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The holomorph of *Parasarcopodium* (*Stachybotryaceae*), introducing *P. pandanicola sp. nov.* on *Pandanus* sp.

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Abstract

Collections of microfungi on *Pandanus* species (*Pandanaceae*) in Krabi, Thailand resulted in the discovery of a new species in the genus *Parasarcopodium,* producing both its sexual and asexual morphs. In this paper, we introduce *P. pandanicola sp. nov*., with an illustrated account. Evidence for the new species is provided by distinct morphology and phylogenetic analyses. This is also the first report of the sexual morph of *Parasarcopodium*. The phylogenetic trees used Maximum Likelihood and Bayesian analyses of combined LSU, SSU, TEF1 and RPB2 sequence data to show the placement of the new species in *Stachybotryaceae*.

Key words: filiform conidia, *Hypocreales*, *Pandanaceae*, sexual morph

Introduction

The order *Hypocreales* (*Sordariomycetes*) was introduced by Lindau (1897), with *Hypocreaceae* (De Notaris 1844) as the type family. Members of the *Hypocreales* are highly diverse and distributed worldwide, in temperate regions they far exceed those known for the tropics (Põldmaa 2011). Maharachchikumbura *et al.* (2015) accepted nine families (i.e., *Bionectriaceae*, *Clavicipitaceae*, *Cordycipitaceae*, *Hypocreaceae*, *Nectriaceae*, *Niessliaceae*, *Ophiocordycipitaceae*, *Stachybotryaceae*, *Tilachlidiaceae*) in *Hypocreales* based on their backbone tree for *Sordariomycetes*.

Stachybotryaceae was introduced with *Stachybotrys* (Corda 1837) as the type genus and *S. chartarum* (Ehrenb.) S. Hughes as the type species (Wang *et al.* 2015). The family was established to accommodate the genera *Myrothecium*, *Peethambara* and *Stachybotrys*. In earlier publications, these three genera were classified in the order *Hypocreales*, genera *incertae sedis*, although phylogenetic studies have shown them to be separated from other families in *Hypocreales* (Castlebury *et al*. 2004, Summerbell *et al.* 2011). Recently, Maharachchikumbura *et al.* (2015) provide outline of *Sordariomycetes* with all genera in the family.

Parasarcopodium is a hyphomycetous genus, which was introduced by Mel'nik *et al.* (2004) with *P. ceratocaryi* Mel'nik *et al*. as the type species. The type species was collected from *Ceratocaryum decipiens* (*Restionaceae*) in the fynbos of the Cape Floristic Region of South Africa. *Parasarcopodium* was earlier placed in family *Bionectriaceae* (Mel'nik *et al.* 2004), recently *Parasarcopodium* was including in family *Stachybotryaceae* base on backbone tree for *Sordariomycetes* (Maharachchikumbura *et al.* 2015). The genus is characterized by macronematous, mononematous,

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hyaline to subhyaline, septate, branched conidiophores, monophialidic, cylindro-lageniform or oblong ampulliform conidiogenous cells and cylindrical, aseptate conidia with amorphous mucoid appendages (Mel'nik *et al.* 2004).

In this study, we collected the sexual and asexual morphs of *Parasarcopodium* from the same *Pandanus* sp. host in Krabi, southern Thailand and we describe it as a new species with an illustrated account and phylogenetic analysis.

Materials and methods

Sample collection and specimen examination

Fresh specimens of *Parasarcopodium pandanicola* were collected from Muang District, Krabi Province, Thailand on dead leaves of *Pandanus* sp. (*Pandanaceae*) in December 2014. The leaves were placed in paper bags and taken to the laboratory. They were examined using a Carl Zeiss GmbH (AxioCam ERC 5 S) stereo microscope. Fruiting bodies were rehydrated in water, lactic acid and 5% KOH. Sections were cut using both a razor blade and microtome to observe characters using a Nikon ECLIPSE 80i compound microscope. Photographs were taken with a Canon 600D digital camera mounted on the microscope. All microscopic structures were measured using Tarosoft® Image Framework program v.0.9.0.7.

