Phytotaxa 176 (1): 251–259
www.mapress.com/phytotaxa/

Article

ISSN 1179-3155 (print edition)
PHYTOTAXA
ISSN 1179-3163 (online edition)

http://dx.doi.org/10.11646/phytotaxa.176.1.24

Morphology and phylogeny of *Pseudorobillarda eucalypti sp. nov.*, from Thailand

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Abstract

Pseudorobillarda is a coelomycete genus of Dothideomycetes with appendaged conidia and 15 species epithets. In this study, we isolated four strains of *Pseudorobillarda* from dead leaves in Thailand. DNA sequence data generated from the large subunit (28S) ribosomal DNA (nuLSU) gene was used in phylogenetic studies. The phylogenetic trees generated indicate that *Pseudorobillarda* form a distinct lineage in Dothideomycetes that may eventually require separate family status. The *Pseudorobillarda* strains comprised two distinct species and this is also supported by morphological characteristics. In this paper we introduce a new species of *Pseudorobillarda*, *P. eucalypti* and compare it with other species of the genus. We also deposit data from the internal transcribed spacer (ITS), small subunit (18S) ribosomal DNA (nuSSU),the RNA polymerase II 2nd largest subunit genes (RPB2), Translation Elongation Factor 1-α (TEF1-α) and β-Tubulin (Bt) genes from each strain in GenBank for future studies.

Key words: appendaged coelomycetes, molecular phylogeny, new species

Introduction

The genus *Pseudorobillarda* was introduced by Morelet (1968) for *Robillarda phragmitis* (Cunnell) M. Morelet and *R. muhlenbergiae* (R. Sprague) M. Morelet, with the former species as the type. Sutton (1980), Nag Raj (1993) and Vujanovic & St-Arnaud (2003) presented keys to species in the genus. Presently, the number of described names under *Pseudorobillarda* is 15 (Index Fungorum 2014). Species of *Pseudorobillarda* are known from temperate and tropical countries including Argentina, Canada, Cuba, Germany, India, Nigeria, Thailand, UK, Ukraine, and the USA (Nag Raj 1993, Bianchinotti 1997, Vujanovic & St-Arnaud 2003, Plaingam *et al.* 2005). They have a broad host range, and are saprobic to pathogenic as well as endophytic (Petrini 1986, Nag Raj 1993, Vujanovic & St-Arnaud 2003). *Pseudorobillarda* species occur on both living and dead leaves, stems and bark (Petrini 1986, Vujanovic & St-Arnaud 2003). *Pseudorobillarda* species are also commonly found in soil. Kadowaki *et al.* (2014) recently reported *P. texana* as one of top ten most abundant soil fungi.

Revised descriptions and illustrations of *Pseudorobillarda,* based on morphological characteristics, are available in Plaingam *et al.* (2005). In *Pseudorobillarda,* conidiomata are generally immersed pycnidia with a central ostiole. Paraphyses are present only at the base in some species or mostly absent. Conidiogenous cells are discrete or integrated and conidia fusiform, subcylindrical, ellipsoidal or naviculate, 0–4-euseptate, with 2–9 apical appendages. Plaingam *et al.* (2005) clearly showed that *Pseudorobillarda* differs from *Robillarda* in conidium ontogeny and conidial appendages. Species of *Pseudorobillarda* are mainly distinguished by presence or absence of paraphyses and conidial features such as position, shape, length/size and septation (Nag Raj 1993). For example, *P. sojae* Uecker & Kulik and *P. siamensis* Plaingam, Somrith. & E.B.G. Jones lack paraphyses; whereas *P. texana* Nag Raj has unicellular conidia; *P. indica* Nag Raj, Morgan-Jones & W.B. Kendr. and *P. magna* Bianchin. possess multi-septate (mostly 3-euseptate) conidia; those in *P. siamensis* are broadly fusiform to ellipsoidal with pointed

Accepted by Eric McKenzie: 25 March 2014; published: 20 Aug. 2014

bases (Bianchinotti 1997, Plaingam 2002, 2005, Vujanovic & St-Arnaud 2003). So far, no sexual state is linked to any of *Pseudorobillarda* species (Hyde *et al.* 2011, Wijayawardene *et al.* 2012). Molecular data support the placement of *Pseudorobillarda* in *Pleosporomycetidae,* Dothideomycetes where they form a well-supported monophyletic group with *Farlowiella carmichaeliana* as sister group and basal to the *Mytilinidiales* Seutrong *et al.* 2009), while *Robillarda* cluster within *Amphisphaeriaceae*, Sordariomycetes. Species of *Pseudorobillarda* and *Robillarda* are clearly phylogenetically unrelated (Rungjindamai *et al.* 2012).

