

## ***Stigmatomyces* aff. *limnophorae* on dipteran hosts in Peninsular Malaysia**

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**ABSTRACT**—Flies parasitized by *Laboulbeniales*, captured in Selangor state, were examined and identified as *Boettcherisca javanica*, *Boettcherisca* sp., and *Hypopygiopsis violacea*. The fungus was identified as *Stigmatomyces* aff. *limnophorae* based on morphology and phylogenetic analysis of sequences of the partial nuclear small and large subunit ribosomal RNA genes. This fungus represents a new record for Malaysia; and these are the first reports of *Boettcherisca* and *Hypopygiopsis* as hosts for any laboulbenialean species.

**KEY WORDS**—*Calliphoridae*, *Laboulbeniomycetes*, *Sarcophagidae*, southeastern Asia.

### **Introduction**

Fungi in *Laboulbeniales* (Ascomycota: *Laboulbeniomycetes*) are ectoparasitic fungi that are obligatorily associated with arthropods as ectoparasites (Haelewaters & al. 2012, Melo & Melo 2019, Blackwell & al. 2020). These fungi are characterized by the presence of a three-dimensional thallus (plural: thalli), instead of hyphae with mycelial growth like many other fungi (Blackwell & al. 2020). Representatives of three arthropod subphyla

(*Chelicerata*, *Myriapoda*, *Hexapoda*) and various insect orders including *Coleoptera*, *Diptera*, *Hemiptera*, and *Hymenoptera* are known as hosts for these fungi (reviewed in Haelewaters & al. 2021). In the past 40 years, only two species *Laboulbeniales* have been reported in Malaysia: *Laboulbenia admirabilis*, found on the body of an unidentified *Spaniocelyphus* (*Diptera*) in Pahang state (Lee & Majewski 1986), and *Diphymyces sabahensis*, on three *Ptomaphagus* spp. (*Coleoptera*) in Sabah state (Haelewaters & al. 2014).

*Stigmatomyces* sensu lato is a large, paraphyletic genus of 176 species on dipteran hosts (Haelewaters & al. 2020a; Species Fungorum 2020). Species within this heterogeneous assemblage (sensu lato) are parasites on hosts in many different families—including *Anthomyiidae*, *Calliphoridae*, *Chamaemyiidae*, *Diopsidae*, *Drosophilidae*, *Ephydriidae*, *Fanniidae*, *Muscidae*, *Nycteribiidae*, *Sarcophagidae*, *Sphaeroceridae*, and *Streblidae* (Thaxter 1901, 1905, 1917; Rossi 1998; Hyde & al. 2019; Haelewaters & al. 2018, 2020a). Thus far, five species of *Stigmatomyces* sensu stricto have been reported in Malaysia: *S. dacinus*, *S. limosinoides*, *S. tortimasculus*, and *S. venezuelae* in Malaysian Borneo (Thaxter 1915, 1918); and *S. neurochaetae* in Peninsular Malaysia (Sugiyama & Majewski 1985; Rossi & Weir 2007). Note that Thaxter (1915) reported *S. stilici* from Malaysian Borneo and Sugiyama & Majewski (1985) reported *S. orientalis* from Peninsular Malaysia—both associated with staphylinid beetles (*Coleoptera*: *Staphylinidae*) and both species later recombined in *Zeugandromyces*.

Here, we provide the first records of *Stigmatomyces* aff. *limnophorae* from Malaysia. Our material was studied based on morphological characters and sequence data. The parasitized fly genera are for the first time reported in the literature as hosts for *Laboulbeniales*.

