Cryptogamie, Mycologie, 2014, 35 (2): 139-149

Novel *Pestalotiopsis* species from Thailand point to the rich undiscovered diversity of this chemically creative genus

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Abstract – Two novel species, *Pestalotiopsis shorea* from *Shorea obtusa* and *P. simitheae* from *Pandanus* sp. were isolated in Thailand. They are introduced in this paper on the basis of morphological and molecular characteristics. *P. shorea* differs in morphology from its closely related species, *P. adusta*, by its shorter, tubular apical appendages and brown median cells; *P. simitheae* can be separated from its sister species, *P. theae,* by its shorter apical appendages, larger length/width ratio and colony with irregular edge and being orange on reverse of PDA plates. Phylogenetic analysis based on a combined sequence dataset from the internal transcribed spacer (ITS), partial β-tubulin and partial translation elongation factor 1-alpha (*tef1*) gene loci, confirms their distinct phylogenetic positions in the genus with strong support.

Amphisphaeriaceae / Coelomycetes / New species / *Pandanus* / phylogeny / *Shorea* / taxonomy

INTRODUCTION

Pestalotiopsis is a coelomycetous genus in the family Amphisphaeriaceae and its sexual state has been identified as *Pestalosphaeria* Barr (1975, 1990, Kang *et al*., 1998, 1999). The conspicuous character of this genus is five-celled, fusiform

doi/10.7872/crym.v35.iss2.2014.139

conidia, with three-coloured median cells, hyaline end cells, and one or more apical appendages. Species of this genus are ubiquitous in tropical and temperate ecosystems (Maharachchikumbura *et al*., 2011, 2012) and may infect a wide range of plants and cause diseases (Maharachchikumbura *et al*., 2013a, b; Zhang *et al*., 2012a, b, 2013). *Pestalotiopsis* species have often been isolated as endophytes and due to their ability to switch life-modes, many pathogens or endophytes may persist as saprobes (Hu *et al*., 2007; Maharachchikumbura *et al*., 2013c). The genus *Pestalotiopsis* has attracted considerable attention owing to its ability to produce a large array of novel bioactive secondary metabolites with potential medicinal use (Aly *et al*., 2010; Ko *et al*., 2011a, b; Xu *et al*., 2010; Debbab *et al*., 2011, 2012, 2013).

In this paper, based on morphological characters and combined phylogenetic analyses of three gene loci (ITS, β-tubulin and *tef1*), we introduce two new species from Thailand, *Pestalotiopsis shorea* and *P. simitheae* which were isolated from *Shorea obtusa* and *Pandanus odoratissimus* respectively.

MATERIALS AND METHODS

Isolation and morphological studies. Leaves of *Shorea obtusa* and *Pandanus odoratissimus* were collected from Chiang Mai Province and Suratthani Province in Thailand respectively. Pure colonies of the fungi were obtained by single spore culture technique (Maharachchikumbura *et al*., 2013d). The colony was transferred to 2% potato-dextrose agar (PDA) medium and incubated at room temperature (25°C). The morphology of fungal colonies was recorded following the method of Hu *et al*. (2007). Fungal mycelia and spores were observed under a light microscope and photographed. Methods of examination, photography and isolation were outlined in Maharachchikumbra *et al*. (2012). All microscopic measurements were done with Tarosoft image framework (v. 0.9.0.7) with 30 conidial measurements.