Description of cultures

Malt extract agar (MEA) was used for single spore isolation following the method described in Chomnunti *et al.* (2014). Single spore isolation from sexual morph and asexual morph were located under the stereomicroscope, aseptically transferred to MEA plates after spores/conidia germinating and incubated at room temperature for 6 weeks. Sexual culture were subcultured and transferred to water agar (WA) media containing sterile toothpicks and pine needles (Phookamsak *et al.* 2015) and incubated at room temperature for 3 months to induce the asexual morph. After 3–5 weeks, on the WA cultures were checked for asexual structures. Herbarium specimens were deposited in Mae Fah Luang University Herbarium (MFLU), Chiang Rai, Thailand and Kunming Institute of Botany Academia Sinica (HKAS). The ex-type living cultures were deposited in the Mae Fah Luang University Culture Collection (MFLUCC). FacesofFungi numbers (FoF) and Index Fungorum (IF) numbers were obtained as explained in Jayasiri *et al.* (2015) and Index Fungorum 2016.

DNA extraction, PCR amplification and DNA sequencing

Isolates of sexual morphs and asexual morphs were grown on MEA for 6 weeks at room temperature. The fungal mycelium was scraped off and transferred to 1.5 ml micro centrifuge tubes. The fungal genomic DNA extraction was followed as detailed by Thambugala *et al*. (2015). The partial large subunit nuclear rDNA region (LSU) was amplified by using primer pairs LROR and LR5 (Vilgalys & Hester 1990). The partial small subunit nuclear rDNA region (SSU) was amplified by using primer pairs NS1 and NS4 (White *et al*. 1990). The partial RNA polymerase second largest subunit region (RPB2) was amplified by using primers fRPB2-5F and fRPB2-7cR (Liu *et al*. 1999). The amplification reactions were carried out with the following protocols Thambugala *et al.* (2015) and Daranagama *et al.* (2015), amplifications were performed in 25 μL of PCR mixtures containing 9.5 μL ddH_2O, 12.5 μL 2×PCR Master Mix (TIANGEN Co., China), 1 μL of DNA template, 1 μL of each primer (10 μM). Conditions of amplification for all regions consisted an initial denaturation step of 5 min at 95 °C and final elongation step of 10 minutes at 72 °C. For the LSU amplification, the 35 cycles consisted of denaturation at 94 °C for 1 minute, annealing at 53 °C for 50 seconds and elongation at 72 °C for 1.30 minute, for the SSU amplification, the 37 cycles consisted of denaturation at 94 °C for 1 minute, annealing at 54 °C for 50 seconds and elongation at 72 °C for 1 minute and for the RPB2 amplification, the 35cycles consisted of denaturation at 95 °C for 45 seconds, annealing at 57 °C for 50 seconds and elongation at 72°C for 1.30 minute. The PCR products were observed on 1% agarose electrophoresis gels stained with Ethidium bromide. Purification and sequencing of PCR products were carried at using the abovementioned PCR primer at Invitrogen Biotechnology Co., China.

Phylogenetic analyses

Sequences generated from this study were analyzed with similar sequences obtained from GenBank (Table 1). Single sequence alignments (LSU, SSU, TEF1, RPB2) were generated with MAFFT v.6.864b (Katoh & Standley 2015: http://mafft.cbrc.jp/alignment/server/) and manually improved with BioEdit v.7.2.5 (Hall 2004). Phylogenies used maximum likelihood (ML) analyses and Bayesian inference (BI) analyses for the dataset. Maximum likelihood

analysis (RAxML) was carried out using raxmlGUI v.0.9b2 (Silvestro & Michalak 2010) with bootstrap support for the branches generated with 1,000 replicates. A GTR substitution models comprised with a discrete gamma distribution (Silvestro & Michalak 2012).

TABLE 1. Taxa used in the phylogenetic analyses and their GenBank accession numbers (ex-type strains are in bold, the new taxon is indicated with an asterisk).

