MycoKeys 33: 1–23 (2018)
doi: 10.3897/mycokeys.33.23341
http://mycokeys.pensoft.net

RESEARCH ARTICLE

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New nematicidal and antimicrobial secondary metabolites from a new species in the new genus, *Pseudobambusicola thailandica*

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Academic editor: *I. Schmitt* | Received 31 December 2017 | Accepted 14 March 2018 | Published 22 March 2018

Citation: Rupcic Z, Chepkirui C, Hernández-Restrepo M, Crous PW, Luangsa-ard JJ, Stadler M (2018) New nematicidal and antimicrobial secondary metabolites from a new species in the new genus, *Pseudobambusicola thailandica*. MycoKeys 33: 1–23. https://doi.org/10.3897/mycokeys.33.23341

Abstract

During the course of a study on the functional biodiversity of the mycobiota inhabiting rainforests in Thailand, a fungal strain was isolated from a plant sample and shown to represent an undescribed species, as inferred from a combination of morphological and molecular phylogenetic methods. Molecular phylogenetic analyses, based on four DNA loci, revealed a phylogenetic tree with the newly generated sequences clustering in a separate branch, together with members of the Sulcatisporaceae (Pleosporales, Ascomycota). The Thai specimen morphologically resembled *Neobambusicola strelitziae* in having pycnidial conidiomata with phialidic conidiogenous cells that produce both fusoid-ellipsoid macroconidia and subcylindrical microconidia. However, the new fungus, for which the name *Pseudobambusicola thailandica* is proposed, differs from *N. strelitziae* in having conidiomata with well-defined necks, the presence of globose to subglobose thick-walled cells adjacent to conidiomata and the production of chlamydospores in culture. When cultures of *P. thailandica,* growing on water agar, were confronted with *Caenorhabditis elegans* nematodes, worms approaching the fungal mycelia were killed. This observation gave rise to a study of its secondary metabolites and six novel and two known compounds were isolated from submerged cultures of *P. thailandica*. The structures of metabolites 1–6, for which the trivial names thailanones A–F are

proposed, were elucidated using a combination of spectral methods, including extensive 1 and 2D NMR analysis and high resolution mass spectrometry. Compounds 4 and 8 showed strong nematicidal and weak antifungal activity, whereas all other tested compounds showed moderate to weak nematicidal activity but no significant effects in the serial dilution assay against various fungi and bacteria. Compounds 1 and 8 also inhibited growth of the pathogenic basidiomycete *Phellinus tremulae* in a plate diffusion assay.

Keywords
Antifungal agent, deoxyphomalone, monocerin, nematode-antagonism, nematicide, phylogeny

Introduction

Fungi are regarded as prolific sources of secondary metabolites with prominent and selective biological activities that can serve as a basis for development of new antimicrobials, agrochemical pesticides and other useful compounds (Bills and Gloer 2016, Karwehl and Stadler 2017). In particular, the mycobiota of tropical countries are widely unexplored and can still yield a plethora of novel chemical entities. In recent years, many novel compounds with, for example, antimicrobial (Helaly et al. 2016, 2017, Richter et al. 2016, Kuephadungphan et al. 2017), cytotoxic (Surup et al. 2017) and antioxidative (Kuhnert et al. 2015) effects were isolated in the authors' laboratory from tropical fungi. Furthermore, fungi represent a rich source of nematicidal compounds because they are both prey and natural antagonists of nematodes. Thus, understanding the chemical basis for fungi nematode interactions offers natural biocontrol strategies (Anke et al. 1995). According to Degenkolb and Vilcinskas (2016), approximately 700 species of nematophagous fungi have been described so far and four ecophysiological categories have been proposed. However, little has been done to screen for metabolites in nematophagous fungi or, for that measure, nematicidal metabolites in other fungi since the first studies of this kind during the 1990s (Stadler et al. 1993a, b, 1994).

Environmentally compatible and low-cost alternatives to chemical control measures for phytoparasitic nematodes are urgently needed and these must not affect vertebrates, crops and other non-target organisms. Highly specific, preferably soil-borne antagonists are best suited for this purpose (Degenkolb and Vilcinskas 2016).

In this context, fungi isolated from nature were examined for morphological features and by ITS sequencing. The strains that turned out to belong to well-studied, ubiquitous mycotoxin-producing genera (in particular Trichocomaceae and Hypocreaeae) were discarded. Those strains that belong to less studied phylogenetic lineages were selected for studies of their antagonistic activities. They were first tested using a water agar assay to detect nematicidal effects and, in parallel, extracts were prepared and checked in an agar plate diffusion assay for antifungal and nematicidal activities. Herein, the authors report the discovery of a new genus and species *Pseudobambusicola thailandica* and its six novel and two known secondary metabolites, including their isolation, structure elucidation and biological activity.

Materials and methods

Fungal isolation

During a fungal exploration in Thailand in 2015, an unrecognised fungus was found growing on a twig of an unidentified plant. The twig was incubated in a damp chamber and treated according to Castañeda-Ruiz et al. (2016). Single conidial isolates were established from sporulating conidiomata in Petri dishes containing water agar (WA; Difco agar 5 g, tap water 1 l). Colonies were sub-cultured on potato carrot agar (PCA; potatoes 20 g; carrots 20 g; agar 20 g; distilled water 1 l) and oatmeal agar (OA; oatmeal 30 g; agar 18 g; distilled water 1 l) as described previously (Hernández-Restrepo et al. 2017). Herbarium type material and the ex-type strain are maintained at the BIOTEC Bangkok herbarium (BBH) and at the BIOTEC culture collection (BCC; both Pathum Thani, Thailand), respectively.

Morphology

Morphological features were characterised from colonies growing on OA or on synthetic nutrient-poor agar (SNA; Nirenberg 1976) supplemented by fragments of autoclaved pine needles and incubated at 25 °C under continuous near-ultraviolet light to promote sporulation. Colony colours were assessed according to the charts of Rayner (1970). Micromorphological descriptions and measurements for 30 replicates of relevant features were carried out from mature conidiomata and conidia mounted in lactic acid 90%. Photomicrographs were made following Hernández-Restrepo et al. (2017).

DNA isolation, amplification and sequences analyses

Genomic DNA was extracted from fungal colonies growing on MEA using the Wizard® Genomic DNA purification kit (Promega, Madison, USA) following the manufacturer's protocols. The nuclear rDNA operon spanning the 3' end of the 18S nrRNA gene, the first internal transcribed spacer (ITS1), the 5.8S nrRNA gene, the second ITS region (ITS2) and approximately 900 bp of the 5' end of the large subunit of the nrRNA gene (LSU), part of the RNA polymerase II second largest subunit gene (*rpb2*) and part of the translation elongation factor 1-α gene (*tef1*) were amplified following Hernández-Restrepo et al. (2016). The programme SeqMan Pro v. 10.0.1 (DNASTAR, Madison, WI, USA) was used to obtain consensus sequences for each DNA region. Blast searches using ITS and LSU sequences were performed and the closest matches and related taxa were retrieved from GenBank and included in the phylogenetic analyses (Table 1, See Suppl. material 1). Alignments were produced with MAFFT v. 7 (Katoh and Standley 2013), checked and refined using MEGA v. 6 (Tamura et al. 2013) and SequenceMatrix (Vaidya et al. 2011).

Table 1. Isolates and GenBank accession numbers used in the phylogenetic analyses.

Taxa	Strain number[1]	GenBank accession numbers[2]				References
		ITS	LSU	*rpb2*	*tef1*	
Alternaria tenuissima	CBS 918.96	–	KC584311	KC584435	KC584693	Woudenberg et al. 2013
Bambusicola didymospora	MFLUCC 10-0557	KU940116	KU863105	KU940163	KU940188	Dai et al. 2017
B. loculata	MFLU 15-0056	KP761732	KP761729	KP761715	KP761724	Dai et al. 2015
B. pustulata	MFLUCC 15-0190	KU940118	KU863107	KU940165	KU940190	Dai et al. 2017
B. splendida	MFLUCC 11-0611	KU940121	KU863110	KU940168	–	Dai et al. 2017
Coniothyrium palmicola	CBS 161.37	JX681086	JX681086	–	–	Verkley et al. 2014
Dendrothyrium longisporum	CBS 824.84	JX496115	JX496228	–	–	Verkley et al. 2014
Dydimella exigua	CBS 183.55	NR135936	EU754155	GU357800	KR184187	De Gruyter et al. 2009, Schoch et al. 2009, Kim et al. 2016
Keissleriella culmifida	KT 2308	–	AB807591	–	AB808570	Tanaka et al. 2015
K. quadriseptata	KT 2292	NR145135	AB807593	–	AB808572	Tanaka et al. 2015
Latorua caligans	CBS 576.65	NR132923	KR873266	–	–	Crous et al. 2015a
Leptosphaeria doliolum	CBS 505.75	JF740205	GQ387576	KY064035	GU349069	De Gruyter et al. 2013, Schoch et al. 2009
Lophiostoma arundinis	AFTOL-ID 1606	–	DQ782384	DQ782386	DQ782387	Schoch et al. 2009
Macrodiplodiopsis desmazieri	CBS 140062	NR132924	KR873272	–	–	Crous et al. 2015a
Magnicamarosporium iriomotense	KT 2822	AB809640	AB807509	–	AB808485	Tanaka et al. 2015
Massarina phragmiticola	CBS 110446	–	DQ813510	–	–	Kodsueb et al. 2007
Montagnula bellevaliae	MFLUCC 14-0924	KT443906	KT443902	–	–	Hongsanan et al. 2015
M. scabiosae	MFLUCC 14-0954	KT443907	KT443903	–	–	Hongsanan et al. 2015
Murilentithecium clematidis	MFLUCC 14-0562	KM408757	KM408759	KM454447	KM454445	Wanasinghe et al. 2014
Neobambusicola strelitziae	CBS 138869	NR 137945	KP004495	–	**MG976037**	Crous et al. 2014, this study
Palmiascoma gregariascomum	MFLUCC 11-0175	KP744452	KP744495	KP998466	–	Liu et al. 2015
Parabambusicola bambusina	H 4321	–	AB807536	–	AB808511	Tanaka et al. 2015
Paraconiothyrium brasiliense	CBS 122851	JX496036	JX496149	–	–	Verkley et al. 2014
Phoma herbarum	CBS 615.75	NR135967	EU754186	KP330420	KR184186	Aveskamp et al. 2009, Chen et al. 2015
Pleurophoma ossicola	CPC 24985	KR476737	KR476770	–	–	Crous et al. 2015b

Taxa	Strain number[1]	GenBank accession numbers[2]				References
		ITS	LSU	*rpb2*	*tef1*	
Polyschema congolensis	CBS 542.73	–	EF204502	EF204486	–	Shenoy et al. 2010
P. terricola	CBS 301.65	–	EF204504	EF204487	–	Shenoy et al. 2010
Pseudobambusicola thailandica sp. nov.	BCC 79462	**MG926559**	**MG926560**	**MG926561**	**MG926562**	This study
Pseudoleptosphaeria etheridgei	CBS 125980	NR111620	JF740291	–	–	De Gruyter et al. 2013
Pseudoxylomyces elegans	KT 2887	–	AB807598	–	AB808576	Tanaka et al. 2015
Setoseptoria arundinacea	KT 552	–	AB807574	–	AB808550	Tanaka et al. 2015
Stemphylium vesicarium	CBS 191.86	KC584239	JX681120	KC584471	KC584731	Woudenberg et al. 2013
Sulcatispora acerina	KT 2982	LC014597	LC014610	–	LC014615	Tanaka et al. 2015
S. berchemiae	KT 1607	AB809635	AB807534	–	AB808509	Tanaka et al. 2015
Trematosphaeria pertusa	CBS 122368	NR132040	FJ201990	FJ795476	KF015701	Zhang et al. 2009

[1] BCC: BIOTEC Culture Collection, Thailand; CBS: Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; CPC: Culture collection of Pedro Crous, housed at CBS; KT and H: Culture collection of K. Tanaka and K. Hirayama, housed at the National Institute of Agrobiological Science, Japan (MAFF); MFLU: Mae Fah Laung University Herbarium, Chiang Rai, Thailand; MFLUCC: Mae Fah Luang University Culture Collection, Chiang Rai, Thailand.

[2] ITS: internal transcribed spacer regions 1 & 2 including 5.8S nrRNA gene; LSU: large subunit of the nrRNA gene, *rpb2*: partial RNA polymerase II second largest subunit gene; *tef1*: partial translation elongation factor 1-α gene. Sequences generated in the present study are in **bold.**

Individual alignments for each locus and the concatenated four-loci dataset were analysed by maximum likelihood (ML) with gamma model of rate heterogeneity using the RAxML HPC BlackBox v. 8.2.8 (Stamatakis 2014) online server of the Cipres Science gateway portal (Miller et al. 2010). The maximum likelihood search option was used to search for the best-scoring tree after bootstrapping. By default, the RAxML BlackBox calculates statistical support for branches by rapid bootstrap analyses of 1000 replicates (Stamatakis 2014). Bootstrap support (bs) values ≥ 70 % were considered significant. Incongruence amongst datasets was tested by visual inspection of all groups with ≥ 70 % bs in partial trees of each locus to search for potentially conflicting groups. A Markov Chain Monte Carlo (MCMC) algorithm was used to generate phylogenetic trees with Bayesian probabilities from the concatenated four-loci dataset using MrBayes v. 3.2.6 (Ronquist et al. 2012). Two analyses of four MCMC chains were run from random trees, trees were sampled every 100 generations and 25 % of them were discarded as the burn-in phase. Posterior probabilities (pp) were determined from the remaining trees. The sequences generated during this study and the alignments used in the phylogenetic analyses were deposited in GenBank and TreeBASE, respectively.

Chromatography and spectral methods

1D and 2D nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance III 700 spectrometer with a 5 mm TXI cryoprobe (^{1}H 700 MHz, ^{13}C 175 MHz) and a Bruker Avance III 500 (^{1}H 500 MHz, ^{13}C 125 MHz) spectrometer, UV spectra were recorded with a Shimadzu UV-2450 UV–Vis spectrophotometer and optical rotations were measured on a Perkin-Elmer 241 polarimeter. Analytical HPLC was carried out on an Agilent 1200 Series, equipped with degasser, binary pump SL, autosampler and connected to a diode array detection/light scattering detector Corona Ultra RS. A Waters C18 Acquity UPLC BEH column (2.1 × 50 mm, 1.7 µm) was used as stationary phase. The mobile phase consisted of H_2O + 0.1% formic acid (solvent A) and acetonitrile + 0.1% formic acid (solvent B) with the following gradient: 0–0.5 min 5% B, 0.5–20 min 100% B, 20–30 min 100% B; injection volume was 2 µl, flow rate 600 µl/min.

HPLC-ESI-MS spectra were recorded on an ion trap mass spectrometer [scan range 100–2000 m/z, capillary voltage 4000 V, dry temperature 250 °C] (amaZon speed, Bruker) and HR-ESIMS spectra on a time-of-flight (TOF) MS [scan range 250–25000 m/z, capillary voltage 4500 V, dry temperature 200 °C] (MaXis, Bruker). In parallel, UV/Vis spectra in the range of 200–600 nm were recorded.

Chemicals and solvents were obtained from AppliChem GmbH (Darmstadt, Germany), Avantor Performance Materials (Deventer, Netherlands), Carl Roth GmbH & Co. KG (Karlsruhe, Germany) and Merck KGaA (Darmstadt, Germany) in analytical and HPLC grade.

Fermentation and extraction

A seed culture was prepared as follows: five mycelial plugs (0.5 × 0.5 cm^2) were cut from actively growing colonies maintained on YM 6.3 agar (malt extract 10 g/l, D-glucose 4 g/l, yeast extract 4 g/l, agar 20 g/l, pH 6.3 before autoclaving) and placed into a 500 mL Erlenmeyer flask containing 200 mL Q6½ medium (D-glucose 2.5 g/l, glycerol 10 g/l, cotton seed flour 5 g/l, pH 6.3) and incubated on a rotary shaker for 96 hours at 24 °C and 140 rpm. 20 mL of the seed culture were added into 10 × 1000 ml sterile Erlenmeyer flasks with 500 ml of Q6 ½ medium (5 l total) and incubated on a rotary shaker (288 hours, 24 °C, 140 rpm).

Biomass and supernatant were separated by means of centrifugation and filtration. The mycelia were extracted twice with acetone (2 l), the extract was evaporated *in vacuo* and the remaining aqueous phase extracted with equal amounts of ethyl acetate three times. One percent (1 %) of Amberlite XAD-16N was given to the culture broth and stirred for 1 h. After filtration, the XAD resin was extracted as described above. 220 mg and 88 mg of mycelial and supernatant crude extracts were obtained, respectively.

Isolation of the compounds 1–8

The supernatant crude extract was fractionated on preparative HPLC (Gilson GX270 Series HPLC system). The reversed phase C18 column (Nucleodur 150/40, 10 μm, 110 Å; with a precolumn VP 100/10; Macherey-Nagel) was used as a stationary phase and the mobile phase was composed of deionised water + TFA 0.05 % (Milli-Q, Millipore, Schwalbach, Germany; solvent A) and acetonitrile (ACN) + TFA 0.05 % (solvent B). The fractionation was accomplished with the following gradient: 15 % of B isocratic for 5 min, followed by a linear increase to 80 % B over 30 min, afterwards increasing to 100% B in 5 min and thereafter isocratic conditions at 100 % for 5 min. In total, 7 compounds were obtained from the supernatant crude extract: Compound **1** (thailanone A; 1 mg) was obtained at the retention time t_R = 6 min, compound **2** (thailanone B; 1 mg) at t_R = 4.3 min, compound **3** (thailanone C; 1.3 mg) at t_R = 6.4 min, compound **4** (thailanone D; 1 mg) at t_R = 8.1 min; compound **5** (thailanone E; 4.2 mg) at t_R = 8.2, compound **6** (thailanone F; 1.6 mg) at t_R = 8.6 min) and compound **7**; monocerin (7.8 mg) at t_R = 9.1 min. The mycelial crude extract was chromatographed in a similar manner as described above, yielding 77.8 mg of deoxyphomalone (**8**, t_R = 11.2 min) but none of the other compounds.

Evaluation of antimicrobial activities

Minimum inhibitory concentrations (MIC) of compounds **1**–**8** were determined in serial dilution assays against *Bacillus subtillis* DSM10, *Mucor plumbeus* MUCL 49355 and *Candida tenuis* MUCL 29892 as described previously by Chepkirui et al. (2016). The assays were carried out in 96-well microtiter plates in YMG (yeast-malt-glucose) medium for filamentous fungi and yeasts and MH (Müller-Hinton) medium for the bacterium. For all tested compounds, the starting concentration was 100 μg/mL and final 0.78 μg/mL.

Water agar plate assay

The fungal cultures were tested in the water agar plate assay against *Caenorhabditis elegans* nematodes (wild type strain, see Ashrafi et al. 2017), in a similar manner as previously described by Stadler et al. (1994). After 3–7 days, nematicidal effects became visible by many dead and immotile nematodes in the vicinity of the mycelia. Fungal colonies exhibiting toxic effects were selected for submerged cultivation and production/isolation of nematicidal compounds.

Microtiter plate assay for nematicidal activities

The nematicidal activity against *C. elegans* of all isolated compounds was determined by a slightly modified method (Stadler et al. 1994, Kuephadungphan et al. 2017 and Ashrafi et al. 2017). *C. elegans* was inoculated monoxenically on nematode agar (soy peptone 2 g/l, NaCl 1 g/l, agar-agar 20 g/l) and, after autoclaving, the following ingredients were added as sterile filtered solutions: cholesterol (1 mg/mL dissolved in EtOH) 0.5 ml, 1M $CaCl_2$ 1 mll, 1M $MgSO_4$ 1 ml, 40 mM potassium phosphate buffer 12.5 ml; pH 6.8) with living *Escherichia coli* DSM498 (1 ml of a suspension containing approximately 10 cells/ml, pre-inoculated for 12 h at 37 °C) and the plates were incubated at 21 °C for 4–5 days. Thereafter, nematodes were washed down from the plates with M9 buffer (3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl and, after autoclaving, the addition of 1 ml 1 M $MgSO_4$). Finally, a nematode suspension of approximately 500 nematodes/ml in M9 buffer was prepared and used in the microtiter plate assay.

The assay was performed in 24-well microtiter plates at four concentrations (100, 50, 25 and 12.5 µg/ml) for each compound. Ivermectin was used as a positive control, while methanol was used as a negative control. The plates were incubated at 20 °C in the dark and nematicidal activity was recorded after 18 h of incubation and expressed as LD_{90} (i.e. concentration causing over 90 % immobility of the nematodes).

Antifungal activity assay against *Phellinus tremulae*

Growth inhibition of *Phellinus tremulae* CBS 123.40 for compounds 1–8 was tested according to the modified protocol published by Ayer and Jimenez (1994). The assay was performed in 24-well microtiter plates where 1 mL of YM agar was added in each well and thereafter the compounds were dissolved in methanol (100, 50, 25 and 12.5 µg/ml) and added to the wells. Shortly after the media solidified, 0.5 × 0.5 mm^2 agar plugs of actively growing colonies of *Ph. tremulae* CBS 123.40, grown on a YM 6.3 agar plate, were placed in each well of the microtiter plate. Nystatin and methanol were used as positive and negative controls, respectively, together with control wells without additives. Inhibition of the radial growth of the colonies of *Ph. tremulae* CBS 123.40 relative to the control was recorded as a positive result. The radial growth was measured after 3, 5, 7 and 9 d.

Phytotoxic activity assay

Phytotoxic activities were carried out by germination and seedling growth bioassay against *Setaria italica* and *Lepidum sativum* according to the protocol from Anke et al. (1989). The amount of 100 µg/paper disc of compound was tested; as a positive control herbicide methyl vilogen dichloride hydrate was used. The negative controls were the seeds only and the solvent alone (the one used for dissolving the compounds).

Results and discussion

Molecular phylogenetic analysis

The combined dataset consisted of 35 taxa with 3126 characters of which 396 bp corresponded to ITS, 853 bp to LSU, 904 bp to *rpb2* and 973 bp to *tef1*. The alignment had 100% representation for LSU, 74% for ITS, 46% for *rpb2* and 57% for *tef1*. The phylogenetic tree (Fig. 1) shows two fully supported main clades, corresponding to the sub-orders Massarineae and Pleosporineae (Pleosporales, Dothideomycetes). In the Massarineae, eleven clades representing families are shown, i.e., Bambusicolaceae (96%, 1 pp), Coniothyriaceae (100%, 1 pp), Didymosphaeriaceae, Latoruaceae (100%, 1 pp), Lentitheciaceae (97%, 1 pp), Macrodiplodiopsidaceae, Massarinaceae (100%, 1 pp), Montagnulaceae (100%, 1 pp), Parabambusicolaceae, Sulcatisporaceae (100%, 1 pp) and Trematosphaeriaceae and an additional subclade comprising *Pseudoxylomyces elegans*. In the phylogenetic tree (Fig. 1), the sequence data of the new species indicate a systematic position in an independent branch in the Sulcatisporaceae close to *Magnicamarosporium eriomotense* without any support.

Taxonomy

***Pseudobambusicola* Hern.-Restr. & Crous, gen. nov.**
MycoBank: MB824299

Etymology. The name reflects its morphological similarity of the type species to the asexual morphs of *Bambusicola* and *Neobambusicola*.

Type species. *Pseudobambusicola thailandica* Hern.-Restr. & Crous.

Diagnosis. Differs from *Neobambusicola* in having conidiomata with a neck, the presence of globose to subglobose thick-walled cells adjacent to the conidiomata and the production of chlamydospores in culture.

Mycelium composed of hyaline to pale brown, septate, smooth to slightly verruculose, hyphae. *Conidiomata* pycnidial, semi- or entirely immersed in the agar, solitary or aggregated, erumpent, globose with a neck, opening via central ostiole, dark brown, surrounded by dark brown, smooth to slightly verruculose hyphae, at the base globose to subglobose, thick-walled cells often present. *Conidiophores* reduced to conidiogenous cells. *Conidiogenous cells* phialidic with periclinal thickening at the conidiogenous locus, subcylindrical to ampulliform, hyaline, smooth. *Conidia* exposed in white, mucous drops at the ostioles of the pycnidia, composed by macro- and microconidia. *Macroconidia* produced in white, mucous heads, solitary, fusoid-ellipsoid, apex bluntly to subobtusely rounded, tapering to a distinctly truncate base, prominently guttulate, hyaline, smooth, 0–3-septate. *Microconidia* produced in the same pycnidia as macroconidia, solitary, oblong to cuneiform, non-guttulate to slightly guttulate, hyaline, smooth, aseptate. *Chlamydospores* brown, terminal at the tips of vegetative hyphae, in chains. *Sexual morph* not observed.

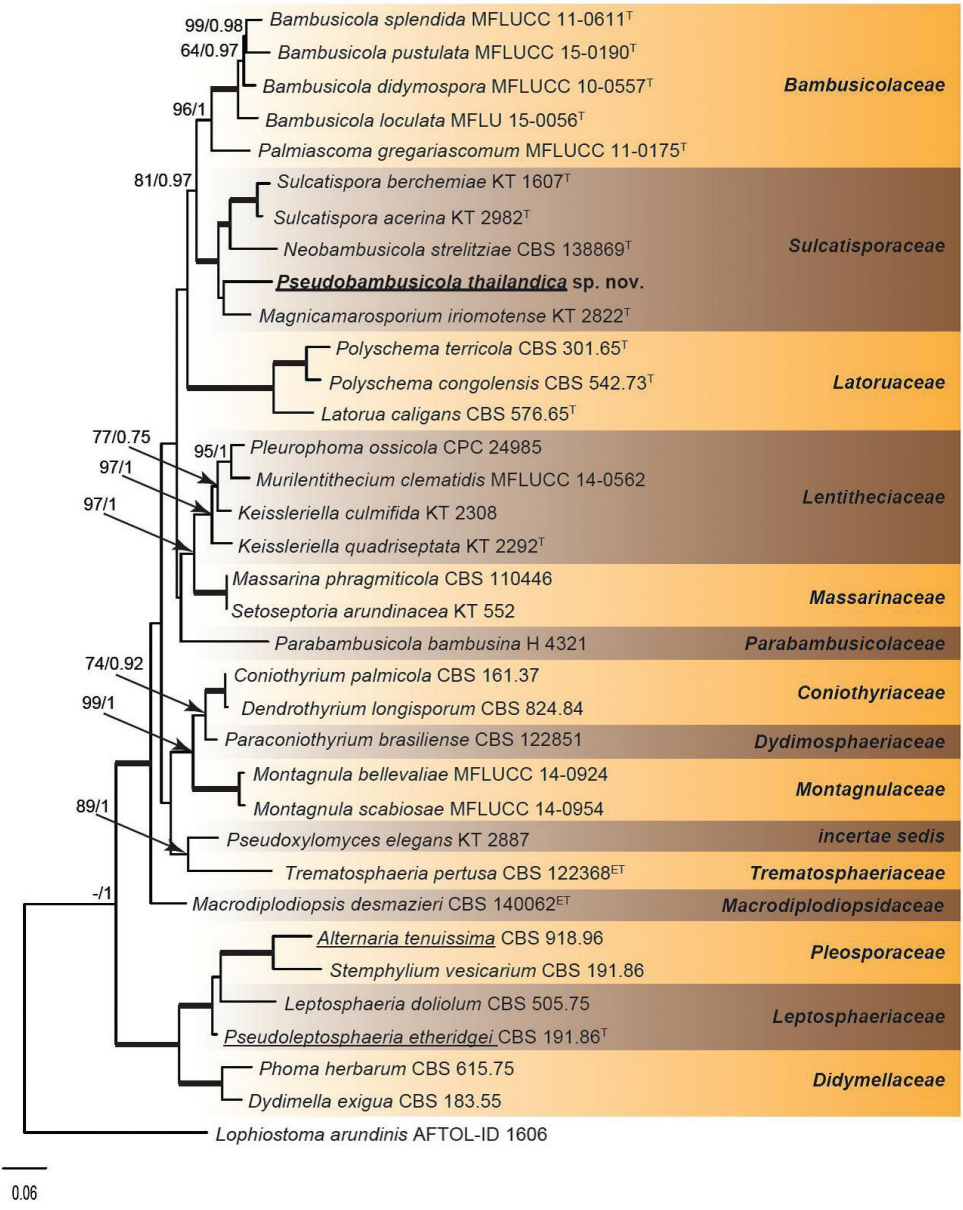

Figure 1. Phylogenetic tree (RAxML) inferred from the DNA sequence data of four loci (ITS, LSU, *tef1* and *rpb2*) of *Pseudobambusicola thailandica* and related species in Pleosporales (Dothideomycetes). The new taxon is indicated in **bold**. Taxa reported to produce deoxyphomalone are indicated by an underlined. Maximum likelihood bootstrap values ≥ 70 % and Bayesian posterior probabilities ≥ 0.95 are shown at the nodes and the scale bar indicates the number of expected mutations per site. Clades with 100 BML and 1 PP are indicated by thickened lines . The tree was rooted to *Lophiostoma arundinis* (AFTOL-ID 1606). ^{T} = ex-type strain; ^{ET} = epitype strain.

Pseudobambusicola thailandica **Hern.-Restr. & Crous, sp. nov.**
MycoBank MB824300

Etymology. The epithet refers to Thailand, where this species was collected.

Type. THAILAND. Lop Buri Province: Chai Badan, Wang Kan Lueang Arboretum, Wang Kan Lueang Waterfall, on twig (unidentified), 14 Jul 2015, M. Hernández-Restrepo, MHR 1534 (holotype: BBH 42022!, culture ex-type BCC 79462!).

Description of fungal structures on SNA. *Mycelium* composed by hyaline to pale brown, septate, smooth to slightly verruculose, hyphae, 1–2.5 µm wide. *Conidiomata* pycnidial, semi- or entirely immersed in the agar, solitary or aggregated, erumpent, globose, sometimes with a neck, opening via central ostiole, dark brown, 63–360 µm diam., sometimes with a cylindrical neck 50–125 × 40–50 µm, opening via central ostiole; at the base of the conidiomata are often present globose to subglobose cells, thick-walled, 5–9 µm wide; conidiomata surrounded by dark brown, smooth to slightly verruculose hyphae, 2–2.5 µm wide. *Conidiophores* reduced to conidiogenous cells. *Conidiogenous cells* phialidic with periclinal thickening, subcylindrical to ampulliform, hyaline, smooth, 6.5–7 × 2.5–4 µm. *Conidia* exposed in white, mucous drops at the ostiole of pycnidia, composed by macro- and microconidia. *Macroconidia* produced in white, mucous heads, solitary, fusoid-ellipsoid, apex bluntly to subobtusely rounded, tapering to a distinctly truncate base, mostly straight, but sometimes slightly curved, prominently guttulate, hyaline, smooth, 0–3-septate, 10–20 × 2–4(–6) µm. *Microconidia* produced in the same pycnidia with macroconidia, solitary, oblong to cuneiform, non-guttulate to slightly guttulate, hyaline, smooth, aseptate, 2–4(–5.5) × 1–2 µm, apex rounded, base truncate. *Chlamydospores* brown, terminal, in chains, 16–38 × 5–6 µm. *Sexual morph* not observed.

Culture characteristics. Colonies on OA at 25 °C reaching 24 mm diam. in 2 weeks, elevated, with dense cottony mycelium at the centre, mouse grey, margin whitish, effuse to fimbriate; reverse dark mouse grey.

Notes. *Pseudobambusicola* is introduced here for a pycnidial coelomycete producing two kinds of conidia. Morphologically, it is similar to the species of *Bambusicola* and *Neobambusicola*. However, asexual morphs in *Bambusicola* are characterised by brown or pale brown conidia and annellidic rather than phialidic conidiogenous cells and hyaline conidia as in *Pseudobambusicola* (Dai et al. 2012, 2017). *Neobambusicola* is a monotypic genus erected for *N. strelitziae*, first described from South Africa growing on necrotic leaf tissue associated with *Phyllachora strelitziae* (Phyllachoraceae, Phyllachorales, Sordariomycetes) (Crous et al. 2014). Both genera are similar in having pycnidial conidiomata and phialidic conidiogenous cells that produce fusoid-ellipsoid macro- and subcylindrical microconidia. However, in the new genus, the conidiomata are surrounded by dark brown, smooth to slightly verruculose hyphae and, in mature conidiomata, a cylindrical neck is often present; furthermore, chlamydospores can be present in culture. Although both genera belong to the Sulcatisporaceae (Pleosporales, Dothideomycetes), they are placed in different clades, *Neobambusicola* is more closely

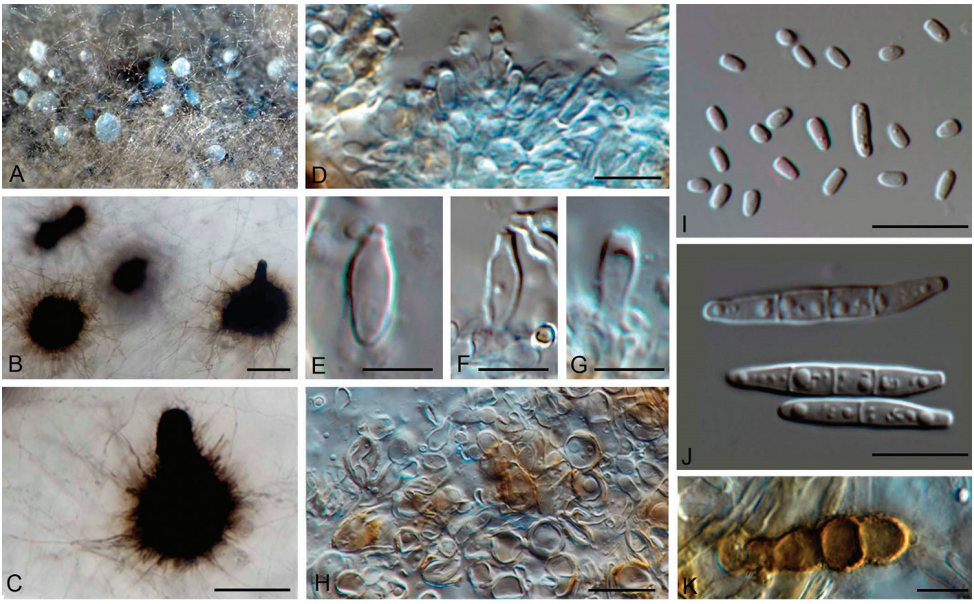

Figure 2. *Pseudobambusicola thailandica* (BCC 79462) on SNA. **A** Colony overview **B–C** Pycnidia **D–G** Conidiogenous cells **H** globose to subglobose cells, thick-walled, at the base of the conidiomata **I** Microconidia **J** Macroconidia **K** Chlamydospores. Scale bars: 200 µm (**B**), 100 µm (**C**), 10 µm (**D, H, I–K**), 5 µm (**E–G**).

related to *Sulcatispora* (100 %, 1 pp), while *Pseudobambusicola* was placed in a distinct branch with *Magnicamarisporium* (Fig. 1). Additionally, based on LSU, ITS and *tef1* sequences, *P. thailandica* is 97 % (KP004495) and 83 % (KP004467) and 93 % similar to *N. strelitziae*, respectively.

Water agar plate assay

Out of 66 fungal strains investigated, 18 exhibited antagonistic activity towards nematodes in the water agar assay. Of those, 3 strains produced compounds with nematicidal activity in submerged culture, while in 5 strains, antimicrobial activity was observed. Extracts from *P. thailandica* (BCC 79462) submerged fermentation displayed strong activity towards nematodes and were subjected to extensive chromatographic studies as described in the Experimental part.

Structure elucidation of compounds 1–8

Fractionation of the crude extracts obtained from submerged cultures of *P. thailandica* (BCC 79462) resulted in the identification of six previously undescribed polyketides for which the authors propose the trivial names thailanones A–F (**1**–**6**) and two known

compounds, monocerin and deoxyphomalone, **7** and **8** (see chemical structures in Fig. 3). The NMR spectroscopic data are compiled in Tables 2 and 3 and the spectra and chromatograms are compiled in the Suppl. material 1.

Compound **1** (thailanone A) was isolated as a white solid from the supernatant with the molecular formula $C_{12}H_{18}O_4$ and four degrees of unsaturation established from the HRMS data. ^{13}C and DEPT NMR data revealed the presence of 12 carbons in the molecule: three methyl groups, four methylene groups and five quaternary carbons (Table 2). ^{1}H NMR spectra on the other hand revealed the presence of two methyl triplets at δ 0.88 (H-11) and δ 0.93 (H-7) together with a methoxy group singlet resonating at δ 4.05 (H-12).

