Studies in Mycology

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. brunneiperitheciata*, *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, PRB2) strongly support these new species of *Ophiocordyceps* in the *Ophiocordycipitaceae*. They differ from species previously described species *Ophiocordyceps acicularis*, *O. atewensis*, *O. cochlidiicola*, and *O. crinalis*, 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. brunneiperitheciata* 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.

INTRODUCTION

The occurrence of species complexes – morphologically similar but genetically different cryptic closely related species – can underestimate the 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, Kauserud *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 perithecium morphology. Ascospores may remain intact (whole) or disarticulate into part-spores 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 entomogenous nutritional mode and has arisen multiple times. For example, torrubielloid species in the three families have been distinguished from *Cordyceps* Fr. species by the presence of superficial perithecia on a very thin subiculum. Some species in *Cordycipitaceae*, such as *Akanthomyces coccidioperitheciatus* (Kobayasi & Shimizu) Spatafora, Kepler & B. Shrestha, *A. tuberculatus* (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 (*Conoideocrella* D. Johnson, G.H. Sung, Hywel-Jones & Spatafora, *Orbiocrella* 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 *Cordyceps* having asci with conspicuous apical caps and whole ascospores with distinct septation. The type of *Ophiocordyceps* is *Ophiocordyceps blattae* (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
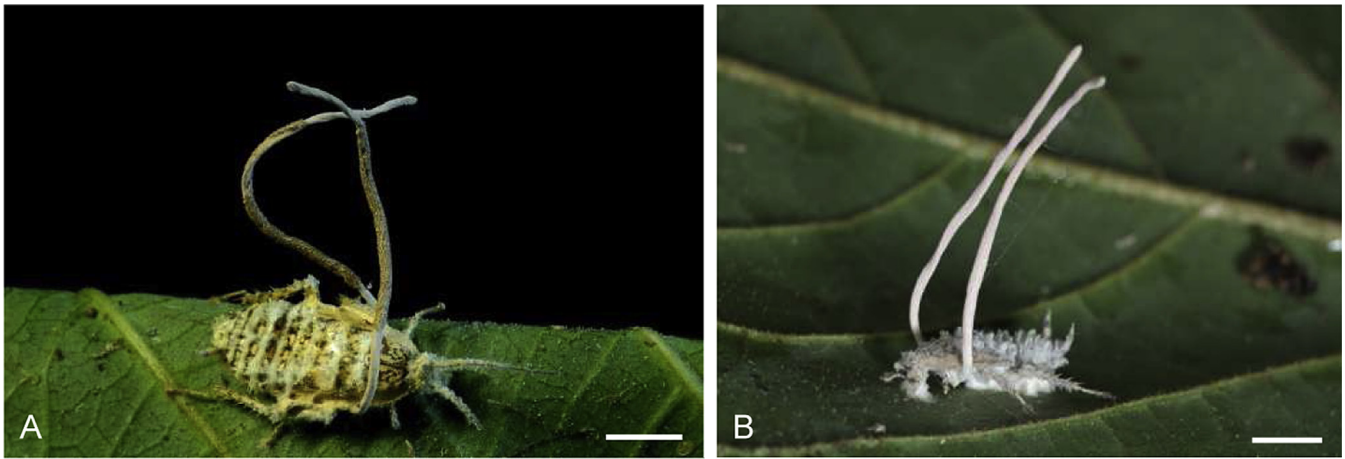
Fig. 1. *Ophiocordyceps blattae,* the type species of *Ophiocordyceps,* on forest cockroach (Blattodea, Blattellidae) from Nam Nao National Park, Phetchabun, Thailand. **A.** Sexual morph. **B.** Asexual morph. Scale bars: A, B = 2.5 mm.

ordinal in arrangement. Ascospores usually cylindrical, multi-septate, either disarticulating into part-spores or remain whole after discharge. Asexual morphs associated with *Ophiocordyceps* include *Hirsutella, Hymenostilbe, Paraisaria* Samson & B.L. Brady and *Syngliocladium* Petch (Sung *et al.* 2007). Quandt *et al.* (2014) proposed to suppress *Hymenostilbe, Paraisaria* and *Syngliocladium* because these genera are younger and contain fewer taxa than *Hirsutella* or *Ophiocordyceps.* They also proposed to suppress *Hirsutella* in favour of *Ophiocordyceps* due to the unavailability of *H. entomophila,* the type of *Hirsutella,* the observation of the *Hirsutella*-like morphology in *Harposporium,* and *Polycephalomyces,* as well as in the *Clavicipitaceae* (*Helicocollum* Luangsa-ard, Mongkols., Noisrip. & Thanakitp., *Aschersonia* Mont.), the differential in number of combinations that were made – 178 for *Ophiocordyceps vs.* 77 for *Hirsutella,* and the desire to maintain "*Cordyceps*" in the name *O. sinensis,* which is an important fungus in traditional Chinese medicine.

Species in *Ophiocordyceps* are found on a wide range of insect hosts and almost exclusively they are entomopathogenic. Insect orders they attack include Coleoptera, Diptera, Hemiptera, Hymenoptera, Isoptera, Lepidoptera, Neuroptera, and Odonata. Collectively, they can infect various stages of the insect life cycle, from larva, pupa to nymph stages, as well as adults (Kobayasi 1941, Mains 1958, Shrestha *et al.* 2016). Examples of *Ophiocordyceps* species that infect adult insects are *O. irangiensis* (Moureau) G.H. Sung *et al.* and *O. halabalaensis* Luangsa-ard *et al.* on ants, *O. dipterigena* (Berk. & Broome) G.H. Sung *et al.* on flies, *O. nutans* (Pat.) G.H. Sung *et al.* on stinkbugs, *O. odonatae* (Kobayasi) G.H. Sung *et al.* on dragonflies, and *O. sphecocephala* (Klotzsch ex Berk.) G.H. Sung *et al.* on wasps. Those found mainly on larvae and pupae are *O. sinensis* (Berk.) G.H. Sung *et al.*, *O. acicularis* (Ravenel) Petch on Lepidoptera larva, *O. ravenelii* (Berk. & M.A. Curtis) G.H. Sung *et al.*, and *O. melolonthae* (Tul. & C. Tul.) G.H. Sung *et al.* on Coleoptera larva. Stromata of these *Ophiocordyceps* species occur in exposed environments such as in the leaf litter (*O. acicularis, O. nutans, O. irangiensis*, *O. sphecocephala*), on the underside of leaves (*O. unilateralis* (Tul. & C. Tul.) Petch, *O. pseudolloydii* (H.C. Evans & Samson) G.H. Sung *et al.*), on stems and or found buried under the ground (*O. acicularis* (Ravenel) Petch, *O. barnesii* (Thwaites) G.H. Sung *et al. O. communis* Hywel-Jones & Samson) and in wood (*O. brunneipunctata* (Hywel-Jones) G.H. Sung *et al.*).

Ophiocordyceps has a panglobal distribution, although the 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 4 000 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, 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 characterization, such as synnemata, 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 mm^2 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 1–2 wk at 20 °C, without shaking. Mycelium was then harvested by filtration and washed several times with sterile distilled water. Filtered mycelium was lyophilized to be used for DNA extraction.

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 LROR 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 1× PCR buffer, 200 µM of each of the four dNTPs, 2.5 mM MgCl$_2$, 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 (Swofford 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 3 580 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 3 580 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).

Table 1. List of specimens and their GenBank accession numbers used in this study.

