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Phaeosaccardinula coffeicola and *Trichomerium chiangmaiensis*, two new species of Chaetothyriales (Eurotiomycetes) from Thailand

Maharachchikumbura SSN[1,2], Haituk S[1], Pakdeeniti P[1], Al-Sadi AM[2], Hongsanan S[3], Chomnunti P[3], Cheewangkoon R[1]

[1] *Entomology and Plant Pathology Department, Faculty of Agriculture, Chiang Mai University*
[2] *Department of Crop Sciences, College of Agricultural and Marine Sciences, Sultan Qaboos University, P.O. Box 34, 123 Al-Khoud, Oman*
[3] *Center of Excellence in Fungal Research, Mae Fah Luang University, Chiang Rai 57100, Thailand*

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Abstract

Two species of sooty mould-like taxa, were obtained from living leaves of *Coffea arabica* plants collected in Chiang Mai Province, Thailand. Differences in phenotypic and phylogenetic characteristics based on combined large subunit nuclear ribosomal DNA and internal transcribed spacer sequences indicated that the two isolates represent two novel species (*Phaeosaccardinula coffeicola* and *Trichomerium chiangmaiensis*) within the order Chaetothyriales. *Phaeosaccardinula coffeicola* (Chaetothyriaceae) is distinguished morphologically from related species by its smaller asci and ascospores. *Trichomerium chiangmaiensis* (Trichomeriaceae) is morphologically distinguishable from its phylogenetically related species by its smaller conidial arms. Detailed taxonomic descriptions and illustrations of the new species are provided.

Key words – Chaetothyriaceae – *Coffea arabica* – new species – sooty mould – Trichomeriaceae

Introduction

The order Chaetothyriales was introduced by Barr (1976) for Loculoascomycetes that presence of with periphysoids (apical paraphyses) in the ascomata (Winka et al. 1998). Barr (1987) accepted Chaetothyriaceae, Coccodiniaceae, Herpotrichiellaceae, Metacapnodiaceae, Microtheliopsidaceae, Pyrenotrichaceae, Strigulaceae and Trichopeltidaceae as families of Chaetothyriales. In the classification of Wijayawardene et al. (2018), ten families are accepted in Chaetothyriales: Chaetothyriaceae, Coccodiniaceae, Cyphellophoraceae, Epibryaceae, Herpotrichiellaceae, Lyrommataceae, Microtheliopsidaceae, Pyrenotrichaceae, Strelitzianaceae and Trichomeriaceae. Members belonging to the order Chaetothyriales are morphologically highly diverse both in their ascomycetous sexual morphs and dematiaceous hyphomycetous asexual morphs (Gueidan et al. 2008, Chomnunti et al. 2012a, Dong et al. 2018). Species of Chaetothyriales are common in tropical and temperate ecosystems and may cause plant diseases, have also been recorded as an opportunistic human and animal pathogens, often isolated as saprobes, or occur as lichenized taxa or epilithic fungi (Gueidan et al. 2008, Chomnunti et al. 2012a, Réblová et al. 2013, Liu et al. 2015, Hongsanan et al. 2016, Hyde et al. 2016, Teixeira et al. 2017, Dong et al. 2018).

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Corresponding Author: Ratchadawan Cheewangkoon – e-mail – ratchadawan.c@cmu.ac.th

The Chaetothyriaceae was introduced by Hansford (1946) and members belonging to the family are saprotrophic or biotrophic (Chomnunti et al. 2012a, 2014, Hongsanan et al. 2016). The Chaetothyriaceae includes 16 genera, including the foliar epiphytic genus *Phaeosaccardinula*, which has superficial ascomata, with a dark, non-setose pellicle, saccate, bitunicate asci and muriform, hyaline to brownish ascospores (Batista et al. 1962, Hughes et al. 1976, Yang et al. 2014). *Phaeosaccardinula* was described by Hennings (1905) with *P. diospyricolai* as the type species. Many of the species belonging to the *Phaeosaccardinula* were later transferred to the genera *Limacinula* and *Treubiomyces* (Reynolds 1971, 1983) and it has an estimated 14 species (Kirk et al. 2008, Yang et al. 2014, Wijayawardene et al. 2017).