HMBC correlations of H-4 to C-1/C-2/C-3-C-5/C-6, H-6 to C-1/C-2/C-2/C-7 and H-9 to C-5/C-8/C-10/C-11 indicated the presence of an isohumulone moiety differing in the ring substitution (Fig. 4). Furthermore, HMBC correlations between H-11 to C-10/C-9 and H-7 to C-2/C-6 were observed. These correlations were further supported by the COSY correlations observed between H-10 and H-9/H-11 and H-6 and H-7. The methoxy proton H-12 showed a HMBC correlation to C-3. There-

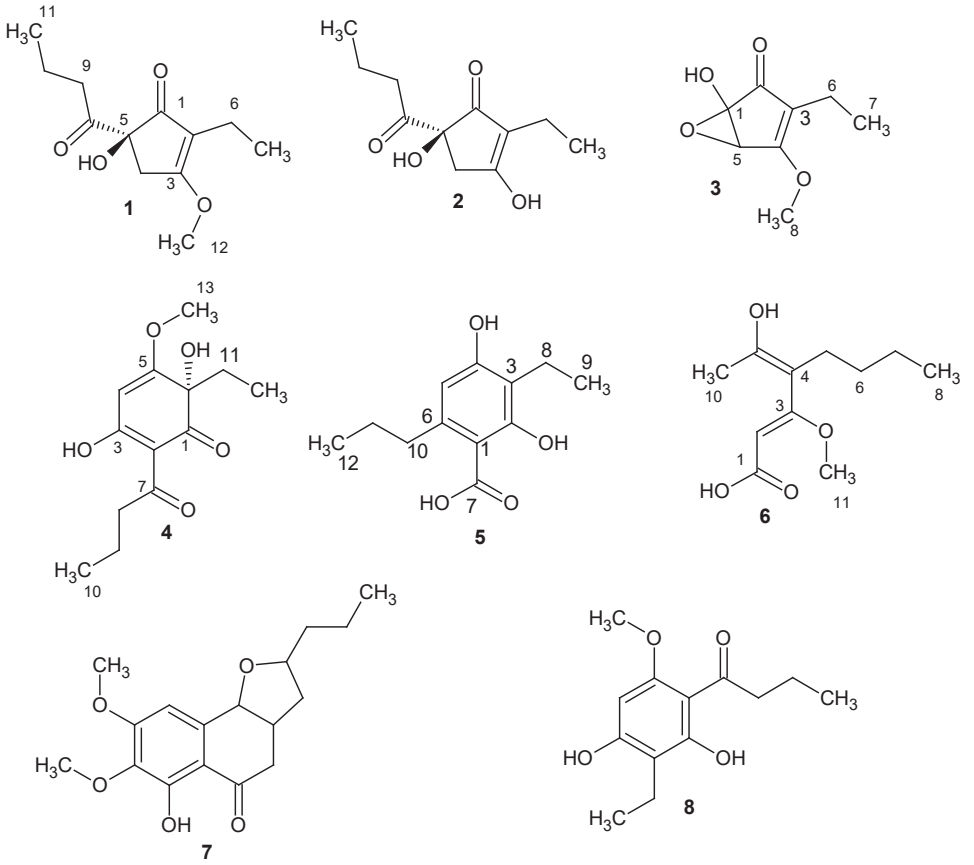

Figure 3. Chemical structures of thailanones A–F (**1–6**), monocerin (**7**) and deoxyphomalone (**8**).

Table 2. NMR spectroscopic data for compounds **1–3** in D_6-acetone (^{1}H NMR at 700 MHz; ^{13}C at 500 MHz).

	1			2			3	
No.	**^{13}C**	**DEPT**	**^{1}H/HSQC**	**^{13}C**	**^{1}H/HSQC**		**DEPT**	
1	202.6	C		202.6		83.9	C	
2	120.2	C		119.0		194.1	C	
3	187.9	C		191.2		120.3	C	
4	38.9	CH_2	2.61 (s), 3.29 (s)	43.8	2.41 (s) 2.88 (s)	170.1	C	
5	86.3	C		84.4		58.3	CH	3.46 (s)
6	15.5	CH_2	2.11 (q), J= 7.53 Hz	15.2	2.11 (q), J= 7.53 Hz	16.7	CH_2	2.16 (q), J=7.53 Hz
7	12.7	CH_3	0.93 (t), 7.53 Hz	12.8	0.93 (t), 7.53 Hz	13.2	CH_3	0.78 (t), J=7.53 Hz
8	210.7	C		210.1		57.4	CH_3	3.84 (s)
9	40.1	CH_2	2.61 (m), 2.74(m)	38.9	2.53 (dt), J=7.10, 17.96 Hz 2.68 (m), J=7.10, 17.96 Hz			
10	17.8	CH_2	1.55 (m)	17.6	1.55 (m)			
11	14.0	CH_3	0.88 (t), 7.42 Hz	13.9	0.86 (t), 7.31 Hz			
12	58.3	CH_3	4.05 (s)					

fore, the structure of compound **1** was established as 5-butanoyl-2-ethyl-5-hydroxy-3-methoxycyclopent-2-en-1-one.

Compound **2** (thailanone B) was obtained from the supernatant as a white solid. From the HR mass spectrum, its molecular formula was deduced as $C_{11}H_{16}O_4$ with four degrees of unsaturation. Analysis of the ^{1}H NMR and ^{13}C NMR spectra of 2 suggested a closely related structure to that of 1 with the difference being the absence of the methoxy group at C-3. Further, the HMBC and COSY correlations observed were similar to those observed for 1. Hence, the structure was elucidated as (5S)-5-butanoyl-2-ethyl-3,5-dihidroxycyclopent-2-en-1-one.

The white solid compound **3** (thailanone C) with the molecular formula $C_8H_{10}O_4$ and 4 degrees of unsaturation deduced from HR mass spectrum was isolated from the supernatant. The 1D and 2D NMR data of **3** suggested that the molecule has a closely related structure to **1** with one of the side chains missing. Analysis of the ^{1}H NMR spectrum indicated the presence of a triplet at δ 0.78 (H-7) and a singlet at δ 3.84 (H-8) for methyl and methoxy groups, respectively. A COSY correlation was observed between H-6/H-7. Further, H-7 exhibited HMBC correlations to C-3/C-6, while H-6 was correlating to C-2/C3/C-4/C-7 in the HMBC spectra. H-5 on the other hand showed HMBC correlations to C-1/C-2/C-3/C-4. The epoxide ring was assigned based on the chemical shifts of C-1 (δ 86.3) and C-5 (δ 58.3) and also the established molecular formula. The methoxy group showed HMBC a correlation to C-4 (δ 170.1). The structure of **3** was established as 3-ethyl-1-hydroxy-4-methoxy-6-oxabicyclo[3.1.0]hex-3-en-2-one.

Compound **4** (thailanone D) was isolated as white solid. The molecular formula $C_{13}H_{18}O_5$ was deduced from the HRMS data. ^{13}C and DEPT NMR data indicated

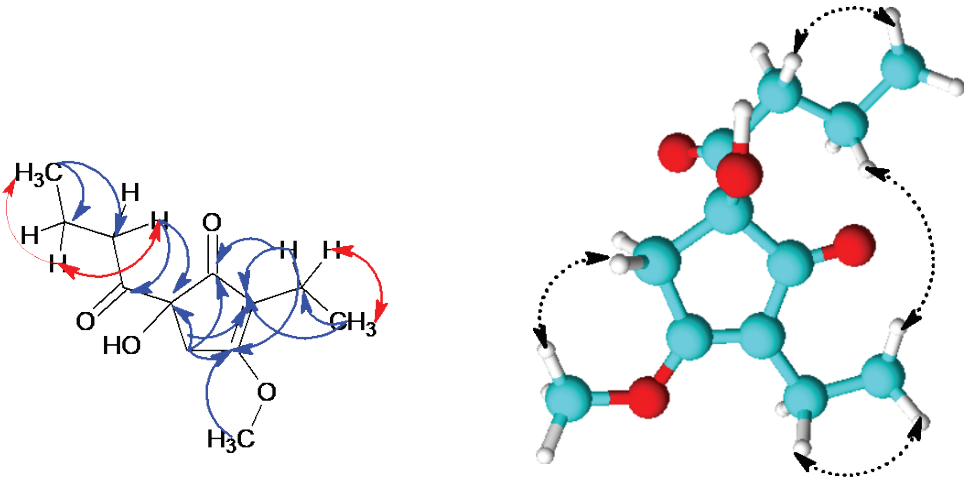

Figure 4. COSY, HMBC and ROESY correlations of **1.**

the presence of two methyl groups, a methoxy group, three methylene groups and five quaternary carbons in the molecule (Table 3). The ^{1}H NMR spectra revealed 2 methyl triplets at δ 0.79 (C-12) and δ 0.96 (C-10). In addition, a methoxy singlet was observed at δ 3.94 (C-13). Networks of COSY correlations were observed between H-9 and H-8/H-10 and H-11 to H-12. In the HMBC spectra, H-4 showed correlations to C-2/C-3/C-5/C-6. Proton H-8 was correlating to C-2/C-7/C-9 /C-10, while the methyl protons H-10 were correlating to C-8/C-9 in the HMBC spectra. Further, H-11 showed HMBC correlations to C-1/C-5/C-6/C-12. The hydroxy group singlet at δ 4.44 showed HMBC correlations to C-1/C-5/C-6/C-11. Cross peaks in the ROESY spectra between the methoxy proton H-13 (δ 3.94) and the aromatic proton H-4 (δ 5.56) were observed. No ROESY correlations were observed between the hydroxy group proton (δ 4.44) and H-11/H-12/H-13. Hence the relative stereochemistry at C-6 can be assigned as S. The structure of compound **4** was elucidated as (6S)-2-butanoyl-6-ethyl-3,6-dihidroxy-5-methoxycyclohexa-2,4-dien-1-one.

The white solid compound **5** (thailanone E) showed the molecular formula $C_{12}H_{16}O_4$ as deduced from HRMS data. The 1D and 2D NMR data of 5 suggested a closely related structure to 4 with the difference being in the ring substitution: The C-7 to C-10 chain and the carbon resonating δ 79.1 in 4 were missing. Analysis of the COSY spectra revealed correlations of H-8 to H-9 and H-11 to H-10/H-12. HMBC correlations of H-5 to C-1/C-3/C-4/C-10, H-8 to C-2/C-3/C-4/C-9, H-10 to C-1/C-5/C-6/C-11/C-12 and H-12 to C-10/C-11 were observed. Hence, the structure of the compound 5 was elucidated as 3-ethyl-2,4-dihydroxy-6-propylbenzoic acid.

Compound **6** (thailanone F) was obtained from the supernatant as a white solid with the molecular formula $C_{11}H_{18}O_4$ established from HRMS data. Analysis of the ^{1}H NMR data revealed a methyl group triplet at δ 0.91 (H-8), a methyl group singlet at δ 2.19 (H-10) and a methoxy singlet at 3.87 (H-11). HMBC correlations of H-2

Table 3. NMR spectroscopic data for compounds **4**–**6** in D_6-acetone (^{1}H NMR at 700 MHz; ^{13}C at 500 MHz).

		4		5			6		
No.	**^{13}C**	**DEPT**	**^{1}H/HSQC**	**^{13}C**	**DEPT**	**^{1}H/HSQC**	**^{13}C**	**DEPT**	**^{1}H/HSQC**
1	196.36	C	–	104.2	C		164.3	C	
2	106.4	C	–	164.7	C		88.7	CH	5.42(s)
3	190.6	C	–	116.1	C		171.9	C	
4	96.0	CH	5.56 (s)	160.7	C		112.3	C	
5	177.9	C	–	111.1	CH	6.36(s)	25.0	CH_2	2.35 (t), J=7.74 Hz
6	79.1	C	–	146.2	C		32.8	CH_2	1.40 (p), J=7.31Hz
7	203.2	C	–	174.7	C		23.6	CH_2	1.34 (m),
8	41.5	CH_2	2.92 (m)	16.8	CH_2	2.64 (q), J=7.53 Hz	14.7	CH_3	0.91 (t), J=7.31 Hz
9	19.34	CH_2	1.65 (m)	13.7	CH_3	1.08 (t), J=7.53 Hz	159.3	C	
10	14.25	CH_3	0.96 (t), J=7.31Hz	39.3	CH_2	2.86 (t), 7.31Hz	17.7	CH_3	2.19 (s)
11	36.12	CH_2	1.79 (dq), J=7.53, 13.34 1.92 (dq), J=7.53, 13.34	26.0	CH_2	1.59 (sext), J=7.31 Hz			
12	8.2	CH_3	0.79 (t), J=7.53	14.6	CH_3	0.93 (t), J=7.31			
13	57.7	CH_3	3.94 (s)						
		OH	4.44 (s)						

to C-1/C-3/C-4 and H-10 to C-4/C-9 were recorded. Furthermore, HMBC correlations between H-5 and C-3/C-4/C-6/C-7/C-9, H-6 and C-4/C-5/C-7/C-8, H-7 and C-5/C-6/C-8 and H-8 and C-6/C-7 were observed. These correlations were further supported by the COSY correlations of H-6 to H-5/H-7 and H-7 to H-6/H-8. Cross peaks between H-2 (5.42) and methoxy protons H-11 (δ 3.87) were not observed in the ROESY spectra, indicating that the olefinic bond at position two had E configuration. The olefinic bond between C-4 (δ 112.3) and C-9 (δ 159.3) was assigned E configuration, since H-5 (δ 2.35) and H-10 (δ 2.19) also did not correlate in the ROESY spectra. The structure of the compound 6 was established as (2Z, 4E)-4-(1-hydroxyethylidene)-3-methoxyoct-2-enoic acid.

Monocerin (**7**) and deoxyphomalone (**8**) were identified by comparing their NMR and HRMS data with those reported in literature (Aldridge et al. 1970, Ayer and Jimenez 1994, respectively). Monocerin was reported before as a potent herbicide and insecticide against Johnson grass and woolly aphids, respectively (Grove et al. 1979, Robeson et al. 1982), while deoxyphomalone has been reported from other pleosporalean fungi like *Alternaria*. To the best of the authors' knowledge, it has not been reported previously from a species of the Sulcatisporaceae.

Biological activity

The results of the biological assays that were performed to detect antibacterial, antifungal and nematicidal activities are summarised in Table 4. Compound **6** was

Table 4. Biological activities of compounds **1**–**8**.

Compounds	Antimicrobial activity MIC (μg/mL)		Nematicidal activity LD_{90} (μg/mL)	Antifungal activity (% growth inhibition at ≤ 12.5 μg/mL)
	Bacillus subtillis **DSM10**	***Mucor plumbeus*** **MUCL 49355**	***Caenorhabditis elegans***	***Phellinus tremulae*** **CBS 123.40**
Thailanone A (**1**)	≤ 50	–	≤ 50	50
Thailanone B (**2**)	–	–	≤ 25	28.6
Thailanone C (**3**)	–	–	≤ 25	31.4
Thailanone D (**4**)	–	≤ 25	≤ 12.5	28.6
Thailanone E (**5**)	–	–	≤ 50	25.
Thailanone F (**6**)	–	–	≤ 25	41.4
Monocerin (**7**)	–	–	–	28.6
Deoxyphomalone (**8**)	≤ 12.5	≤ 25	≤ 12.5	50
Standards				
Nystatin [#]	–	≤ 0.782	–	100
Ciprofloxacin [††]	≤ 0.78	–	–	–
Ivermectin [‡‡]	–	–	≤ 12.5	–
Methanol	–	–	–	–

No activity against *Candida tenuis, Setaria italica* and *Lepidum sativum* was observed for any of tested compounds up to concentrations of 100 μg/mL. [#] Nystatin-antifungal reference; [††] Ciprofloxacin-antibacterial reference; [‡‡] Ivermectin-nematicidal reference

moderately active against *M. plumbeus* with MIC of 25 μg/ml, while deoxyphomalone (**8**) exhibited moderate activities against *B. subtilis* and *M. plumbeus* with MIC values of 12.5 and 25 μg/ml, respectively. Compounds **1**–**6** and **8** were also the only compounds with significant activities against *M. plumbeus.* The results by Ayer and Jimenez (1994), regarding the antifungal activity of phomalone and its deoxy derivative, were also repeated in the serial dilution assay. Compounds **2**–**7** failed to significantly inhibit growth of *Ph. tremulae*, whereas the new phomalone derivative **1** showed moderate inhibition and was more weakly active than the known compound **8**. No phytotoxic effects in plant germination and seedling growth bioassay with *S. italica* and *L. sativum* at 100 μg/paper disk were observed for any of tested compounds.

Compound **7** was reported as a potent herbicide and insecticide against Johnson grass and woolly aphids, respectively (Grove et al. 1979, Robeson et al. 1982). It was first isolated from a fungus described as *Phoma etheridgei* (Hutchison et al. 1994) and recently, **7** was also isolated from *Alternaria tenuissima* (Pleosporaceae) and the bioactivity was tested against *E. coli* (Anyanwu and Sorensen 2015). It has previously been reported to be active against the pathogenic basidiomycete *Phellinus tremulae*, which infects different species of poplar (Ayer and Jimenez 1994). This fungus causes extensive damage to hardwoods in North America and, in Canada, *Ph. tremulae* seriously reduces the economic value of *Populus tremuloides* (Trifonov et al. 1992). This prompted the authors to re-evaluate the compound

in an antifungal assay against *Ph. tremulae*, in which all purified metabolites from *P. thailandica* were tested.

Moreover, the phytotoxic activity of terrein and congeners on plant growth and induction of lesions on fruit surfaces were previously investigated by Zaehle et al. (2014). Terrein, a major metabolite of *Aspergillus terreus,* resembles **1** and **7** in its chemical structure. The authors therefore performed a phytotoxicity assay, but they did not find significant effects of these compounds on germination and shoot/root elongation.

Conclusion

In the course of this investigation of the fungal specimens collected in the rainforest of Thailand, several nematode-antagonistic strains were detected. The use of nematodes as test organisms can detect bioactivity from the compounds that are not detected by whole-cell-based screening for antimicrobial activities. As an outcome of the antihelmintic screening, six new compounds (thailanones 1–6) and two known compounds, deoxyphomalone (7) and monocerin (8) were isolated and further evaluated regarding their antifungal activity. Even though these results are just preliminary and the biological activities of the new compounds are rather moderate, they are very likely to play an important chemo-ecological role in the natural habitat of the fungal producer organisms, e.g. to protect against nematode predation. The authors have not yet tried to detect the metabolites on water agar in the presence of nematodes because of the experimental limitations that would first need to be overcome. The moderate activity of the new compounds (as compared to, for example, the standard ivermectin, which is at least ten times more active) probably precludes their adoption as a nematicidal agent that could serve as a candidate for an antihelmintic drug or an agrochemical nematicide. On the other hand, the fungus might turn out to be a candidate for a biocontrol agent to act as an antagonist of pathogenic nematodes and fungi.

Acknowledgments

This project has received funding from the European Union's Horizon 2020 research and innovation programme (RISE) under the Marie Skłodowska-Curie grant agreement No 645701, Project acronym "GoMyTri" (lead beneficiaries JJL, PWC and MS). Financial support by the German Academic Exchange Service (DAAD) and the Kenya National Council for Science and Technology (NACOSTI), who provided a personal PhD stipend for CC is gratefully acknowledged. We are grateful to Cäcilia Bergmann, Vanessa Stiller and Anke Skiba for expert technical assistance and to Christel Kakoschke for recording NMR spectra.

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MycoKeys 33: 25–67 (2018)
doi: 10.3897/mycokeys.33.23670
http://mycokeys.pensoft.net

RESEARCH ARTICLE

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Identification of endophytic fungi from leaves of Pandanaceae based on their morphotypes and DNA sequence data from southern Thailand

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Academic editor: *T. Lumbsch* | Received 16 January 2018 | Accepted 16 March 2018 | Published 28 March 2018

Citation: Tibpromma S, Hyde KD, Bhat JD, Mortimer PE, Xu J, Promputtha I, Doilom M, Jun-Bo Yang, Tang AMC, Karunarathna SC (2018) Identification of endophytic fungi from leaves of Pandanaceae based on their morphotypes and DNA sequence data from southern Thailand. MycoKeys 33: 25–67. https://doi.org/10.3897/mycokeys.33.23670

Abstract

The authors established the taxonomic status of endophytic fungi associated with leaves of Pandanaceae collected from southern Thailand. Morphotypes were initially identified based on their characteristics in culture and species level identification was done based on both morphological characteristics and phylogenetic analyses of DNA sequence data. Twenty-two isolates from healthy leaves were categorised into eight morphotypes. Appropriate universal primers were used to amplify specific gene regions and phylogenetic analyses were performed to identify these endophytes and established relationships with extant fungi. The authors identified both ascomycete and basidiomycete species, including one new genus, seven new species and nine known species. Morphological descriptions, colour plates and phylogenies are given for each taxon.

Keywords
Ascomycetes, Basidiomycota, biodiversity, phylogenetic analysis

Introduction

Endophytic fungi are beneficial to their host plants and have the ability to produce bioactive compounds that have applied uses (Fisher et al. 1994; Strobel et al. 2004; Gunatilaka 2006; Arnold et al. 2007; Saikkonen et al. 2010; Aly et al. 2010; Lin et al. 2010; Rajulu et al. 2011; Chowdhary et al. 2015). Research on endophytic fungi began approximately 30 years ago and has intensified over the past 20 years (Thomson et al. 1997; Arnold et al. 2000; Stone et al. 2000; Hyde and Soytong 2008; Lumyong et al. 2009). This rising interest in endophytic fungi dates back to Bills' 1996 novel concept that mycelia sterilia isolates could be assigned to groups based on their degree of similarity in colony surface texture (Rodrigues 1994; Fisher et al. 1995; Lodge et al. 1996; Brown et al. 1998; Taylor et al. 1999; Umali et al. 1999; Fróhlich et al. 2000). Lacap et al. (2003) used molecular data to demonstrate the reliability of Bill's 1996 concept based on the cultural approach. Guo et al. (2000, 2003) found that morphological characteristics were insufficient to identify most endophyte isolates, especially when they do not sporulate and so DNA sequence data were used for identification of these taxa. Although this has been followed by numerous authors using ITS sequence data analysis, the use of ITS alone is not accurate (Promputtha et al. 2005). Subsequent studies have shown that multi-gene analyses are needed to identify endophytes (Ko et al. 2011).

Endophytic fungal strains have been isolated from many different plants including trees, vegetables, fruits, cereal grains and other crops (Rosenblueth and Martinez-Romero 2006). Dickinson (1976) published the first study of endophyte - leaf associations. However, there has been less research on the endophytic fungi associated with the leaves of tropical plants (Promputtha et al. 2007). The high species diversity of endophytic fungi makes their study a pressing research area. Globally, endophytic fungi were estimated to comprise 7 % of the 1.5 million species of fungi (Hawksworth 2001; Chowdhary et al. 2015). The actual numbers may be far higher. Recently, Hawksworth and Lucking (2017) estimated that there are 2.2 to 3.8 million fungal taxa. Endophytes are expected to be numerous because their host-specificity will drive diversification and they can occupy several niches, including that of pathogens and saprobes (Zhou and Hyde 2001). Several studies have investigated the relationships between endophytes and saprotrophs and also between endophytes and pathogens (Petrini 1991; Yanna and Hyde 2002; Ghimire and Hyde 2004; Photita et al. 2004; Hyde et al. 2006).

The authors have been investigating saprobic and endophytic fungi associated with Pandanaceae (Tibpromma et al. 2016a, b, c, 2017a, b) and, in this study, taxonomic details are presented regarding the endophytic fungi that were isolated. Pandanaceae are monocotyledonous plants. Their associated endophytic fungi were first studied by McKenzie et al. (2002), with further research conducted by Thongkantha et al. (2008), Bungihan et al. (2011), Ariffin (2013), Bungihan et al. (2013) and Eskandarighadikolaii et al. (2015).

The objectives of the present study were to establish the endophytic fungal community on selected Pandanaceae collected in southern Thailand. The authors isolated 22 endophytic isolates and sorted them in morphotypes and identified the taxa based on DNA sequence analyses. Both ascomycete and basidiomycete genera were identified, including one new genus, seven new species and nine known species. The recommendations of Jeewon and Hyde (2016) were followed when introducing the new species based on molecular data.

Materials and methods

Sample collection and fungal isolation

Healthy mature leaves of *Pandanus* and *Freycinetia* species (Pandanaceae, Figure 1) were collected from Chumphon (10°57'38.2"N 99°29'21.8"E) and Ranong (9°55'15.9"N, 98°38'30.7"E) provinces of southern Thailand during the rainy season (December) of 2016. Leaves with physical damage or showing signs of pathogenic infection were excluded from the study. In total, more than 100 healthy leaves were placed in Ziploc plastic bags, preserved with ice and transported to the laboratory. Leaves were randomly cut into 0.5 cm size pieces (10 pieces/leaf) using a hole puncher under aseptic conditions. These sections were soaked in 95 % ethanol for 1 minute, then in 3 % sodium hypochlorite solution for 3 minutes and finally in 95 % ethanol for 30 seconds. All samples were rinsed with sterile distilled water and dried on sterile tissue paper. Leaf sections were placed in Malt Extract Agar (MEA), Potato Dextrose Agar (PDA) and Water Agar (WA). They were incubated at room temperature (25-30 °C) for 1-3 days. If hyphal tips of any fungal colony appeared during incubation, the colony was transferred to new PDA plates and incubated to obtain pure cultures.

Cultures and identification

The above methods resulted in 22 isolates which were separated into morphotypes based on visual assessment of the similarity of the cultures (Bills 1996; Umali et al. 1999; Fróhlich et al. 2000; Lacap et al. 2003). All of these cultures were grown on Potato Dextrose Agar (PDA). Growth rate measurements are shown in Table 1 with colony colour defined with the Methuen Handbook of Colour (Kornerup and Wanscher 1967). New taxa were examined in pure culture, allowing photographs, records of morphological characteristics and descriptions to be recorded. Herbarium specimens were prepared from cultures that were dried in silica gel. The holotypes were deposited in the Mae Fah Luang University Herbarium (Herb. MFLU), Chiang Rai, Thailand and in the Kunming Institute of Botany Academia Sinica (HKAS), Kunming, China. The ex-types cultures were deposited in the Mae Fah Luang University Culture Collection (MFLUCC) with duplicates deposited in the BIOTEC Culture Collection

Figure 1. Habitats of the host plants: **a, b** *Pandanus* spp. **c, d** *Freycinetia* spp.

Laboratory (BCC) and the Kunming Institute of Botany Culture (KMUCC). New taxa were registered in Facesoffungi (FoF) (Jayasiri et al. 2015) and MycoBank (Crous et al. 2004).

DNA extraction, PCR amplification, and sequencing

Genomic DNA was extracted from pure fungal cultures using Biospin Fungal Genomic DNA extraction Kit–BSC14S1 (BioFlux, P.R. China). Polymerase chain reaction (PCR) was used to amplify partial gene regions of Internal Transcribed Spacers (ITS), 28S ribosomal RNA (LSU), 18S ribosomal RNA (SSU), RNA polymerase II second largest subunit (RPB2), β-tubulin (Tub2), Actin (ACT), Glyceraldehyde-3- Phosphate Dehydrogenase (GADPH), Chitin synthase 1 (CHS-1) and Translation Elongation Factor 1-alpha (TEF1) using primers as shown in Table 1. The total volume of PCR mixtures for amplifications were 25 µl containing 8.5 µl ddH_2O, 12.5 µl 2× Easy Taq PCR Super Mix (mixture of Easy Taq TM DNA Polymerase, dNTPs and optimised buffer (Beijing Trans Gen Biotech Co., Chaoyang District, Beijing, PR China), 2 µl of DNA template, 1 µl of each forward and reverse primers (10 pM). The quality of PCR

Table 1. Details of genes/loci with PCR primers and protocols.

Gene/Loci	PCR primers (Forward/Reverse)	References
LSU	LROR/LR5	Vilgalys and Hester 1990
ITS	ITS5/ITS4	White et al. 1990
SSU	NS1/NS4	White et al. 1990
TEF1	983F/2218	Rehner 2001
	728F/986R	Carbone and Kohn 1999
RPB2	fRPB2-5f/fRPB2–7cR	Liu et al. 1999
β-tubulin	BT2a/BT2b	Glass and Donaldson 1995
	T1/T2	O'Donnell and Cigelnik 1997
Actin	512F/783R	Carbone and Kohn 1999
CHS-1	79F/354R	Carbone and Kohn 1999
GADPH	Gpd1/Gpd2	Myllys et al. 2002
	GDF/GDR	Templeton et al. 1992

products was checked on 1 % agarose gel electrophoresis stained with 4S green nucleic acid (Life Science Products & Services, Cat. No: A616694). Purification and sequencing of PCR products were carried out by Sangon Biotech Co., Shanghai, China.

Phylogenetic analysis

The sequence data generated during this study were the subject of BLAST searches in the nucleotide database of GenBank (www http://blast.ncbi.nlm.nih.gov/) to determine their most probable closely related taxa. Sequence data were retrieved from GenBank based on recent publications. Raw forward and reverse sequences were assembled using Geneious Pro.v4.8.5. Sequence alignments were carried out with MAFFT v.6.864b (Katoh and Standley 2016) and alignments were manually improved where necessary. The sequence datasets were combined using BioEdit v.7.2.5 (Hall 2004). Maximum Likelihood (ML) and Bayesian Inference (BI) analyses were performed for the sequence dataset. The phylogenetic trees were configured in FigureTree v. 1.4 (Rambaut and Drummond 2008) and edited using Microsoft Office PowerPoint 2007 and Adobe Illustrator CS3 (Adobe Systems Inc., USA).

Results and discussion

Identification of morphotypes

Twenty-two fungal isolates from *Pandanus* and *Freycinetia* species were recovered and these mycelia sterilia were separated into eight morphotypes based on the similarity of their culture characteristics, as summarised in Table 2 (Bills 1996; Umali et al. 1999; Fróhlich et al. 2000; Lacap et al. 2003).

Table 2. Culture characteristics of the 22 strains (8 morphotypes) of mycelia sterilia on PDA.

Morpho-types	Isolate code	Host	Size (cm) of colony		Shape	Colour		Mycelium	Edge
			3 days	7 days		Above	Reverse		
1	PE05	*Pandanus* sp.	4.6	>A	Circular	4A1	4A2	Aerial	Undulate
	PE09	*Pandanus* sp.	4.6	>A	Circular	6D3	6B3	Aerial	Entire
	PE15	*Pandanus* sp.	>A	>A	Circular	5B2	5B3	Flat	Entire
2	PE10	*Pandanus* sp.	1.1	3.2	Irregular	4C1	4A3	Aerial	Undulate
	PE60	*Pandanus* sp.	1.6	3.8	Irregular	4B1	4A3	Aerial	Undulate
	FE46	*Freycinetia* sp.	2.1	5.6	Irregular	5B2	5A2	Aerial	Undulate
	FE42	*Freycinetia* sp.	1.5	5	Irregular	4A1	4A3	Aerial	Undulate
	FE43	*Freycinetia* sp.	1.4	4.2	Irregular	5D4	5C4	Flat	Undulate
	PE75	*Pandanus* sp.	1.6	5	Circular	6A1/6D3	6A1/6F5	Aerial	Undulate
	PE84	*Pandanus* sp.	1.5	3.8	Circular	5F4	5F7	Aerial	Curled
	FE98	*Freycinetia* sp.	1.3	3.1	Irregular	5B2	5D5	Flat	Filamentous
3	PE25	*Pandanus* sp.	>A	>A	Circular	5E1	5F2	Aerial	Entire
4	PE26	*Pandanus* sp.	3.1	7.2	Irregular	5B3	5B5	Aerial	Undulate
	PE52	*Pandanus* sp.	1.2	2.9	Circular	5A2	5A3	Aerial	Undulate
5	PE35	*Pandanus* sp.	1.1	2.7	Filamentous	8E2	8F2	Aerial	Filamentous
6	PE92	*Pandanus* sp.	5.1	>A	Irregular	4B1	4A6	Aerial	Curled
	PE37	*Pandanus* sp.	2.3	7.9	Circular	4A1	4B3	Aerial	Curled
	FE88	*Freycinetia* sp.	2.9	6.2	Circular	5D3	5B2	Flat	Undulate
	PE77	*Pandanus* sp.	4.2	7.1	Irregular	6B1/6E1	6B2	Aerial	Undulate
	FE41	*Freycinetia* sp.	>A	>A	Irregular	4D2	4F6	Flat	Filamentous
7	PE58	*Pandanus* sp.	<B	1.7	Circular	4F2	4F8	Aerial	Entire
8	FE101	*Freycinetia* sp.	<B	2	Circular	4B2	4A3	Aerial	Entire

Notes: >A Completely covering plate, <B Less than 1 cm

Phylogenetic analysis

Based on phylogenetic analysis, 22 fungal isolates were identified for 16 species. These include one new genus, seven new species and nine known species. All sequences obtained from this study are summarised in Table 3.

Basidiomycota R.T. Moore
Agaricomycetes Doweld
Polyporales Gäum., 1926

Polyporaceae Fr. ex Corda

Remarks. The family *Polyporaceae* was introduced by Fr. ex Corda (1839) and includes 92 genera and 636 species (Kirk et al. 2008). According to Cannon and Kirk (2007), the species in this family are characterised by poroid, irregular or lamellate hymenophores and are saprobes. Recent phylogenetic analyses of *Polyporaceae* are by Binder

Table 3. Species of endophytes obtained in this study.

No.	Original code	Species name	Culture collection no.
1	PE26	*Alternaria burnsii*	MFLUCC 17-0582
2	PE58	*Cladosporium endophyticum*	MFLUCC 17-0599
3	PE09	*Colletotrichum pandanicola*	MFLUCC 17-0571
4	FE88	*Colletotrichum fructicola*	MFLUCC 17-0555
	PE84		MFLUCC 17-0613
5	PE77	*Diaporthe pandanicola*	MFLUCC 17-0607
6	PE37	*Diaporthe siamensis*	MFLUCC 17-0591
7	FE41	*Endomelanconiopsis freycinetiae*	MFLUCC 17-0547
8	FE42	*Endopandanicola thailandica*	MFLUCC 17-0548
	FE43		MFLUCC 17-0549
	FE46		MFLUCC 17-0551
	PE10		MFLUCC 17-0572
	PE60		MFLUCC 17-0600
9	PE25	*Lasiodiplodia theobromae*	MFLUCC 17-0581
10	PE52	*Massarina pandanicola*	MFLUCC 17-0596
11	FE98	*Meyerozyma caribbica*	MFLUCC 17-0556
	PE75		MFLUCC 17-0606
12	FE101	*Mycoleptodiscus endophytica*	MFLUCC 17-0545
13	PE05	*Pestalotiopsis jiangxiensis*	MFLUCC 17-0567
14	PE92	*Pestalotiopsis microspora*	MFLUCC 17-0619
15	PE15	*Phanerochaete chrysosporium*	MFLUCC 17-0575
16	PE35	*Phyllosticta capitalensis*	MFLUCC 17-0589

et al. (2013) and Hyde et al. (2017). In this study, a new endophytic genus, *Endopandanicola* with *En. thailandica* as the type species was discovered. In addition to the new genus, *Phanerochaete chrysosporium* was also identified.

Endopandanicola **Tibpromma & K.D. Hyde, gen. nov.**
MycoBank number: MB823835
Facesoffungi number: FoF03900

Etymology. Named after its habitat as an endophyte of *Pandanus.*

Type species. *Endopandanicola thailandica* Tibpromma & K.D. Hyde

Culture characteristics. Colonies on PDA (PE60), superficial, initially white, later becoming yellow-white, smooth at the surface, irregular, with undulate margin, flossy to velvety; reverse white to yellow-white. Generative hyphae simple-septate, branched, sub-hyaline, thin-walled.

Notes. *Endopandanicola* formed a single, well-supported clade (100 % in ML, 100 % in MP), which is distinct as compared to other genera in *Polyporaceae* (Figure 3). This genus comprises resupinate or crust polypores that live inside leaves or wood as endophytes and do not form fruiting bodies (sexual morph), but form flat mycelia. More collections of *Pandanus* are needed in the future to locate the sexual morph of *Endopandanicola*.

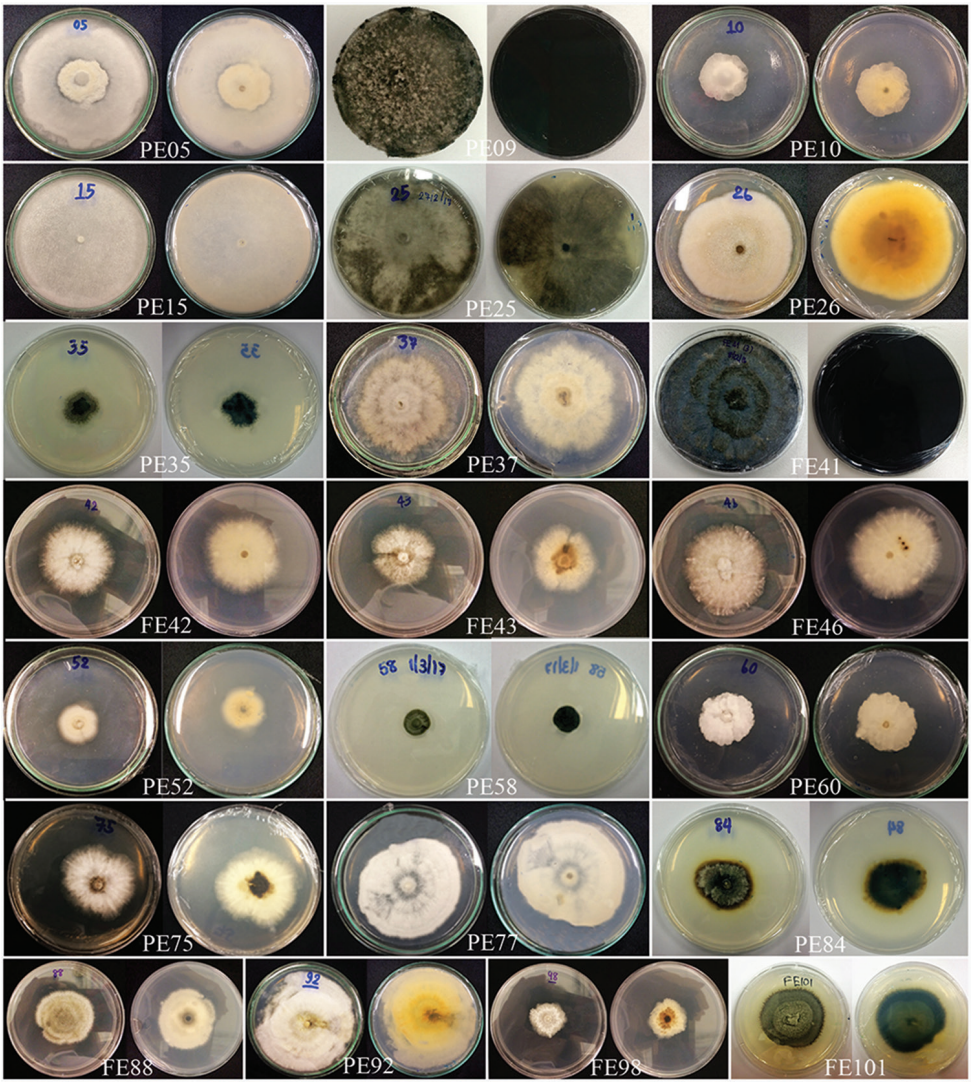

Figure 2. All cultures from this study are grown on PDA at room temperature after 7 days (original codes are written at the bottom of each picture).