Taxon	*Culture Accession*	*GenBank Accession*			
		LSU	*SSU*	*TEF1*	*RBP2*
Albosynnema elegans	**GB3101**	**AF193226**	**-**	**-**	**-**
Ascopolyporus polychrous	**PC546**	**AY886546**	**-**	**DQ118745**	**DQ127236**
Bionectria ochroleuca	AFTOL-ID 187	DQ862027	DQ862044	DQ862029	DQ862013
Claviceps purpurea	**AEG 97-2**	**AF543789**	**AF543765**	**AF543778**	**-**
Cordyceps militaris	**OSC 93623**	**AY184966**	**AY184977**	**DQ522332**	**-**
Cosmospora coccinea	**AR2741**	**AY489734**	**AY489702**	**AY489629**	**-**
Elaphocordyceps capitata	OSC 71233	AY489721	AY489689	AY489615	DQ522421
Elaphocordyceps japonica	OSC 110991	DQ518761	DQ522547	DQ522330	DQ522428
Epichloe typhina	ATCC 56429	-	-	AF543777	DQ522440
Hydropisphaera peziza	**GJS92-101 = BPI802846**	**AY489730**	**AY489698**	**AY489625**	**-**
Hymenostilbe aurantiaca	OSC 128578	DQ518770	DQ522556	DQ522345	DQ522445
Hyperdermium pulvinatum	PC602	DQ118738	-	-	-
Hypocrea americana	AFTOL-ID 52	AY544649	AY544693	DQ471043	-
Hypocrea rufa	**DAOM JBT1003**	**JN938865**	**JN939042**	**-**	**-**
Hypocrella discoidea	**BCC 8237**	**DQ384937**	**-**	**DQ384977**	**DQ452461**
Melanopsamma pomiformis	**ATCC 18873**	**AY489709**	**AY489677**	**AY489604**	**-**
Myrothecium acadiense	CCFC221473	AY283563	-	-	-
Myrothecium cinctum	ATCC 22270	AY489710	AY489678	AY489605	EF692512
Myrothecium gramineum	BCC9458	FJ825379	FJ825369	-	-
Myrothecium inundatum	**IMI158855**	**AY489731**	**AY489699**	**AY489626**	**-**
Myrothecium leucotrichum	AR3506	AY489707	AY489675	AY489602	-
Myrothecium macrosporum	MFLUCC 11-0392	KP744491	-	-	-
Myrothecium roridum	ATCC 16297	AY489708	AY489676	AY489603	-
Myrothecium verrucaria	ATCC 9095	AY489713	AY489681	AY489608	EF692514
Nectria cinnabarina	CBS 114055	U00748	-	AF543785	DQ522456
Neonectria ramulariae	**CBS 151.29**	**HM042436**	**HQ840414**	**-**	**DQ789792**
Niesslia exilis	CBS 560.74	AY489720	AY489688	AY489614	-
Nitschkia tetraspora	GKML148N	FJ968987	-	FJ969011	FJ968936
Ophiocordyceps sinensis	YN09-64	JX968033	JX968028	-	JX968013
Parasarcopodium ceratocaryi	**CBS 110664**	**AY425026**	**-**	**-**	**-**
Parasarcopodium pandanicola*	**MFLUCC 15-0676**	**KU708267**	**KU708269**	-	**KU708271**
Parasarcopodium pandanicola*	**MFLUCC 15-0677**	**KU708268**	**KU708270**	**-**	**KU708272**
Peethambara spirostriata	CBS 110115	AY489724	AY489692	AY489619	-

...Continued on next page

TABLE 1. (Continued)

Taxon	*Culture Accession*	*GenBank Accession*			
		LSU	*SSU*	*TEF1*	*RBP2*
Peethambara sundara	**CCFC52196**	**AY283546**	**-**	**-**	**-**
Roumegueriella rufula	GJS 91-164	EF469082	EF469129	EF469070	EF469116
Scopinella solani	CBS 770.84	AY015632	AY015621	-	-
Shimizuomyces paradoxus	**EFCC 6279**	**EF469084**	**EF469131**	**-**	**EF469117**
Sphaerostilbella berkeleyanan	CBS 102308	U00756	AF543770	AF543783	DQ522465
Stachybotrys bisbyi	CBS 363.58	AY554250	-	-	-
Stachybotrys chartarum	HGUP 0479	KC305347	-	-	-
Stachybotrys chlorohalonata	UAMH6417	AY489712	AY489680	AY489607	-
Stachybotrys echinata	HGUP 0468	KC305358	-	-	-
Stachybotrys elegans	DAOM 225565	JN938867	JN939040	-	-
Stachybotrys microspora	HGUP 0203	KC305349	-	-	-
Stachybotrys microspora	HGUP 0120	KC305353	-	-	-
Stachybotrys sp.	HGUP 0201	KC305354	-	-	-
Stachybotrys sp.	HGUP 0146	KC305345	-	-	-
Stachybotrys subreniformis	HGUP 1051	KC305348	-	-	-
Stachybotrys subsimplex	ATCC 32888	AY489711	AY489679	AY489606	-
Stachybotrys zeae	HGUP 0143	KC305346	-	-	-
Stephanonectria keithii	**GJS92-133**	**AY489727**	**AY489695**	**AY489622**	**-**
Torrubiella wallacei	CBS 101237	AY184967	AY184978	EF469073	EF469119
Trichoderma viride	**GJS89-127**	**AY489726**	**-**	**-**	**-**
Valsonectria pulchella	**SMH1193**	**AY346304**	**-**	**-**	**AY780199**
Viridispora diparietispora	ATCC MYA 627	AY489735	AY489703	AY489630	-