During studies on microfungi of Thailand, we isolated four appendaged strains of *Pseudorobillarda*. The strains are described and subjected to phylogenetic analysis. In this paper, we introduce a new species and further report *P. siamensis* from Thailand.

Materials and methods

Collection, isolation, cultivation and morphology

Fallen and decomposing leaves were collected from various localities in Thailand from November 2011 to October 2012, placed in plastic Zip lock bags and brought to the laboratory. The leaf litter was observed under a stereomicroscope to locate the fruiting bodies. If the fruiting bodies were immature, the specimens were incubated in a sterile moist chamber (plastic containers with sterile tissue paper soaked with sterile distilled water) and examined at intervals. The specimens were divided into two parts. The first part was used to study the morphology of fruiting body with its contents and for isolation of the fungus in pure culture. The second part of the sample was deposited at MFLU herbarium (Mae Fah Luang University, Thailand).

Cultivation

Single-spore isolation was performed as prescribed by Chomnunti *et al.* (2011). A pycnidium was sliced with a razor blade and the inner contents released in a drop of water. The water was spread on potato dextrose agar (PDA) incorporated with an antibiotic (ampicillin, 1.25 mg/100 ml). The plates were incubated overnight. Germinating conidia were located under a stereomicroscope and aseptically transferred onto fresh PDA plates. The pure cultures were incubated at room temperature for 2–5 days and sub-cultured onto fresh PDA plates. Colonies were cut into 15 mm cubes and suspended in 2 ml screw cap microcentrifuge tube either with water for storage at 4˚C or with 10% glycerol for storage at -20˚C. The stock cultures are maintained at MFLU culture collection (MFLUCC), Mae Fah Luang University, Thailand. The cultures suspended in 2 ml screw cap micro-centrifuge tube with liquid RG medium are stored at -80˚C (as per protocol and media available on *Podospora anserina* Genome Project website: http://podospora.igmors.u-psud.fr/) at the Institute of Genetics and Microbiology (IGM, NTCL code), University Paris-Sud 11, France. The pure cultures were used for further molecular analysis.

Specimens were sectioned free-hand using a sharp razor-blade. The sections were put in a drop of water and examined under a light microscope. A thin section of the fruiting body was chosen for photomicrographs. All taxonomically significant fungal parts were examined carefully and photomicrographs taken by Eclipse 80i (Nikon) photographic unit fitted to a microscope. The fungal parts were measured using an Image Frame Work program (Version 0.9.7). Besides water, 70% lactic acid or lacto-phenol cotton blue was used as mountant or stains. Photo-plates were prepared using the Photoshop CS5 program. Colony characters and growth rate studies were done on PDA, M2 (medium composition is available at http://podospora.igmors.u-psud.fr/) and V8 media and cultures incubated at 27˚C. Finally, growth rate was measured after 7 days.

DNA, PCR amplification and molecular analyses

The genomic DNA was extracted following the protocol described by Lecellier & Silar (1994). Primers used to amplify ITS, nuSSU, nuLSU, RPB2, TEF1-α and Bt genes are described in Table 1. PCR reaction mixtures (50 µL) contained 5.0 µL 10 × PCR buffer as recommended by the manufacturer, 1 µL each primer (10 µM), 2.0 µL dNTP (5 µM), 0.5 µL Taq polymerase (5.0 U/µL), 1 µL DNA template and 40.5 µL ddH_2O. The PCR reaction parameters were as follows: ITS cycling conditions with denaturation at 94˚C for 5 min, followed by 30 cycles of denaturation at 94˚C for 1 min, annealing at 52˚C for 1 min and elongation at 72˚C for 1 min, with a final extension step at 72˚C for 10 min. And on another region, the cycling conditions of denaturation at 96˚C for 2 min, 35 cycles of denaturation at 94˚C for 45 sec, The annealing period depended on primer at 45˚C (nuSSU, RPB2 and Bt), 49˚C