Code	Species	GenBank accession no.				References
		LSU	EF1	RPB1	RPB2	
ARSEF 5473	*Hirsutella nodulosa*	KM652117	KM652000	KM652040	–	Simmons *et. al.* 2015
ARSEF 996	*H. satumaensis*	KM652125	KM652008	KM652047	–	Simmons *et. al.* 2015
ARSEF 2227	*H. subulata*	KM652130	KM652013	KM652051	–	Simmons *et. al.* 2015
OSC 110987	*Ophiocordyceps acicularis*	EF468805	EF468744	EF468852	–	Sung *et. al.* 2007
OSC 110988	*O. acicularis*	EF468804	EF468745	EF468853	–	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. brunneiperitheciata*	MF614660	MF614642	–	MF614686	This study
TBRC 8100	*O. brunneiperitheciata*	MF614658	MF614643	–	MF614685	This study
TBRC 8099	*O. brunneiperitheciata*	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. cochlidiicola*	KJ878884	KJ878965	KJ878998	–	Quandt *et. al.* 2014
NHJ 12581	*O. communis*	EF468831	EF468775	–	EF468930	Sung *et. al.* 2007
NHJ 12582	*O. communis*	EF468830	EF468771	–	EF468926	Sung *et. al.* 2007
GDGM 17327	*O. crinalis*	KF226253	KF226256	KF226255	EU149925	Wang *et. al.* 2014
KEW 53484	*O. entomorrhiza*	EF468809	EF468749	EF468857	EF468911	Sung *et. al.* 2007
TBRC 8094	*O. geometridicola*	MF614647	MF614631	MF614664	MF614678	This study
TBRC 8095	*O. geometridicola*	MF614648	MF614632	MF614663	MF614679	This study
EFCC 8572	*O. gracilis*	–	EF468751	EF468859	–	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. konnoana*	–	EF468753	EF468861	EF468916	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 22861	*O. multiperitheciata*	MF614656	MF614640	MF614670	MF614683	This study
BCC 69008	*O. multiperitheciata*	MF614657	MF614641	–	MF614682	This study
OSC 110993	*O. melolonthae*	–	DQ522331	DQ522376	–	Sung *et. al.* 2007
EFCC 9247	*O. nigrella*	EF468818	EF468758	EF468866	EF468920	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. pauciovoperitheciata*	MF614649	MF614636	MF614665	MF614672	This study
TBRC 8097	*O. pauciovoperitheciata*	MF614650	MF614635	MF614667	MF614671	This study
TBRC 8098	*O. pauciovoperitheciata*	MF614651	MF614634	MF614666	MF614674	This study
TBRC 8106	*O. pauciovoperitheciata*	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
GZUHHN8	*O. ramosissimum*	–	KJ028014	KJ028017	–	Wen *et. al.* 2014
GZUH2012HN12	*O. ramosissimum*	–	KJ028016	KJ028020	–	Wen *et .al.* 2014
NHJ 12529	*O. rhizoidea*	EF468824	EF468765	EF468872	EF468922	Sung *et. al.* 2007
NHJ 12522	*O. rhizoidea*	EF468825	EF468764	EF468873	EF468923	Sung *et. al.* 2007
KEW 27083	*O. robertsii*	EF468826	EF468766	–	–	Sung *et. al.* 2007

Table 1. (Continued).

Code	Species	GenBank accession no.				References
		LSU	EF1	RPB1	RPB2	
NBRC 100946	*O. rubiginosiperitheciata*	JN941436	AB968581	–	AB968543	Ban *et. al.* 2015
NBRC 106966	*O. rubiginosiperitheciata*	JN941437	AB968582	–	AB968544	Ban *et. al.* 2015
EFCC 7287	*O. sinensis*	EF468827	EF468767	EF468874	EF468924	Sung *et. al.* 2007
NHJ 12525	*O. spataforae*	EF469078	EF469063	EF469092	EF469111	Sung *et. al.* 2007
OSC 128575	*O. spataforae*	EF469079	EF469064	EF469093	EF469110	Sung *et. al.* 2007
BCC 86480	*O. spataforae*	MG831746	MG831747	MG831748	MG831749	This study
NBRC 101752	*O. sphecocephala*	JN941445	AB968591	–	AB968552	Ban *et. al.* 2015
NBRC 101753	*O. sphecocephala*	JN941446	AB968592	–	AB968553	Ban *et. al.* 2015
OSC 111000	*O. stylophora*	DQ518766	DQ522337	DQ522382	DQ522433	Sung *et. al.* 2007
OSC 110999	*O. stylophora*	EF468837	EF468777	EF468882	EF468931	Sung *et. al.* 2007
NBRC 106968	*O. tricentri*	AB968423	AB968593	–	AB968554	Ban *et. al.* 2015
OSC 111003	*O. variabilis*	EF468839	EF468779	EF468885	EF468933	Sung *et. al.* 2007
ARSEF 5365	*O. variabilis*	DQ518769	DQ522340	DQ522386	DQ522437	Sung *et. al.* 2007
GZUHHN13	*O. xuefengensis*	–	KC631790	KC631795	–	Wen *et. al.* 2013
GZUH2012HN13	*O. xuefengensis*	–	KC631792	KC631797	–	Wen *et. al.* 2013
NHJ 6209	*Orbiocrella petchii*	EU369039	EU369023	EU369061	EU369081	Johnson *et. al.* 2009
NHJ 5318	*O. petchii*	EU369040	EU369021	EU369062	EU369080	Johnson *et. al.* 2009

