



Clavatispora thailandica gen. et sp. nov., a novel taxon of *Venturiales* (Dothideomycetes) from Thailand

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Abstract

An unusual wood-inhabiting fungus was found in northern Thailand. The combination of characters of this taxon, viz. setose, ostiolate ascomata, bitunicate asci, dark brown, wedge-shaped to clavate, muriform ascospores, and a hyphomycetous asexual state producing ellipsoidal, septate, highly constricted, holoblastic conidia is not found in any hitherto described genus of Dothideomycetes. An LSU phylogeny showed this taxon to be clearly placed in the family *Sympoventuriaceae*, *Venturiales*. The name *Clavatispora thailandica* gen. and sp. nov., is introduced to accommodate this taxon, which is described and illustrated and compared with other genera in this family.

Key words: bitunicate asci, hyphomycetous asexual state, molecular phylogeny, muriform ascospores, *Sympoventuriaceae*

Introduction

Pleosporales is the largest order in the class Dothideomycetes that comprises 41 families and 181 genera (Hyde *et al.* 2013). In this order, the family *Pleosporaceae* contains several genera, e.g. *Clathrospora*, *Lewia*, *Platysporoides*, *Pleospora*, and *Pyrenophora* that have brown muriform ascospores (Sivanesan 1984, Barr 1987, 1990a, Zhang *et al.* 2012, Hyde *et al.* 2013). Species with muriform ascospores are also found in *Aigialaceae*, *Cucurbitariaceae*, *Halojulellaceae*, *Montagnulaceae*, *Platystomaceae*, *Pleomassariaceae*, *Shiraiaceae* and *Teichosporaceae*, thus this character has evolved on numerous occasions even outside Pleosporales (e.g. *Gloniopsis*, *Hysterobrevium*, *Hysterographium* and other genera in *Hysteriales*). Barr (1990a) placed species with muriform ascospores in 12 families within the Pleosporales, i.e. *Arthopyreniaceae*, *Cucurbitariaceae*, *Dacampiaceae*, *Hysteriaceae*, *Leptosphaeriaceae*, *Lophiostomataceae*, *Micropeltidaceae*, *Phaeosphaeriaceae*, *Phaeotrichaceae*, *Pleomassariaceae*, *Pleosporaceae* and *Pyrenophoraceae*. Species in these families have differing ascomata characters and asexual states (Barr 1990a, 1990b, Zhang *et al.* 2012, Hyde *et al.* 2013).

In our ongoing study of Dothideomycetes of Thailand (Boonmee *et al.* 2011, 2012, Chomnunti *et al.* 2011, 2012a,b, Liu *et al.* 2011, 2012), we collected an intriguing species with ascospores resembling *Alternaria*-like conidia and bitunicate asci indicative of *Pleosporaceae*. The superficial, solitary, ascomata with dark, septate setae, forming on a basal subiculum, and bright-coloured ostiole, indicated that this fungus differed from other dictyosporous genera in families of the Pleosporales (Simmons 1986, Barr 1987, 1990a, 1992). The fungus also developed a *Trichocladium*-like asexual state in culture. The aim of this paper is to introduce this unusual ascomycete, which is described as *Clavatispora thailandica* gen. nov., sp. nov. It is only described and illustrated but also compared with morphologically similar genera. We sequenced our isolate to establish the phylogenetic placement of our taxon. The trees generated using LSU region place our new genus in the *Sympoventuriaceae*, *Venturiales*.

Materials and methods

Collection, examination and isolation of fungi

Dead herbaceous stems were randomly collected from Doi Tung forest, Chiang Rai, Thailand. Microscopic studies were carried out and the fungus illustrated following the procedures outlined in Boonmee *et al.* (2011). Type specimens are deposited at the herbarium of Mae Fah Luang University (MFLU), Chiang Rai, Thailand and cultures are deposited at Mae Fah Luang University Culture Collection (MFLUCC), BIOTEC Culture Collection (BCC) and IFRD culture collections, International Fungal Research & Development Centre, Kunming, China, the latter under MTA no. 4/2010.