(TEF1-α) and 50˚C (nuLSU) for 45 sec and elongation at 72˚C for 2 min, with a final extension step at 72˚C for 10 min. The sequencing was carried at Beckman Coulter Genomics (Danvers, MA and Grenoble, France). Newly derived DNA sequences were deposited at GenBank (Benson *et al.* 2010). The phylogenetic analyses were done using sequence data of select fungal species obtained from GenBank (Maharachchikumbura *et al*. 2012 & Hyde *et al.* 2013). A maximum likelihood (ML) analyses was performed with a user-friendly, graphical, front-end software, raxmlGUI version 1.3 (Silvestro & Michalak 2012) using the settings of Maharachchikumbura *et al*. (2013). Phylogenetic trees were viewed FigTree v1.4.0 (http:// tree.bio.ed.ac.uk/software/figtree/) and prepared in Adobe Illustrator CS3.

TABLE 1. Regions and primers detail in this study.

Region	Primer (5'–3')	Reference
ITS	ITS1 (F): TCC GTA GGT GAA CCT GCG G	White *et al.* 1990
	ITS4 (R): TCC TCC GCT TAT TGA TAT GC	White *et al.* 1990
nuSSU	NS1 (F): GTA GTC ATA TGC TTG TCT C	White *et al.* 1990
	NS8 (R): TCC GCA GGT TCA CCT ACG GA	White *et al.* 1990
nuLSU	LROR (F): GTA CCC GCT GAA CTT AAG C	Rehner & Samuels 1994
	LR7 (R): TAC TAC CAC CAA GAT CT	Vilgalys & Hester 1990
RPB2	fRPB2-5F (F): GA(T/C) GA(T/C) (A/C)G(A/T) GAT CA(T/C) TT(T/C) GG	Liu *et al.* 1999
	fRPB2-7cR (R): CCC AT(A/G) GCT TG(T/C) TT(A/G) CCC AT	Liu *et al.* 1999
TEF1-α	EF1 (F): ATG GGT AAG GA(A/G) GAC AAG AC	O'Donnell *et al.* 1998
	EF2 (R): GGA (G/A)GT ACC AGT (G/C)AT CAT GTT	O'Donnell *et al.* 1998
Bt	T1 (F): AAC ATG CGT GAG ATT GTA AGT	O'Donnell & Cigelnik 1997
	T22 (R): TCT GGA TGT TGT TGG GAA TCC	O'Donnell & Cigelnik 1997

Results

DNA analyses

A total of 24 DNA sequences were generated from ITS, nuSSU, nuLSU, RPB2, Bt and TEF1-α regions. Sequences are deposited in GenBank under the accession numbers listed in Table 2. The dataset of nuLSU contained 88 strains representing 21 taxa belonging to Dothideomycetes with *Schismatomma decolorans* (Arthoniomycetes) as the outgroup, and consisted of 822 total characters, including gaps. Most of the isolates had variation in colony morphology, thus making it hard to differentiate between *Pseudorobillarda* species. The nuLSU phylogeny shows *Pseudorobillarda* species to be placed between the order *Tubeufiales* and *Mytilinidiales*. We did maximum parsimony (MP) analyzes (results not shown) and maximum likelihood analysis, and note that the position of the *Pseudorobillarda* group is not stable. In the tree, *Pseudorobillarda* sp. (strain MFLUCC 12-0414) clustered with *P. siamensis* (BCC 12513) with 100% support indicating that our strain in also *P. siamensis.* Two other strains (MFLUCC 12-0316 and MFLUCC 12-0422) were basal between *P. siamensis* and *P.* cf. *sojae* (BCC 20495), but with less support. *P. sojae* is the name given to an isolate from Thailand by Plaingam *et al.* (2005), but we suspect that these taxa are not related to *P. sojae* which is a pathogen of soybean. Further collections and careful study are needed to resolve the status of these strains which may represent new species. *Pseudorobillarda* sp. (strain MFLUCC 12-0417) clusters basal to the above taxa and is clearly unrelated. The species has unicellular conidia and sparse paraphyses and unlike any other species in *Pseudorobillarda.* It is therefore introduced as new species *P. eucalypti*.

