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The asexual morph of *Trichomerium gloeosporum*

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Abstract

Trichomerium species are sooty molds that develop superficially on the host surface. There is little morphological and molecular data for the genus. The asexual morphs of *Trichomerium* have been poorly studied, with also lack molecular data. Several researchers have noted that *Tripospermum* is possibly the asexual morph of *Trichomerium* species. In this study, we collected *Trichomerium*-like taxa from leaves in northern Thailand. The sexual structures found on the specimen were identified as *Trichomerium gloeosporum*, and *Tripospermum*-like conidia were also associated with the sexual morph. Cultures were obtained from single *Tripospermum*-like conidia and sequenced. Phylogenetic analyses generated from maximum likelihood and Bayesian analyses of combined LSU and ITS sequence data demonstrate that our strains isolated from *Tripospermum*-like conidia clustered with strains of *T. gloeosporum* with high statistical support. A description of the asexual morph of *T. gloeosporum* with molecular data is provided.

Key words – Asexual morph – Trichomeriaceae – sooty molds

Introduction

The genus *Trichomerium* was introduced by Spegazzini (1918), and had been regarded as belonging to the order Capnodiales (Dothideomycetes). *Trichomerium* species feed on honey dew from insect excretions (Chomnunti et al. 2012a) and are characterized by forming hyphal networks on the surface of plants, globose to subglobose ascomata, with setae surrounding the upper part of ascomata, bitunicate asci, and septate, hyaline ascospores, with or without mucilaginous sheaths (Chomnunti et al. 2012a). *Trichomerium* species are referred as sooty molds, and similar to species in Capnodiaceae and Chaetothyriaceae based on their black hyphal networks (Chomnunti et al. 2012a, b, 2014). However, *Trichomerium* differs from other species in these two families in having an apical ascal ring, ascospores with or without sheaths and in their septation (Chomnunti et al. 2012a, b, 2014). Chomnunti et al. (2012a) demonstrated that *Trichomerium* species clustered separately from Capnodiaceae and Chaetothyriaceae, but in Chaetothyriales (Eurotiomycetes) based on their phylogenetic tree. Therefore, *Trichomerium* was introduced as a new family (Chomnunti et al. 2012a). Réblová et al. (2013) accepted species of Trichomeriaceae as a part of Chaetothyriaceae. Isola et al. (2016) and Nascimento et al. (2016) also discovered that some extremotolerant fungi growing on rock surfaces were phylogenetically related to species of

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Trichomerium. Most of these species have been found on rocks, and were slow-growing (Isora et al. 2016). The taxonomic position of non-sporulating species is uncertain, however, they have been placed in Trichomeriaceae and Chaetothyriaceae based on phylogenetic analyses (Tsuneda et al. 2011, Réblová et al. 2013, Hubka et al. 2014, Isola et al. 2016). Sequence data from *Arthrocladium* species were provided, and they are phylogenetically related to *Trichomerium* (Nascimento et al. 2016). However, Nascimento et al. (2016) found that one of their *Arthrocladium* isolates caused a fatal disseminated infection in a human, with GATA-2 immune defect (Egenlauf et al. 2015), while two other isolates were as inhabitants of rotten wood. Thus, Nascimento et al. (2016) noted that Trichomeriaceae mainly comprises epiphytic and epilithic organisms, and the placement of the family in phylogenetic tree presently comprises species of *Arthrocladium*, *Bradymyces*, *Exophiala*, *Knufia*, and unidentified species of *Chaetothyriales*. The asexual morph of *Trichomerium* species is uncertain and several researchers did note that *Trichomerium* possibly had *Tripospermum* morphs (Chomnunti et al. 2012a, Crous et al. 2015). There was no molecular evidence to support this idea, until Crous et al. (2015) confirmed this association when introducing a new species *Trichomerium dioscoreae* with *Tripospermum*-like morphs. Their blast results showed that the new species was closely related to *Trichomerium gloeosporum*, but was a distinct new species (ITS, 92%). Below we report on the asexual morph of *Trichomerium gloeosporum* and resolve the confusion with a phylogenetic tree.