Endopandanicola thailandica **Tibpromma & K.D. Hyde, sp. nov.**
MycoBank number: MB823836
Facesoffungi number: FoF03901
Figure 4

Etymology. named after Thailand, the country where the fungus was first discovered.

Holotype. MFLU 18-0021

Culture characteristics. Colonies on PDA (Figure 2 PE10, FE42, FE43, FE46 and PE60), superficial, initially white, later becoming yellow-white, irregular, with un-

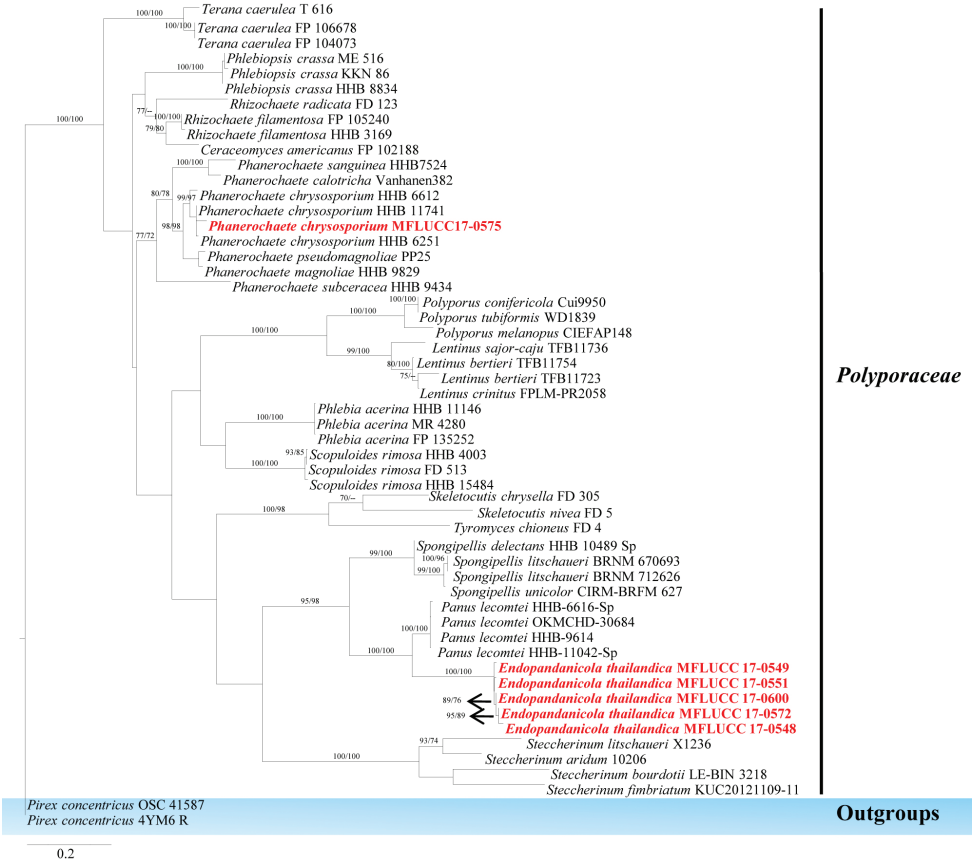

Figure 3. Phylogram generated from maximum likelihood analysis based on ITS sequence data. Maximum parsimony (left) and maximum likelihood (right) bootstrap support values are given above/below the nodes. The newly generated sequences are in red text. The tree is rooted with *Pirex concentricus.*

dulate margin, smooth with flossy to velvety; reverse white to yellow-white. Generative hyphae simple-septate, branched, with clamp connections, sub-hyaline, thin-walled, 1.5–3.5 µm wide.

Material examined. THAILAND, Chumphon, Pathio District, on healthy leaves of *Pandanus* sp. (Pandanaceae), 1 December 2016, S. Tibpromma PE60 (MFLU 18-0021, **holotype**); HKAS100856, paratype, ex-type living cultures, MFLUCC 17-0600 = KUMCC 17-0295; Chumphon, Pathio District, 1 December 2016, S. Tibpromma PE10, living culture, MFLUCC 17-0572; Ranong, Muang, Muang District, 3 December 2016, S. Tibpromma FE42, living culture, MFLUCC 17-0548; FE43, living culture, MFLUCC 17-0549 = KUMCC 17-0264; FE46, living culture, MFLUCC 17-0551 = KUMCC 17-0265.

GenBank numbers. ITS; MFLUCC 17-0545=MG646961, MFLUCC 17-0548=MG646964,MFLUCC17-0549=MG646963,MFLUCC17-0551=MG646962, MFLUCC 17-0572=MG646959, MFLUCC 17-0600=MG646960.

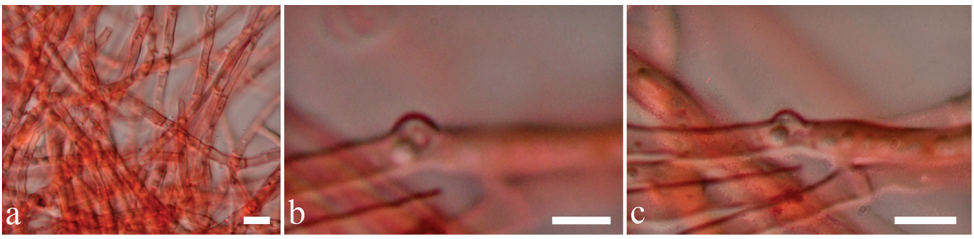

Figure 4. *Endopandanicola thailandica* (MFLU 18-0021, holotype). **a** Mycelia masses **b, c** Clamp connections. Scale bars: 10 μm (**a**), 5 μm (**b, c**).

Notes. *Endopandanicola* is introduced and typified by *En. thailandica* which is represented by six isolates and is described as a novel species based on its asexual morph. The phylogenetic analysis of ITS sequence data showed that this species clustered together with *Panus,* but there is a high level of statistical support for its separation (100% in ML, 100% in MP) (Figure 3).

Phanerochaete chrysosporium Burds., in Burdsall & Eslyn, Mycotaxon 1(2): 124 (1974)

Culture characteristics. Colonies on PDA (Figure 2, PE15), superficial, white, surface smooth with flat media surface, circular, with entire edge; reverse white to yellow-white.

GenBank numbers. ITS=MG646957.

Notes. Burdsall and Eslyn (1974) introduced *Phanerochaete chrysosporium* which was collected on dead wood of *Platanus wrightii* in the USA. Phylogenetic analysis of ITS sequence data shows this taxon groups with *Phanerochaete chrysosporium* (sequences obtained from GenBank) that had been collected from different hosts. The phylogenetic placement of this species is shown in Figure 3.

Ascomycota Whittaker
Dothideomycetes O.E. Erikss. & Winka

Botryosphaeriales C.L. Schoch, Crous & Shoemaker

Remarks. The order *Botryosphaeriales* was introduced by Schoch et al. (2006) with *Botryosphaeriaceae* as the type family. *Botryosphaeriales* is a diverse order with a worldwide distribution, comprising species that vary from endophytes to pathogens (Slippers and Wingfield 2007; Phillips et al. 2013; Chethana et al. 2016; Daranagama et al. 2016; Dissanayake et al. 2016; Konta et al. 2016a, b; Linaldeddu et al. 2016a, b, c; Manawasinghe et al. 2016; Zhang et al. 2017). Currently, nine families are recognised, namely, *Aplosporellaceae, Botryosphaeriaceae, Endomelanconiopsisaceae, Melanopsaceae, Phyllostictaceae, Planistromellaceae, Pseudofusicoccumaceae, Saccharataceae* and *Septorioideaceae*

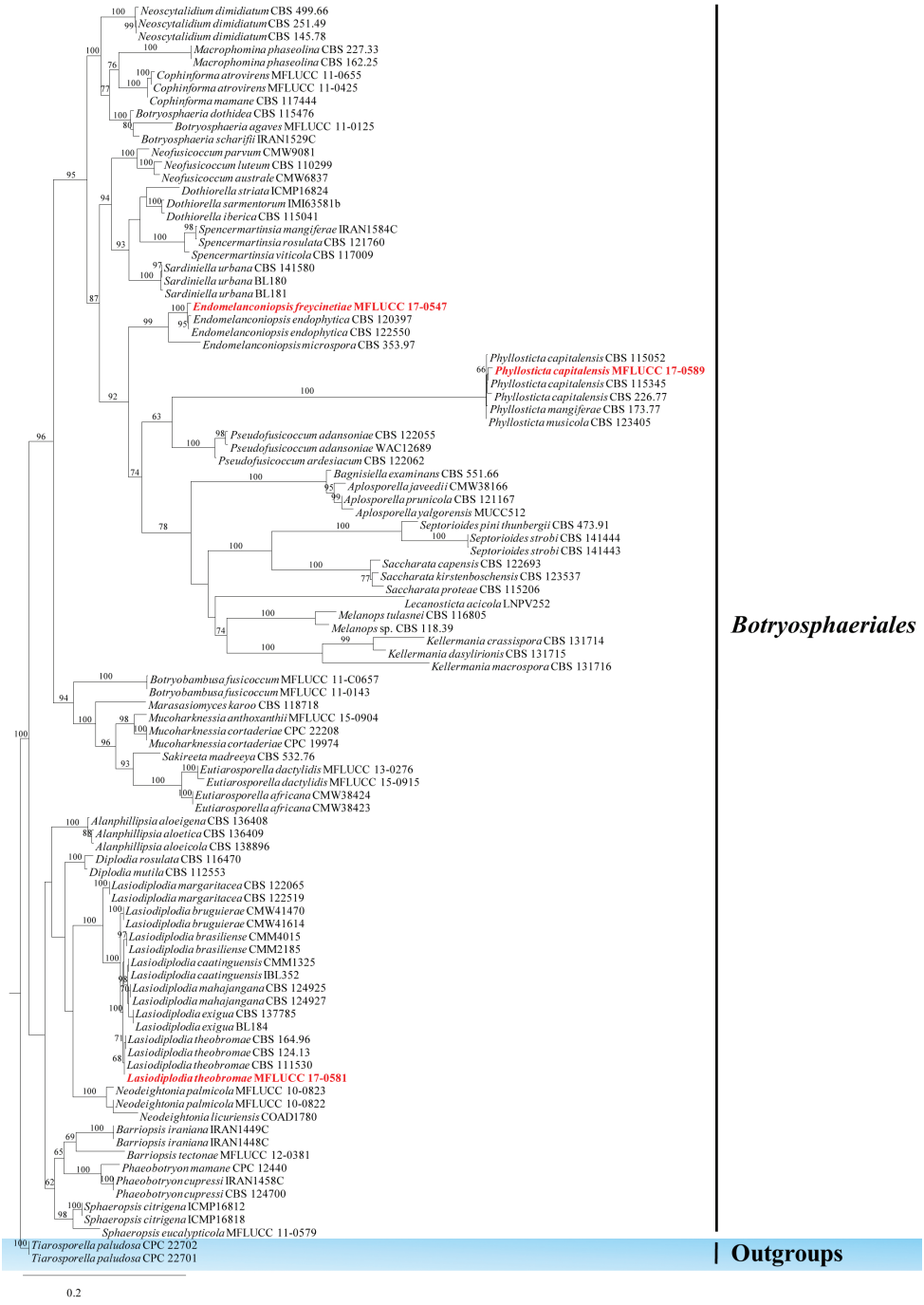

Figure 5. Phylogram generated from maximum likelihood analysis based on ITS, LSU and TEF1 sequenced data. Maximum likelihood bootstrap values are given above/below the nodes. The newly generated sequences are in red bold. The tree is rooted with *Tiarosporella paludosa.*

(Schoch et al. 2006; Minnis et al. 2012; Wikee et al. 2013; Slippers et al. 2013; Wyka and Broders 2016; Dissanayake et al. 2016; Yang et al. 2017). In this study, *Endomelanconiopsis freycinetiae* is introduced as a new species and reports are provided on *Phyllosticta capitalensis* and *Lasiodiplodia theobromae*.

Endomelanconiopsis freycinetiae **Tibpromma & K.D. Hyde, sp. nov.**
MycoBank number: MB823837
Facesoffungi number: FoF03902
Figure 6

Etymology. name referring to the host genus on which the fungus was found (*Freycinetia*).

Holotype. MFLU 18-0002

Culture characteristics. Colonies on PDA (Figure 2, FE41), superficial, initially white-grey with flat mycelium on media with dark centre, later becoming dark olivaceous with circular rings and flossy at the margin; reverse dark olivaceous. Generative hyphae simple-septate, branched, sub-hyaline to brown, cylindrical, guttulate, thick-walled. Not sporulating in culture (Figure 6).

Material examined. THAILAND, Ranong, Muang, on healthy leaves of *Freycinetia* sp. (Pandanaceae), 3 December 2016, S. Tibpromma FE41 (MFLU 18-0002, holotype); HKAS100853, paratype, ex-type living cultures, MFLUCC 17-0547 = KUMCC 17-0292.

GenBank numbers. ITS=MG646955, LSU=MG646948, TEF1=MG646983, β-tubulin=MG646924.

Notes. *Endomelanconiopsis freycinetiae* is closely related to the endophytic fungus *En. endophytica*. Therefore, the culture characteristics of these two taxa were compared and it was found that, in *En. endophytica*, at first the hyphae are colourless, immersed, later becoming olivaceous in the centre with irregular concentric rings; aerial mycelia are dark olivaceous or grey when dense; shiny black when the aerial mycelia are loose (Rojas et al. 2008) whereas aerial mycelia of *En. freycinetiae* has dark olivaceous, circular rings and flossy surface (Figure 2, FE41). Nucleotide base pairs of ITS and TEF1 were also compared and it was found that there are differences (ITS 3 bp, TEF1 8 bp).

Phyllosticta capitalensis **Henn., Hedwigia 48: 13 (1908)**

Culture characteristics. Colonies on PDA (Figure 2, PE35), superficial, dark olivaceous with filamentous hyphae and raised edge; reverse dark olivaceous. Sporulating in culture after 2 months.

GenBank numbers. ITS=MG646954, LSU=MG646953, TEF1=MG646982.

Notes. *Phyllosticta capitalensis* (Hennings 1908) is known as an endophytic taxon and a minor plant pathogen. It has a worldwide distribution and has been recorded

Figure 6. *Endomelanconiopsis freycinetiae* (MFLU 18-0002, holotype). **a–d** Mycelia masses. Scale bars: 20 μm (**a–c**), 10 μm (**d**).

on 70 plant families (Baayen et al. 2002; Okane et al. 2003; Motohashi et al. 2009; Wikee et al. 2013). The isolate recovered herein clusters with reasonable ML bootstrap support with other *P. capitalensis* isolates (Figure 5). Morphological examination also depicts similar morphs and hence it is identified as *P. capitalensis*.

***Lasiodiplodia theobromae* (Pat.) Griffon & Maubl., Bull. Soc. Mycol. Fr. 25: 57 (1909)**

Culture characteristics. Colonies on PDA (Figure 2, PE25), superficial, initially white with flat mycelium on media, later becoming dark, circular, flossy and velvety; reverse dark. Not sporulating in culture.

GenBank numbers. ITS=MG646970, LSU=MG646945, SSU=MG646976, TEF1=MG646984.

Notes. Morphological and phylogenetic data supported placement of this isolate as *Lasiodiplodia theobromae*. The phylogenetic analysis showed the isolate groups with *Lasiodiplodia theobromae*. Nucleotide base pairs of published sequences of *Lasiodiplodia theobromae* (strain EucN188, CBS 111530, PHLO9, CDFA145) were also compared with the sequence and found that the nucleotide base pairs of the ITS gene are 100% similar.

Capnodiales Woron., 1925
Cladosporiaceae Castell. & R.G. Archibald

***Cladosporium* Link, 1816**

Remarks. The genus *Cladosporium* (*Cladosporiaceae*, *Capnodiales*) is a large genus of the Ascomycota (Wijayawardene et al. 2017). The genus comprises species that are saprobes, endophytes and pathogens. A few species have been documented as being etiologic agents in vertebrate hosts (David 1997; Bensch et al. 2012, 2015; Crous et al. 2014). In this study, a new species of *Cladosporium* is described, with high bootstrap support in the phylogenetic analysis (Figure 7).

***Cladosporium endophyticum* Tibpromma & K.D. Hyde, sp. nov.**
MycoBank number: MB823838
Facesoffungi number: FoF03903
Figure 8

Etymology. named after its status as an endophytic fungus.

Holotype. MFLU 18-0005

Description. *Colonies* on PDA attaining 9 cm diam. in six weeks at room temperature, slow growing, dark olivaceous. *Mycelium* superficial and immersed composed of septate, branched, 2.3–4.5 µm wide, sub-hyaline, with smooth and thick-walled hyphae. **Sexual morph** Undetermined. **Asexual morph** *Conidiophores* 6–10 µm high, 3–4 µm diam. ($\bar{x}$ = 8.24 × 3.52 µm, n = 10), terminal and intercalary, cylindrical or sub-cylindrical, darkened conidiogenous loci. *Conidia* 3–6 × 2–4 µm ($\bar{x}$ = 3.64 × 2.75 µm, n = 30), forming long branched chains, hyaline to pale-olivaceous, smooth and thin-walled, aseptate, globose to ovoid with rounded ends.

Culture characteristics. Colonies on PDA (Figure 2, PE58), superficial, dark olivaceous with dark-grey centre, irregular, undulate with wrinkled and raised on surface media; reverse dark olivaceous. Generative hyphae simple-septate, branched, sub-hyaline, guttules, thick-walled (Figure 8).

Material examined. THAILAND, Chumphon, Pathio District, on healthy leaves of *Pandanus* sp. (Pandanaceae), 1 December 2016, S. Tibpromma PE58 (MFLU 18-0005, **holotype**); HKAS100855, paratype, ex-type living cultures, MFLUCC 17-0599 = KUMCC 17-0294.

GenBank numbers. ITS=MG646956, LSU=MG646949, SSU=MG646981, TEF1=MG646988.

Notes. *Cladosporium endophyticum* was isolated as an endophyte from *Pandanus* sp. in Thailand. In the phylogenetic analysis of combined gene sequence data of ITS, LSU, SSU and TEF1, the new taxon *Cladosporium endophyticum* is sister to *C. halotolerans* (Figure 7), but well-separated with high bootstrap support (90% in ML). Moreover, the morphology of this new taxon was compared with *Cladosporium halotolerans* which has brown to dark

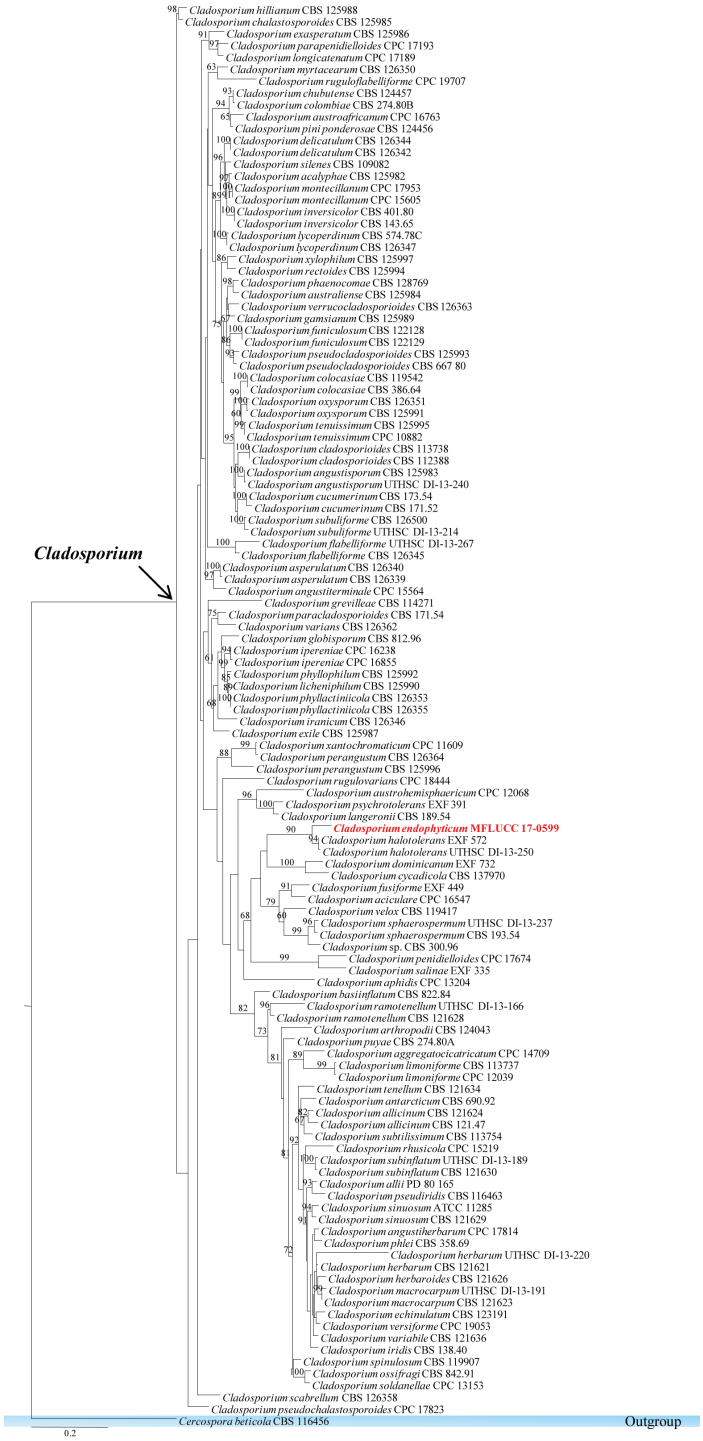

Figure 7. Phylogram generated from maximum likelihood analysis based on ITS, TEF1 and Actin sequenced data. Maximum likelihood bootstrap is given above/below the nodes. The newly generated sequences in red bold. The tree is rooted with *Cercospora beticola.*

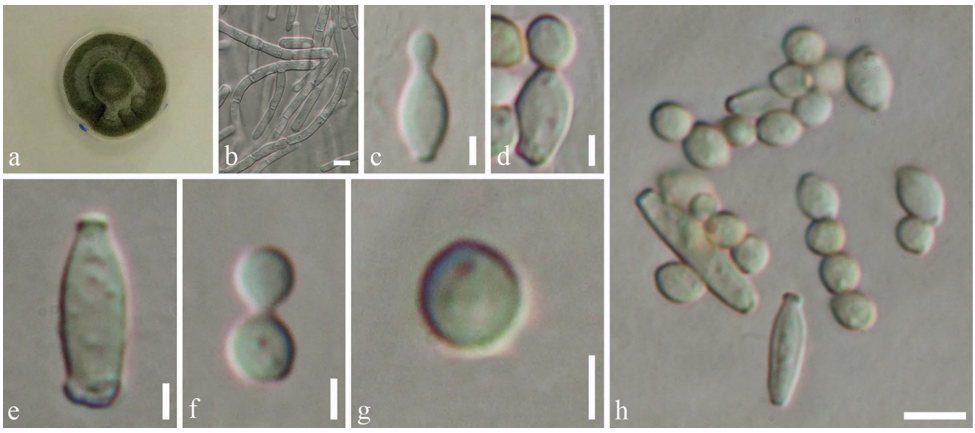

Figure 8. *Cladosporium endophyticum* (MFLU 18-0005, holotype). **a** Colony on MEA media **b** Mycelium masses **c–e** Conidia and conidiogenous cells **f, g** Conidia **h** Conidia and conidiogenous cells. Scale bars: 5 μm (**b–h**), 10 μm (**h**).

brown, subglobose to globose with verrucose, less often short-ovoid conidia, narrower at both ends (Zalar et al. 2007), while *C. endophyticum* has globose to ovoid, hyaline to pale-olivaceous conidia with rounded ends. Here, the authors introduce the new species *C. endophyticum* and provide an updated phylogenetic tree for the genus *Cladosporium*.

Pleosporales Luttr. ex M.E. Barr, 1987

Massarinaceae Munk.

Remarks. The family *Massarinaceae* was introduced by Munk (1956) under *Pleosporales* together with *Cucurbitariaceae* and *Didymosphaeriaceae*. Later, Barr (1987) segregated *Massarinaceae* under *Lophiostomataceae* based on morphology, while based on multigene phylogenetic analysis Schoch et al. (2009) also showed *Massarinaceae* is a distinct family in order *Pleosporales*. Recently, Zhang et al. (2009, 2012) recognised *Massarinaceae* as a distinct lineage based on both morphology and molecular phylogeny. In this study, a new species of endophytic *Massarina,* based on morphological and phylogenetic support, is introduced from *Pandanus* sp. in Thailand.

Massarina pandanicola Tibpromma & K.D. Hyde, sp. nov.

MycoBank number: MB823839
Facesoffungi number: FoF03904
Figure 10

Etymology. name referring to the host genus of the plant on which the fungus was first discovered (*Pandanus*).

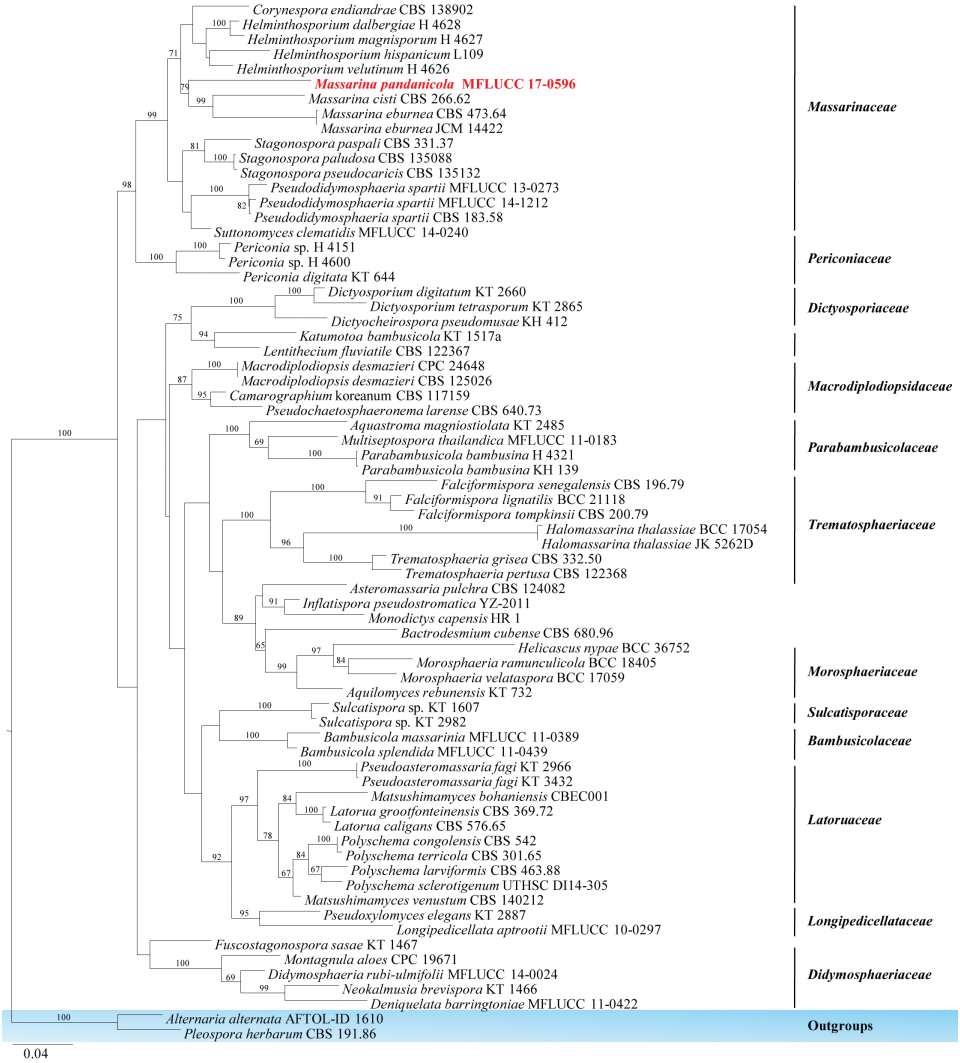

Figure 9. Phylogram generated from maximum likelihood analysis based on ITS, TEF1, SSU, LSU and RPB2 sequenced data. Maximum likelihood bootstrap values are given above/below the nodes. The newly generated sequences in red bold. The tree is rooted with *Alternaria alternata* and *Pleospora herbarum*.

Holotype. MFLU 18-0004

Description. *Colonies* on PDA attaining 9 cm diam. in 4 weeks at room temperature, slow growing, white to yellow-white. *Mycelium* superficial and immersed composed of septate, branched, 2.5–7 µm wide, sub-hyaline, with smooth and thick-walled hyphae. **Sexual morph** Undetermined. **Asexual morph** *Conidiophores* 12–25 µm high, 8–14 µm diam. ($\bar{x}$ = 15.12 × 10.45 µm, n = 10), enteroblastic, phialidic, cylindrical or sub-cylindrical, sub-hyaline. *Conidia* 3–5 × 1–3 µm ($\bar{x}$ = 4.34 × 1.75 µm, n = 30), cylindrical, hyaline, smooth and thin-walled, aseptate, rounded ends, guttulate, without sheet or appendages.

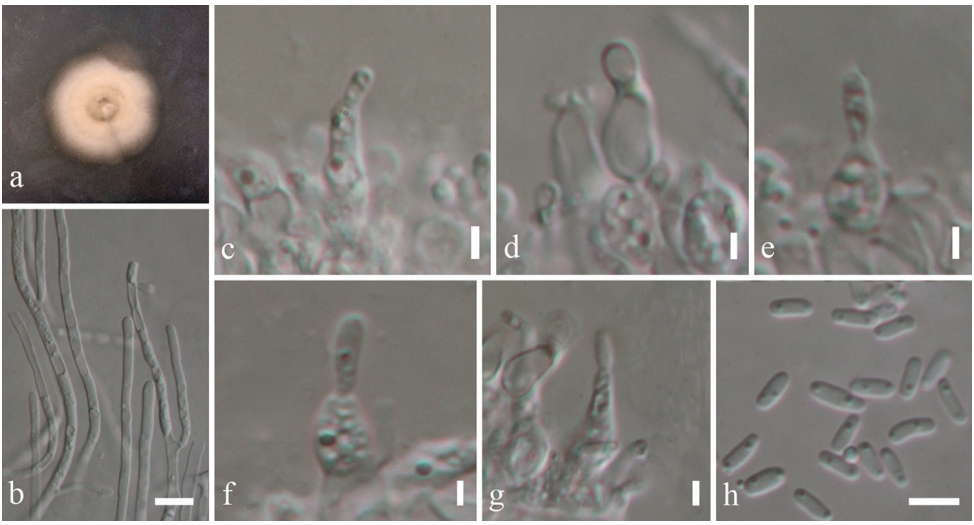

Figure 10. *Massarina pandanicola* (MFLU 18-0004, holotype). **a** Colony on MEA media **b** Mycelium masses **c–g** Conidia and conidiogenous cells **h** Conidia. Scale bars: 20 μm (**b**), 2 μm (**c–g**), 5 μm (**h**).

Culture characteristics. Colonies on PDA (Figure 2, PE52), superficial, white to yellow-white, irregular, undulate with smooth and raised on surface media; reverse yellow-white. Generative hyphae simple-septate, branched, sub-hyaline, with guttulate cells, thin-walled. Sporulating in culture within 3 months (Figure 10).

Material examined. THAILAND, Chumphon, Pathio District, on healthy leaves of *Pandanus* sp. (Pandanaceae), 1 December 2016, S. Tibpromma PE52 (MFLU 18-0004, **holotype**); HKAS100854, paratype, ex-type living cultures, MFLUCC 17-0596 = KUMCC 17-0293.

Genbank numbers. ITS=MG646958, LSU=MG646947, SSU=MG646979, TEF1=MG646986.

Notes. The genus *Massarina* has been known as a phylogenetically diverse group in the order Pleosporales based on molecular data (Liew et al. 2002) and most members in *Massarina* except for the type species (*M. eburnea*) are morphologically variable. The taxon, *Massarina pandanicola* collected from *Pandanus* sp. in Thailand is introduced here as a new species with both morphology and phylogeny support. The morphology of the taxon showed similar conidia with *Massarina eburnean* (Tanaka et al. 2015), but based on phylogenetic analysis of combined ITS, LSU, SSU and TEF1 gene sequence data, the new taxon *M. pandanicola* is well-separated from other species in *Massarina* (Figure 9) with high bootstrap support (79 % in ML). This is the first record of *Massarina* from *Pandanus* sp.

Pleosporaceae Nitschke

Remarks. The family *Pleosporaceae* was introduced by Nitschke (1869) and is the largest family of the order *Pleosporales* (Hyde et al. 2013; Ariyawansa et al. 2015b; Liu et al. 2017). Members of this family can be endophytes, aquatic or terrestrial saprobes, plant

pathogens or opportunistic animal pathogens (Sivanesan 1984; Carter and Boudreaux 2004). A backbone tree for *Pleosporaceae* was provided by Ariyawansa et al. (2015a). In this study, *Alternaria burnsii* is reported from a *Pandanus* sp. host in Thailand.

Alternaria burnsii Uppal, Patel & Kamat, Indian J. Agric. Sci. 8: 49 (1938)

Culture characteristics. Colonies on PDA (Figure 2, PE26), superficial, white-orange to cream, circular, entire edge, smooth, flossy, velvety and raised on surface media; reverse yellow-white at the margin and yellow-brown in centre. Not sporulating in culture.

GenBank numbers. ITS=MG646973, LSU=MG646952, TEF1=MG646987.

Notes. *Alternaria burnsii* was introduced by Uppal et al. (1938) from India on *Cuminum cyminum.* This species has a close phylogenetic relationship with *Alternaria tomato* and *A. jacinthicola* (Woudenberg et al. 2015). Results from phylogenetic analysis show that the authors' collection belongs to *Alternaria burnsii* with a relatively high bootstrap support (89% in ML) (Figure 11). Nucleotides across the ITS regions of *Alternaria burnsii* CBS 108.27 and the isolates were compared and the authors noted that they are identical.

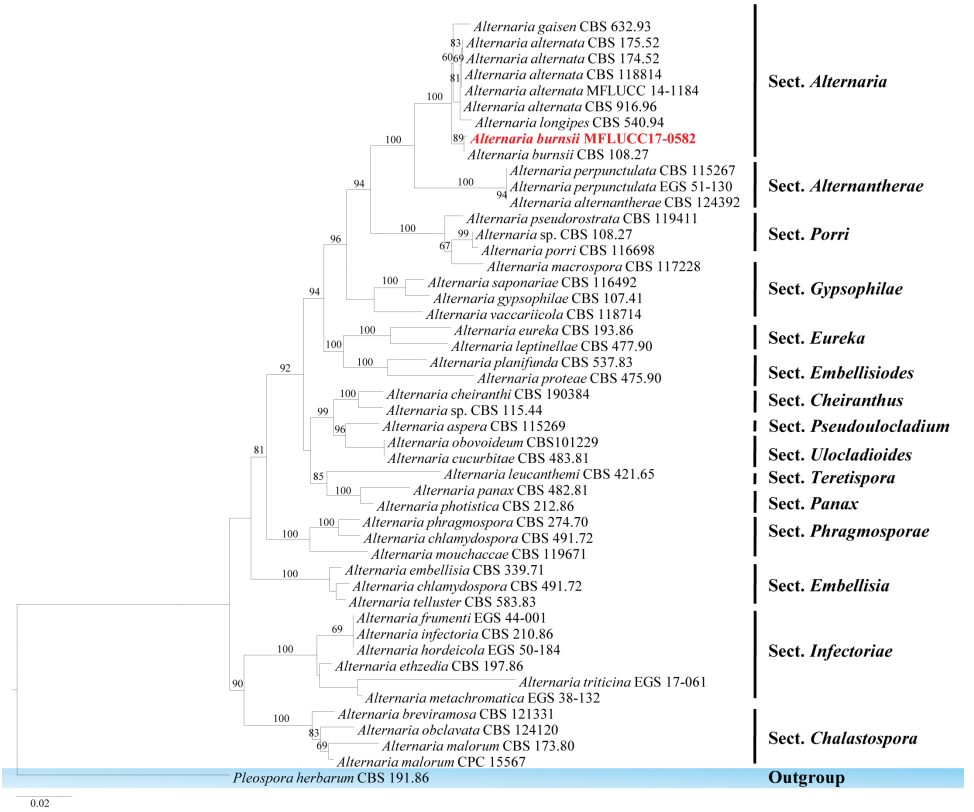

Figure 11. Phylogram generated from maximum likelihood analysis based on ITS, TEF1, LSU and RPB2 sequence data. Maximum likelihood bootstrap values are given above/below the nodes. The newly generated sequences are in red bold. The tree is rooted with *Pleospora herbarum.*

Sordariomycetes O.E. Erikss. & Winka
Diaporthales Nannf.

Diaporthaceae Höhn. ex Wehm.