## Material & methods

### Collection & identification of flies

An entomological survey was conducted in September 2019 in the state of Selangor, Peninsular Malaysia, to investigate the biodiversity of carrion flies. Chicken liver (200 g, 2d old) was used as bait and flies were collected using sweep nets at two different forests in the town of Rawang. Collected adult flies were then placed in a cloth-lid jar and brought back to the Parasitology Laboratory, Institute for Medical Molecular Biotechnology, Universiti Teknologi MARA (UiTM) in Sungai Buloh. The flies were incubated at  $-4^{\circ}\text{C}$  for 15 minutes, after which they were pinned and dried at room temperature. During microscopic examination for species determination, we observed four fly specimens (out of >100 observed) with thalli of



*Laboulbeniales*. These specimens were carefully examined and photographed using an Olympus SZ51 stereomicroscope equipped with a digital camera and CellD Imaging Software. The parasitized adult flies were identified using Kurahashi & al. (1997) and Kurahashi & Samerjai (2018).

#### Microscopic study of *Laboulbeniales*

Parasitized flies were shipped to Purdue University for microscopic study of the *Laboulbeniales* (by J.L.). Thalli were taken from the host fly using a BioQuip #1208SA entomological pin dipped in Hoyer's medium (30 g arabic gum, 200 g chloral hydrate, 16 ml glycerol, 50 ml ddH<sub>2</sub>O). Thalli were mounted in Amann's medium applying a double coverslip technique using Solakryl BMX as outlined in Liu & al. (2020). Microscope mounts were viewed at 200–400× using an Olympus BH2 bright field compound microscope. Line and stipple drawings were made with PITT artist pens based on photomicrographs taken with an Olympus SC30 camera and cellSens 1.18 imaging software. Permanent slides are deposited at PUL (Kriebel Herbarium) under numbers PUL F25943–F25950.

#### DNA extraction, PCR amplification, sequencing

DNA was extracted from 2–4 thalli of *Stigmatomyces* using the REPLI-g Single Cell Kit with modifications by Haelewaters & al. (2019). The nuclear ribosomal RNA small (SSU) and (LSU) large subunits were amplified using primer pairs NSL1/NSL2 for SSU (Haelewaters & al. 2015), and LR0R/LR5 and LIC24R/LR5 for LSU (Vilgalys & Hester 1990, Hopple 1994, Miadlikowska & Lutzoni 2000). The DNA was amplified using an Eppendorf pro S Mastercycler in 25 µL volumes containing 12.5 µL 2× MyTaq Mix (Bioline, Swedesboro, New Jersey), 9.5 µL ddH<sub>2</sub>O, 1.0 µL forward and reverse primer, and 1.0 µL DNA. Cycling conditions—for SSU: initial denaturation at 95 °C for 5 min; 40 cycles of denaturation at 95 °C for 30 sec, annealing at 55 °C for 45 sec, extension at 72 °C for 45 sec; and final extension at 72 °C for 1 min and for LSU: initial denaturation at 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 30 sec, annealing at 50 °C for 45 sec, extension at 72 °C for 1 min; and final extension at 72 °C for 7 min. The PCR amplicons were sent to Genewiz (South Plainfield, New Jersey) for purification and sequencing. Raw sequence reads were assembled and edited in Gene Codes Sequencher 5.2.3. Sequences were deposited at the National Center for Biotechnology Information (NCBI) GenBank database; accession numbers MT341792–MT341794 (SSU) and MT341789–MT341791 (LSU). These sequences were then BLAST searched against NCBI's nucleotide collection to establish a rough relationship with existing sequences.

#### Sequence alignments & phylogenetic analysis

SSU and LSU sequences of *Stigmatomyces* species representing the same clade (clade IV sensu Haelewaters & al. 2020a) were downloaded from GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>). *Gloeandromyces dickii* was selected as outgroup. Details for all isolates are presented in TABLE 1. Sequences of both regions

TABLE 1. Isolates and sequences used in phylogenetic analysis.

