Novel and interesting Ophiocordyceps spp. (Ophiocordycipitaceae, Hypocreales) with superficial perithecia from Thailand

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Abstract: Ophiocordyceps is a heterogeneous, species-rich genus in the order Hypocreales (Sordariomycetes, Ascomycota) that includes invertebrate-pathogenic taxa. In this study, seven new species in Ophiocordyceps producing superficial perithecia infecting various insect hosts (Lepidoptera, Hemiptera) are described from Thailand – Ophiocordyceps brunneinigra, O. bruneiperitheciata, O. geometridicola, O. multiperitheciata, O. pauciovoperitheciata, O. pseudoacicularis and O. spataforae. Phylogenetic analyses based on multigene loci comprising the large subunit of the ribosomal DNA (LSU), partial sequences of elongation factor 1-alpha (TEF) and the largest and second largest subunit of the RNA polymerase (RPB1, RPB2) strongly support these new species of Ophiocordyceps in the Ophiocordycipitaceae. They differ from species previously described species Ophiocordyceps acicularis, O. atewensis, O. cochliidicola, and O. crinallis, in the shape and sizes of distinguishing characters such as perithecia, ascospores and conidia. We also report a new record of O. macroacicularis in Thailand.

Key words: Cryptic species, 7 new taxa, Ophiocordyceps, Taxonomy.

Taxonomic novelties: New species: Ophiocordyceps brunneinigra Tasanathai, Thanakitpipattana, Khonsanit & Luangsa-ard, O. bruneiperitheciata Tasanathai, Thanakitpipattana, Khonsanit & Luangsa-ard, O. geometridicola Tasanathai, Thanakitpipattana, Khonsanit & Luangsa-ard, O. multiperitheciata Tasanathai, Thanakitpipattana, Khonsanit & Luangsa-ard, O. pauciovoperitheciata Tasanathai, Thanakitpipattana, Khonsanit & Luangsa-ard, O. pseudoacicularis Tasanathai, Thanakitpipattana, Khonsanit & Luangsa-ard, O. spataforae Tasanathai, Thanakitpipattana, Khonsanit & Luangsa-ard.

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INTRODUCTION

The occurrence of species complexes – morphologically similar but genetically different cryptic closely related species – can underestimatethe diversity for a wide range of taxa in Kingdom Fungi. Molecular studies and barcoding efforts have routinely unmasked so many cryptic species and have revealed this as a common phenomenon in the Fungi (Vilgalys & Sun 1994, Dettman et al. 2003, Kaiserud et al. 2007, Pažoutová et al. 2015). Despite this realization, however, the primary step a mycologist often performs when specimens are collected is to study the phenotypic characteristics and initially classify them based on these traits.

Among invertebrate-pathogenic fungi, the phenotypic traits used to describe species are ascospore morphology, size and shape, and the orientation of perithecia, colour and texture of Stromata, conidial shapes and sizes, as well as phialide morphology (Petch 1934, Mains 1959, Kobayasi & Shimizu 1980, Evans et al. 2011, Tasanathai et al. 2016, Luangsa-ard, Mongkolsamrit, Thanakitpipattana, et al. 2017, Luangsa-ard, Mongkolsamrit, Noisripoom, et al. 2017). Past classifications and current alpha taxonomy place special emphasis on correlations between character states associated between ascospore and perithecial morphogy. Ascospores may remain intact (whole) or disarticulate into part-sporoes after discharge. Perithecia may be immersed, pseudo-immersed or superficial and may be ordinal (vertically attached) or presented at an oblique angle in arrangement along the stroma.

The occurrence of superficial perithecia is a phenotypic trait that can be found in all three hypocrealean families Clavicipitaceae, Cordycipitaceae and Ophiocordycipitaceae with endomogenous nutritional mode and has arisen multiple times. For example, torriellidoid species in the three families have been distinguished from Cordycyces Fr. species by the presence of superficial perithecia on a very thin subiculum. Some species in Cordycipitaceae, such as Akanthomyces coecidiopthecithiatus (Kobayasi & Shimizu) Spatafora, Kepler & B. Shrestha, A. tuberculosis (Lebert) Spatafora, Kepler & B. Shrestha and Cordyceps thaxterii Mains also produce superficial perithecia loosely distributed along the stroma. In Clavicipitaceae, genera on scale insects mainly have superficial perithecia (Coniocepharella D. Johnson, G.H. Sung, Hywel-Jones & Spatafora, Orbicrella D. Johnson, G.H. Sung, Hywel-Jones & Spatafora) but are formed on thin subiculum on or surrounding the scale insect. However, the majority of species with superficial perithecia are found in the Ophiocordycipitaceae.

Ophiocordyceps Petch is a large genus with 223 accepted species names (Spatafora et al. 2015). It was erected originally by Petch (Petch 1924, 1931) for species of Cordycyces having asci with conspicuous apical caps and whole ascospores with distinct septation. The type of Ophiocordyceps is Ophiocordyceps blitae (Petch) Petch (Petch 1931) (Fig. 1). The majority of the species possess darkly pigmented stromata or subiculum, especially those with Hirsutella Pat. asexual morphs while some species produce brightly coloured stromata with Hymenostilbe Petch asexual morph. The stromata can be tough, wiry, fibrous or pliant. Perithecia could be superficial to completely immersed, oblique or
tropics and subtropics seem to be where the highest species diversity has been reported (Petch 1933, 1937, Kobayasi 1941, Tzean et al. 1997, Ban et al. 2015). Ophiocordyceps sinensis could be found in the high altitudes of the Himalayas up to 4000 m above sea level (Shrestha & Sung 2005, Negi et al. 2014). In Thailand alone, 35 species in Ophiocordyceps including asexual morphs have been recorded (BIOTEC Culture Collection).

During our study of the diversity of invertebrate-pathogenic fungi, Ophiocordyceps species with superficial perithecia were found in the leaf litter, on the underside of leaves, as well as on stems of plants in national parks in central and northern Thailand. We investigated the differences of the morphological characters of collected specimens and studied the phylogenetic relationships between these sexual morphs and other members of the genus, such as the asexual morph, Hirsutella, using a combined analysis of four loci (TEF, RPB1, RPB2, LSU).