Remarks. The family *Diaporthaceae* was introduced by von Höhnel (1917) and was placed in the order Diaporthales. This family comprised two *Diaporthe* genera (*Phomopsis* and *Mazzantia*) (Wehmeyer 1975; Castlebury et al. 2002). Later, *Diaporthaceae* was given the synonym *Valsaceae* (Barr 1978). Based on DNA sequence data, some other genera have been placed in *Diaporthaceae* (Dai et al. 2014; Voglmayr and Jaklitsch 2014). Recently, Maharachchikumbura et al. (2015) and Senanayake et al. (2017) listed further genera that belong to *Diaporthaceae*. In this study, a new and a known species of *Diaporthe* from Pandanaceae hosts in Thailand is reported.

Diaporthe pandanicola **Tibpromma & K.D. Hyde, sp. nov.**
MycoBank number: MB823840
Facesoffungi number: FoF03905
Figure 13

Etymology. Name referring to the host genus on which the fungus was first discovered (*Pandanus*).

Holotype. MFLU 18-0006

Culture characteristics. Colonies on PDA (Figure 2, PE77), superficial, white, circular with entire edge, smooth and raised on surface media, flossy and velvety; reverse yellow-white, 9 cm diam. in 10 days. Generative hyphae simple-septate, branched, sub-hyaline, cells with guttules, thin-walled, 1.5–7 µm wide. Not sporulating in culture (Figure 13).

Material examined. THAILAND, Chumphon, Pathio District, on healthy leaves of *Pandanus* sp. (Pandanaceae), 1 December 2016, S. Tibpromma PE77 (MFLU 18-0006, **holotype**); HKAS100858, paratype, ex-type living cultures, MFLUCC 17-0607 = KUMCC 17-0297.

GenBank numbers. ITS=MG646974, β-tubulin=MG646930, ACT=MG646930.

Notes. *Diaporthe* species are plant pathogens, endophytes or saprobes (Carroll 1986; Garcia-Reyne et al. 2011; Udayanga et al. 2011, 2012, 2014, Hyde et al. 2014). Here, a new species *Diaporthe pandanicola* is introduced based on phylogeny support. Based on phylogenetic analysis, the new species was well-separated from closely related species of *Diaporthe* (61% in ML, 0.97 in PP). However, this isolate is an endophytic fungus and did not sporulate in culture during 5 months (Figure 13).

Diaporthe siamensis **Udayanga, X.Z. Liu & K.D. Hyde, 2012**

Culture characteristics. Colonies on PDA (Figure 2, PE37), superficial, white to yellow-white, irregular, curled and raised on media surface, flossy; under surfaceyellow-white.

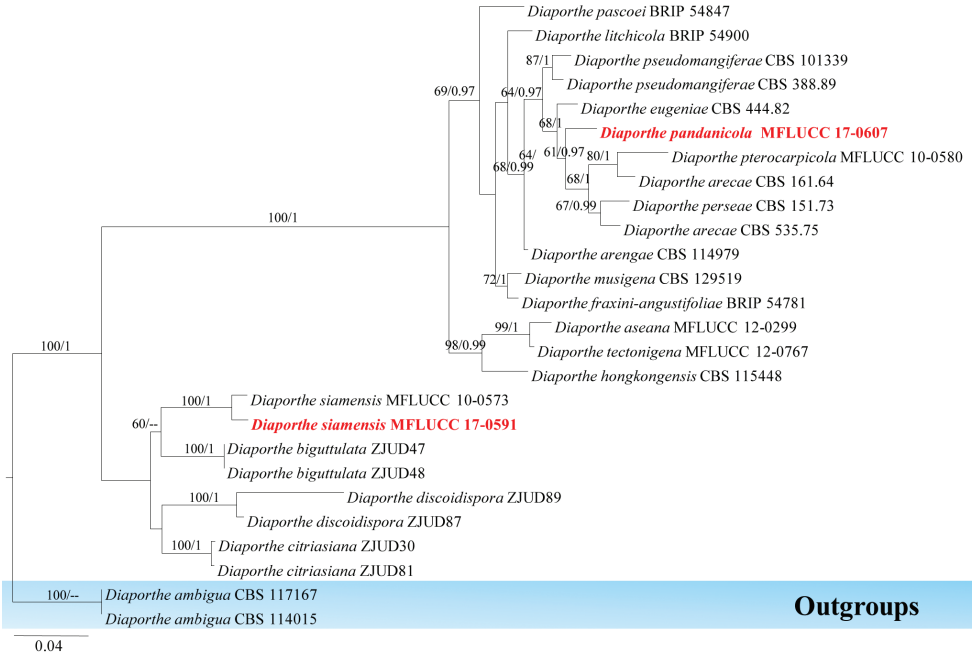

Figure 12. Phylogram generated from maximum likelihood analysis based on ITS, TEF1 and β-tubulin sequenced data. Maximum likelihood (left) and Bayesian inference (right) bootstrap values are given above/below the nodes. The newly generated sequences are in red bold. The tree is rooted with *Diaporthe ambigua.*

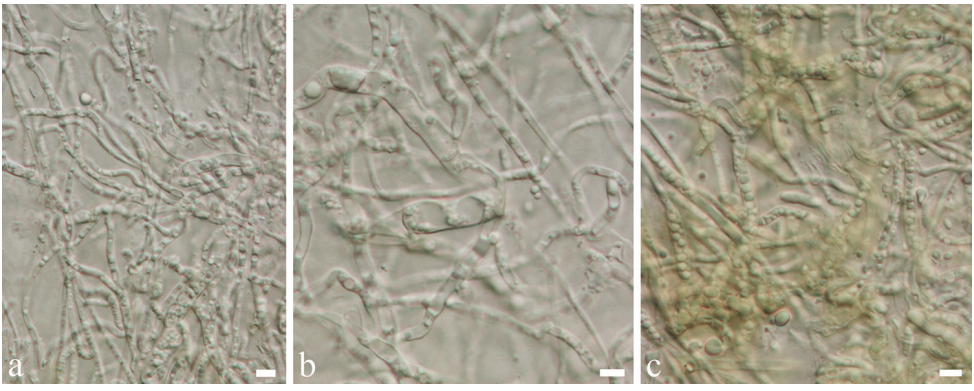

Figure 13. *Diaporthe pandanicola* (MFLU 18-0006, holotype). **a–c** Mycelia masses. Scale bars: 5 μm (**a–c**).

GenBanknumbers.ITS=MG646975,TEF1=MG646989,β-tubulin=MG646925, ACT=MG646940.

Notes. In the phylogenetic analysis, the authors' collection grouped with *Diaporthe siamensis* MFLUCC 10-0573 with high statistical values of 100% in ML and 1.00 in PP. *Diaporthe siamensis* is an endophytic fungus collected from a Pandanaceae host in Thailand.

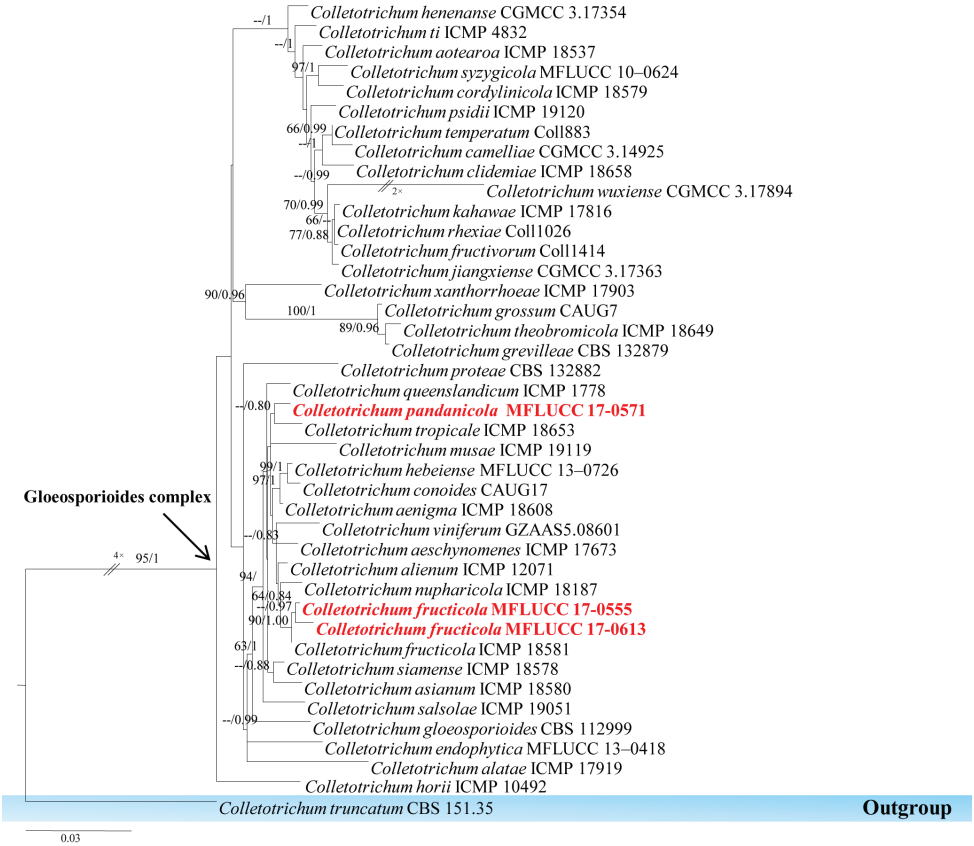

Figure 14. Phylogram generated from maximum likelihood analysis based on combined ITS, Actin, β-tubulin, GADPH and CHS-1 sequenced data. Maximum likelihood (left) and Bayesian inference (right) bootstrap values are given above/below the nodes. The newly generated sequences are in red text. The tree is rooted with *Colletotrichum truncatum.*

Glomerellales Chadef. ex Réblová et al.

Glomerellaceae Locq. ex Seifert & W. Gams, in Zhang et al. (2007)

Remarks. The family *Glomerellaceae* was introduced by Locquin (1984), but was invalidly published. To date, most *Glomerellaceae* have been recorded to be pathogens (Maharachchikumbura et al. 2016b). Earlier studies reported that the position of the family *Glomerellaceae* was not stable (Zhang et al. 2006; Kirk et al. 2001; Kirk et al. 2008). Réblová et al. (2011) resolved the placement of *Glomerellaceae* by using phylogenetic analysis of combined ITS, LSU, SSU and RPB2 sequence data. Recently, the family *Glomerellaceae* was established based on the genus *Glomerella* (Zhang et al. 2006), which had been given a synonym under its asexual morph *Colletotrichum* (Maharachchikumbura et al. 2015). Recently, Jayawardena et al. (2016) provided notes

on currently accepted species of *Colletotrichum.* In this study, the authors introduce a new endophytic *Colletotrichum* species and report a known species of endophytic *Colletotrichum* from gloeosporioides species complex based on morphology and phylogenetic analysis.

***Colletotrichum fructicola* Prihast., L. Cai & K.D. Hyde, 2009**

Culture characteristics. Colonies on PDA (Figure 2, PE84, 88), superficial, white to olivaceous in the beginning and later become olivaceous to dark-olivaceous, circular, entire edge, smooth, dense and raised on surface media; reverse dark-olivaceous. Sporulating in culture after 1 month.

GenBank numbers. MFLUCC 17-0613 ITS=MG646968, β-tubulin=MG646927, GAPDH=MG646932, CHS-1=MG646937, ACT=MG646939. MFLUCC 17-0555 ITS=MG646969, β-tubulin=MG646928, GADPH=MG646933, CHS-1=MG646936, ACT=MG646944.

Notes. The gloeosporioides species complex is mainly plant pathogens (Weir et al. 2012) and some species are endophytes (Liu et al. 2015). *Colletotrichum fructicola* has a wide host range (Weir et al. 2012) and was originally reported from coffee berries in Thailand (Prihastuti et al. 2009). In this study, the authors followed Jayawardena et al. (2016) and identify the collection as *Colletotrichum fructicola* which was isolated from a Pandanaceae host. Based on phylogenetic analysis, this taxon grouped with *Colletotrichum fructicola* with 90 % in ML and 1.00 in PP. The ITS, β-tubulin, GAPDH, CHS-1 and ACT DNA nucleotide comparison showed that the taxon and other strains of *Colletotrichum fructicola* Prihast., L. Cai & K.D. Hyde have 100% similarity.

***Colletotrichum pandanicola* Tibpromma & K.D. Hyde, sp. nov.**
MycoBank number: MB823841
Facesoffungi number: FoF03906
Figure 15

Etymology. name referring to the host genus (*Freycinetia*).

Holotype. MFLU 18-0003

Description. Colonies on PDA attaining 9 cm diam. in 7 days at room temperature, dark-grey. **Sexual morph** Undetermined. **Asexual morph** *Conidiophores* hyaline, smooth-walled, cylindrical to slightly inflated. *Conidia* 9–18 µm high, 4–8 µm diam. ($\overline{x}$ = 13.39 × 5.35 µm, n = 20), hyaline, cylindrical with rounded ends tapering slightly towards the base, smooth, septate, guttulate.

Culture characteristics. Colonies on PDA (Figure 2, PE09), superficial, white in the beginning and later becoming dark-grey, circular, entire edge, smooth, flossy, velvety and raised on surface media; reverse dark. Sporulating in culture after 1 month.

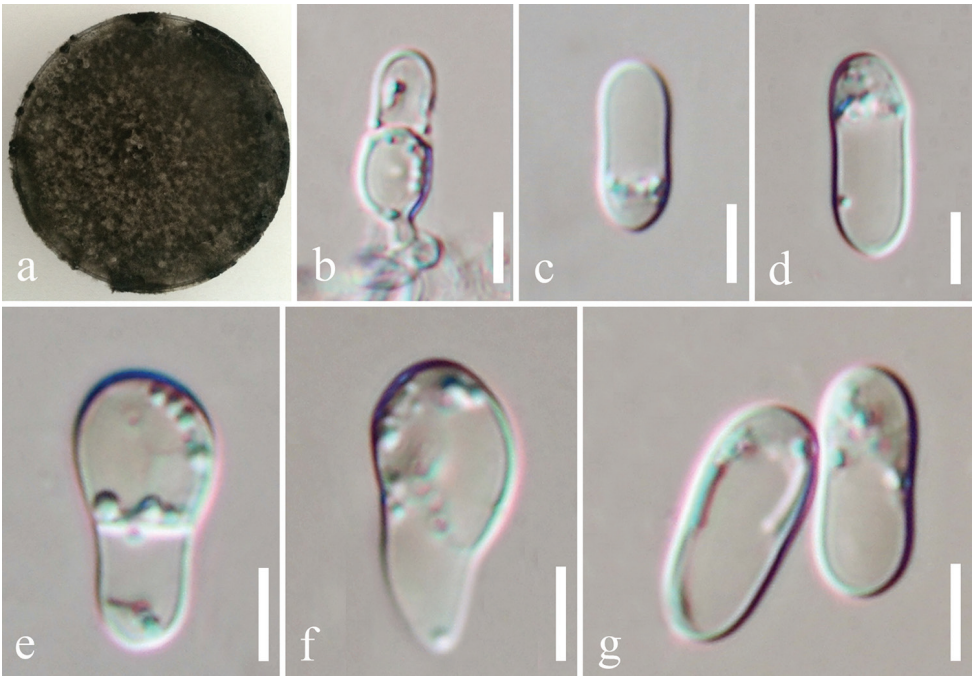

Figure 15. *Colletotrichum pandanicola* (MFLU 18-0003, holotype). **a** Colony on PDA media **b** Conidia and conidiogenous cells **c–g** Conidia on PDA culture. Scale bars: 5 μm (**b**), 2 μm (**c–g**).

Material examined. THAILAND, Chumphon, Pathio District, on healthy leaves of *Pandanus* sp. (Pandanaceae), 1 December 2016, S. Tibpromma PE09 (MFLU 18-0003, **holotype**); GZAAS 16-0145, paratype, ex-type living cultures, MFLUCC 17-0571.

GenBank numbers. ITS=MG646967, β-tubulin=MG646926, GAPDH=MG646931, CHS-1=MG646935, ACT=MG646938.

Notes. *Colletotrichum pandanicola* is introduced here as a new species in the gloeosporioides species complex based on morphological and phylogenetic data. The phylogenetic analysis shows that this new taxon is well-separated from other known *Colletotrichum* species (Figure 14). The authors also compared nucleotides of β-tubulin, GAPDH, CHS-1 and ACT and found that there are differences between *Colletotrichum tropicale* and this new species (β-tubulin 7 bp, GAPDH 11 bp, CHS-1 7 bp and ACT 3 bp).

Magnaporthaceae P.F. Cannon

Remarks. The family *Magnaporthaceae* was introduced by Cannon (1994) and was placed as a family within the class Sordariomycetes (Kirk et al. 2001; Lumbsch and Huhndorf 2007). According to Thongkantha et al. (2009), the placement of the taxa *Magnaporthaceae* has long been problematic due to a lack of convincing morphological

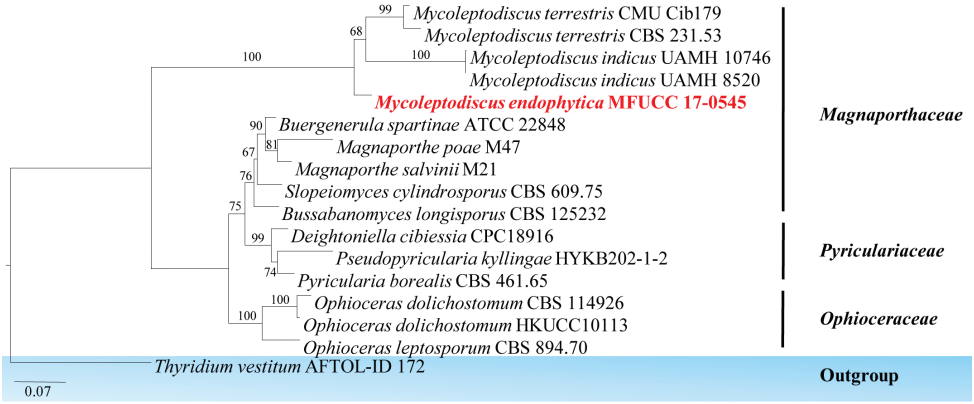

Figure 16. Phylogram generated from maximum likelihood analysis based on combined ITS, LSU, SSU and TEF1 sequenced data. Maximum parsimony bootstrap values are given above/below the nodes. The newly generated sequences are in red bold. The tree is rooted with *Thyridium vestitum.*

characteristics and inconclusive molecular data. Thongkantha et al. (2009) established a new order, *Magnaporthales*, to accommodate *Magnaporthaceae*, based on a combination of morphological characteristics and the phylogenetic analysis of combined sequence data. Maharachchikumbura et al. (2015) provided an updated outline of the family *Magnaporthacea*e with 20 genera, which included both sexual and asexual morphs. In this study, *Mycoleptodiscus endophyticus* is introduced as a new species.

Mycoleptodiscus endophyticus **Tibpromma & K.D. Hyde, sp. nov.**
MycoBank number: MB823842
Facesoffungi number: FoF03907
Figure 17

Etymology. Named after its original habitat as an endophytic fungus.

Holotype. MFLU 18-0001

Culture characteristics. Colonies on PDA (Figure 2, FE101), superficial, dark olivaceous with circular rings with filiform edge and rough and raised on media surface; reverse dark olivaceous. *Mycelium* composed of branched, pale-brown to dark-brown, thick-walled, guttulate, hyphae, with cells sub-globose to ovoid in shape. Not sporulating in culture.

Material examined. THAILAND, Ranong, Muang, on healthy leaves of *Freycinetia* sp. (Pandanaceae), 3 December 2016, S. Tibpromma FE101 (MFLU 18-0001, **holotype**); HKAS100847, paratype, ex-type living cultures, MFLUCC 17-0545 = KUMCC 17-0263.

GenBank numbers. LSU=MG646946, SSU=MG646978, TEF1=MG646985.

Notes. *Mycoleptodiscus* Ostaz. (1968) belongs to *Magnaporthaceae*, *Magnaporthales.* Since 1968, there have been 17 records of *Mycoleptodiscus* in Index Fun-

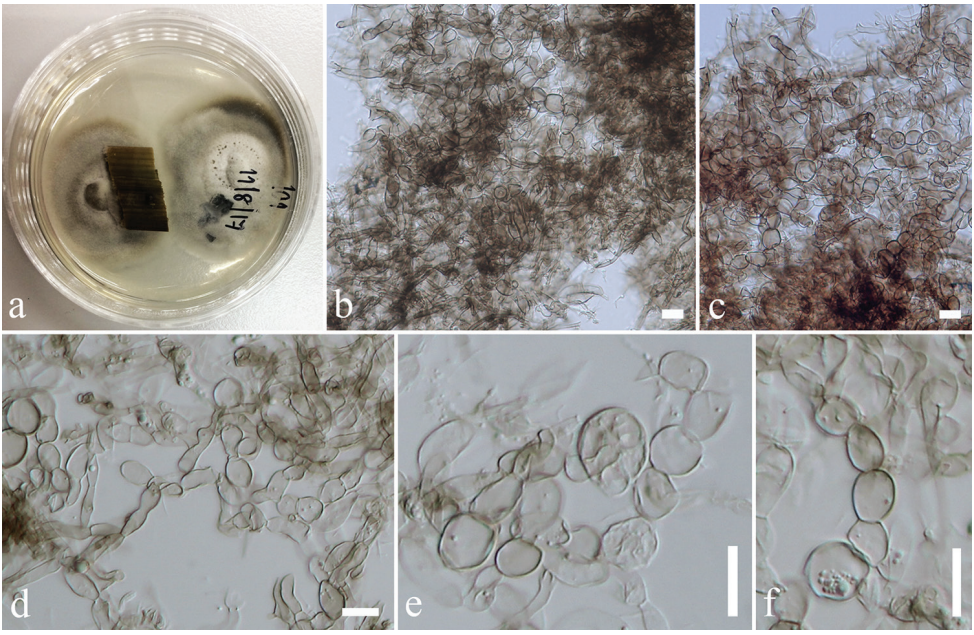

Figure 17. *Mycoleptodiscus endophyticus* (MFLU 18-0001, holotype). **a** Colony on MEA media **b, c** Mycelia masses **d–f** Vegetative hyphae in culture. Scale bars: 10 μm (**b–d**), 5 μm (**e, f**).

gorum. Most of these species were described without molecular data. In this study, a new species *Mycoleptodiscus endophyticus* is introduced, based on culture characteristics and phylogenetic analysis (100 % in ML). *Mycoleptodiscus endophyticus* was found as an endophytic fungus on leaves of *Freycinetia* sp; *Mycoleptodiscus freycinetiae* Whitton, K.D. Hyde & McKenzie was found as a saprobic fungus on the same host but there was no molecular data available to confirm this identification. The authors were unable to compare the morphological differences between the new taxon and *Mycoleptodiscus freycinetiae*, because only culture characteristics are presented here for this new taxon (Fig. 17).

Sporocadaceae Corda, 1842

Remarks. *Sporocadaceae* was introduced by Corda (1842) with *Pestalotiopsis*-like asexual morphs and confirmed by Senanayake et al. (2015). Members of *Sporocadaceae* are saprobes, endophytes or foliar pathogens in tropical and temperate regions (Jeewon et al. 2004; Tanaka et al. 2011). *Pestalotiopsis* can be found as saprobes or pathogens worldwide (Jeewon et al. 2002, 2003; Maharachchikumbura et al. 2011, 2012, 2013, 2014a, b, 2016a, c). Recently, Chen et al. (2017) provided updates for this genus based on morphology and phylogeny. In this study, two known species of *Pestalotiopsis* from Pandanaceae hosts were isolated.

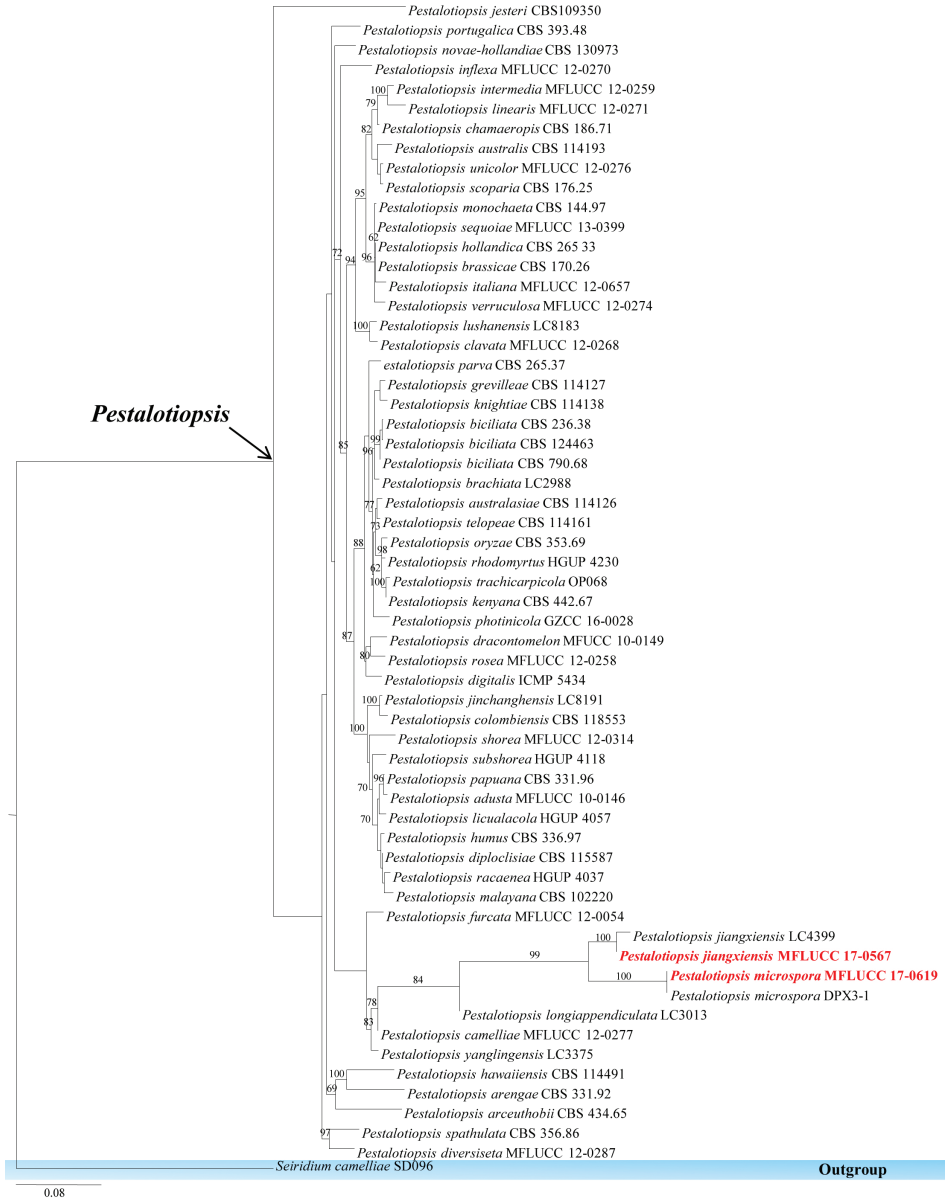

Figure 18. Phylogram generated from maximum likelihood analysis based on the combination of ITS, β-tubulin and TEF1 sequenced data. Maximum parsimony bootstrap is given above/below the nodes. The newly generated sequences are in red bold. The tree is rooted with *Seiridium camelliae.*

Pestalotiopsis jiangxiensis F. Liu & L. Cai, 2017

Culture characteristics. Colonies on PDA (Figure 2, PE05), superficial, white at the margin with yellow-white in the centre, with circular to undulate at the edge and raised

and dense aerial mycelia on surface; reverse yellow-white. Sporulating in culture after 2 months.

GenBank numbers. ITS=MG646966, ACT=MG646942, GAPDH=MG646934, β-tubulin=MG646929.

Notes. The authors' collection from Pandanaceae host in Thailand was identified as *Pestalotiopsis jiangxiensis*. This taxon grouped with *Pestalotiopsis jiangxiensis* LC4399 which is collected from *Eurya* sp., with high bootstrap support of 100% in ML.

Pestalotiopsis microspora (Speg.) G.C. Zhao & N. Li, 1995

Culture characteristics. Colonies on PDA (Figure 2, PE92), superficial, white to yellow-white, edge irregular, flossy and velvety; under surface yellow-white to yellow. Sporulating in culture after 2 months.

GenBank numbers. ITS=MG646965, ACT=MG646943.

Notes. *Pestalotiopsis microspora* was isolated from a Pandanaceae host in Thailand. This strain clusters with *Pestalotiopsis microspora* DPX3-1 with a strong bootstrap support.

Saccharomycetes

Debaryomycetaceae Kurtzman & M. Suzuki

Remarks. *Debaryomycetaceae* was introduced by Kurtzman and Suzuki in 2010 and was typified by *Debaryomyces* Klöcker. *Meyerozyma* belongs to family *Debaryomycetaceae* and was detailed in Kurtzman and Suzuki (2010). In this study, *Meyerozyma caribbica* was found on a Pandanaceae host as an endophytic fungus. Species identification was confirmed by DNA sequence data.

Meyerozyma caribbica (Vaughan-Mart., Kurtzman, S.A. Mey. & E.B. O'Neill) Kurtzman & M. Suzuki, Mycoscience 51(1): 8 (2010)

Culture characteristics. Colonies on PDA (Figure 2, PE75, 98), superficial, white to yellow-white, rings with irregular, undulate edge and curled, raised on the surface media; reverse yellow-white to yellow at the margin and dark-brown at the centre. Sporulating in culture after 2 months.

GenBank numbers. MFLUCC 17-0556 ITS=MG646971, LSU=MG646950, SSU=MG646977. MFLUCC 17-0606 ITS=MG646972, LSU=MG646951, SSU=MG646980.

Notes. *Meyerozyma caribbica* collected in this study is represented by two endophytic isolates from Pandanaceae. Phylogenetic analysis also supported the identification of this sample as *Meyerozyma caribbica*.

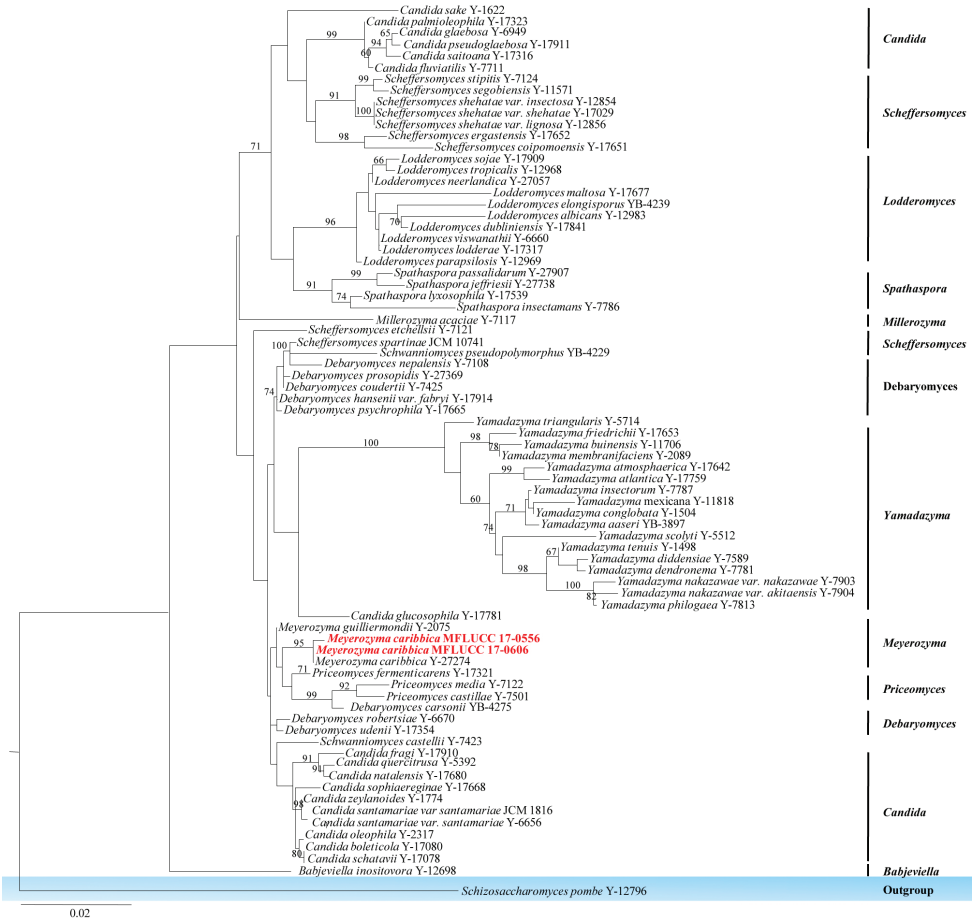

Figure 19. Phylogram generated from maximum likelihood analysis based on combined LSU and SSU sequence data. Maximum parsimony bootstrap is given above/below the nodes. The newly generated sequences are in red text. The tree is rooted with *Schizosaccharomyces pombe.*

Conclusion

In this study on fungal endophytes found on leaves of Pandanaceae, it was found that the taxa belonged to both Ascomycota and Basidiomycota. The majority of the taxa were Ascomycota, as found in most previous endophytic studies (Crozier et al. 2006; Selim et al. 2017). In classical mycology, most endophytic fungi were described based on their morphological features (Barseghyan and Wasser 2010). However, there are difficulties in identifying ascomycetes to the species level based only on morphological features (Lu et al. 2012), because they have only a small set of morphological characteristics and exhibit homoplasy (Barseghyan and Wasser 2010).

The 22 endophytic fungal strains found in this study were chiefly identified using their microscopic characteristics and DNA sequence data and holotype materials in the

form of dried cultures. Future studies are however needed to recollect the taxa which are sporulating to describe sexual and asexual characteristics (*sensu* Lacap et al. 2003). In this study, 22 endophytes were isolated and sorted into eight morphotype based on colony characteristics. The authors, however, subjected all isolates to phylogenetic analysis and found they belong to 16 different taxa. The taxa were sorted roughly into morphotypes, but they did not reflect the actual species. Several isolates of this study did not sporulate, but are introduced as new species because DNA sequence comparison and multi-gene phylogenetic analyses provided sufficient evidence to show that they are distinct taxa (Jeewon and Hyde 2016). The new taxa are, however, code compliant, as they are provided with MycoBank numbers, full descriptions, colour photographss and illustrations.

The species composition of endophytic microorganisms is likely to depend on the plant age, genotype, sampled tissue, host type and season of isolation (Rosenblueth and Martinez-Romero 2006). Promputtha et al. (2007) showed that endophytic species can change their ecological strategies and adopt a saprotrophic lifestyle. However, it was found that for the cultures of some endophytic fungal species, mycelia are the only visible morphological structures. According to these conclusions, the authors agree with Petrini (1991), Yanna and Hyde (2002), Ghimire and Hyde (2004) and Hyde et al. (2006) regarding the relationships between fungal endophytes and saprobic fungi. However, the use of next-generation sequencing (NGS) (Shendure and Ji 2008) is another option for identification of fungal species that cannot be cultured *in vitro* and has now become popular. These methods have also been applied to large-scale culture-independent molecular biological methods (Zoll et al. 2016). Future developments in technology are likely to produce further novel methods that mycologists could apply to the field of taxonomy (e.g. Hawksworth and Lucking 2017).

Acknowledgements

We would like to thank Molecular Biology Experimental Centre at Kunming Institute of Botany for their help with sequencing work. Saowaluck Tibpromma thanks the Mushroom Research Foundation (MRF), Chiang Rai, Thailand for financial support. Kevin D. Hyde thanks Mae Fah Luang University for the grant "Biodiversity, phylogeny and role of fungal endophytes of Pandanaceae" (Grant number: 592010200112) and the Chinese Academy of Sciences (project number 2013T2S0030), for the award of Visiting Professorship for Senior International Scientists, at Kunming Institute of Botany and Chiang Mai University. Andrew Stevenson, Fiona Worthy, Sajeewa Maharachchikumbura, Danushka Sandaruwan, Chada Norphanphoun, Asha Dissanayake, Ruvishika Jayawardena and Kasun Thambugala are thanked for their help and valuable suggestions. Samantha C. Karunarathna thanks Yunnan Provincial Department of Human Resources and Social Security funded postdoctoral project (number 179122) and National Science Foundation of China (NSFC) project code 31750110478. Pe-

ter E. Mortimer thanks the National Science Foundation of China (NSFC) project codes 41761144055 and 41771063. Fiona Worthy in the World Agroforestry Centre (ICRAF), Kunming Institute of Botany, China is thanked for English editing.

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MycoKeys 33: 69–84 (2018)
doi: 10.3897/mycokeys.33.23911
http://mycokeys.pensoft.net

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An updated checklist of the lichens of St. Eustatius, Netherlands Antilles

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Academic editor: *G. Rambold* | Received 26 January 2018 | Accepted 17 March 2018 | Published 3 April 2018

Citation: Aptroot A, Stech M (2018) An updated checklist of the lichens of St. Eustatius, Netherlands Antilles. MycoKeys 33: 69–84. https://doi.org/10.3897/mycokeys.33.23911

Abstract

In the course of a multi-taxon biodiversity inventory for the island of St. Eustatius, lichens were collected from 11 plots representing different vegetation types. From these collections, 126 lichen species are reported, 54 of which are new reports for St. Eustatius. Most species could be identified to species level based on morphological and chemical characters. In a few cases, mtSSU DNA sequences were generated for a preliminary molecular identification and future phylogenetic studies. In total, 263 identified lichen species are currently known from St. Eustatius, as well as some additional genera with yet unidentified species and lichenicolous fungi.