Trichomeriaceae was introduced by Chomnunti et al. (2012a) and placed in Chaetothyriales based on analysis of LSU and ITS sequence data. The family comprises a single genus, *Trichomerium*, with its type species *T. coffeicola*. Chomnunti et al. (2012a) proposed *T. foliicola* as the type species of *Trichomerium*, since it has been impossible to obtain the holotype specimen of *T. coffeicola* and also no molecular data exists in worldwide databases for this species or genus. The foliar epiphyte genus *Trichomerium* occurs superficially on living leaves of a variety of plants (Chomnunti et al. 2012a). It is estimated that the genus includes 23 species (Kirk et al. 2008), however, only seven species have sequence data. Trichomeriaceae was synonymized under Chaetothyriaceae by Réblová et al. (2013) based on ITS-β-tubulin-LSU-SSU-RPB2-mcm7 phylogeny and assessment of ITS secondary structures. However, Trichomeriaceae was accepted as a distinct family in most subsequent studies based on multi-gene phylogenetic analysis (Gueidan et al. 2014, Liu et al. 2015, Hyde et al. 2016).

We have been carrying out a taxonomic survey of foliar epiphytes on *Coffea arabica* (coffee) plants in Thailand and in the present study, two new species, *Phaeosaccardinula coffeicola* and *Trichomerium chiangmaiensis*, are introduced. Evidence for the new species is based on their unique morphological characters, as well as the support from phylogenetic analyses of combined LSU and ITS sequence data.

Materials & Methods

Isolation and identification of fungi

Fungi with sooty mould-like colonization were collected in 2014 from coffee leaves in Chiang Mai Province, Thailand. Specimens were taken to the laboratory in envelopes. Measurements and descriptions of sections of the ascomata/conidiomata, asci and ascospores/condia were carried out by immersing ascomata/conidiomata in water or in Shear's reagent. Unless otherwise stated, all photographs and measurements were made from material mounted in water and 95% lactic acid. Slides were viewed under a Nikon SMZ745T stereo microscope and (Axiovision Zeiss Scope-A1) microscope where diagnostic morphological features were recorded photomicrographs and measurements were taken. The fungal species present on the substrate were isolated using a single spore culture technique. In short, an ascomata/ conidiomata was immersed in 300 µl of sterile distilled water on a slide and left for a few minutes, so that the ascospores were discharged. An ascospore suspension was made, small drops were placed on water agar (WA) in Petri-dishes and kept at room temperature for 8–12 h for ascospores to germinate; single germinating ascospores were transferred to potato dextrose agar (PDA) plates. The plates were incubated at 25 °C for 15 to 20 days. After a month, the growing cultures were used for molecular work. Holotype specimens are deposited in herbarium of Chiang Mai University and living cultures are deposited in Sultan Qaboos University culture collection (SQUCC). Facesoffungi and Index Fungorum numbers are registered as outlined in Jayasiri et al. (2015), Index Fungorum (2018).

DNA isolation, amplification and Sequencing

Total genomic DNA was extracted directly using a DNA extraction kit (E.Z.N.A. Forensic DNA Kit), following the protocols in the manufacturer's instructions. For nucleotide sequence

comparisons, the internal transcribed spacer region (ITS) and a segment of the large subunit rDNA (LSU) regions were amplified using primer pairs ITS4/ITS5 (White et al. 1990) and LROR/LR5 (Vilgalys & Hester 1990), respectively. The thermal cycling program followed Maharachchikumbura et al. (2014). Sequencing of the PCR amplicons was conducted using the same primers used for the amplification reactions. The PCR products were verified by staining with ethidium bromide on 1 % agarose electrophoresis gels. DNASTAR Lasergene SeqMan Pro v.8.1.3 was used to obtain consensus sequences from sequences generated from forward and reverse primers and these were subsequently lodged in GenBank (Table 1).