MATERIALS AND METHODS

Fungal materials and isolation

The specimens for this study were collected in various national parks in central and northern Thailand composed of hill and tropical evergreen forests. The stems, undersides and upper sides of leaves and leaf litter in the forest were scanned for fungi growing on dead insects. Collected materials were placed in plastic boxes and returned to the laboratory. The materials were examined under a stereo microscope (Olympus SZ61). For the isolation of the sexual morph: the fertile head or stroma containing mature perithecia was placed over a potato dextrose agar (PDA: potato 200 g/L, dextrose 20 g/L, agar 20 g/L) plate so that the stroma was above and did not touch the agar surface. These were placed in a plastic box with moist tissue paper and examined daily for discharged ascospores. Discharged ascospores were carefully removed with a sterile needle from the agar and transferred to a new PDA plate. Pure cultures were transferred onto two kinds of media: PDA, potato sucrose agar plus (PSA: potato 200 g/L, sucrose 20 g/L, agar 20 g/L) plate so that the stroma was above and did not touch the agar surface. These were placed in a plastic box with moist tissue paper and examined daily for discharged ascospores. Discharged ascospores were carefully removed with a sterile needle from the agar and transferred to a new PDA plate. Pure cultures were transferred onto two kinds of media: PDA, potato sucrose agar plus (PSA: potato 200 g/L, sucrose 20 g/L, calcium carbonate 5 g/L, agar 20 g/L) and incubated at 20 °C in the dark for 20 d before being deposited in the BIOTEC Culture Collection (BCC) or the Thailand Biological Resource Center (TBRC). Specimens were either air-dried or dried in an electric food dryer (50–55 °C) overnight and stored in plastic boxes for deposit in the BIOTEC
Bangkok Herbarium (BBH). For identification of the insect host, specimens were viewed under the stereo microscope.

In the isolation of the asexual morph: a flame-sterilized inoculation needle was used to pick out conidia from sporulating structures, conidia were transferred to PDA plates and these were incubated in a plastic box at room temperature and examined daily using a stereomicroscope (Olympus SZ61) for germinated conidia. These were then treated in the same way as the sexual morph isolations.

**Morphological study**

Descriptions of the sexual morph are based on host material, while those of the asexual morph are based on sporulating structures on pure culture. Critical fungal structures for characterisation, such as synnema, perithecia, asci, ascospores, phialides and conidia, were mounted in a lactophenol cotton blue solution and measured using a light microscope (Olympus CX31). Twenty to fifty individual length and width measurements were taken, and the amount of variability is provided as average ± standard deviation with absolute minima and maxima in parentheses. For detailed morphological comparisons of conidia, phialides and colony coloration, cultures were grown on PDA and PSA, incubated at 20 °C in darkness in an incubator within 20 d. The colour of freshly collected specimens, and cultures incubated on PDA, PSA and for 20 d at 20 °C, was described according to Kornerup & Wanscher (1963). Nomenclatural novelties and descriptions were deposited in MycoBank. Insect hosts were examined under the stereomicroscope for identification.

**Cultivation of fungi for molecular work**

For DNA extraction purposes, starter cultures on PDA plates were prepared. After ca. 2 wk, the plates were checked for contaminants and 5–10 of 5 mm² agar blocks from pure cultures were inoculated into sterile Erlenmeyer flasks containing 50 mL Sabouraud dextrose broth (Difco) (SDB: peptic digest of animal tissue 5 g/L, pancreatic digest of casein 5 g/L, dextrose 40 g/L) and incubated for 1 wall in darkness in an incubator within 20 d. The colour of freshly collected specimens, and cultures incubated on PDA, PSA and for 20 d at 20 °C, was described according to Kornerup & Wanscher (1963). Nomenclatural novelties and descriptions were deposited in MycoBank. Insect hosts were examined under the stereomicroscope for identification.

**DNA extraction, PCR and sequencing**

Genomic DNA was extracted from lyophilized mycelium of fungal strains by a modified CTAB method as previously described (Luangsa-ard et al. 2004, 2005). Four nuclear gene regions were amplified and sequenced. Regions sequenced were from the large subunit of the ribosomal DNA (nrLSU), translation elongation factor 1-α (TEF), the largest subunit of RNA polymerase II (RPB1) and the second largest subunit of RNA polymerase II (RPB2). The nrLSU was amplified with the primer pairs LORR and LR7 (Rehner & Samuels 1994). The TEF was amplified and sequenced with the primers 983F and 2218R (Rehner & Buckley 2005). The largest subunit of RNA polymerase II (RPB1) was amplified with the primers CRPB1 and RPB1Cr (Rehner & Samuels 1994). The second largest subunit of RNA polymerase II (RPB2) was amplified and sequenced with the primers RPB2-5F2 and RPB2-7Cr (Rehner & Samuels 1994). Amplifications were done in 50 μL volumes consisting of 1x PCR buffer, 200 μM of each of the four dNTPs, 2.5 mM MgCl₂, 0.4 M Betaine, 1 U Taq DNA Polymerase, recombinant (Thermo Scientific, US) and 0.5 μM of each primer. Sequencing primers used were the same as the amplification primers. PCR conditions were set as in Sung et al. (2007).

All PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer’s instructions. Purified PCR products were sequenced by Macrogen Inc., Korea.

**Sequence alignment and phylogenetic analyses**

The sequences generated in this study were supplemented with additional sequences from known literature and GenBank blast searches for the construction of the phylogenetic tree. The list is shown in Table 1. Each generated sequence was checked for ambiguous bases and assembled in BioEdit v.7.2.5 (Hall 2004) and multiple alignment was done using MUSCLE 3.6 software (Edgar 2004) and was manually adjusted. To determine the closest matches with the O. superficialis/O. acicularis group, the DNA sequences of our isolates were compared to sequences in the GenBank database by BLAST search. The final alignment of the combined dataset was used to analyse maximum parsimony (MP), Bayesian criteria and maximum likelihood.

Parsimony analyses were implemented in PAUP 4.0b10 (Swoford 2002) and heuristic searches were performed using the following options: 100 replicates of random sequence addition, tree bisection reconnection (TBR) branch swapping with MulTrees option in effect. Gaps were treated as missing data and uninformative sequences were excluded from the data prior to analysis. Relative support of the resulting trees were determined by 1 000 bootstrap replications on informative characters only with 10 replicates of random sequence addition and TBR branch swapping algorithm. From these separate analyses all members of clades with 70 % bootstrap support were chosen and sequences were assembled for a combined analysis. Bayesian analysis was performed using MrBayes 3.0b4 (Ronquist & Huelsenbeck 2003) using the same methodologies described in Mongkolsamrit et al. (2009). Prior to this test, MrModeltest v. 2.2 (Nylander 2004) was used to determine the best nucleotide substitution model for use in Bayesian analysis. After the best nucleotide substitution model was determined, Bayesian analysis was conducted using Markov chain Monte Carlo (MCMC) using a GTR+I+G model. Four default chains were sampled every 100 generations and run for a total of 3M generations. Bayesian posterior probabilities (PP) were calculated on the posterior distribution of trees excluding the initial set of burn-in trees. Maximum likelihood analysis was performed using RAxML-HPC2 (Stamatakis 2014) under CIPRES Science Gateway (Miller et al. 2010); nodal supports were assessed with 1 000 replicates of rapid bootstrapping (Stamatakis et al. 2008).