Keywords

Biodiversity inventory, lichens, mtSSU, St. Eustatius

Introduction

Sint Eustatius is a small island (21 km^2) in the northern Leeward Islands part of the West Indies. It is one of the six islands of the Netherlands Antilles and, since 2010, a special municipality of the Netherlands. Sint Eustatius is roughly divided into three parts, the Northern Hills, the urbanised central area ('Cultuurvlakte') and the southern part dominated by the steep dormant volcano The Quill (600 m elev.). Although the whole island of St. Eustatius has been heavily impacted by human activities, the northern and

southern parts are nowadays designated as National Parks with varied vegetation types especially on the slopes of The Quill. The latter comprise, for example, thorny woodlands, deciduous to evergreen seasonal forests, dry evergreen forest, montane thickets and elfin woodland (Stoffers 1956). A re-classification of the vegetation of St. Eustatius, based on cluster analysis of sample plots, resulted in 13 vegetation types characterised by different combinations of individual vascular plant species (Freitas et al. 2014).

Just as in almost all other groups of organisms, lichens are most diverse in the tropics (Sipman and Aptroot 2001). On St. Eustatius, they are commonly present on various substrates, including tree bark and twigs, siliceous rock, limestone, dead wood and living leaves. Despite their abundance, the lichen flora of St. Eustatius is still incompletely known. The authors are aware of only nine publications citing in total 14 lichen species from St. Eustatius, *viz. Phyllopsora corallina* (Eschw.) Müll. Arg. and *P. parvifoliella* (Nyl.) Müll. Arg. (Brako 1991), *Anisomeridium excellens* (Müll. Arg.) R.C. Harris (Harris 1995), *Syncesia glyphysoides* (Fée) Tehler (Tehler 1997), *Pseudopyrenula subnudata* Müll. Arg. (Harris 1998, as *P. diluta* var. *degenerans* Vain.), *Stirtonia neotropica* Aptroot, described based on material from St. Eustatius and Costa Rica (Aptroot 2009), *Syncesia subintegra* Sipman, described based on material from St. Eustatius, as well as *S. farinacea* (Fée) Tehler, *S. glyphysoides* and *S. graphica* (Fr.) Tehler (Sipman 2009), *Roccella gracilis* Bory (Aptroot and Schumm 2011), *Dirina paradoxa* (Fée) Tehler (Tehler et al. 2013), as well as *Astrothelium bicolor* (Taylor) Aptroot & Lücking, *A. phlyctaena* (Fée) Aptroot & Lücking and *Pseudopyrenula subnudata* Müll. Arg. (Aptroot and Lücking 2016).

The main source of information about the lichen flora of St. Eustatius is the online portal 'Plants and Lichens of St. Eustatius' (Boom et al. 2009). It contains a list with 209 lichen species, based on specimens collected by H. Sipman and W.R. Buck in 2008, identified by H. Sipman and mostly hosted in B (some in NY). In addition, some identified specimens are present in various other herbaria, some of which can be searched online. For instance, the database of BR cites the following identified specimens from St. Eustatius, collected by R. Hensen in 1991 and identified by the first author: *Megalaria bengalensis* Jagadeesh Ram & Aptroot, *Porina mastoidea* (Ach.) Müll. Arg. and *Sticta xanthotropa* (Kremp.) D.J. Galloway.

In 2015, a plot-based, multi-taxon biodiversity inventory of St. Eustatius was carried out by Naturalis Biodiversity Center, the European Invertebrate Survey (EIS) and different Dutch non-governmental organisations, together with St. Eustatius National Parks Foundation (STENAPA) and students from different Dutch universities. Here, the lichen records of that inventory are reported and an updated checklist of the lichens known from St. Eustatius is presented.

Materials and methods

As part of a multi-taxon inventory, lichens were collected on St. Eustatius from 11 plots (25 m × 25 m) in different main vegetation types according to Freitas et al. (2014). Two plots (H1, H2) were situated in the Northern Hills area, eight (M1–M5,

M7–M9) on and around The Quill in the southern part of the island and one (U1) in the central urban area. Details concerning the location and vegetation characteristics of the plot locations and the multi-taxon sampling approach are described in van Andel et al. (2016). Lichens were collected using a knife or hammer and chisel and subsequently air-dried and stored in paper bags.

Specimens were observed and identified by the first author using an Olympus SZX7 stereomicroscope and an Olympus BX50 compound microscope with interference contrast, connected to a Nikon Coolpix digital camera. Sections were mounted in tap water, in which all measurements were also taken. The chemistry of selected specimens was investigated by thin-layer chromatography (Orange et al. 2001), using solvent A.

DNA analysis based on mitochondrial ribosomal small subunit (mtSSU) sequences was carried out for ten unidentifiable or provisionally identified specimens of good quality (indicated in Table 1). Although the nuclear ribosomal ITS region is the generally accepted fungal DNA barcode locus (Schoch et al. 2012), mtSSU was chosen since more mtSSU than ITS sequences have yet been published for several of the genera or families to which the respective specimens putatively belong.

Genomic DNA was extracted using the NucleoMag 96 Plant kit (Macherey-Nagel) on the KingFisher Flex Purification System (ThermoFisher Scientific). The mtSSU region was PCR-amplified following Zoller et al. (1999) in terms of primers (mrSSU1/mrSSU3R) and the PCR protocol. PCR products were purified and sequenced at BaseClear B.V. (www.baseclear.com) using the amplification primers. Sequences were assembled and edited using Geneious v8.1.8 (Biomatters Ltd.) and subjected to a BLAST search (Altschul et al. 1990) against the GenBank database (megablast; considering, where possible, BLAST results with E value of 0.0 and query cover >90 %). Sequences are available in GenBank under accession numbers MH028639–MH028646.

To compile an updated list of the lichens of St. Eustatius, literature and internet sources were exhaustively consulted for previous reports and previous collectors were contacted for additional information.

Results and discussion

In total, 126 lichen species (and one identifiable lichenicolous fungus) were found in 243 collections (Table 1). The vast majority (113 species) could be identified to species level based on morphological and chemical characters, even though no identification book exists for any region nearby. However, many species have been described from other islands in the Caribbean, which can be expected to have many species in common. These were often already described in the 19th century and partly never studied again, but illustrations of their types are increasingly available. The authors also had access to various unpublished sources, such as the unpublished keys, descriptions and specimen citation (by H. Sipman) that was the basis of the internet checklist of St. Eustatius lichens and keys to the lichens from Puerto Rico (Harris 1989) and Guadeloupe (Øvstedal 2010), the latter with many illustrations of type and other specimens.

Table 1. Lichenised and lichenicolous fungi recorded in 11 plots on St. Eustatius. Species names in earlier publications are indicated in brackets. Author names are given in Table 2. Plots **H1** and **H2** are situated in the Northern Hills, **M2–M9** on and around the volcano The Quill in the southern part of the island and **U1** in the central urban area. Substrates occupied by each taxon are indicated per plot; **b**: bark, **k**: limestone, **l**: leaves, **r**: siliceous rock, **s**: soil, **w**: wood. Asterisks indicate first records for St. Eustatius (asterisks in brackets indicate additional taxa that are not yet identified to species level). Black dots (•) indicate specimens from which DNA was extracted.

Taxon	H1	H2	M1	M2	M3	M4	M5	M7	M8	M9	U1
(*)*Acanthothecis* sp. •				b							
Alyxoria culmigena (*Opegrapha herbarum*)	r						w		b		
**Alyxoria varia*	b		b							b	b
**Amandinea multispora*										b	
**Anisomeridium subprostans*				b							
Anisomeridium tamarindi	b										
(*)*Anisomeridium* sp. corticate c. pycnidia •					b						
**Anisomeridium terminatum*							b				
Arthonia antillarum		b				b					
Arthonia caribaea	b										
Arthonia conferta	b	b				b	b	b	b	b	
Arthonia cyrtodes				b							
Arthonia minuta					b					b	
**Arthonia parantillarum*	b				b						
Arthothelium macrothecum				b	b						
**Bacidia medialis*					b, r	r					
(*)*Bacidia* sp. apotheciate •							b, w				
(*)*Bacidia* sp. sorediate •					b						
Bactrospora denticulata	b				b	b			b	b	
**Bactrospora jenikii*					b						
**Bogoriella annonacea*							b		b		
**Brigantiaea leucoxantha*				b							
Buellia dejungens		r				r	r	r		r	
**Buellia griseovirens*							w		w		
Buellia mamillana (*Buellia glaziouana*)									r		
Caloplaca leptozona		r					r				
**Caloplaca obscurella*							w				
Coenogonium linkii			b	b							
**Coenogonium saepincola*							w				
Coenogonium strigosum				b							
Coniocarpon cinnabarinum (*Arthonia cinnabarina*)				b							
**Crespoa carneopruinata*	b, r										
**Cresponea flava*	r										
**Cryptothecia punctosorediata*					b						
Cryptothecia striata				b	b, r						
(*)*Cryptothecia* sp. isidiate •			b								
(*)*Cryptothecia* sp. sterile •					b						
**Dactylospora saxatilis* (lichenicolous on *Pertusaria praetervisa*)		r									
Dichosporidium nigrocinctum			b, r	b, r							

Taxon	H1	H2	M1	M2	M3	M4	M5	M7	M8	M9	U1
**Dictyomeridium amylosporum*	b						b				
Diorygma hieroglyphicum				b							
Diorygma poitaei				b							
**Diorygma pruinosum*				b							
**Endocarpon pallidulum*		r				r		r, k	r, s	r	
Enterographa pallidella		b				b					
Enterographa subserialis									b		
Flakea papillata			b	b, r, w	b, r		r				
Glyphis scyphulifera									b		
Graphis caesiella					b						
**Graphis cincta*					b						
Graphis dendrogramma				b			b				
**Graphis librata*										b	
Gyalolechia bassiae (*Caloplaca bassiae*)						b	b, r				
**Hafellia curatellae*										b	
Hyperphyscia adglutinata						b				b, r	b
Lathagrium neglectum (*Collema neglectum*)									b		
Lecanora legalloana	r				r	r	r	r			
**Lecanora leproplaca*					b		b		b		
Lecanora leprosa							r				
Lecanora prosecha	r	r				r	r	r	r		
Lecanora sulfurescens	r										
**Lepraria finkii*			b								
**Leprocollema nova-caledonianum*						w					
Letrouitia domingensis				b							
Malmidea piperis (*Malcolmiella piperis*)				b							
**Malmidea psychotrioides*				b							
Malmidea vinosa (*Malcolmiella vinosa*)			b								
Mazosia carnea (*Mazosia ocellata*)				b	b						
(*)*Melaspilea* sp. (lichenicolous on *Pyrenula dissimulans*)						b					
**Microtheliopsis uleana*			l								
**Mycoporum eschweileri*				b	b	b			b	b	
Nyungwea anguinella (*Enterographa anguinella*)					b						
**Opegrapha astraea*						b	b				
**Opegrapha lithyrgiza*	r										
**Opegrapha quintana*					b						
(*)*Opegrapha* sp.								k			
Peltula bolanderi		r									
Peltula obscurans		r				r				r	
Pertusaria coccopoda						r		r			
Pertusaria praetervisa	r	r									
**Pertusaria texana*									b		
Pertusaria xanthodes								b			
**Phaeographis crispata*	b										
Phaeographis dendritica									b		
**Phyllopeltula corticola*								b			
Phyllopsora corallina			b		b, r	r					
Physcia atrostriata				b							

Taxon	H1	H2	M1	M2	M3	M4	M5	M7	M8	M9	U1
**Physcia erumpens*										b	
**Physcia integrata*						b	b				
Physcia sorediosa						b					
**Porina conspersa*				b, r	r						
Porina epiphylla			l								
Porina internigrans				b	b						
**Porina leptalea*					r						
Porina nucula					b, r						
**Porina rubentior*			l								
Porina tetracerae			b	b	r						
**Porina thaxteri*			l								
(*)*Psorotichia* cf. *americana*							r				
**Pyrenopsis antillarum*		r									
**Pyrenula adacta*					b		b	b	b		
Pyrenula breutelii (*Pyrenula macularis*)							b			b	
Pyrenula cocoes	b				b	b	b		b		
**Pyrenula cruenta*					b						
**Pyrenula dissimulans*	b				b	b	b	b			
Pyrenula nitidula					b						
Pyxine cocoes						b, r	r	r	r		b
**Ramalina stoffersii*				r							
**Rinodina antillarum*						r					
**Rinodina colobinoides*						b		b			
Rinodina pyxinoides	r	r				r	r		r	r	
Sarcographa cf. *tricosa* •					b						
Squamulea subsoluta (*Caloplaca subsoluta*)				r		r		r		r	
**Staurolemma dussii*					b						
**Sticta xanthotropa*			r	r							
**Stigmatochroma gerontoides*									w		
(*)*Stigmidium* cf. *schaereri*								k			
**Strigula decipiens*	r										
**Strigula phaea*					b, r						
Strigula smaragdula			l								
**Syncesia decussans*							b				
**Thelenella luridella*						r		r	r		
(*) *Thelidium* cf. *decipiens* •								k			
(*) *Verrucaria* cf. *dolosa* •							r				
**Verrucaria nigrescens*								r			
(*) *Wetmoreana* cf. *appressa* •		r								r	

Somewhat to the authors' surprise, as many as 54 (almost 50 %) of the identified species are new records for St. Eustatius. This includes mostly relatively common and widespread tropical or Neotropical species, but also some rare species, notably *Staurolemma dussii* (Vain.) P.M. Jørg. & Henssen, which was so far only known from its type from Guadeloupe. Furthermore, it is remarkable that *Cresponea flava* (Vain.) Egea & Torrente was found on siliceous rock. The presence of so many additional species within the limited surface area of the plots, totalling 6875 m^2 (0.03% of the total island surface), suggests that the exploration of the lichen flora of St. Eustatius has not yet been

exhaustive. However, no clearly undescribed species were found in the material and the number of species described based on material from St. Eustatius remains low with two, *viz*. *Stirtonia neotropica* (Aptroot 2009) and *Syncesia subintegra* (Sipman 2009).

Several specimens could not be identified with certainty in the present material but represent additional species (and in several cases additional genera). These are, for instance, Lichinaceae and Verrucariaceae, of which the taxonomy of the tropical taxa is incompletely known. Rather than describing them as new, they were listed with the name of the species that is morphologically most similar, preceded by "cf". The BLAST results from the mtSSU sequences obtained from eight of these specimens in most cases allowed preliminary insights into their phylogenetic position.

The sequence of the *Anisomeridium* specimen with only conidia from St. Eustatius receives the highest BLAST hits with other representatives of the Monoblastiaceae in Nelsen et al. (2009, 2011), *viz. Anisomeridium ubianum* (Vain.) R.C. Harris, *A.* cf. *willeyanum* (R.C. Harris) R.C. Harris, *Megalotremis verrucosa* (Makhija & Patw.) Aptroot and *Trypetheliopsis kalbii* (Lücking & Sérus.) Aptroot. The low sequence identities of 86–93% clearly indicate that the St. Eustatius specimen belongs to another species in that family, but too few mtSSU sequences are yet available for a more precise molecular identification.

In the Graphidaceae, the top five BLAST hits for the specimen of *Acanthothecis* sp. were all with *Acanthothecis peplophora* (M. Wirth & Hale) E. Tripp & Lendemer specimens (97% identity), whereas the identity with the sequence of the type species of *Acanthothecis*, *A. hololeucoides* (Nyl.) Staiger & Kalb, was only 89%. The specimen from St. Eustatius thus most probably does not belong to *Acanthothecis* s.str., but may represent a species of '*Acanthothecis* 2' in the *Carbacanthographis* clade (cf. Rivas Plata et al. 2013, Medeiros et al. 2017). The *Sarcographa* cf. *tricosa* specimen received BLAST hits of 97% identity with *Sarcographina glyphiza* (Nyl.) Kr.P. Singh & D.D. Awasthi and *Pallidogramme chlorocarpoides* (Nyl.) Staiger, Kalb & Lücking, both situated in the Graphioideae tribe Graphidae p.p. clade of Rivas Plata et al. (2013). However, another GenBank sequence of *P. chlorocarpoides,* as well as several species of other genera of same clade, were 96 % identical, including the single other specimen of *S. tricosa* in GenBank (but not the species of the *Sarcographa* s.str. clade *sensu* Rivas Plata et al. 2013). The identity of the St. Eustatius specimen thus remains ambiguous based on the presently available mtSSU sequence data.

Both the apotheciate and sorediate *Bacidia* specimens are closest to sequences of species of the *Toninia-Bacidia* p.p. clade in Miadlikowska et al. (2014), the former to *Toninia sedifolia* (Scop.) Timdal (94 % identity) and the latter to *Bacidia californica* S. Ekman and *B. phacodes* Körb. (88–89 % identity), respectively. Consequently, they do not belong to *Bacidia* s.str., which forms a separate clade (including the type species, *B. rosella* (Pers.) De Not.) in Miadlikowska et al. (2014).

In the Verrucariaceae, *Verrucaria* was resolved as polyphyletic and *Thelidium* mixed with *Polyblastia*, *Staurothele* p.p. and *Verrucaria* p.p. (*Polyblastia* clade) in molecular phylogenetic reconstructions (Gueidan et al. 2007, Muggia et al. 2010, Thüs et al. 2011). The sequence of the *Thelidium* cf. *decipiens* specimen from St. Eustatius, how-

Table 2. Updated checklist of the lichens of St. Eustatius.

Species	References
Lichens	
Acarospora chrysops (Tuck.) H.Magn.	Boom et al. (2009) as *Acarospora dissipata* H.Magn.
Alyxoria culmigena (Lib.) Ertz	Boom et al. (2009) as *Opegrapha herbarum* Mont., present study
Alyxoria ochrocheila (Nyl.) Ertz & Tehler	Boom et al. (2009) as *Opegrapha ochrocheila* Nyl.
Alyxoria varia (Pers.) Ertz & Tehler	present study
Amandinea efflorescens (Müll. Arg.) Marbach	Boom et al. (2009)
Amandinea multispora (Kalb & Vězda) Marbach	present study
Amandinea prospersa (Nyl.) Elix & H. Mayhofer	Boom et al. (2009) as *Buellia prospersa* (Nyl.) Riddle
Anisomeridium americanum (A.Massal.) R.C. Harris	Boom et al. (2009)
Anisomeridium excellens (Müll. Arg.) R.C. Harris	Boom et al. (2009), Harris (1995)
Anisomeridium subprostans (Nyl.) R.C. Harris	present study
Anisomeridium tamarindi (Fée) R.C. Harris	Boom et al. (2009), present study
Anisomeridium terminatum (Nyl.) R.C. Harris	present study
Anisomeridium tuckerae (R.C. Harris) R.C. Harris	Boom et al. (2009)
Arthonia antillarum (Fée) Nyl.	Boom et al. (2009), present study
Arthonia caribaea (Ach.) A. Massal.	Boom et al. (2009), present study
Arthonia conferta (Fée) Nyl.	Boom et al. (2009), present study
Arthonia cyanea Müll. Arg.	Boom et al. (2009)
Arthonia cyrtodes Nyl.	Boom et al. (2009), present study
Arthonia minuta Vain.	Boom et al. (2009), present study
Arthonia parantillarum Aptroot	present study
Arthothelium macrothecum (Fée) A. Massal.	Boom et al. (2009), present study
Astrothelium bicolor (Taylor) Aptroot & Lücking	Boom et al. (2009) as *Trypethelium nitidiusculum* (Nyl.) R.C. Harris, Aptroot and Lücking (2016)
Astrothelium phlyctaena (Fée) Aptroot & Lücking	Boom et al. (2009) as *Trypethelium ochroleucum* (Eschw.) Nyl., Aptroot and Lücking (2016)
Bacidia medialis (Tuck.) Zahlbr.	present study
Bactrospora denticulata (Vain.) Egea & Torrente	Boom et al. (2009), present study
Bactrospora jenikii (Vězda) Egea & Torrente	present study
Bactrospora myriadea (Fée) Egea & Torrente	Boom et al. (2009)
Baculifera intermedioides Marbach	Boom et al. (2009)
Blastenia brittonii Zahlbr.	Boom et al. (2009) as *Caloplaca brittonii* (Zahlbr.) ined.
Bogoriella annonacea (Müll. Arg.) Aptroot & Lücking	present study
Brigantiaea leucoxantha (Spreng.) R. Sant. & Hafellner	present study
Brownliella cinnabarina (Ach.) S.Y. Kondr., Kärnefelt, A. Thell, Elix, J.Kim, A.S.Kondr. & J.-S.Hur	Boom et al. (2009) as *Caloplaca cinnabarina* (Ach.) Zahlbr.
Buellia boergesenii Imshaug	Boom et al. (2009)
Buellia dejungens (Nyl.) Vain.	Boom et al. (2009), present study
Buellia griseovirens (Turner & Borrer ex Sm.) Almb.	present study
Buellia mamillana (Tuck.) W.A. Weber	Boom et al. (2009) as *Buellia glaziouana* (Kremp.) Müll. Arg., present study
Buellia posthabita (Nyl.) Zahlbr.	Boom et al. (2009)
Bulbothrix scortella (Nyl.) Hale	Boom et al. (2009)
Bulbothrix suffixa (Stirt.) Hale	Boom et al. (2009)
Byssoloma leucoblepharum (Nyl.) Vain.	Boom et al. (2009)
Caloplaca diplacia (Ach.) Riddle	Boom et al. (2009)
Caloplaca leptozona (Nyl.) Zahlbr.	Boom et al. (2009), present study
Caloplaca obscurella (J. Lahm) Th. Fr.	present study
Canoparmelia martinicana (Nyl.) Elix & Hale	Boom et al. (2009)
Carbacanthographis triphoroides (M. Wirth & Hale) Lücking	Boom et al. (2009)

Species	References
Chapsa cinchonarum (Fée) Frisch	Boom et al. (2009)
Chrysothrix xanthina (Vain.) Kalb	Boom et al. (2009)
Cladonia corymbites Nyl.	Boom et al. (2009)
Cladonia didyma (Fée) Vain.	Boom et al. (2009)
Cladonia subradiata (Vain.) Sandst.	Boom et al. (2009)
Coccocarpia palmicola (Spreng.) Arv. & D.J. Galloway	Boom et al. (2009)
Coccocarpia pellita (Ach.) Müll. Arg.	Boom et al. (2009)
Coenogonium interpositum Nyl.	Boom et al. (2009)
Coenogonium leprieurii (Mont.) Nyl.	Boom et al. (2009)
Coenogonium linkii Ehrenb.	Boom et al. (2009), present study
Coenogonium saepincola Aptroot, Sipman & Lücking	present study
Coenogonium strigosum Rivas Plata, Lücking & Chaves	Boom et al. (2009), present study
Coenogonium subdilutum (Malme) Lücking, Aptroot & Sipman	Boom et al. (2009)
Coniocarpon cinnabarinum DC.	Boom et al. (2009) as *Arthonia cinnabarina* (DC.) Wallr., present study
Cratiria lauricassiae (Fée) Marbach	Boom et al. (2009)
Crespoa carneopruinata (Zahlbr.) Lendemer & B.P. Hodk.	present study
Cresponea flava (Vain.) Egea & Torrente	present study
Cresponea leprieurii (Mont.) Egea & Torrente	Boom et al. (2009)
Cresponea proximata (Nyl.) Egea & Torrente	Boom et al. (2009)
Cryptolechia carneolutea (Tuck.) A. Massal.	Boom et al. (2009)
Cryptothecia megalocarpa (Müll. Arg.) R. Sant.	Boom et al. (2009)
Cryptothecia punctosorediata Sparrius	present study
Cryptothecia striata G. Thor	Boom et al. (2009), present study
Dichosporidium nigrocinctum (Ehrenb.) G. Thor	Boom et al. (2009), present study
Dictyomeridium amylosporum (Vain.) Aptroot, M.P. Nelsen & Lücking	present study
Diorygma hieroglyphicum (Pers.) Staiger & Kalb	Boom et al. (2009), present study
Diorygma poitiaei (Fée) Kalb, Staiger & Elix	Boom et al. (2009), present study
Diorygma pruinosum (Eschw.) Kalb, Staiger & Elix	present study
Diploschistes actinostomus (Ach.) Zahlbr.	Boom et al. (2009)
Diploschistes aeneus (Müll. Arg.) Lumbsch	Boom et al. (2009)
Diploschistes prominens (Vain.) Lumbsch	Boom et al. (2009)
Dirina paradoxa (Fée) Tehler	Boom et al. (2009) as *Dirina approximata* subsp. *hioramii* (B. de Lesd.) Tehler, Tehler et al. (2013)
Dirinaria aegialita (Ach.) B.J. Moore	Boom et al. (2009)
Endocarpon pallidulum (Nyl.) Nyl.	present study
Enterographa compunctula (Nyl.) Redinger	Boom et al. (2009)
Enterographa multilocularis (Müll. Arg.) Sparrius	Boom et al. (2009)
Enterographa pallidella (Nyl.) Redinger	Boom et al. (2009), present study
Enterographa perez-higaredae Herrera-Camp. & Lücking	Boom et al. (2009)
Enterographa sipmanii Sparrius	Boom et al. (2009)
Enterographa subserialis (Nyl.) Redinger	Boom et al. (2009), present study
Eremothecella microcephalica Sipman	Boom et al. (2009)
Fellhanera santessonii Barillas & Lücking	Boom et al. (2009)
Fissurina dumastii Fée	Boom et al. (2009)
Flakea papillata O.E. Erikss.	Boom et al. (2009), present study
Glyphis cicatricosa Ach.	Boom et al. (2009)
Glyphis scyphulifera (Ach.) Staiger	Boom et al. (2009), present study
Graphis caesiella Vain.	Boom et al. (2009), present study
Graphis chondroplaca (Redinger) Lücking	Boom et al. (2009)
Graphis cincta (Pers.) Aptroot	present study

Species	References
Graphis dendrogramma Nyl.	Boom et al. (2009), present study
Graphis furcata Fée	Boom et al. (2009)
Graphis glaucescens Fée	Boom et al. (2009)
Graphis librata C. Knight	present study
Graphis lineola Ach.	Boom et al. (2009)
Graphis tenella Ach.	Boom et al. (2009)
Graphis tenellula Vain.	Boom et al. (2009)
Gyalectidium filicinum Müll. Arg.	Boom et al. (2009)
Gyalolechia bassiae (Ach.) Søchting, Frödén & Arup ex Ahti	Boom et al. (2009) as *Caloplaca bassiae* (Ach.) Zahlbr., present study
Hafellia bahiana (Malme) Sheard	Boom et al. (2009)
Hafellia curatellae (Malme) Marbach	present study
Herpothallon aurantiacoflavum (B. de Lesd.) Aptroot, Lücking & G.Thor	Boom et al. (2009)
Heterodermia albicans (Pers.) Swinscow & Krog	Boom et al. (2009)
Heterodermia galactophylla (Tuck.) W.L. Culb.	Boom et al. (2009)
Heterodermia lutescens (Kurok.) Follmann	Boom et al. (2009)
Heterodermia obscurata (Nyl.) Trevis.	Boom et al. (2009)
Heterodermia squamulosa (Degel.) W.L. Culb.	Boom et al. (2009)
Heterodermia verrucifera (Kurok.) W.A. Weber	Boom et al. (2009)
Hyperphyscia adglutinata (Flörke) H. Mayrhofer & Poelt	Boom et al. (2009), present study
Hyperphyscia minor (Fée) D.D. Awasthi	Boom et al. (2009)
Lathagrium neglectum (Degel.) Otálora, P.M. Jørg. & Wedin	Boom et al. (2009) as *Collema neglectum* Degel., present study
Lecanactis epileuca (Nyl.) Tehler	Boom et al. (2009)
Lecanora galactiniza Nyl.	Boom et al. (2009)
Lecanora legalloana Elix & Øvstedal	Boom et al. (2009), present study
Lecanora leproplaca Zahlbr.	present study
Lecanora leprosa Fée	Boom et al. (2009), present study
Lecanora prosecha Ach.	Boom et al. (2009), present study
Lecanora sulfurescens Fée	Boom et al. (2009), present study
Leiorreuma exaltatum (Mont. & Bosch) Staiger	Boom et al. (2009)
Lepraria finkii (B. de Lesd.) R.C. Harris	present study
Leprocollema novacaledonianum A.L. Sm.	present study
Leptogium austroamericanum (Malme) C.W. Dodge	Boom et al. (2009)
Leptogium azureum (Sw.) Mont.	Boom et al. (2009)
Leptogium cyanescens (Rabenh.) Körb.	Boom et al. (2009)
Leptogium marginellum (Sw.) Gray	Boom et al. (2009)
Letrouitia domingensis (Pers.) Hafellner & Bellem.	Boom et al. (2009), present study
Leucodecton bisporum (Nyl.) Sipman & Lücking	Boom et al. (2009)
Leucodecton compunctum (Ach.) A. Massal.	Boom et al. (2009)
Loflammia gabrielis (Müll. Arg.) Vezda	Boom et al. (2009)
Malmidea piperis (Spreng.) Kalb, Rivas Plata & Lumbsch	Boom et al. (2009) as *Malcolmiella piperis* (Spreng.) Kalb & Lücking, present study
Malmidea psychotrioides (Kalb & Lücking) Kalb, Rivas Plata & Lumbsch	present study
Malmidea vinosa (Eschw.) Kalb, Rivas Plata & Lumbsch	Boom et al. (2009) as *Malcolmiella vinosa* (Eschw.) Kalb & Lücking, present study
Mazosia carnea (Eckfelt) Aptroot & M. Cáceres	Boom et al. (2009) as *Mazosia ocellata* (Nyl.) R.C. Harris, present study
Mazosia phyllosema (Nyl.) Zahlbr.	Boom et al. (2009)
Megalaria bengalensis Jagadeesh Ram & Aptroot	Hensen (BR)
Melanotrema meiospermum (Nyl.) Frisch	Boom et al. (2009)
Microtheliopsis uleana Müll. Arg.	present study

Species	References
Mycoporum eschweileri (Müll. Arg.) R.C. Harris	present study
Myriostigma candidum Kremp.	Boom et al. (2009) as *Cryptothecia candida* (Kremp.) R. Sant.: incorrect report
Myriotrema myriotremoides (Nyl.) Hale	Boom et al. (2009)
Myriotrema olivaceum Fée	Boom et al. (2009)
Nyungwea anguinella (Nyl.) Aptroot	Boom et al. (2009) as *Enterographa anguinella* (Nyl.) Redinger, present study
Ocellularia depressa (Mont.) Hale	Boom et al. (2009)
Ocellularia interposita (Nyl.) Hale	Boom et al. (2009)
Ocellularia terebrata (Ach.) Müll. Arg.	Boom et al. (2009)
Opegrapha astraea Tuck.	present study
Opegrapha lithyrgiza Vain.	present study
Opegrapha quintana Redinger	present study
Pannaria prolificans Vain.	Boom et al. (2009)
Parmotrema crinitum (Ach.) M. Choisy	Boom et al. (2009)
Parmotrema endosulphureum (Hillmann) Hale	Boom et al. (2009)
Parmotrema praesorediosum (Nyl.) Hale	Boom et al. (2009)
Parmotrema tinctorum (Nyl.) Hale	Boom et al. (2009)
Parmotrema ultralucens (Krog) Hale	Boom et al. (2009)
Peltula bolanderi (Tuck.) Wetmore	Boom et al. (2009), present study
Peltula obscurans (Nyl.) Gyeln.	Boom et al. (2009), present study
Pertusaria coccopoda Vain.	Boom et al. (2009), present study
Pertusaria leioplacella Nyl.	Boom et al. (2009)
Pertusaria praetervisa Vain.	Boom et al. (2009), present study
Pertusaria texana Müll. Arg.	present study
Pertusaria xanthodes Müll. Arg.	Boom et al. (2009), present study
Phaeographis crispata Kalb & Matthes-Leicht	present study
Phaeographis dendritica (Ach.) Müll. Arg.	Boom et al. (2009), present study
Phaeographis intricans (Nyl.) Staiger	Boom et al. (2009)
Phaeographis scalpturata (Ach.) Staiger	Boom et al. (2009)
Phyllopeltula corticola (Büdel & R. Sant.) Kalb	present study
Phyllopsora chlorophaea (Müll. Arg.) Zahlbr.	Boom et al. (2009)
Phyllopsora corallina (Eschw.) Müll. Arg.	Brako (1991), Boom et al. (2009), present study
Phyllopsora glaucescens Timdal	Boom et al. (2009)
Phyllopsora parvifoliella (Nyl.) Müll. Arg.	Brako (1991), Boom et al. (2009)
Physcia atrostriata Moberg	Boom et al. (2009), present study
Physcia crispa Nyl.	Boom et al. (2009)
Physcia erumpens Moberg	present study
Physcia integrata Moberg	present study
Physcia sinuosa Moberg	Boom et al. (2009)
Physcia sorediosa (Vain.) Lynge	Boom et al. (2009), present study
Physcia tenuis Moberg	Boom et al. (2009)
Platythecium colliculosum (Mont.) Staiger	Boom et al. (2009)
Platythecium leiogramma (Nyl.) Staiger	Boom et al. (2009)
Polymeridium quinqueseptatum (Nyl.) R.C. Harris	Boom et al. (2009)
Porina conspersa Malme	present study
Porina epiphylla (Fée) Fée	Boom et al. (2009), present study
Porina internigrans (Nyl.) Müll. Arg.	Boom et al. (2009), present study
Porina leptalea (Durieu & Mont.) A.L. Sm.	present study
Porina mastoidea (Ach.) Müll. Arg.	Hensen (BR), Boom et al. (2009)
Porina nitidula Müll. Arg.	Boom et al. (2009)
Porina nucula Ach.	Boom et al. (2009), present study
Porina octomera (Müll. Arg.) F.Schill.	Boom et al. (2009)

Species	References
Porina rubentior (Stirt.) Müll. Arg.	present study
Porina tetracerae (Ach.) Müll. Arg.	Boom et al. (2009), present study
Porina thaxteri R. Sant.	present study
Pseudochapsa dilatata (Müll. Arg.) Parnmen, Lücking & Lumbsch	Boom et al. (2009) as *Chapsa dilatata* (Müll. Arg.) Kalb
Pseudopyrenula subgregaria Müll. Arg.	Boom et al. (2009)
Pseudopyrenula subnudata Müll. Arg.	Harris (1998) as *Pseudopyrenula diluta* (Fée) Müll. Arg. var. *degenerans* Vain, Boom et al. (2009) as *Pseudopyrenula diluta*, Aptroot and Lücking (2016)
Pyrenopsis antillarum Vain.	present study
Pyrenula adacta Fée	present study
Pyrenula astroidea (Fée) R.C. Harris	Boom et al. (2009)
Pyrenula bahiana Malme	Boom et al. (2009) as *Pyrenula concatervans* (Nyl.) R.C. Harris
Pyrenula breutelii (Müll. Arg.) Aptroot	Boom et al. (2009) as *Pyrenula macularis* (Zahlbr.) R.C. Harris, present study
Pyrenula cinerea Zahlbr.	Boom et al. (2009)
Pyrenula cocoes Müll. Arg.	Boom et al. (2009), present study
Pyrenula confinis (Nyl.) R.C. Harris	Boom et al. (2009)
Pyrenula cruenta (Mont.) Vain.	present study
Pyrenula dissimulans (Müll. Arg.) R.C. Harris	present study
Pyrenula duplicans (Nyl.) Aptroot	Boom et al. (2009)
Pyrenula leucostoma Ach.	Boom et al. (2009)
Pyrenula mamillana (Ach.) Trevis.	Boom et al. (2009) as *Pyrenula xyloides* (Eschw.) Müll. Arg.
Pyrenula massariospora (Starbäck) R.C. Harris	Boom et al. (2009)
Pyrenula microtheca R.C. Harris	Boom et al. (2009)
Pyrenula nitidula (Bres.) R.C. Harris	Boom et al. (2009), present study
Pyrenula septicollaris (Eschw.) R.C. Harris	Boom et al. (2009)
Pyxine cocoes (Sw.) Nyl.	Boom et al. (2009), present study
Ramalina anceps Nyl.	Boom et al. (2009)
Ramalina complanata (Sw.) Ach.	Boom et al. (2009)
Ramalina dendroides (Nyl.) Nyl.	Boom et al. (2009)
Ramalina furcellata (Ach.) Zahlbr.	Boom et al. (2009)
Ramalina stoffersii Sipman	present study
Rinodina antillarum Vain.	present study
Rinodina colobinoides (Nyl.) Müll. Arg.	present study
Rinodina pyxinoides Vain.	Boom et al. (2009), present study
Roccella gracilis Bory	Boom et al. (2009), Aptroot and Schumm (2011)
Roccellographa circumscripta (Leight.) Ertz & Tehler	Boom et al. (2009) as *Peterjamesia circumscripta* (Taylor) D. Hawksw.
Sarcographa heteroclita (Mont.) Zahlbr.	Boom et al. (2009)
Sarcographa labyrinthica (Ach.) Müll. Arg.	Boom et al. (2009)
Sarcographa ramificans (Kremp.) Staiger	Boom et al. (2009)
Sarcographa tricosa (Ach.) Müll. Arg.	Boom et al. (2009), present study
Sclerophyton elegans Eschw.	Boom et al. (2009)
Sclerophyton trinidadense Sparrius	Boom et al. (2009)
Sporopodium phyllocharis (Mont.) A. Massal.	Boom et al. (2009)
Squamulea subsoluta (Nyl.) Arup, Søchting & Frödén	Boom et al. (2009) as *Caloplaca subsoluta* (Nyl.) Zahlbr., present study
Staurolemma dussii (Vain.) P.M. Jørg. & Henssen	present study
Stegobolus auberianus (Mont.) Frisch & Kalb	Boom et al. (2009)
Stegobolus granulosus (Tuck.) Frisch	Boom et al. (2009)
Stegobolus subcavatus (Nyl.) Frisch	Boom et al. (2009)
Sticta xanthotropa (Kremp.) D.J. Galloway	Hensen (BR), present study

Species	References
Stigmatochroma gerontoides (Stirt.) Marbach	present study
Stirtonia neotropica Aptroot	Aptroot (2009)
Strigula decipiens (Malme) P.M. McCarthy	present study
Strigula macrospora Vain.	Boom et al. (2009)
Strigula nemathora Mont.	Boom et al. (2009)
Strigula obducta (Müll. Arg.) R.C. Harris	Boom et al. (2009)
Strigula phaea (Ach.) R.C. Harris	present study
Strigula smaragdula Fr.	Boom et al. (2009), present study
Synalissa lichinella Vain.	Boom et al. (2009)
Syncesia decussans (Nyl.) Tehler	present study
Syncesia farinacea (Fée) Tehler	Boom et al. (2009), Sipman (2009)
Syncesia glyphysoides (Fée) Tehler	Tehler (1997), Boom et al. (2009), Sipman (2009)
Syncesia graphica (Fr.) Tehler	Boom et al. (2009), Sipman (2009)
Syncesia subintegra Sipman	Boom et al. (2009), Sipman (2009)
Teloschistes flavicans (Sw.) Norman	Boom et al. (2009) as *Teloschistes flavicans* var. *crocea* (Ach.) Müll. Arg.
Thalloloma hypoleptum (Nyl.) Staiger	Boom et al. (2009)
Thelenella luridella (Nyl.) H. Mayrhofer	present study
Thelotrema porinoides Mont. & Bosch	Boom et al. (2009)
Toninia submexicana B. de Lesd.	Boom et al. (2009)
Trapelia coarctata (Sm.) M. Choisy	Boom et al. (2009)
Usnea baileyi (Stirt.) Zahlbr.	Boom et al. (2009)
Varicellaria velata (Turner) I. Schmitt & Lumbsch	Boom et al. (2009)
Verrucaria nigrescens Pers.	present study
Xanthoparmelia succedans Elix & J. Johnst.	Boom et al. (2009)
Lichenicolous fungus	
Dactylospora saxatilis (Schaer.) Hafellner (lichenicolous on *Pertusaria praetervisa*)	present study
Additional genera (species uncertain)	
Acanthothecis sp.	present study
Bacidina sp.	present study
Melaspilea sp. (lichenicolous fungus)	present study
Psorotichia sp.	present study
Stigmidium sp. (lichenicolous fungus)	present study
Thelidium sp.	present study
Wetmoreana sp.	present study

ever, is closest to the *Catapyrenium-Placidiopsis-Verrucaria* p.p. (*V. caerulea* DC., *V. praetermissa* (Trevis.) Anzi) clade (Muggia et al. 2010) with sequence identities of 96–97 %. The placement of the *Verrucaria* cf. *dolosa* specimen is more difficult to assess, since its sequence shows lower similarities of 92–94 % to representatives of different Verrucariceae genera, such as *Agonimia*, *Bagliettoa* and *Verrucaria* spp.

