Ophiocordyceps flavida sp. nov. (Ophiocordycipitaceae), a new species from Thailand associated with Pseudogibellula formicarum (Cordycipitaceae), and their bioactive secondary metabolites

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Abstract
During a diversity study of entomopathogenic fungi in an agricultural ecosystem, two fungi were collected, isolated, and identified based on molecular phylogenetic analyses of three nuclear loci (LSU, TEF1, and RPB1) combined with morphological data. In this study, one novel species is described, Ophiocordyceps flavida, and a new record of Pseudogibellula formicarum for Thailand. Ophiocordyceps flavida morphologically resembles the Hirsutella anamorph of Ophiocordyceps pruinosa by having a mononematous character of the conidiophores and the same insect host (Hemiptera: Cicadellidae). Pseudogibellula formicarum is found to occur simultaneously with O. flavida, producing white conidiophores on the host. Additionally, secondary metabolites of both fungi were investigated and the major compound in O. flavida was identified as 2-[2-(4-chlorophenyl)ethyl]-2-(1,1-dimethylethyl)-oxirane. Pseudogibellula formicarum from Ghana and Thailand produces 6-methoxy-1H-indole-3-carbonitrile as a main component. These compounds are known from chemical synthesis or as products of biotransformation, respectively. However, they were obtained in our study as genuine fungal metabolites for the first time and may even constitute chemotaxonomic markers for the respective species.

Keywords Entomopathogenic fungi · Phylogenetics · Taxonomy · Systematics

Introduction
The genus Ophiocordyceps was erected by Petch (1931) with O. blattae as type. Species in Ophiocordyceps have diverse morphologies, including fibrous, hard, pliant to wiry, and dark to light colored stromata with perithecia that are superficial or immersed with an ordinal or oblique arrangement (Kobayasi 1941; Mains 1958; Sung et al. 2007). The hosts of Ophiocordyceps are found in various stages of the insect’s life cycle, occurring on orders Blattodea, Coleoptera, Diptera, Hemiptera, Hymenoptera, Lepidoptera, Megaloptera, and Odonata (Kobayasi 1941; Mains 1958; Sung et al. 2007; Sanjuan et al. 2015; Shrestha et al. 2016; Khonsanit et al. 2018; Luangsa-ard et al. 2018; Tasanathai et al. 2019, 2020; Thanakitpipattana et al. 2020). The dominant anamorph associated with Ophiocordyceps is Hirsutella Patouillard followed by Hymenostilbe Petch and Syngliocladium Petch. Paraisaria Samson & B.L. Brady was previously described as an anamorph of Ophiocordyceps, but this genus is now resurrected and segregated from Ophiocordyceps based on distinct morphological characters and molecular phylogeny (Mongkolsamrit et al. 2019).

Leafhoppers and planthoppers are plant feeders belonging to the order Hemiptera, which are considered insect pests in agriculture and forestry. Hirsutella was erected by Patouillard (1892), based on the type species H. entomophila Pat. Previous studies showed that Hirsutella species are commonly found associated with these hoppers. Most species are
mononematous (e.g., *O. pruinosa* D. Johnson, G.H. Sung, Hywel-Jones & Spatafora), and a few species occasionally produce synnemata (e.g., *Hirsutella citriforis* Speare). The conidiogenous cells of *Hirsutella* spp. are monophialidic or polyphialidic producing conidia on phialides. Teleomorph and anamorph stages occurring on hoppers have been reported co-existing on the same or on different insect hosts. For example, Hywel-Jones observed both teleomorph and anamorph (*Hirsutella versicolor* Petch) stages in *O. pruinosa* on the same insect host, and a single collection in which only the anamorph was present (Hywel-Jones 1997).

*Pseudogibellula* was established by Samson and Evans (1973) for *Gibellula formicarum* Mains as *Pseudogibellula formicarum*. In the original description, the insect host of *Gibellula formicarum* was an ant collected from Liberia (Mains 1949). *Pseudogibellula* is morphologically similar to *Gibellula* in producing synnemata scattered on the insect cadaver, giving rise to white or brown conidio-phores arising from mycelium covering the host or from synnemata. However, *Pseudogibellula* differs from *Gibellula* in the way its conidia are produced. The conidia in *Pseudogibellula* are produced singly from sym-podial and polyblastic conidiogenous cells whereas *Gibellula* produces conidia in chains from phialides (Samson et al. 1988). Additionally, *Pseudogibellula* occurs on a wide range of insect hosts whereas *Gibellula* is found exclusively on spiders. Samson et al. (1989) described *Torrubiella pseudogibellulae* infecting *Palthyreus tarsatus* from Ghana as the teleomorph associated with *P. formicarum*. The hosts of *P. formicarum* from Ghana were reported as Hymenoptera and Hemiptera (Samson and Evans 1973). In a taxonomic paper on *Ophiocordyceps* (Ophiocordycipitaceae, Ascomycota), Spatafora et al. (2015) proposed *Ophiocordyceps pseudogibellulae* (Samson, Reenen & H.C. Evans) B. Shrestha, G.H. Sung & Spatafora as a new combination for *Torrubiella pseudogibellulae* Samson, Reenen & H.C. Evans in which *Pseudogibellula formicarum* was included as its syno-nym without including this species in the phylogenetic analyses of Ophiocordycipitaceae. However, there was no formal transfer of the genus from Cordycipitaceae to Ophiocordycipitaceae.

During a diversity study on entomopathogenic fungi in an organic orchard ecosystem, we discovered a mononematous *Hirsutella* sp. attacking an unidentified leafhopper on the underside of pomelo leaves (*Citrus maxima*). The macroscopic morphologies of the natural samples of *Hirsutella* sp. resemble *H. versicolor*, the anamorph of *O. pruinosa*. Additionally, we also found another fungus developing white conidio-phores together with the *Hirsutella* sp. on the host. We attempted to identify these two fungi by investigating their molecular phylogeny and morphological characters. *Hirsutella* sp. did not match *Hirsutella versicolor* or any *Hirsutella* species. The second fungus was identified as *Pseudogibellula formicarum*. The aims of this study are to describe the fungus producing *Hirsutella* anamorph as a new species, *O. flavida* and to establish a new record of *P. formicarum* from Thailand. We confirm the position of *P. formicarum* in the Cordycipitaceae. Finally, an investigation of the secondary metabolites produced by both fungi is presented.