The model of evolution was performed using MrModeltest 2.2 (Nylander 2004). Posterior probabilities (PP) (Rannala & Yang 1996, Zhaxybayeva & Gogarten 2002) were determined by Markov Chain Monte Carlo sampling (MCMC) in MrBayes v. 3.0b4 (Huelsenbeck & Ronquist 2001). Six simultaneous Markov chains were run for 5,100,000 generations and trees were sampled every 100th generation. The first 10,200 trees, representing the burn-in phase of the analyses were discarded, while the remaining trees (40,800) were used for calculating posterior probabilities in the majority rule consensus tree. Bayesian posterior probabilities (BYPP) equal or greater than 0.95 are given at the node. Phylograms were figured in Treeview (Page 1996) and reorganized in Microsoft power point (2007) and Adobe Photoshop version CS3 (Adobe Systems, USA). Sequences derived in this study were deposited in GenBank, and the alignments in TreeBASE (www.treebase.org) submission ID: 18869.

Results

Phylogenetic analyses

Fifty-five strains are included in the combined LSU, SSU, TEF1 and RPB2 analyses with *Nitschkia tetraspora* (GKML 148N) as the outgroup taxon. The dataset comprised 3,920 characters after alignment. A best scoring RAxML tree is shown in Fig. 1 with bootstrap support values of ML and values of the posterior probabilities (PP). The phylogenetic trees obtained from ML analysis gave similar results as previous studies in Maharachchikumbura *et al.* (2015). The

multi-gene showed *Parasarcopodium pandanicola* clustering with *Parasarcopodium ceratocaryi* with moderate bootstrap support (60% ML and 0.99 PP of multi-gene analyses) and separated from other genera in *Stachybotryaceae* (Fig 1). *Parasarcopodium pandanicola* shares morphological features similar to *P. ceratocaryi* (asexual morph) with conidia that are hyaline, cylindrical, aseptate and distinctly guttulate (Figs 3, 4).