DNA extraction and sequencing. Total genomic DNA was extracted from fresh mycelia scraped from the margin of the PDA plate incubated at 25°C for 5-8 days using a modified protocol of Doyle & Doyle (1987) and Lee & Taylor (1990). The ITS region of rDNA fragment were amplified using primer pairs ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTA TTGATATGC-3') (White *et al*., 1990); partial β-tubulin gene region was amplified with primer pairs BT2A (5'-GGTAACCAAATCGGTGCTGCTTTC-3') and BT2B (5'-ACCCTCAGTGTAGTGACCCTTGGC-3') (Glass & Donaldson 1995; O'Donnell & Cigelnik 1997); *tef1* was amplified using the primer pairs EF1-526F (5'-GTCGTYGTYATYGGHCAYGT-3') and EF1-1567R (5'-ACHGTRCCRAT ACCACCRATCTT-3') (Rehner 2001). Polymerase Chain Reaction (PCR) was performed with the 25 μL reaction system containing 19.5 μL of double distilled water, 2.5 μL of 10 × Taq buffer with $MgCl_2$, 0.5 μL of dNTP (10 mM each), 0.5 μL of each primer (10 μM), 0.25 μL Taq DNA polymerase (5 U/μL), and 1.0 μL of DNA template. The thermal cycling program followed Maharachchikumbura *et al*. (2012). The DNA sequences generated from this study are deposited at GenBank (Table 1).

Table 1. Sequences used for phylogenetic analysis

Taxon	*Isolates*	*GenBank Accession Number*		
		ITS	β-*tubulin*	*tef1*
P. adusta (Ellis & Everh.) Steyaert	ICMP6088	JX399006	JX399037	JX399070
P. adusta	MFLUCC10-146	JX399007	JX399038	JX399071
P. anacardiacearum Y.M. Zhang, Maharachch. & K.D. Hyde	IFRDCC2397	KC247154	KC247155	KC247156
P. asiatica Maharachch. & K.D. Hyde	MFLUCC12-0286	JX398983	JX399018	JX399049
P. camelliae Y.M. Zhang, Maharachch. & K.D. Hyde	MFLUCC12-0277	JX399010	JX399041	JX399074
P. camelliae	MFLUCC12-0278	JX399011	JX399042	JX399075
P. chinensis Maharachch. & K.D. Hyde	MFLUCC12-0273	JX398995	–	–
P. chrysea Maharachch. & K.D. Hyde	MFLUCC12-0261	JX398985	JX399020	JX399051
P. chrysea	MFLUCC12-0262	JX398986	JX399021	JX399052
P. clavata Maharachch. & K.D. Hyde	MFLUCC12-0268	JX398990	JX399025	JX399056
P. clavispora (G.F. Atk.) Steyaert	IFRDCC2391	KC537808	KC537822	KC537815
P. clavispora	MFLUCC12-0280	JX398978	JX399013	JX399044
P. clavispora	MFLUCC12-0281	JX398979	JX399014	JX399045
P. coffeae-arabicae Y. Song, K. Geng, K.D. Hyde & Yong Wang bis	HGUP4015	KF412647	KF412641	KF412644
P. coffeae-arabicae	HGUP4019	KF412649	KF412643	KF412646
P. diversiseta Maharachch. & K.D. Hyde	MFLUCC12-0287	JX399009	JX399040	JX399073
P. ellipsospora Maharachch. & K.D. Hyde	MFLUCC12-0283	JX398980	JX399016	JX399047