### Materials and methods

#### Collection and isolation

Two species of fungi occurring simultaneously on leaf-hoppers (Hemiptera) were collected on the underside of pomelo leaves (*Citrus maxima*) in an organic orchard ecosystem in Samut Songkhram Province, Thailand. The specimens were collected in two different seasons, the first collection was during the rainy season in June 2016, and the second was during the dry period in January 2017. They were collected carefully so as not to lose the host, and were put in small plastic boxes and transported to the laboratory for isolation. The materials were examined under a stereo microscope (Olympus SZ61). For the isolation from anamorphs, a flame-sterilized inoculation needle was used to remove conidia from sporulating structures to potato dextrose agar plates (PDA; freshly diced potato 200 g, dextrose 20 g, agar 15 g, in 1 L distilled water). Pure cultures were deposited at the BIOTEC Culture Collection (BCC). The leafhopper hosts were identified by morphology. The fungal specimens were dried in an electric food dryer (50–55 °C) overnight and stored in plastic boxes before storage at the BIOTEC Bangkok Herbarium (BBH), National Biobank of Thailand.

#### Morphological observation

Fungal structures, such as phialides and conidia, were mounted in lactophenol cotton blue solution and measured using a light microscope (Olympus CX31). They were photographed using an Olympus DP70 Digital Camera mounted on an Olympus BX51 (Olympus) compound microscope and SZX12 (Olympus) stereo microscope as well as a Hitachi scanning electron microscope (Model SU8020). The cultures were grown on PDA for study of important morphological characters such as conidia, phialides, and colony coloration. The color of fresh specimens and cultures incubated on PDA for 21 days at 25 °C was described and codified following the Sixth Royal Horticultural Society (R.H.S.) Colour Chart.
Molecular phylogenetic analyses

Genomic DNA was harvested from mycelial mass on PDA using a modified cetyltrimethyl-ammonium bromide (CTAB) method as described previously in Mongkolksamrit et al. (2009). The partial gene regions of three nuclear loci, including nuc 28S rDNA (large subunit ribosomal DNA: LSU), translation elongation factor-1α gene (TEF1), and the largest subunit of RNA polymerase II (RPB1), were sequenced. The primer pairs and thermocycler conditions for PCR amplifications used in this study followed Mongkolksamrit et al. (2018). The purified PCR products were sequenced with the same PCR amplification primers for Sanger dideoxy sequencing.

The DNA sequences generated in this study were checked for ambiguous bases using BioEdit v. 7.2.5 (Hall 2004) and then submitted to GenBank. Table 1 shows the list of LSU, TEF1, and RPB1 sequences generated in this study as well as those of other taxa from previous studies. Phylogenetic analyses were performed, including maximum parsimony (MP), Bayesian inference (BI), and maximum likelihood (ML). The MP analysis was conducted on the combined dataset using PAUP 4.0a168 (Swofford 2003) (http://paup.phylosolutions.com/) adopting random addition sequences (10 replications) with gaps being treated as missing data. The nodes in the best MP topologies were evaluated via 1000 bootstrap replicates. The Bayesian inference was performed using MrBayes v.3.2.7a (Ronquist et al. 2012) with the GTR model. Four topologies were evaluated via 1000 bootstrap replicates. RAxML and BI were run on XSEDE in CIPRES Portal (www.phylo.org). Phylogenetic trees were visualized in TreeView v.1.6.6 (Page 1996). The sequence alignment for the dataset used in this study is provided in Supplementary Information.

Instrumentation for spectral measurements

Analytical HPLC profiles were obtained using a Dionex Ultimate 3000 HPLC system [column 2 × 55 mm, 3 μm, C18 Puropher® (Merck)] and Agilent 1260 UHPLC Infinity Systems [column 2.1 × 50 mm, 1.7 μm, C18 Acquity UPLC BEH (Waters)]. High-resolution electrospray ionization mass spectrometry (HRESIMS) was obtained using an Agilent 1200 series HPLC system with an electrospray ionization time-of-flight mass spectrometer (ESI-TOF-MS; Maxis, Bruker) module [column 2.1 × 50 mm, 1.7 μm, C18 Acquity UPLC BEH (Waters)]. Semi-preparative HPLC was performed on a Waters HPLC system equipped with a Waters 600 controller and Waters 2996 photodiode array detector. Nuclear magnetic resonance (NMR) spectra were obtained using a Bruker Avance III 500 MHz and 700 MHz spectrometers.

Fermentation and HPLC analytical of crude extracts

Three strains of Ophiocordyceps flavida sp. nov. (BCC 84254, BCC 84255, and BCC 84256) and seven strains of Pseudogibellula formicarum (BCC 84247, BCC 84249, BCC 84251, BCC 84257, BCC 84259, CBS 871.72, and CBS 433.73) were grown on PDA for 21 days. Five agar plugs (1 × 1 cm) with mycelia were cut and transferred into 3 × 250 mL Erlenmeyer flasks containing 50 mL Potato dextrose broth (PDB, Difco) and incubated for 3 weeks at 25 °C under static condition. Pseudogibellula formicarum BCC 81493 was grown exclusively on yeast-malt glucose (YMG) medium (Rupcic et al. 2018). Five mycelial plugs (1 × 1 cm) from the growing colonies on the agar plate were transferred into 1 × 500 mL Erlenmeyer flasks containing 200 mL of YMG medium. Flasks were incubated on a shaker at 120 rpm for 8 days at 24 °C.

The broth and fungal mycelia obtained from culture were extracted together with ethyl acetate (100 mL) to yield a crude analyte. The fungal extracts from O. flavida and P. formicarum were chemically analyzed on a Dionex Ultimate 3000 HPLC system with diode array detector using a reverse phase column with gradient condition (Puropher® C18, 2 × 55 mm, 3 μm; 0-100% MeCN/H2O over 10 min, 100% MeCN over 2 min, then reversed back to 100% H2O in 1 min and equilibrated at 100% H2O over 2 min). For the mobile phase, 0.05% formic acid was added to both acetonitrile and deionized water.