The result of MrModeltest selected the General Time Reversible (GTR) model with proportion in invariable sites (I) and gamma distribution (G) (GTR+I+G; Lanave *et al.* 1984) as the best-fit model by AIC in MrModeltest 2.2. The parameters included base frequencies—A = 2.235, C = 3.122, G = 2.740, T = 1.904—and the rate matrix for the substitution model: [AC] = 0.8818, [A–G] = 2.9135, [A–T] = 0.7804, [C–G] = 0.8889, [C–T] = 5.8768, [G–T] = 1.000. For the among-site variation the proportion of invariable sites (I) was 0.4237, and the 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 "burn-in" trees; the consensus of the remaining 10 K trees resulted in identical topology (−lnL 31844.8164) 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 subterranean 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 & Luangsa-ard **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, unchanged 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, 250–320 × 10–15 µm, with thickened apical cap, 6–7 µm in diam. *Ascospores* hyaline, filiform, 220–300 × 3–5 µm, remain whole after discharge, multiseptate. Conidiogenous structures on the stroma in a discontinuous layer at the terminal end which arise perpendicular to the surface, brown. *Conidiogenous cells* monophialidic, 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, funiculose, attaining a diameter of 12–14 mm within 20 d at 20 °C. Colonies orange grey (5A2) to olive (3E8) bearing conidiogenous cells and conidia of the *Hirsutella* asexual morph. Colony reverse dark brown (6F8) after 30 d. *Conidiogenous cells* monophialidic, 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.
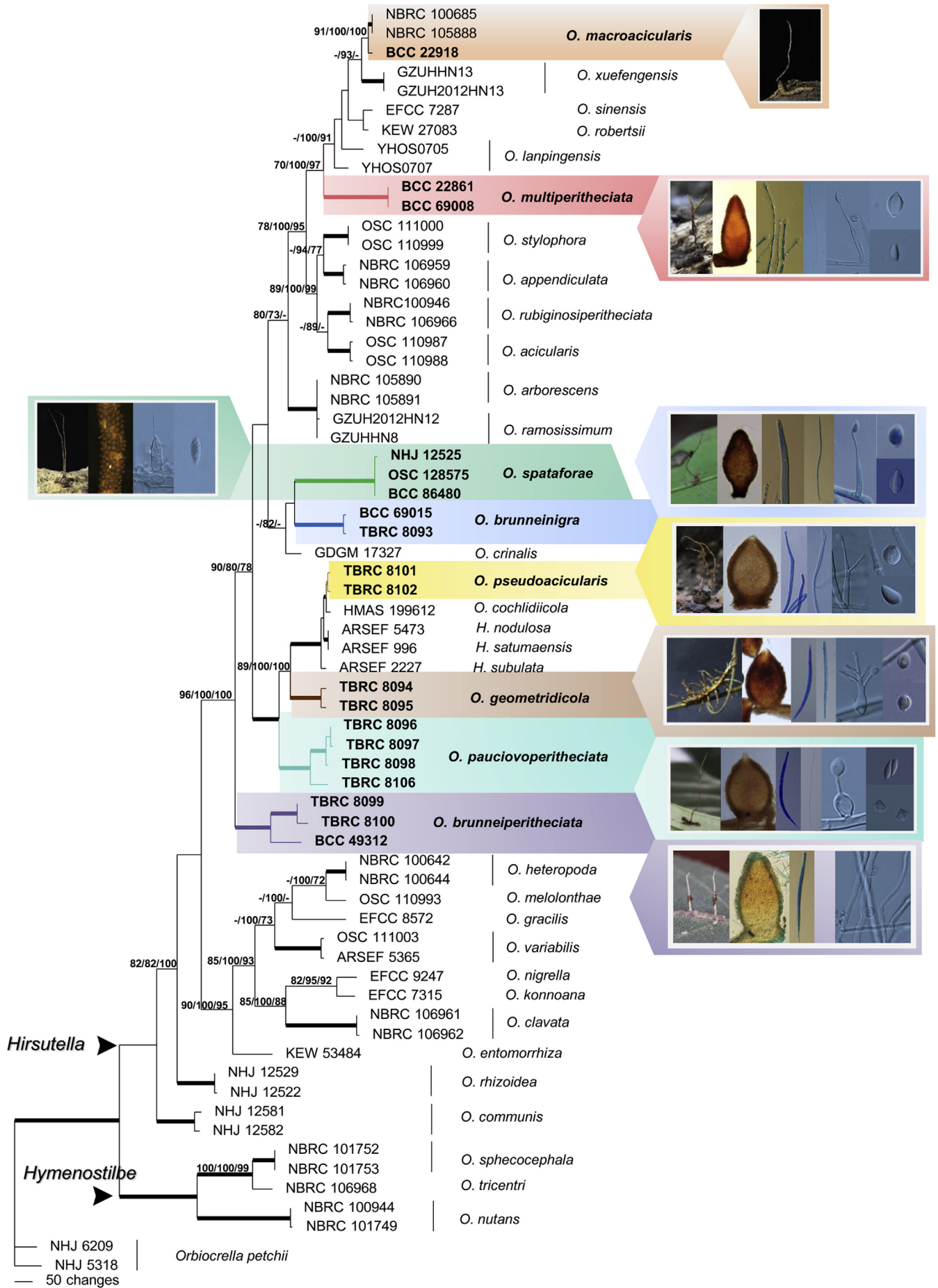
Fig. 2. Phylogenetic relationships of *Ophiocordyceps* specimens collected in Thailand with superficial perithecia in comparison with other species of *Ophiocordyceps* inferred from the analyses of a combined data set for the partial genes LSU, TEF, RPB1 and RPB2 based on Maximum Parsimony, Bayesian analysis and RAxML. Numbers above lines at significant nodes present MP bootstrap values, Bayesian posterior probabilities of 100 and Maximum Likelihood bootstrap values.
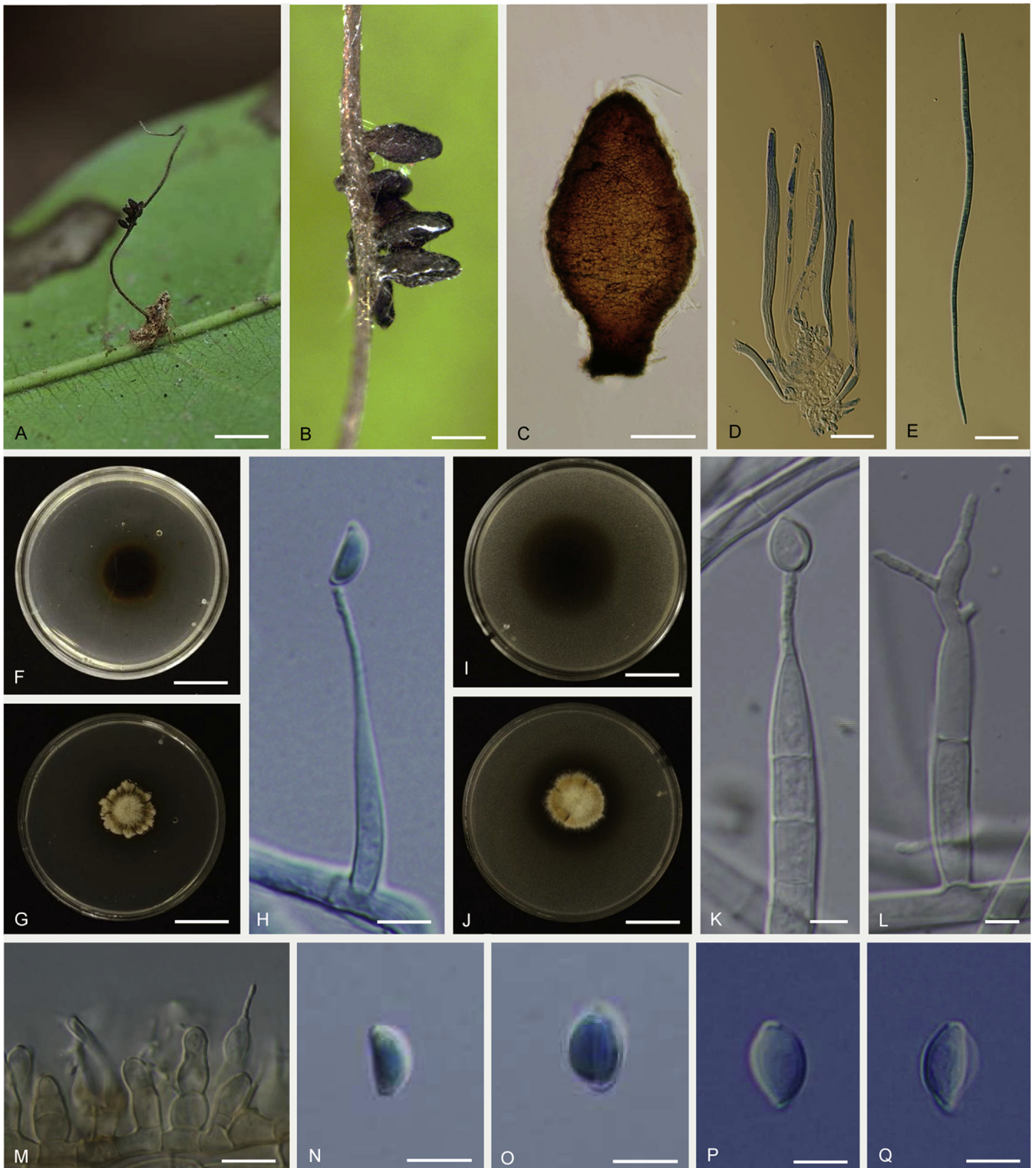
Fig. 3. *Ophiocordyceps brunneinigra* (BBH 38015). **A.** Stromata arising from host. **B.** Superficial perithecia. **C.** Perithecium. **D.** Asci with ascospores. **E.** Ascospore. **F–H.** On PDA, **F.** Colony reverse. **G.** Colony obverse. **H.** Monophialidic conidiogenous cell with conidium. **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.