Single spore isolation was carried out following the methods outlined by Chomnunti *et al.* (2011). Germinating ascospores were transferred to fresh malt extract agar (MEA, Difco Laboratories, Detroit, Michigan, USA) and incubated at 28°C for one week in darkness. When colonies appeared, subcultures were made onto fresh MEA media at room temperature. Morphology and cultural characters of the fungus were recorded based on MEA cultures after 60 days (Boonmee *et al.* 2011, 2012).

Molecular procedures

Fresh mycelium was scraped from the surface of colonies grown on MEA for 60 days at 28°C. DNA extraction, PCR amplification and sequencing of LSU region using primer pair LROR/LR5 was performed under the conditions described in Boonmee *et al.* (2011). Sequencing was done in Shanghai Sangon Biological Engineering Technology & Services Co., China and new sequences generated in this study were blasted to search for related taxa in GenBank database (www.ncbi.nlm.nih.gov/blast/).

Phylogenetic analyses

Sequence data were aligned with Clustal W (Thompson *et al.* 1994) and manually adjusted using BioEdit (Hall 1999). Most of the taxa used in this study are derived from Schoch *et al.* (2009) and Zhang *et al.* (2011). The LSU dataset for showing the placement of *Clavatispora thailandica* comprises 40 taxa including *Acrospermum compressum* (*Acrospermales*) as an outgroup (Table 1). Poorly aligned regions on nucleotide multiple alignments were removed using Gblock 0.91b (Castresana 2000), following nonconservative settings; the maximum number of contiguous nonconserved positions was set to 10, the minimum length blocks set to 5, allowing for gap positions in half the sequences. The construction of the maximum likelihood (ML) analysis using RAxML version 7.6.3 (Stamatakis 2006, Stamatakis *et al.* 2008) as part of the “RAxML-HPC BlackBox on TG tool” was performed at the CIPRES Science Gateway V. 3.3 (<http://www.phylo.org/portal2/>, Miller *et al.* 2010). RAxML rapid bootstrapping and subsequent ML search used distinct model/data partitions with joint branch length optimization, executing 1,000 rapid bootstrap inferences and thereafter a thorough ML search. All free model parameters were estimated by RAxML and ML estimate of 25 per site rate categories. Likelihood of final tree were evaluated and optimized under GAMMA. GAMMA Model parameters were estimated to an accuracy of 0.1000000000 Log Likelihood units. Every 100th tree was saved. Phylogenetic trees were illustrated in Treeview (Page 1996).

Bayesian posterior probability analysis was constructed with the MrBayes 3.1.2 run on XSEDE at the CIPRES webportal (Ronquist & Huelsenbeck 2003), using the parameter setting of 2 parallel runs, 4 chains, run for 4,000,000 generations, sample frequency every 1,000 generations and all other parameters were left as default. The 50% majority rule consensus tree was created from the remaining trees in Treeview (Page 1996).

Results

Phylogenetic studies

The RAxML phylogenetic tree (Fig. 1) was constructed through analysis of the LSU gene region and comprised 40 taxa and 754 aligned characters out of 4% of ambiguous characters. The RAxML phylogram received the best scoring tree with a final ln value of -4136.348470. The isolate of *Clavatispora thailandica* (MFLUCC 100107) clustered with *Scolecobasidiella avellanea* (CBS 772.73) and grouped within the family *Symptoventuriaceae* with high support values (98% BS and 1.00 PP). Additionally, taxa from the genera *Ochroconis* and *Scolecobasidium* formed a sister-group to *Clavatispora*. To confirm the placement of new genus we built a further tree including

Dothideomycetes and some species of *Trichocladium* (*Sordariales*) (tree not shown). Results indicate that *Clavatispora* belongs to order *Venturiales* and is well separated from taxa in *Pleosporales*.

TABLE 1. Taxa included in the phylogenetic study.