P. ellipsospora	MFLUCC12-0284	JX398981	JX399015	JX399046
P. ericacearum Y.M. Zhang, Maharachch. & K.D. Hyde	IFRDCC2439	KC537807	KC537821	KC537814
P. foedans (Sacc. & Ellis) Steyaert	CGMCC3.9178	JX398989	JX399024	JX399055
P. foedans	CGMCC3.9123	JX398987	JX399022	JX399053
P. foedans	CGMCC3.9202	JX398988	JX399023	JX399054
P. furcata Maharachch. & K.D. Hyde	MFLUCC12-0054	JQ683724	JQ683708	JQ683740
P. gaultheria Y.M. Zhang, Maharachch. & K.D. Hyde	IFRD411-014	KC537805	KC537819	KC537812
P. inflexa Maharachch. & K.D. Hyde	MFLUCC12-0270	JX399008	JX399039	JX399072
P. karstenii (Sacc. & P. Syd.) Steyaert	IFRDCCOP013	KC537806	KC537820	KC537813
P. keteleeria Y. Song, K.D. Hyde & Y. Wang	MFLUCC13-0915	KJ503820	KJ503821	KJ503822
P. intermedia Maharachch. & K.D. Hyde	MFLUCC12-0259	JX398993	JX399028	JX399059
P. linearis Maharachch. & K.D. Hyde	MFLUCC12-0271	JX398992	JX399027	JX399058
P. magna Maharachch. & K.D. Hyde	MFLUCC12-652	KF582795	KF582793	KF582791
P. rhododendri Y.M. Zhang, Maharachch. & K.D. Hyde	IFRDCC2399	KC537804	KC537818	KC537811
P. rhodomyrtus Y. Song, K. Geng, K.D. Hyde & Yong Wang bis	HGUP4230	KF412648	KF412642	KF412645
P. rosea Maharachch. & K.D. Hyde	MFLUCC12-0258	JX399005	JX399036	JX399069
P. samarangensis Maharachch. & K.D. Hyde	MFLUCC12-0233	JQ968609	JQ968610	JQ968611
P. saprophyta Maharachch. & K.D. Hyde	MFLUCC12-0282	JX398982	JX399017	JX399048
P. simitheae Y. Song, N. Tangthirasunun, K.D. Hyde & Y. Wang	MFLUCC12-0121	KJ503812	KJ503815	KJ503818
P. simitheae	MFLUCC12-0125	KJ503813	KJ503816	KJ503819
P. shorea Y. Song, N. Tangthirasunun, K.D. Hyde & Y. Wang	MFLUCC12-0314	KJ503811	KJ503814	KJ503817
P. steyaertii Mordue	IMI192475	KF582796	KF582794	KF582792
P. theae (Sawada) Steyaert	MFLUCC12-0055	JQ683727	JQ683711	JQ683743
P. theae	SC011	JQ683726	JQ683710	JQ683742
P. trachicarpicola Y.M. Zhang & K.D. Hyde	MFLUCC12-0263	JX399000	JX399031	JX399064
P. trachicarpicola	MFLUCC12-0264	JX399004	JX399035	JX399068
P. trachicarpicola	MFLUCC12-0265	JX399003	JX399034	JX399067
P. trachicarpicola	MFLUCC12-0266	JX399002	JX399033	JX399066
P. trachicarpicola	MFLUCC12-0267	JX399001	JX399032	JX399065
P. trachicarpicola	IFRDCC2403	KC537809	KC537823	KC537816
P. trachicarpicola	OP068	JQ845947	JQ845945	JQ845946
P. umberspora Maharachch. & K.D. Hyde	MFLUCC12-0285	JX398984	JX399019	JX399050
P. unicolor Maharachch. & K.D. Hyde	MFLUCC12-0275	JX398998	JX399029	JX399063
P. unicolor	MFLUCC12-0276	JX398999	JX399030	–
P. verruculosa Maharachch. & K.D. Hyde	MFLUCC12-0274	JX398996	–	JX399061
Seiridium sp.	SD096	JQ683725	JQ683709	JQ683741