**RESULTS**

**Phylogeny**

We obtained 62 new sequences from 17 specimens (Table 1). The combined dataset of 66 taxa and four genes consisted of 3 880 bp (TEF 988 bp, LSU 917 bp, RPB1 705 bp, RPB2 920 bp). Sequences of Orbiocrella petchii (Hywel-Jones) D. Johnson et al. in the Clavicipitaceae were used as the outgroup. The combined dataset included 3 880 characters, 2 020 of which are constant, 189 are variable and parsimony-uninformative, while 1 371 are parsimony-informative. The maximum parsimony analyses resulted in eleven equally most parsimonious trees, of which one is shown in Fig. 2 (tree length, 5 847 steps; CI, 0.416; RI, 0.727; RC, 0.303; HI, 0.584).
| Code     | Species                        | LSU     | EF1     | RPB1    | RPB2    | References         |
|----------|--------------------------------|---------|---------|---------|---------|--------------------|
| ARSEF 5473 | Hirsutella nudulosa            | KM652117| KM652000| KM652040| –       | Simmons et. al. 2015 |
| ARSEF 996  | H. satsumaensis                | KM652125| KM652008| KM652047| –       | Simmons et. al. 2015 |
| ARSEF 2227 | H. subulata                    | KM652130| KM652013| KM652051| –       | Simmons et. al. 2015 |
| OSC 110987 | Ophiocordycaps acicularis     | EF688065| EF68744  | EF68852  | –       | Sung et. al. 2007  |
| OSC 110988 | O. acicularis                  | EF68804  | EF68745  | EF68853  | –       | Sung et. al. 2007  |
| NBRC 106959 | O. appendiculata              | JN941412| AB968578| –         | AB968540| Ban et. al. 2015   |
| NBRC 106960 | O. appendiculata              | JN941413| AB968577| –         | AB968539| Ban et. al. 2015   |
| NBRC 105890 | O. arborescens                | AB968415| AB968573| –         | AB968535| Ban et. al. 2015   |
| NBRC 105891 | O. arborescens                | AB968414| AB968572| –         | AB968534| Ban et. al. 2015   |
| BCC 69015  | O. brunneinigra               | MF614653| MF614637| –         | MF614680| This study         |
| TBRC 8093 | O. brunneinigra               | MF614654| MF614638| MF614668| MF614681| This study         |
| BCC 49312  | O. brunneinientheciata        | MF614660| MF614642| –         | MF614686| This study         |
| TBRC 8100  | O. brunneinientheciata        | MF614658| MF614643| –         | MF614685| This study         |
| TBRC 8099  | O. brunneinientheciata        | MF614659| MF614644| –         | MF614684| This study         |
| NBRC 106961 | O. clavata                    | JN941414| AB968586| –         | AB968547| Ban et. al. 2015   |
| NBRC 106962 | O. clavata                    | JN941415| AB968587| –         | AB968548| Ban et. al. 2015   |
| HMAS 199612 | O. cochlidiocola              | KJ878984| KJ878965| KJ878998| –       | Quandt et. al. 2014 |
| NHU 12581  | O. communis                   | EF68831 | EF68775  | –         | EF68930 | Sung et. al. 2007  |
| NHU 12582  | O. communis                   | EF68830 | EF68771  | –         | EF68926 | Sung et. al. 2007  |
| GDGM 17327 | O. crinalis                    | KF226253| KF226256| KF226255| EU149925| Wang et. al. 2014   |
| KEW 53484  | O. entomorrhiza               | EF68809 | EF68749  | EF68857  | EF68911 | Sung et. al. 2007  |
| TBRC 8094  | O. geometricidcola            | MF614647| MF614631| MF614664| MF614678| This study         |
| TBRC 8095  | O. geometricidcola            | MF614648| MF614632| MF614663| MF614679| This study         |
| EFCC 8572  | O. gracilis                   | –       | EF68875  | EF68859  | –       | Sung et. al. 2007  |
| NBRC 100642 | O. heteropoda                 | JN941421| AB968594| –         | AB968555| Ban et. al. 2015   |
| NBRC 100644 | O. heteropoda                 | JN941423| AB968596| –         | AB968557| Ban et. al. 2015   |
| EFCC 7315  | O. konoana                    | –       | EF68873  | EF68861  | EF68916 | Sung et. al. 2007  |
| YHOS0705  | O. lanpingensis               | KC417460| KC417462| KC417464| KC456333| Chen et. al. 2013   |
| YHOS0707  | O. lanpingensis               | KC417461| KC417463| KC417465| –       | Chen et. al. 2013   |
| NBRC 105888 | O. macroacicularis            | AB968417| AB968575| –         | AB968537| Ban et. al. 2015   |
| NBRC 100685 | O. macroacicularis            | AB968416| AB968574| –         | AB968536| Ban et. al. 2015   |
| BCC 22918 | O. macroacicularis            | MF614655| MF614639| MF614669| MF614675| This study         |
| BCC 22661 | O. multiperretheciata         | MF614656| MF614640| MF614670| MF614683| This study         |
| BCC 69008  | O. multiperretheciata         | MF614657| MF614641| –         | MF614682| This study         |
| OSC 110993 | O. melolonthae               | –       | DQ522331| DQ522376| –       | Sung et. al. 2007  |
| EFCC 9247  | O. nigrella                   | EF68818 | EF68758  | EF68866  | EF68920 | Sung et. al. 2007  |
| NBRC 100944 | O. nutans                    | JN941428| AB968588| –         | AB968549| Ban et. al. 2015   |
| NBRC 101749 | O. nutans                    | JN941429| AB968589| –         | AB968550| Ban et. al. 2015   |
| TBRC 8096  | O. pauciocopertheciata        | MF614649| MF614636| MF614665| MF614672| This study         |
| TBRC 8097  | O. pauciocopertheciata        | MF614650| MF614635| MF614667| MF614671| This study         |
| TBRC 8098  | O. pauciocopertheciata        | MF614651| MF614634| MF614666| MF614674| This study         |
| TBRC 8106  | O. pauciocopertheciata        | MF614652| MF614633| –         | MF614673| This study         |
| TBRC 8101  | O. pseudoacicularis           | MF614645| MF614629| MF614662| MF614676| This study         |
| TBRC 8102  | O. pseudoacicularis           | MF614646| MF614630| MF614661| MF614677| This study         |
| GZUHNNH8  | O. ramossissimum              | –       | KJ028014| KJ028017| –       | Wen et. al. 2014   |
| GZUH2012HNN12 | O. ramossissimum          | –       | KJ028016| KJ028020| –       | Wen et. al. 2014   |
| NHU 12529  | O. rhizidea                   | EF68824 | EF68765  | EF68872  | EF68922 | Sung et. al. 2007  |
| NHU 12522  | O. rhizidea                   | EF68825 | EF68764  | EF68873  | EF68923 | Sung et. al. 2007  |
| KEW 27083  | O. robertsi                   | EF68826 | EF68766  | –        | –       | Sung et. al. 2007  |

Table 1. List of specimens and their GenBank accession numbers used in this study.
The result of MrModeltest selected the General Time Reversible (GTR) model with proportion in invariable sites (I) and gamma distribution shape parameter was 0.7730. This model was used in MrBayes v.3.0B4 and RAxML v. 8.2.10. Bayesian analyses resulted in 3 K trees; the consensus of the remaining 10 K trees resulted in identical topology (~InL 31844.1614) as the Maximum Parsimony tree.