Finally, the mtSSU sequence of the *Wetmoreana* cf. *appressa* specimen from St. Eustatius is difficult to interpret, since it matches more closely with sequences of the Xanthorioideae (sequence identity 97–99 %) than with Teloschistoideae, in which *Wetmoreana* is placed (e.g. Arup et al. 2013).

The lichen flora of St. Eustatius can be characterised as lowland, relatively dry Caribbean. As can be seen from Table 1, most species were found on one substratum

type, but some are less specialised. Also, there is a marked difference between the lichens of the different plots and the three main areas on St. Eustatius (Northern Hills, central urban area, The Quill). However, the authors refrain from performing statistical comparisons of the lichen diversity between plots, since the number of plots per main area differs and is still low and the sampling strategy was devised by specialists of other organism groups. Nevertheless, the lichen data will be useful for an island-wide, plot-based comparison of diversity amongst all organism groups sampled during the 2015 inventory.

In Table 2, an updated checklist is presented of the lichens of St. Eustatius, citing only identified species, but based on all available sources and with their taxonomy (nomenclature and sometimes species concept) updated where necessary. According to this list, a total of 263 species are currently known from St. Eustatius. As a side effect of revising the existing records, one record becomes questionable, *viz*. *Myriostigma candidum* Kremp., which is not known from the Neotropics. It is intended to continue the exploration of the lichens of this island in the near future.

Acknowledgements

This research was funded by Naturalis Biodiversity Center. Fieldwork was co-organized by T. van Andel, B. van der Hoorn and J. Miller (Naturalis) and facilitated by the Caribbean Netherlands Science Institute (CNSI), St. Eustatius National Parks (STENAPA), DCNA Island Conservationists H. Madden and A. van Zanten and field guide C. Gibbs. Students E. Haber, T. Huijts, C. Posthouwer, T. Verheijden, R. Vogel and S. Zwartsenberg of the Leiden University course Tropical Biodiversity and Field Methods as well as R. Butôt (Naturalis) contributed to collecting. Student participation was facilitated by the Alberta Mennega Stichting and the Van Eeden Fonds. Koos Biesmeijer facilitated the Naturalis Caribbean programme and participated in the fieldwork. Marlon Murray and the St. Eustatius Historical Foundation allowed us to sample the urban plot. Molecular lab work was carried out by R. Butôt. We are indebted to H. Sipman (Berlin) for providing a copy of the unpublished keys, descriptions and specimen citation that was the basis for the internet checklist by Boom et al. (2009).

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MycoKeys 33: 85–102 (2018)
doi: 10.3897/mycokeys.33.24704
http://mycokeys.pensoft.net

RESEARCH ARTICLE

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The Northeast Chinese species of *Psathyrella* (Agaricales, Psathyrellaceae)

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Academic editor: *M.P. Martín* | Received 27 February 2018 | Accepted 1 April 2018 | Published 13 April 2018

Citation: Yan J-Q, Bau T (2018) The Northeast Chinese species of *Psathyrella* (Agaricales, Psathyrellaceae). MycoKeys 33: 85–102. https://doi.org/10.3897/mycokeys.33.24704

Abstract

Twenty seven species of *Psathyrella* have been found in Northeast China. Amongst them, *P. conica*, *P. jilinensis*, *P. mycenoides* and *P. subsingeri* are described as new species, based on studying morphological characteristics and phylogenetic analyses. Detailed morphological descriptions, line drawings and photographs of the new species are presented. Phylogenetic analysis of the nuclear ribosomal internal transcribed spacer (ITS) region and an identification key to the 27 *Psathyrella* species occuring in Northeast China are provided.

Keywords

Basidiomycete, new taxon, phylogenetic analysis, taxonomy

Introduction

Psathyrella (Fr.) Quél. is one of the large genera of Agaricales Underw. which consists of 1,030 records in Index Fungorum (http://www.indexfungorum.org), comprising approximately 500 species (Smith 1972; Kits van Waveren 1985; Örstadius and Kundsen 2012;). It is characteristic of fragile basidiomata, hygrophanous pileus, brown to

black brown spore print, always present cheilocystidia and basidiospores smooth or rarely granulose or with myxosporium, fading to greyish in concentrated sulphuric acid (H_2SO_4).

The studies of this genus mainly focused on Europe and North America in recent years (Romagnesi 1952; Smith 1972; Kits van Waveren 1985; Nagy et al. 2011; Örstadius and Kundsen 2012; Örstadius et al. 2015). In China, 51 names (*Psathyrella* s.l.) were reported, including four new species (Chiu 1973; Bi et al. 1985; Bi et al. 1987; Bi 1991; Wang and Bau 2014). Amongst them, 21 species can be found in Northeast China which includes Helongjiang Province, Jinlin Province, Liaoning Province and the northeast of Inner Mongolia Autonomous Region (Wang 2014).

Due to the morphological plasticity of the *Psathyrella*, some species cannot be distinguished clearly and many names have been combined (Örstadius and Kundsen 2012). Therefore, the aim of this study is to clarify the diversity of *Psathyrella* in Northeast China by traditional taxonomy and molecular phylogenetic analysis. The examined specimens (from 1997 to 2017) are deposited in the Herbarium of Mycology, Jilin Agricultural University (HMJAU). As a result of morphological and molecular observations, 27 species of *Psathyrella* were identified, and of which *P. conica*, *P. jilinensis*, *P. mycenoides* and *P. subsingeri* were reported as new species. Molecular phylogenetic affinities of the 27 species based on the nuclear ribosomal internal transcribed spacer (ITS) region and an identification key to them are provided.

Materials and methods

Morphological studies

Specimens are deposited in the Herbarium of Mycology, Jilin Agricultural University (HMJAU). Macroscopic characteristics were recorded from fresh specimens. Colour codes are from Kornerup and Wanscher (1978). Samples for microscopic examination were mounted in water and 5% aqueous KOH. Amyloid reactions were diagnosed in Melzer's reagent. Thirty basidiospores, cystidia and basidia were measured for each collection. The basidiospores quotient (Q=L/B) was calculated from measurements of basidiospores.

DNA extraction and sequencing

The NuClean Plant Genomic DNA kit (CWBIO) was employed for DNA extraction and PCR amplification from dried specimens. PCR was performed using a touchdown programme (Yan and Bau 2017) and the ITS region was amplified with the primer pair ITS1 and ITS4 (White et al. 1990). The details of sequenced specimens are given in Table 1. The DNA sequencing was done by Comate Bioscience Co., Ltd., Changcun City, China.

Table 1. Sequenced specimens used in phylogenetic analysis.

Taxa	Voucher	Locality	GenBank accession no. (ITS)
P. amaura (Berk. & Broome) Pegler	HMJAU 37810	Jilin: Qiupi Village, Tonghua City	MG734724
P. bipellis (Quél.) A.H. Sm.	HMJAU 25349	Jilin: Jilin Agricultural University	MG734722
P. borealis	HMJAU 37924	Inner Mongolia Autonomous Region: Mangui Town	MG734743
P. borealis A.H. Sm.	HMJAU 37911	Jilin: Changbai Mountain National Nature Reserve	MG734746
P. boreifasciculata Kytöv. & Liimat.	HMJAU 27556	Heilongjiang: Nanwenghe National Nature Reserve	KX901850
P. candolleana	HMJAU 37994	Jilin: Dayangcha, Erdaobaihe Town	MG734719
P. candolleana (Fr.) Maire	HMJAU 37994	Liaoning: Wulong Mountain	MG734720
P. conica	HMJAU 22096	Jilin: Lushuihe Town, Baishan City	MG734713
P. conica	HMJAU 37846 Type	Jilin: Changbai Mountain National Nature Reserve	MG734739
P. conica	HMJAU 37905	Jilin: Changbai Mountain National Nature Reserve	MG734745
P. effibulata Örstadius & E. Ludw.	HMJAU 37832	Jilin: Jingyuetan National Scenic Area	MG734727
P. fennoscandica Örstadius & E. Larss.	HMJAU 37918	Heilongjiang: Shuanghe National Nature Reserve	MG734723
P. gordonii	HMJAU 35984	Jilin: Jilin Agricultural University	KX901852
P. gordonii (Berk. & Broome) A. Pearson & Dennis	HMJAU 35983	Jilin: Jilin Agricultural University	KY120974
P. jilinensis	HMJAU 37822 Type	Jilin: Changbai Mountain National Nature Reserve	MG734717
P. jilinensis	HMJAU 37824	Jilin: Changbai Mountain National Nature Reserve	MG734721
P. lutensis (Romagn.) M.M. Moser	HMJAU 37840	Inner Mongolia Autonomous Region: Huihe National Nature Reserve	MG734748
P. luteopallida A.H. Sm.	HMJAU 5148	Jilin: Zuojia Town, Jilin City	MG734736
P. mammifera	HMJAU 21908	Jilin: Mahutou Mountain, Changchun City	MG734734
P. mammifera (Romagn.) Courtec.	HMJAU 37882	Jilin: Changbai Mountain National Nature Reserve	MG734740
P. mycenoides	HMJAU 37888 Type	Jilin: Jilin Agricultural University	MG734730
P. mycenoides	HMJAU 37993	Jilin: Jilin Agricultural University	MG734731
P. obtusata	HMJAU 37307	Jilin: Changbai Mountain National Nature Reserve	KY224080
P. obtusata (Pers.) A.H. Sm.	HMJAU 37310	Jilin: Changbai Mountain National Nature Reserve	KY224081
P. panaeoloides (Maire) Arnolds	HMJAU 23696	Jilin: Lushuihe Town, Baishan City	MG734733
P. pertinax (Fr.) Örstadius	HMJAU 6830	Jilin: Changbai Mountain National Nature Reserve	MG734735
P. phegophila	HMJAU 37848	Jilin: Songjiang Town	MG734738
P. phegophila	HMJAU 37804	Heilongjiang: Shengshan National Nature Reserve	MG734726
P. phegophila Romagn.	HMJAU 28267	Inner Mongolia Autonomous Region: Baiyin'aobao National Nature Reserve	MG734728
P. piluliformis (Bull.) P.D. Orton	HMJAU 37922	Heilongjiang: Shuanghe National Nature Reserve	MG734716
P. pygmaea (Bull.) Singer	HMJAU 37850	Jilin: Changbai Mountain National Nature Reserve	MG734744
P. senex (Peck) A.H. Sm.	HMJAU 4450	Inner Mongolia Autonomous Region: Hulunbeier City	MG734732

Taxa	Voucher	Locality	GenBank accession no. (ITS)
P. singeri A.H. Sm.	HMJUA 37867	Jilin: Changbai Mountain National Nature Reserve	MG734718
P. spintrigeroides	HMJAU 37820	Jilin: Changbai Mountain National Nature Reserve	MG367203
P. spintrigeroides P.D. Orton	HMJAU 37901	Jilin: Changbai Mountain National Nature Reserve	MG734737
P. squamosa	HMJAU 37816	Heilongjiang: Nanwenghe National Nature Reserve	MG367206
P. squamosa (P. Karst.) A.H. Sm.	HMJAU 35923	Jilin: Lushuihe Town, Baishan City	MG734729
P. subsingeri	HMJAU 37814	Yunnan: Yeya Lake	MG734714
P. subsingeri	HMJAU 37811	Jilin: Jilin Agricultural University	MG734715
P. subsingeri	HMJAU 37913 Type	Jilin: ingyuetan National Scenic Area	MG734725
P. subsingeri	HMJAU 37915	Henan: Boerdeng National Forest Park	MG734742
P. subspadiceogrisea	HMJAU 35992 Type	Jilin: Changbai Mountain National Nature Reserve	KY678465
P. subspadiceogrisea T. Bau & J.Q. Yan	HMJAU 35996	Jilin: Changbai Mountain National Nature Reserve	KY678466
P. subterrestris A.H. Sm.	HMJAU 37885	Jilin: Changbai Mountain National Nature Reserve	MG734747
P. subterrestris	HMJAU 37887	Jilin: Songjiang Town	MG734741

Data analyses

ITS1+5.8S+ITS2 sequences of 27 species were tested with BLAST in GenBank. Fifty five sequences were downloaded from GenBank, including 21 type species of *Psathyrella*, based on BLAST results and referred to the recent studies (Nagy et al. 2013; von Bonsdorff et al. 2014; Örstadius et al. 2015; Yan and Bau 2017). A total of 103 ITS sequences were aligned using MAFFT 7.205 (Katoh and Standley 2013). The aligned ITS dataset consisted of 643 nucleotide sites (including gaps). The best model (GTR+I+G) was selected by AIC in MRMODELTEST 2.3 (Nylander 2004). Bayesian Inference (BI) was performed with MRBAYES 3.2.6 and four Markov Chains (MCMC) were run for three million generations, sampling every 300th generation. The first 25% trees were discarded (Ronquist and Huelsenbeck 2003). Maximum likelihood analysis was performed with IQTREE 1.5.6 (Nguyen et al. 2014).

Results

The phylogenetic tree (Figure 1) shows that all studied materials fall into *Psathyrella*, with a high statistical support value (BPP=1). It is divided into 14 clades. Most of them have a high statistical support value (BPP≥0.95, Bootstrap≥75), except /fibrillosa I and /fibrillosa II.

Four new species are separated into individual lineages (BPP=1, Bootstrap=100) and are independent from the close taxa. *Psathyrella conica* forms a distinct lineage in /fibrillosa II; *P. jilinensis* belongs to /fibrillosa II and groups together with *P. borealis*; *P. mycenoides* belongs to /prona and is closely related to *P. lilliputana* Örstadius & E. Larss.; and *P. subsingeri* forms a distinct lineage in /candolleana.

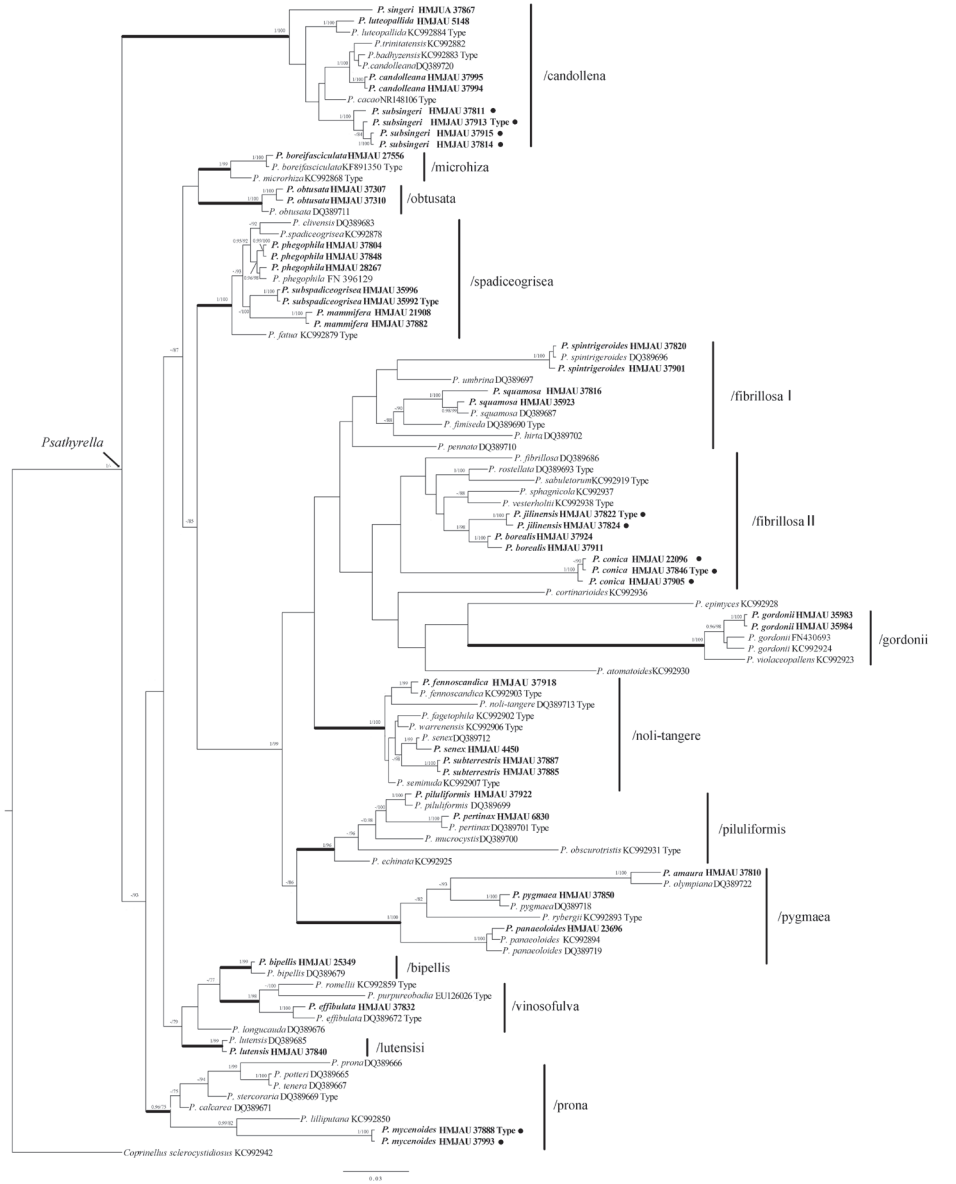

Figure 1. Bayesian and Maximum Likelihood tree inferred from partial ITS sequence data (BPP≥0.95, Bootstrap≥75 are indicated). The tree is rooted with *Coprinellus sclerocystidiosus* (M. Lange & A.H. Sm.) Vilgalys, Hopple & Jacq. Johnson. Newly generated sequences appear in bold. ● indicates newly described species.

The positions of some species are firstly supplemented: *P. amaura* belongs to /pygmaea and is very close to *P. olympiana* A.H. Sm.; *P. borealis* belongs to /fibrillosa II. *P. mammifera* belongs to /spadiceogrisea; *P. singeri* A.H. Sm. belongs to /candolleana; and *P. subterrestris* belongs to /noli-tangere.

Taxonomy

Psathyrella conica **T. Bau & J.Q. Yan, sp. nov.**
MycoBank: MB823858
Figs 2a–b, 3

Diagnosis. Pileus campanulate to conical, with a subacute to obtuse umbo in early stage. Lamellae 3.0–5.0 mm broad, close. Basidiospores 7.8–8.8 × 4.0–4.5(–5.0) µm, germ pore indistinct or absent. Pleurocystidia numerous, narrowly utriform, with obtuse to broad obtuse or slightly subcapitate at apex. Cheilocystidia scattered.

Holotype. CHINA. Jilin Province, Yanbian Korean Autonomous Prefecture, Antu County, Changbai Mountain, 30 Jun 2017, HMJAU 37846.

Etymology. Name refers to the conical pileus.

Description. Pileus 12–45 mm, campanulate to conical, with a subacute to obtuse umbo in early stage, hygrophanous, chestnut (7D4–7D6), becoming dirty white with slightly yellowish-brown (6C5–6C6) as drying, striate indistinctly. Veil with a thin coating of white to dirty white (6A1–6B1) fibrils, evanescent. Context dirty white with slightly pink (6B4–6B5), about 3.0 mm thick at stipe centre. Lamellae 3.0–5.0 mm broad, close, adnate to slightly adnexed, coffee-cream (6C4–6C6); edges white (6A1), saw-toothed under 20× magnifier. Stipe 34–85 × 2.0–7.0 mm, cylindrical, slightly

Figure 2. Basidiomata of *Psathyrella* species. **a–b** *Psathyrella conica* **c–e** *Psathyrella jilinensis* **f** *Psathyrella mycenoides* **g–i** *Psathyrella subsingeri*; Bars: 10 mm (**a, c, d, f–h**). Photographs **a–e, g–i** by Jun-Qing Yan; Photograph **f** by Tolgor Bau.

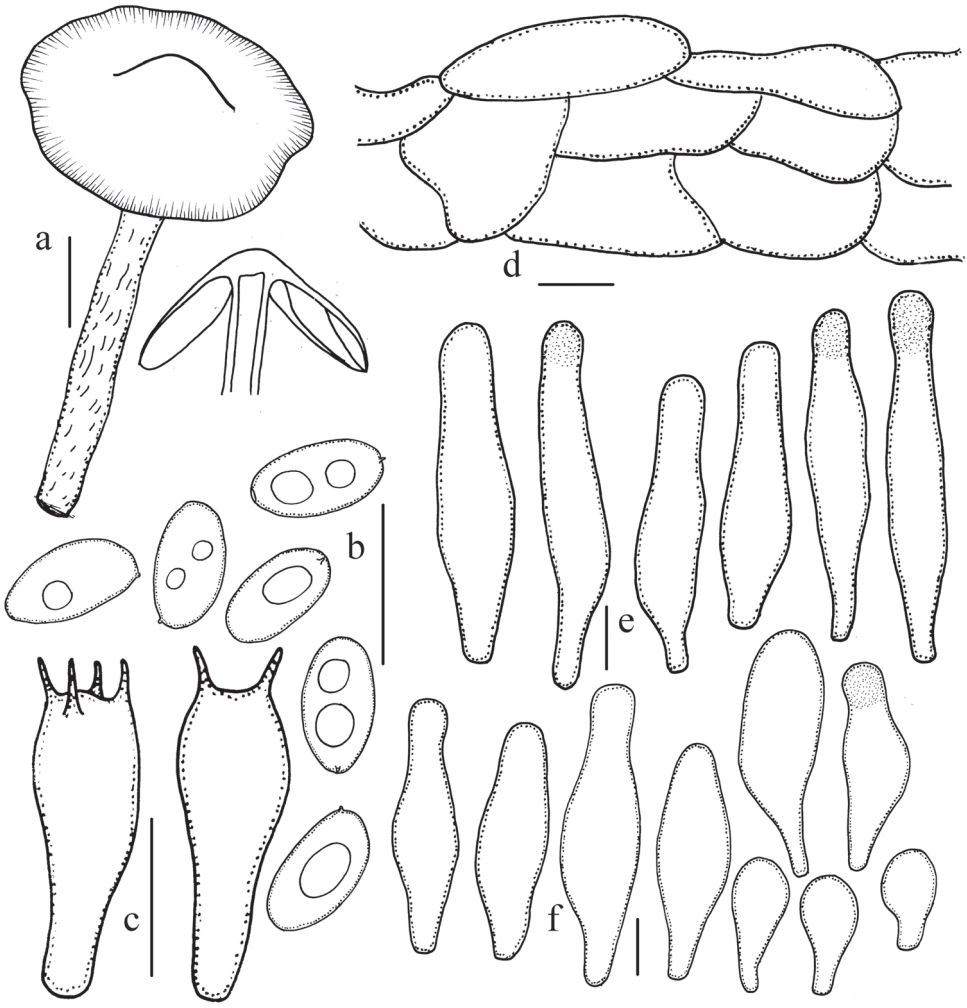

Figure 3. Microscopic features of *Psathyrella conica* (HMJAU 37846). **a** Basidiomata **b** Basidiospores **c** Basidia **d** Pileipellis **e** Pleurocystidia **f** Cheilocystidia. Bars: 10 mm (**a**); 10 μm (**b–f**). Drawing by Jun-Qing Yan.

expanded or not at base, white, with slightly brown at base, hollow, equal, surface covered with white (6A1) fibrils in early stage, evanescent. Odour and taste indistinctive.

Basidiospores 7.8–8.8 × 4.0–4.5(–5.0) μm, Q=1.8–2.1(–2.3), oblong-ellipsoid to oblong, in profile slightly flattened on one side, pale yellowish-brown in water, yellowish-brown to brown in 5% potassium hydroxide (KOH), inamyloid, smooth, with 1–2 guttulate, germ pore indistinct or absent. Basidia 20–25 × 7.3–9.8 μm, clavate, hyaline, 4- or 2-spored. Pleurocystidia 43–61 × (8.5–)9.8–12 μm, numerous, narrowly utriform, thin-walled, hyaline, with obtuse to broad obtuse or slightly subcapitate, sometimes adhering subhyaline deposits. Cheilocystidia scattered, similar to pleurocystidia, 24–39 × 8.5–12 μm; spheropedunculate or clavate cells abundant, 20–29 ×

12–18 μm. Trama of gills irregular, up to 20 μm broad. Pileipellis consisting of 2–3 cells deep layer of subglobose cell, 25–37 μm broad. Clamps present.

Habit and habitat. Solitary to scattered on rotten wood or humus in mixed forests.

Other specimens examined. Jilin Province, Baishan City, Fusong County, Lushuihe town, 7 Jul 2004, HMJAU 4969; 29 Jun 2005, HMJAU 4923; 25 Jun 2009, HMJAU 22096; Yanbian Korean Autonomous Prefecture, Antu County, Changbai Mountain, 23 Jun 2012, HMJAU 25342; 4 Jul 2015, HMJAU 37826; 29 Jun 2017, HMJAU 37847, HMJAU 37904; 6 Aug 2017, HMJAU 37905.

Psathyrella jilinensis T. Bau & J.Q. Yan, sp. nov.

MycoBank: MB823856
Figs 2c–e, 4

Diagnosis. Pileus paraboloid to convex, margin at first appendiculate with adhering patches of white evanescent inner veil. Lamellae 2.0–5.0 mm broad, moderately close. Basidiospores (5.8–)6.3–7.3(–7.8) × (2.9–)3.4–4.4 μm, germ pore absent or indistinct. Pleurocystidia fusiform to narrowly fusiform. Cheilocystidia similar to pleurocystidia. Cheilocystidia and pleurocystidia covered by hyaline, hemispherical amorphous incrustation at apex.

Holotype. CHINA. Jilin Province: Changbai Mountain, Antu County, Yanbian Korean Autonomous Prefecture, 42°23'51"N, 126°05'47"E, 760 m alt., 7 Jul 2015, HMJAU 37822.

Etymology. Name refers to the type locality where the new species was collected.

Description. Pileus 17–45 mm, paraboloid to convex, hygrophanous, reddish-brown (8E5–8E6) at centre, pale yellowish-brown (7C6–7D7) at margin in early stage, yellowish-brown (6B5–6C5), striate up to 1/2 from margin at maturity, becoming slightly brown (7C5–7D6) as pileus dries. Veil white (6A1), thin, fibrillose, at first as appendiculate inner veil or adhering patches at pileus margin, evanescent. Context white (6A1), thin, very fragile, about 2.0 mm thick at centre. Lamellae 2.0–5.0 mm broad, moderately close, adnate, greyish to greyish-brown (7C1–7C3); edges saw-toothed under 20× magnification. Stipe 40–50 × 3.0–7.0 mm, white (6A1), cylindrical, hollow, surface covered with slight white (6A1) evanescent fibrils. Odour and taste indistinctive.

Basidiospores (5.8–)6.3–7.3(–7.8) × (2.9–)3.4–4.4 μm, Q= (1.4–)1.8–2.0(–2.3), oblong-ellipsoid, in profile flattened on one side, pale brown in water, brown in 5% KOH, gradually becoming greyish-brown, inamyloid, smooth, germ pore absent or indistinct, about 0.9 μm wide (if it can be observed). Basidia 15–17 × 6.0–7.0 μm, clavate, hyaline, 4 or 2-spored. Pleurocystidia fusiform, narrowly fusiform, rarely narrowly utriform, thin-walled or slightly thick-walled, apex obtuse to subacute, hyaline, covered by hyaline, hemispherical amorphous incrustation, which can dissolve in 5% KOH. Cheilocystidia 37–51 × 8.5–12 μm, similar to pleurocystidia, hyaline, covered

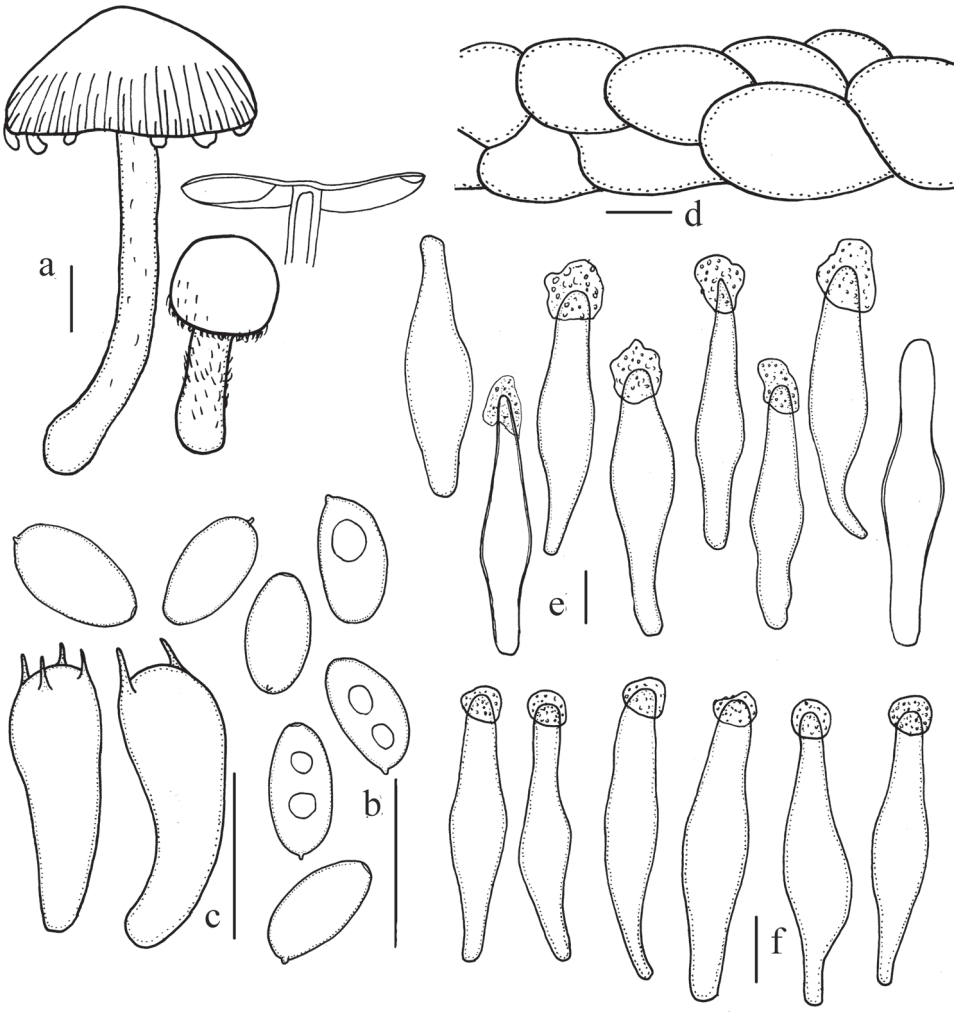

Figure 4. Microscopic features of *Psathyrella jilinensis* (HMJAU 37822). **a** Basidiomata **b** Basidiospores **c** Basidia **d** Pileipellis **e** Pleurocystidia **f** Cheilocystidia. Bars: 10mm (**a**); 10μm (**b–f**). Drawing by Jun-Qing Yan.

with amorphous incrustation at apex. Trama of gills parallel to hyphae, up to 15 μm broad. Pileipellis consisting of 2–3 cells deep layer of subglobose cell, 20–30 μm broad. Veil composed of cylindrical hyphae, 8.5–10 μm broad. Clamps present.

Habit and habitat. Solitary to scattered on rotten wood or humus in mixed forests.

Other specimens examined. Jilin Province, Baishan City, Fusong County, Lushuihe town, 27 Jun 2009, HMJAU 22099; 9 Jul 2015, HMJAU 37823; Yanbian Korean Autonomous Prefecture, Antu County, Changbai Mountain, 23 Jun 2012, HMJAU 25351; 31 Aug 2012, HMJAU 25351; Dayangcha, 6 Jul 2015, HMJAU 37824.

***Psathyrella mycenoides* T. Bau, sp. nov.**
MycoBank: MB823857
Figs 2f, 5

Diagnosis. Pileus 4.0–5.0 mm, hemispherical to convex. Stipe slender. Basidiospores 8.8–9.2(–9.7) × 4.9–5.4 μm, germ pore distinct, but small. Pleurocystidia scattered, fusiform to lageniform with an obtuse apex. Cheilocystidia lageniform, with an obtuse apex or clavate to spheropedunculate with a long or short mucronate apex.

Holotype. CHINA. Jilin Province, Changchun City, Jilin Agricultural University, 43°48'36"N, 125°24'25"E, 220 m alt., 10 Sep 2016, HMJAU 37888.

Etymology. Name refers to its macroscopic characteristics similar to *Mycena*.

Description. Pileus 4.0–5.0 mm, hemispherical to convex, dirty white with pinkish (7A4–7B5), hygrophanous, striate up to centre from margin. Veil not observed. Context very thin and very fragile, about 0.5 mm thick at stipe centre. Lamellae 1.5–2.0 mm broad, adnate to slightly adnexed, pale brown (7C3–7C4), edges saw-toothed under 20× magnification. Stipe slender, 25–30 × 0.5–1.0 mm, hygrophanous, subhyaline, cylindrical, hollow, equal, fragile, evanescently pruinose at apex.

Basidiospores 8.8–9.2(–9.7) × 4.9–5.4 μm, Q=1.6–2.0, ellipsoid to oblong- ellipsoid, in profile flattened on one side, pale yellowish-brown in water, becoming dark grey to dark brown in 5% KOH, germ pore distinct, but small, about 0.9 μm broad. Basidia 15–17 × 8.8–10 μm, clavate, hyaline, 4- or 2-spored. Pleurocystidia 37–56 × 12–17 μm, scattered, fusiform to lageniform with an obtuse apex, thin-walled and hyaline. Cheilocystidia numerous, 29–44 × 9.8–17 μm, hyaline, lageniform with an obtuse apex or clavate to spheropedunculate, with long or short mucronate apex, rarely spheropedunculate. Trama of gills irregular, hyphae up to 10 μm broad. Pileipellis hymeniderm, cells 20–30 μm broad. Clamps present.

Habit and habitat. Solitary to scattered on humus in mixed forests.

Other specimens examined. CHINA. Jilin Province, Changchun City, Jilin Agricultural University, 12 Sep 2016, HMJAU 37993.

***Psathyrella subsingeri* T. Bau & J.Q. Yan, sp. nov.**
MycoBank: MB823855
Figs 2g–i, 6

Diagnosis. Pileus 15–40 mm, paraboloid to conical. Lamellae 2.0–4.0 mm broad, close. Basidiospores 5.8–7.8(–8.8) × 3.9–4.4(–5.0) μm, very pale, nearly hyaline or slightly yellow in water and 5% KOH. Germ pore absent. Pleurocystidia absent. Cheilocystidia utriform to predominantly spheropedunculate.

Holotype. CHINA. Jilin Province, Changchun City, Jingyuetan National Scenic Area, 43°47'38"N, 125°26'55"E, 200 m alt., 25 Jun 2017, HMJAU 37913.

