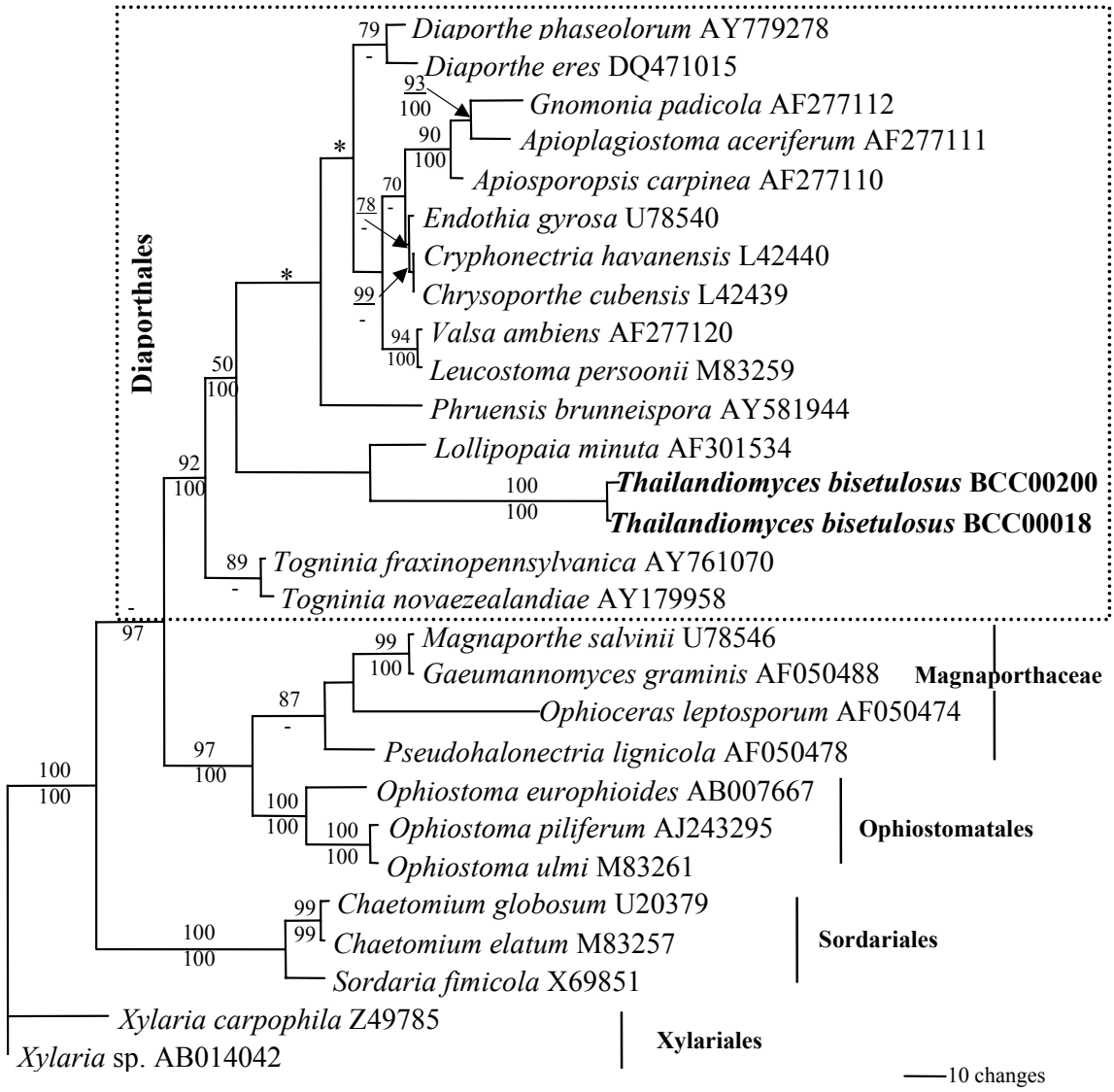


Fig. 19. One of eight MPTs resulted from maximum parsimony analysis from partial SSU rDNA sequences. Bootstrap values from maximum parsimony higher than 50% and Bayesian posterior probabilities greater than 95% are given above and below each branch. Scale bar indicates 10 character state changes. Low supported clades are indicated by an asterisk (*).

and 3-4 weeks, respectively. Initially the anamorph was isolated from conidia as reported in Pinruan *et al*. (2004d). In this study, single ascospore were isolated which gave rise to the anamorph and the teleomorph.

