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A new species of *Chaetothyria* on branches of mango, and introducing Phaeothecoidiaceae fam. nov.

Hongsanan S^{1,2,3}, Zhao RL⁴, Hyde KD^{1,2,3}

¹World Agroforestry Centre, East and Central Asia, Kunming 650201, Yunnan, PR China

²Key Laboratory of Economic Plants and Biotechnology, Kunming Institute of Botany, Chinese Academy of Sciences, Lanhei Road No 132, Panlong District, Kunming, Yunnan Province, 650201, PR China

³Center of Excellence in Fungal Research, Mae Fah Luang University, Chiang Rai, 57100, Thailand

⁴The State Key Laboratory of Mycology, Institute of Microbiology, Chinese Academy of Science No.3 1st Beichen West Rd., Chaoyang District, Beijing 100101, PR China

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Abstract

The new family Phaeothecoidiaceae, introduced in this paper, comprises several species which cause sooty blotch and flyspeck diseases of several economic fruits. This results in quality issues with fruits and plants, due to the black thallus and small black dots coating the surface. Most species of Phaeothecoidiaceae are biotrophs and are unculturable without the host material, and direct-sequencing is difficult because of the very small and flattened thyriothecia. Therefore, this fungal group is relative poorly known due to limited sampling and few in-depth studies. "Microthyriales"-like taxa appearing as small black dots on the surface of mango trees were collected in northern Thailand. Taxa were studied based on morphological characters and molecular analyses. Maximum likelihood and Bayesian analyses of combined ITS, LSU and SSU sequence data, indicated that the collection from branches of mango is a previously undescribed species. The new species, *Chaetothyria guttulata*, is introduced in this paper with descriptions and illustrations.

Key words – Capnodiales – Dothideomycetes – Flyspeck – Phylogeny – Sooty blotch

Introduction

Sooty blotch and flyspeck disease does not directly damage fruits, but causes marketability problems in economic plants by blemishing fruits and reduces the photosynthesis ability of plants (Miñarro & Dapena 2012, Zhang et al. 2015). There are several epiphytic fungi which cause sooty blotch and flyspeck disease on the epicuticular wax layer of fruits, leaves and stems of various plants (Batzer et al. 2005, 2010, Yang et al. 2010, Cooley et al. 2011, Gleason et al. 2011, Miñarro & Dapena 2012, Hao et al. 2013, Zhang et al. 2015). Sooty blotch and flyspeck disease fungi have a wide distribution, and are common in humid temperate regions (Yang et al. 2010, Singtripop et al. 2016). Both diseases can occur together since they are favoured by similar environmental conditions (Wilcox 1994). Sooty blotch differs from flyspeck in having colonies comprising dark mycelial mats, whereas flyspeck has shiny, black, rounded, sclerotia-like bodies and lacks a mycelial mat (Batzer et al. 2005, Frank et al. 2010, Gleason et al. 2011, Mayfield et al. 2013). More

than 60 taxa of sooty blotch and flyspeck disease fungi are presently known (Díaz Arias et al. 2010). Sooty blotch and flyspeck disease is generally poorly known due to limited sampling and few in-depth studies. Furthermore, identifying sooty blotch and flyspeck disease fungi in culture is difficult due to the very small and flattened thyriothecia (Hickey 1960).

Many sooty blotch and flyspeck disease species belong in the order Microthyriales which are foliar epiphytes and mainly form small, inconspicuous, black dots on the surface of hosts (Wu et al. 2011, Hongsanan et al. 2014a, 2015a). Microthyriales is placed in the class Dothideomycetes (Hyde et al. 2013), and is characterized by flattened thyriothecia appearing as small black dots, with thyriothecial setae in some species, various ostiolar forms, while the basal wall is poorly-developed. Asci are bitunicate, and ascospores are uni- to multi-septate and hyaline or brown (Arnaud 1918, Luttrell 1973, von Arx & Müller 1975, Barr 1987, Kirk et al. 2008, Hyde et al. 2013, Hongsanan et al. 2014a, Singtripop et al. 2016). There are two families accepted in Microthyriales based on morphology, which are Microthyriaceae and Micropeltidaceae (Hyde et al. 2013, Hongsanan et al. 2014a, Wijayawardene et al. 2014, Ariyawansa et al. 2015). The family Microthyriaceae includes foliar biotrophs and saprotrophs, with easily removed thyriothecia, cell walls arranged in parallel rows from a prominent central ostiole to the margin and poorly-developed bases, fusiform to cylindrical or elongate asci, and hyaline 2-celled ascospores (DoIDGE 1942, Müller & von Arx 1962, Hofmann 2010, Wu et al. 2011, Hyde et al. 2013, Ariyawansa et al. 2015). Micropeltidaceae includes biotrophs and saprotrophs which are usually found on the lower surface of leaves as black-blue or greenish to black thyriothecia, which are poorly-developed at the base, walls comprising interwoven hyphae, with central ostioles, hyaline and mostly multi-septate ascospores (Clements & Shear 1931, Lumbsch & Huhndorf 2010, Wu et al. 2011, Hyde et al. 2013, Hongsanan et al. 2015b).