TABLE 2. Accession number of *Pseudorobillarda* spp. from this study.

Species	MFLUCC no	MFLU no	GenBank accession number					
			ITS	nuLSU	nuSSU	Bt	RPB2	TEF1-α
Pseudorobillarda eucalypti	12-0417	13-0275	KF827449	KF827455	KF827461	KF827488	KF827494	KF827482
P. siamensis	12-0414	13-0273	KF827448	KF827454	KF827460	KF827487	KF827493	KF827481
Pseudorobillarda sp.	12-0316	13-0268	KF827447	KF827453	KF827459	KF827486	KF827492	KF827480
	12-0422	13-0278	KF827451	KF827457	KF827463	KF827490	KF827496	KF827484

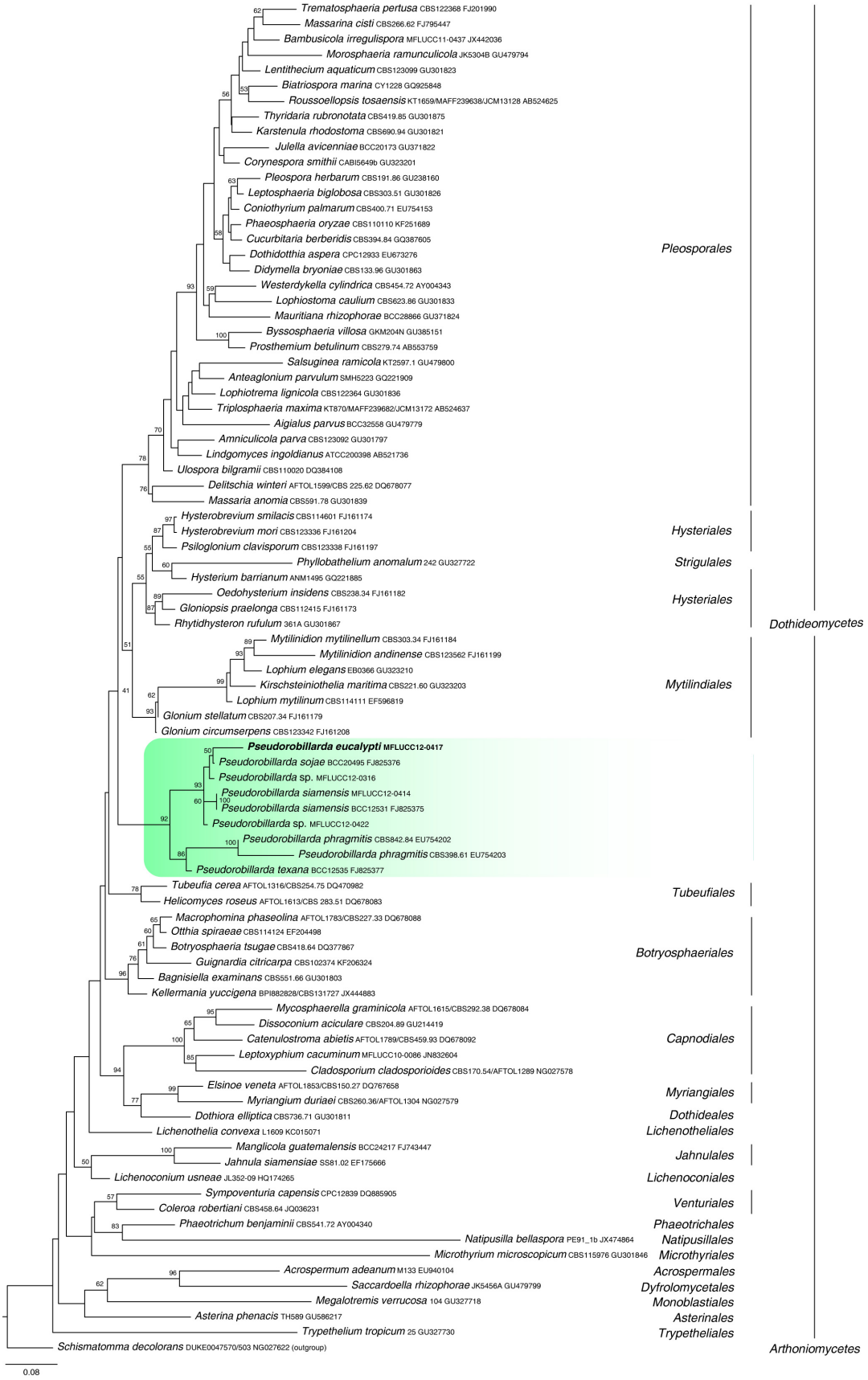

FIGURE 1. The maximum likelihood majority rule consensus tree for the analyzed *Pseudorobillarda* and related taxa. RAxML bootstrap support values above 50% (ML) are given at the nodes. Phylogeny tree is rooted to *Schismatomma decolorans.*

Taxonomy

Pseudorobillarda M. Morelet, Bull. Soc. Sci. nat. Arch. Toulon et du Var 175: 5 (1968)
Synonymy:

Neokellermania Punith., Nova Hedwigia 34(1 & 2): 85 (1981)
Pseudorobillarda Nag Raj, Morgan-Jones & W.B. Kendr., Ann. Soc. Sci. Nat. Arch. Toulon et du Var 50(4): 862 (1972)

Type species:—*Pseudorobillarda phragmitis* (Cunnell) M. Morelet, Bull. Soc. Sci. nat. Arch. Toulon et du Var 175: 6 (1968)

≡ *Robillarda phragmitis* Cunnell, Trans. Br. mycol. Soc. 41(4): 405 (1958)

Pseudorobillarda siamensis Plaingam, Somrithipol & E.B.G. Jones, Nova Hedwigia 80 (3–4): 337 (2005) (Fig. 2)

Type species:—Holotype BBH 7303!