Species	Culture no.	GenBank Accession number
		LSU
<i>Acrospermum compressum</i>	M 151	EU940084
<i>Apiosporina collinsii</i>	CBS 118973	GU301798
<i>Apiosporina morbosa</i>	dimosp	EF114694
<i>Caproventuria hanliniana</i>	ATCC 96019	AF050290
<i>Caproventuria hystrioides</i>	CBS 117727	EU035459
<i>Clavatispora thailandiaca</i>	MFLUCC 100107	KF770458
<i>Coleroa robertiani</i>	CBS 458.64	JQ036231
<i>Fusicladium africanum</i>	CPC 12828	EU035423
<i>Fusicladium amoenum</i>	CBS 254.95	EU035425
<i>Fusicladium intermedium</i>	CBS 110746	EU035432
<i>Fusicladium proteae</i>	CPC18282	JN712551
<i>Gibbera conferta</i>	CBS 191.53	GU301814
<i>Metacoleroa dickiei</i>	medipc	EF114695
<i>Metacoleroa dickiei</i>	Kruys503 (UPS)	DQ384100
<i>Ochroconis calidifluminalis</i>	IFM54738	AB385698
<i>Ochroconis constricta</i>	NBRC9375	AB564619
<i>Ochroconis gallopava</i>	IFM54737	AB272164
<i>Ochroconis humicola</i>	NBRC32054	AB564618
<i>Ochroconis tshawytschae</i>	CBS100438	GU328005
<i>Protoventuria barriae</i>	CBS 300.93	JQ036232
<i>Protoventuria major</i>	CBS 114594	JQ036233
<i>Scolecobasidiella avellanea</i>	CBS 772.73	EF204505
<i>Scolecobasidium cateniphorum</i>	P050	EU107309
<i>Scolecobasidium terreum</i>	P043	EU107306
<i>Scolecobasidium tricladiatum</i>	P051	EU107286
<i>Scolecobasidium variabile</i>	P041	EU107310
<i>Scolecobasidium verruculosum</i>	P044	EU107308
<i>Spilocaea pomi</i>	CBS176.42	GU348998
<i>Symptoventuria capensis</i>	CPC 12839	DQ885905
<i>Symptoventuria capensis</i>	CBS 120136	DQ885906
<i>Tyrannosorus pinicola</i>	CBS 124.88	DQ470974
<i>Venturia alpina</i>	CBS 373.53	EU035446
<i>Venturia asperata</i>	ATCC34052	EF114711
<i>Venturia carpophila</i>	ICMP5402	AY849967
<i>Venturia chlorospora</i>	Kruys 502 UPS	DQ384101
<i>Venturia inaequalis</i>	CBS 815.69	GU301878
<i>Venturia inaequalis</i>	ATCC 60070	EF114712
<i>Venturia populina</i>	CBS 256.38	GU323212
<i>Venturia pyrina</i>	ATCC38995	EF114714
<i>Veronaeopsis simplex</i>	CBS 588.66	EU041877