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, Cicadallidae, attached to the underside of living leaves of forest shrubs (*Ardisia sanguinolenta*) and small trees.
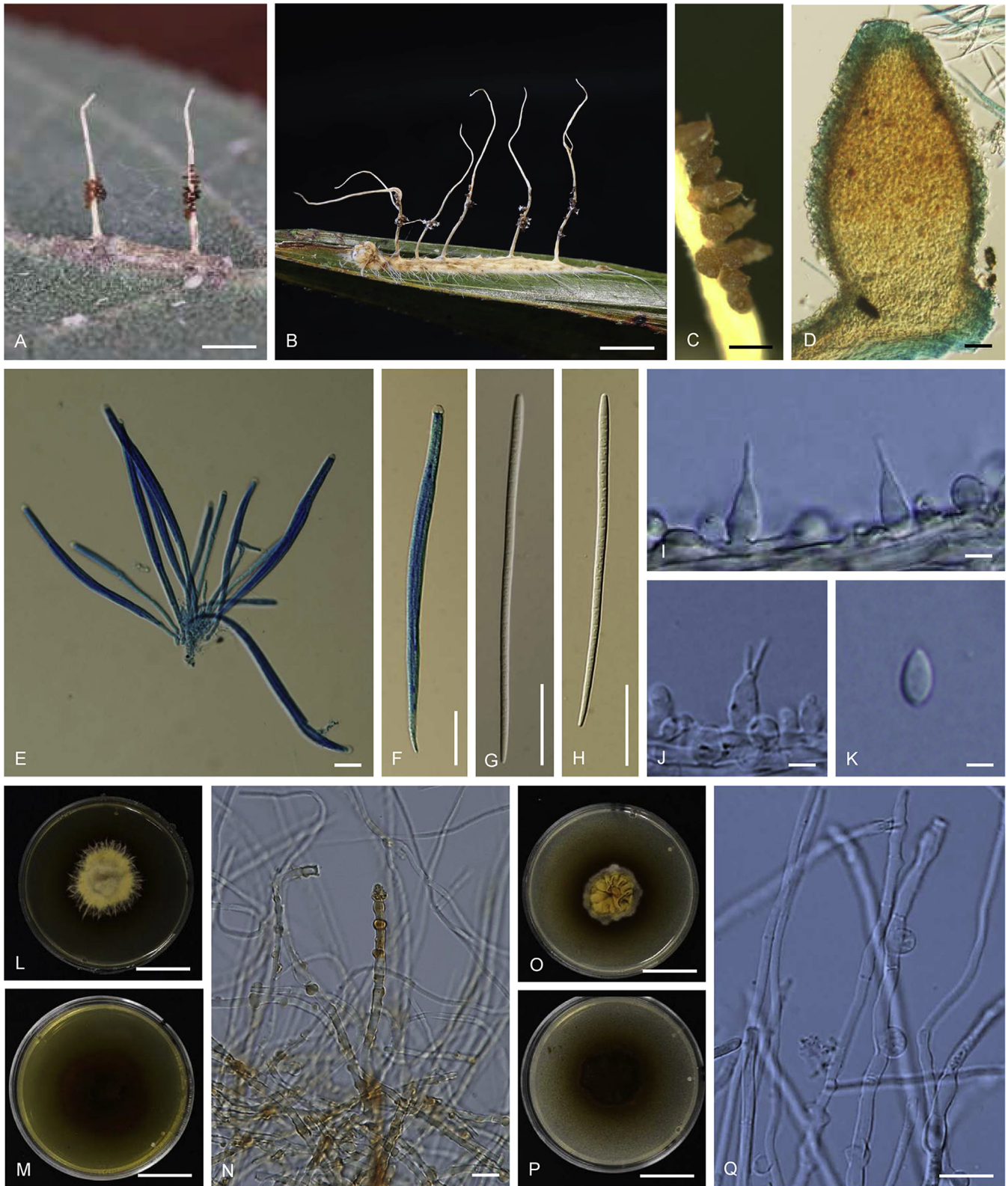
Fig. 4. *Ophiocordyceps brunneiperitheciata*. **A–B.** Stromata arising from host (A: BBH 36142, B: BBH 38841). **C.** Superficial perithecia. **D.** Perithecium. **E–F.** Asci with ascospores. **G–H.** Ascospores multiseptate. **I–J.** Part of stroma showing conidiogenous cells. **K.** Conidia. **L–N.** On PDA, **L.** Colony obverse. **M.** Colony reverse. **N.** Chlamydospores. **O–Q.** On PSA, **O.** Colony obverse. **P.** Colony reverse. **Q.** Chlamydospores. Scale bars: A, B = 2 mm; C = 1 mm; D = 30 μm; E, F = 20 μm; G, H = 30 μm; I = 4 μm; J = 3 μm; K = 2 μm; L, M = 8 mm; N, Q = 10 μm; O, P = 8 mm.

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

Specimens examined: **Thailand**, Nakhon Ratchasima Province, Khao Yai National Park, at 14°711′N, 101°421′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°711′N, 101°421′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 perithecium. 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*

Samson, H.C. Evans & Hoekstra. *O. brunneinigra* resembles *C. atewensis* in the sparse production of perithecia on a stand, whole filiform ascospores and occur on Hemipteran hosts but differs from *C. atewensis* in producing small, ovoid perithecia (560–650 × 200–240 μm), containing small cylindrical asci (250–320 × 10–15 μm) and filiform ascospores (220–300 × 3–5 μm). In *C. atewensis*, the perithecia are also ovoid subglobose but bigger, 600–1100 × 400–600 μm, and the asci are cylindrical to slightly fusiform, 400–600 × 9–12 μm, with whole, multiseptate, filiform ascospores, measuring 5–11 × 3–3.5 μm each cell (Samson *et al.* 1982).

Ophiocordyceps brunneiperitheciata Tasanathai, Thanakitpipattana, Khonsanit & Luangsa-ard, **sp. nov.** MycoBank MB822099. Fig. 4.

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

Stromata: Two to several, simple, wiry to pliant or 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, 350–400 × 180–200 μm. *Asci* hyaline, cylindrical, 8-spores, 125–175 × 6–8 μm. *Ascospores* 110–160 × 3–4 μm, filiform, whole, multiseptate.

Asexual morph terminal, pale grey (1B1) to dark grey (1F1). *Conidiogenous cells* monophialidic, hyaline, smooth, 12–16 μm long, 3–4 μm wide at the base and 0.5–1 μm wide at neck. *Conidia* hyaline, smooth, fusiform, 4–6 × 2 μm, without a mucous sheath.

Culture characteristics: Colonies on PDA very slow growing attaining a diameter of 8 mm in 20 d at 20 °C. Colonies light yellow (4A4) to reddish yellow (4A7), velvety; colony reverse dark brown (7F8). Colonies on PDA did not produce any conidiogenous structures after 1 mo. Chlamydospores observed on PDA after 1 mo.

Colonies on PSA very slow growing attaining a diameter of 8 mm in 20 d at 20 °C. Similar to colonies on PDA, no asexual morph was seen in culture. Chlamydospores observed on PSA after 1 mo.

Habitat: On small Lepidoptera larvae, 8–10 mm long, 0.5–1 mm wide.

Geographic distribution: Thailand, known from Nam Nao National Park and Hala Bala Wildlife Sanctuary.

Specimens examined: **Thailand**, Phetchabun Province, Nam Nao National Park, at 16°768′N, 101°671′E, on Lepidoptera larva, 3 Jul. 2013, K. Tasanathai, S. Mongkolsamrit, W. Noisripoom, P. Srikitikulchai & A. Khonsanit **(holotype** BBH 36142; culture ex-type TBRC 8100). **Thailand**, Phetchabun Province, Nam Nao National Park, at 16°768′N, 101°671′E, on Lepidoptera larva, 4 Jul. 2013, K. Tasanathai, S. Mongkolsamrit, W. Noisripoom, P. Srikitikulchai & A. Khonsanit (BBH 36150, TBRC 8099), Narathiwat Province, Hala Bala Wildlife Sanctuary, at 5°928′E, 101°883′E, on Lepidoptera larva, 24 Aug. 2011, A. Khonsanit (BBH 38841, BCC 49312).

Note: *Ophiocordyceps brunneiperitheciata* is similar to *O. pauciovoperitheciata* in parasitizing small Lepidoptera larvae on the underside of leaves and the production of loosely scattered perithecia on the middle part of the stroma. It differs from *O. pauciovoperitheciata* in the production of multiple stromata and the colour and shape of the perithecia.

Ophiocordyceps geometridicola Tasanathai, Thanakitpipattana, Khonsanit & Luangsa-ard, **sp. nov.** MycoBank MB822033. Fig. 5.

Etymology: Refers to the family of Lepidoptera, Geometridae.

Stromata: Numerous, up to 20, simple, wiry to flexuous, 5–8 mm long, 1 mm wide on twig, unchanged in 3 % KOH. *Perithecia* superficial, loosely aggregated, covering lower part to apex of stroma, ordinal in arrangement, ovoid, brown-dark brown, 280–400 × 240–290 μm. *Asci* hyaline, cylindrical, 8-spored, 150–190 × 5–8 μm. *Ascospores* hyaline, cylindrical to vermiform, remaining whole after discharge with distinct septation, 120–150 × 2–2.5 μm.