Phylogenetic analysis

The sequences generated in this study were supplemented by additional sequences obtained from GenBank, based on BLAST searches and the literature. Multiple sequence alignments were generated with MEGA v.7.0.26 (Kumar et al. 2016) and the alignment was visually improved where necessary. Phylogenetic analyses of the sequence data consisted of Maximum Parsimony (MP) and Maximum Likelihood (ML) analyses of the aligned datasets. A maximum likelihood analysis was performed using RAxMLGUI v. 1.3 (Silvestro & Michalak 2012). The optimal ML tree search was conducted with 1,000 separate runs, using the default algorithm of the program from a random starting tree for each run. The final tree was selected among suboptimal trees from each run by comparing likelihood scores under the GTR+GAMMA substitution model evolution by MrModeltest 2.2 (Nylander 2004). The MP analysis was performed with PAUP v.4.0b10 (Swofford 2003). Trees were inferred by using the heuristic search option with TBR branch swapping and 1,000 random sequence additions. The maximum number of retained trees was limited to 5,000, branches of zero length were collapsed and all multiple equally most parsimonious trees were saved. Tree length (TL), consistency index (CI), retention index (RI), rescaled consistency index (RC), homoplasy index (HI), and log likelihood (−ln L) (HKY model) values were calculated. The robustness of the equally most parsimonious trees was evaluated by 1,000 bootstrap replications (Felsenstein 1985) resulting from a maximum parsimony analysis, each with 10 replicates of random stepwise addition of taxa. The resulting trees were printed with MEGA v.7.0.26 (Kumar et al. 2016) and the layout was made with Adobe Illustrator CS v.6.

Table 1 Details of the strains included for molecular study and newly generated sequences are indicated in bold

Taxon	**Isolate**	**GenBank accessions**	
		LSU	**ITS**
Aphanophora eugeniae	CBS 124105	FJ839652	NR_132829
Bradymyces alpinus	CCFEE 5493	GU250396	NR_132844
Brycekendrickomyces acaciae	CBS 124104	FJ839641	NR_132828
Camptophora hylomeconis	CBS 113311	-	NR_132881
Capronia mansonii	CBS 101.67	NR_121262	AY004338
Ceramothyrium ficus	MFLUCC 15-0229	KT588600	KT588602
Ceramothyrium ficus	MFLUCC 15-0228	KT588599	NR_154800
Ceramothyrium menglunense	MFLUCC 16-1874	KX524146	NR_154813
Ceramothyrium podocarpi	CPC 19826	KC005795	NR_120227
Ceramothyrium thailandicum	MFLUCC 10-0008	KP324930	NR_137768
Cladophialophora minourae	CBS 556.83	FJ358235	AY251087
Cyphellophora fusarioide	MUCL 44033	KC455252	NR_132879
Cyphellophora guyanensis	MUCL 43737	KC455253	NR_132880
Cyphellophora olivacea	CBS 122.74	KC455260	KC455247
Cyphellophora oxyspora	CBS 698.73	KC455262	NR_132883

Table 1 Continued.

Taxon	Isolate	GenBank accessions	
		LSU	**ITS**
Cyphellophora reptans	CBS 113.85	JQ766493	NR_121346
Epibryon diaphanum	M122	EU940101	-
Epibryon hepaticola	M10	EU940091	JX298885
Epibryon interlamellare	M223	EU940135	-
Epibryon plagiochilae	M187	EU940124	-
Exophiala eucalyptorum	CBS 121638	KC455258	KC455245
Exophiala xenobiotica	CBS 115831	FJ358246	AY857539
Fonsecaea monophora	CBS 102243	FJ358247	EU938579
Knufia cryptophialidica	DAOM 216555	JN040500	JN040500
Knufia epidermidis	CBS 120353	NG_042475	NR_111330
Knufia perforans	CBS 885.95	JN040506	NG_042586
Leptoxyphium madagascariense	CBS 124766	GQ303308	GQ303277
Metulocladosporiella musae	CBS 161.74	DQ008161	DQ008137
Metulocladosporiella musicola	CBS 113873	DQ008135	DQ008159
Minimelanolocus aquaticus	MFLUCC 15-0414	KR215612	NR_154181
Minimelanolocus submersus	KUMCC 15-0206	KX789215	KX789212
Phaeococcomyces aloes	CPC 21873	KF777234	NR_132069
Phaeococcomyces catenatus	CBS 650.76	-	AF050277
Phaeosaccardinula coffeicola	**COF25**	MH345729	MH345730
Phaeosaccardinula dendrocalami	IFRDCC 2649	KF667244	NR_132894
Phaeosaccardinula dendrocalami	IFRDCC 2663	KF667246	KF667243
Phaeosaccardinula ficus	MFLUCC 10-0009	HQ895837	NR_132850
Phaeosaccardinula multiseptata	IFRDCC 2639	KF667246	KF667243
Strelitziana malaysiana	CPC 24874	KR476766	KR476731
Trichomerium bambusae	MFLU 16-2286	-	KX845435
Trichomerium deniqulatum	MFLUCC 10-0884	JX313660	JX313654
Trichomerium chiangmaiensis	**COF18**	MH345728	MH345731
Trichomerium eucalypti	CBS 143443	MG386121	MG386068
Trichomerium foliicola	MFLUCC10-0078	JX313661	NR_144963
Trichomerium gleosporum	MFLUCC10-0087	JX313662	JX313656
Trichomerium siamensis	MFLUCC 12-0105	-	KP744468
Vonarxia vagans	CPC 15152	FJ839673	FJ839637
Vonarxia vagans	CBS 123533	FJ839672	NR_132830