The phylogenetic trees recovered from maximum parsimony, Bayesian inference and maximum likelihood analyses had identical topologies and similar well-supported clades by bootstrap and posterior probabilities (Fig. 2). A clade comprising Ophiocordyceps species with brightly coloured fleshy terminal fertile stromata producing Hymenostilbe asexual morph that breaks into 64 part-spores is formed sister to Ophiocordyceps species with Hirsutella asexual morphs, mainly producing whole ascospores (Fig. 2). The subtropical species O. rhizoidea (Höhn.) Petch and O. communis Hywel-Jones & Samson are sister taxa to the remaining species in the Hirsutella group. Although the inner nodes with the Hirsutella group do not have good support, seven independent terminal clades having 100 % bootstrap support and posterior probability within the Hirsutella group were formed and thus represent new species in Ophiocordyceps.

**Taxonomy**

As a result of morphological comparisons and phylogenetic analyses of 17 strains along with sequences from 49 taxa obtained from GenBank, seven new Ophiocordyceps species, and one new record for Thailand are recognized in Fig. 2. This section contains new species descriptions, specimen and culture information, with annotations.

*Ophiocordyceps brunneinigra* Tasanathai, Thanakitpipattana, Khonsanit & Luangsaard sp. nov. MycoBank MB822031. Fig. 3.

**Etymology:** Named after the dark brown to black colour of the perithecia

Stroma: Single, cylindrical and flexuous, arising from between the head and the thorax of host, brown-dark brown, 30–40 × 2 mm, unchanced in 3 % KOH. Perithecia superficial, sparse, up to 10, loosely aggregated, arising from middle part of stroma, ovoid with a distinct base, dark brown-black, 560–650 × 200–240 μm. Asci hyaline, cylindrical, 8-spores, 250–300 × 10–15 μm, with thickened apical cap, 6–7 μm in diam. Ascospores hyaline, filiform, 220–300 × 3–5 μm, remain whole after discharge, multisepitate. Conidigenous structures on the stroma in a discontinuous layer at the terminal end which arise perpendicular to the surface, brown. *Conidigenous cells* monopodial, hyaline, smooth. *Phialides* (14.5–)15–18 (–19) μm, phialide base (8–) 8.5–11.5 (–13) × 4–5 μm, phialide neck (4.5–)5–7 (–9) × 1 μm. *Conidia* hyaline, smooth, obovoid to falcate, 5–7 × 2–3 μm, embedded in a mucous sheath.

**Culture characteristics:** Colonies on PDA growing very slowly, funicolose, attaining a diameter of 12–14 mm within 20 d at 20 °C. Colonies orange grey (5A2) to olive (3E8) bearing conidigenous cells and conidia of the *Hirsutella* assexual morph. Colony reverse dark brown (6F8) after 30 d. *Conidigenous cells* monopodial, arising from hyphae laterally or terminally, hyaline, smooth, tapering gradually or abruptly into slender neck. *Phialides* (30–)35.5–43.5 (–50) μm, phialide base (16–) 23.5–31.5 (–35) × 3–3.5 (–4) μm, phialide neck (7–) 9–14 (–18) × 1 μm, rough and warty. *Conidia* hyaline, 1-celled, smooth-walled, lemon shaped to falcate, (6–) 6.5–8 (–9) × 3–4 μm, embedded in a mucous sheath. Chlamydospores not observed.

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**Table 1.** (Continued).

| Code     | Species                        | LSU | EF1   | RPB1 | RPB2 | References     |
|----------|--------------------------------|-----|-------|------|------|----------------|
| NBRC 100946 | O. rubiginosiperitheciata      |     |       |      |      |                |
| NBRC 106966 | O. rubiginosiperitheciata      |     |       |      |      |                |
| EFCC 7287  | O. sinensis                    |     |       |      |      |                |
| NHJ 12525  | O. spatetofarai                |     |       |      |      |                |
| OSC 128575 | O. spatetofarai                |     |       |      |      |                |
| BCC 86480  | O. spatetofarai                |     |       |      |      |                |
| NBRC 101752 | O. sphecocephala              |     |       |      |      |                |
| NBRC 101753 | O. sphecocephala              |     |       |      |      |                |
| OSC 111000 | O. stylphora                   |     |       |      |      |                |
| OSC 110999 | O. stylphora                   |     |       |      |      |                |
| NBRC 109688 | O. tricentri                  |     |       |      |      |                |
| OSC 111003 | O. variabilis                  |     |       |      |      |                |
| ARSEF 5365 | O. variabilis                  |     |       |      |      |                |
| GZUHNN13  | O. xuefengensis                |     |       |      |      |                |
| GZUH2012HNN13 | O. xuefengensis           |     |       |      |      |                |
| NHJ 6209  | Orbiocrella pitchii            |     |       |      |      |                |
| NHJ 5318  | O. pitchii                     |     |       |      |      |                |
Fig. 2. Phylogenetic relationships of Ophiocordyceps specimens collected in Thailand with superficial perithecia in comparison with other species of Ophiocordyceps inferred from the analyses of a combined data set for the partial genes LSU, TEF, RPB1 and RPB2 based on Maximum Parsimony, Bayesian analysis and RAxML. Numbers above lines at significant nodes present MP bootstrap values, Bayesian posterior probabilities of 100 and Maximum Likelihood bootstrap values.
Colonies on PSA growing very slowly, attaining a diameter of 10 mm within 20 d at 20 °C, producing abundant yellowish white (4A2) to greyish yellow (4C7) aerial mycelium on the surface of the medium; reverse yellowish brown (5F8) after 30 d bearing conidiogenous cells and conidia of the *Hirsutella* asexual morph.

Conidiogenous cells monophialidic or polyphialidic, arising from hyphae laterally or terminally, hyaline, smooth. *Phialides* (18–) 22–30(–33) μm, phialide base (13–) 16–22(–24) × 4 μm tapering to a thin neck, (5–) 5.5–9 (–10) × 1 μm, nonseptate or sometimes septate at the base. *Conidia* hyaline, smooth, ovoid, 6–8 × 2–4 μm surrounded by mucous sheath. Chlamydospores observed on PSA after 20 d.