The genus *Chaetothyria* causes flyspeck disease on various plants worldwide, such as *C. musarum* (Speg.) Theiss. and *C. panamensis* (F. Stevens & Dorman) Arx, which occurs on banana and *Oncoba laurina* (Müller & von Arx 1962) respectively. *Chaetothyria* was introduced by Theissen (1913), the type species is *C. musarum*. A reference type specimen and sequence data generated from ITS, LSU and SSU sequence data were provided by Singtripop et al. (2016). The phylogenetic placement of *Chaetothyria* is now shown to lie within Capnodiales (Singtripop et al. 2016, and this study). The morphological characters are similar to Micropeltidaceae in Microthyriales; but differs in having inconspicuous superficial hyphae (sometimes this may be absent) and thyriothecia with setae (Reynolds & Gilbert 2005). Ascospores in Micropeltidaceae mostly have up to two septa, while *Chaetothyria* has 1-septate ascospores. However, *Chaetothyria* is placed in the new family Phaeothecoidiaceae (Capnodiales), based on its phylogenetic placement. *Chaetothyria* contains 14 records in Index Fungorum (2016), and sequence data for only two species are available in GenBank.

In this study, we introduce a new species, *Chaetothyria guttulata* in the new family Phaeothecoidiaceae (Capnodiales). The new taxon is compared morphologically with other species in the genus *Chaetothyria*, in Micropeltidaceae, and in Capnodiales. The introduction of *Chaetothyria* is also supported by molecular analysis of the ITS, LSU and SSU sequence data.

Materials & Methods

Isolation and morphology study

“Microthyriales”-like taxa were collected from Chiang Rai Province in northern Thailand and ascomata were studied by free-hand section under a stereomicroscope. A compound microscope was used to observe asci and ascospore characters. Slides were preserved in lactoglycerol after photographing (Nikon 80i). Measurements were determined using Tarosoft (R) Image Frame Work v. 0.9.7. Single spore isolation was carried out using the methods in Chomnunti et al. (2014). The type specimen of the new species is deposited in the Mae Fah Luang University Herbarium (MFLU), Chiang Rai, Thailand, and ex-type cultures in Mae Fah Luang University

Culture Collection (MFLUCC), and in Kunming Institute of Botany (KIB). Faces of Fungi numbers and Index Fungorum numbers are provided (Jayasiri et al. 2015, Index Fungorum 2016).

DNA isolation, amplification and sequencing

Fungal isolates were grown on potato dextrose agar (PDA) for 15 days at 25–30 °C with dark/ 12 h with light. Genomic DNA was extracted from mycelium using a Forensic Genomic DNA Extraction Kit (OMEGA Bio-tek); following the instructions. The conditions for the polymerase chain reaction (PCR) were determined using the primer pairs ITS1/ITS4 to amplify internal transcribed spacer region (ITS), LROR/LR5 to amplify the large subunit region (LSU), and NS1/NS4 to amplify the small subunit region (SSU). The amplification conditions were as described in Hongsanan et al. (2014b). PCR products were visualized on 1% agarose electrophoresis gels, and sequencing followed Li et al. (2015). Sequences generated for the new species are deposited in GenBank.