Isolation of secondary metabolites

Pseudogibellula formicarum BCC 81493 was grown on YMG medium (200 mL) for 8 days under static condition. The culture broth was obtained after the separation from the fungal mycelia and extracted three times with ethyl acetate (3 × 300 mL) to give a crude material (3.8 mg). Fungal mycelia were extracted with methanol and sonicated for 1 hour to obtain a crude substance (6 mg). Both fractions were analyzed on an Agilent 1260 UHPLC Infinity Systems using a previously described gradient condition method (Noumeur et al. 2017), and were discovered to possess very similar HPLC chromatograms. Isolation of pure compounds was performed on a Waters HPLC system with the mobile phase composed of acid-free deionized water and acid-free acetonitrile. The combined broth and mycelial extracts were purified by semi-preparative HPLC using a reversed-phase column (Phenomenex Luna C18, 21.2 × 250 mm, 10 μm; a flow rate
| Species                        | Strain     | Host/Substratum                  | GenBank Accession no. |
|-------------------------------|------------|----------------------------------|-----------------------|
|                               |            |                                  | LSU                   |
| Akanthomyces aculeatus        | HUA 772    | Lepidoptera; Sphingidae          | KC519370              |
| Akanthomyces aculeatus        | HUA 186145 | –                                | MF416520              |
| Ascopolyporus polychrous      | P.C. 546   | Plant                            | DQ118733              |
| Ascopolyporus villosus        | ARSEF 6355 | Plant                            | AY886544              |
| Blackwellomyces cardinalis    | OSC 93609T | Lepidoptera (larva)              | AY184962              |
| Blackwellomyces pseudomilitaris| BCC 1919T | Lepidoptera (larva)              | MF416534              |
| Beauveria bassiana            | ARSEF 1564 | Lepidoptera; Arctiidae           | –                     |
| Beauveria bassiana            | ARSEF 7518 | Hymenoptera; Pamphilidae         | –                     |
| Cordyceps farinosa            | CBS 111113T| –                                | –                     |
| Cordyceps fumosorosea         | CBS 244.31 | Butter                           | MG665230              |
| Cordyceps fumosorosea         | CBS 107.10 | –                                | MG665227              |
| Cordyceps piperis             | CBS 116719 | Hemiptera; scale insect          | AY466442              |
| Drechmeria balanoides         | CBS 250.82 | Nematoda                         | MH873239              |
| Drechmeria gunnii             | BCC 16025  | Arachnid; Araneae                | MF416548              |
| Drechmeria sinensis           | CBS 567.95 | Nematoda                         | MH874175              |
| Gibellula clavulifera var. alba| ARSEF 1915 | Arachnid; *Euophrys* sp.         | DQ518777              |
| Gibellula gamssii             | BCC 25798  | Arachnid; Araneae                | MH152542              |
| Gibellula gamssii             | BCC 27968T | Arachnid; Araneae                | MH152539              |
| Gibellula leiotus             | OSC 76404  | Lepidoptera (larva)              | AF339522              |
| Harposporium harposporiferum  | ARSEF 4472 | –                                | AF339519              |
| Hevansia arachnophila         | NHJ 10469  | Arachnid; Araneae                | EU369031              |
| Hevansia novoguineensis       | CBS 610.80 | Arachnid; Araneae                | MH394646              |
| Hevansia novoguineensis       | NHJ 13161  | Arachnid; Araneae                | –                     |
| Hirsutella cf. haptospora     | ARSEF 2228 | Diptera; Itonidae                | KM652118              |
| Hirsutella citiformis         | ARSEF 1035 | Hemiptera; Cixiidae              | KM652153              |
| Hirsutella citiformis         | ARSEF 1446 | Hemiptera; Cixiidae              | KM652154              |
| Hirsutella cryptosclerotium   | ARSEF 4517T| Hemiptera; Pseudococcidae        | KM652109              |
| Hirsutella fusiformis         | ARSEF 5474 | Coleoptera; Curculionidae        | KM652110              |
| Hirsutella gigantea           | ARSEF 30   | Hymenoptera; Pamphilidae         | –                     |
| Hirsutella guyana             | ARSEF 878  | Hemiptera; Cicadellidae          | KM652111              |
| Hirsutella haptospora         | ARSEF 2226T| Acari; Uropodina                 | –                     |
| Hirsutella homalodiscata      | –          | Hemiptera                        | DQ075674              |
| Hirsutella illustris          | ARSEF 5539 | Hemiptera; Aphididae             | KM652112              |
| Hirsutella kirchneri          | ARSEF 5551 | Acari; Eriophyidae               | KM652161              |
| Hirsutella lecanicola         | ARSEF 8888 | Hemiptera; Coccidae              | KM652114              |
| Hirsutella liboensis          | ARSEF 9603 | Lepidoptera; Cossidae            | KM652115              |
| Hirsutella necatrix           | ARSEF 5549 | Acari                            | KM652116              |
| Hirsutella nudolosa           | ARSEF 5473 | Lepidoptera; Pyralidae           | KM652117              |
| Hirsutella radiata            | ARSEF 1369 | Diptera                          | –                     |
| Hirsutella repens nom. inval. | ARSEF 2348 | Hemiptera; Delphacidae           | KM652120              |
| Hirsutella rhossiliensis      | ARSEF 2931 | Tylenchida; Heteroderida         | KM652121              |
| Hirsutella satumaensis        | ARSEF 996  | Lepidoptera; Pyralidae           | KM652125              |
| Hirsutella sp.                | ARSEF 7578 | Hymenoptera; Formicidae          | –                     |
| Hirsutella sp.                | ARSEF 8378 | Hemiptera; Cixiidea              | KM652127              |
| Hirsutella stilbelliformis var. myrmicarum | IMI 39639 | Hymenoptera; Formicidae          | –                     |
| Hirsutella strigosa           | ARSEF 2044 | Hemiptera; Delphacidae           | KM652128              |
| Hirsutella strigosa           | ARSEF 2197 | Hemiptera; Cicadellidae          | KM652129              |
| Species | Strain | Host/Substratum | GenBank Accession no. |
|---------|--------|-----------------|----------------------|
| *Hirsutella subramanianii var. myrmicarum* | IMI 396400 | Hymenoptera; Formicidae | – EU797598 – |
| *Hirsutella subulata* | ARSEF 2227 | Lepidoptera; Microlepidoptera | KM652130 KM652013 KM652051 |
| *Hirsutella thompsonii* | ARSEF 253 | Acari; Eriophyidae | KM652133 KM652016 – |
| *Hirsutella thompsonii var. vinacea* | ARSEF 254 | Acari; Eriophyidae | KM652149 KM652028 KM652062 |
| *Hirsutella versicolor* | ARSEF 1037 | Hymenoptera; Membracidae | – KM652150 KM652029 |
| *Hyperdermium pulvinatum* | P.C. 602 | Hemiptera; Scale insect | AF242353 DQ118746 DQ127237 |
| *Lecanicillium antillanum* | CBS 350.85 | Hymenomycetes; Agaric | – KM652150 KM652029 |
| *Lecanicillium tenuipes* | CBS 309.85 | Arachnid; Araneae | AF339526 DQ522369 DQ522386 |
| *Neotorrubiella chinghridicola* | BCC 39684 | Orthopterida | – KM652150 KM652029 |
| *Neotorrubiella chinghridicola* | BCC 80733 | Orthopterida | – KM652150 KM652029 |
| *Ophiocordyceps acicularis* | OSC 12858 | Coleoptera | – KM652150 KM652029 |
| *Ophiocordyceps blattae* | BCC 34765 | Blattodea | – MT533484 MT533478 |
| *Ophiocordyceps flavida* | OSC 12857 | Cicadidae | – MT533478 |
| *Ophiocordyceps ravenelii* | HKAS 102447 | Formicidae | – KJ879902 |
| *Ophiocordyceps sp. A* | TNS-F-30044 | Phyllophaga sp. | – |
| *Paraisaria gracilis* | EFCC 3101 | Lepidoptera (larva) | – |
| *Paraisaria heteropoda* | EFCC 10125 | Lepidoptera; Cicadidae | – |
| *Paraisaria heteropoda* | EFCC 8572 | Lepidoptera; Cicadidae | – |
| *Paraisaria heteropoda* | EFCC 10125 | Lepidoptera; Cicadidae | – |
| *Paraisaria heteropoda* | EFCC 8572 | Lepidoptera; Cicadidae | – |
| *Paraisaria heteropoda* | EFCC 10125 | Lepidoptera; Cicadidae | – |
| *Paraisaria heteropoda* | EFCC 8572 | Lepidoptera; Cicadidae | – |
| *Paraisaria heteropoda* | EFCC 10125 | Lepidoptera; Cicadidae | – |
| *Paraisaria heteropoda* | EFCC 8572 | Lepidoptera; Cicadidae | – |
10 mL/min, 10% MeCN/H₂O over 5 min, 10–100% MeCN/H₂O over 35 min, 100% MeCN over 20 min) to afford the major compound, 6-methoxy-1H-indole-3-carbonitrile (4.3 mg), at the retention time (Rt) = 29–30 min.