SPECIES	ISOLATE	COUNTRY	SSU	LSU	REFERENCE
<i>Gloeandromyces dickii</i>	D.Haelew. 1323b	Panama	MG958011	MH040582	Haelewaters & al. 2018
<i>Stigmatomyces borealis</i>	AW-979	USA	JN835186	—	A. Weir (unpublished)
<i>S. chamaemyiae</i>	D.Haelew. 1137a	Portugal	MH040564	—	Haelewaters & al. 2018
	D.Haelew. 1137c	Portugal	MH040565	—	Haelewaters & al. 2018
<i>S. limnophorae</i>	AW-785	USA	AF407576	—	Weir & Blackwell 2001b
<i>S. aff. limnophorae</i>	D.Haelew. 1802c	Malaysia	MT341792	MT341789	This study
	D.Haelew. 1802d	Malaysia	MT341793	MT341790	This study
	D.Haelew. 1820e	Malaysia	MT341794	MT341791	This study
<i>S. protrudens</i>	AW-793	USA	AF298232	AF298234	Weir & Blackwell 2001a
<i>S. rugosus</i>	—	—	AF431759	—	Weir & Hughes 2002
	D.Haelew. 1138a	Portugal	MH040563	—	Haelewaters & al. 2018

were aligned with MUSCLE (Edgar 2004) on the Cipres Science Gateway web portal (Miller & al. 2010). For both datasets, the appropriate nucleotide substitution model was selected by considering the corrected Akaike Information Criterion (AICc) using ModelFinder Plus (Kalyaanamoorthy & al. 2017). Models selected were HKY+F+I (SSU, -lnL = 2420.547) and TIM2+F+I (LSU, -lnL = 1837.675). SSU and LSU aligned datasets were combined using MEGA7 (Kumar & al. 2016). A Maximum likelihood analysis of the concatenated two-locus dataset was performed using IQ-TREE (Nguyen & al. 2015) with partitioned models (Chernomor & al. 2016) and ultrafast bootstrapping with 1000 replicates (Hoang & al. 2018). The best-scoring tree was visualized in FigTree 1.4.3 (<http://tree.bio.ed.ac.uk/software/figtree/>) and edited in Adobe Illustrator 24.1.1.

Host identification

The infected insect hosts (n = 4) belonged to two families, *Calliphoridae* (n = 1) and *Sarcophagidae* (n = 3). The calliphorid fly was identified as *Hypopygiopsis violacea* (FIG. 1). Two sarcophagid flies were identified as *Boettcherisca javanica* and the third as *Boettcherisca* sp. (FIGS 2, 3). All four specimens bore thalli at their abdominal segments (TABLE 2).



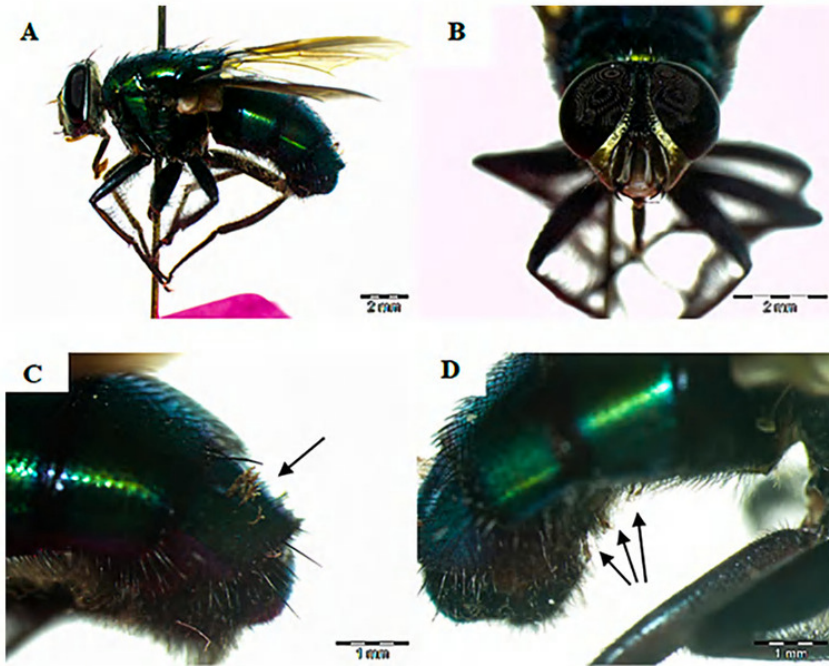


FIG. 1. Adult fly of *Hypopygiopsis violacea* (Diptera: Calliphoridae): A. Habitus of *H. violacea* at 0.8× magnification. B. The silver white facial tomentum. C. *Stigmatomyces* aff. *limnophorae* thalli on 5th tergite (black arrow). D. *Stigmatomyces* aff. *limnophorae* thalli on sternites 3 and 4 (black arrows). Scale bars: A, B = 2 mm; C, D = 1 mm.