Phylogenetic analysis

Sequences that relate to *Chaetothyria* species were obtained from GenBank following previous publications to supplement the dataset (Table 1, Ariyawansa et al. 2015, Hongsanan et al. 2015b, Singtripop et al. 2016). Forty sequences were downloaded and are included with the newly generated sequences from this study, *Chaetothyria guttulata* (Fig. 1). *Venturia inaequalis* (Cooke) G. Winter was selected as outgroup taxon (Table 1). The phylogenetic tree for ITS sequence data was generated separately from the phylogenetic tree of combined LSU and SSU sequence data (Fig. 2). The data set were aligned by using MAFFT (Katoh et al. 2009), checked and aligned manually using Bioedit (Hall 1999). Maximum likelihood analysis was carried out by raxmlGUIv.0.9b2 (Silvestro & Michalak 2012). The search strategy was set to bootstrapping with GTRGAMMA model analysis. The number of replicates was inferred using the stopping criterion (Pattengale et al. 2009). Maximum likelihood bootstrap values were produced from 1,000 repetitions by RAxML analysis, and the values which are equal or greater than 50% are given to the left of each node (Figs. 1, 2). The MrModeltest 2.2 was used to perform the model of evolution (Nylander 2008). Posterior probabilities were set by MCMC sampling in MrBayes v3.1.2 (Huelsenbeck & Ronquist 2001, Zhaxybayeva & Gogarten 2002), following Cai et al. (2006, 2008). Posterior probabilities values (PP) which are equal or greater than 0.90 are given at the right of each node (Figs 1, 2). Phylogenetic trees were viewed and arranged using MEGA v5.2.1 (Tamura et al. 2011).

Results

Molecular phylogeny

The LSU and SSU sequence data from taxa in Capnodiaceae, Dissoconiaceae, Euantennariaceae, Micropeltidaceae, Mycosphaerellaceae, Myriangiaceae Schizothyriaceae and Teratosphaeriaceae were included in the phylogenetic analysis; *Venturia inaequalis* was used as outgroup taxon (Table 1). The best tree of RAxML maximum likelihood (ML) is provided with Bayesian values at each node (Fig. 1). Phylogenetic analyses indicate that nine stains from Micropeltidaceae cluster within Capnodiales (93% ML, 1.0 PP support), however, they are morphologically similar to Microthyriales; the result is similar to previous studies (Ismail et al. 2016, Singtripop et al. 2016). Four stains from the genus *Chaetothyria* cluster within the clade of Micropeltidaceae in Capnodiales, and are closely related to *Stomiopeltis versicolor* (Desm.) Arx. In combined LSU and SSU tree, *Chaetothyria guttulata* is closely related to *C. musarum* which is a reference type species of the genus and was found on *Musa* sp. (97% ML, 1.0 PP). The phylogenetic tree generated from ITS sequence data also shows that *C. guttulata* is a new species (Fig. 2). In both phylogenetic trees, *Chaetothyria guttulata* is a distinct species. Consequently, we introduce *C. guttulata* as a new species of *Chaetothyria*.

Table 1. Taxa used in the phylogenetic analysis with GenBank accession numbers (LSU and SSU) and species voucher/culture numbers.