Secondary metabolites were obtained from three strains of *O. flavida* (BCC 84254, BCC 84255, and BCC 84256) as follows. Culture broth and mycelia were combined and then extracted three times with ethyl acetate (80 mL) to yield a crude extract. Crude extracts from three fungal strains were analyzed on a Dionex Ultimate 3000 HPLC system with a previously mentioned analytical gradient condition method. HPLC profiles from these three strains were shown to be similar with one major and a few minor peaks. The isolation of the major component was performed on a Waters HPLC system and the mobile phase was composed of acid-free solvents. The combined broth and mycelial extracts (22.9 mg) were purified by semi-preparative HPLC using a reversed-phase column (VDSpher PUR 100 C18-E, 20 × 250 mm, 10 μm; a flow rate 10 mL/min, 30% MeCN/H₂O for 7 min, 30–80% MeCN/H₂O for 38 min, 80–100% MeCN/H₂O for 10 min, 100% MeCN for 30 min) to afford the major component, 2-[2-(4-chlorophenyl)ethyl]-2-(1,1-dimethylethyl)-oxirane (2.4 mg), (Rt) = 54–55 min.

**Biological characterization**

The compound 6-methoxy-1H-indole-3-carbonitrile was tested for antimicrobial affects against several bacteria and fungi and against mammalian cells as reported previously, using the same protocols (Cheng et al. 2019; Rupcic et al. 2018; Sandargo et al. 2020). The other compound 2-[2-(4-chlorophenyl)ethyl]-2-(1,1-dimethylethyl)-oxirane (2.4 mg) unfortunately decayed before the bioassays could be carried out. Hence, no antimicrobial and cytotoxic activities are reported. List of test microorganisms used are in Supplementary Information.