Asexual morph terminal, pale grey (1B1) to dark grey (1F1), 5–7 mm long, 0.5–1 mm wide, *Conidiogenous cells* monophialidic, in a continuous layer perpendicular to the stroma, arising from hyphae, hyaline, smooth-walled. *Phialides* (10–) 12–16(–18) μm, phialide base (5–)6.5–9(–12) × 4–5 μm, phialide neck (3–)5–7(–8) × 0.5–1 μm. *Conidia* hyaline, smooth-walled, fusiform, 6–7(–8) × 2–3 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 *Hirsutella* asexual morph. *Conidiogenous cells* monophialidic, sometimes polyphialidic, 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 rough 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 greyish green (1D4). Mycelium hyaline, septate, smooth-walled. *Conidiogenous cells* monophialidic or polyphialidic, hyaline, tapering gradually or abruptly into a long slender neck. *Phialides* (22–)24.5–32(–37) μm, phialide base (10–)14–19(–20) × 3–4.5(–6) μm, phialide neck 9–14(–20) × 1 μm, with numerous branching on the neck (Fig. 5L). *Conidia* hyaline, aseptate, smooth walled, oval to lemon shaped, (3–)4.5–6(–7) × (2–)2.5–4 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, Khao Yai National Park, at 14°711′N, 101°421′E, on larva of Lepidoptera, on twig of dicotyledonous plant, 8 Sep. 2015, K. Tasanathai, N. Kobmoo, W. Noisripoom & D. Thanakitpipattana, **(holotype** BBH 40665; culture ex-type TBRC 8095). **Thailand**, Chiang Rai Province, Phlu Kaeng Waterfall, at 20°26′N, 100°406′E, on larva of Lepidoptera, on twig of dicotyledonous plant, 18 Jan. 2009, K. Tasanathai, S. Mongkolsamrit, T. Chohmee, A. Khonsanit, P. Srikitikulchai & R. Promharn (BBH 31388, TBRC 8094).