Discussion

Phylogenetic analyses of SSU and LSU rDNA sequences showed that *Thailandiomyces bisetulosus* is well positioned in the order *Diaporthales* (*Sordariomycetes, Sordariomycetidae*) (Zhang *et al*., 2006; Hibbett *et al*., 2007). However *Th*. *bisetulosus* can not be assigned to any family at this time. Characters *Th*. *bisetulosus* shares with members of the *Diaporthales* include its saprobic habitat on decaying plant material, partially-immersed ascomata, long periphysate necks, unbranched paraphyses that deliquesce early in development, unitunicate asci that float free within the centrum and asci with a refractive, apical J-ring (Barr, 1991; Samuels and Blackwell, 2001).

The *Diaporthales* comprise phylogenetic groups based on morphological and molecular

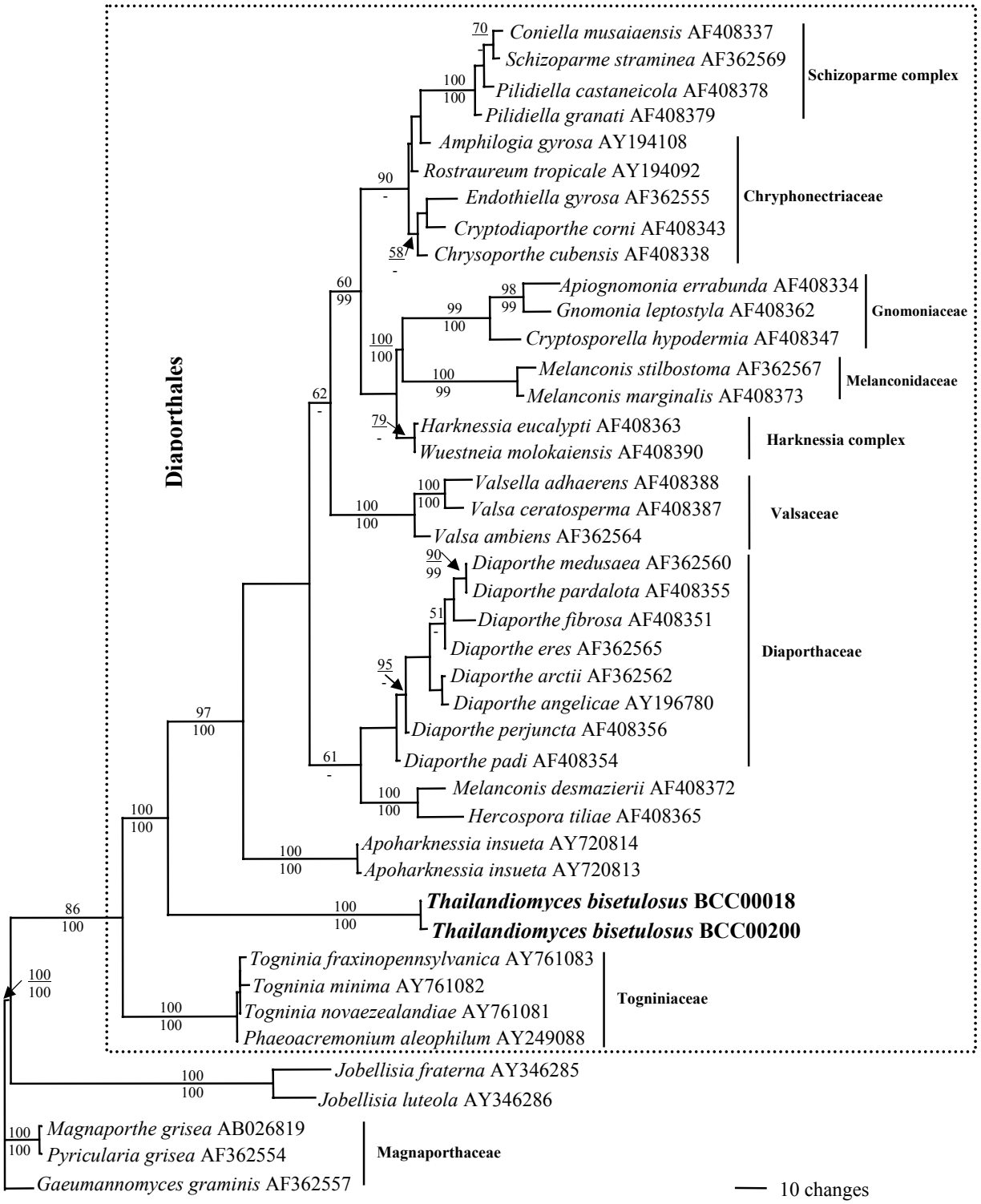


Fig. 20. One of 24 MPTs resulted from maximum parsimony analysis from partial LSU rDNA sequences. Bootstrap values from maximum parsimony higher than 50% and Bayesian posterior probabilities greater than 95% are given above and below each branch. Scale bar indicates 10 character state changes.