### Table 1 (continued)

| Species | Strain | Host/Substratum | GenBank Accession no. |
|---------|--------|-----------------|----------------------|
|         |        |                 | LSU | TEF1 | RPB1 |
| *Perennicordyceps prolifica* | TNS-F-18481 | Hemiptera | KF049631 | KF049686 | KF049648 |
| *Perennicordyceps prolifica* | TNS-F-18547 | Hemiptera | KF049632 | KF049687 | KF049649 |
| *Pseudogibellula formicarum* | CBS 433.73 | Hymenoptera; *Paathothe myces flavida* | MH872442 | MT533481 | MT533475 |
| *Pseudogibellula formicarum* | CBS 871.72 | Hemiptera; *Ricani a mediana* | MH878295 | MT63565 | MT533474 |
| *Pseudogibellula formicarum* | BCC 81493 | *Ophiocordyceps flavida* | MT512652 | MT63566 | MT533472 |
| *Pseudogibellula formicarum* | BCC 84257 | *Ophiocordyceps flavida* | MT512653 | MT533480 | MT533473 |
| *Purpureocillium lilacinum* | CBS 284.36 | Soil | FR775484 | EF468792 | EF468898 |
| *Purpureocillium lilacinum* | CBS 431.87 | *Meloidogyne* sp. | EF468844 | EF468791 | EF468897 |
| *Purpureomyces ramosopulvinatus* | EFCC 1424 | Coleoptera | JF415970 | JF410012 | JN049888 |
| *Samsoniella aurantia* | TRBC 7271 | Lepidoptera | MF140728 | MF140846 | MF140791 |
| *Samsoniella aurantia* | TRBC 7272 | Lepidoptera | MF140727 | MF140845 | – |
| *Simplicillium formicae* | MFLUCC 18-1379 | Hymenoptera; *Ant* | MK766512 | MK926451 | MK882623 |
| *Simplicillium lanosonivum* | CBS 704.86 | *Hemileia vastatrix* | AF339553 | DQ522335 | DQ522406 |
| *Simplicillium unilateralis* | MFLUCC 18-1385 | *Ophiocordyceps unilateralis* | MK752849 | MK926450 | MK882622 |
| *Tolypocladium capitatum* | NBRC 100997 | – | JN941401 | AB968597 | JN924748 |
| *Tolypocladium capitatum* | OSC 71233 | *Elaphomyces* sp. | AY489721 | AY489615 | AY489649 |

The accession numbers marked in bold font refer to sequences new in this study or have been generated by our group in Thailand

1 Sanjuan et al. (2014), 2 Kepler et al. (2017), 3 Chaverri et al. (2005), 4 Bischoff et al. (2005), 5 Sung and Spatafora (2004), 6 Spatafora et al. (2007), 7 Rehner et al. (2011), 8 Mongkolsamrit et al. (2018), 9 Vu et al. (2019), 10 Sung et al. (2001), 11 Castlebury et al. (2004), 12 Kuypers et al. (2016), 13 Johnson et al. (2009), 14 Mongkolsamrit et al. (2020), 15 Simmons et al. (2015), 16 Boucias et al. (2006), 17 Sullivan et al. (2000), 18 Thanakittipattana et al. (2015), 19 Luangsa-ard et al. (2018), 20 Sung et al. (2007), 21 Quandt et al. (2014), 22 Kepler et al. (2011), 23 Wei et al. (2019), 24 Kepler et al. (2013), 25 Perdomo et al. (2013), 26 Kepler et al. (2012), 27 Luangsa-ard et al. (2017), 28 Schoch et al. (2012), 29 Ban et al. (2015) T Ex-type culture
Results

Molecular phylogeny

We generated 6 LSU, 8 TEF1, and 8 RPB1 sequences from three strains of *O. flavida*, two strains of *O. blatta*, two strains of *P. formicarum* from Thailand and two strains of *P. formicarum* from Ghana (CBS 433.73 and CBS 871.72) (Table 1). *Purpureomyces khaoyaensis* (Hywel-Jones) Luangs-a-ard, Samson & Thanakitipipattana (BCC 1375 and BCC 14290) in the Clavicipitaceae was used as the outgroup.

Phylogenetic analyses (Fig. 1) strongly support *P. formicarum* from Thailand (BCC 81493, BCC 84257) and from Ghana (CBS 433.73, CBS 871.72) as a monophyletic clade and as members of Cordycipitaceae with strong support (RAxML 100%, MP 100%, BPP 100%). *O. flavida* is nested in *Ophiocordyceps* with strong support (RAxML 100%, MP 100%, BPP 100%). Additionally, analyses of *O. flavida* with related species in *Ophiocordyceps* strongly support (RAxML 100%, MP 100%, BPP 100%) *O. flavida* as a distinct clade that formed independently and did not group with any known species occurring on leafhoppers or planthoppers (Fig. 2). The species descriptions based on morphological characters of *O. flavida* as a novel species and *P. formicarum* from Thailand are given below. Comparison of LSU sequence of the only available sequence data for *P. formicarum* that can also be found on the underside of leaves. However, our phylogenetic analyses (Fig. 2) clearly show that *O. flavida* forms its own and separate clade from *Hirsutella versicolor* ARSEF 1037. Another species reported on cicadellid hosts is *Hirsutella homalodiscae* nom. prov. occurring on leafhoppers (Hemiptera) that can be found on the underside of leaves. This species differs from *O. flavida* in having longer phialides (30.9 ± 4.5 μm) and in the cirriform to amygdaliform shape of its conidia (Boucias et al. 2006).

Notes: On the natural host, *Ophiocordyceps flavida* closely resembles the anamorph of *O. pruinosa* (Petch) D. Johnson, G.H. Sung, Hywel-Jones & Spatafora (= *Hirsutella versicolor*) in having yellow to brown mycelium and fusiform to globose conidia occurring on leafhoppers (Hemiptera) that can be found on the underside of leaves. However, our phylogenetic analyses (Fig. 2) clearly show that *O. flavida* formed its own and separate clade from *Hirsutella versicolor* ARSEF 1037. Another species reported on cicadellid hosts is *Hirsutella homalodiscae* nom. prov. occurring on *Homalodisca coagulata* (glassy-winged sharpshooter) causing epizootics in southeastern USA. This species differs from *O. flavida* in having longer phialides (30.9 ± 4.5 μm) and in the cirriform to amygdaliform shape of its conidia (Boucias et al. 2006).

Taxonomy

**Ophiocordyceps flavida** Mongkolsamrit, Noisripoom, Pumiputikul & Luangs-ard, sp. nov. (Fig. 3).

Mycobank: MB838111

**Holotype**: Thailand, Samut Songkhram Province, Boonmee Orchard, on leafhopper (Hemiptera: Cicadellidae: *Tartessus ferrugineus*), underside of pomelo leaves (*Citrus maxima*), K. Tasanathai, P. Srikitikulchai, R. Promharn, S. Mongkolsamrit, 31 January 2017, SM 2060 (BBH 42349, paratype), ex-paratype culture BCC 84254; SM 02068 (BBH 42357), BCC 84263.