Saprobic on dead leaves. *Conidiomata* 99–123 ($\bar{x}$ = 111) µm high, 135–153 ($\bar{x}$ = 142) µm diam., pycnidial, globose to obclavate, unilocular, separate to gregarious, immersed in the substratum, with a central long ostiolar canal on each conidioma. *Conidiomata wall* 11–23 ($\bar{x}$= 17) µm wide, 3–5-layered, dark brown to black, with thick-walled cells, of *textura angularis*. *Paraphyses* lacking. *Conidiophores* absent. *Conidiogenous cells* phialidic, sometimes proliferating 2–3 times, cylindrical, hyaline, smooth, deliquescing at maturity. *Conidia* 16–21 ($\bar{x}$ = 19) × 6–8 ($\bar{x}$ = 7) µm, mean conidium length/width ratio = 3:1, cylindrical to fusiform, smooth-walled, hyaline, rounded at both ends, with 4–5 extracellular appendages, arising from splitting of the conidial sheath.

Colonies cream or light yellow from above and reverse, with sparse to medium mycelium, flat, irregular form, undulate margin, and attaining a diam of 39 mm on PDA in 7 days at 27˚C.

Host substratum/locality:—On leaf of dicotyledons, Thailand.

Material examined:—THAILAND. Nakhonratchasima: Khao Yai, on unidentified dead leaves, 16 June 2012, *N. Tangthirasunun* (MFLU 13-0273!); ex-type living culture = MFLUCC 12-0414, NTCL 082-3, ICMP 20059.

Notes:—This species is morphologically typical of *P. siamensis* and clusters with the type strain in the phylogenetic tree with 100% support.

Pseudorobillarda eucalypti N. Tangthirasunun & K.D. Hyde, *sp. nov.* MycoBank: MB807475 (Fig. 3)

Differs from its closest known relative, *P. siamensis* in having paraphyses, and smaller conidia 15–18 ($\bar{x}$ =17) × 4–5 ($\bar{x}$ = 5) µm in *P. eucalypti* and 16–21 ($\bar{x}$ = 19) × 6–8 ($\bar{x}$ = 7) µm in *P. siamensis.*

Holotype:—MFLU 13-0275!