Ophiocordyceps multiperitheciata Tasanathai, Thanakitpipattana, Khonsanit & Luangsa-ard **sp. nov.** MycoBank MB822030. Fig. 6.
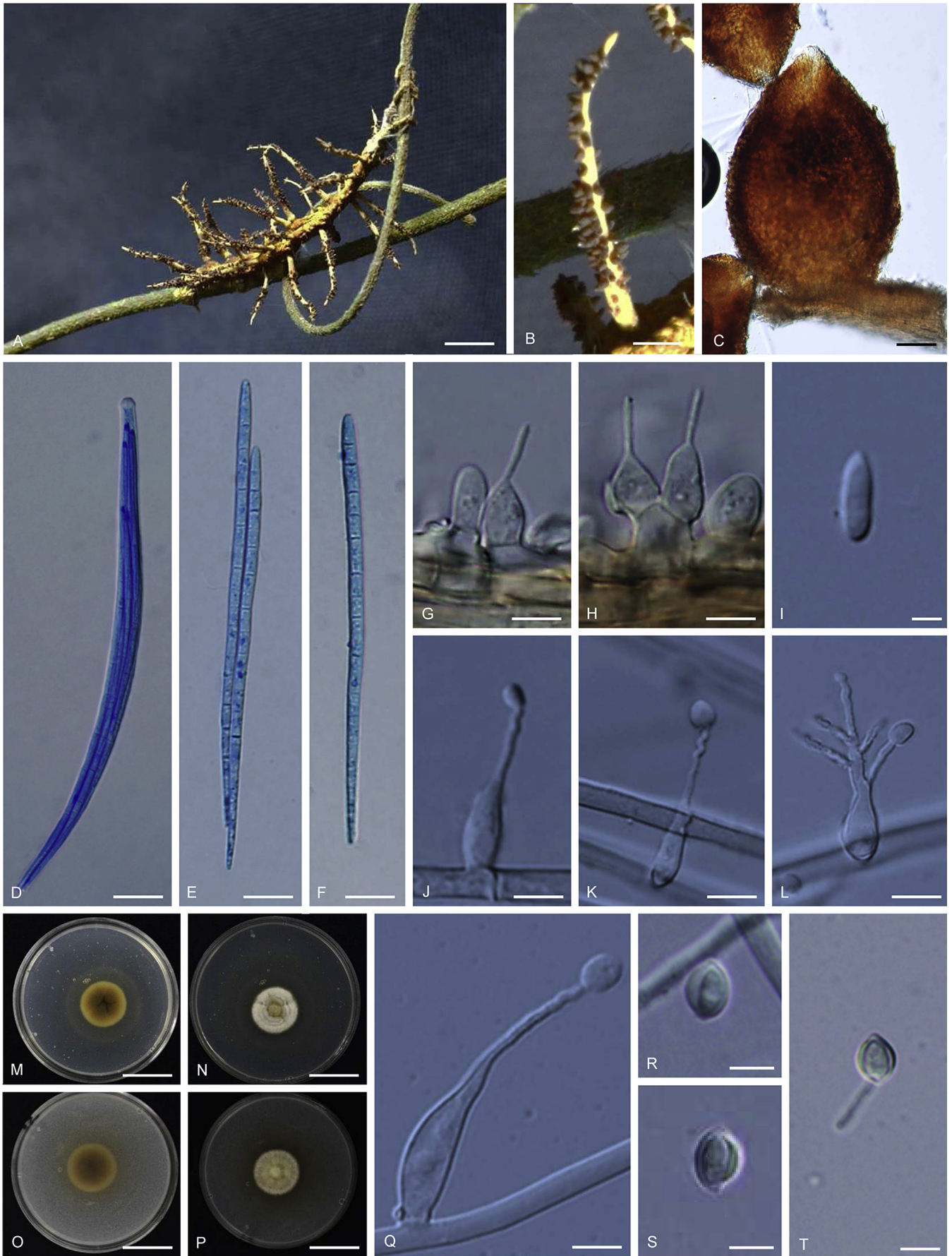
Fig. 5. *Ophiocordyceps geometridicola* (BBH 40665). **A.** Stromata arising from host. **B.** Superficial perithecia. **C.** Perithecium. **D.** Ascus with ascospores. **E–F.** Non-disarticulating ascospores. **G–H.** Part of stroma showing conidiogenous cells. **I.** Conidium. **M–N, J–L.** On PDA, **M.** Colony reverse. **N.** Colony obverse. **J–K.** Mono-phialidic conidiogenous cell with conidia. **L.** Polyphialidic conidiogenous cell with conidia. **O–P, Q–T.** On PSA, **O.** Colony reverse. **N.** Colony obverse. **O.** 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.
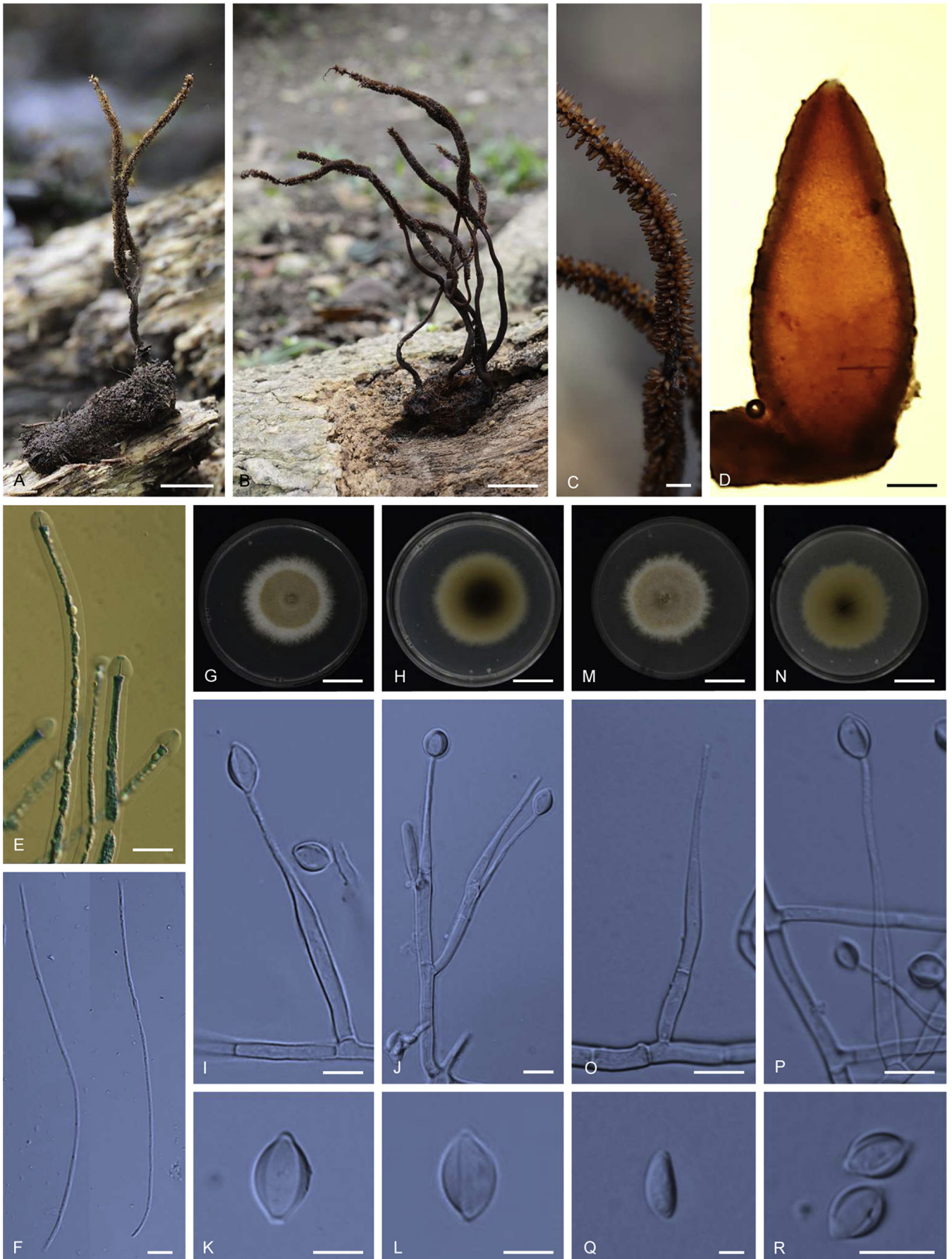
Fig. 6. *Ophiocordyceps multiperitheciata*. **A–B.** Stromata arising from host (A: BBH 38308, B BBH 18817). **C.** Superficial perithecia. **D.** Perithecium. **E.** Asci. **F.** Ascospores. **G–L.** On PDA, **G.** Colony obverse. **H.** Colony reverse. **I.** Monophialidic conidiogenous cell with conidia. **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.
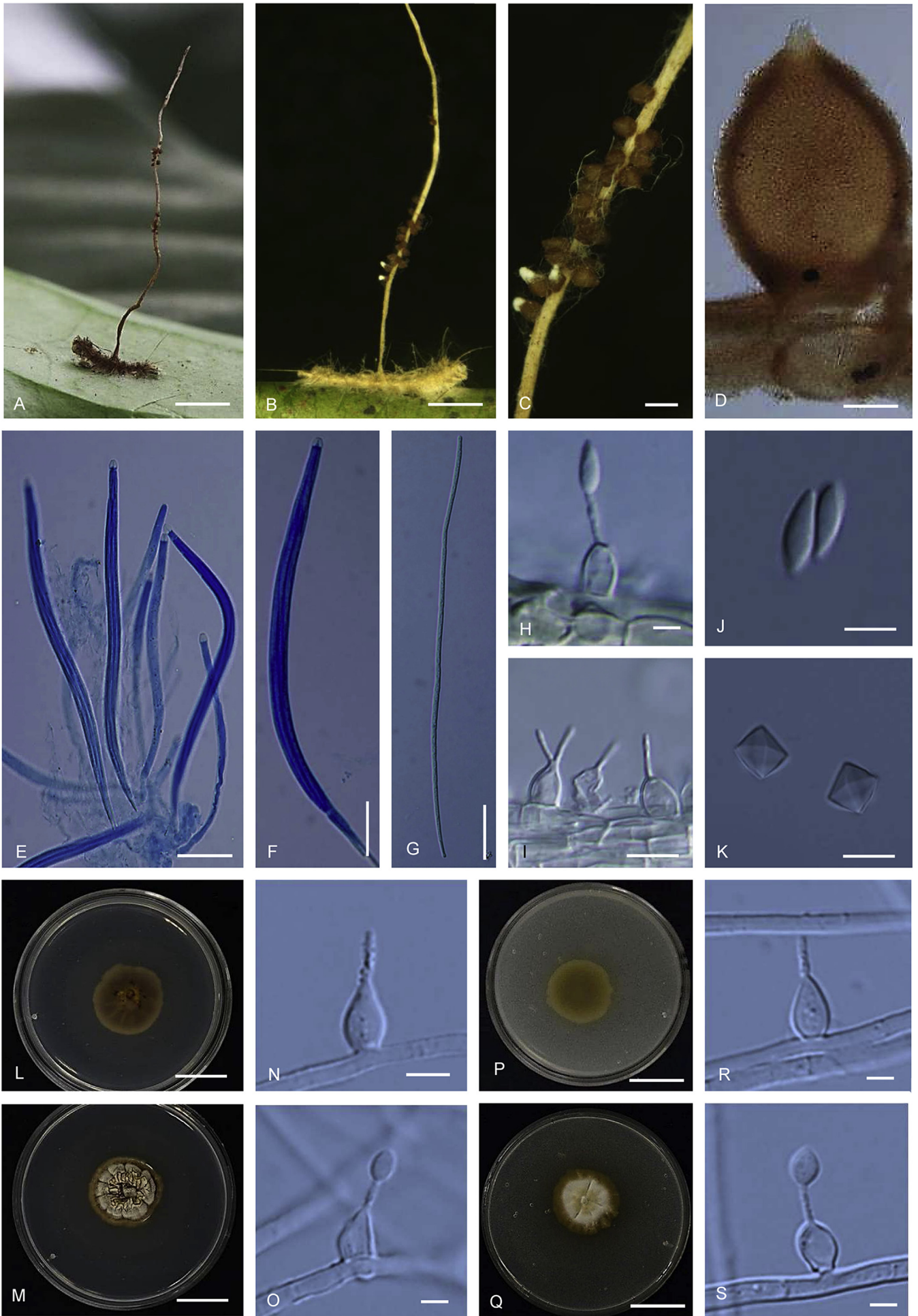
Etymology: Refers to the multiple perithecia on the stroma.

Stromata: Several, cylindrical, branched dentritic, 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. *Peritheca* 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, multiseptate.

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 centre 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 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–)19–28(–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°498′N, 98°361′E, on Lepidoptera larva, in the leaf litter, 29 Oct. 2013, K. Tasanathai, P. Srikikitulchai, A. Khonsanit, W. Noisripoom, D. Thanakitpi-pattana & S. Watcharapayungkit, **(holotype** BBH 38308; culture ex-type BCC 69008). **Thailand**, Chiang Mai Province, Doi Inthanon National Park, at 18°498′N, 98°36′E, on Lepidoptera larva, in the leaf litter, 7 Sep. 2006, K. Tasanathai, S. Mongkolsamrit, P. Srikitikulchai, B. Thongnuch, R. Ridkaew & C. Chuaseeharonnachai (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 *Fallopia japonica* Houtt. (Ban *et al.* 2015).

Ophiocordyceps pauciovoperitheciata Tasanathai, Thana-kitpipattana, Khonsanit & Luangsa-ard, **sp. nov.** MycoBank MB822034. Fig. 7.