**Etymology**: Describes the pale yellow color of the fresh specimens.

**Anamorph**: Mycelium covering loosely the leafhopper with compact pale yellow to orange (22A) hyphae and surrounded by a radiating mat over the leaf. *Conidiogenous* structures phialidic, borne directly and singly on hyphae. Phialides cylindrical or flask-shaped, basal portion, (7)9.5–14(20) × 2–4 μm, tapering into a long neck, 4–6(10) × 1 μm. Conidia smooth-walled, one-celled, hyaline, fusiform to globose, apiculate on distal end, 2–5 × 2–3 μm.

**Culture characteristics**: Colonies on PDA attaining a diam of 8 mm in 14 days, cottony with high mycelial density, greyish purple. Mycelium smooth-walled, septate, hyaline. *Conidiogenous* structures phialidic, borne directly and singly on hyphae. Phialides (8)10–18(20) × 3–4 μm, with cylindrical basal portion, tapering predominantly into a long neck or occasionally producing two to three necks, (3)4–7(10) × 1 μm. Conidia smooth-walled, one-celled, hyaline, fusiform to globose, apiculate on distal end, 2–3 × 2–3 μm.

**Distribution**: Found in western Thailand.

**Additional specimens examined**: Thailand, Samut Songkram Province, Boonmee Orchard, on leafhopper (Hemiptera: Cicadellidae: *Tartessus ferrugineus*), underside of pomelo leaves (*Citrus maxima*), K. Tasanathai, P. Srikitikulchai, R. Promharn, S. Mongkolsamrit, 31 January 2017, SM 2060 (BBH 42349, paratype), ex-paratype culture BCC 84255; SM 2057 (BBH 42424), BCC 84252; SM 2058 (BBH 42347), BCC 84253; SM 2059 (BBH 42348), BCC 84254; SM 02068 (BBH 42357), BCC 84263.

**Notes**: On the natural host, *Ophiocordyceps flavida* closely resembles the anamorph of *O. pruinosa* (Petch) D. Johnson, G.H. Sung, Hywel-Jones & Spatafora (= *Hirsutella versicolor*) in having yellow to brown mycelium and fusiform to globose conidia occurring on leafhoppers (Hemiptera) that can also be found on the underside of leaves. However, our phylogenetic analyses (Fig. 2) clearly show that *O. flavida* formed its own and separate clade from *Hirsutella versicolor* ARSEF 1037. Another species reported on cicadellid hosts is *Hirsutella homalodiscae* nom. prov. occurring on *Homalodisca coagulata* (glassy-winged sharpshooter) causing epizootics in southeastern USA. This species differs from *O. flavida* in having longer phialides (30.9 ± 4.5 μm) and in the cirriform to amygdaliform shape of its conidia (Boucias et al. 2006).

**Pseudogibellula** Samson & H.C. Evans, Acta Bot. Neerl. 22: 524 (1973)

**Circumscription**: The genus is emended here consisting of one species in the node defined in Fig. 1 as the reference phylogeny for the *Pseudogibellula* clade in Cordycipitaceae, which contains *P. formicarum*. The descriptions from the teleomorph are taken from Samson et al. (1989). The morphological character associated with the teleomorph includes the presence of distinct white powdery conidia from scattered conidiophores arising from mycelium covering the host. *Conidiogenous* cells...
Fig. 1 RAxML tree showing phylogenetic relationships within Cordycipitaceae and Ophiocordycipitaceae. The dataset included 81 taxa generated from combined LSU, TEF1, and RPB1 DNA sequence data. Numbers at the significant nodes represent RAxML/MP bootstrap values/Bayesian posterior probabilities, multiplied by 100. Bold lines in the tree represent 100% of the three values.
Fig. 2  RAxML tree showing the placement of Ophiocordyceps flavida and related species in Ophiocordyceps. Dataset included 46 taxa generated from the combined LSU, TEF1, and RPB1 DNA sequence dataset. Numbers at the significant nodes represent RAxML/MP bootstrap values/Bayesian posterior probabilities, multiplied by 100. Bold lines in the tree represent 100% of three values. Fungi found on leafhoppers or planthoppers are marked with bold triangle.
flask-shaped to cylindrical, inconspicuous scars, conidia one-celled, hyaline, smooth.

Type: *Pseudogibellula formicarum* (Mains) Samson & H.C. Evans  
Basionym: *Gibellula formicarum* Mains, Mycologia 41: 309 (1949).  
Synonyms: *Torrubiella pseudogibellulae* Samson, van Reenen & H.C. Evans, Stud. Mycol. 31: 127 (1989).  
*Ophiocordyceps pseudogibellulae* (Samson, Reenen & H.C. Evans) B. Shrestha, G.H. Sung & Spatafora, IMA Fungus 6: 361 (2015).

The description and illustrations provided herein are based on *Pseudogibellula formicarum* specimens collected from Thailand (Fig. 4).

**Anamorph:** Occurring with *Ophiocordyceps flavida* on *Tartessus ferrugineus* (Hemiptera, Cicadellidae) on the underside of *Citrus maxima* leaf (pomelo). *Mycelium* becoming cream to white due to presence of white powdery conidia, mononematous. *Conidiophores* arising from the mycelium covering and surrounding the host, up to 180 μm in length, 3–5 μm wide, septate, rough, terminating in a small vesicle, globose, 4–5 μm in diam. *Conidial heads* radiating, biseriate, 40–60 μm diam. *Conidiogenous branches* swollen, globose, 3–5 μm in diam. *Conidiogenous cells* flask-shaped to cylindrical, with conspicuous rachises at the upper part, 4–8 × 1.5–2 μm. *Conidia* hyaline, smooth, cylindrical, or narrowly obovoid, 2–6 × 1–2 μm.

**Culture characteristics:** Colonies on PDA attaining a diam of 10 mm in 21 days, cottony with high mycelial density, white. *Mycelium* smooth-walled, hyaline, sporulating poorly. *Conidiogenous cells* arising from aerial hyphae, solitary, ovoid with conspicuous rachises at the upper part of conidiogenous cells, 4–8 × 2–5 μm. *Conidia* hyaline, cylindrical, or narrowly obovoid, 3–6 × 1–2.5 μm.