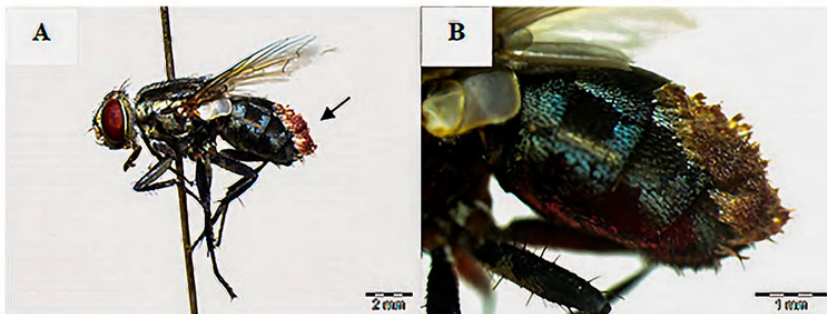


FIG. 2. Adult fly of *Boettcherisca* sp. (Diptera: Sarcophagidae): A. Heavy infection with *Stigmatomyces* aff. *limnophorae* at abdominal tergites 4 and 5 (black arrow); B. Close-up view of the parasitized tergites 4 and 5 at 2.5× magnification. Scale bars: A = 2 mm; B = 1 mm.

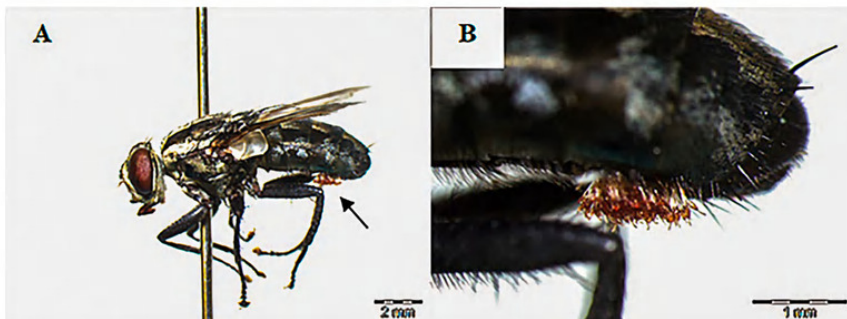


Fig. 3. Adult fly of *Boettcherisca javanica* (Diptera: Sarcophagidae): A. *Stigmatomyces* aff. *limnophorae* thalli on abdominal sternites 4 and 5 (black arrow); B. Tuft of *S.* (aff.) *limnophorae* thalli on abdominal sternites 4 and 5 at 3.2× magnification. Scale bars: A = 2 mm; B = 1 mm.

TABLE 2. Host specimens examined and position of thalli on the host body.

DIPTERAN HOST SPECIES	FAMILY	LOCATION OF THALLI
<i>Hypopygiopsis violacea</i> ♀	<i>Calliphoridae</i>	Tergite 5; sternites 3, 4 (FIG. 1C, D)
<i>Boettcherisca</i> sp. ♀	<i>Sarcophagidae</i>	Abdominal tergites 3, 4 (FIG. 2)
<i>Boettcherisca javanica</i> ♂	<i>Sarcophagidae</i>	Abdominal sternites 4, 5 (FIG. 3)