Species	Voucher/culture numbers	Accession numbers		
		LSU	SSU	ITS
<i>Austroafricana associata</i>	CBS 120732	KF901829	-	KF901512
<i>Capnobotryella renispora</i>	CBS 215.90	GU214399	AY220613	AY220613
<i>Capnodium coffeae</i>	CBS 147.52	GU214400	DQ247808	AJ244239
<i>Chaetothyria guttulata</i>	MFLUCC15-1080	KU358917	KU358916	KX372277
<i>Chaetothyria guttulata</i>	MFLUCC15-1081	KU358914	KU358915	KX372276
<i>Chaetothyria musarum</i>	MFLUCC15-0383	KU710171	KU710174	KX372275
<i>Devriesia strelitziae</i>	CBS 122379	GU301810	GU296146	EU436763
<i>Dissoconium aciculare</i>	CBS 204.89	GU214419	GU214523	AY725520
<i>Dissoconium dekkeri</i>	CBS 342.86	JN232431	-	-
<i>Dothistroma septosporum</i>	CBS:112498	GQ852597	JX901744	JX901744
<i>Hortaea werneckii</i>	4263	JX141471	JX141470	DQ336709
<i>Houjia yanglingensis</i>	YHLB20	GQ433630	-	GQ433629
<i>Houjia yanglingensis</i>	YHJN13	GQ433631	-	GQ433628
<i>Leptoxyphium cacuminum</i>	MFLUCC10-0049	JN832602	JN832587	
<i>Mycosphaerella ellipsoidea</i>	CBS:110843	GQ852602	AY725545	AY725545
<i>Mycosphaerella endophytica</i>	CBS:114662	GQ852603	DQ302953	DQ302953
<i>Mycosphaerella irregulariramosa</i>	CBS:111211	GQ852609		KF901706
<i>Mycosphaerella keniensis</i>	CBS:111001	GQ852610	-	-
<i>Mycosphaerella punctiformis</i>	CBS 113265	NG027571	AY490775	AY490763
<i>Myriangium duriaei</i>	CBS 260.36	NG027579	AF242266	-
<i>Myriangium hispanicum</i>	CBS 247.33	GU301854	GU296180	-
<i>Phaeothecoidiella illinoisensis</i>	CBS:125223	GU117901	-	GU117897
<i>Phaeothecoidiella missouriensis</i>	CBS:118959	GU117903	-	GU117899
<i>Phragmocapnias asiticus</i>	MFLUCC10-0062	JN832612	JN832597	-
<i>Phragmocapnias betle</i>	MFLUCC10-0053	JN832606	JN832591	-
<i>Phragmocapnias betle</i>	MFLUCC10-0050	JN832605	JN832590	-
<i>Pseudoveronaea ellipsoidea</i>	MI3 34F1a	JQ622103	-	FJ425205
<i>Pseudoveronaea obclavata</i>	UIF3	AY598916	-	AY598877
<i>Ramichloridium apiculatum</i>	CBS 400.76	EU041851	EU041794	EU041794
<i>Rasutoria pseudotsugae</i>	rapssd	EF114704	EF114729	EF114687
<i>Rasutoria tsugae</i>	ratstk	EF114705	EF114730	EF114688
<i>Schizothyrium pomi</i>	CUA1a	AY598895	-	EF164898
<i>Schizothyrium pomi</i>	Flyspeck1924-Zj001	AY598894	-	AY598848
<i>Scorias spongiosa</i>	MFLUCC10-0084	JN832586	JN832601	-
<i>Scorias spongiosa</i>	AFTOL-ID 1594	DQ678075	DQ678024	-
<i>Stomiopeltis versicolor</i>	GA3 23C2b	FJ147163	-	FJ438375
<i>Venturia inaequalis</i>	CBS 815.69	GU301878	GU296204	-
<i>Zygothiala cryptogama</i>	KY1 1.2A1c	EF164902	-	EF164900
<i>Zygothiala tardicrescens</i>	MWA1a	EF164901	-	AY598856
<i>Zygothiala wisconsinensis</i>	MSTA8a	AY598897	-	AY598853

Taxonomy

Phaeothecoidiaceae K.D. Hyde & Hongsanan, fam. nov.

Index Fungorum number: IF552766; Facesoffungi number: FoF02883

Pathogenic on host surface. Sexual morph: *Thallus* very thin, covering surface of host with dark brown hyphae. *Superficial hyphae* septate, not constricted at the septum, branched, brown to dark brown. *Thyriothecia* superficial, circular, flattened, base poorly developed, thin-walled, brown to dark brown, central ostiole present, thyriothecial setae present. *Setae* arising from the surface of thyriothecia, brown to dark brown, smooth-walled. *Upper wall* dark brown of cells of *textura epidermoidea*. *Peridium* comprising cells of *textura angularis* or flattened cells, inner layer hyaline, outer layer dark brown or reddish brown. *Hamathecium* hyaline, pseudoparaphyses. *Asci* 8-spored,

bitunicate, subcylindrical to obovoid, pedicellate or apedicellate, ocular chamber. *Ascospores* ellipsoid, 1-septate, hyaline. Asexual morph: Hyphomycetous. *Colonies* effuse to punctiform. *Mycelium* septate, branched, brown to dark brown, warty hyphae, sometimes encased in a mucilaginous sheath. *Endoconidia* phragmospores inside hyphae, pale brown to brown, aseptate, thin-walled, subcylindrical to broadly ellipsoid, roughened upon release; sometimes with a thin, inconspicuous septum. *Conidiophores* reduced to conidiogenous cells, intercalary, dark-brown, subcylindrical to cuneiform, with conidiogenesis holoblastic to phialidic. *Conidia* broadly ellipsoid to subcylindrical or obclavate, truncate at base, rounded at apex, transversely septate, brown, tapering to a cuneiform with a truncate hilum at base.