Phylogenetic analysis. Sequences were optimized manually to allow maximum alignment and maximum sequence similarity as detailed in Maharachchikumbura *et al*. (2012) (Table. 1). Maximum parsimony analysis was performed using PAUP* 4.0b10 (Swofford 2002). Parsimony trees were inferred using the heuristic search option with TBR branch swapping and 1,000 random sequence additions, with all characters equally weighted and all gaps treated as missing data. Maxtrees were set up to 5,000, branches of zero length were collapsed and all multiple parsimonious trees were saved. Tree length [TL], consistency index [CI], retention index [RI], rescaled consistency index [RC] and homoplasy index [HI] were calculated. The robustness of the most parsimonious trees was evaluated by 1,000 bootstrap replications resulting from maximum parsimony analysis, each with 10 replicates of random stepwise addition of taxa (Felsenstein 1985). The Kishino-Hasegawa tests (Kishino & Hasegawa 1989) were performed to determine whether the trees inferred under different optimality criteria were meaningfully different.

RESULTS

The aligned data matrix for combined ITS, β-tubulin and *tef1* datasets consisted of 53 sequences representing 34 isolates of *Pestalotiopsis*, with a *Seiridium* sp. as the outgroup taxon. Sequences used in this study are listed in Table 1. The alignment comprised 1562 characters including gaps (ITS: 1-564, β-tubulin: 565-1,028 and *tef1*: 1,029-1,562), among which, 994 characters were constant, 171 variable characters were parsimony-uninformative and 397 were parsimony-informative. The parsimony analysis of the data matrix resulted in seven equally parsimonious trees and the first tree (TL=1313, CI=0.596, RI=0.886, RC=0.529, HI=0.404) is shown in Fig. 1. *Pestalotiopsis shorea* forms a well-separated sub clade together with *P. adusta*, *P. rosea*, *P. rhodomyrtus* and *P. trachicarpicola*. *Pestalotiopsis shorea* displayed a close relationship with *P. adusta*. *Pestalotiopsis simitheae* forms a well separate clade apart from its sister species *P. theae*.

TAXONOMY

Pestalotiopsis shorea Y. Song, N. Tangthirasunun, K.D. Hyde & Yong Wang bis, **sp. nov.** **Fig. 2**

MycoBank: **MB 808046**
Holotype: MFLU13-0267
Etymology: *Shorea*, refers to the genus name of the host plant.

Saprobic on wing of seeds of *Shorea obtusa*. Sexual state: Unknown. Asexual state: ***Conidiomata*** pycnidial, globose, solitary, black; Conidia exuding in cirrus from pycnida, slimy, dark brown. ***Conidiophores*** often indistinct, simple, reduce to conidiogenous cells, hyaline. ***Conidiogenous cells*** discrete, clavate, septate, thin-walled, hyaline, proliferation once or twice. ***Conidia*** fusiform, straight to slightly curved, 4-septate, 18.5-25 × 5-7 μm (mean = 21 × 5.5 μm); basal cell obconic to cylindrical, hyaline, verruculose, 2-5 μm long (mean = 4 μm); three