**Taxonomy**

*Stigmatomyces* aff. *limnophorae* Thaxt.,

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FIG. 4

Thallus hyaline, dark amber brown that gradually attenuates as the cell wall thickens; 647 µm long from foot to perithecial tip. Cell II ≤2 times longer than cell I, striate on the cell surface. Cell III longer than wide, rounded externally, not protruding abruptly below basal cell of appendage. Appendage free, slender, elongated, about as long as the perithecial venter; distal portion distinctly curved; bearing a single or multiple antheridia. Antheridia, short, broad, slightly recurved. Perithecium amber brown, 220 × 64 µm; venter relatively small, ellipsoid, subsymmetrical, with wall cells powdered by darker maculation, spirally twisted, separated by corresponding number of well-defined longitudinal ridges, somewhat oblique; neck 96 × 16 µm, abruptly distinguished from venter.

MATERIAL EXAMINED/SEQUENCED—PENINSULAR MALAYSIA: SELANGOR, Gombak District, RAWANG, forested area, 3.281°N 101.261°E, 4 m a.s.l., ex. chicken liver, 11 Sep. 2019, leg. N.A. Nur Aliah & N. Azmiera, on ♀ *Boettcherisca* sp. D. Haelew. 1796 [host label] (slides PUL F25943, PUL F25944, PUL F25945); 3.296°N 101.611°E, 69 m a.s.l., ex. chicken liver, 7 Sep. 2019, leg. N.A. Nur Aliah & N. Azmiera, on ♂ *Boettcherisca javanica* Lopes, D. Haelew. 1801 [host label] (slides PUL F25947, PUL F25948); on ♂ *B. javanica*, D. Haelew. 1802 [host label] (slides PUL F25949, PUL F25950) isolate 1802c [4 mature thalli] GenBank MT341792, MT341789; isolate 1802d [3 mature thalli] GenBank MT341793, MT341790; isolate 1802e [2 perithecia] GenBank MT341794, MT341791); 3.296°N 101.611°E, 101 m a.s.l., ex. chicken liver, 7 Sep. 2019, leg. N.A. Nur Aliah & N. Azmiera, on ♀ *Hypopygiopsis violacea* (Macquart), D. Haelew. 1800 [host label] (slide PUL F25946).

**Phylogenetic results**

All three newly generated SSU sequences share highest similarity (99.62%) with *Stigmatomyces chamaemyiae* (GenBank MH040565), followed by