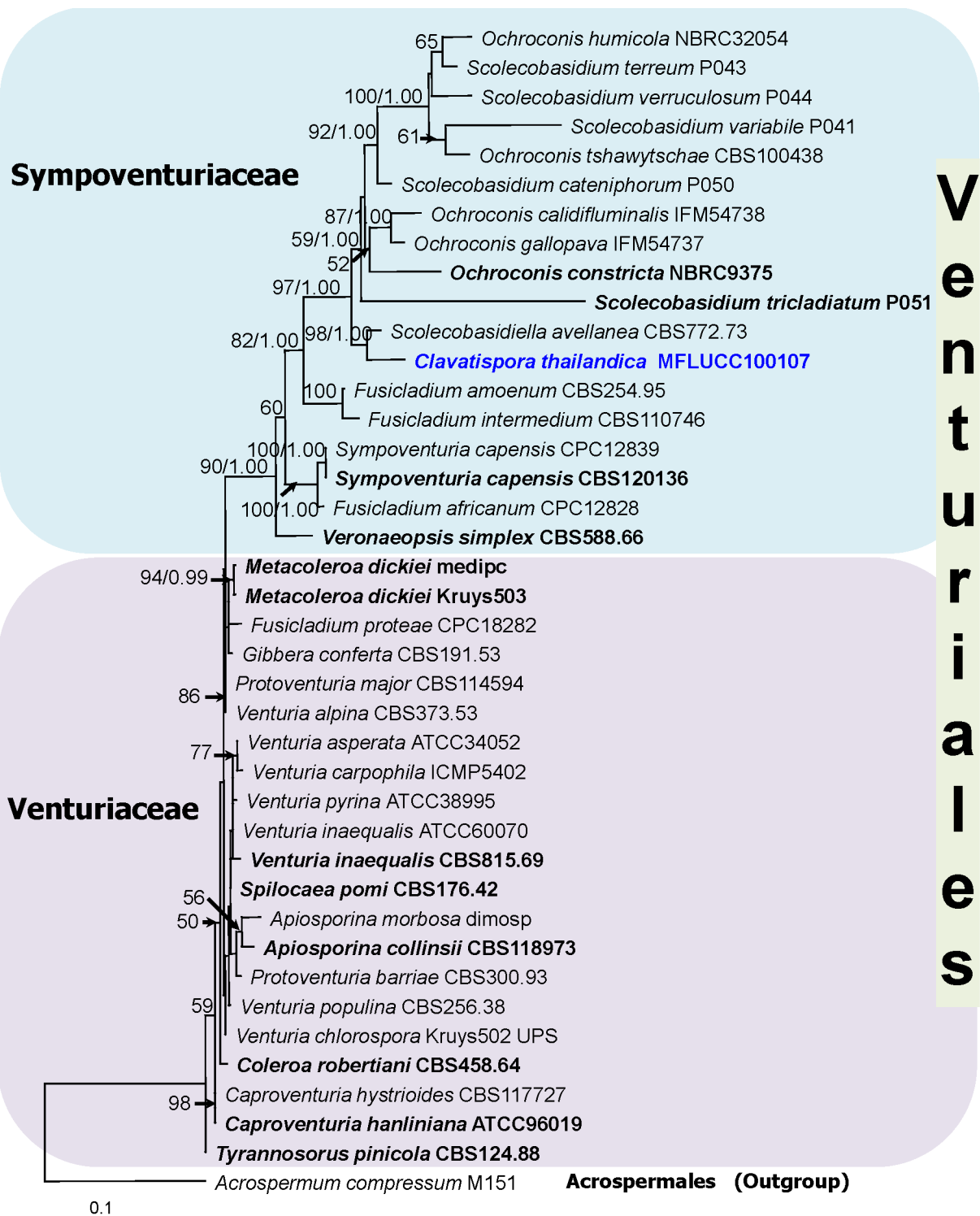


FIGURE 1. RAxML phylogenetic tree based on the LSU sequence data. Bootstrap support values for maximum likelihood ($\geq 50\%$ BS) and Bayesian posterior probabilities (≥ 0.95 PP) are shown in the tree (values below these thresholds not shown). The tree was rooted with *Acrospermum compressum* (*Acrospermales*) as outgroup. The new taxon is highlighted in blue and types are in bold.

Taxonomy

Clavatispora S. Boonmee & K.D. Hyde, *gen. nov.* MycoBank: MB 805923

Etymology:—“*clavatispora*,” name recognizes the clavate ascospores.

Type species:—*Clavatispora thailandica* S. Boonmee & K.D. Hyde

Saprobic on dead stems of herbaceous plants. Sexual state: *Ascomata* superficial, solitary, scattered on substrate, developing on subiculum of brown hyphae, subglobose to globose, dark brown, covered with dark brown, thick-walled, septate setae, and strands of radiating fused hyphae at base, with a barely raised, bright, central ostiole. *Peridium* comprising 6–8 layers of dark brown cells of *textura angularis*, almost black at outside. *Hamathecium* of ca 2 µm wide, anastomosing, septate, branched pseudoparaphyses, hyaline, embedded in gelatinous matrix. *Asci* bitunicate, subclavate to broadly obovoid, thick-walled, with a short pedicel, apically rounded, lacking an ocular chamber. *Ascospores* overlapping 2–3-seriate, clavate, asymmetrical, yellowish to reddish brown, becoming dark brown at maturity, muriform, constricted at septa, tapering towards a subacute base, with basal cell pale brown or brownish, smooth-walled. Asexual state: directly developing on MEA. *Mycelium* superficial, pale to moderately dark brown, composed of septate, branched, thin- to thick-walled, smooth-walled, 1–3 µm wide hyphae. *Conidiophores* erect, developing on hyphae, brown or light brown, septate, smooth, sometimes branched. *Conidiogenous cells* holoblastic, subglobose. *Conidia* ellipsoidal to subglobose, pale brown to brown, guttulate, rounded at apex, sub-acute at base, trans-septate, constricted and darkened at septa, smooth-walled.