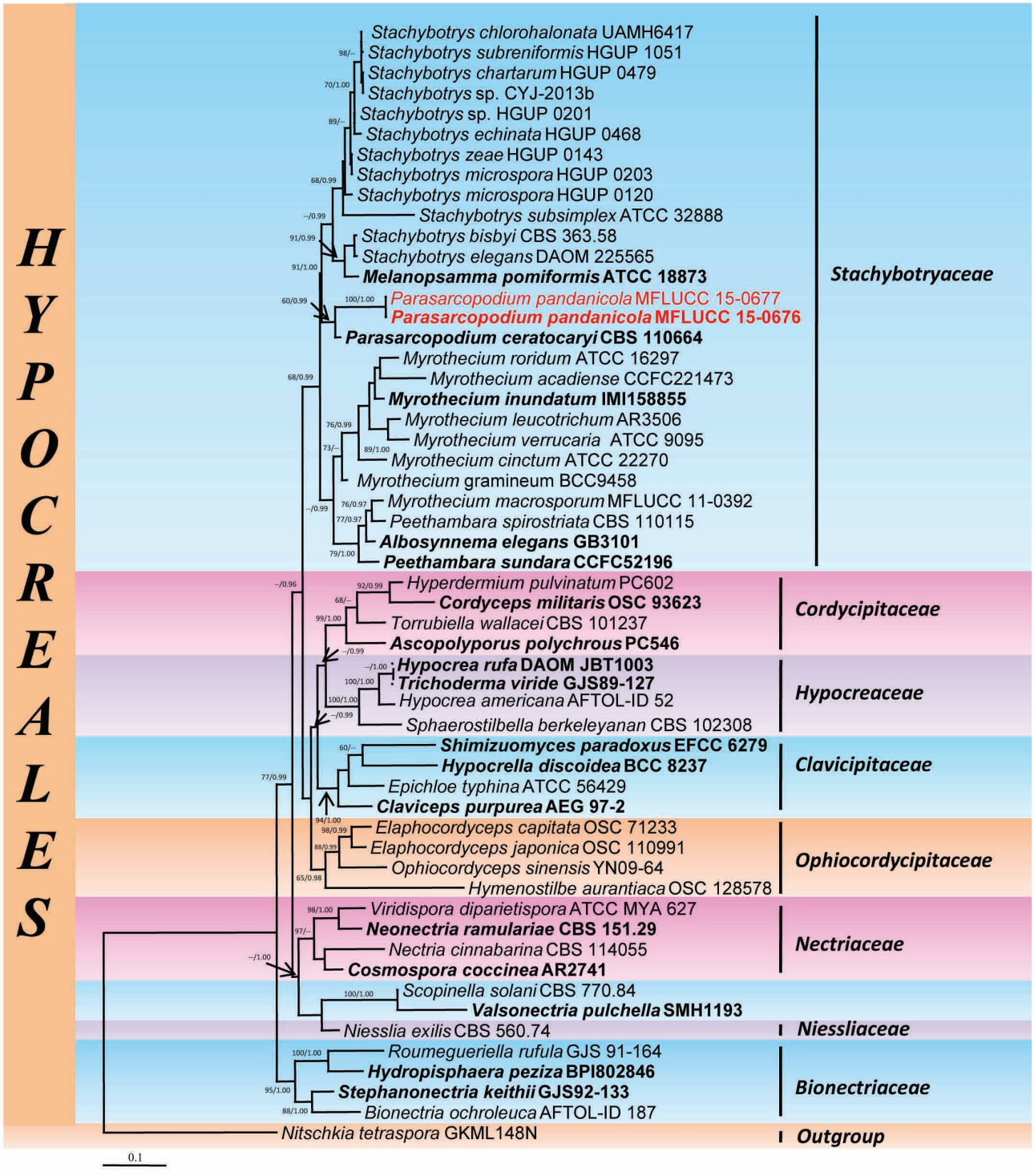

FIGURE 1. The best scoring RAxML tree based on combined LSU, SSU, TEF1 and RPB2 sequenced data of taxa from the order *Hypocreales*. Bootstrap support values for maximum likelihood greater than 60 % and Bayesian posterior probabilities greater than 0.95 are given at the nodes. The tree is rooted to *Nitschkia tetraspora* (GKML148N). Ex-type strains are in bold. The newly generated sequences are in red.

FIGURE 2. ***Parasarcopodium pandanicola*** **(**MFLU15-3280, **holotype)**. **a** Host of the fungus (*Pandanus* sp., *Pandanaceae*). **b** Appearance of fungus on host surface. **c** Cross section of ascoma. **d** Ostioles with periphysoids. **e** Section of peridium. **f** Pseudoparaphyses. **g–h** Asci. **i–m** Ascospores. **n** Germinated ascospore. Scale bars: b=200 µm, c=20 µm, d–e=10 µm, f=2 µm, g–h=10 µm, i–n=5 µm.

Taxonomy

Parasarcopodium pandanicola Tibpromma & K.D. Hyde, ***sp. nov.*** (Fig. 2, 3)
Index Fungorum number: IF 551710, *FacesofFungi number*: FoF 01627

Etymology:—named for its occurrence on the host plant family (*Pandanaceae*).

Holotype:—MFLU 15–3280.