Materials & Methods

Morphology and isolation

Specimens of "*Trichomerium*"-like taxa were collected in Chiang Rai, Thailand. The morphology was observed and photographed under a stereomicroscope. Micromorphological characters were studied under a compound microscope, and photographed using a Nikon80i. Measurements were determined using Tarosoft(R) Image Frame Work v. 0.9.7. Slides were preserved in lactoglycerol. A spore suspension was obtained, and dropped onto the surface of the PDA plate. The plate was incubated 12 hour of light/12 hours dark at room temperature (25−28 °C) and observed every 12 hours. Germinated spores were transferred onto the surface of fresh PDA (Chomnunti et al. 2014). Herbarium specimens are deposited in the Mae Fah Luang University Herbarium (MFLU), Chiang Rai, Thailand, and living cultures is deposited in both Mae Fah Luang University Culture Collection (MFLUCC). Faces of fungi numbers and Index Fungorum numbers are as explained in Jayasiri et al. (2015) and Index Fungorum (2016).

Phylogenetic analyses

Fungal isolates were grown on PDA for 7 days at 25 °C. Genomic DNA was extracted from the growing mycelium using the Biospin Fungus Genomic DNA Extraction Kit-BSC14S1 (BioFlux®, P.R. China); following the instructions of the manufacturer (Hangzhou, P.R. China). Polymerase chain reaction (PCR) was carried out using known primer pairs LROR/LR5 to amplify the large subunit rDNA region, and ITS1/ITS4 to amplify internal transcribed spacer region (White et al. 1990). The amplification was performed following the instructions, and were set up for initial denaturation of 5 minutes at 95 °C, followed by 35 cycles of 45 seconds at 94 °C, 45 seconds at 52 °C and 90 seconds at 72 °C, final extension period of 10 minutes at 72 °C. Products of PCR were checked on 1% agarose electrophoresis gels stained with ethidium bromide, and sequenced by Majorbio Co., China. DNA. Primer sequences and database are available in GenBank.

Sequence data of related strains based on BLAST results were downloaded from GenBank to supplement the dataset (Table 1). The data set was aligned with our new strains using MAFFT (Katoh et al. 2009), and checked manually using Bioedit (Hall 1999). *Ceramothyrium thailandicum* was selected as the outgroup taxon. Maximum likelihood analysis was performed by using raxmlGUIv.0.9b2 (Silvestro & Michalak 2012). The search strategy was set to rapid boot strapping and the analysis was carried out using GTRGAMMA model of nucleotide substitution. Maximum likelihood bootstrap values equal or greater than 70% are given as the first set of numbers above the nodes (Fig. 1). The model of evolution was performed by using MrModeltest 2.2 (Nylander et

al. 2008). Posterior probabilities (PP) (Rannala & Yang 1996, Zhaxybayeva & Gogarten 2002) were determined by Markov Chain Monte Carlo sampling (MCMC) in MrBayesv 3.1.2 software (Huelsenbeck & Ronquist 2001). Six simultaneous Markov chains were run for 1,000,000 generations and trees were sampled every 100th generation. Thus, 10,000 trees were obtained. The first 2,000 trees were discarded based on the result from Tracer software. The remaining 8,000 trees were performed for calculating posterior probabilities (Cai et al. 2006, 2008). Bayesian posterior probabilities (BYPP) equal or greater than 0.95 are given as the second set of numbers above the nodes (Fig. 1).

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

Species	Voucher/culture	Accession numbers	
		LSU	ITS
Arthrocladium fulminans	CBS 136243	KT337444	KT337439
Arthrocladium tardum	CBS 127021	KT337446	KT337441
Arthrocladium tardum	CBS 134919	KT337447	KT337442
Arthrocladium tropicale	CBS 134926	KT337445	KT337440
Chaetothyriales sp.	CR08/2-1	FJ538960	FJ538960
Chaetothyriales sp.	CR08/2-2	FJ538959	FJ538959
Chaetothyriales sp.	M-Mo2	HQ634636	HQ634636
Chaetothyriales sp.	CN-Cre-Bo3-2	HQ634620	HQ634620
Chaetothyriales sp.	CN-Phe1-1	HQ634622	HQ634622
Chaetothyriales sp.	CN-Cre-Bo1-4	HQ634614	HQ634614
Chaetothyriales sp.	M-Camp4	HQ634626	HQ634626
Cladophialophora modesta	CBS 985.96	KF928485	KF928421
Cladophialophora mycetomatis	CBS 454.82	KC809991	EU137293
Trichomerium deniqulatum	MFLUCC10-0884	JX313660	NR_132965
Trichomerium dioscoreae	CBS:138870	KP004496	KP004468
Trichomerium foliicola	MFLUCC10-0078	JX313661	JX313655
Trichomerium foliicola	MFLUCC10-0073	JX313658	JX313652
Trichomerium foliicola	MFLUCC10-0058	JX313659	JX313653
Trichomerium foliicola	MFLUCC10-0054	JX313657	JX313651
Trichomerium gloeosporum	MFLUCC10-0087	JX313662	JX313656
Trichomerium gloeosporum	MFLUCC15-0209	KY381953	KY381954
Trichomerium gloeosporum	MFLUCC15-0208	KY381951	KY381952