Type species:—*Clavatispora thailandica* S. Boonmee & K.D. Hyde, *sp. nov.* MycoBank: MB 805924 (Figs 2–3)

Holotype:—THAILAND. Chiang Rai: Mae Fah Luang, Doi Tung, elev. ca. 1000 m., on dead stems of unidentified herbaceous plants, 10 June 2009, *Saranyaphat Boonmee* DT04 (MFLU 100038!)—ex-type living culture = MFLUCC 100107 = BCC 38863 = IFRD 2170.

Sequence data:—LSU = KF770458, SSU = KF770457 and TEF1a = KF770459.

Etymology:—The specific epithet “*thailandica*” refers to the country from where the fungus was collected.

Saprobic on dead stems of herbaceous plants. Sexual state: *Ascomata* (115–)130–135 µm high × (120–)135–150(–160) µm diam (\bar{x} = 130 × 140 µm, n = 5), superficial, solitary, scattered on substrate, developing on subiculum of brown hyphae, subglobose to globose, dark brown, covered with dark brown, thick-walled, septate setae, and strands of radiating fused hyphae at base, with a barely raised, bright central ostiole under light microscope. *Peridium* 16–21 µm wide, comprising 6–8 layers of dark brown cells of *textura angularis*, almost black at outside. *Hamathecium* of ca 2 µm wide, anastomosing, septate, branched pseudoparaphyses, hyaline, embedded in gelatinous matrix. *Asci* 90–110 × 24–30 µm (\bar{x} = 100 × 28 µm, n = 20), 8-spored, bitunicate, fissitunicate, subclavate to broadly obovoid, thick-walled, with a short pedicel, apically rounded, lacking an ocular chamber. *Ascospores* 32–44 × 10–13 µm (\bar{x} = 37 × 11 µm, n = 20), overlapping 2–3-seriate, clavate, slightly curved, asymmetrical, yellowish to reddish brown, becoming dark brown at maturity, 4–7(–8) transversely septate, with 1–3 vertical septa in some cells, constricted at septa, tapering towards a subacute base, with basal cell pale brown or brownish, smooth-walled. Asexual state: single spore isolate growing on MEA. *Mycelium* slightly raised, pale to moderately dark brown, composed of septate, branched, thin- to thick-walled, smooth-walled, 1–3 µm wide hyphae. *Conidiophores* erect, developing on hyphae, brown or light brown, septate, smooth, sometimes branched, (5–)10–15 µm long. *Conidiogenous cells* holoblastic, subglobose. *Conidia* (14.5–)15–18(–21) × 5–7(–8) µm (\bar{x} = 17 × 6 µm, n = 20), ellipsoidal to subglobose, pale brown to brown, guttulate, rounded at apex, sub-acute at base, 3-septate, 1–2-septate when immature, constricted and darkened at septa, smooth-walled.

Cultural characteristics:—Ascospores germinating on WA within 12 h and germ tubes produced from spore cells. Colonies on MEA slow growing, reaching 7 mm diameter in 1 week at 28°C, low convex, slightly effuse hairy, edge entire, dark brown. Aerial mycelium, radiating outwards, superficial, septate.

Notes:—The dark brown ascomata, bitunicate asci and muriform brown ascospores of *Clavatispora thailandica* are similar to characteristics of many genera of *Pleosporales* and some taxa in *Capnodiales* and *Hysteriales* (Barr 1990, Boehm *et al.* 2009, Zhang *et al.* 2012, Hyde *et al.* 2013). *Clavatispora* can be distinguished from other genera by its solitary ascomata, covered by superficial, dark setae, a bright ostiole and hyphomycetous asexual state. The hyphomycetous conidia of the asexual state, derived from single ascospores, developed directly on MEA within 60 days. The asexual conidia which formed chains, resemble species of *Trichocladium*, i.e. *T. constrictum* (Goh & Hyde 1999), but *C. thailandica* differs in having branched conidiophores, light pigmented conidia and cells with conspicuous guttules (Fig. 3E–L). *Trichocladium constrictum* has not been sequenced, however, *T. asperum* (generic type) and *T. opacum* are not phylogenetically related to *C. thailandica* (data not shown).