**Distribution:** Found in western Thailand.

**Additional specimens examined:** Thailand, Samut Songkhram Province, Boonmee Orchard, occur simultaneously with *O. flavida* on leafhopper (Hemiptera: Cicadellidae: Tartessus ferrugineus), underside of pomelo leaves (*Citrus maxima*), D. Thanakitpipattana, J. Luangsa-ard, K. Tasanathai, S. Mongkolsamrit, 1 June 2016, SM2010, (BBH 42310, BCC 81493), *idem.*, K. Tasanathai, P. Sritikitkulchai, R. Promham, S. Mongkolsamrit, 31 January 2017, SM2052 (BBH 42342), BCC 84247; SM2054 (BBH 42344), BCC 84249; SM 2056 (BBH 42345), BCC 84251; SM 2062 (BBH 42351), BCC 84257; SM 2063 (BBH 42352), BCC 84258; SM 2064 (BBH 42353), BCC 84259; SM 2065 (BBH 42354), BCC 84260; SM 2066 (BBH 42355), BCC 84261; SM 2067 (BBH 42356), BCC 84262.

**Notes:** Based on the macromorphologies of the natural specimens, *Pseudogibellula formicarum* collected from Thailand closely resembles *Beauveria* by producing powdery white conidia covering its host. In Thailand, *P. formicarum* grows with *O. flavida*, possibly as a mycoparasite. Samson and Evans (1973), who reported *P. formicarum* found in Ghana as a parasitic fungus on insect hosts (Hemiptera: Cercopidae, Rictanidae and Hymenoptera: Ponerinae, Myrmiciniae), also recognized it as a strongly competitive fungus on insect substrates that frequently exploits ant cadavers killed by other fungal pathogens. It was found to be especially associated with *Ophiocordyceps australis* (Speg.) G.H. Sung.
J.M. Sung, Hywel-Jones & Spatafora where it directly grows on the stroma, suggesting it could be a mycoparasite. The specimens from Ghana produced synnemata arising from hosts while the conidiogeneous structures of specimens from Thailand were mononematous.

**Identification of the major metabolite from Ophiocordyceps flavida**

The HPLC profile of the crude extract of *Ophiocordyceps flavida* (BCC 84254, BCC 84255, and BCC 84256) showed one major metabolite peak (Rt = 8.7 min) with a few other minor metabolites evident in the chromatogram (Fig. 5a). Additional purification by semi-preparative HPLC led to the isolation of the major metabolite, which could be identified as 2-[2-(4-chlorophenyl)ethyl]-2-(1,1-dimethylethyl)-oxirane (Fig. 6a). The structure of the isolated compound was elucidated based on the interpretation of 2D NMR spectroscopic data including $^{1}$H–$^{1}$H correlation spectroscopy (COSY), distortionless enhancement by polarization transfer (DEPT), heteronuclear single-quantum correlation spectroscopy (HSQC), and heteronuclear multiple-bond correlation spectroscopy (HMBC). The core structure of the molecule could be established using proton-carbon correlations from these experiments. The molecular formula was shown to be $C_{14}H_{19}ClO$ and the pattern of mass spectrum peaks further confirmed the presence of a chloride atom in the molecule.

**Identification of the major metabolite from Pseudogibellula formicarum**

HPLC profiles of the broth and mycelial extract of *Pseudogibellula formicarum* (BCC 81493) showed one similar major metabolite peak (Rt = 6.4 min) with a UV absorption pattern characteristic for indoles chromophors (Fig. 7). Further purification by semi-preparative HPLC led to the isolation of this major metabolite as 6-methoxy-1H-indole-3-carbonitrile (Fig. 6b). The structure of the isolated compound...
was elucidated from the interpretation of 2D NMR spectroscopic data including $^1$H–$^1$H COSY, HSQC, and HMBC. Its molecular formula was confirmed as C$_{10}$H$_8$N$_2$O by HRESIMS and the UV spectrum also matched that of the corresponding synthetic compound with characteristic absorption bands at 221, 271, and 290 nm. This substance has been reported to exhibit antifungal activity against *Alternaria brassicicola* (Pedras and Abdoli 2013).

Seven additional strains of *P. formicarum* including BCC 84247, BCC 84249, BCC 84251, BCC 84257, BCC 84259, CBS 871.72, and CBS 433.73 were cultivated on PDB in small scale (50 mL) and the derived crude extracts were chemically evaluated. All *P. formicarum* strains demonstrated the same major metabolite at the retention time of 5.0 min (Fig. 5b), which was identified as 6-methoxy-1H-indole-3-carbonitrile, as inferred from a comparison with

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**Fig. 5** Comparison of HPLC-(DAD)-UV chromatograms of the crude extracts from (a) *Ophiocordyceps flavida* and (b) *Pseudogibellula formicarum*.
the isolated pure metabolite from *P. formicarum* BCC 81493. Additional HPLC profiles of *O. flavida* and *P. formicarum* along with 1H–NMR and HRESIMS spectra of isolated metabolites are provided in the Supplementary Information.

The biological activity of 6-methoxy-1H-indole-3-carbonitrile and its anamorph was identified as *Hirsutella versicolor* (Petch 1939). G.H. Sung, Hywel-Jones & Spatafora occurs on leafhoppers (Cicadellidae). Both species occur on leafhoppers (Petch on an unknown species of leafhopper from Sri Lanka (Petch 1932). This species was originally described from Sri Lanka by Petch. Hirsutella citriformis Speare was reported from New Zealand, Puerto Rico, and the Hawaiian Islands. Both *Ophiocordyceps pruinosa* and *H. citriformis* were documented in Thailand on adult hoppers (Cicadellidae and Delphacidae, respectively) (Hywel-Jones 1997). Recently, Luangsa-ard et al. (2018) reported two novel species from Thailand, *Ophiocordyceps spataporae* Tasan., Thanakit., Khons. & Luangsa-ard occurring on planthoppers (Fulgoridae) and *Ophiocordyceps brunneinigra* Tasan., Thanakit., Khons. & Luangsa-ard found on leafhoppers (Cicadellidae).