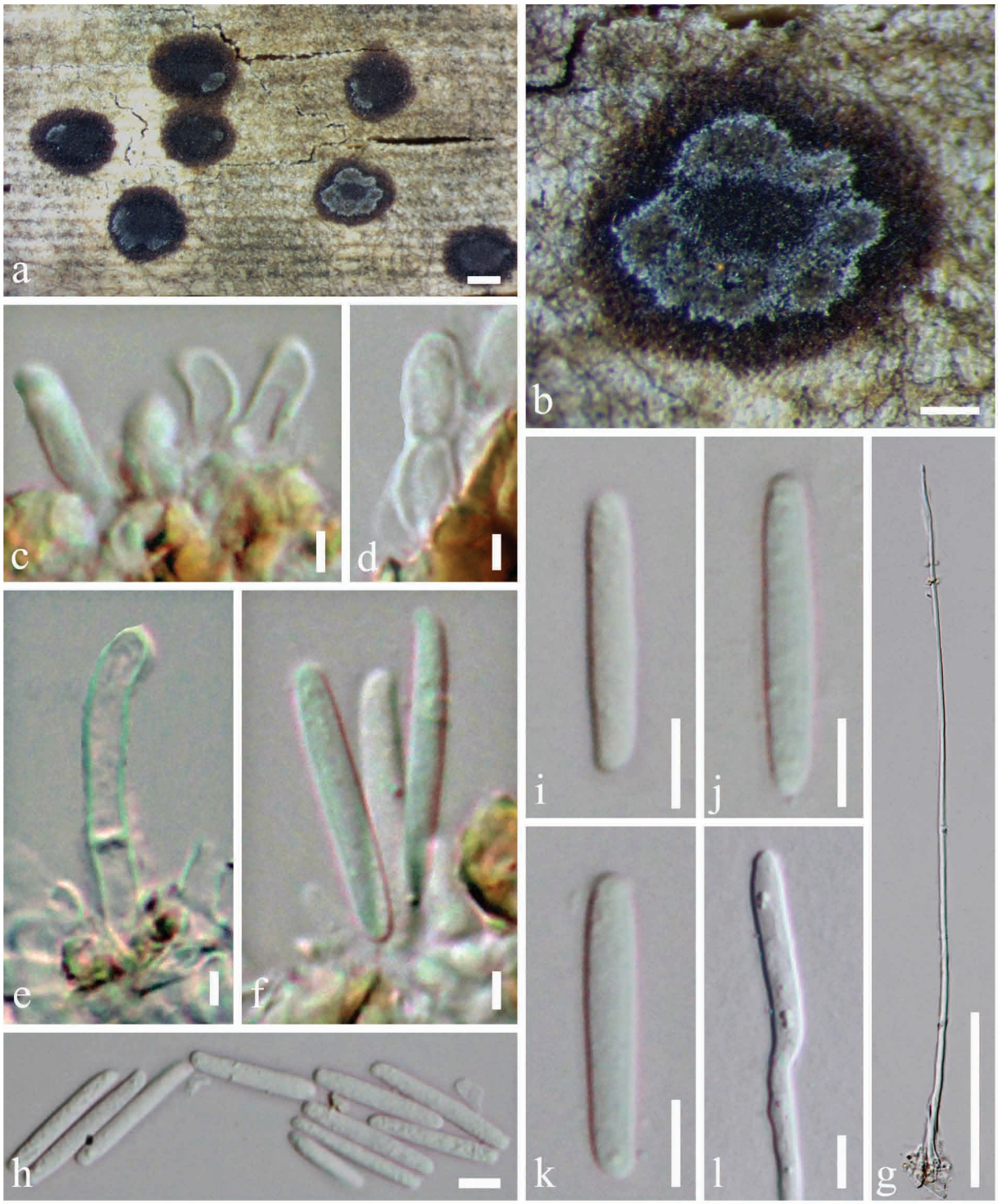

FIGURE 3. ***Parasarcopodium pandanicola*** **(**MFLU15-3280, **holotype)**. **a–b** Specimen with stroma on *Pandanus* sp. **c–f** Conidiophores. **g** Papilla**. h–k** Hyaline conidia. **l** Germinating conidium. Scale bars: a = 500 µm, b = 200 µm, c–f = 2 µm, g = 20 µm, h–l = 5 µm.