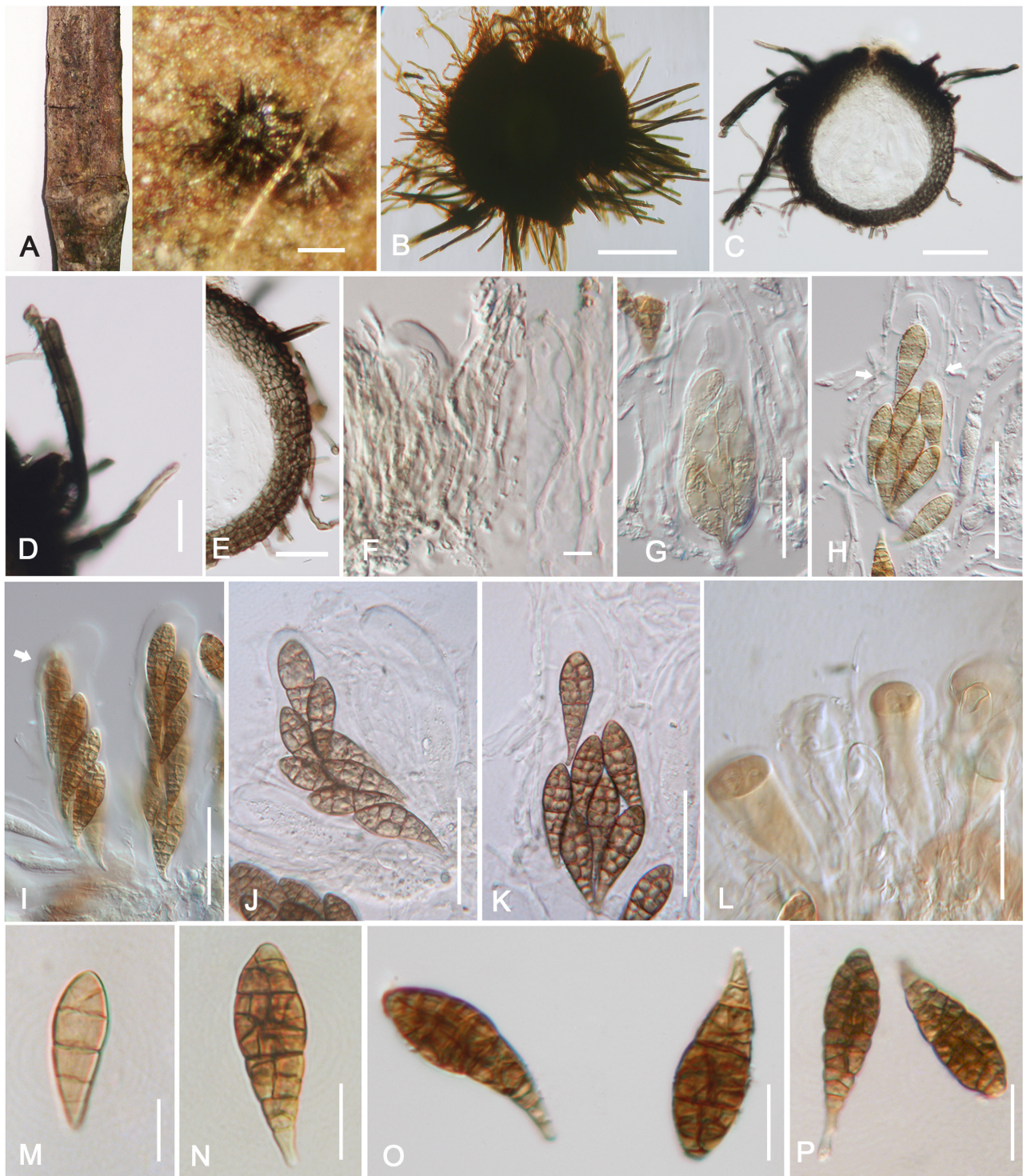


FIGURE 2. Sexual state of *Clavatispora thailandica* (MFLU100038, holotype). A. Substrate and ascomata appearance. B. Squash mount of ascoma. C. Section of ascoma. Note the fused hyphae forming hairs at the base. D. Setae. E. Peridium. F. Pseudoparaphyses. G–H. Immature bitunicate asci with young developing ascospores. I–K. Mature asci. Fissitunicate dehiscence arrowed in H and I. L. Characteristic dehiscence of asci after release of ascospores. M–P. Young and mature ascospores. Scale bars: A–C, G–L = 50 μm , D–E = 20 μm , F = 5 μm , M–P = 10 μm .