Type: *Phaeothecoidiella* Batzer & Crous, in Yang et al., Persoonia 24: 30 (2010)

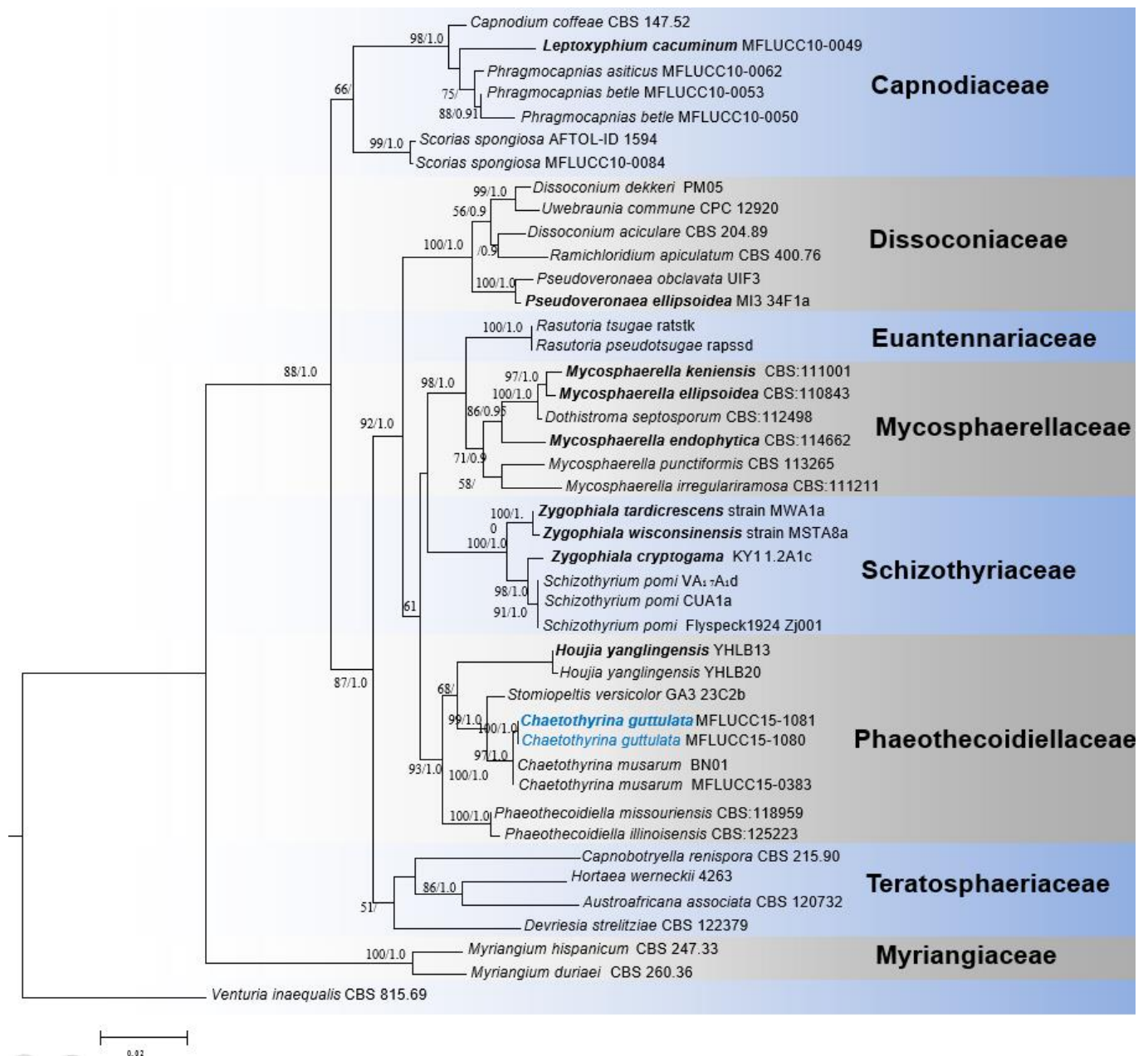


Figure 1 Maximum likelihood phylogenetic tree generated from RAxML analysis of combined LSU and SSU sequence data. The first set of numbers above the nodes are RAxML bootstrap values. The second set of numbers above the nodes are Bayesian posterior probabilities. Strain numbers are indicated after species names. Types strains are in black bold, sequence data of new species are in blue.