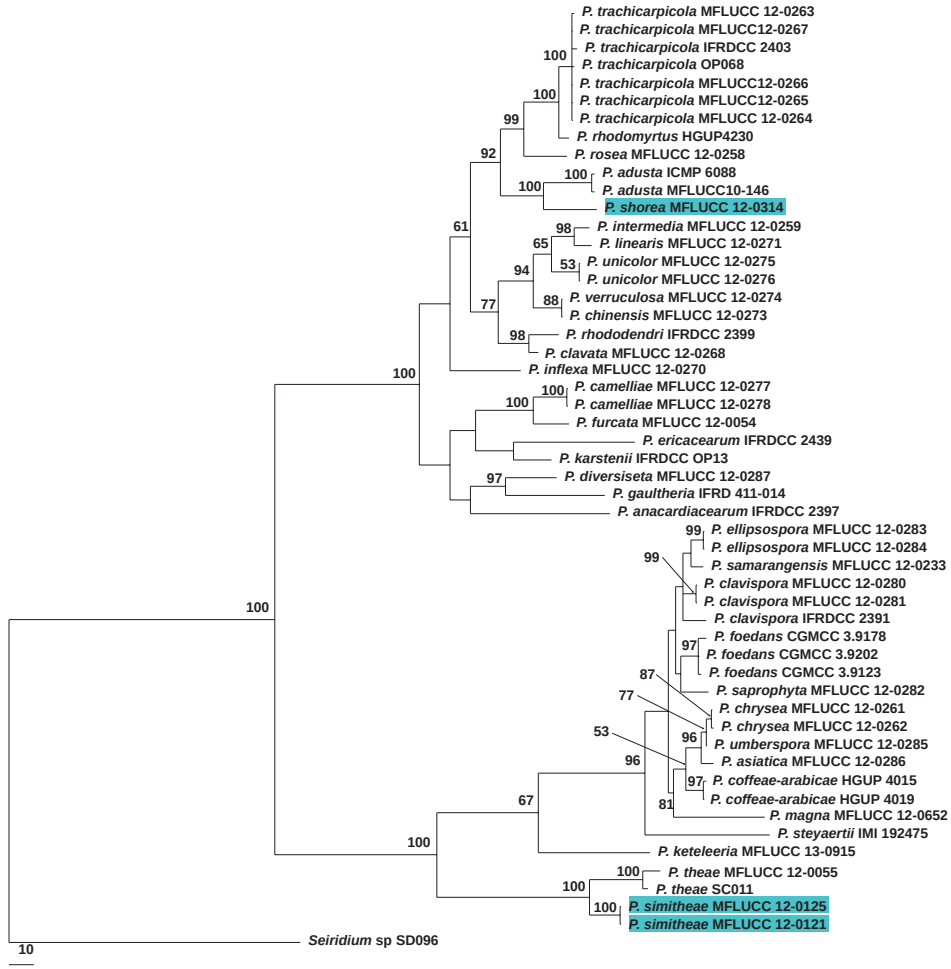

Fig. 1. Maximum parsimony phylogram generated from combination of ITS, β-tubulin and *tef1* sequences. Data were analyzed with random addition sequence, unweighted parsimony and treating gaps as missing data. *Seiridium* sp. was used as the outgroup taxon. Bootstrap values higher than 50% are shown.

median cells doliiform to cylindrical, constricted at the septa, concolorous, brown, septa and periclinal walls darker than the rest of the cell, wall rugose, together 10-16 µm long (mean = 13 µm) second cell from base 3-5 µm (mean = 4 µm); third cell 3-5 µm (mean = 4 µm); fourth cell 3.5-5.5 µm (mean = 4.5 µm); apical cell hyaline, obconic to cylindrical, 3-5 µm long (mean = 4 µm); with 1-3 tubular apical appendages (mostly 2), arising from the apical crest, unbranched, 5-12 µm long (mean = 7 µm); basal appendage filiform, unbranched, 4-10 µm long (mean = 6 µm).

Colonies growing fast on PDA at 25°C, edge undulate, white, dense aerial mycelium on surface, fruiting bodies black, concentric; reverse of culture orange.

Material examined: THAILAND. Chiang Mai Province, dead wing of seed of *Shorea obtusa*, May 2012, N. Tangthirasunun NTCL071-1 (MFLU13-0267, **holotype**); ex-type living culture MFLUCC 12-0314= ICMP 20195).

Distribution and habitat: on dead wing of seed of *Shorea obtusa*, Chiang Mai Province, Thailand.

Fig. 2. *Pestalotiopsis shorea* (holotype). **A. B.** Dead seed and wings of *Shorea obtusa* with fungus. **C.** Acervuli (Conidia coming in cirrus from pycnidia). **D-F.** Conidiogenous cells. **G-I.** Conidia. **J.** Germinating conidium. **K. L.** Colony on PDA, **K.** from above, **L.** from reverse. Scale bars: D-J = 10 μm.

Notes: *Pestalotiopsis shorea* is recognized as a distinct species based on morphological characters and phylogenetic analysis. In phylogenetic analyses, *P. shorea* forms a well-separated branch apart from *P. adusta* which was isolated from a refrigerator door PVC gasket in Fiji (Maharachchikumbura *et al*., 2012). *P. shorea* however, can be distinguished from *P. adusta* by its shorter, tubular apical appendages and brown median cells. In addition, conidiogenous cells of *P. shorea* are clavate, while in *P. adusta* they are filiform (Maharachchikumbura *et al*., 2012).

Pestalotiopsis simitheae Y. Song, N. Tangthirasunun, K.D. Hyde & Yong Wang bis, **sp. nov.** **Fig. 3**

MycoBank: **MB 808047**

Holotype: MFLU 13-0305

Etymology: *Simi* is the abbreviation of similis which means similar in Latin, *simitheae* refers to the morphologically similar to *Pestalotiopsis theae*.