Sequence data placed *Clavatispora* in the order *Venturiales* but it differs in morphology from most other genera. The only genus in *Venturiales* with setose ascomata similar to *Clavatispora* is *Trichodothis* (Zhang *et al.* 2011). However, *Trichodothis* differs in having stromatic ascomata, oval asci and 2-celled, hyaline to pale brown, ornamented ascospores. Molecular data are needed to confirm the placement of *Trichodothis* in *Sympoventuriaceae* as it is rather atypical of the family. The presence of stromatic ascomata covered by spine-like setae, cylindrical

asci, 2-celled and hyaline, smooth ascospores are also found in *Gibbera conferta* and *Venturia compacta* (Peck 1873, Petrak 1947). Furthermore, LSU data analysis paired *G. conferta* with an isolate of *Fusicladium proteae* in the *Venturiaceae* clade (Fig. 1). *Venturia compacta* lacks DNA sequence data. *Acantharia*, *Coleroa*, *Metacoleroa*, *Protoventuria* and *Pyrenobotrys* also have setose ascomata, but ascospores of these genera are 1-septate, hyaline or coloured, with smooth or verrucose walls (Barr 1989, Carris & Poole 1993). Phylogenetic analysis based on LSU sequence data shows that *Clavatispora* clusters with the hyphomycete genus *Scolecobasidiella* in *Sympoventuriaceae* as well as *Ochroconis*, *Scolecobasidium*, *Fusicladium* and *Veronaeopsis*. The asexual state of *Clavatispora* has 1–3-phragmoseptate, smooth conidia that are deeply constricted at septa, whereas in other genera the conidia and conidiogenous apparatus are mostly verrucose to denticulate.