Results

Phylogenetic analyses

New nucleotide sequences generated in this study were deposited in GenBank (Table 1). Results of the individual LSU and ITS trees (results not shown) indicated that there was no significant conflict between the topologies and respective clades in each loci. Therefore, the LSU and ITS loci were concatenated into a single dataset for analysis. After exclusion of ambiguously aligned regions, the final dataset included 48 isolates with an alignment length of 1724 characters (including gaps). With parsimony analysis, 942 characters were constant and 595 parsimony-informative. The parsimony analysis of the data matrix resulted in 12 equally parsimonious trees and the first tree (TL = 3,370 steps, CI = 0.396, RI = 0.609, HI = 0.604, RC = 0.241). A best

scoring RAxML tree resulted with the value of Likelihood: –16510.941529. The ML analyses showed similar tree topologies and was congruent to those obtained in MP analysis and the phylogenetic tree resulting from the analysis of two loci concatenated is rooted using *Leptoxyphium madagascariense* (CBS 124766) (Fig. 1). The phylogenetic tree of the partial LSU and ITS loci produced by using ML and MP analysis shows that the families of the order Chaetothyriales generally resolved into well supported clades. *Trichomerium chiangmaiensis* sp. nov. formed a distinct clade which is sister to species including *Trichomerium foliicola*, *Trichomerium gleosporum* and *Trichomerium eucalypti*. Phylogenetic analysis observation that *Phaeosaccardinula coffeicola* is distinct evolutionary entity and was recognized as basal lineage for all other *Phaeosaccardinula* isolates.