Trichomerium gloeosporum Chomnunti & K.D. Hyde
Facesoffungi number FoF02826

Systematic placement: Ascomycota, Pezizomycotina, Eurotiomycetes, Chaetothyriomycetidae, Chaetothyriales, Chaetothyriaceae.

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. Sexual morph: *Ascomata* 100−117 high × 115−120 μm diam. ($\bar{x}$ = 115 × 118 μm, n = 5), superficial on the surface of hosts, solitary to clustered, subglobose to globose, brown to dark brown, with abundant setae. *Ascomatal setae* 105−122 × 4−6 μm ($\bar{x}$ = 120 × 5 μm, n = 20), septate, dark brown or olivaceous, straight, tapering toward the apex. *Peridium* 14−21 μm wide ($\bar{x}$ = 18 μm, n = 10), two layers, the outer layer of brown to dark brown cells of *textura prismatica*, inner layer of pale brown to hyaline of flattened cells. *Hamathecium* aparaphysate. *Asci* 58−62 × 20−24 μm ($\bar{x}$ = 60 × 22 μm, n = 10), 8-spored, bitunicate, ellipsoidal to cylindrical, with a short pedicel, with an apical ring. *Ascospores* 20−22 × 5−7 μm ($\bar{x}$ = 21 × 6 μm, n = 20), 2-seriate, hyaline, fusoid, 2−3-septate, rounded at the ends, with a mucilaginous sheath (description of sexual morph modified from Chomnunti et al. 2012a and own observations). Asexual morph: *Conidiophores* reduced to

conidiogenous cells, conidia arising directly from hyphae. *Conidia* solitary, hyaline to pale brown, or grayish, giving rise to 3−4 lateral arms from a central cell. *Conidial arms* 29−35 × 5−7 µm ($\bar{x}$ = 32 × 6 µm, n = 10), pale brown to grayish, 2−5-septate, not constricted or slightly constricted, darker at the septa, subcylindrical, tapering to the apex, with rounded ends, smooth-walled.

Culture on PDA: Conidia germinating on PDA at 22−25 °C for 12 h dark/ 12 h light, germ tubes appearing from each branch of conidia, hyaline to bluish, but becoming dark brown to black. Colonies reaching 1 cm diameter after 4 days on PDA at 22−25 °C, colonies superficial to erumpent, velvety surface, dark brown, reddish-brown at the margin, dark brown to grayish sparse aerial hyphae outer region. Conidia produced in PDA after 7 days incubation.

Material examined: THAILAND, Chiang Rai Province, Tasud, STK resort, on living leaves of *Gardenia* sp. (Rubiaceae), 21 January 2015, S. Hongsanan STK07 (MFLU 16-2885), living culture, MFLUCC 15-0208, MFLUCC 15-0209. THAILAND, Chiang Rai Province, Mae Khao Tom, on living leaves of *Ficus annulata* (Moraceae), February 2013, S. Hongsanan CC1 (MFLU 16-2926), living culture, MFLUCC 13-0780, MFLUCC 13-0789.