Associated with dead leaves of *Pandanus odoratissimus*. Sexual state: Unknown. Asexual state: ***Conidiomata*** pycnidial, globose, solitary or integrated, black. ***Conidiophores*** indistinct, often reduce to conidiogenous cells. ***Conidiogenous cells*** simple, filiform, smooth, thin-walled, hyaline. ***Conidia*** fusiform, straight to slightly curved, 4-septate, 22-30 × 5-6.5 µm (mean = 26 × 6 µm); basal cell conic to obconic, hyaline, verruculose, 3-7 µm long (mean = 5 µm); three median cells, doliiform to cylindrical, slightly constricted at the septa, concolorous, olivaceous, septa and periclinal walls darker than the rest of the cell, verruculose, together 14-19.5 µm long (mean = 17 µm) second cell from base 4.5-7 µm (mean = 6 µm); third cell 4-6.5 µm (mean = 5 µm); fourth cell 4-6 µm (mean = 5.5 µm); apical cell hyaline, obconic to cylindrical, 3.5-5.5 µm long (mean = 4.5 µm); with 2-4 tubular apical appendages (mostly 3), arising from the apical crest, 14.5-26.5 µm long (mean = 21.5 µm), slightly swollen at the tip, unbranched; basal appendage mostly present, unbranched, centric, filiform, 4-6.5 µm long (mean = 5 µm).

Colonies growing fast on PDA at 25°C, edge irregular, white, dense aerial mycelium on surface, fruiting bodies black, gregarious; reverse of culture orange.

Material examined: THAILAND. Suratthani Province, Khao Sok, dead leaves of *Pandanus odoratissimus*, December 2011, N. Tangthirasunun NTPL 001-3 (MFLU 13-0305 **holotype**); ex-type living culture MFLUCC 12-0121 = ICMP 20197; THAILAND, Chiang Mai Province, Doi Suthep, living leaves of *Pandanus odoratissimus*, February 2012, N. Tangthirasunun NTPL 007-1.1, culture MFLUCC 12-0125.

Distribution and habitat: on dead leaves of *Pandanus odoratissimus*, Thailand.

Notes: *Pestalotiopsis simitheae* is a distinct species in the genus in terms of morphology and phylogeny. In phylogenetic analyses, *Pestalotiopsis simitheae* forms a well-separated clade as sister position to *P. theae*. *Pestalotiopsis theae* however, can be distinguished from *P. simitheae* by its longer apical appendages (22.5-31 µm) (Maharachchikumbura *et al*., 2013d) and wider conidia (22.5-28 × 6.7-8.2). In addition, the characteristics of their colonies on PDA plates are also largely different, *P. simitheae* has irregular edge and is orange in reverse, while *P. theae* has a fimbriate edge and is yellowish-white in reverse. Furthermore, *P. keteleeria* can be separated from *P. simitheae* by its shorter (7.5-21 µm), unknobbed apical appendages and wider conidia (18.5-24 × 7-9.5) (Song *et al*.,

2014). *Pestalotiopsis simitheae* was isolated from dead leaves and we could not perform any pathogenicity tests. Therefore, it would be interesting to see how these species perform in pathogenicity tests in future research.