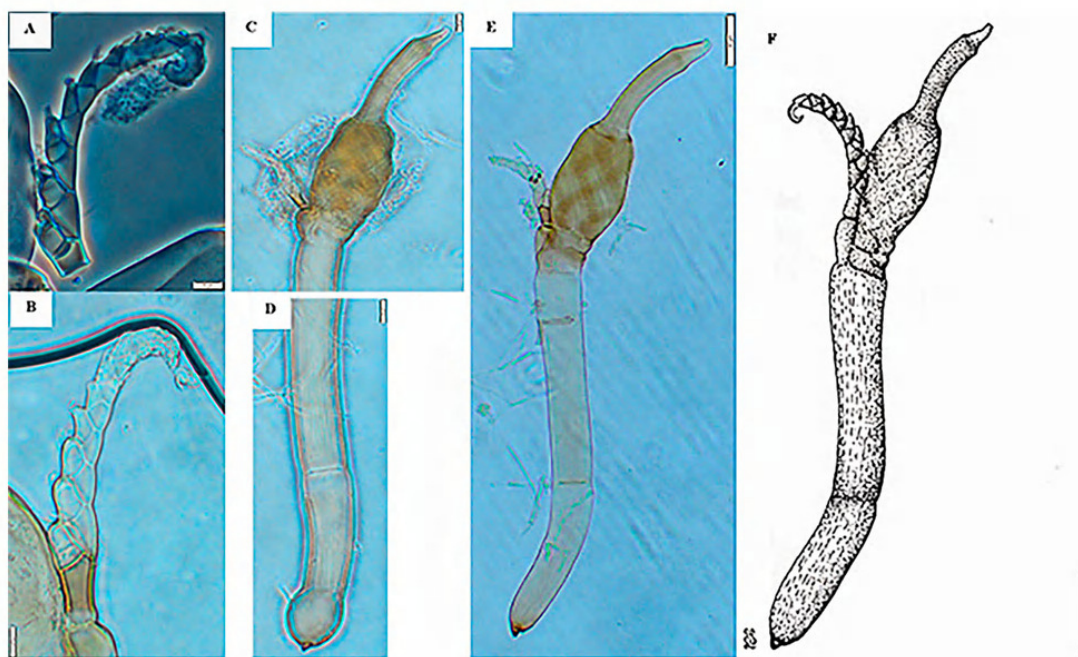


Fig. 4. *Stigmatomyces* aff. *limnophorae*: A [PUL F25945], B [PUL F25948]. Details of primary appendage; C [PUL F25950]. Detail of spirally twisted perithecium; D [PUL F25950]. Receptacular cells I and II with longitudinally striped ornamentation; E [PUL F25950]. Habitus of mature thallus; F. Stipple drawing. Scale bars: A, B = 10 µm; C, D = 20 µm; E = 50 µm; F = 100 µm.

*S. rugosus* (MH040563) with 98.39–98.54% similarity (TABLE 1). Based on morphology, we thought the fungus might represent *S. limnophorae*, but that species was not among the BLAST results despite the availability of an SSU sequence in GenBank (AF407576; isolate AW-785). Comparison of our Malaysian SSU sequences with *S. limnophorae* AW-785, however, shows a 99.08–99.36% similarity. All newly generated LSU sequences are most closely related to *Gloeandromyces nycteribiidarum* (MH040566) with 85.78% similarity (TABLE 1). The highest percentage similarity (87.30%) with any *Stigmatomyces* species is with *S. protrudens* (AF298234), but with only over a 42% query cover.

The three Malaysian isolates group together in a maximum-supported clade that also includes *S. limnophorae* AW-785 and two *S. chamaemyiae* isolates from Portugal. The branch length among the Malaysian isolates and between the USA isolate AW-785 is very short. As a result, we believe the Malaysian fungus may be identified as *S. aff. limnophorae*.

## Discussion

Fungal thalli were removed from all four fly specimens and subsequently identified as *Stigmatomyces* aff. *limnophorae* based on morphological (FIG. 4)