Etymology. Name refers to its microscopic characteristics similar to *P. singeri*.

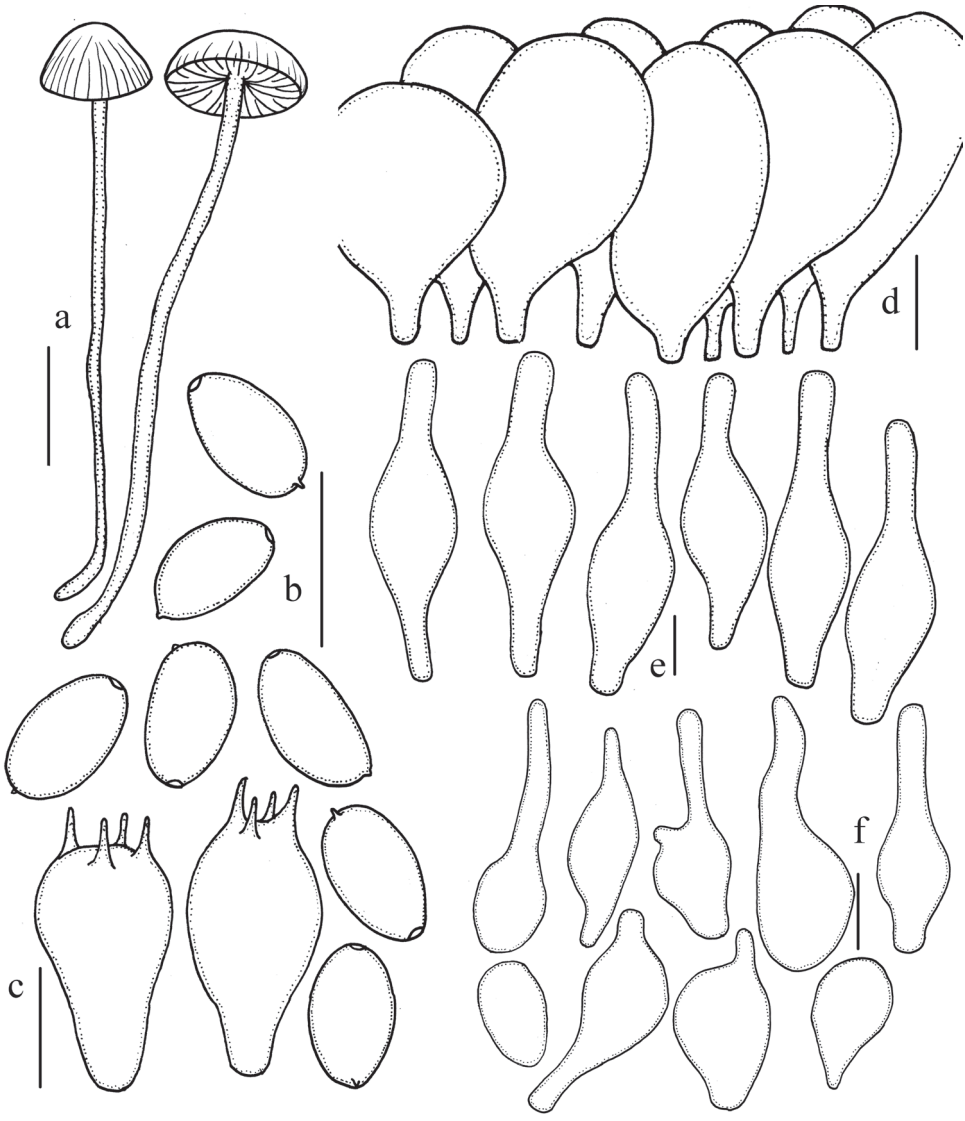

Figure 5. Microscopic features of *Psathyrella mycenoides* (HMJAU 37888). **a** Basidiomata **b** Basidiospores **c** Basidia **d** Pileipellis **e** Pleurocystidia **f** Cheilocystidia. Bars: 10mm (**a**); 10μm (**d–f**). Drawing by Jun-Qing Yan.

Description. Pileus 15–40 mm, paraboloid to conical, obtuse or slightly umbonate at disc, hygrophanous, dark reddish-brown (8E7–8F8) or faint yellowish-brown (5C5–5C4), becoming yellowish-brown (6D5–6D6) as pileus dries, striate indistinct. Veil present in early stage, thin, white (6A1), fibrillose, evanescent. Context white (6A1), thin and very fragile, about 2.5 mm thick at stipe centre. Lamellae 2.0–4.0 mm broad, close, adnate, pale brown (6C4–6C5), edges white (6A1), saw-toothed under

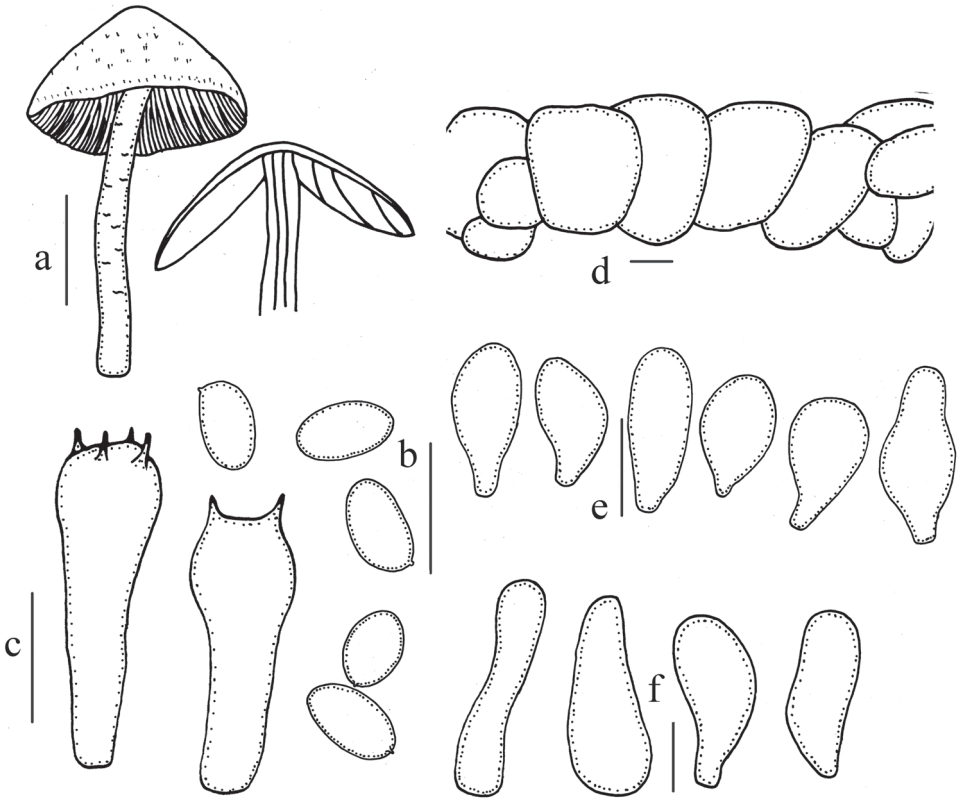

Figure 6. Microscopic features of *Psathyrella subsingeri* (HMJAU 37913). **a** Basidiomata **b** Basidiospores **c** Basidia **d** Pileipellis **e** Cheilocystidia **f** Caulocystidia. Bar: 10 mm (**a**); 10 μm (**b–f**). Drawing by Jun-Qing Yana.

20× magnifier. Stipe 35–50 × 3.0–4.5 mm, cylindrical, hollow, equal, fragile, covered with slight white (6A1) fibrils, which fall off easily. Spore print chocolate (7E7–7E8). Odour and taste indistinctive.

Basidiospores 5.8–7.8(–8.8) × 3.9–4.4(–5.0) μm, Q=1.4–2.0, ellipsoid to oblong-ellipsoid, in profile flattened on one side, very pale, nearly hyaline or slightly yellow in water and 5% KOH, inamyloid, smooth. Germ pore absent. Basidia 15–22 × 7.3–9.8 μm, 4- or 2-spored, clavate, hyaline. Pleurocystidia absent. Cheilocystidia utriform to spheropedunculate, rarely clavate to fusiform with an obtuse to broadly obtuse apex, thin-walled, hyaline. Caulocystidia 26–37 × 9.8–15 μm, rarely, various, clavate, utriform, thin-walled, hyaline. Trama of gills irregular, up to 15 μm broad. Pileipellis consisting of 1–2 cells, deep layer of subglobose cell, 20–32 μm broad. Clamps present.

Habit and habitat. Solitary to scattered on terrestrial or humus in mixed forests.

Other specimens examined. Henan Province, Xinyang City, Boerdeng National Forest Park, 16 Jul 2017, HMJAU 37915; Xian Mountain, 15 Jul 2017, HMJAU

37931; Jilin Province, Changchun City, Jilin Agricultural University, 21 Jun 2016. HMJAU 37811; Jingyuetan National Scenic Area, 25 Jun 2017, HMJAU 37914; 7 Jul 2017, HMJAU 37849; Tonghua City, Qiupi Village, 6 Aug 2015, HMJAU 37812, HMJAU 37813; Yunnan Province, Yeya Lake, 7 Aug 2016, HMJAU 37814; 6 Aug 2017, HMJAU 37852; 23 Aug 2017, HMJAU 37962.

Discussion

These phylogenetic results are very much in congruence with the study of Larsson and Örstadius (2008) and Örstadius et al. (2015), except /fibrillosa, which separates to two lineages (/fibrillosa I and /fibrillosa II). As only ITS sequences were analysed in this study, this accounts for the difference and the very low support value (BPP<0.3). Four new species are separated into individual lineages (BPP=1) and distinct from other closely related taxa.

Psathyrella conica is a distinct lineage in fibrillosa II, which is independent from any other related taxa. Morphologically, it can be classified in subsection *Spadiceogriseae* (Kits van Waveren 1985). Only *P. clivensis* (Berk. & Broome) P. D. Orton does not have a germ pore in this subsection, but basidiospores of *P. clivensis* are obviously broader, 8–10 × 5.5–6.5 µm and ellipsoid to ovoid (Kits van Waveren 1985). It can also be classified in section *Fatuae* (Smith 1972), some species having sturdy stipe and utriform cystidia, but they can be clearly distinguished from *P. conica* by other micromorphology. *Psathyrella acadiensis* A.H. Sm. has smaller basidiospores, which are only up to 6.0 µm long; *P. albocinerascens* A.H. Sm. has an obvious germ pore and white pileus in the early stage; *P. amarella* A.H. Sm. and *P. spadiceogrisea* (Schaeff.) Maire have an obvious germ pore; *P. vesiculocystis* A.H. Sm. has pedicellate-pleurocystidia (Smith 1972). Furthermore, *P. terrestris* Natarajan has aspects of *P. conica*, whose pileus is umbonate, but it has broadly utriform pleurocystidia and its basidiospores are dark brown, subglobose and up to 8.5 µm broad (Natarajan 1978).

Psathyrella jilinensis grouped together with *P. borealis* in /fibrillosa II. However, *P. borealis* has an obvious germ pore. Morphologically, it can be classified in section *Hydrophilae* by basidiospores rarely exceeding 7.5 µm and the presence of pleurocystidia. There are hardly any other species in the section that match the characteristics of *P. jilinensis*. The pleurocystidia of *P. atomatoides* (Peck) A.H. Sm. do not have amorphous incrustation. Basidiospores of *P. cortinarioides* P.D. Orton and *P. pertinax* have a clearly truncated base. Cystidia of *P. umbrina* Kits van Wav. have subacute apex and their basidiospores are broader, up to 4.5–5.5 µm (Kits van Waveren 1985; Örstadius and Kundsen 2012). Furthermore, *P. cokeri* (Murrill) A.H. Sm., *P. pennata* and *P. subsimilissima* A.H. Sm. have some similar aspects of *P. jilinensis*, but *P. cokeri* (Murrill) A.H. Sm. and *P. subsimilissima* A.H. Sm. do not have amorphous incrustation (Smith 1972) and *P. pennata* grows on burnt soil, its basidiospores being larger and narrowly amygdaloid (Örstadius and Kundsen 2012).

Psathyrella mycenoides belongs to /prona and is placed close to *P. lilliputana*. However *P. lilliputana* has larger (9.5–11 × 5.0–6.0 μm) and snout-like basidiospores (Örstadius et al. 2015). Morphologically, more than 10 species of *Psathyrella* have very small basidiomata, whose pileus rarely exceeds 10 mm, but they can be separated by obvious characteristics as follows: *P. byssina* (Murrill) A.H. Sm. and *P. scheppingensis* Arnolds have smaller basidiospores, which rarely exceed 7.5 μm (Smith 1972; Arnolds 2003); *P. coprinoides* A. Delannoy, Chiaffi, Courtec. & Eyssart. and *P. tenuicula* (P. Karst.) Örstadius & Huhtinen have pileocystidia and slender basidiospores (Örstadius and Huhtinen 1996; Delannoy et al. 2002); the coprophilous fungi of *P. granulose* Arnolds have utriform cystidia (Arnolds 2003); basidiospores of *P. liciosae* Contu & Pacioni are partly phaseoliform in side view and ochraceous-brown in 5% KOH (Contu and Pacioni 1998); *P. minima* Peck has very distant lamellae (Peck 1878); and basidiospores of *P. psilocyboides* A.H. Sm. are truncated at the base (Smith 1972).

Psathyrella subsingeri belongs to /candollena. Only *P. luteopallida* and *P. singeri* have nearly hyaline basidiospores pores in this clade. However, the basidiospores of *P. luteopallida* are longer than 8.0 μm. The basidiospores of *P. singeri* are broader, up to 5.5 μm (Smith 1972). Morphologically, *P. subsingeri* belongs to section *Spintrigerae* with basidiospores less than 9.0 μm and absent pleurocystidia (Kits van Waveren 1985). Its cheilocystidium is similar to *P. submicrospora* Heykoop & G. Moreno [= *Coprinopsis submicrospora* (Heykoop & G. Moreno) Örstadius & E. Larss.], but basidiospores of *P. submicrospora* are predominantly amygdaliform (Heykoop and Moreno 2002). It also can be classified in series *Atricastaneae* (Smith 1972). There are only three species in the series that match the characteristic of subhyaline to hyaline basidiospores in water or 5% KOH. However, they can be separated as follows: the basidiospores of *P. atricastanea* (Murrill) A.H. Sm are truncate; *P. albipes* A.H. Sm. and *P. subhyalinispora* (Murrill) A.H. Sm. differ in having an obvious germ pore (Smith 1972). Furthermore, *P. aequatoriae* Singer has subhyaline to hyaline basidiospores, but differs by smaller and sometimes papillate pileus. *Psathyrella olympiana* and *P. bipellis* [= *P. odorata* (Peck) Sacc.] have aspects of *P. subsingeri* in macroscopic characteristics, whose pileus are reddish-brown, but have pleurocystidia (Örstadius and Kundsen 2012).

Key to species of *Psathyrella* in Northeast China

1	Pleurocystidia absent	**2**
–	Pleurocystidia present	**5**
2	Basidiospores brown in 5% KOH	***P. candolleana***
–	Basidiospores very pale, subhyaline in 5% KOH	**3**
3	Basidiospores predominantly longer than 8.0 μm	***P. luteopallida***
–	Basidiospores shorter	**4**
4	Basidiospores up to 5.5 μm broad	***P. singeri***
–	Basidiospores up to 4.5 μm broad	***P. subsingeri***

5 Basidiospores longer than 10 μm, pleurocystidia utriform to clavate, sometimes with yellowish-brown inclusions ..... ***P. bipellis***
– Not as above **6**
6 Germ pore always or predominantly distinctly visible **7**
– Germ pore absent or predominantly indistinctly visible **19**
7 Pleurocystidia rarely, lageniform, shorter than 40 μm, clamps absent... ***P. effibulata***
– Not as above **8**
8 Basidiospores up to 7.0 μm long **9**
– Basidiospores longer **10**
9 Pleurocystidia up to 35 μm long, mostly with distinct crystals .... ***P. pygmaea***
– Pleurocystidia up to 65 μm long, without crystals ..... ***P. piluliformis***
10 Basidiomata densely caespitose, cheilocystidia fusiform or mucronat, basidiospores 8.5–9.8 × 4.6–5.1 μm ..... ***P. boreifasciculata***
– Not as above **11**
11 Pleurocystidia mostly fusiform or lageniform **12**
– Pleurocystidia mostly utriform, narrowly utriform or clavate **14**
12 Basidiomata vary small, pileus up to 5.0 mm, cheilocystidia with long or short mucronate ..... ***P. mycenoides***
– Not as above **13**
13 Grown on sphagnum, basidiospores 8.8–9.2 × 4.4–5.0 μm ..... ***P. borealis***
– Terrestrial or basidiomata attached to bits of woody debris, basidiospores 7.3–8.8 × 4.1–4.4 μm ..... ***P. subterrestris***
14 Basidiospores distinctly triangular ..... ***P. panaeoloides***
– Not as above **15**
15 Pleurocystidia often with in ammonia greenish deposits, basidiospores 8.8–9.7 × 4.4–4.9 μm ..... ***P. lutensis***
– Not as above **16**
16 Cheilocystidia clavate to spheropedunculate, pleurocystidioid cheilocystidia scattered ..... ***P. phegophila***
– Pleurocystidioid cheilocystidia numerous **17**
17 Basidiospores up to 6.0 μm broad ..... ***P. fennoscandica***
– Basidiospores up to 5.0 μm broad **18**
18 Veil strongly developed and flocculose, pleurocystidia utriform or clavate ..... ***P. gordonii***
– eil with a thin coating of fibrils, pleurocystidia narrowly utriform ..... ***P. senex***
19 Pleurocystidia distinctly thick-walled or slightly thick-walled, covered with distinct crystals, basidiospores up to 9 μm long **20**
– Not as above **21**
20 Pleurocystidia utriform, distinctly thick-walled ..... ***P. amaura***
– Pleurocystidia fusiform, slightly thick-walled or thin-walled ..... ***P. jilinensis***
21 Pleurocystidia fusiform, lageniform, with obtuse or subacute apex **22**
– Pleurocystidia utriform, narrowly utriform, with obtuse to broad obtuse apex **25**

22 Cheilocystidia mucronate, basidiospores ellipsoid, pale brown in 5% KOH ..... ***P. obtusata***
– Not as above **23**
23 Basidiospores (6.8–)7.3–7.8(–8.8) × 3.4–4.9 μm, base often broadly truncate, in profile often phaseoliform ..... ***P. pertinax***
– Not as above **24**
24 Basidiospores oblong to oblong-ellipsoid, pleurocystidia thin-walled ..... ***P. squamosa***
– Basidiospores ellipsoid, pleurocystidia slightly thick-walled .... ***P. spintrigeroides***
25 Basidiospores reddish-brown in water ..... ***P. mammifera***
– Basidiospores yellowish-brown or pale yellowish-brown in water **26**
26 Pileus often with subacute or obtuse umbo, basidiospores 7.8–8.8 × 4.0–4.5(–5.0) μm, oblong to oblong-ellipsoid ..... ***P. conica***
– Pileus without umbo, basidiospores 6.8–7.8 × 3.9–4.9 μm, ellipsoid, rarely oval ..... ***P. subspadiceogrisea***

Acknowledgments

This work is supported by the 111 Project (grant numbers D17014) and the Ministry of Education Innovation Team (grant numbers IRT1134 and IRT-15R25). We sincerely thank Mr. Bai Wang (Changbai Mountain Academy of Sciences, Jilin, China) for his kind help during field work.

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MycoKeys 33: 103–124 (2018)
doi: 10.3897/mycokeys.33.23703
http://mycokeys.pensoft.net

RESEARCH ARTICLE

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Two new species and one new record of the genus *Tylopilus* (Boletaceae) from Indian Himalaya with morphological details and phylogenetic estimations

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Academic editor: *K. Hosaka* | Received 18 January 2018 | Accepted 28 March 2018 | Published 13 April 2018

Citation: Chakraborty D, Vizzini A, Das K (2018) Two new species and one new record of the genus *Tylopilus* (Boletaceae) from Indian Himalaya with morphological details and phylogenetic estimations. MycoKeys 33: 103–124. https://doi.org/10.3897/mycokeys.33.23703

Abstract

Tylopilus himalayanus and *T. pseudoballoui* are described as new species from two Himalayan states (Sikkim and Uttarakhand) in India. *Tylopilus himalayanus* is characterised by a unique combination of features: reddish- or brownish-grey to purplish-grey then brown to reddish-brown or darker pileus, absence of olive or violet tinges on stipe surface, angular pores, stipe without reticulum or rarely with a faint reticulum restricted to the very apex, bitter taste of the context and positive macrochemical colour reaction of the stipe context with KOH (dark orange) and $FeSO_4$ (dark green), medium sized (10.9–14.4 × 3.9–4.9 µm) basidiospores and occurrence under coniferous trees; *T. pseudoballoui* is distinguished by orange-yellow to brown-yellow sticky pileus, pale yellow pore surface with pinkish hues that turns pale to greyish-orange on bruising; angular pores, stipe concolorous to pileus with pruinose but never reticulate surface, ixocutis pattern of pileipellis and occurrence under broadleaf trees. Another species, *T. neofelleus*, which was reported earlier from China and Japan, was also collected from Sikkim and reported for the first time from India. All three species are described with morphological details and two-locus based (nrLSU and nrITS) phylogenetic data.

Keywords

Agaricomycetes, Basidiomycota, Boletales, macrofungi, phylogeny, Sikkim, taxonomy

Introduction

The genus *Tylopilus* P. Karst., one of the less attractive to eye-catching ectomycorrhizal taxa (associated mainly with Fagales and Pinaceae) in the family Boletaceae, is featured by its dry, glabrous to subvelvety pileus, white to greyish pore surface usually becoming flesh pink to purple-brown at maturity, immutable to slightly brownish or becoming blue-green context on bruising, solid stipe with pruina or reticulation over the surface, absence of annulus or veil, flesh-pink to dull flesh-ochre spore print, smooth pink-coloured basidiospores, presence of pleurocystidia and absence of clamp-connections (Smith and Thiers 1971, Wolfe 1979, Singer 1986, Wu et al. 2014). Further, this genus was divided into two subgenera namely, *T*. subg. *Tylopilus* and *T*. subg. *Porphyrellus* (Smith and Thiers 1971, Singer 1975). The former subgenus is characterised by "spores with pale cinnamon-yellow to pale yellow walls in KOH and IKI; context usually unchanging or rust coloured on injury; context not turning red-brown in KOH", whereas the latter is characterised by "spores with dark brown walls in KOH and IKI; context usually turning blue-green on injury then becoming red-brown and, in some taxa, the context becoming red-brown in KOH" (Wolfe 1979). From all over the world (mostly from North America, Australia, Asia, Africa and Europe), about 75 species are reported (Kirk et al. 2008, Magnago et al. 2017). Like some other morphology-based genera in Boletaceae, the traditional concept of *Tylopilus* (*Tylopilus* s.l.) was split and has given birth to a few other genera with the recent advancement of multi-locus phylogeny. *Tylopilus* s.l. appeared as polyphyletic and evolved in 11 different lineages during the course of evolution (Nuhn et al. 2013, Wu et al. 2014). Thus, taxonomic placement of the members of this genus are still floating and many previously considered *Tylopilus* species are shifted into new genera such as *Zangia* Yan C. Li & Zhu L. Yang, *Australopilus* Halling & Fechner and *Harrya* Halling, Nuhn & Osmundson (Li et al. 2011, Halling et al. 2012). According to Wu et al. (2014), all the 11 clades consisting of the members of *Tylopilus* come under five subfamilies (Austroboletoideae, Leccinoideae, Boletoideae, *Pulveroboletus* group and Zangioideae) in Boletaceae and *Tylopilus* s.s., typified by *Tylopilus felleus* (Bull.) P. Karst., is placed within the subfamily Boletoideae.

The entire Indian Himalayan region comes under one ("Himalaya") of the globally acclaimed biodiversity hotspots and thus has immensely diverse mycobiota (including macrofungi) apart from its myriad flora and fauna. A wide range of phytogeographic variations with the presence of large numbers of ectomycorrhizal host plants, cold to warm monsoon, favourable humidity and plenty of rainfall, supports the growth of ectomycorrhizal mushrooms of Boletaceae. However, due to the lack of mushroom-explorers or mushroom-taxonomists, most of the areas of Indian Himalaya remain unexplored in terms of Boletaceae (only 80 species belonging to 23 genera, while more than 1050 species from 66 genera are reported from the world) (Chakraborty et al. 2017). During macrofungal surveys to different forested areas of Eastern Himalaya (Sikkim) and Western Himalaya, three interesting members of *Tylopilus* were collected separately. Detailed macro- and micromorphological studies followed by phylogenetic

analyses based on nrLSU and nrITS sequences, confirm the novelty of two of them and are proposed here as *T. himalayanus* and *T. pseudoballoui*, whereas the third one appeared as conspecific to *T. neofelleus* (a species so far reported from Japan and China, Gelardi et al. 2015) and is reported as a new record for Indian mycobiota.

Materials and methods

Morphological study

Macromorphological characters and habitat details were noted from fresh, young to mature basidiomata in the field and in base-camp. After recording the macromorphological characters, basidiomata were dried with a field drier. Photographs of these fresh and dry basidiomata and microphotographs were taken with the aid of Canon Power Shot SX 50HS, Canon SX 220 HS and Nikon-DS-Ri1 (dedicated to Nikon Eclipse N*i* compound microscope) cameras. Colour codes and terms are mostly from Methuen Handbook of Colour (Kornerup and Wanscher, 1978). Micromorphological characters were observed with compound microscopes (Nikon Eclipse N*i*-U and Olympus CX 41). Sections from dry specimens were mounted in a mixture of 5% KOH, 1% Phloxine and 1% Congo red or in distilled water. Micromorphological drawings were prepared with a drawing tube (attached to the Nikon Eclipse N*i* microscope) at 1000×. The basidium length excludes that of the sterigmata. Basidiospore measurements were recorded in profile view from 30 basidiospores. Spore measurements and length/width ratios (Q) are recorded here as: minimum–**mean**–maximum. Herbarium codes follow Thiers (continuously updated).

DNA extraction, polymerase chain reaction (PCR) and sequencing

Genomic DNA (for all the species) was extracted from 100 mg of dry basidiomata using the InstaGeneTM Matrix Genomic DNA isolation kit (Biorad, USA) following the manufacturer's instructions. PCR amplification primers were ITS1 and ITS4 (nrITS region) and LR0R and LR7 (nrLSU region) (White et al. 1990). PCR amplification on "ABI Veriti" thermal cycler protocols for nrITS and nrLSU regions were after Das et al. (2017). The PCR products were then purified using the QIAquick PCR Purification Kit (QIAGEN, Germany) before they were sent for sequencing. Both strands of the PCR fragments were sequenced on the 3730xl DNA Analyzer (Applied Biosystems, USA) using the amplifying primers and assembled using Sequencer (Gene Codes Corporation, USA). The nrITS and nrLSU sequences for DC 16-64 (MG777524 and MG777529), DC 16-63 (MG777523 and MG777525), DC 17-31 (MG799323 and MG799326), DC 17-25 (MG799322 and MG799328), DC 17-30 (MG799329 and MG799327) and DC 17-35 (MG799324 and MG799325), respectively, were deposited in GenBank.

Phylogenetic analyses

The nrLSU and nrITS datasets were assembled according to recent previous studies on this genus (Gelardi et al. 2015, Magnago et al. 2017) and from BLAST (Altschul et al. 1997) searches in GenBank (Clark et al. 2016). As most *Tylopilus* collections in GenBank are not provided with both molecular markers, we were unable to establish a combined nrITS+nrLSU dataset and so have opted for present separate nrLSU and nrITS phylogenetic inferences. These (nrITS and nrLSU) sequences were aligned separately in MAFFT 7.305 (Katoh and Standley 2013). For the nrLSU dataset, *Xanthoconium sinense* (KT990666 and KT990664) and *X. purpureum* (KT990663) from Boletaceae were used as outgroup taxa. Similarly, for the nrITS dataset, two sequences from *Gyroporus* (KX869874, GQ166901), another genus in Boletales (Gyroporaceae), were used as the outgroup. Phylip file formats were created in AliView (Larsson 2014) using default settings. Phylogenies were reconstructed using Maximum Likelihood (ML) in RAxML 7.2.6 (Stamatakis 2006) in GTRGAMMA substitution model. All parameters in the ML analyses used the default settings in RAxML and Maximum Likelihood bootstrap percentage (MLB) were obtained using nonparametric bootstrapping with 1000 replicates. Additionally (to generate supplementary data), nrLSU and nrITS sequences were also phylogenetically analysed using Bayesian analysis. The best-fit models of nucleotide evolution for nrLSU and nrITS datasets (TIMef and TrNef+G, respectively) were obtained in MrModeltest 3.7 (Posada and Crandall 1998). Bayesian inferences were computed independently twice in MrBayes v.3.2.2 (Ronquist et al. 2012), under TIMef (for nrLSU) and TrNef+G (for nrITS) models, respectively. Bayesian posterior probabilities values (BPP) were calculated in two simultaneous runs with the Markov Chain Monte Carlo (MCMC) algorithm (Larget and Simon 1999). Markov chains were run for 1000000 generations, saving a tree every 100th generation. These analyses were terminated when the average standard deviation of split frequencies fell below 0.01. The first 25% of trees was discarded as burn-in (Hall 2004). The convergence of runs was visually assessed using Trace function in Tracer version 1.6.0 (Rambaut et al. 2013).

Results

Phylogenetic inferences

The nrLSU- and nrITS-based phylogenetic analyses (Figs 1–2 and Suppl. materials 1–2) consist of 76 and 42 sequences, respectively. In our nrLSU based ML and BI analyses (Figs 1 and Suppl. material 1, respectively), the two Indian collections of *T. himalayanus* (DC 17–25 and DC 17–31) clustered together and appeared sister (MLB = 100%, BPP = 1) to the North American *T. intermedius* (HQ161875) and *T. rubrobrunneus* (HQ161875). However, our species with its two sequences (MG799328 and MG799326) is recovered

Figure 1. nrLSU based phylogram generated from Maximum likelihood (ML) analysis under GTR-GAMMA substitution model depicting the placement of *Tylopilus neofelleus, T. pseudoballoui* and *T. himalayanus* within *Tylopilus* s.s. Two species of *Xanthoconium* (*X. sinense* and *X. purpureum*) were used as outgroup taxa. ML Bootstrap percentage (MLB) derived from this analysis (MLB >50%) are shown above or beneath the branches. Two novel species and a new record for Indian mycobiota are highlighted in bold and red font. GenBank accession no. and country name (when available) for each species are shown after the species name.

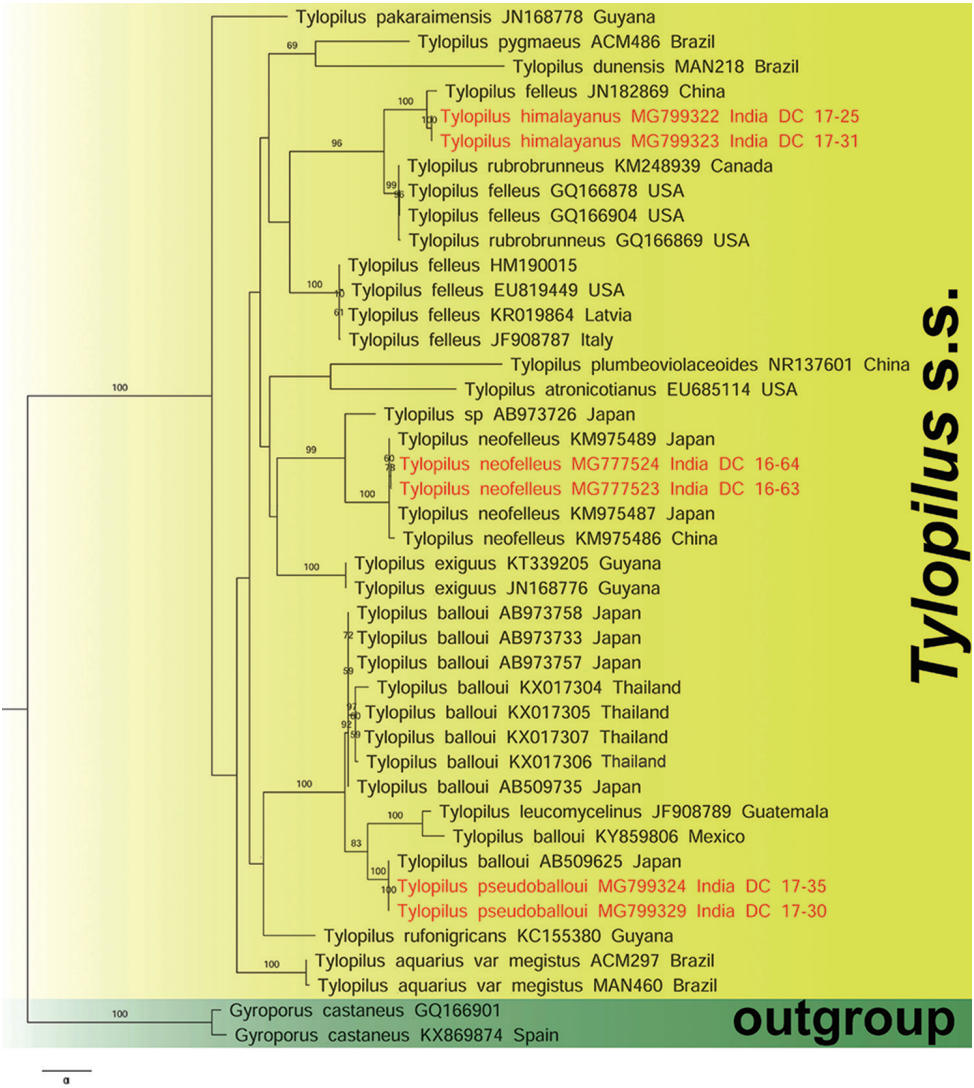

Figure 2. nrITS based phylogram generated from Maximum Likelihood (ML) analysis under GTR-GAMMA substitution model depicting the placement of *Tylopilus neofelleus, T. pseudoballoui* and *T. himalayanus* within *Tylopilus* s.s. Two sequences of *Gyroporus castaneus* were used as outgroup. ML Bootstrap percentage (MLB) derived from this analysis (MLB >50%) are shown above or beneath the branches. Two novel species and a new record for Indian mycobiota are highlighted in bold and red font. GenBank accession no. and country name (when available) for each species are shown after the species name.

as a distinct species. In these same analyses, the two Indian specimens of *T. pseudoballoui* (DC 17–30 and DC 17–35) are sister (MLB = 98%, BPP = 1) to a strongly supported clade (MLB = 99%, BPP = 1) formed by six sequences named as "*T. balloui*" or "*T.* aff. *balloui*" (EU430740, KX017298, KF112458, KX017295, KX017296, KX017297) from Asia. However, our Indian collections (MG799325 and MG799327) are recovered

as a distinct species. Our other two Indian collections of *T. neofelleus* (DC 16–45 and DC 16–63) clustered along with all the Asian counterparts (KM975495, HQ326936, KM975496, KM975497, KM975494, KF000101, KF112450) in a strongly supported clade (MLB = 100%, BPP = 1), indicating its conspecificity.

Similarly, in our nrITS-based ML and BI analyses (Figs 2 and Suppl. material 2, respectively), the two Indian collections of *T. himalayanus* (DC 17–25 and DC 17–31), along with a collection of China (JN182869, wrongly labelled as "*Tylopilus felleus*"), appeared sister (MLB = 96%), in the ML analysis, or close, in the BI analysis, to a clade consisting of two *T. rubrobrunneus* sequences (KM248939 from Canada, GQ166869 from USA) and two "*T. felleus*" from USA (GQ166878, GQ166904). However, our collection is recovered as a separate species. The two Indian specimens of *T. pseudoballoui* (DC 17-30 and DC 17-35) clustered strongly (MLB = 100%, BPP = 1) with a Japanese sequence of "*T. balloui*" (AB509625) and appeared as sister (MLB = 83%), in the ML analysis, to a clade consisting of one Mexican collection (represented by KY859806 and labelled as "*Tylopilus ballouii*") and *Tylopilus leucomycelinus* (JF908789) from Guatemala and as sister (BPP = 0.85) whereas, in the BI analysis, to a clade (MLB = 100%) formed by eight Asian sequences of "*T. balloui*", four from Japan (AB973733, AB973757, AB973758, AB973735) and four from Thailand (KX017304, KX017306, KX017305, KX017307). However, our species is recovered as a distinct species. Finally, as in the nrLSU analysis, here also the two Indian collections of *T. neofelleus* are strongly clustered (MLB = 100%, BPP = 1) with three Asian counterparts (KM975487 and KM975489 from Japan, KM975486 from China), showing their conspecificity.

Taxonomy

Tylopilus himalayanus **D. Chakr., K. Das & Vizzini, sp. nov.**
MycoBank: MB823975
Figs 3, 4

Holotype. India. Sikkim: East District, Upper Chandmari, 1977 m alt., N27°23'13.7", E88°46'42.9", 26 Aug 2017, *D. Chakraborty & K. Das*, DC 17-25 (CAL 1649).

Diagnosis. Distinct from all allied taxa by a combination of sequence data (nrITS and nrLSU), reddish- or brownish-grey to purplish-grey, then brown to reddish-brown pileus in basidiomata, absence of olive or violet tinges on stipe surface, presence of angular pores, stipe without reticulum or rarely with a faint reticulum restricted to the very apex, bitter taste of the context, positive reaction of the stipe context with KOH (dark orange) and $FeSO_4$ (dark green) and medium sized (10.9–14.4 × 3.9–4.9 µm) basidiospores.

Etymology. Referring to Indian Himalaya, the type locality.