Saprobic on dead leaves. *Conidiomata* 132–154 ($\bar{x}$ = 140) µm high, 158–214 ($\bar{x}$ = 198) µm diam., pycnidial, globose to obclavate, unilocular, separate to gregarious, immersed in the substratum, ostiolate, with a central long ostiolar canal. *Conidiomata wall* 22–47 ($\bar{x}$ = 32) µm wide, of two layered, with outer thick-walled, black to carbonaceous, 2–3-layer cells of *textura angularis* and inner, thin-walled, 5–6-layer colourless cells of *textura angularis* and *prismatica* . *Paraphyses* sparse, composed of filamentous, septate, hyaline hyphae. *Conidiophores* absent. *Conidiogenous cells* phialidic, sometimes proliferating 2–3 times, cylindrical, hyaline, smooth, deliquescing at maturity. *Conidia* 15–18 ($\bar{x}$ = 17) × 4–5 ($\bar{x}$ = 5) µm; mean conidium length/width ratio = 4:1, cylindrical to fusiform, smooth-walled, hyaline, rounded at both ends, 1–4 extracellular appendages, arising subapically of the conidial sheath.

Colonies mycelium white when young, yellow to light orange from above and reverse when aged, with sparse to medium mycelium, flat, circular or round form, smooth or entire margin, and attaining a diam of 36 mm on PDA in 7 days at 27˚C.

Material examined:—THAILAND. Sakaeo, Pang Sida: on dead leaves of eucalyptus, 17 June 2012, *N. Tangthirasunun* (MFLU 13-0275!); ex-type living culture = MFLUCC 12-0417, NTCL 085, ICMP 20061.

Notes:—The closest relative to *P. eucalypti* in the phylogenetic tree (Fig. 1) is *P.* cf. *sojae*. The former however, has ellipsoidal to fusiform conidia with both ends rounded and appendages at the apex. In *P. siamensis* the conidia are wider and rounded at both ends. The evolution within family *Pleosporomycetidae* has shown the order

Pleosporales to be close to *Hysteriales* and *Mytilinidiales* clades (Schoch *et al.* 2009). This finding is similar to previous studies using multigene phylogenies (Boehm *et al.* 2009). Phylogenetic evidence confirms that *Pseudorobillarda* is distinct from *Robillarda* which sits in the order *Xylariales* (Sordariomycetes) (Rungjindamai *et al.* 2012). The present phylogenetic study clearly showed *Pseudorobillarda* is in Dothidoemycetes and *Pseudorobillarda* separated into a separate clade. However inside the Dothidoemycetes, placement of the *Pseudorobillarda* is not stable. Since the placement requires more work, and until additional collections and sequence data are available we prefer not to introduce a new family for *Pseudorobillarda.*