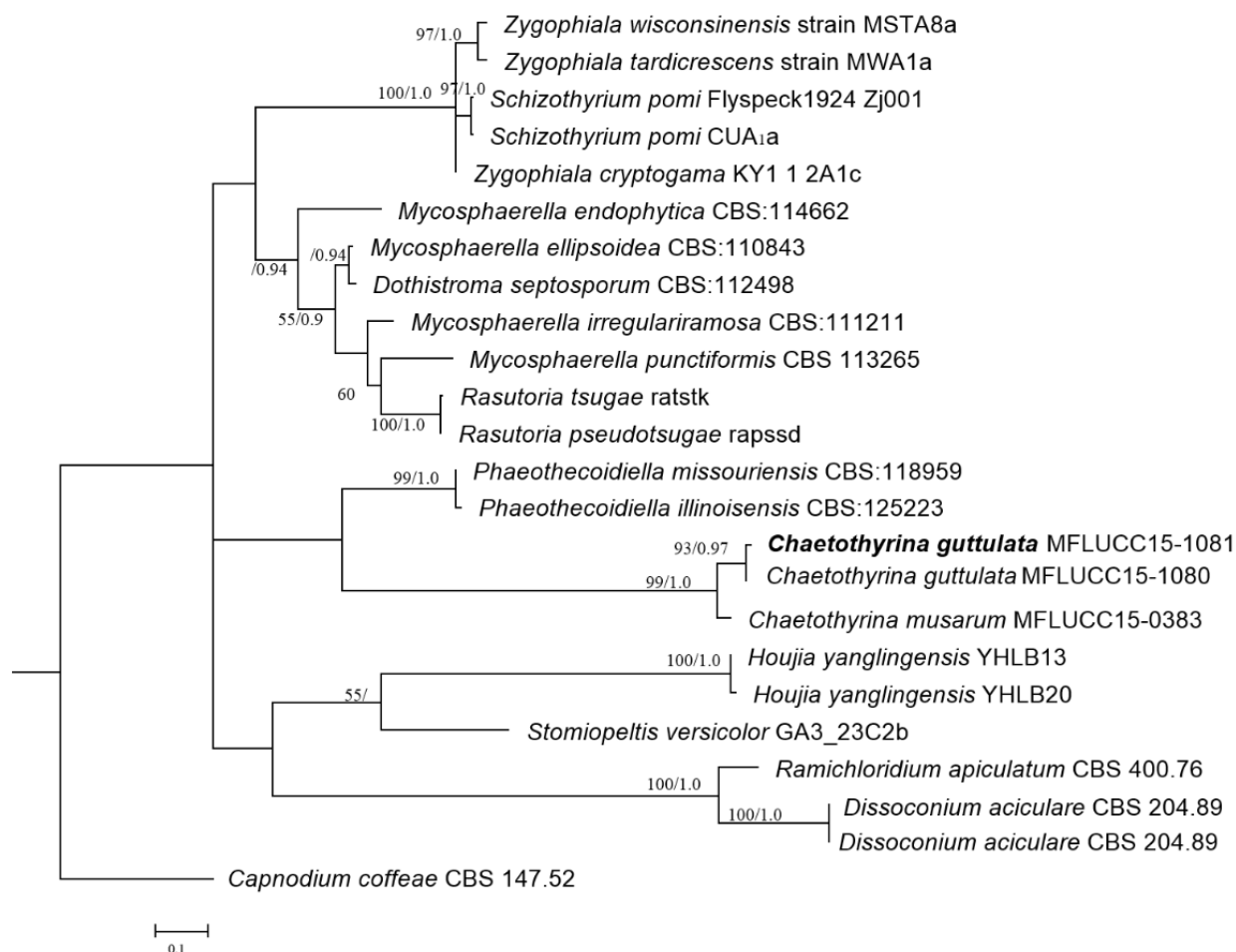


Figure 2 – Maximum likelihood phylogenetic tree generated from RAxML analysis of ITS sequence data. The first set of numbers above the nodes are RAxML bootstrap values. The second set of numbers above the nodes are Bayesian posterior probabilities. Strain numbers are indicated after species names. The type sequence data of new species is in bold.

Notes: *Houjia* and *Phaeothecoidiella* were introduced and placed in Micropeltidaceae, Microthyriales based on their morphology by Yang et al. (2010). However, phylogenetic analysis showed that these taxa formed a distinct lineage sister to the familial clades of Dissoconiaceae, Mycosphaerellaceae and Schizothyriaceae in Capnodiales (Yang et al. 2010). In our analysis, we confirmed the placement of *Chaetothyria*, *Houjia* and *Phaeothecoidiella* and introduce the new family Phaeothecoidiellaceae to accommodate these taxa.

Chaetothyria guttulata Hongsanan & K.D. Hyde, sp. nov.

Index Fungorum number: IF551933, Facesoffungi number: FoF01884, Fig. 3

Etymology: *guttulata* referring to the ascospores which have 2-guttules in each cell.

Type: MFLU16-0299.