Description. Pileus 71–120 mm diam., initially convex then plano-convex to applanate, surface dry, matte to somewhat subvelvety, faintly areolate, brownish-grey, dull red, reddish-grey to purplish-grey or greyish-magenta (11–13B–C2–3) when young, gradually brown to reddish-brown (7E4–9D4) or darker, paler greyish-yellow (4C4) towards

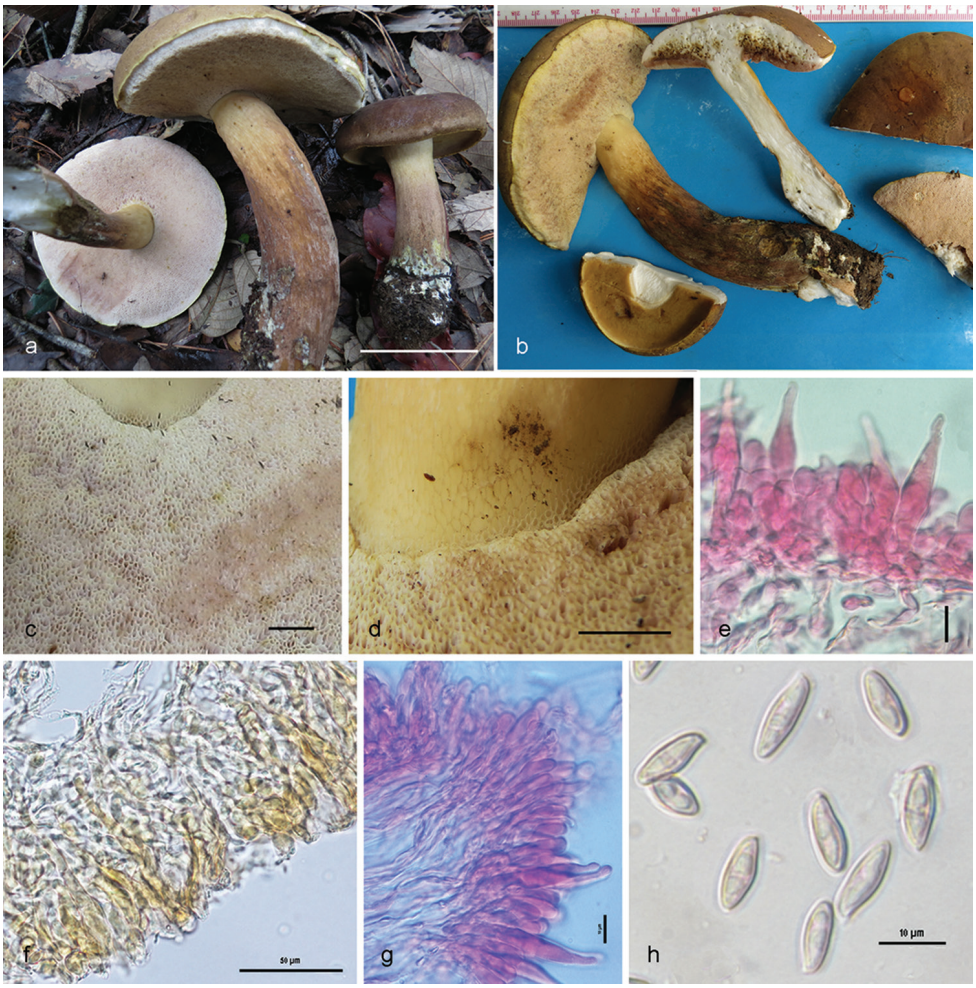

Figure 3. *Tylopilus himalayanus* (DC 17-25, holotype). **a, b** Fresh basidiomata in the field and in base-camp **c** Pore surface after bruising **d** Surface of stipe apex with reticulation **e** Pleurocystidia **f** Pileipellis **g** Tube edge **h** Basidiospores. Scale bars: 50 µm (**f**); 10 µm (**e, g, h**); 5 mm (**c, d**); 5 cm (**a**).

margin, pale yellow (2A3) at margin; margin entire, decurved to plane with a narrow flap of tissue, blond (4C4). Pore surface greyish-yellow (3C4) when young, pinkish-white (8A2) with age, turning greyish-brown (6D3) on bruising; pores angular, 2/mm. Tubes adnexed to subdecurrent, 5–6 mm long, whitish-brown to brownish, light brown to brown (26B2–3) on bruising. Stipe 95–155 × 20–32 mm, mostly subclavate, hollow, pale yellow (1–2A3) at apex, brownish towards base but never violaceous; surface usually without any reticulum, but sometimes faintly reticulate at apex (1/8th from the juncture), the rest longitudinally striate. Context up to 16 mm thick in pileus, milk white (1A2), unchanging when exposed. Stipe context turning dark green with $FeSO_4$, dark orange with 5% KOH, orange with 10% NH_4OH. Taste bitter. Spore print not obtained.

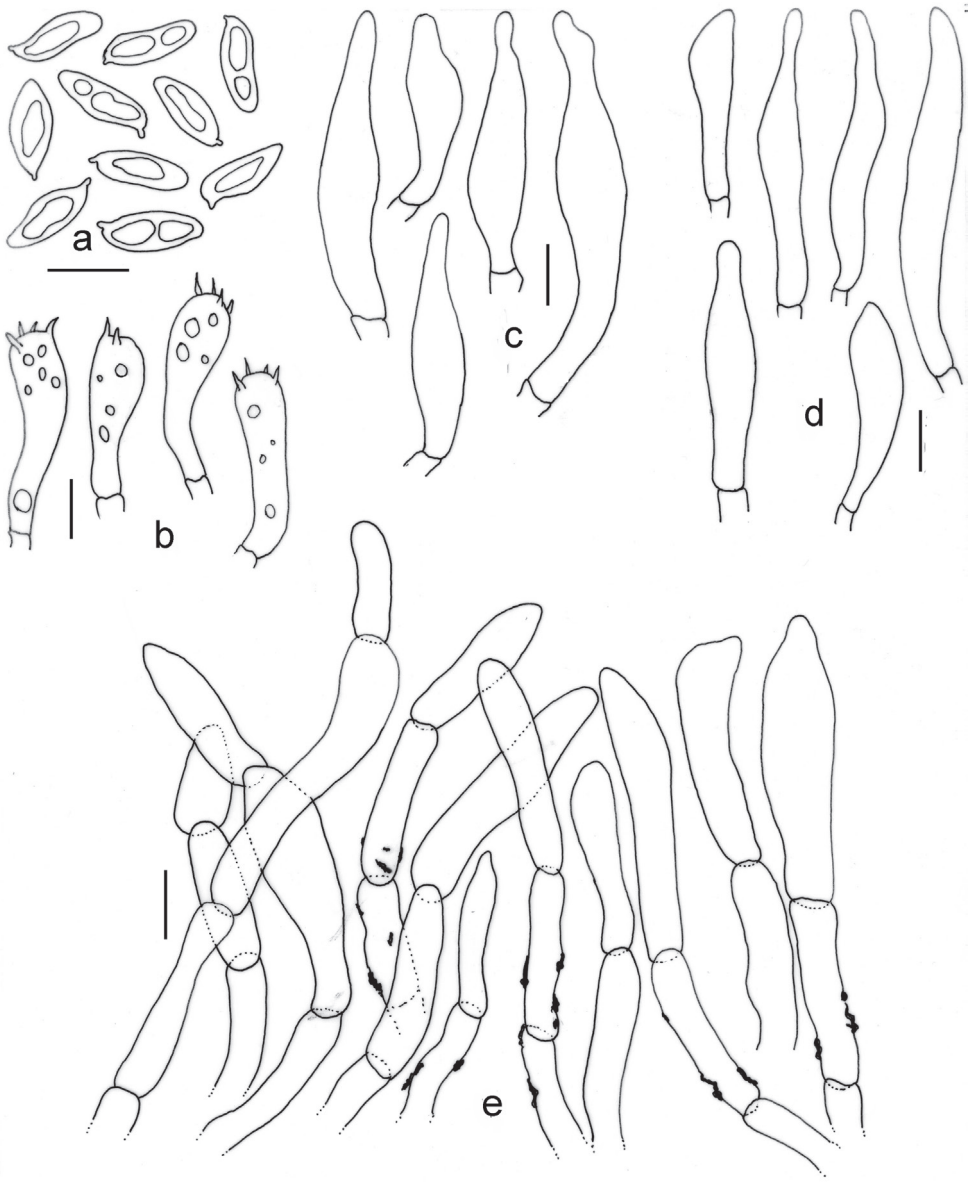

Figure 4. *Tylopilus himalayanus* (DC 17-25, holotype). **a** Basidiospores **b** Basidia **c** Pleurocystidia **d** Cheilocystidia **e** Pileipellis. Scale bars: 10 μm (**a–e**).

Basidiospores 10.9–**12.5**–14.4 × 3.9–**4.5**–4.9 μm, (n = 30; Q = 2.51–**2.75**–3.25), elongated to fusiform, inequilateral, thin-walled, smooth under light microscope. Basidia 30–40 × 9–10 μm, four-spored, clavate. Pleurocystidia 27–54 × 8–10.5 μm, emergent up to 30 μm, fusoid to ventricose, appendiculate. Tube edge sterile; cheilocystidia 32–48 × 5.2–8 μm, common, clavate to cylindrical, subfusoid to ventricose. Hymenophoral tra-

ma divergent, hyphae septate, gelatinous, up to 6 µm wide. Pileipellis a trichoderm, up to 150 µm thick, composed of erect hyphae, somewhat interwoven, encrusted, brown pigmented; terminal elements 20–50 × 5–10 µm, cylindrical to subcylindrical, sometimes subfusoid, content brown pigmented. Stipitipellis a cutis, made up of sub-parallel repent hyphae; caulocystidia not observed; caulobasidia similar to that of hymenial basidia.

Specimens examined. India. Sikkim: Upper Chandmari, 1977 m alt., N27°23'13.7", E88°46'42.9", under *Pinus* sp. in temperate mixed forest, 26 Aug 2017, *D. Chakraborty & K. Das*, DC 17-31 (CAL); Uttarakhand: Champawat district, Abbot mount, 1933 m alt., N29°25.465', E80°06.422', under *Cedrus deodara* in temperate coniferous forest, 18 Jul 2017, A. Ghosh, KD B-03 (CAL).

Tylopilus pseudoballoui K. Das, D. Chakr & Vizzini, sp. nov.

Mycobank: MB823977
Figs 5, 6

Type. INDIA. Sikkim, South District, Maenam WLS (Maenum 3), 2136 m alt., N27°15'34.7" E88°21'25.7", 23 Aug 2017, *Quercus* spp., *D. Chakraborty & K. Das*, DC 17-30 (CAL 1651)

Diagnosis. Distinct from all allied taxa by sequence data (nrITS and nrLSU) and morphologically by its sticky orange-yellow pileus surface, pale yellow pore surface which turns to pale orange or greyish-orange when bruised and absence of reticulation on stipe surface.

Etymology. referring to its being a look-alike of *T. balloui*, a North American species.

Description. Pileus 60–150 mm diam., initially convex then plano-convex, surface sticky, orange-yellow to brownish-yellow (5B–C8), paler at margin; margin entire, plain, without any sterile flap of tissue. Pore surface pale yellow (3A3), turning pale orange to greyish-orange (6A–B3) on bruising; pores angular, 5–8/mm. Tubes subdecurrent, 6–10 mm long, yellowish-white, brownish on bruising. Stipe 55–110 × 20–40 mm, mostly subclavate, solid, concolorous with pileus; surface pruinose, never reticulate; basal mycelium white. Context 20 mm thick in pileus, chalky white (1A1), unchanging on exposure but turning turquoise grey (24D2–D1) with $FeSO_4$ (chalk), pale yellow (4A3) with 5% KOH, yellowish-grey (4B3) in Guaiacol. Pileus surface brownish-red (8C8–7) on bruising, turning dark green to greenish-grey (25E–F3–2) in FeSO4, vivid yellow (3A8) in KOH, unchanging in NH_4OH. Stipe 55–110 × 20–40 mm, mostly subclavate, solid, concolorous with pileus; surface pruinose, never reticulate; basal mycelium white. Odour pleasant. Taste slightly pungent. Spore print not obtained.

Basidiospores 6.4–**7.4**–9.9 × 3.8–**4.5**–5.7 µm (n = 30, Q = 1.5–**1.73**–2.04), ellipsoidal, thin-walled, smooth under light microscope. Basidia 22– 30 × 8–10 µm, four-spored, clavate. Pleurocystidia 40–54 × 7–10 µm, emergent up to 30 µm, fusoid to ventricose, appendiculate, yellow pigmented or hyaline, mostly with dense globular to oily content. Tube edge fertile; cheilocystidia 33–55 × 7–10 µm, common, clavate to

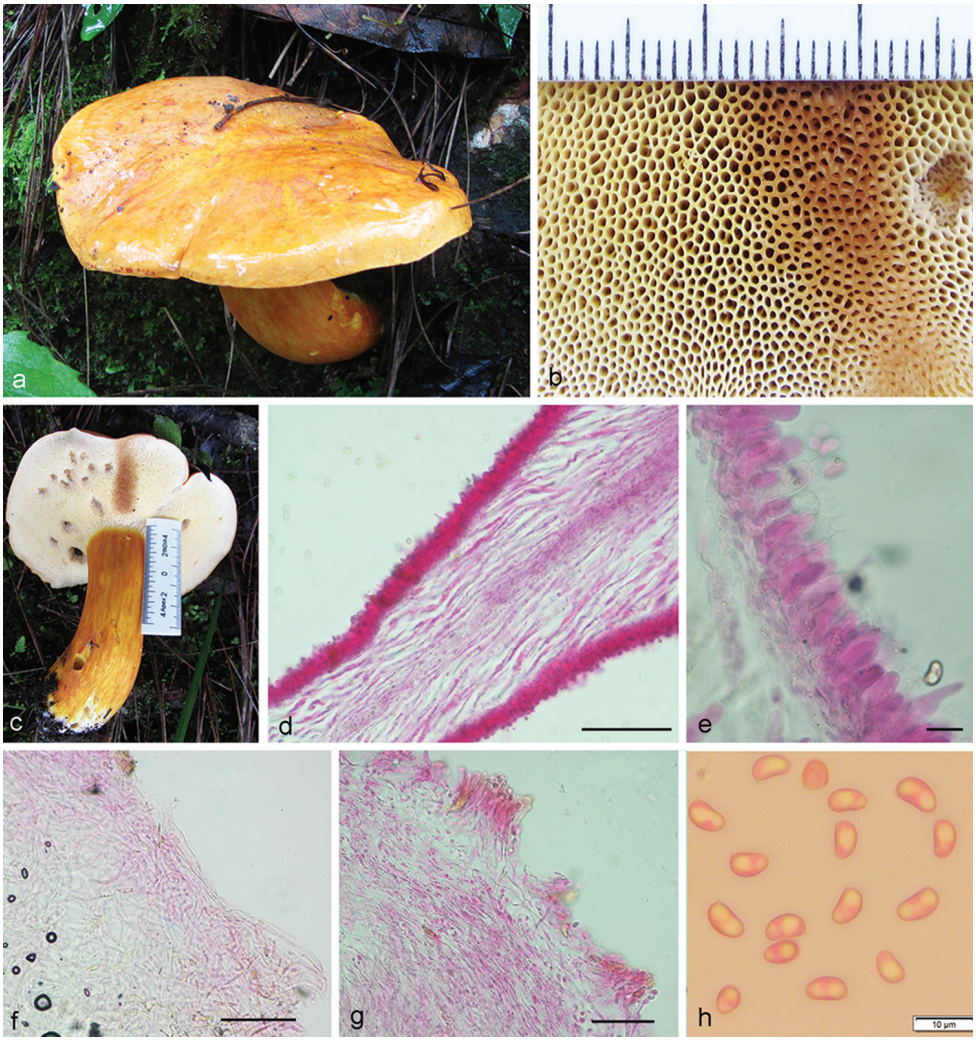

Figure 5. *Tylopilus pseudoballoui* (DC 17-30, holotype). **a, c** Fresh basidiomata in the field **b** Pore surface after bruising **d** Hymenophoral trama **e** Pleurocystidia **f** Pileipellis **g** Stipitipellis **h** Basidiospores. Scale bars: 100 µm (**d, f**); 50 µm (**g**); 10 mm (**e, h**).

cylindrical, subfusoid to ventricose. Hymenophoral trama divergent, hyphae septate, gelatinous, up to 5 µm wide. Pileipellis an ixocutis up to 150–280 µm thick, composed of subparallel to suberect, somewhat interwoven hyphae; terminal elements 20–70 × 6–11 µm, cylindrical to subcylindrical, sometimes subfusoid, content orange-brown pigmented. Stipitipellis up to 150 µm thick, fertile, composed of basidia and cystidia in several clusters; caulobasidia similar to that of hymenial basidia; caulocystidia 40–76 × 10–12 µm, broadly clavate to subclavate or appendiculate.

Specimens examined. India. Sikkim: South District, Maenam WLS (Maenam 3), 2136 m alt., N27°15'34.7", E88°21'25.7", 23 Aug 2017, *Quercus* sp., *D. Chakraborty*

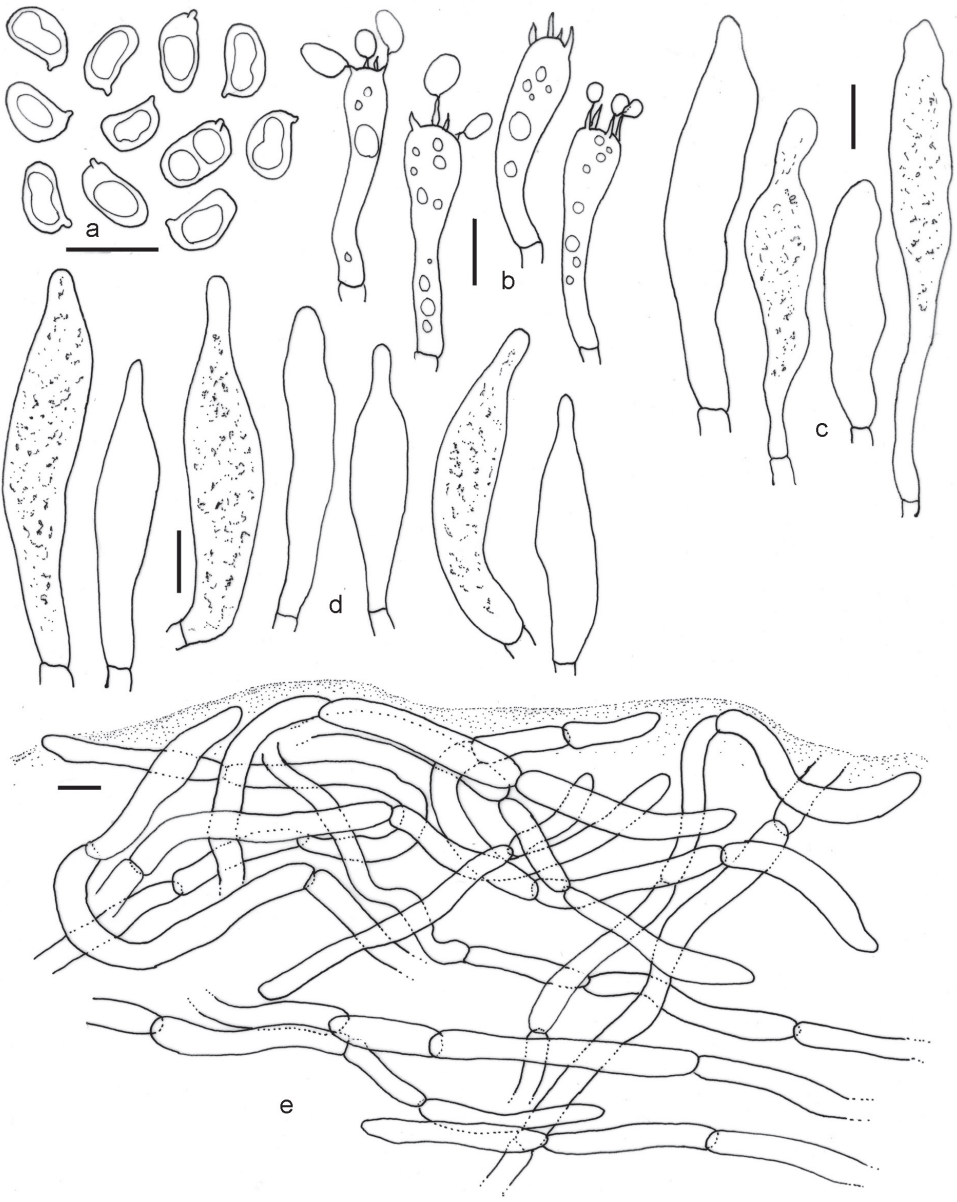

Figure 6. *Tylopilus pseudoballoui* (DC 17-30, holotype). **a** Basidiospores **b** Basidia **c** Caulocystidia **d** Pleurocystidia **e** Pileipellis. Scale bars: 10 μm (**a–e**).

& K. Das, DC 17-35 (CAL); Uttarakhand: Champawat district, Abbot mount, 1885 m alt., N29°25.466', E80°06.085', 18 July 2017, A. Ghosh, KD B-02 (CAL). Uttarakhand: Pauri district, 1971 m alt., N30°02.874', E79°08.221', 4 Aug 2017, *K. Das*, KD 17-24 (CAL).

Figure 7. *Tylopilus neofelleus* (DC 16-63). **a, b** Fresh basidiomata in the field and in basecamp **c** Stipe surface with reticulation **d** Pileipellis **e** Hymenial layer showing basidia and pleurocystidia **f** Tube edge **g** Basidiospores. Scale bars: 50 µm (**d, f**); 10 µm (**e, g**).

Tylopilus neofelleus Hongo, J. Jpn. Bot. 42: 154 (1967)

Figs 7, 8

Description. Pileus 70–120 mm broad, convex to broadly convex; surface distinctly tomentose to subvelvety, dry, subviscid when wet; reddish-brown (8–9F4) when young, rosewood (9C5) to vinaceous-brown (16C5) with maturity, fawn (7E4) towards margin. Pores yellowish-white or cream with a pinkish tinge, orange-grey (6B2) with age; pore 2–3/mm, rounded. Tubes 10–15 mm, adnate-sinuate, white in colour,

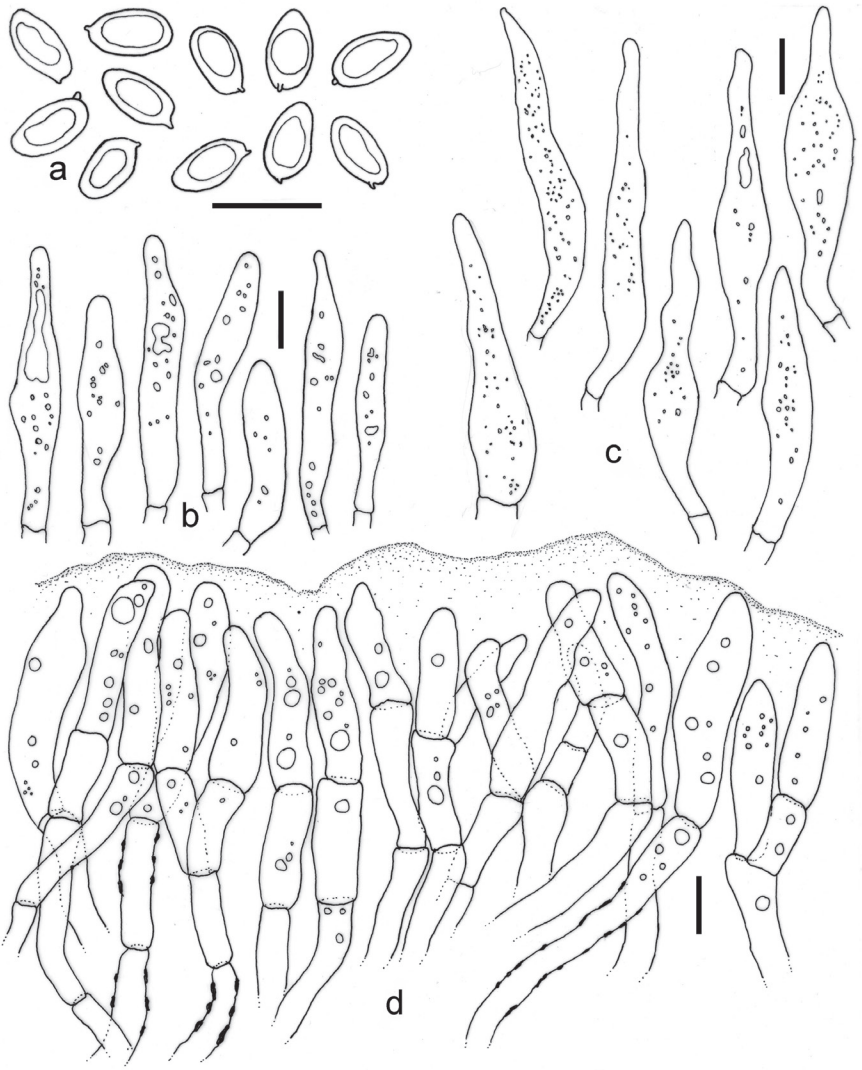

Figure 8. *Tylopilus neofelleus* (DC 16-63). **a** Basidiospores **b** Pleurocystidia **c** Caulocystidia **d** Pileipellis. Scale bars: 10 μm (**a–d**).

yellow-brown or orange white with maturity. Stipe 60–100 × 18–22 mm, cylindrical, solid, surface dry, glabrous to subvelutinous, typically distinctly reticulate at apex, reticulation greyish-ruby (12C–D4) to dark ruby (12F5); surface pinkish brown to vinaceous or violaceous, dark brown to reddish-brown with maturity. Context chalky white, but pinkish-brown when exposed. Spore print not obtained.

Basidiospores 10–**11.5**–13.5 × 4–**4.6**–5.2 μm (n = 30, Q = 2.05–**2.48**–2.76), ellipsoid to narrowly subfusoid, inequilateral, smooth, thin-walled. Basidia 30–36 × 10–11 μm, 4-spored, clavate, thin-walled, hyaline or pale grey in KOH. Pleurocystidia

35–66 × 14–24 µm, scattering and numerous, fusoid-ventricose or subclavate, with orange brown contents. Cheilocystidia 33–38 × 9–12 µm, ventricose to fusoid, shorter and smaller than pleurocystidia thin-walled, with orange brown contents. Pileipellis 100–150 µm thick, an ixotrichoderm of suberect, branched, septate hyphae; terminal elements ventricose to fusoid, vaculolated, 28–50 × 12–14 µm, with granular yellowish to brown orange contents in KOH; subterminal elements mostly with incrustations. Stipitipellis 35–65 µm, fertile, composed of basidia and cystidia. Caulocystidia 52–63 × 8–13 µm, fusoid to subfusoid, ventricose to ventricose-rostrate or narrowly cylindrical, content granular. Clamp connection absent in all tissues.

Habitat. Under *Castanopsis* sp. in temperate broadleaf forest.

Known distribution. Japan (Kawamura 1954; Hongo 1960; Imazeki et al. 1970, 1988; Takahashi 1986; Gelardi et al. 2015), China (Ying and Zang 1994; Li and Song 2000; Wang et al. 2004; Fu et al. 2006; Wu et al. 2011; Gelardi et al. 2015), Russia (Vasil'jeva 1978) and New Guinea (Hongo 1973).

Specimens examined. India. Sikkim: East district, Fambonglo WLS, 2021 m alt., N27°21'47.5" E88°34'13.2", 26 Aug 2016, *D. Chakraborty & K. Das*, DC 16-63 (CAL); ibid., *D. Chakraborty & K. Das*, DC 16-64 (CAL).

Discussion

Our first novel species in *Tylopilus* s.s. (Wu et al. 2014), i.e. *T. himalayanus*, is featured by its brown, reddish-brown to purplish-grey, dry pileus, angular pores, stipe usually without reticulum even though sometimes with faintly reticulate apex, but longitudinally striate throughout, white unchanging context on exposure, bitter taste, sterile tube edge and trichodermic structure of pileipellis. Morphologically, *T. rubrobrunneus* Mazzer & A.H. Sm., *T. felleus* (Bull.) P. Karst., *T. neofelleus* Hongo and *T. intermedius* A.H. Sm. & Thiers resemble *T. himalayanus*. *Tylopilus rubrobrunneus* (originally reported from North America) differs from this species by its olive tinge on stipe surface, pileus surface with vinaceous tinges, rounded pores, negative colour reaction with KOH or NH_4OH on context (Mazzer and Smith 1967, Smith and Thiers 1971, Grund and Harrison 1976, Both 1993, Bessette et al. 2010, 2016). Similarly, *T. intermedius* differs from the present Indian species by possessing a distinctively whitish pileus that stains pinkish buff to brown with age and context (pileus) that turns pinkish with $FeSO_4$, but remains unchanged with KOH (Smith and Thiers 1971, Both 1993, Bessette et al. 2010, 2016). Some other members of this genus, such as *T. felleus* (originally described from Europe and known from India as well without checking its conspecificity through phylogeny), *T. neofellus* (originally reported from Japan but reported here for the first time from India), *T. plumbeoviolaceus* Snell & Dick (originally reported from North America but also known from this country without verifying its conspecificity through phylogeny) and *T. violatinctus* T.J. Baroni & Both (originally reported from North America), can also be separated from *T. himalayanus* morphologically: *T. felleus* has brownish pileus and distinctively reticulate stipe (Lannoy and Estadès 2001, Mu-

ñoz 2005); *T. neofellus* and *T. plumbeoviolaceus* possess reddish-brown to violaceous-brown pileus and reticulate purplish-violaceous stipe (Snell and Dick 1941, Smith and Thiers 1971, Grund and Harrison 1976, Both 1993, Lakhanpal 1996, Bessette et al. 2010, 2016, Gelardi et al. 2015); *T. violatinctus* is easily distinguished by the more brightly coloured, bluish-violet to lilac-lavender or purple-greyish pileus, bruising dark rusty-violet when handled, the stipe turning yellowish on bruising, pileus surface and context staining yellowish-brown and negative to pinkish-brown with KOH, respectively, small basidiospores [(5.6–)7–9(–10) × 3–4 µm] and the growth in mixed woodlands possibly with *Quercus*, *Fagus* or *Picea*, in any case not in association pine or cedar trees (Baroni and Both 1998, Ortiz-Santana et al. 2007, Bessette et al. 2010).

Tylopilus balloui, as currently circumscribed (pileus orange-yellow and short elliptical, pale-coloured basidiospores), represents a species complex rather than a single species, based on morphological data (Watling and Gregory 1988, Li and Watling 1999, Watling and Lee 1999, Watling and Li 1999, Watling 2001a, b, Watling et al. 2006) and molecular phylogenetic inference (Halling et al. 2008, Osmundson and Halling 2010, Magnago et al. 2017 and our analyses, Figs 1–2, Suppl. materials 1–2). Due to its spore shape, *Boletus balloui* Peck was previously considered as a *Gyrodon* (Snell 1941), a *Gyroporus* (Horak 2011) or a *Rubinoboletus* (Heinemann & Rammeloo 1983), but recent molecular studies (Halling et al. 2008; Osmundson and Halling 2010, Trappe et al. 2013, Wu et al. 2014, Magnago et al. 2017, Orihara and Smith 2017) confirmed its position in *Tylopilus* s.s. The true *T. balloui* has to be restricted only to the North American collections (Halling et al. 2008, Osmundson and Halling 2010). Our second novel species, i.e. *T. pseudoballoui*, a distinct species in this complex, is characterised by robust basidiomata with sticky orange to brownish-yellow pileus; pale yellow pore surface that turns to greyish-orange to orange on bruising, angular pores; concolorous stipe, pruinose, never reticulate; context white, unchanging on bruising or when exposed; pileipellis an ixocutis with somewhat interwoven hyphae; possessing two types of hymenial cystidia (hyaline and pigmented with yellowish globular to oily content); and occurrence under *Quercus* spp. In the field, the present species can be confused with its closest look-alike *T. balloui* (Peck) Singer which was originally reported from North America. However, *T. balloui* differs from the Indian species by possessing a dry pileus surface (sticky in *T. pseudoballoui*), white to dingy-white pores and a context turning pinkish-tan on exposure. (Smith and Thiers 1971, Wolfe 1981, Both 1993, Bessette et al. 2010, 2016, Osmundson and Halling 2010). *Tylopilus oradivensis* Osmundson & Halling, described recently from Costa Rica, possesses longer spores, (7.6–)8.2–12(–13.6) × (2.6–)3-4(–4.4) µm and a dry pileus surface, (Osmundson and Halling 2010). *Tylopilus leucomycelinus* (Singer & M.H. Ivory) R. Flores & Simonini from Honduras and Guatemala, has a dry, fibrillose to squamulose pileus surface, abundant white basal mycelium, smaller spores, (5.8–)6.1–6.7(–7.3) × (3.4–)3.8–4.3(–4.9) µm and is associated with *Pinus* spp. (Singer et al. 1983, Flores Arzù and Simonini 2000). Moreover, our twofold phylogenetic analysis clearly separates *T. pseudoballoui* (also known from Japan as clearly indicated in our Figs 2 and

Suppl. material 2). *Boletus balloui* var. *fuscatus* Corner from Malaysia, is morphologically similar to the Indian collection but the former differs by its narrower stipe (width 7–24 mm at apex, 3-12 mm at base), fawn-ochraceus pileus surface, dull purple brown pore surface on bruising, vinaceous to dull purple context on exposure (context unchanging in *T. pseudoballouii*), sterile stipitipellis and low land distribution (1300 mm alt.) (Corner 1972). *Tylopilus viscidulus* (Pat. & Baker) Watl. & Lee also known from Malaysia, differs from the Indian species by its pale cream coloured pileus and stipe, smaller size of basidiomata (pileus 25–40 mm diam. and stipe 20–35 × 8–15 mm), pale brown colour of context on exposure and presence of lageniform cystidia (Patouillard and Baker 1918, Watling and Lee 1999). Finally, *Rubinoboletus balloui* var. *viscidus* T.H. Li & Watling from Australia is distinguished by a smaller pileus (up to 70 mm broad), context turning pinkish on cutting and longer spores, 7.5–11.0 × 4.0–4.8 µm (Li and Watling 1999, Watling and Li 1999).

The combination of morphological features in Indian collections of *T. neofelleus* and two-fold phylogeny (MG777529, MG777525 in Figs 1 and Suppl. material 1; MG777524, MG777523 in Figs 2 and Suppl. material 2) attest the conspecificity of these collections with their Chinese or Japanese counterparts. *Tylopilus neofelleus* (= *T. microsporus* S.Z. Fu, Q.B. Wang & Y.J. Yao fide Gelardi et al. 2015) is closely related to *T. felleus* (Bull.) P. Karst. and *T. plumbeoviolaceus* (Snell & E.A. Dick) Snell & E.A. Dick. However, *T. felleus* (originally reported from Europe, Munoz 2005) has a brown pileus with olive-grey colour and distinctively brown reticulation on its yellowish stipe-surface, while *T. plumbeoviolaceus* (originally reported from North America) has a deep violet-purplish, then purple-brown to dull cinnamon-brown pileus. Micromorphologically, basidiospores of *T. plumbeoviolaceus* are distinctively longer [10–13(–14) × 3–4(–5.5) µm, than those of *T. neofelleus* (Smith & Thiers 1971, Bessette et al. 2000, 2006, Gelardi et al. 2015). *Tylopilus plumbeoviolaceoides* T.H. Li, B. Song & Y.H. Shen, described from China, differs in the darkly coloured pileus and stipe ranging from dark violaceous to brown-vinaceous, the context turning pinkish to purplish when cut, and usually longer and somewhat narrower spores [(7.5–) 8.5–10.5(–12) × (2.5–)3.0–3.8(–4.2) µm] (Li 2011, Gelardi et al. 2015). Finally, *T. himalayanus* is distinct from *T. neofelleus* by the absence of purplish-violaceous tinges on the stipe surface and of a well-developed reticulum.

Acknowledgements

Two (DC & KD) of us are grateful to the Director, Botanical Survey of India (BSI), Kolkata and Scientist-in-Charge, BSI, Gangtok for providing facilities and to Aniket Ghosh (H.N.B. Garhwal University, Uttarakhand) for sending the collections of *Tylopilus* from Uttarakhand and helping us in many ways. Literature provided by A.C. Magnago (Brazil) was highly appreciated. Field assistance to KD rendered by M.E. Hembrom (Central National Herbarium, BSI, Howrah, India) is duly acknowledged.

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Supplementary material 1

Figure S1

Authors: Dyutiparna Chakraborty, Alfredo Vizzini, Kanad Das

Data type: (measurement/occurence/multimedia/etc.)

Explanation note: nrLSU based Bayesian phylogram inferred from MrBayes under TIMef model of nucleotide evolution. Two species of *Xanthoconium* (*X. sinense* and *X. purpureum*) were used as outgroup taxa. Posterior probabilities values (BPP) are indicated above or below the respective branches. New taxa or new record for Indian mycobiota are shown in bold and red in the phylogram.

Link: https://doi.org/10.3897/mycokeys.33.23703.suppl1

Supplementary material 2

Figure S2

Authors: Dyutiparna Chakraborty, Alfredo Vizzini, Kanad Das

Data type: (measurement/occurence/multimedia/etc.)

Explanation note: nrITS based Bayesian phylogram inferred from MrBayes under TrNef+G model of nucleotide evolution. *Gyroporus castaneus* was used as outgroup species. Posterior probabilities values (BPP) are indicated above or below the respective branches. New taxa or new record for Indian mycobiota are shown in bold and red in the phylogram.

Link: https://doi.org/10.3897/mycokeys.33.23703.suppl2