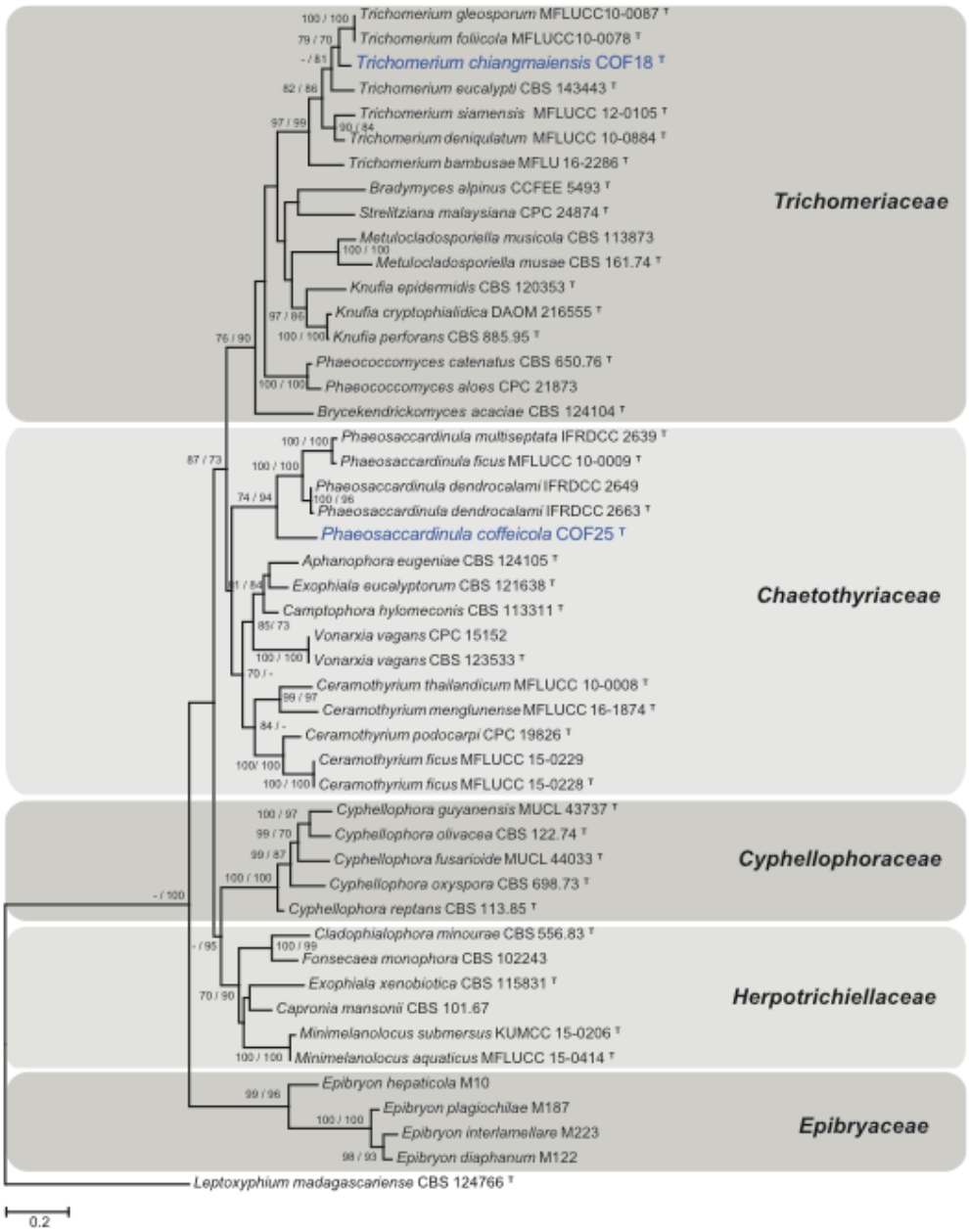

Figure 1 – Consensus tree resulting from a maximum likelihood analysis of a combined LSU and ITS sequence alignment for taxa of Chaetothyriaceae, Trichomeriaceae and other species in Chaetothyriales. Families are indicated in coloured blocks. RAxML bootstrap support values (MLB above 70 %) and maximum parsimony bootstrap support values (MPB above 70 %) are given at the nodes (MLB/MPB). The scale bar represents the expected number of changes per site. The tree is rooted to *Leptoxyphium madagascariense* (CBS 124766). The new sequences used in this study are in blue and type sequences are indicate by ^{T}.

Taxonomy

Phaeosaccardinula coffeicola Maharachch., Haituk & Cheew., sp. nov. Fig. 2

Index Fungorum number: IF554763; Facesoffungi number: FoF04392

Etymology – *coffeicola* referring to the host on which the taxon was found.

Foliar epiphytes growing on the upper surface of living leaves forming a soot-like coating. *Sexual morph*: Mycelium superficial, black, hyphae-like, dark brown to black, reticulate to branched, constricted at the septa. *Ascomata* 171–215 × 134–172 μm ($\bar{x}$ = 193 × 153 μm, n = 10), scattered on upper surface of living leaves of *Coffea arabica*, superficial, globose, dull black, lacking setae, thick-walled, inwardly of hyaline of *textura prismatica*, dark brown to brown towards the outside, comprised 3−4 layers of *textura angularis*. *Asci* 37–54 × 10–26 μm ($\bar{x}$ = 46 × 18 μm, n = 10), 4–6-spored, bitunicate, oblong-ellipsoid when young, subglobose to oval when mature, with short pedicel, ocular chamber not visible in mature asci. *Ascospores* 24–33 × 8–12 μm ($\bar{x}$ = 29 × 10 μm, n = 10) overlapping 2–4-seriate, hyaline, olivaceous green at the septa of mature ascospores, oblong-ellipsoid, muriform, with 5–7 transversal septa and 3–5 longitudinal septa, constricted at the septum, with mucilaginous sheath (absent in some mature ascospores) germ tubes developing from numerous cells. *Asexual morph*: Undetermined.