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

Stroma: Solitary, simple, wiry to pliant 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. *Peritheca* 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 cell* 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 (5D8-5F8). *Conidiogenous cells* monophialidic or poly-phialidic 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(–6) µm, phialide neck (2–)4–6.5(–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–)4–6.5(–9) × 0.5–1 µm, rough and tiny warty. *Conidia* hyaline, 1-celled, smooth-walled, oval, 5–6 × 3 µm, without a mucous sheath.

Habitat: On small Lepidoptera larva, 7 mm long, 2 mm wide, found on underside of leaves of forest plants.

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

Specimens examined: **Thailand**, Nakhon Ratchasima Province, Khao Yai National Park, at 16°203′N, 99°321′E, on Lepidoptera larva on the underside of leaves of dicotyledonous plants, 2 Oct. 2007, K. Tasanathai, S. Mongkolsamrit, P. Srikitikulchai, B. Thongnuch, R. Ridkaew & A. Khonsanit **(holotype** BBH 29403; culture ex-type TBRC 8106). **Thailand**, Nakhon Ratchasima Province, Khao Yai National Park, at 14°711′N, 101°421′E, on Lepidoptera larva, 20 June 2012, K. Tasanathai, S. Mongkolsamrit, A. Khonsanit, W. Noisripoom, P. Srikitikulchai, K.

Fig. 7. *Ophiocordyceps pauciovoperitheciata*. **A–B.** Stroma arising from host (A: BBH 29403, B: BBH 32730). **C.** Superficial perithecia. **D.** Perithecium. **E–F.** Asci with ascospores. **G.** Ascospore. **H–I.** Part of stroma showing conidiogenous cells with conidia. **J.** Conidia. **K.** Crystals on PDA. **L–O** on PDA, **L.** Colony reverse. **M.** Colony obverse. **N–O.** Monophialidic conidiogenous cell with conidia. **P–S** on PSA, **P.** Colony reverse. **Q.** Colony obverse. **R–S.** Monophialidic conidiogenous cell with conidia. Scale bars: A, C = 2 mm; B = 4 mm; D = 70 µm; E = 30 µm; F, G = 20 µm; H, I–K, N, O = 5 µm; L, M = 8 mm; P, Q = 10 mm; R, S = 4 µm.

Sansatchanon & R. Somnuk (BBH 32730, TBRC 8096), Nakhon Ratchasima Province, Khao Yai National Park, at 16°203′N, 99°321′E, on Lepidoptera larva, 7 Oct. 2009, K. Tasanathai, P. Srikitikulchai, S. Mongkolsamrit, T. Chohmee, R. Ridkaew, P. Puyngain & M. Sudhadham (BBH 27112, TBRC 8097), Nakhon Ratchasima Province, Khao Yai National Park, at 14°711′N, 101°421′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. brunneiperitheciata* in the production of sparsely distributed perithecia along the stromata. With the exception of *O. brunneinigra* which occurs on Hemiptera, the three other species are found on the same host (Lepidoptera). *O. pauciovoperitheciata* differs from the three species in the colour, shape and size of the perithecia.

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

Etymology: 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. *Asci* 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, 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 (5A2) 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°711′N, 101°421′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°711′N, 101°421′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 stromata and the size of the perithecia (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 Tasanathai, Thanakitpipattana, 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 planthoppers (*Fulgoridae*, *Hemiptera*) and on *Coleoptera.*

Geographical distribution: Thailand and USA.

The material examined (NHJ 12525, BBH 9271) has immature superficial, brown to dark brown perithecia. Stromata up to two, up 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°102′N, 102°192′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, Hywel-Jones & Spatafora. Both *O. spataforae* and *O. crinalis* possess longer stromata compared to *O. brunneinigra*. However, *O. crinalis* has numerous stromata compared with both *O. spataforae* and *O. brunneinigra*. *O. crinalis* and *O. spataforae* were found in the leaf litter while *O. brunneinigra* was found on the underside of leaves (Wang *et al.* 2014). In Thailand, *O. spataforae* was found only on fulgorid plant hoppers while in the USA it was reported on Coleoptera. This is not a common species in Thailand. The first specimen was collected in 2003 while the second sighting and collection was made in 2017.