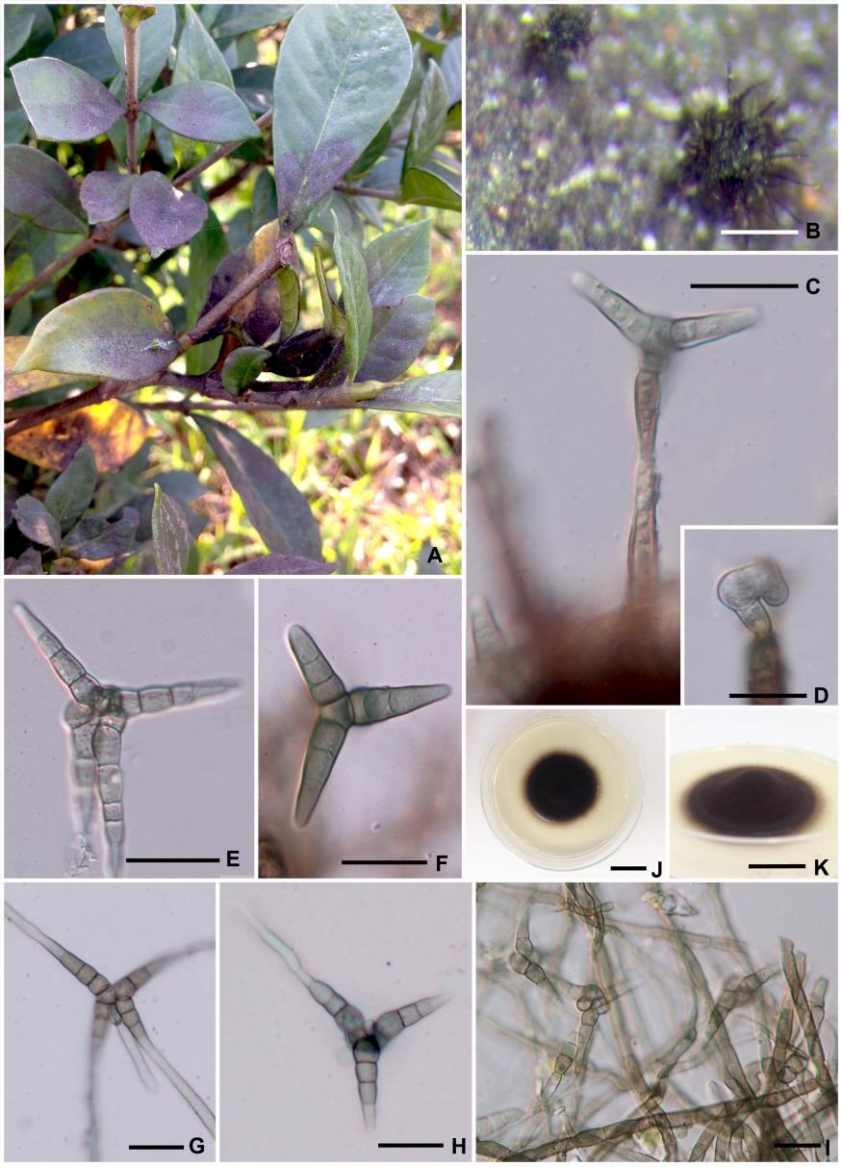

Fig. 1 – *Trichomerium gloeosporum*. A. Sooty molds on host. B. Ascomata of *Trichomerium gloeosporum* (sexual morph). C. Conidia arising from conidiophore which are reduced to conidiogenous cells. D. Immature conidia. E, F. Conidia. G, H. Conidia germinating on media. I. Conidia with septate hyphae. J, K. Colonies on media. Scale bars: C, E-I = 20 µm, d = 10 µm, J, K = 1 cm.

Notes: The asexual morph characters found in this study are identical to *Tripospermum* in having branched conidia, giving rise to 3−4 lateral arms from a central cell. The combined of LSU and ITS sequence data obtained from "*Tripospermum-like*" conidia indicate that our isolates are the same species as *T. gloeosporum* with 90% ML and 1.0 PP support, even thought they were isolated from "*Tripospermum-like*" conidia. The sexual morph associated with *Tripospermum-like* conidia in our specimens is morphologically typical to *T. gloeosporum* and thus confirms the linkage.