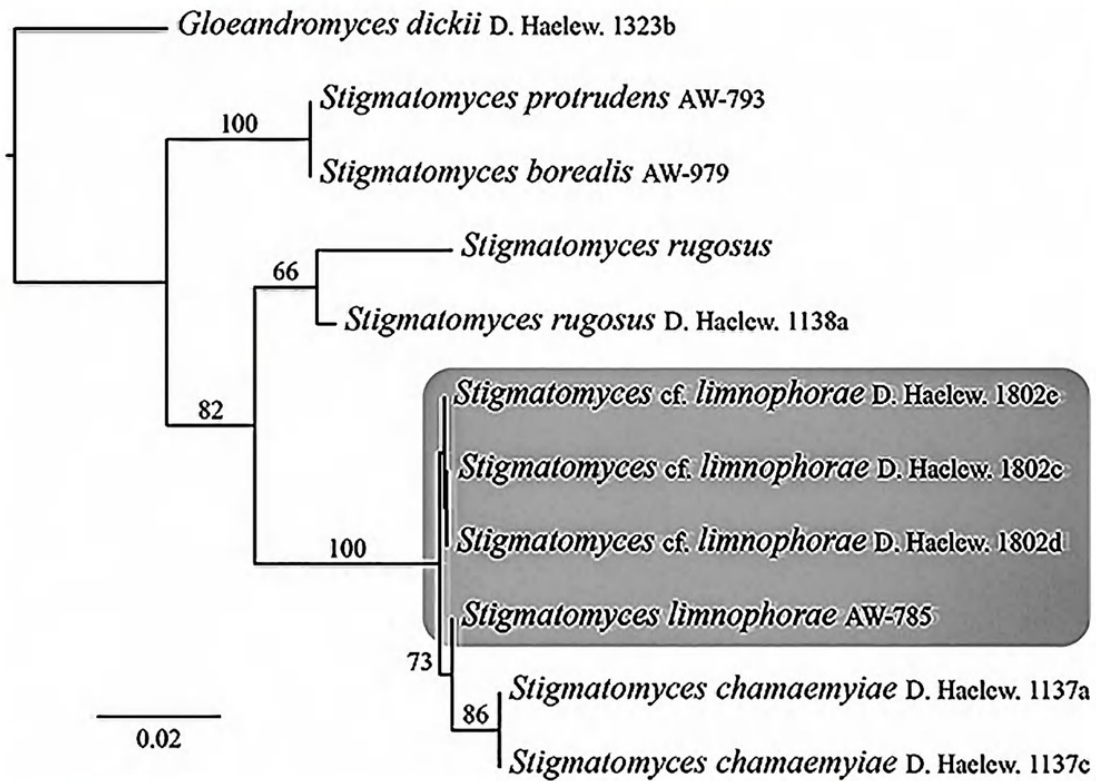


Fig. 5. Phylogeny of *Stigmatomyces* isolates reconstructed from a combined SSU–LSU rDNA dataset, with *Gloeandromyces dickii* as outgroup. Shown is the best-scoring tree ( $-\ln L = 4263.622$ ) as a result of maximum likelihood inference performed with IQ-TREE. For each node, the ML bootstrap (if  $>60$ ) is presented at the branch leading to that node. *Stigmatomyces* aff. *limnophorae* is highlighted in grayscale.

and phylogenetic (FIG. 5) analyses. The global distribution of *S. limnophorae* is presented in TABLE 3. *Stigmatomyces limnophorae* was described from a *Limnophora* fly (*Muscidae*) in California, USA. Isolate AW-785 originated from a *Muscidae* gen. sp. indet. collected in Louisiana, USA (Weir & Blackwell 2001b). This isolate will be regarded as representing the species until fungal sequences are obtained from a muscid fly identified to genus-level collected in the type locality of Berkeley, California. Morphologically our material resembles typical *S. limnophorae* morphology (Thaxter 1901, 1908), but our phylogenetic placement of the Malaysian isolates sister to *S. chamaemyiae* + *S. limnophorae* AW-785, suggests that they might represent another species closely related to *S. limnophorae*. Since the vast majority of thalli were heavily damaged, we were unable to describe the material accurately based on all morphological features.



TABLE 3. World distribution of *Stigmatomyces limnophorae*, with host species and reference of first report.