Culture characters – Conidia germinating on PDA at 25 °C for 24 h, Colonies on PDA growing 2 cm diam after 60 days, surface brown, spreading, velvety, dense floss on the surface, pale brown and rough at the margin. No asexual state produced on PDA after 60 days.

Material examined – Thailand, Chiang Mai Province, Khun Chang Khian Highland Research Station, on living leaves of *Coffea arabica* L. (Rubiaceae), 21 December 2014, S.S.N Maharachchikumbura (COF25, holotype), – ex-type culture in SQUCC 12166.

Notes – *Phaeosaccardinula coffeicola* forms a separate cluster in the combined phylogeny, as basal sister to a group including *P. dendrocalami*, *P. ficus* and *P. multiseptata* which were isolated from on living leaf of *Dendrocalamus brandisii*, on living leaf of *Ficus* sp. and on living leaf of *Dillenia pentagyna* respectively (Chomnunti et al. 2012b, Yang et al. 2014). It differs from its closest phylogenetic neighbours; *P. dendrocalami* (asci = 57−70 × 27−41 μm; ascospores = 37−49 × 11−15 μm), *P. ficus* (asci = 120–185 3 49–64 μm; ascospores = 33–56 × 10–17 μm) and *P. multiseptata* (asci = 55–69 × 30–40 μm; ascospores = 38–47 × 13–16 μm) by its smaller asci and ascospores (asci = 37−54 × 10−26 μm; ascospores = 24−33 × 8−12 μm).

Trichomerium chiangmaiensis Maharachch., Pakdeeniti & Cheew., sp. nov. Fig. 3

Index Fungorum number: IF554764; Facesoffungi number: FoF04393

Etymology – named after the region where it was isolated.

Epiphytic or *saprobic* on the upper surface of leaves. *Superficial hyphae*, branched, septate, slightly constricted at the septa, pale brown to dark brown, hyphal networks cover the surface of hosts.Foliar epiphytes growing on the upper surface of living leaves forming a soot-like coating Asexual morph: *Stroma* none. Setae and hyphopodia absent. *Conidiophore* micronematous, mononematous; usually very short, pale brown, thick denticles develop from swollen hyphal cells but are not cut off by septa. *Conidiogenous* cells monoblastic, integrated, intercalary, determinate, cylindrical, denticulate; denticles stout, cylindrical. *Conidia arms* 39–58 × 7–9 μm ($\bar{x}$ = 50 × 8 μm, n = 10), solitary, dry, pleurogenous, branched, with usually 4 arms, sometime up to 5 arms, 3−5-septate, pale olivaceous or brown, smooth; arms subulate, multiseptate, tapering to the apex, with rounded ends; conidia also have a fifth branch of 1–2 cells tapering to a subobtuse apex. Sexual morph: Undetermined.

Culture characters – Conidia germinating on PDA at 25 °C for 18 hours, germ tubes appearing from each branch of conidia, hyaline to bluish, but becoming dark brown to black. Colonies reaching 1 cm diameter after 7 days on PDA at 25 °C, colonies, velvety surface, dark

brown pale brown at the margin, dark brown sparse aerial hyphae outer region. Conidia produced in PDA after 15 days incubation.