DISCUSSION

This study advanced our understanding of the genotypic variation among closely related species of *Ophiocordyceps* with
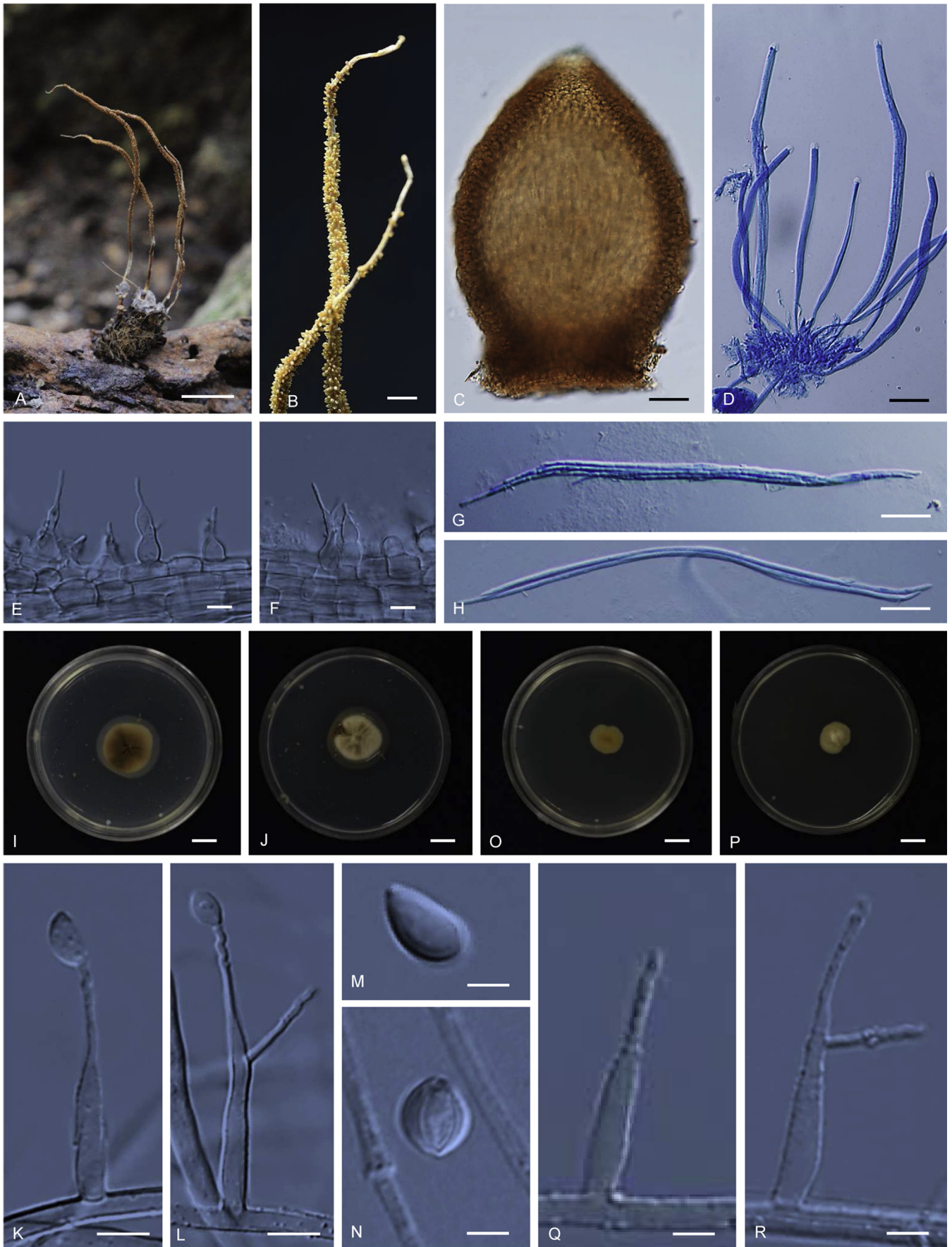
Fig. 8. *Ophiocordyceps pseudoacicularis* (BBH 32211). **A.** Stroma arising from host. **B.** Superficial perithecia. **C.** Perithecium. **D.** Asci with ascospores. **E–F.** Part of stroma showing conidiogenous cells. **G-H.** Ascospore. **I–N.** On PDA, **I.** Colony reverse. **J.** Colony obverse. **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.
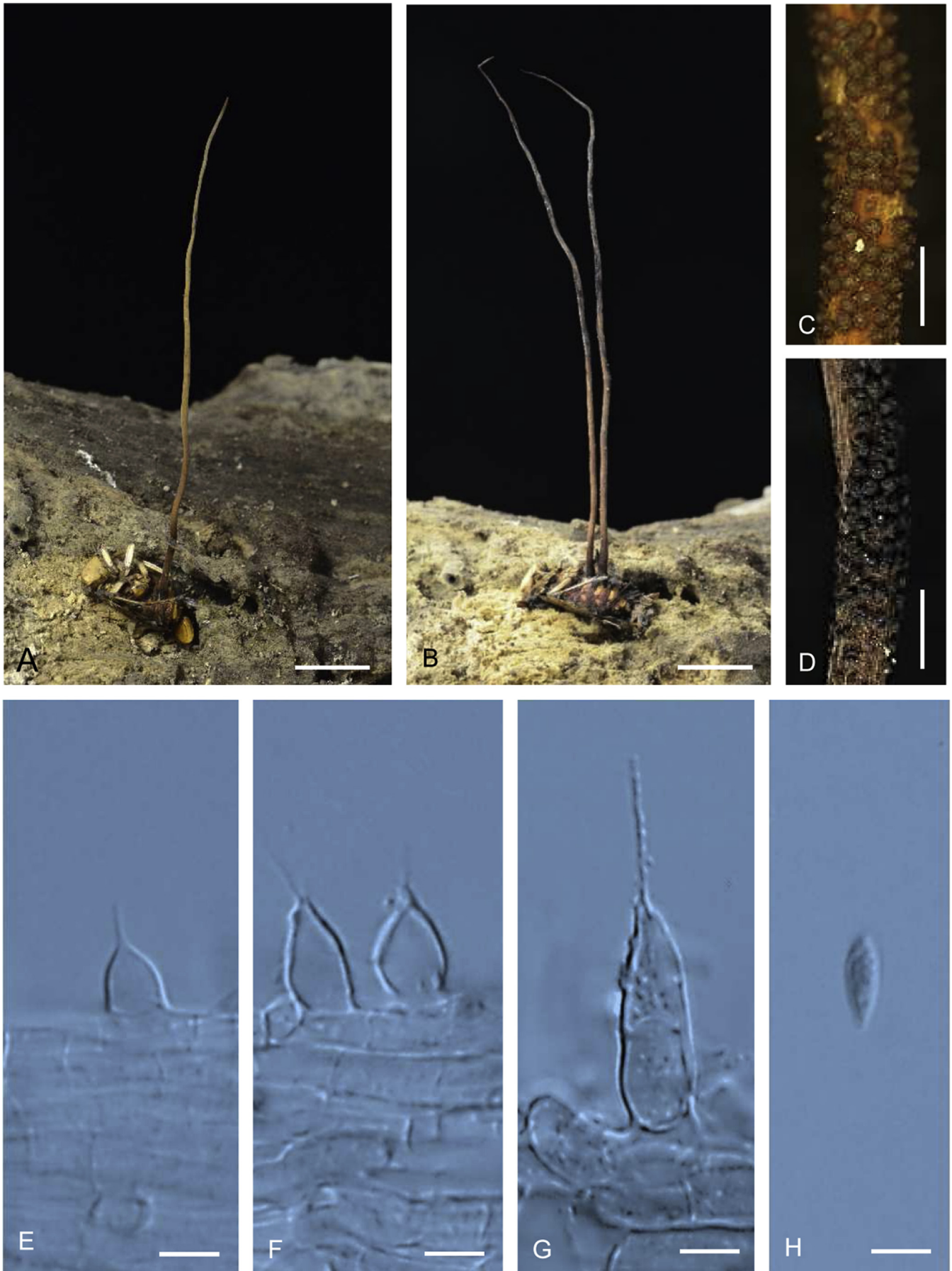
Fig. 9. *Ophiocordyceps spataforae*. **A, B.** Stromata arising from host: **A.** Immature stroma with only *Hirsutella* asexual state (BBH 43464). **B.** Stromata containing immature perithecia (BBH 43466). **C, D.** Part of stroma showing developing superficial perithecia. **C.** Fresh collection, **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 seem 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), Luangsa-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 *Hirsutella* (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 phylogenies, not only the fungal barcode marker and an additional gene, should be incorporated routinely in taxonomic research. In our analyses using these four combined loci, we recognize seven new species in *Ophiocordyceps* including one new record of *Ophiocordyceps macroacicularis* in Thailand. Our study also revealed that *O. ramosissimum* T.C. Wen, J.C. Kang & K.D. Hyde and *O. arborescens* S. Ban, Sakane & Nakagiri are conspecific based on analysis of multiple loci. The use of these four loci helped to improve the delimitation of the species, especially in the absence of published data of asexual morphs from the natural specimens. Older records have mostly overlooked or ignored collecting data of the asexual morph, assuming a sterile apex instead of conidia-filled discontinuous layer of conidiogenous cells which arise perpendicular to the surface of a filiform stroma. A significant result of this study is the addition of the number of species in *Ophiocordyceps* and thus the number of sequenced ex-type cultures and asexual morph records.

Specimens found on the underside of leaves (*O. brunneinigra, O. pauciovoperitheciata, O. brunneiperitheciata*) produce loosely distributed and sparse perithecia, with up to 30 perithecia developing in the central part of the stromata, while two species found in the leaf litter produce abundant and tightly packed perithecia (*O. multiperitheciata, O. pseudoacicularis*) starting from the base of the stroma up to the apex. *O. spataforae,* also found in the leaf litter, was immature and still had very few perithecia along the bottom centre of the stroma. The perithecia of specimens on twigs (*O. geometridicola*) produce loosely distributed perithecia along the entire stroma.

Ophiocordyceps brunneinigra belong to a well-supported clade with *O. crinalis* and *O. spataforae.* It differs from *O. spataforae* and *O. crinalis* in the ecology of the host. *O. spataforae* and *O. crinalis* are both found in the leaf litter while *O. brunneinigra* infects insects on the underside of leaves. Three other new species, *O. brunneiperitheciata, O. geometridicola* and *O. pauciovoperitheciata* are attached to the underside of leaves like *O. brunneinigra*. *O. brunneinigra* and *O. pauciovoperitheciata* both produce a single filiform stroma with sparsely distributed perithecia. They differ from each other in the host and shape of the perithecia. *O. pauciovoperitheciata* produces crystals in cultures on PDA while no other newly collected specimen shows this trait. *O. brunneiperitheciata* produces two or more filiform stromata and does not form conidiogenous cell in culture. It produces chlamydospores on PSA, like *O. brunneinigra.*

Ophiocordyceps pseudoacicularis grouped with *O. cochlidiicola* in the *Hirsutella nodulosa* clade *sensu* Simmons *et al.* (2015). It is the second sexual morph to be found in the *H. nodulosa* group and the macro morphology is similar to *O. cochlidiicola.* A significant difference in ascospore morphology was revealed following microscopic examination. In our collections *O. pseudoacicularis* 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* Luangsa-ard, Tasan. & Thanakitp. where a whole ascospore/part-spore disjunction has been reported (Tasanathai *et al.* 2016).

Ophiocordyceps pseudoacicularis 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 asci and ascospores, and the host. *O. pseudoacicularis* has shorter asci and ascospores and infect Lepidoptera larvae instead of Coleoptera larvae. *O. geometridicola* shares similarity with the *H. nodulosa* group in producing undulate phialides.

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, focussing on invertebrate-pathogens worldwide to facilitate exact comparisons among collections of species deemed to have a cosmopolitan distribution.

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