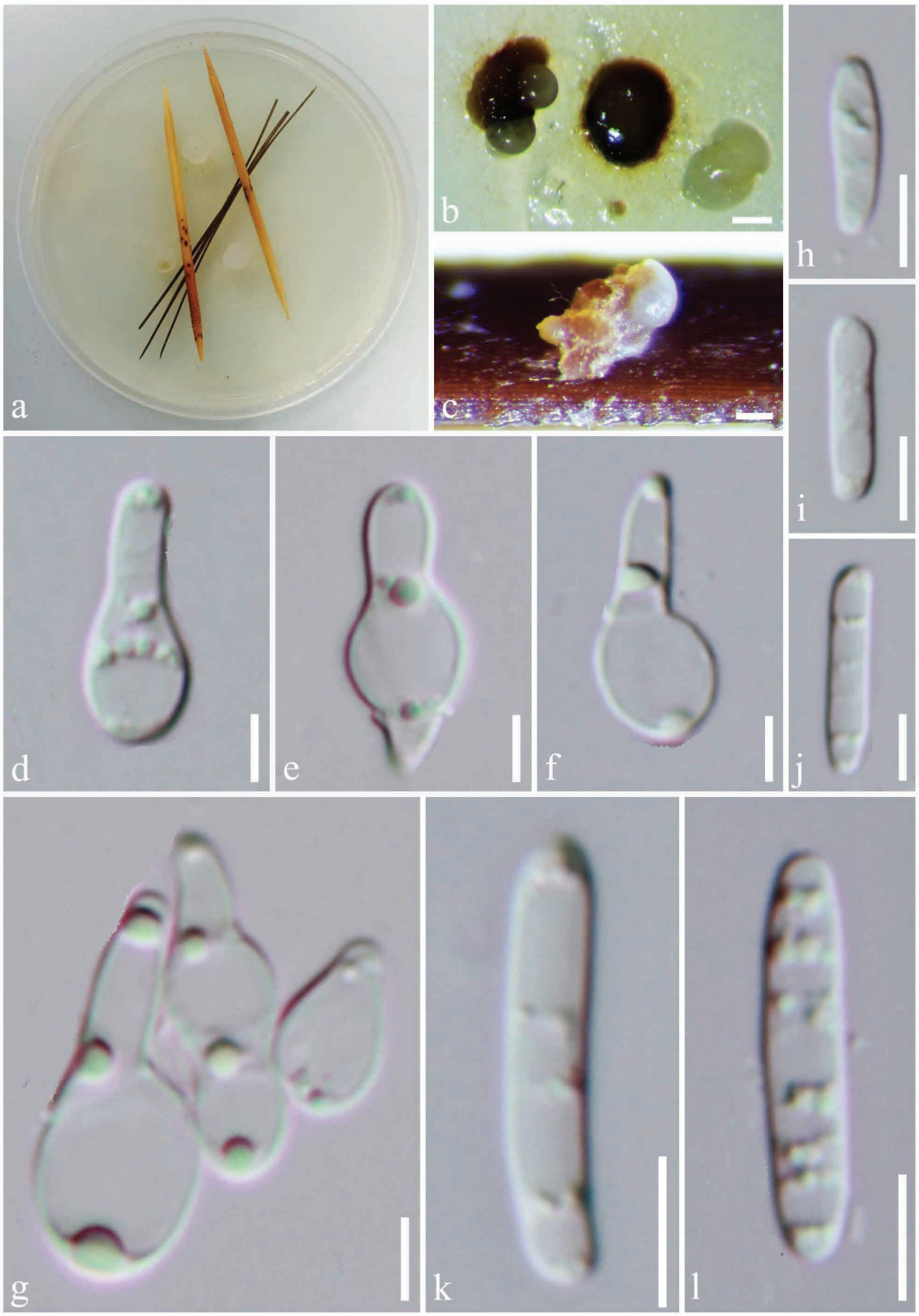

FIGURE 4. ***Parasarcopodium pandanicola*** **(**MFLUCC15-0677, **ex-type culture)**. **a** Asexual morph on water agar (WA) after 2 weeks (method from Phookamsak *et al*. 2015). **b** Conidiomata produced on WA. **c** Conidiomata produced on bamboo stalk. **d–g** Conidiophores. **h–l** Hyaline conidia. Scale bars: b = 200 µm, c = 100 µm, d–g = 2 µm, h–l = 5 µm.