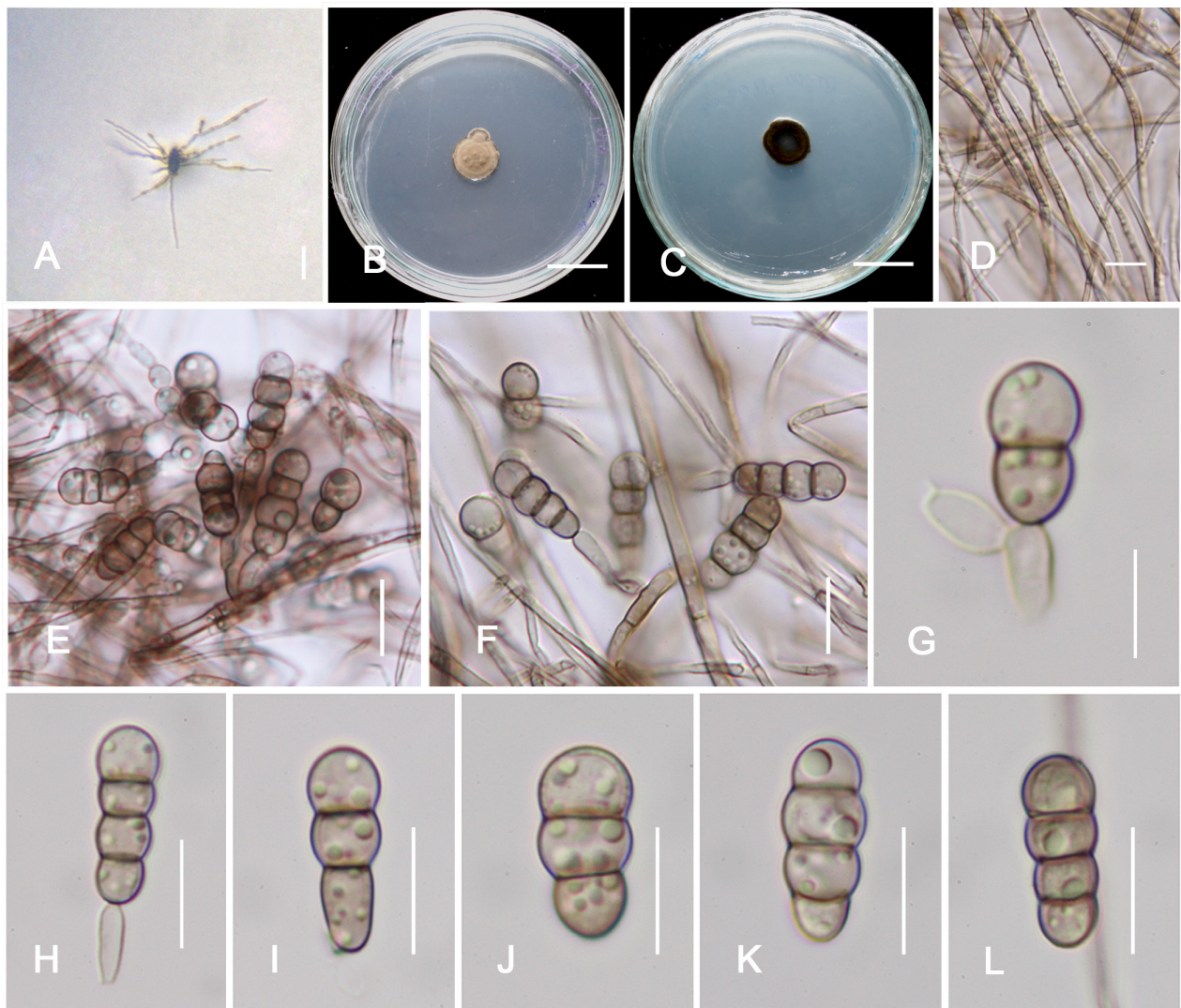


FIGURE 3. Asexual state of *Clavatispora thailandica* (MFLUCC100107, extype living culture). A. Germinating ascospore. B–C. Colonies on MEA, upper surface and lower surface. D. Vegetative hyphae in culture. E–H. Conidiophores with conidia. I–L. Conidia. Scale bars: A = 10 μ m, B–C = 1 cm, D = 5 μ m, E–L = 10 μ m.

Discussion

Clavatispora thailandica, discovered on dead stems of an unidentified herbaceous plant in northern Thailand, is characterized by its setiferous black ascomata, bitunicate asci with fissitunicate dehiscence, with a shrunken ectotunica, endotunica and coloured plasmalemma layers, and clavate, dark brown, muriform ascospores. The unique ascospore features suggest this species belongs in the *Pleosporales*. However, molecular studies place *C.*

thailandica in the order *Venturiales* (Dothideomycetes), although its morphological characters are rather different from all taxa in *Venturiales* (Zhang *et al.* 2012). Members of the order *Pleosporales* are distantly related (data not shown). The phylogenetic analysis based on LSU sequence data show the placement of *Clavatispora* in family *Symptoventuriaceae*, where it clusters with *Scolecobasidiella avellanea* (Fig. 1). Most taxa in *Symptoventuriaceae* are hyphomyceteous, and, with the exception of *Symptoventuria*, the sexual state of most species is unknown (Crous *et al.* 2007, Seifert *et al.* 2011, Samerpitak *et al.* 2014). *Clavatispora* also produced a hyphomyceteous state in culture characterized by erect, coloured, septate, sometimes branched conidiophores and ellipsoidal to subglobose, light coloured, guttulate, 3-septate, deeply constricted conidia (Fig. 3). The asexual state of *Clavatispora*, which is *Trichocladium*-like, differs from all genera in the same clade *i.e.* *Fusicladium*, *Ochroconis*, *Scolecobasidiella*, *Scolecobasidium* and *Veronaeopsis* (Abbott 1927, Ellis 1971, 1976, Fothergill 1996, Schubert *et al.* 2003, Seifert *et al.* 2011, Martin-Sanchez *et al.* 2012, Zhang *et al.* 2012, Samerpitak *et al.* 2014). In the light of these unique morpho-molecular features, the taxonomic affinity of *Clavatispora thailandica* as a new genus and species is confirmed.

Acknowledgements

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