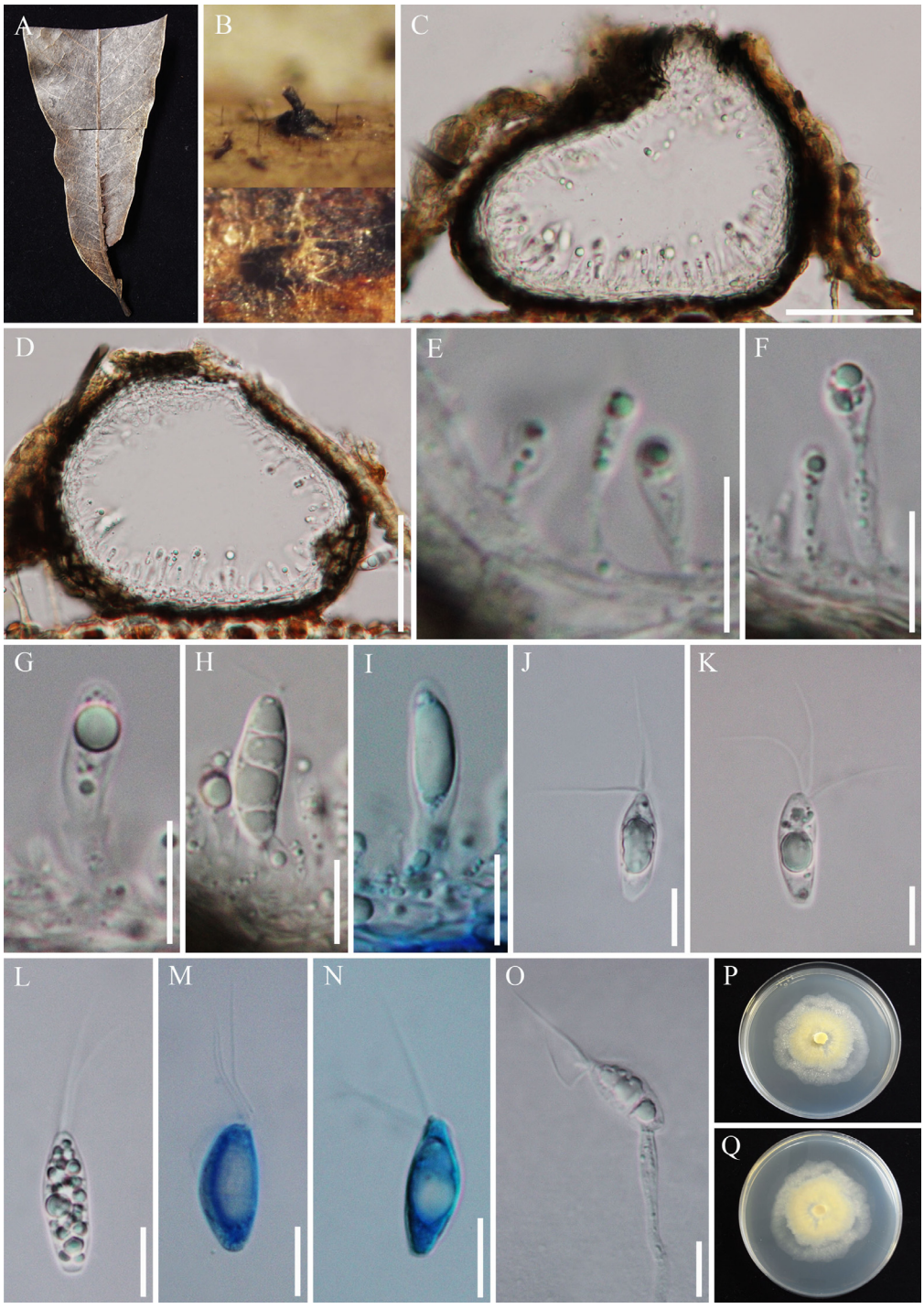

FIGURE 2. *Pseudorobillarda siamensis* (MFLUCC 12 - 0414): A. Specimen on dead leaf. B. Conidiomata on the host surface. C–D. Longitudinal section of a conidioma. E–H. Conidiogenous cells with developing conidia. I. Conidiogenous cells with developing conidia stained with lactophenol cotton blue. J–L. Conidia. M–N. Conidia stained with lactophenol cotton blue. O. Germinating conidium. P–Q. Colonies on PDA; P, from top; Q, from reverse. Scale bars: C–D = 50 µm; E–O = 10 µm.