*Habitat:* On Hemiptera, Cicadellidae, attached to the underside of living leaves of forest shrubs (*Ardisia sanguinolenta*) and small trees.

Fig. 3. *Ophiocordyceps brunneinigra* (BBH 38015). A. Stromata arising from host. B. Superficial perithecia. C. Perithecium. D. Asci with ascospores. E. Ascospore. F–H. On PDA. F. Colony reverse. G. Colony obverse. H. Monophialidic conidiogenous cell with conidium. I–L. On PSA. I. Colony reverse. J. Colony obverse. K. Monophialidic conidiogenous cell with conidium. L. Polyphialidic conidiogenous cell. M. Part of stroma showing conidiogenous cells. N. Conidium without mucous sheath showing lemon shaped on PDA. O. Conidia with mucous sheath on PDA. P–Q. Conidia with mucous sheath on PSA. Scale bars: A = 5 mm; B = 10 mm; C = 100 μm; D = 40 μm; E = 30 μm; F, G = 15 mm; H, N, O = 8 μm; I, J = 10 mm; K, L, P, Q = 4 μm; M = 10 μm.
Geographic distribution: Thailand, only from Khao Yai National Park.

Specimens examined: Thailand, Nakhon Ratchasima Province, Khao Yai National Park, at 14°71'N, 101°42'E, on Hemiptera, on the underside of living leaf of forest plant, 27 Nov. 2013, S. Mongkolsamrit, P. Srikitikulchai, A. Khonsanit, W. Noisripoom & D. Thanakitpipattana (holotype BBH 38015; culture ex-type TBRC 8093). Thailand, Nakhon Ratchasima Province, Khao Yai National Park, at 14°71'N. 101°42'E, on Hemiptera, on the underside of living leaf of forest plant, 25 Nov. 2013, S. Mongkolsamrit, P. Srikitikulchai, A. Khonsanit, W. Noisripoom & D. Thanakitpipattana (BBH 37875, BCC 69015).

Note: Ophiocordyceps brunneinigra has a uniquely shaped peritheciun. Although it is ovoid, it possesses a prominent, small constriction at the base connecting it to the stroma, like a stand (Fig. 3C). This feature is also present in Cordyceps atewensis.
Ophiocordyceps brunneiperitheciata Tasanathai, Thanakitpattana, Khonsanit & Luangsa-ard, sp. nov. MycoBank MB822039. Fig. 4.

Eymology: Refers to the brown colour of the perithecia on the host.

Stromata: Two to several, simple, wiry to fibrous, 4–8 mm long, 0.5–1 mm wide on the underside of leaves of forest plants, unchanged in 3 % KOH. Perithecia superficial, sparse, covering the lower middle part of stroma, loosely scattered, ordinal in arrangement, ovoid, brown-dark brown, 120–160 × 3–4 mm. Asci hyaline, cylindrical to vermiculose, (10–12) × (4–6) μm, without a mucous sheath.

Culture characteristics: Colonies on PDA growing very slowly, attaining a diameter of 6 mm within 20 d at 20 °C. Colonies white (2A1) to yellowish white (2A2), velvety to funiculose, colony reverse greyish yellow (1B4) to olive (1E4) bearing conidiogenous cells and conidia of the Hirutella asexual morph. Conidiogenous cells monophiliadic, sometimes polyphiliadic, with up to 2 lateral necks arising from hyphae laterally or terminally, hyaline, smooth, tapering gradually or abruptly into a short slender neck. Phialides 30–36(–40) μm, phialide base (16–17.5–22(–25) × 3–4(–5) μm, phialide neck and warty, (10–11)–15.5(–20) × 1 μm. Conidia hyaline, 1-celled, smooth-walled, oval to lemon shaped, 5–6 × 3–4 μm, without a mucous sheath.

Colonies on PSA growing very slowly, attaining a diameter of 6 mm within 20 d at 20 °C. Colonies white (2A1) to yellow white (2A2), funiculose; colony reverse greyish yellow (1B4) to olive (1E4) bearing conidiogenous cells and conidia of the Hirutella asexual morph. Conidiogenous cells monophiliadic, sometimes polyphiliadic, with up to 2 lateral necks arising from hyphae laterally or terminally, hyaline, smooth, tapering gradually or abruptly into a short slender neck. Phialides 30–36(–40) μm, phialide base (16–17.5–22(–25) × 3–4(–5) μm, phialide neck and warty, (10–11)–15.5(–20) × 1 μm. Conidia hyaline, 1-celled, smooth-walled, oval to lemon shaped, 5–6 × 3–4 μm, without a mucous sheath.

Habitat: On Geometridae larva (Lepidoptera) found on twig and stems of forest plants.

Geographic distribution: Thailand, known from Khao Yai National Park and Phlu Kaeng Waterfall, Chiang Rai Province.

Specimens examined: Thailand, Nakhon Ratchasima Province, Kha Yai National Park, at 14°711′N, 101°421′E, on Lepidoptera larva, 8 Sep. 2015, K. Tasanathai, N. Kobmoo, W. Noisripoom & D. Thanakitpattana, (holotype BBH 40665; culture ex-type TBRC 8095), Thailand, Chiang Rai Province, Phu Kaeng Waterfall, at 20°28′N, 100°40′E, on Lepidoptera, on twig of dicotyledonous plant, 8 Sep. 2015. K. Tasanathai, N. Kobmoo, W. Noisripoom & D. Thanakitpattana, (holotype BBH 40665; culture ex-type TBRC 8095), Thailand, Chiang Rai Province, Phu Kaeng Waterfall, at 20°28′N, 100°40′E, on Lepidoptera, on twig of dicotyledonous plant, 8 Jan. 2009. K. Tasanathai, S. Mongkolasmat, T. Chohmee, A. Khonsanit, P. Srikitikulchai & R. Promharn (BBH 31388, TBRC 8094).