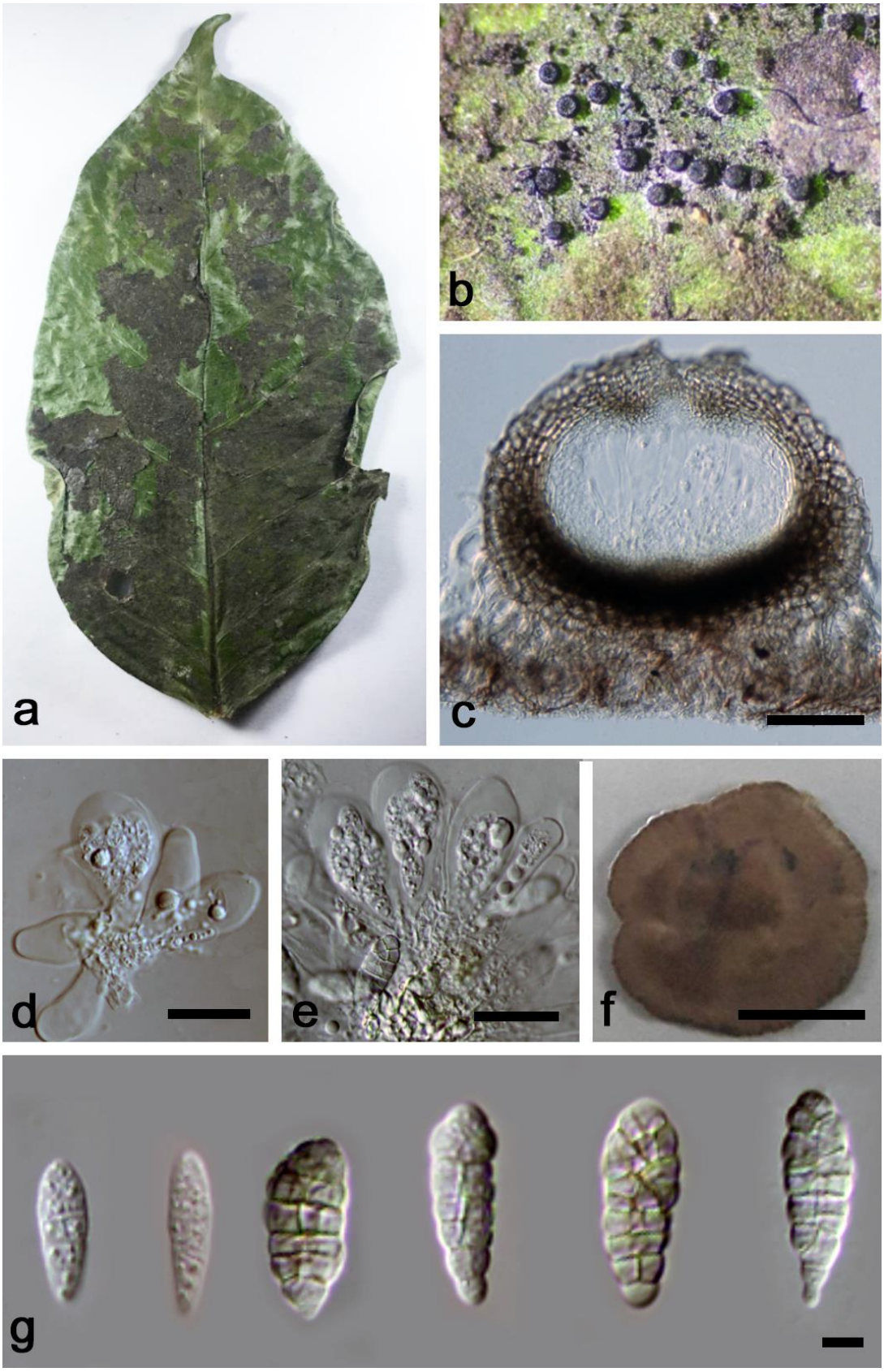

Figure 2 – *Phaeosaccardinula coffeicola* (COF25, holotype). a–b Sooty molds on leaf surface. c Vertical sections of ascoma. d–e Asci. f Colonies on media. g Ascospores. Scale bars: c–e, = 30 µm, g = 5 µm.

Material examined – Thailand, Chiang Mai Province, Mae Rim, Nong Hoi Royal Project, on living leaves of *Coffea arabica* L. (Rubiaceae), 15 December 2014, S.S.N Maharachchikumbura (COF18, holotype) – ex-type culture in SQUCC 12167.

Notes – *Trichomerium chiangmaiensis* is introduced as a new species based on its asexual morphs isolated from the living leaves of *Coffea arabica*. *Trichomerium chiangmaiensis* phylogenetically closely related to *T. eucalypti*, *T. foliicola* and *T. gleosporum*. The genus *Trichomerium* has *Tripospermum* asexual morphs (Crous et al. 2014) and the asexual morph of *T. chiangmaiensis* is comparable with *T. eucalypti* and *T. gloeosporum*. *Trichomerium chiangmaiensis* can be distinguished from *T. eucalypti* (conidial arms size = 30–80 × 8–10 µm) by its smaller conidial arms (size = 39−58 × 7−9 µm). Furthermore, the microconidia present on the *T. eucalypti* (Crous et al. 2017) were not observed on the *T. chiangmaiensis*. *Trichomerium gloeosporum i*s distinct from *T. chiangmaiensis* by its smaller conidial arms (size = 29−35 × 5−7 µm), longer basal, and apical appendages.