data: *Gnomoniaceae, Melanconidaceae, Valsaceae, Diaporthaceae, Togniniaceae* and a new family *Cryphonectriaceae* (Castlebury *et al*., 2002; Gryzenhout *et al*., 2006). However, recent sequence data highlights a number of lineages that can not be referred to these families: *Schizoparme* and *Harknessia* complexes (Castlebury *et al*., 2002; Gryzenhout *et al*., 2006), *Apoharknessia insueta, Hercospora tiliae, Melanconis desmazierii, Jobellisia* species and *Th. bistulosus* (Fig. 20).

All *Diaporthe* species included in the SSU/LSU analyses formed a well-supported clade (Figs 19-20). However, morphological and molecular results show that *Th. bisetulosus* is not closely related to *Diaporthe* species (*Diaporthaceae*), the genus it morphologically most resembles. *Diaporthe* comprises circa 780 species (Index Fungorum, http://www.indexfun gorum. org/ Names/Names.asp) and a number of these have appendaged ascospores: *D. dakotensis*, *D. decedens*, *D. laschii*, *D. oxyspora*, *D. pruni* and *D. salsuginosa* (Wehmeyer, 1933; Vrijmoed *et al*., 1994; Wong and Hyde, 2001). Ascospores of *Th. bisetulosus* are distinct from these species in spore measurements, lacking a mucilaginous sheath but with bipolar appendages. Ascomata of *Th. bisetulosus* are partially immersed in the substratum to superficial, while in *Diaporthe* species they are generally stromatic. A further difference between *Th. bisetulosus* and *Diaporthe* spp. is in their anamorphs, the former with *Craspedodidymum licualae* as its anamorph, while *Diaporthe* species universally have a *Phomopsis* anamorph.

Craspedodidymum has 12 species but no teleomorph has been linked to any of these, prior to this study. Pinruan *et al*. (2004d) described three new *Craspedodidymum* species from the same locality as *Th. bisetulosus* one of which is its anamorph, *C. licualae*. Subsequently, Huhndorf and Fernández (2005) described five *Chaetosphaeria* species with *Craspedodidymum*-like anamorphs, but did not identify any of them to species. *Craspedodidymum licualae* differs from these *Craspedodidymum*-like species in the morphology of the conidia and conidiogenous cells. *Craspedodidymum*-like anamorphs of *Chaetosphaeria ellisii*, *Ch. raciborskii* and *Ch. rubicunda*, have phialides formed directly on the hyphae without forming distinct conidiophores. The conidia of *Ch. ellisii*, *Ch. raciborskii* and *Ch. rubicunda* are globose, while in *C. licualae* they are cylindrical or obovoid. The conidial forms of *Ch. rubicunda* and one collection of *Ch. raciborskii*, also differ in having elongate hyaline appendages. The three *Craspedodidymum* species isolated from peat swamp palm material (Pinruan *et al*., 2004d) do not resemble those illustrated by Huhndorf and Fernández (2005).

Morphologically *Th. bisetulosus* shares few characters in common with *Lollipopaia minuta*. In *L. minuta*, ascomata are born on a stroma, asci are cylindrical, ascospores are filiform, lacking a sheath or appendages. While *Th. bisetulosus* is not stromatic, asci are cylindrical to clavate, ascospores fusoid with apical appendages.

Exploration of new habitats and geographical locations has yielded a number of new and interesting taxa. In Thailand, three genera from freshwater habitats have been discovered with affinities with the *Diaporthales*: *Lollipopaia minuta* on submerged wood with hyaline, septate ascospores; *Phruensis brunneispora* with versicoloured, septate ascospores, and *Th. bisetulosus* with hyaline 1-septate spores, both on palm material from a peat swamp (Inderbitizin and Berbee, 2001; Pinruan *et al*., 2004a; present study). However, their familial position remains unresolved. *Phruensis brunneispora* and *L. minuta* grouped within the *Valsaceae, Diaporthales* (Pinruan *et al*., 2001). The current analysis does not support this with *L. minuta* and *Th. bisetulosus* forming a subclade to the *Diaporthaceae* along with other genera and families of uncertain affinities (e.g. *Apoharknessia, Jobellisia* and *Togninia*). Further collections and sequences from a wider range of genes are required to resolve their taxonomic position.

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