CONTINENT	COUNTRY	HOST (FAMILY)	FIRST REPORT
North & Central America	Cuba	<i>Limnophora arcuata</i> (Muscidae)	Krejzová & Weiser 1968
	Grenada	<i>Anthomyiidae</i> gen. sp. <i>indet.</i>	Thaxter 1917
	Guatemala	<i>Limnophora</i> sp. (Muscidae)	Thaxter 1917
	Jamaica	<i>Leucomelina</i> sp. (Muscidae)	Thaxter 1917
	Mexico	<i>Onesia</i> sp. (Calliphoridae)	Thaxter 1917
	USA	<i>Limnophora</i> sp. (Muscidae) [type]	Thaxter 1901
		<i>Anthomyiidae</i> gen. sp. <i>indet.</i>	Thaxter 1917
South America	Bolivia	<i>Limnophora</i> sp. (Muscidae)	Rossi 1998
	Brazil	<i>Limnophora</i> sp. (Muscidae)	Bergonzo & al. 2004
	Venezuela	<i>Sarcophaga</i> sp. (Sarcophagidae)	Thaxter 1905
Europe	Portugal	<i>Limnophora obsignata</i> (Muscidae)	Rossi & al. 2013
Africa	Cameroon	<i>Anthomyiidae</i> gen. sp. <i>indet.</i>	Thaxter 1917
	Canary Islands	<i>Limnophora obsignata</i> (Muscidae)	Rossi & al. 2013
	Kenya	<i>Rhyncomya forcipata</i> (Rhiniidae)	Rossi & al. 2013
	Morocco	<i>Limnophora obsignata</i> (Muscidae)	Rossi & al. 2013
	Sierra Leone	<i>Lispe desjardinsii</i> (Muscidae)	Rossi & Leonardi 2018
	Uganda	<i>Fainia albitarsis</i> (Rhiniidae)	Rossi & al. 2013
Asia	Indonesia	<i>Lucilia dux</i> (Calliphoridae)	Thaxter 1917
	Philippines	<i>Lucilia dux</i> (Calliphoridae)	Thaxter 1917
	Thailand	<i>Heliographa ceylanica</i> (Muscidae)	Rossi & al. 2013
	Israel	<i>Limnophora quaterna</i> (Muscidae)	Rossi & al. 2013
	Saudi Arabia	<i>Isomyia terminata</i> (Rhiniidae)	Rossi & al. 2013
		<i>Limnophora quaterna</i> (Muscidae)	Rossi & al. 2013
	Taiwan	<i>Sumatria flava</i> (Rhiniidae)	Rossi & al. 2013
	Turkey	<i>Dasyphora albofasciata</i> (Muscidae)	Rossi & al. 2013
Australasia	Australia	<i>Calliphora augur</i> (Calliphoridae)	Rossi & al. 2013

As a result, for the time being, we refer to the species as *S. aff. limnophorae*. Efforts will be ongoing to sample flies using chicken liver and other baits and to collect additional *Stigmatomyces*-infected fly specimens.

*Stigmatomyces limnophorae* was discovered by Thaxter (1901) as a parasite of *Limnophora* sp. [misspelt as “*Limnophorus*”] (*Muscidae*) in California, USA. The fungus appears to be a very widespread and plurivorous species and has been reported on all continents except Antarctica from hosts in different dipteran families including *Anthomyiidae*, *Calliphoridae*, *Muscidae*, *Rhiniidae*, and *Sarcophagidae* (TABLE 3). Despite its wide distribution, the fungus has not yet been reported in Malaysia. *Stigmatomyces limnophorae* has, however, been reported in neighbouring countries—Indonesia, the Philippines, Thailand—on different dipteran hosts such as *Chrysomya megacephala* [as “*Lucilia dux*”] (*Calliphoridae*) and *Heliographa ceylanica* (*Muscidae*) (Thaxter 1917, Rossi & al. 2013). Here we report the occurrence of *S. aff. limnophorae* in Malaysia on different hosts in *Calliphoridae* and *Sarcophagidae*. While it is not uncommon for *S. limnophorae* to parasitize flies in either of these families (TABLE 3), the Malaysian hosts are in genera that have not previously been observed with thalli of *Laboulbeniales*.

Thalli of *Stigmatomyces aff. limnophorae* were always observed at the abdominal segments, which might indicate the original infection site; however, our observations are based on only three host specimens. Limited data are available on *Laboulbeniales*. For example, the impact of infection on their hosts is still poorly studied (Nalepa & Weir 2007, Riddick 2010, Báthori & al. 2017, Haelewaters & al. 2020b). Further exploration of the diversity of ectoparasitic fungi is required to gain a complete picture of their distribution and interactions with insect hosts.

In summary, we report two new hosts for *Stigmatomyces limnophorae*, a complex species able to infect wide number of species within different genera. This could be caused by evolution and adaption of the fungi for survival in different genera on various continents. As our sample was identified morphologically as *S. limnophorae* but phylogenetically as sister to *S. chamaemyiae* + *S. limnophorae* AW-785 (suggesting a different species), we could postulate that hybridization is occurring between these two species, thereby explaining adaptation of these fungi to different host species and within wide range of genera. Additional research is required to support this hypothesis.

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