Ophiocordyceps multiperitheciata Tasanathai, Thanakitpattana, Khonsanit & Luangsa-ard sp. nov. MycoBank MB822030. Fig. 6.
Fig. 5. *Ophiocordyceps geometridicola* (BBH 40665). A. Stromata arising from host. B. Superficial perithecia. C. Perithecium. D. Ascus with ascospores. E–F. Non-disarticulating ascospores. G–H. Part of stroma showing conidiogenous cells. I. Conidium. M–N. J–L. On PDA. M. Colony reverse. N. Colony obverse. J–K. Monophialidic conidiogenous cell with conidia. L. Polymphialidic conidiogenous cell with conidia. O–P, Q–T. On PSA. O. Colony reverse. P. Colony obverse. Q. Monophialidic conidiogenous cell with conidia. R–T. Conidia with mucous sheath. Scale bars: A = 5 mm; B = 1 mm; C = 60 μm; D, J, K = 10 μm; E, F = 15 μm; G, H = 5 μm; I = 2 μm; L = 5 μm; M–P = 6 mm; Q = 6 μm; R–T = 4 μm.
Fig. 6. *Ophiocordyceps multiperitheciata*. A–B. Stromata arising from host (A: BBH 38308, B BBH 18817). C. Superficial perithecia. D. Perithecium. E. Asci. F. Ascospores. G–L. On PDA. G. Colony obverse. H. Colony reverse. I. Monophialidic conidiogenous cell with conidia. J. Polyphialidic conidiogenous cell with conidia. K–L. Conidia with mucous sheath. M–R. On PSA. M. Colony obverse. N. Colony reverse. O–P. Monophialidic conidiogenous cell. Q. Conidium without mucous sheath showing lemon shaped. R. Conidia with mucous sheath. Scale bars: A, B = 15 mm; C = 2 000 μm; D = 150 μm; E = 15 μm; F = 40 μm; G, H, M, N = 10 mm; I, J, O, P, R = 10 μm; K, L = 6 μm; Q = 4 μm.
**Eymology:** Refers to the multiple perithecia on the stroma.

**Stromata:** Several, cylindrical, branched dendritic, 75–110 mm long, 1–1.5 mm wide, dark brown (7F8) to black, sometimes with a sterile tip, on larvae of Lepidoptera in the leaf litter, unchanged in 3 % KOH. *Penthecia* superficial, gregarious, distributing unequally on upper three-fourths of the stroma, ordinal in arrangement, narrowly ovoid, brown (6D6) to dark brown (7F8), 990–1200 × 350–450 μm. Asci hyaline, cylindrical, 8-spores, 400–600 × 6–7.5 μm. Ascospores hyaline, filiform, 470–660 × 1.5–2.5 μm, remaining whole after discharge, multisepitate.

**Culture characteristics:** Colonies on PDA growing slowly, flat and velvety in the middle, attaining a diameter of 16–22 mm within 20 d at 20 °C, light brown (5D6) in the centre and white (5A1) funiculose growth at the periphery of the colony. Colony reverse olive brown (4F8) in the center and olive brown (4D7) at the edges. Aerial mycelia dense on the edges of the colony. *Conidiogenous cells* monophialidic or polyphialidic, arising from hyphae laterally or terminally, hyaline, cylindrical to lanceolate, tapering gradually or abruptly into a long slender neck. *Phialides* (35–43–57–70) μm, phialide base (22–25–34–40) × (3–4–5–5) μm, phialide neck simple to branched, (12–16–27–37) × 1–2 μm, rough to warty, nonseptate or sometimes septate below the phialide base. *Conidia* hyaline, 1-celled, smooth walled, oval to lemon shaped, (8–) 9–11–(14) × (5–)6–7–(8–) μm, embedded in a mucous sheath.

Colonies on PSA growing slowly, velvety and flat, attaining a diameter of 20 mm within 2 d at 20 °C, greyish yellow (4C5) to (4C3) with funiculose growth at the edges of the colony. Colony reverse olive brown (4F8) in the center and olive brown (4D7) at the colony edges. *Conidiogenous cells* monophialidic or polyphialidic, arising from hyphae laterally or terminally, hyaline, cylindrical to lanceolate, tapering gradually or abruptly into a long slender neck. *Phialides* (41–)50–61.5–(65) μm, phialide base (25–)27.5–35(–40) × (4–)3.5–4.5(–5) μm, phialide neck rough to warty, (10–)19–26(–31) × 1–1.5 μm. *Conidia* hyaline, 1-celled, smooth-walled, oval to lemon shaped, (7–)9–10.5(–12) × 4–5 μm, embedded in a mucous sheath, (7–)9–10.5(–12) × (5–)5.5–7.5(–9) μm.

**Habitat:** On Lepidoptera larva in the leaf litter of forest floor.

**Geographic distribution:** Thailand, only known from Doi Inthanon National Park.

**Specimens examined:** Thailand, Chiang Mai Province, Doi Inthanon National Park, at 18°49′N, 98°36′E, on Lepidoptera larva, in the leaf litter, 29 Oct. 2013, K. Tasanathai, P. Sritikitkulchai, A. Khonsanit, W. Noisripoom, D. Thanakitpi–N, 98°36′E, on Lepidoptera larva, in the leaf litter, 7 Sep. 2006, K. Tasanathai, S. Mongkolansom, P. Sritikitkulchai, B. Thongruch, R. Rikdkaew & C. Chuursechanornchai (BBH 18817, BCC 22861).

**Note:** *Ophiocordyceps multiperitheciata* has only been found in Doi Inthanon at 2 000–2 500 m above sea level. It resembles *O. macroacicularis* S. Ban, T. Sakane & Nakagiri in gross morphology and host but differs in having bigger perithecia and asci (perithecia, 410–760 × 260–420 μm; asci, 235–310 μm). *O. multiperitheciata* was found in the leaf litter while *O. macroacicularis* was found on lepidopteran larvae that inhabit the wood or roots of *Fullopia japonica* Houtt. (Ban et al. 2015).

**Ophiocordyceps pauciovertheciatea** Tasanathai, Thanakitpipattana, Khonsanit & Luangs-ard, sp. nov. MycoBank MB822034. Fig. 7.

**Eymology:** Named after the few ovoid shapes of the perithecia along the stroma.

**Stroma:** Solitary, simple, wavy to piliferous or fibrous, arising from any point on the insect, dark grey (1F1)–dark brown (7F7), 14 mm long, 1 mm wide on the underside of leaves of forest plants, unchanged in 3 % KOH. *Penthecia* superficial, sparse, up to 20, covering middle part to apex of stroma, loosely scattered, vertically placed, ovoid, brown, 300–400 × 210–300 μm. Asci hyaline, cylindrical, 8-spores, 140–220 × 5–7.5 μm. Ascospores 135–215 × 2–3 μm, filiform, remaining whole after discharge, no septation.

**Asexual morph, terminal, pale grey (1B1)-dark grey (1F1), 4 mm long.** *Conidiogenous cells* monophialidic, rarely polyphialidic, hyaline, smooth. *Phialides* (7–9–10–11) μm, phialide base (4–)5–6(–7) × (3–)3.5–4(–5) μm, phialide neck (2–)3.5–5 × 0.5–1 μm. *Conidia* hyaline, smooth-walled, fusiform, 5–7.5(–9) × 2–3 μm, with a mucous sheath.