Saprobic on leaves of *Pandanus* sp. **Sexual morph**: *Ascomata* 90–115 µm high, 85–110 µm diam. ($\overline{x}$ =103.2 × 98.7 µm, n=10), scattered or clustered, superficial on thin subiculum, distinct, uniloculate, solitary, globose, ostioles central with periphysoids, without hairs, smooth-walled, black. *Peridium* 11–25 µm wide, thick-walled, composed of 3–14 layers, of brown to reddish brown cells, arranged in a *textura angularis* to *textura globosa*. *Hamathecium* of numerous, 1.9–3.5 µm wide, septate paraphyses, branched, guttulate, delicate. *Asci* 54–62 × 10–12 µm ($\overline{x}$ =57.6 × 11.1 µm, n=15), 8-spored, unitunicate, cylindrical to cylindric-clavate, long pedicellate with knob-like pedicel, apically rounded with J-apical ring. *Ascospores* 12–17 × 4–5 µm ($\overline{x}$ =14.4 × 5.2 µm, n=15), overlapping 1–2-seriate, fusiform, 1-septate, slightly constricted at septum, upper cell broader and shorter than lower cell, apices obtuse, with distinct guttules, smooth-walled, without a mucilaginous sheath. **Asexual morph from host plant**: Hyphomycetous-like. *Conidiomata* stromatic, sporodochial, superficial on host surface after ascomata deteriorate, scattered, solitary, orange, with long papilla. *Conidiophores* reduced to conidiogenous cells. *Conidiogenous cells* 5–14 µm high, 3–4 µm diam. ($\overline{x}$ =9.1 × 3.6 µm, n=15), simple, smooth, short, enteroblastic, phialidic, ampulliform, hyaline, 1-septate at the base, smooth-walled. *Conidia* 10–20 × 2–3 µm ($\overline{x}$ =15.2 × 2.7 µm, n=40), hyaline, filiform, straight to slightly curved, aseptate, smooth and thick-walled, distinctly guttulate, without appendages.

Culture characteristics:—on MEA reaching 2 cm diam. after 4 weeks at room temperature, circular, entire, wrinkled and flat on media, yellow-white and thick.

Material examined:—THAILAND, Krabi Province, Muang District, on dead leaf of *Pandanus* sp., 4 December 2014, S Tibpromma & KD Hyde, SF14-035 (MFLU 15–3280, **holotype**), HKAS92501 **paratype,** ex-type living culture, MFLUCC 15–0676, MFLUCC 15–0677. *Ibid*. (MFLU15-3410 **isotypes**).

Note:—*Parasarcopodium pandanicola* is introduced as a new species and comprises both sexual and asexual morphs from the same host. The asexual morph has conidia of a similar size to those of *P. ceratocaryi* (10–20 × 2–3 µm in *P. pandanicola* vs. (12)14–18(19.5) × (2)2.5–3 µm in *P. ceratocaryi*), however, *P. ceratocaryi* has a distinctive mucilaginous appendage at either end of the conidia (Mel'nik *et al.* 2004), a feature that is lacking in *P. pandanicola*. The sexual morph was compared with *Stachybotrys oleronensis* (sexual morph) and our new species differs from *S. oleronensis* in having superficial, globose ascomata, without hairs, cylindrical to cylindric-clavate asci and fusiform, 1-septate ascospores, slightly constricted at septum and without a mucilaginous sheath. *Stachybotrys oleronensis* has subglobose to obpyriform ascomata, immersed in host tissues, clavate asci and ellipsoidal to fusiform,, 1-septate ascospores, not constricted at the septum, with 1–2 guttules in each cell (Crous *et al*. 2013).

Discussion

Parasarcopodium pandanicola is described as a new species based on morphological and phylogenetic analyses. Both morphs of *Parasarcopodium pandanicola* were found on the same host and isolated separately via single spore isolation, and later we found same asexual morph in water agar media. The evidence based on the DNA isolated from both asexual and sexual morph on the same host were compared and they are identical. Phylogenetic analyses of multi-gene analyses showed *P. pandanicola* always grouped with the genus *Parasarcopodium* with good bootstrap support (Fig. 1). *Parasarcopodium pandanicola* has different features from the asexual morph of *P. ceratocaryi* (Mel'nik *et al.* 2004) and sexual morph of *Stachybotrys oleronensis* (Crous *et al*. 2013). However, this is also the first record of the sexual morph for *Parasarcopodium* as Mel'nik *et al.* (2004) found only the asexual morph.

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