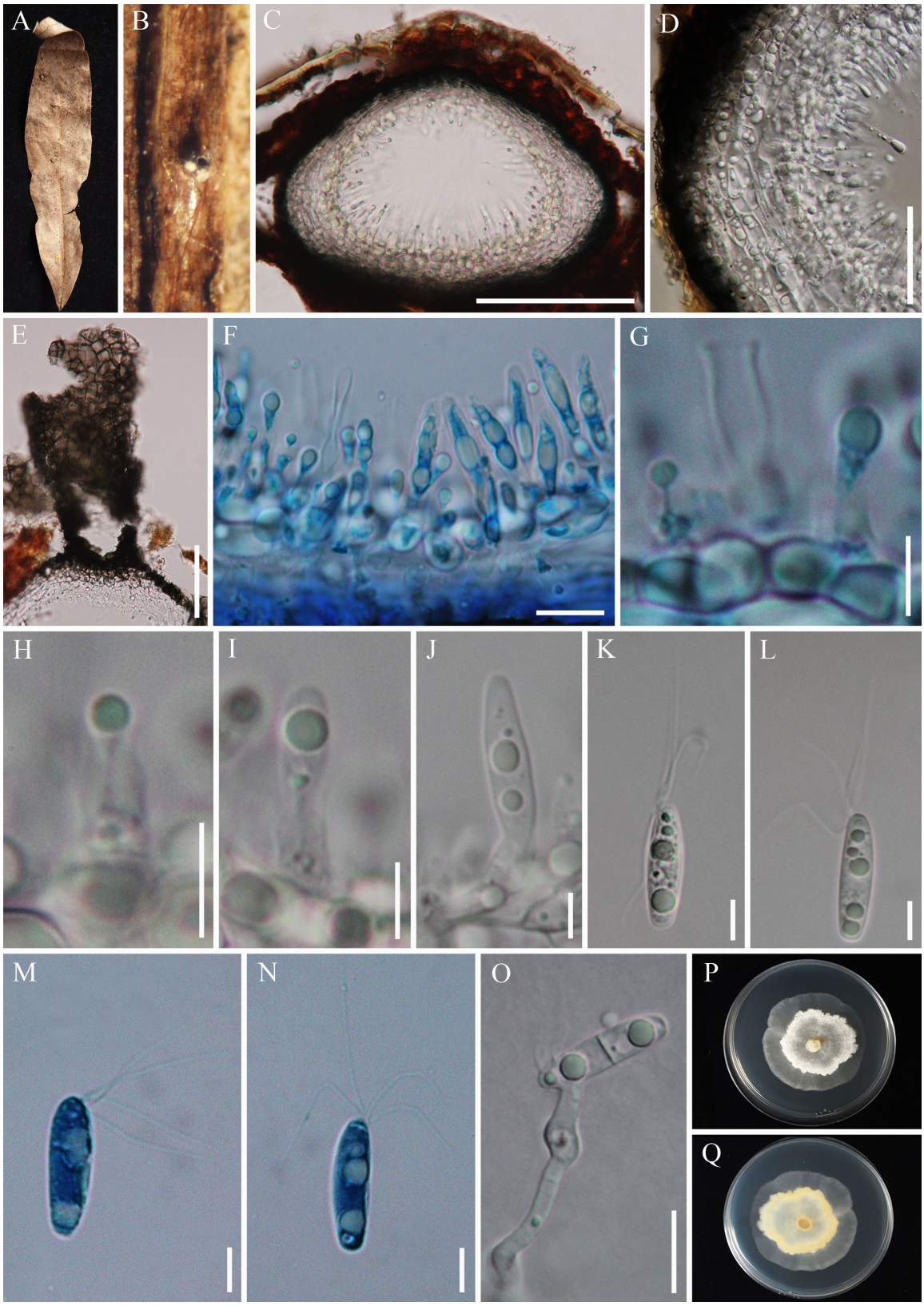

FIGURE 3. *Pseudorobillarda eucalypti* (MFLUCC 12-0417): A. Specimen on dead leaf. B. Conidiomata on host surface. C. Longitudinal section of a conidioma. D. Longitudinal section of a conidioma wall. E. Longitudinal section of ostiole. F. Conidiogenous cells with sparse paraphyses. G. Conidiogenous cells with developing conidia stained with lactophenol cotton blue. H–J. Conidiogenous cells with developing conidia. K–L. Conidia. M–N. Conidia stained with lactophenol cotton blue. O. Germinating conidium. P–Q. Colonies on PDA; P. from top, Q. from reverse. Scale bars: C = 100 µm, D, F, O = 10 µm, E = 50 µm, G–N = 5 µm.

Acknowledgements

We would like to thank our colleagues at the Institute of Excellence in Fungal Research, Mae Fah Luang University, Chiang Rai, Thailand and Institut de génétique et microbiologie (IGM), Université Paris diderot (Paris 7) for all assistance. The financial support received in the form of research grants from the French embassy (Thailand), Mae Fah Luang University grant for *Pestalotiopsis* No. 55201020008 and *Dothideomycetes* No. 56101020032 and the Mushroom Research Foundation, Chiang Rai, Thailand are duly acknowledged.

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