**Culture characteristics:** Colonies on PDA growing slowly, attaining a diameter of 10–12 mm within 20 d at 20 °C. Colonies brownish grey (5C2) to yellowish brown (5E8), velvety with depression on the colony, bearing conidiogenous cells and conidia of the *Hirsutella* asexual morph; colony reverse yellowish brown (5D6–5F8). *Conidiogenous cells* monophialidic or polyphialidic arising from hyphae laterally or terminally, hyaline, smooth-walled. *Phialides* (10–11–15–16) μm, basal portion globose (5–)6–9(–10) × 4.5–5.5(–6) μm, phialide neck (2–)4.6–5.5(–8) × 0.5–1 μm, rough to warty. *Conidia* hyaline, 1-celled, smooth-walled, oval, 5–6 × 3–4 μm, without a mucous sheath. Formation of crystals observed.

Colonies on PSA growing slowly, attaining a diameter of 12–15 mm within 20 d at 20 °C. Colonies greyish yellow (4C5) to olive brown (4E8), velvety with depression in the colony formation, bearing conidiogenous cells and conidia of the *Hirsutella* asexual morph; colony reverse dark yellow (4C8) to olive brown (4E8). *Conidiogenous cells* monophialidic or polyphialidic arising from hyphae laterally or terminally, hyaline, smooth-walled. *Phialides* (10–)11–15(–17) μm, basal portion globose (6–)9(–10) × (3–)4–5(–6) μm, phialide necks (2–)4.5–6.5(–9) × 0.5–1 μm, rough and tiny warty. *Conidia* hyaline, 1-celled, smooth-walled, oval, 5–6 × 3 μm, without a mucous sheath. Formation of crystals observed.
Sansatchanon & R. Somnuk (BBH 32730, TBRC 8096), Nakhon Ratchasima Province, Khao Yai National Park, at 15°20′N, 99°32′E, on Lepidoptera larva, 7 Oct. 2009, K. Tasanathai, P. Srikitikulchai, S. Mongkolsamrit, T. Chohme, R. Ridkaew, P. Puyngain & M. Sudhaibham (BBH 27112, TBRC 8097), Nakhon Ratchasima Province, Khao Yai National Park, at 14°71′11″N, 101°42′1″E, on Lepidoptera larva, 31 Aug. 2010, K. Tasanathai, P. Srikitikulchai, S. Mongkolsamrit, A. Khonsanit, R. Somnuk & K. Sansatchanon (BBH 31409, TBRC 8098).

Note: Ophiocordyceps pauciovoperitheciata shows similarity to O. brunneinigra, O. geometridicola and O. brumineiperitheciata in the production of sparsely distributed perithecia along the stroma.

**Etymology**

Ophiocordyceps *pseudoacicularis* Tsanathanai, Thanakitipattana, Khonsanit & Luangsa-ard sp. nov. MycoBank MB822032. Fig. 8.

**Eymology:** Named after the similarity to *O. acicularis*.

**Stromata**

Solitary to several, cylindrical, 45–70 mm long, 0.5 mm wide, brown to dark brown, on larvae of Lepidoptera in the leaf litter, unchanged in 3 % KOH. Perithecia superficial, densely packed covering lower part and loosely aggregated at the apex of stroma, ordinal in arrangement, ovoid, brown-dark brown, (340–) 343.5–386(–420) × (240–)257–292(–310) μm. Ascii hyaline, cylindrical, 8-spores, (112.5–)129–187(–225) × 5–7.5 μm. Ascospores hyaline, filiform, 152.5–205 × 2 μm, remain whole after discharge, with septation.

Asexual morph terminal, pale grey (1B1)-dark grey (1F1), 4–5 mm long. *Conidiogenous cells* monophialidic or polyphialidic arising from hyphae laterally or terminally, hyaline, smooth. Phialides 12–15(–16) μm, phialide base (6–)6.5–9(–10) × 4–5 μm, phialide neck (2–)4–7(–9) × 0.5–1 μm. Conidia hyaline, smooth, fusiform, 5–6(–7) × 2 μm, surrounded by mucous sheath.

**Culture characteristics:** Colonies on PDA slow growing, attaining a diameter of 20 mm within 20 d at 20 °C. Colonies pale orange (5A3) to light brown (5E7), velvety, colony reverse pale orange (5A3) to light brown (5E7) after 20 d bearing conidiogenous cells and conidia of the *Hirsutella* asexual morph. *Conidiogenous cells* monophialidic or polyphialidic arising from hyphae laterally or terminally, hyaline, smooth. Phialides 22–30 μm, phialide base 12–20 × 3–4 μm, phialide neck, wavy, 8–12 × 1 μm. Conidia hyaline, smooth, obovoid to citriform, 5–7 × 3–4 μm surrounded by mucous sheath. Chlamydospores observed on PDA after 20 d.

Colonies on PSA slow growing, attaining a diameter of 12–15 mm within 20 d at 20 °C. Colonies white (5A1) to pale orange (5A3), colony reverse orange white (5A1) to pale orange (5A3) after 20 d bearing conidiogenous cells and conidia of the *Hirsutella* asexual morph. *Conidiogenous cells* monophialidic or polyphialidic arising from hyphae laterally or terminally, hyaline, rough to warty. Phialides (23–)25–34(–40) μm, phialide base (11–)14–21(–25) × 3–3.5 (–4) μm, phialide neck (6–)7.5–15(–20) × 1 μm. Conidia hyaline, oval to lemon shaped, 6–7 × 3–4 μm.

**Habitat:** On Lepidoptera larva, in the leaf litter of forest.

**Geographic distribution:** Thailand, only known from Khao Yai National Park.

Specimens examined: Thailand, Nakhon Ratchasima Province, Khao Yai National Park, at 14°71′11″N, 101°42′1″E, on Lepidoptera larva, on the leaf litter, 9 Jul. 2012, K. Tasanathai, S. Mongkolsamrit, A. Khonsanit, W. Noisripoom, P. Srikitikulchai, K. Sansatchanon & R. Somnuk (holotype BBH 32211; culture ex-type TBRC 8102). Thailand, Nakhon Ratchasima Province, Khao Yai National Park, at 14°71′11″N, 101°42′1″E, on Lepidoptera larva, on the leaf litter, 3 Aug. 2011, K. Tasanathai, P. Srikitikulchai, S. Mongkolsamrit, A. Khonsanit, K. Sansatchanon & W. Noisripoom (BBH 30689, TBRC 8101).

Note: Ophiocordyceps *pseudoacicularis* shows similarity to *O. acicularis* (Ravenel) Petch in the length of the stroma and the size of the perithecium (Petch 1933). It differs from *O. acicularis* in the presence of slightly smaller ascospores. This species belongs to the *Hirsutella nodulosa* group while *O. acicularis* belong to the *Hirsutella sinensis* group (Simmons et al. 2015). It shares similarities with other species in the same clade by the conidiogenous cells having warty surface and an undulate phialide neck.