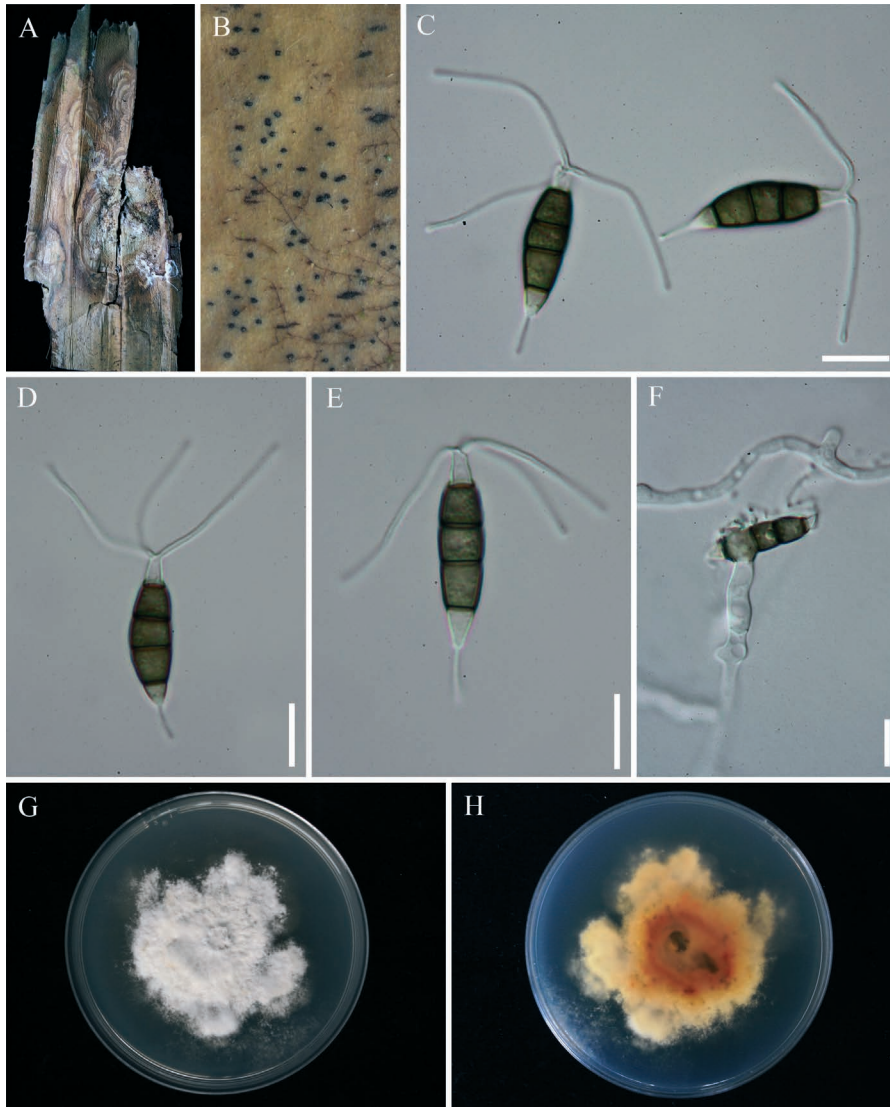

Fig. 3. *Pestalotiopsis simitheae* (holotype). **A.** Fungus on dead leaves of *Pandanus odoratissimus*. **B.** Conidiomata on dead leaves. **C-E.** Conidia. **F.** Germinating conidium. **G. H.** Colony on PDA, **G.** from above, **H.** from below. Scale bars: C-F = 10 μm.

DISCUSSION

Previously, species delimitation for *Pestalotiopsis* was mainly based on the morphological characters of the conidia (Guba 1961; Nag Raj 1993), conidiogenesis (Sutton 1980) and asexual state association (Barr 1975, 1990; Zhu *et al*., 1991; Metz *et al*., 2000) and was much confused. These methods have been confused and ambiguous due to the overlapping of morphological descriptions between different species (Maharachchikumbura *et al*., 2011). Identification of species was therefore subjective and the number of species known in the genus was hard to estimate. Kirk *et al*. (2008) estimated that there were 197 species, while there are many unusual species in the genus that need re-examination and yet to be discover (Maharachchikumbura *et al*., 2013c).

The classification based on host association is not accurate because *Pestalotiopsis* are not highly host-specific, and geographical location is also less informative (Jeewon *et al*., 2003). In present study, coupling of morphological studies with phylogenetic analyses based on the combination of ITS, β-tubulin and *tef1* gene regions were used to resolve *Pestalotiopsis* species. This method produced a well-resolved species boundary, which indicates that the application of molecular data together with morphological characters in taxonomic studies is significant. The suggestion that combined multi-gene data in phylogeny would better resolve the taxonomy of *Pestalotiopsis* (Hu *et al*., 2007; Liu *et al*., 2010; Maharachchikumbura *et al*., 2012) was also confirmed in present study.

Acknowledgements. The authors would like to thank Mushroom Research Foundation, Chiang Mai, Thailand, the National Research Council of Thailand (grant for *Pestalotiopsis* No: 55201020008), and Mae Fah Luang University (grant for *Pestalotiopsis* No: 55101020004); for funding this research. The National Natural Science Foundation of China (grant No: 31360012) is also thanked for supporting this research.

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