From our phylogenetic analyses in Fig. 2, *Ophiocordyceps flavida* is closely related to *Hirsutella versicolor* and shows similar morphological characteristics. Both species occur on leafhoppers that are found on the underside of leaves. The phialides of both species are conoid, cylindrical to flask-shaped and their sizes are in the same range (7–20 × 2–4 μm vs. 8–20 × 2.5–3 μm). However, the conidia in *O. flavida* are fusiform to globose whereas the conidia of *O. pruinosa* (H. versicolor) are narrowly cymbiform or narrow-oval.

**New host record for Pseudogibellula formicarum**

This study is the first report of *Pseudogibellula formicarum* from Thailand. The results of our phylogenetic analyses (Fig. 1) indicated that *Pseudogibellula* is a strongly supported genus in the Cordycipitaceae. In a study by Samson and Evans (1973), *P. formicarum* in Ghana was documented as a generalist insect pathogen found on various insect hosts, such as Hymenoptera (Myrmicinae, Ponerinae) and Hemiptera (Ricaniidae, Cercopidae). Moreover, it is also recognized as a colonizer of insect debris in the soil. Kanga et al. (2004) collected glassy-wing sharpshooter (GWSS) Homalodisca coagulata (Cicadellidae) cadavers that had died on crape myrtle (*Lagerstroemia indica* L.) and holly (*Ilex myrtifolia* Walter) in Mississippi for identification of the primary pathogens of sharpshooters. They confirmed that *P. formicarum* was the cause of the epizootics in sharpshooters (Cicadellidae) in the field after conducting pathogenicity assay. Boucas et al. (2006) also studied outbreaks on the GWSS (*H. coagulata*) from north Florida and southern Georgia. They determined and identified that three fungi, including *Hirsutella homalodiscae* nom. prov., *Pseudogibellula* sp., and *Sporothrix* sp. are pathogens of the glassy-winged sharpshooter. However, there was no report of an association between *Hirsutella homalodiscae* and *Pseudogibellula*. Based on our study, we found the conidiophores of *P. formicarum* occurring simultaneously with *O. flavida* in the natural specimens on leafhoppers (Fig. 4a, 4b). More studies are needed to explain the association between *O. flavida* and *P. formicarum* and to clarify what fungal species caused the epizootics in the leafhopper.

**Secondary metabolites from Ophiocordyceps flavida and Pseudogibellula formicarum**

From the crude extract of *Ophiocordyceps flavida* (BCC 84254, BCC 84255, and BCC 84256), the major secondary metabolite could be isolated and identified as 2-[2-(4-chlorophenyl)ethyl]-2-(1,1-dimethylethyl)-oxirane. The synthesized compound is commercially available, but no report regarding its original natural sources has been confirmed. Nevertheless, we report it here from a fungal source (and as a natural product) for the first time. There is a broad spectrum of usage for this metabolite. The compound is used as raw material in a variety of products and industries such as cosmetics and chemical manufacturing. In addition, agrochemical...
related products including fertilizer and pesticide also employ this substance as the raw material (Martins et al. 2013).

_Pseudogibellula formicarum_ (BCC 81493) was discovered to produce a single metabolite as its major product. The structure elucidation process led to the identification of the isolated substance as the novel fungal metabolite, 6-methoxy-1H-indole-3-carbonitrile. This compound had previously only been reported as a derivative from the biotransformations of the phytoalexin camalexin with antifungal activity against _Alternaria brassicicola_ (Pedras and Abdoli 2013). We confirmed its antifungal activity, although this is weaker than its activity against mammalian cells.

To further explore the metabolite production of _P. formicarum_ BCC 81493, crude extracts from static cultures in YMG medium were also analyzed. The HPLC profiles were similar to previous results from cultures grown in shake flasks. The HPLC profiles from samples of five additional _P. formicarum_ isolates (BCC 84247, BCC 84249, BCC 84251, BCC 84257, and BCC 84259) cultivated in PDB and Q6 media (Cheng et al. 2019) exhibited very similar chromatograms with 6-methoxy-1H-indole-3-carbonitrile as the dominant major metabolite. Moreover, the same major metabolite was identified from BCC 81493 cultivated under different conditions.

A great deal of work by natural product chemists has revealed a vast diversity of secondary metabolites from entomopathogenic fungi (Gibson et al. 2014; Helaly et al. 2019; Isaka et al. 2019; Kuephadungphan et al. 2019; Sonyot et al. 2020; Zhang et al. 2020). While the majority of these studies were aimed at the discovery of new biologically active compounds, some commonly found secondary metabolites have been reported to be suitable as chemotaxonomic markers. For instance, hopane triterpenes are specific for _Hypocrella_ and _Moelleriella_ species, and zeorin commonly occurs on _Conioideocrella_ species on scale insects (Isaka et al. 2009; Isaka et al. 2011). Accordingly, all six strains of _P. formicarum_ from Thailand which have been evaluated so far were found to produce 6-methoxy-1H-indole-3-carbonitrile as their major component. In addition, commercially available _P. formicarum_ CBS 871.72 and CBS 433.73 strains collected from Ghana in Western Africa were found to produce this metabolite. These data indicate that 6-methoxy-1H-indole-3-carbonitrile could be considered as a chemotaxonomic marker for _P. formicarum_, but additional strains including related species remain to be studied in order to assess whether it is species-specific.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11557-021-01683-y.

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Author contribution The specimens in this study were collected by S. Mongkolsamrit, S. Pumiputikul, and J.J. Luangsard. Morphological data were collected by S. Mongkolsamrit and S. Pumiputikul. Molecular data and phylogenetic analyses were performed by W. Noisripoom. Identification of metabolite and bioassay were performed by C. Boonlarppradab and K. Becker.

S. Mongkolsamrit and C. Boonlarp pradab wrote the original draft and review and editing were done by R.A. Samson, M. Stadler, and J.J. Luangsard.

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Data availability All sequence data generated in this study (see Table 1) are available in GenBank (https://www.ncbi.nlm.nih.gov/genbank/).

Declarations Conflict of interest The authors declare no competing interests.

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