Epiphytes appearing as small black dots on the surface of branches of *Mangifera indica*. *Thallus* very thin, especially around the thyriothecia, covering surface of host with dark brown hyphae. *Superficial hyphae* 3 µm wide, septate, not constricted at the septum, branched, brown to dark brown, not dense. Sexual morph: *Thyriothecia* 175–185 × 47–56 µm (\bar{x} =180 × 52 µm, n=5), superficial, solitary, circular, flattened, base poorly developed, thin-walled, brown to dark brown, rounded, easily removed from host surface, central ostiole present at maturity, thyriothecial setae present, but poorly developed. *Setae* 60–85 µm long (\bar{x} =76 µm, n=10), arising from the surface of thyriothecia, straight, unbranched, septate, brown to dark brown, darkened at the septa, hyaline at the apex, smooth-walled. *Ostiole* 28–31 µm diam. (\bar{x} =30 µm, n=5), comprising subcylindrical cells, light brown. *Upper wall* comprising 2–3 layers, inner layer hyaline, outer layer dark brown of

cells of *textura epidermoidea*. *Peridium* 10–14 μm (\bar{x} =11 μm , n=10), comprising cells of *textura angularis* or flattened cells, inner layer hyaline, outer layer dark brown or reddish brown. *Hamathecium* of 2 μm wide, septate, hyaline, pseudoparaphyses. *Asci* 32–37 \times 12–14 μm (\bar{x} =35 \times 13 μm , n=10), 8-spored, bitunicate, subcylindrical to obovoid, short pedicellate or sometimes apedicellate, ocular chamber long when immature. *Ascospores* 14–16 \times 4–5 μm (\bar{x} =15 \times 4.5 μm , n=10), bi to tri-seriate, ellipsoid, 1-septate, constricted at the septum, upper cell wider than lower cell, tapering towards the base, hyaline, with 2 guttules in each cell. Asexual morph: Undetermined.