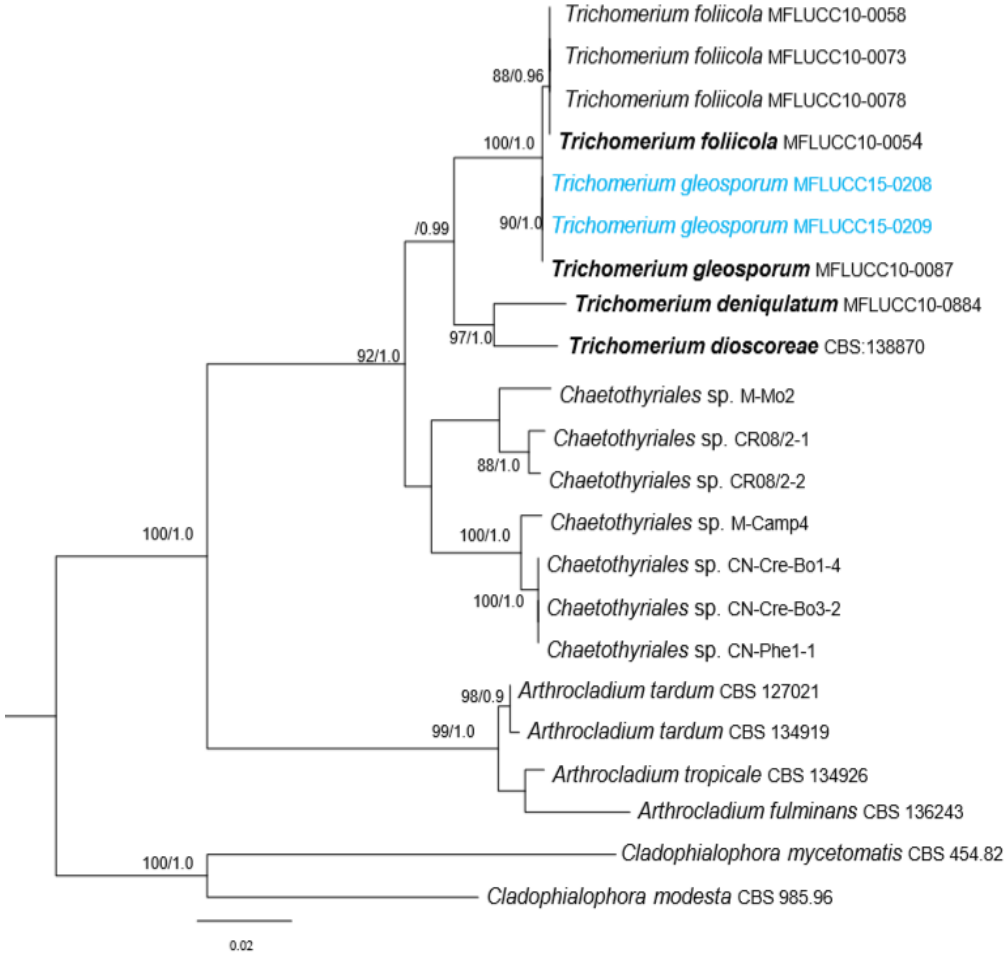

Fig. 2 – RAxML maximum likelihood phylogenetic tree based on combined LSU and ITS sequence data. The first set of numbers above the nodes are RAxML value expressed from 1,000 repetitions with values above 70% shown. The second set of numbers above the nodes are Bayesian posterior probabilities, with values above 0.95 shown. Strain numbers are indicated after species names. New sequence data are in blue bold, and other types are in black bold.

Discussion

Trichomerium is the generic type of Trichomeriaceae (Chomnunti et al. 2012a, b, 2014, Liu et al. 2015), with the type species *Trichomerium coffeicola* (Puttemans) Speg. Twenty-seven species epithets are listed in Index Fungorum (2016). Molecular analyses indicate that species of *Trichomerium* cluster separately from Capnodiaceae and Chaetothyriaceae (Chomnunti et al. 2012a, b, 2014, Yang et al. 2014, and this study) and is included with three other genera in Trichomeriaceae (Nascimento et al. 2016). *Trichomerium gloeosporum* was introduced by Chomnunti et al. (2012a) based on phylogenetic analyses and the distinct morphology of the sexual morph. Crous et al. (2015) collected *Tripospermum* on leaves of *Dioscorea* sp. (Dioscoreaceae), and introduced a new species *Trichomerium dioscoreae* Crous & C. Nakash. based on phylogenetic analyses of LSU and SSU sequence data using a megablast search of GenBank. We collected a *Tripospermum* species on *Gardenia* sp. (MFLU 16-2885) and isolated strains from single conidia (Fig. 1). The strains produce the *Tripospermum* asexual morph in culture (Fig. 1). Our strain differs

from *T. dioscoreae* in having 3−4 lateral arms from a central cell, with 2−5-septa, while *T. dioscoreae* has 2− lateral arms, with 1−2-septa. Ascomata were associated with the *Tripospermum* morph and had the same characters as *Trichomerium gloeosporum*. Molecular analyses based on combined data set of LSU and ITS sequence data indicate that our taxa is the same species with *T. gloeosporum* (90% ML and 1.0 PP support; Fig. 2), which confirms this linkage. In this paper we provide the first description of the asexual morph of *T. gloeosporum*, with sequence data.

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