**Ophiocordyceps spataforae** Tsanathanai, Thanakitipattana, Khonsanit & Luangsa-ard sp. nov. MycoBank MB822068. Fig. 9.

**Etymology:** In honour of Prof. Joseph W. Spatafora, for his contribution to our knowledge of arthropod-pathogenic fungi.

**Habitat:** Found on fulgorid plant hoppers (*Fulgoridae, Hemiptera*) and on Coleoptera.

**Geographical distribution:** Thailand and USA.

The material examined (NHU 12525, BBH 9271) has immature superficial, brown to dark brown perithecia. Stromata up to two, to 45 mm long and 0.5 mm wide, cylindrical, cream to pale brown, emerging from the terminal end of the abdomen and between the thorax and abdomen of a fulgorid (*Fulgoridae, Hemiptera*). Perithecia superficial, ovoid, dark brown. Terminal part of the stroma bearing discontinuous layer of *Hirsutella* phialides and conidia which are perpendicular to the surface of the stroma. Phialides 9–33 μm, phialide base 5–22 × 4–6 μm, phialide neck 4–11 × 0.5 μm. Conidia hyaline, fusiform to ellipsoidal, herbarium material does not have a mucous sheath due to drying, 5–7 × 1.5–3 μm.

Specimen examined: Thailand, Chanthaburi Province, Khao Soi Dao Wildlife Sanctuary, at 13°10′2″N, 102°19′2″E, on fulgorid planthopper, 26 Jul. 2017, W. Himaman, P. Jangsantear & B. Sakolrak (holotype BBH 43466; culture ex-type BCC 86480). Thailand, Chanthaburi Province, Khao Soi Dao Wildlife Sanctuary, on fulgorid planthopper, 20 Jul. 2003, R. Nasit, N.L. Hywel-Jones & JW. Spatafora (BBH 9271).

**Notes:** Ophiocordyceps *spataforae* is closely related to *O. brunneinigra* and *O. crinalis* (Ellis ex Lloyd) G.H. Sung, J.M. Sung, J.M. Sun, N.L. Hywel-Jones & JW. Spatafora (BBH 9271).

**DISCUSSION**

This study advanced our understanding of the genotypic variation among closely related species of *Ophiocordyceps* with...
**Fig. 8.** Ophiocordyceps pseudoacicularis (BBH 32211). A. Stroma arising from host. B. Superficial perithecia. C. Perithecium. D. Asci with ascospores. E–F. Part of stroma showing conidiogenous cells. G–H. Ascospore. I–N. On PDA. I. Colony reverse. J. Colony obverse. K. Monophialidic conidiogenous cell with conidia. L. Polyphialidic conidiogenous cell with conidium. M. Conidium without mucous sheath. N. Conidium with mucous sheath. O–R. On PSA. O. Colony reverse. P. Colony obverse. Q. Monophialidic conidiogenous cell. R. Polyphialidic conidiogenous cell. Scale bars: A, I, J, O, P = 10 mm; B = 2 mm; C = 50 μm; D = 25 μm; E, L–N, Q, R = 5 μm; F = 7 μm; G, H = 20 μm; K = 6 μm.
**Fig. 9.** *Ophiocordyceps spatulata*. **A, B.** Stromata arising from host: **A.** Immature stroma with only *Hirsutella* asexual state (BBH 43464). **B.** Stromata containing immature perithecia (BBH 43466). **C, D.** Part of stroma showing developing superficial perithecia. **C.** Fresh collection. **D.** Herbarium material. **E–G.** Conidiogenous cells. **H.** Conidium. Scale bars: **A, B** = 10 mm; **C, D** = 0.5 mm; **E–G** = 5 μm; **H** = 2 μm.
limited morphological differentiation. The degree of morphological variation among various specimens of Ophiocordyceps with superficial perithecia is so low that a continuum seems to exist to accommodate all in a single species. A broader species concept for this group of fungi seems the logical step, an approach advocated by Samson et al. (1982) in the past, but the application of molecular phylogenetics supports that these are distinct evolutionary species, as also shown in the works of Evans et al. (2011), Luangsara-ard et al. (2011), Kobmoo et al. (2012), Araújo et al. (2015) for the O. unilateralis species complex.

To improve species delimitation and resolution in the genus Ophiocordyceps we generated LSU, TEF, RPB1 and RPB2 sequences and added them to published sequences from various taxonomic studies performed for Ophiocordyceps and Hirselfettia (Quandt et al. 2014, Ban et al. 2015, Simmons et al. 2015, Spatafora et al. 2015) (Fig. 2, Table 1). The frequency with which cryptic species are unmasked with data from multiple genetic loci (and often subsequently confirmed with morphological and/or ecological data) suggests that multi-gene phylogenetic loci (and often subsequently con

specimens have ascospores that remain intact after discharge whilst in O. cochlidiicola the ascospores readily break into fragments (Kobayasi & Shimizu 1982). They both share the same ecology in that they occur on Lepidoptera larvae in the ground. Two or more morphologically similar and closely related taxa are disjunct in distribution when they are widely separated geographically (Chaverri et al. 2008). This disjunction has been shown as a common event among invertebrate-pathogens, especially in Hypocrella and Moelleriella where and Old World/New World disjunction is sometimes observed (Petch 1921, Evans 1982, Evans & Samson 1984, Evans & Hywel-Jones 1990, Chaverri, Bischoff, Evans et al. 2005, Chaverri, Bischoff, Hodge et al. 2005, 2008) or in Nigelia Luangsara-ard, Tasan. & Thanakiltip, where a whole ascospore/part-spore disjunction has been reported (Tasanathai et al. 2016).

Ophiocordyceps pseudaacicularis shows similarity with O. acicularis in the length of the cylindrical stromata, the ovoid shape of the perithecia, and the production of whole ascospores. It differs from O. acicularis in the length of the asc and ascospores, and the host. O. pseudaacicularis has shorter asc and ascospores and infect Lepidoptera larvae instead of Coleoptera larvae. O. geometridicola shares similarity with the H. nodulosa group in producing undulate pialidces.

One of the most common encountered problems related to Ophiocordyceps taxonomy is the availability of sequence material and cultures from type strains. Out of the 223 species accepted in the genus, only 127 have sequences (Sung et al. 2007, Quandt et al. 2014, Ban et al. 2015, Simmons et al. 2015, Spatafora et al. 2015) and cultures available for comparative studies. The lack of standard media to study growth conditions and macro morphology of the asexual morphs needs to be discussed among the various research groups, and standard criteria established, focusing on invertebrate-pathogens worldwide to facilitate exact comparisons among collections of species deemed to have a cosmopolitan distribution.

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