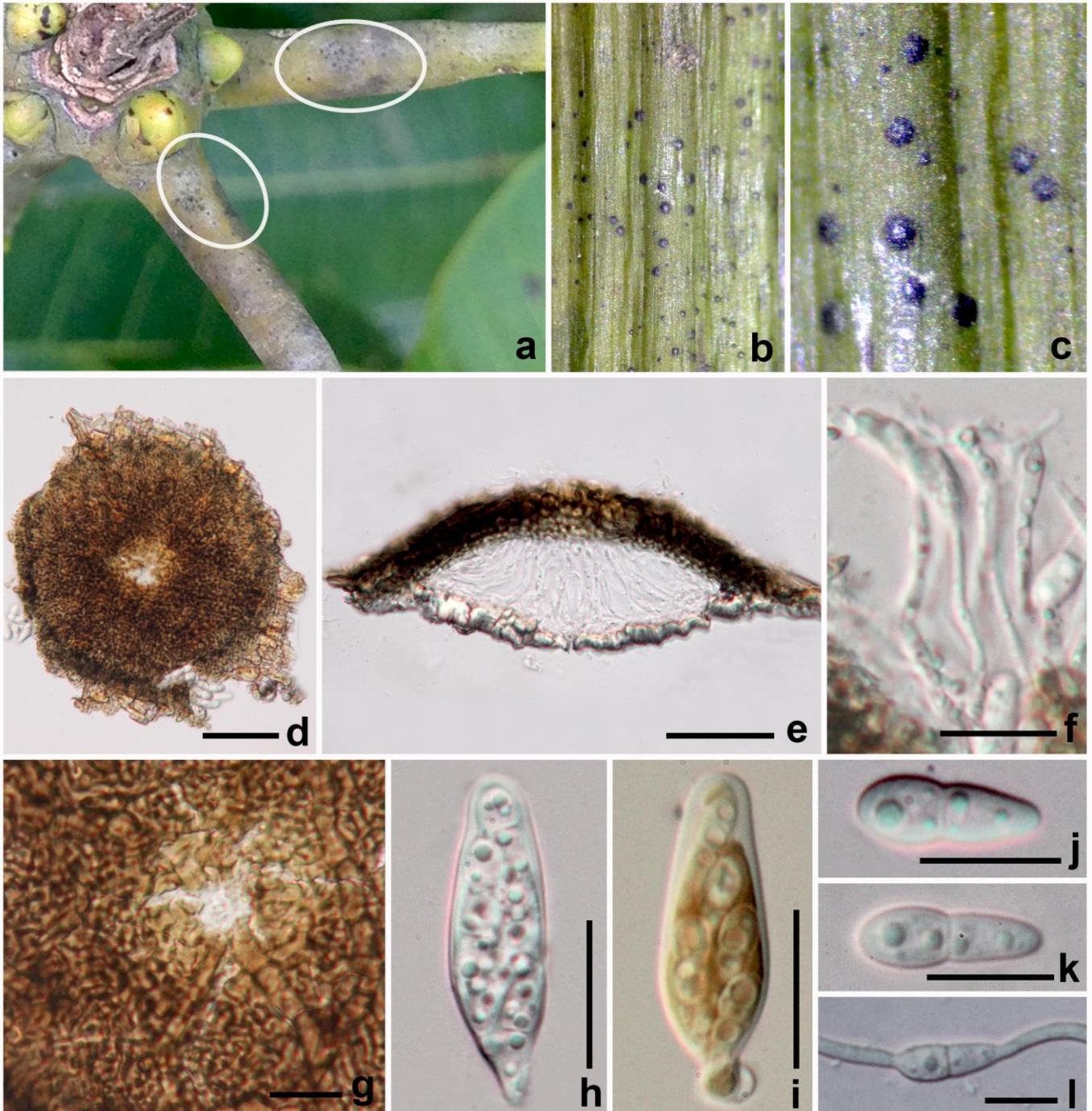


Figure 3 – *Chaetothyria guttulata*.)holotype(. a. Young budding shoot of mango with small black dots of thyriothechia. b, c. Thyriothechia on the surface of young budding shoot of mango. d. Thyriothecium when viewed in squash mount. e. Section through thyriothecium. f. Thyriotheccial setae. g. Hamathecium. h. Ostiole. i. Ascus. j. Ascus in Melzer's reagent. k, l. Ascospores with 2-guttules. m. Germinated ascospore. Scale bars: d, e=50 μm , f-h, k-m=10 μm , i, j=20 μm .

Culture characteristics: Ascospore germinating on PDA at 25–28 °C for 12 h of light/12 h of dark, hyphae germinating from both cells of the ascospore, septate, not constricted and slightly darkened at the septum, hyaline to grayish initially, and become dark grey to black later, darker at the center. Colonies slow growing, reaching 2 cm diam. after 5 days on PDA, colony superficial to erumpent, surface smooth, velvety.

Material examined: THAILAND. Chiang Rai Province: Tasud, Ban Doo, Pong Phra Bat, on branches of *Mangifera indica*, 13 September 2015, S. Hongsanan, MM03 (MFLU16-0299, holotype); *ibid.* (isotype in KUN), ex-type living culture, MFLUCC15-1081; THAILAND. Chiang Rai Province: Tasud, Ban Doo, CRU, on branches of *Mangifera indica*, 13 September 2015, S. Hongsanan, MM02 (MFLU16-0256), living culture, MFLUCC 15-1080.

Discussion

Chaetothyria guttulata is most typical of *C. applanata* (Ellis & G. Martin) M.E. Barr in having two guttules in each cell of the ascospores. However, *C. guttulata* has dark brown thyriothechia, which are slightly darker at the margin and 1-septate ascospores which are narrow at the base and are constricted at the septum, while *C. applanata* has thyriothechia that are blackish brown above and hyaline at the base, 1-septate ascospores with obtuse ends, and lack constrictions at the septum. *Chaetothyria guttulata* is also similar to *C. musarum*, based on its thyriothechia with setae and 1-septate ascospores, although the thyriothechial setae in *C. guttulata* are poorly developed. *Chaetothyria guttulata* has more flattened thyriothechia than *C. musarum*, and has 2 guttules in each cell of the ascospores, with constrictions at the septum, tapering towards the base, while *C. musarum* has aguttulate ascospores, with slight constrictions at the septum, and a basal cell with rounded ends. We also considered the size of both *C. guttulata* and *C. musarum* and found that the ascospores of *C. guttulata* are longer than in *C. musarum*. Phylogenetic analyses of combined LSU and SSU sequence data and the analyses for ITS sequence data demonstrate that *C. guttulata* belongs in *Chaetothyria* and is closely related to *C. musarum* (Figs 1, 2). *Chaetothyria* species clustered with species of *Phaeothecoidiella* and *Houjia* as a distinct clade in Capnodiales although the morphology of *Chaetothyria* is similar to Micropeltidaceae in Microthyriales. Sequence data of *Micropeltis* which is the generic type of Micropeltidaceae shown the phylogenetic placement of this family outside Capnodiales (Wu et al. 2011, Hongsanan et al. 2015a, Singtripop et al. 2016). Thus, we introduce the new family Phaeothecoidiellaceae in this paper to accommodate *Chaetothyria*, *Houjia* and *Phaeothecoidiella* in Capnodiales.

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