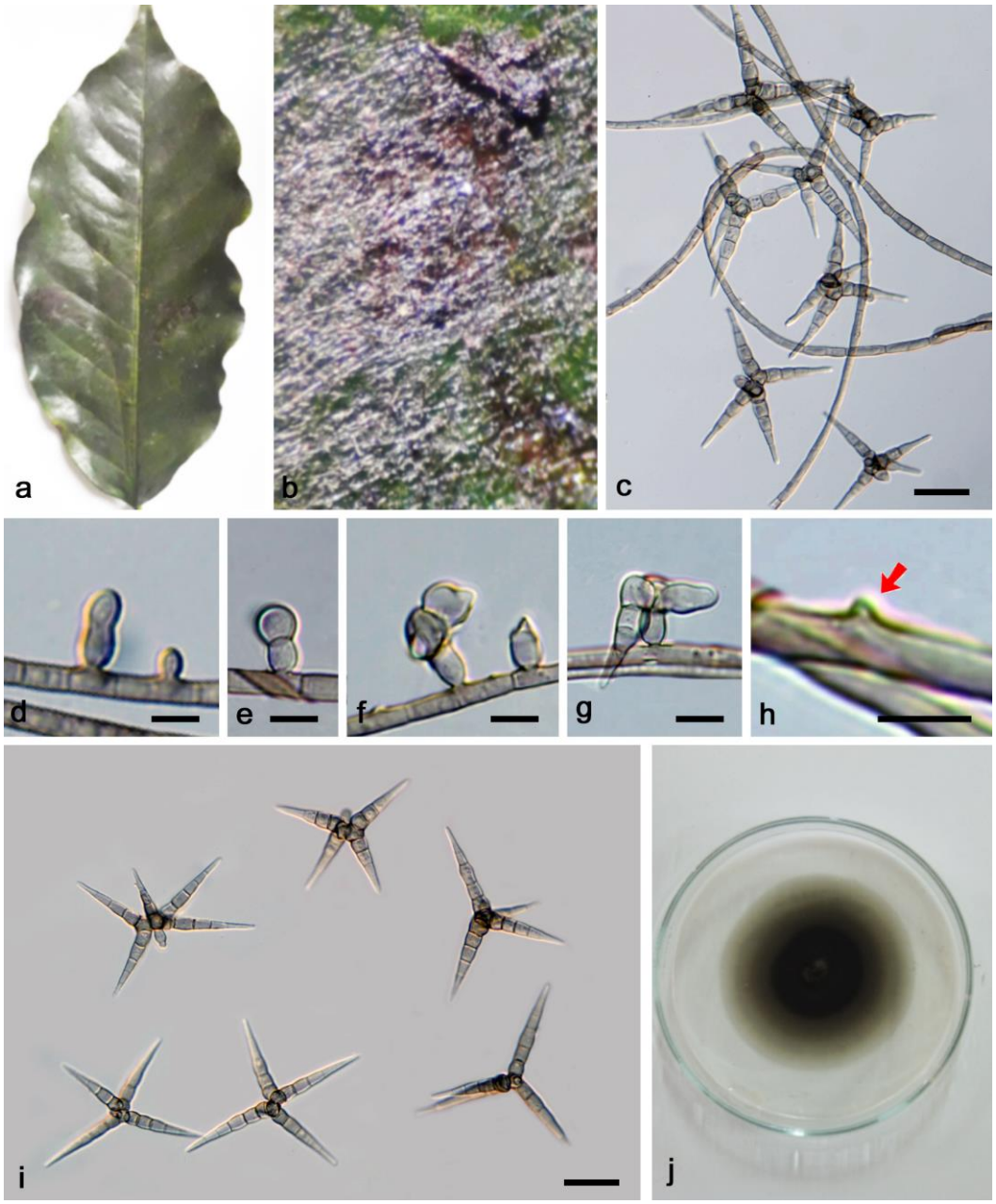

Figure 3 – *Trichomerium chiangmaiensis*. a–b Sooty molds on host. c Conidia and conidiophore. d–g Immature conidia at various stages of development attached to the prostrate mycelium. h Denticulate. i–f Conidia. j Colonies on media. Scale bars: c, i–k = 30 µm, d–h = 10 µm.

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